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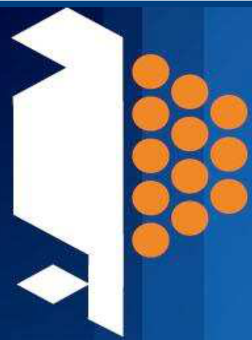

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IN CONJUNCTION
WITH XVI MEETINGOF THE SPANISH SOCIETY
OF HISTOLOGY AND TISSUE ENGINEERING

GRANADA, SPAIN, JUNE 7TH – 10TH 2011

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V EUROPEAN CHAPTER OF THE TISSUE ENGINEERING AND REGENERATIVE MEDICINE INTERNATIONAL SOCIETY (TERMIS)

GRANADA, SPAIN, JUNE 7TH – 10TH 2011
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B. INDUSTRY DAY: CELLS AND TISSUES AS THERAPEUTIC TOOLS

1. ADIPOSE TISSUE DERIVED STEM CELLS IN TISSUE ENGINEERING APPROACHES

Chair: Manuela E. Gomes
Co-chair: Rui L. Reis
Keynote speaker: Jeffrey Gimble
Organizer: Manuela E. Gomes
Synopsis: In 2001 it was reported for the first time the existence of stem cells within the adipose tissue, and since then, this tissue has been gaining an increased importance as a stem cells source for a wide range of potential applications in Tissue Engineering and Regenerative Medicine. Adipose tissue is probably the most abundant and accessible source of adult stem cells and thus it holds great promise for use in tissue repair and regeneration. In fact, Adipose Stem Cells (ASCs), present several advantages over other adult stem cell sources, such as the bone marrow, as they can be obtained in larger quantities, under local anesthesia and with minimal discomfort. Furthermore, it has been demonstrated that adipose tissue-derived stem cells (ASCs) possess multiple differentiation capacities.
Nevertheless, to take full advantage of this cell source for Tissue Engineering applications, current research has been addressing several issues, such as, for example, the differences found in the harvesting methods, differences in fat tissue derived from different anatomic sites and the heterogeneity of the cells population that are obtained using the isolation methods most commonly used do far. Many researcher have focused essentially in their potential use in a number of regenerative medicine approaches, based o their wide availability, possibility of autologous use and differentiation potential.
In summary, the aim of this Symposium is to expose most recent findings and knowledge generated from research on adipose derived stem cells, focused on their application in tissue engineering/regeneration.

(1.KP) CURRENT OPPORTUNITIES AND CHALLENGES IN ADVANCING HUMAN ADIPOSE-DERIVED CELLS TO THE CLINIC

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Subcutaneous fat has emerged as an alternative tissue source for stromal/stem cells in regenerative medicine. Over the past decade, international research efforts have established a wealth of basic science and pre-clinical evidence regarding the differentiation potential and regenerative properties of both freshly processed, heterogeneous stromal vascular fraction (SVF) cells and culture expanded, relatively homogeneous adipose-derived stromal/stem cells (ASCs). The SVF cells and ASC populations display distinct advantages and functional properties making them attractive for either autologous or allogeneic use. Mechanistically, the cells can act via direct differentiation to the tissue of interest and/or as a source of trophic factors. The stage has been set for clinicians to translate adipose-derived cells from the bench to the bedside; however, this process will involve "development" steps that fall outside of traditional "hypothesis-driven, mechanism-based" paradigm. It is important for the tissue engineering community to design and pursue randomized and controlled clinical trials with long term follow up. An evidence-based medical approach will advance the field more effectively than anecdotal or uncontrolled reports. Clinical applications will be further served by standardization and reproducibility of adipose-derived cell therapies with respect to their efficacy and safety.

(1.O1) TNF-TREATED ADIPOSE TISSUE-DERIVED STEM CELLS INCREASE THE MIGRATORY ACTIVITY OF ENDOTHELIAL CELLS IN VITRO

Salamon A (1), Ramer R (2), Adam S (1), Rychly J (3), Peters K (1)
1. Junior Research Group, Department of Cell Biology, Medical Faculty, University of Rostock; 2. Institute of Toxicology and Pharmacology, Medical Faculty, University of Rostock; 3.Department of Cell Biology, Medical Faculty, University of Rostock

Introduction. Adipose tissue-derived stem cells (ASC) express the mesenchymal stem cell (MSC) markers CD44, CD68, CD105 and CD166 and can differentiate along different lineages. Since MSC are known to have immunomodulatory effects and since freshly isolated ASC express the perivascular marker CD34, we investigated whether inflammatory stimulation of ASC influences migration of human dermal microvascular endothelial cells (HDMEC).
Materials and Methods. To this end, we treated ASC with tumor necrosis factor (TNF), transferred the cell culture supernatant to a culture of HDMEC and observed the migratory activity of the endothelial cells in Scratch and Boyden Chamber Assays. ELISA-based techniques were used to find factors that are secreted by ASC.
Results. We found that ASC-conditioned medium significantly increased the migratory activity of HDMEC both in Scratch and Boyden Chamber Assays. Under TNF treatment, ASC-mediated migratory activation of HDMEC was further increased. Out of 31 factors that were analyzed by ELISA-based techniques, ASC were found to secrete 18 to the supernatant, and 13 of those factors were more strongly secreted following TNF treatment.
Conclusion. Our findings indicate that there is an indirect interaction between ASC and HDMEC via diverse soluble factors. Although we can so far not decipher the individual contributions of the large variety of factors involved, we can nevertheless assume that ASC in vivo modify HDMEC-mediated processes such as e. g. wound healing, tissue infiltration by leukocytes or the development of new blood vessels. Therefore, ASC are a promising source for cell-based regenerative therapies.
This work was financially supported by the Ministry of Economy, Labor and Tourism Mecklenburg-Vorpommern and by the European Union (ESF/IV-WM-B34-0006/08).
Keywords. ASC, HDMEC, inflammation, migration

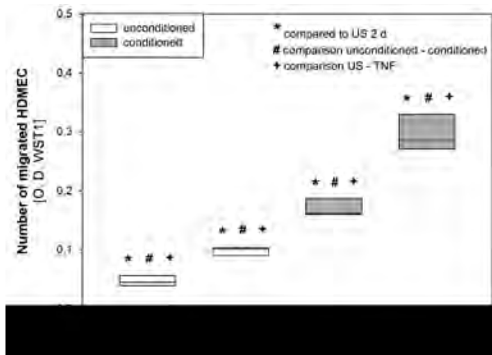

Figure 1: Conditioned medium from TNF-treated ASC significantly increased HDMEC migratory activity. Migration of HDMEC through 8 µm pores and towards ASC-conditioned medium was assessed in a classical Boyden chamber assay.

(1.O2) DEVELOPING OPTIMIZED METHODS FOR CGMP COMPLIANCE IN THE ISOLATION OF HUMAN ADIPOSE-DERIVED STROMAL/STEM CELLS

Carvalho PP (1), Yu G (2), Wu X (2), Dias IR (3), Gomes ME (1), Reis RL (1), Gimble JM (2)

1. 3B's Research Group; 2. Pennington Biomedical Research Center; 3. University of Tras-os-Montes e Alto Douro

Introduction. This study aimed to explore non-animal sources of trypsin-like enzymes as alternatives to porcine trypsin for the passage of ASCs and to determine the effect of time delays on the yield and function of ASCs after collagenase digestion.

Materials and Methods. Differentiation ASCs (P1) were induced with either Adipogenic Medium or Osteogenic Medium for 9-12 days and stained with Oil Red O or Alizarin Red (respectively). Flow Cytometry: hASCs were assessed with CD29, CD34, CD44, CD45, CD73, CD90, CD105 and IgG1 control.

Results. Trypsin alternatives. There is no significant difference between Trypsin and animal-free alternatives tested, in total cell recovered number and their viability; immunophenotype and differentiation capacity in adipogenic and osteogenic lineages is maintained. Lipoaspirate storage, show significant differences between total number of nucleated cells obtained in SVF harvested on day 0 relative to days 1, 2 and 3 (room temperature). There was no significant difference between ASC yields on day 0 and day 1. Flow cytometric analysis showed no significant difference in the immunophenotype of ASCs throughout the four day period. Capacity for adipogenic and osteogenic differentiation remained present in cells harvested up to day 3 although a decrease in the intensity of the staining was evident in days 2 and 3.

Conclusions. We conclude that TrypLE Express and TrypZean can be used in cell culture protocols as effective animal-free alternatives to Trypsin/EDTA. Cell yield, viability and phenotype will remain the same as cells treated with Trypsin/EDTA. Our findings indicate that one can obtain hASCs even 72hrs after surgical procedure but the cell yield and differentiation ability is optimal within the first 24hrs. These studies have relevance to the optimization of GMP methods using ASCs in tissue engineering and regenerative medicine.

Pedro P. Carvalho acknowledges the Portuguese Foundation for Science and Technology (F.C.T.) for his grant (SFRH/BD/44128/2008).

Keywords. Adipose-derived stem cells; lipoaspirates; animal-free; trypsin

	Day 0	Day 1	Day 2	Day 3
CD29	88.0 ± 20.9	96.5 ± 2.	92.7 ± 6.	89.9 ± 13.
CD34	91.6 ± 3.4	91.7 ± 5.	84.9 ± 6.	84.1 ± 5.9
CD44	11.8 ± 4.9	20.3 ± 4.	15.1 ± 11.	8.5 ± 5.6
CD45	17.3 ± 7.4	17.5 ± 3.	8.4 ± 4.2	6.0 ± 3.3
CD73	79.3 ± 9.2	76.1 ± 8.	67.5 ± 21.	75.1 ± 19.
CD90	86.9 ± 5.5	80.9 ± 7.	74.3 ± 26.	68.8 ± 27.
CD10	94.2 ± 5.5	93.9 ± 3.	94.8 ± 6.	96.7 ± 4.

Table 1 – Flow Cytometry data. The percentage of positive cells for each marker is presented as an average value ± SD in a total of four different donors (n=4). Values with significant difference to day 0 are marked with an asterisk***** (p<0.05).

(1.O3) KERATIN BIOMATERIALS SUPRESS PPARΓ EXPRESSION AND ENHANCE HUMAN ADIPOSE DERIVED STEM CELL OSTEOGENESIS

Teli T (1), Van Dyke ME (2)

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Introduction. The combination of autologous, multipotent mesenchymal stem cells (MSC) and a biomaterial carrier has been proposed as a treatment for patients with fractures, osteoporosis, and cancer. However, the target patient population is typically elderly, and significant changes in the multipotency of MSC with age have brought the clinical utility of this treatment paradigm into question. Specifically, peroxisome proliferator-activated receptor gamma 2 (PPARγ2), has been shown to activate adipogenic and suppress osteogenic differentiation pathways in aged mice, thereby limiting the potential effectiveness of regenerative treatments that rely on endogenous and exogenous MSC for bone repair. The goal of this study was to examine the effect of keratin biomaterials on osteogenesis and PPARγ signaling in human adipose derived stem cells (hADSC), a clinically important source of autologous MSC for therapy.

Materials and Methods. Keratin was extracted from human hair obtained from a commercial supplier under oxidative conditions to yield a crude keratose mixture. Fractions of alpha and gamma keratose were purified and added as supplements at 0.03 mg/mL in bone differentiation media (100nM dexamethasone, 50mM ascorbic acid and 10mM β- glycerophosphate). hADSC were isolated from adipose tissue collected from donors undergoing elective abdominoplasty, assayed for CD13, CD44, CD73, CD105 and CD166, and subsequently induced to differentiate towards osteogenic lineage for 21 days. Cultures were assayed for alkaline phosphatase (AP) activity and deposition of calcified mineral. Quantitative RT-PCR assays were performed using osteogenic-specific primers runt-related transcription

factor 2 (Runx2), collagen type I alpha 1 (Col1a1), AP, OC, homeobox protein DLX5 and PPARγ.

Results. AP activity and calcium staining were both increased at day 14 in the crude keratose (C-KOS), alpha-keratose (A- KOS), and gamma-keratose (G-KOS) treated samples compared to controls. qRT-PCR showed large fold changes for differentiating hADSC, particularly for gamma-keratose and crude keratose (which contains gamma-keratose; Figure 1). Also, PPARγ gene expression was down regulated in the presence of all keratose fractions compared to osteogenic media alone at all time points (Figure 2).

Conclusion. Keratose biomaterials have a measurable effect on osteogenesis as evidenced by increase AP activity, calcification, and expression of important osteogenic genes, seemingly through a down regulation of PPARγ expression. This finding suggests that keratose-based bone grafts may have particular efficacy in elderly patients where adipogenesis of autologous MSC used for therapy may be unwittingly favored over osteogenesis.

Disclosures Mark Van Dyke holds stock and is an officer in the company, KeraNetics LLC, who has provided partial funding for this research. Wake forest University Health Sciences has a potential finan

Keywords. Adipose Derived Stem Cells, osteogenesis, keratin biomaterials, PPARγ

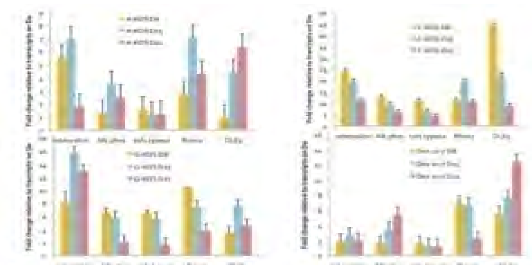

Figure 1. Osteogenic-related gene expression in cultures of hADSC treated with osteogenic media supplemented with keratose fractions at 0.03 mg/mL. Samples labeled as Dex 10-7 received only osteogenic supplements.

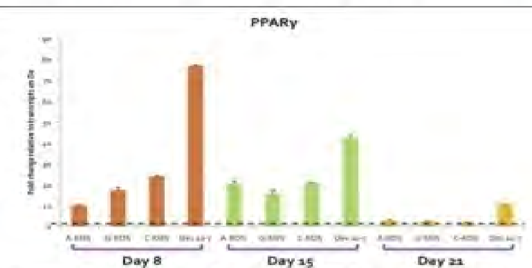

Figure 2. PPARγ gene expression in cultures of hADSC treated with osteogenic media supplemented with keratose fractions at 0.03 mg/mL. Samples labeled as Dex 10-7 received only osteogenic supplements.

(1.O4) ADIPOSE-DERIVED STEM CELLS (ASCS) FROM ANATOMICALLY DIFFERENT SITES DETERMINE PHENOTYPE AND FUNCTION OF SCHWANN-LIKE CELLS FOR PERIPHERAL NERVE REPAIR

Haycock J (1), Kaewkhaw R (1), Scutt A (1)
1. Sheffield University

Adipose-derived stem cells (ASCs) have gained considerable interest as a source for deriving other specific cell types including Schwann cells for treating peripheral nerve injury. However, our hypothesis was that the adipose donor site might influence the differentiation potential of ASCs into Schwann cells, which is presently unknown. This work therefore investigated the differentiation of ASCs harvested from different anatomical sites of: i) subcutaneous; ii) perinephric; and iii) epididymal adipose tissue. We demonstrated that although these cell types shared a common multilineage differentiation potential and cell surface markers, ASCs from anatomically different sites differed in their Schwann cell phenotype and function in stimulating neuronal differentiation in vitro. The up-regulation of S100β, GFAP and p75NGFR was observed in perinephrium-ASCs, while only the expression of S100β or GFAP and p75NGFR was elevated in subcutaneous-ASCs or epididymis-ASCs. Co-culture of ASCs with NG108-15 neuronal cells showed that differentiated ASCs from each source stimulated neurite outgrowth, which was significantly greater than undifferentiated ASCs. In addition, subcutaneous and perinephrium-ASCs stimulated neurite extension and sprouting number more effectively than epididymis-ASCs. High levels of BDNF and NGF were detected in differentiated ASCs in the above co-cultures, but levels of NT-3 were low. We found that through functional blocking studies to BDNF and NGF that complete abrogation resulted, suggesting a major role of these two neurotrophins in particular for stimulating neuronal cell differentiation. Thus, ASCs can be obtained from different anatomical sites and this determines the differentiation potential, Schwann cell like phenotype and extent of function. In conclusion, this work supports the potential of ASCs as an alternative cell source to primary Schwann cells for the local delivery and treatment of peripheral nerve injury.

Keywords. Adipose stem cell; Schwann cell; Nerve

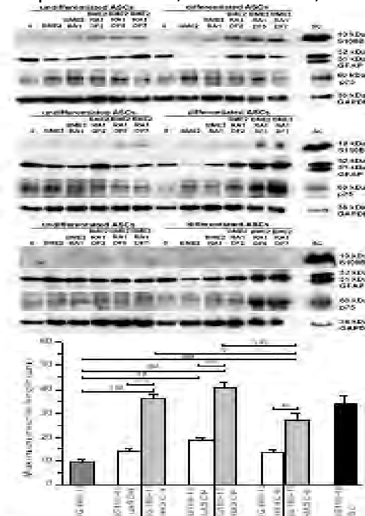

*Figure 1. Differentiation of adipose-derived stem cells (ASCs) into Schwann-like cells. A-C. Western blotting for S100B, GFAP and p75 proteins of A) subcutaneous-, B) perinephrium- and C) epididymis-ASCs. D. The promotion of neurite elongation from NG108-15 neuronal cells by ASCs or primary Schwann cells (SC). *** or *** or*

(1.O5) INTERACTION BETWEEN SHEAR STRESS AND VEGF IN THE INDUCTION OF ENDOTHELIAL DIFFERENTIATION OF HUMAN ADIPOSE – DERIVED STEM CELLS

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Introduction. Adipose tissue represents an abundant and accessible source of adult stem cells with the ability to differentiate into endothelial cells for therapeutic vascularisation and tissue engineering applications. However none of the studies to date have been able to demonstrate differentiated cells displayed a comprehensive range of endothelial characteristics. Herein we combine chemical and mechanical stimulation to investigate the effects of vascular endothelial growth factor (VEGF) and physiological shear stress in promoting the differentiation of human adipose derived stem cells (ADSCs) into endothelial cells.

Materials and Methods. ADSCs were isolated and characterised by immunofluorescence and flow cytometry. Endothelial differentiation was promoted by culturing confluent cells in the presence of 2% foetal calf serum and 50ng/ml VEGF under physiological shear stress (12 dynes) for up to 14 days. Endothelial characteristics were evaluated by immunofluorescence staining for endothelial markers, analysis of acetylated–low density lipoprotein (Ac-LDL) uptake and assessment of tubular formation performed using an in vitro angiogenesis assay.

Results. Human ADSCs treated with VEGF and subjected to shear stress expressed vWF, eNOS and FLT-1 after 7 days and CD31, FLK-1 and VE-cadherin after 14 days. Treated cells also were able to incorporate Ac-LDL as well as form tubular structures on matrigel, unlike control cells. Untreated cells or cells only subject to shear stress did not display any of the noted endothelial characteristics.

Conclusion. Based on these results, we have demonstrated that ADSCs subject to mechanical shear stress and chemical stimulation with VEGF are able to express a comprehensive range of endothelial markers. Our differentiation protocol provides a more efficient strategy to obtain endothelial-like cells for tissue engineering based on autologous, mesenchymal stem cells (MSCs).

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Keywords. ADSCs, Endothelial Differentiation, VEGF, shear stress

(1.O6) CO-CULTURE OF HUMAN PREADIPOZYTES AND ENDOTHELIAL PROGENITOR CELLS FOR NEOVASCULARISATION OF TISSUE ENGINEERED ADIPOSE TISSUE

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Introduction. Tissue engineering of adipose tissue suffers from the major disadvantage of tissue resorption due to an insufficient vascularisation. Thus, a novel strategy to create vascular networks and enhance neovascularisation of tissue engineered adipose tissue might be the co-implantation of preadipozytes with endothelial progenitor cells (EPCs). Here, we investigate the effects of co-culture of preadipozytes and EPCs on EPC sprout formation in vitro and neovascularisation of implants in vivo.

Material and Methods. Preadipozytes were isolated from human fat tissue and EPCs from human peripheral blood. In vitro, the angiogenic effects of preadipozytes on EPCs were analysed in an EPC spheroid sprouting assay. In vivo, investigations to determine the vascularisation of Fibrin implants due to co-culture of preadipozytes and EPC spheroids were performed in a chick embryo chorioallantoic membrane (CAM) assay. After 8 days, the neovascularisation of the implants were evaluated by histological analyses.

Results. In vitro, co-culture with preadipozytes induces significant longer sprout formation in EPCs compared to EPC spheroids alone. In vivo, implants containing preadipozytes and EPC spheroids displayed a significant higher rate of neovascularisation in terms of number and depth compared to preadipozytes or EPC spheroids alone where less or no vessel ingrowth was observed.

Discussion. Co-culture of preadipozytes and EPCs enhances the angiogenic capacities in vitro and in vivo. Thus, this study highlights the importance of cellular contact between preadipozytes and EPCs for neovascularisation of tissue engineered adipose tissue.

Keywords. preadipozytes, endothelial progenitor cells, co-culture, CAM, angiogenesis

(1.O7) ACCELERATION AND AUGMENTATION OF FEMORAL SEGMENTAL BONE HEALING BY ADIPOSE-DERIVED STEM CELLS ENGINEERED BY HYBRID BACULOVIRUS VECTORS CONFERRING SUSTAINED TRANSGENE EXPRESSION

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Introduction. Massive segmental defects arising from trauma or tumor resection remain a challenging clinical problem. To heal massive, segmental bone defects using adipose-derived stem cells (ASCs) which alone cannot heal large defects, we hypothesized that sustained expression of factors promoting bone regeneration (BMP2) and angiogenesis (VEGF) provides continuous stimuli to augment the healing. Baculovirus (BV) holds promise for gene therapy and efficiently transduces stem cells, but it only mediates transient transgene expression.

Materials and Methods. We developed a dual BV system whereby one BV expressed FLP recombinase (BacFLP) while the other hybrid BV harbored an Frt-flanking transgene cassette for ASCs engineering and healing of critical-size segmental bone defects in New Zealand White (NZW) rabbits. Whether the ASCs persistently expressing BMP2/VEGF expedited the healing was assessed by X-ray, PET/CT, μCT, histochemical staining and biomechanical testing.

Results. We confirmed that within ASCs transduced with BacFLP and the hybrid BV, FLP/Frt-mediated recombination occurred in up to 46% of ASCs, leading to cassette excision off the BV genome, formation and persistence of episomal transgene and prolongation of expression to >28 days. Transduction of ASCs with the BMP2-expressing hybrid BV prolonged the BMP2

expression and augmented osteogenesis of ASCs even without osteogenic supplements. ASCs engineered by the hybrid vectors mediating sustained BMP2/VEGF expression healed the critical-size (10 mm) segmental bone defects in 12 out of 12 rabbits in 8 weeks, which remarkably outperform ASCs engineered with BV transiently expressing BMP2/VEGF with respect to healing rate, bone metabolism, bone volume, bone density, angiogenesis and mechanical properties.

Conclusion. These data attested our hypothesis that persistent BMP2/VEGF expression are essential when using ASCs for repairing massive defects. The use of ASCs engineered with the hybrid BV vector represents a novel therapy to treat massive segmental defects necessitating concerted ossification and vascularization.

Keywords. baculovirus, adipose-derived stem cells, segmental bone defect, sustained expression

(1.O8) EVALUATION OF DIFFERENT SCAFFOLD DESIGNS FOR VASCULARIZED ADIPOSE CONSTRUCTS

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Free fat grafts are frequently used in plastic and reconstructive surgery to treat large volume defects e.g. breast reconstruction. Current clinical limitations are however in larger defects which need vascularized fat grafts in order to improve the survival and in addition the need to provide a 3D predictable structure. Recently described innovative scaffold fabrication systems allow patient specific scaffold fabrication and thus engineering of customized fat grafts. We investigated Polycaprolactone (PCL) scaffolds made by fused deposition modeling and Polyurethane (PU) sponges made by solvent casting in a combined in vitro and in vivo study. Scaffolds were evaluated in respect to adipose tissue engineering.

Scaffold structure was analyzed with SEM and µCT. Scaffolds were then seeded with human adipoderived progenitor cells which were obtained from lipoaspirates. Cell seeded constructs were cultured in adipogenic culture media for 2 weeks and were analyzed biochemically and microscopically. Subsequently the constructs were implanted in nude mice for in vivo studies. Femoral artery and vein were dissected and placed upon constructs to mimic a vessel loop for vascularization. Constructs were explanted after 2 and 4 weeks and histologically processed. Adipoderived progenitor cells attached to both scaffolds and showed an increase ($p<0.05$) of metabolic activity in experimental groups. Formation of fat tissue was superior ($p<0.05$) in PU-scaffolds compared to PCL-scaffolds in vitro and in vivo. However vascularization of constructs was equal within all groups.

In conclusion both scaffold systems represent suitable carriers for adipose tissue formation in vitro and in vivo. The advantage of rapid prototyping technology allows production of customized vascularized grafts, which have great potential especially for breast reconstruction.

Keywords. polycaprolactone, polyurethane, vascularized adipose constructs, in vitro, in vivo

(1.O9) CARTILAGINOUS TISSUES ENGINEERED USING HUMAN FAT PAD DERIVED MESENCHYMAL STEM CELLS UNDER ALTERED DIFFERENTIATION CONDITIONS

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Introduction. Adult stem cells from adipose tissue can potentially be used in cell-based therapies for cartilage repair. Previous studies from our lab have shown that functional cartilage tissue can be engineered using porcine infrapatellar fat pad (FP) mesenchymal stem cells (MSCs). It remains unclear if functional tissue can be engineered using MSCs isolated from human osteoarthritic FP tissue. The objective of this study was to determine the influence of oxygen tension during expansion and supplementation conditions during differentiation on the functional properties of cartilage tissues engineered using human FP MSCs.

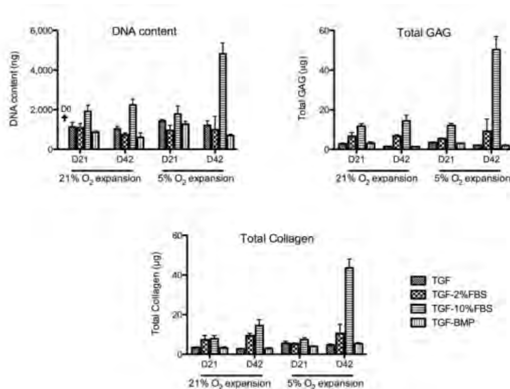
Materials and Methods. Human infrapatellar FP was harvested during total knee replacement following ethical approval. The isolated FP MSCs was expanded under 21% or 5% O2. After expansion to P2, MSCs was seeded in agarose at 10 million cells/ml. Cell pellets (250,000 cells/pellet) were also cultured as a control. Both cell-seeded agarose hydrogels and pellets were cultured at 5% pO2 with chondrogenic differentiation medium with different supplements: (1) TGF-β3 (10ng/ml), (2) TGF-β3+2% fetal bovine serum (FBS), (3) TGF+10% FBS or (4) TGF-β3+BMP-6 (10ng/ml). Constructs were analyzed using DNA, GAG, and collagen assays.

Results. DNA content within the agarose hydrogels increased with the supplement of TGF-β3+10%FBS, in contrast to the reduced DNA content in other groups. The addition of serum also promoted GAG and collagen accumulation. In contrast, in pellet culture, only small differences were observed between constructs supplemented with different factors (data not shown).

Conclusion. Robust chondrogenic differentiation of human FP derived MSCs was observed following agarose encapsulation for constructs supplemented with TGF-β3+10%FBS. FP MSCs appear to respond differently to media supplementation if cultured in hydrogels or pellets.

This work was supported by IRCSET / Sports Surgery Clinic (Dublin) Enterprise Partnership Scheme, Science Foundation Ireland and the European Research Council.

Keywords. human fat pad mesenchymal stem cells; chondrogenesis; differentiation conditions



(1.O10) ADIPOGENESIS IN 3D SPHEROIDS OF ADIPOSE-DERIVED STEM CELLS IS LESS DEPENDENT ON EXOGENOUS STIMULATION THAN IN CONVENTIONAL 2D CULTURE

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Introduction. 3-dimensional (3D) spheroids of human adipose-derived stem cells (hASC) have the potential to serve as building blocks for adipose tissue engineering. They also constitute an alternative model system for basic research, allowing investigations of cellular processes in a more in vivo-like context, representing cell-cell-interactions and the influence of the extracellular matrix more closely than conventional 2-dimensional (2D) culture.

Materials and Methods. Using hASC, a 3D carrier-free spheroid model of human adipose tissue was established utilizing the liquid overlay technique. Characterizing the 3D culture system, differences in the process of adipogenesis between 3D spheroids and 2D culture were addressed on a functional and molecular level by investigating lipid accumulation and gene expression (TaqMan® array).

Results. Applying short-term adipogenic induction (common hormonal cocktail for two days), a strong adipogenic response with a high lipid content on day 14 was observed in 3D spheroids, whereas lipid content was only minimal in 2D culture. Gene expression data reflected these results: In 2D culture, several genes associated with lipid synthesis and transport (FASN, ACLY, FATP1) were very weakly expressed, in contrast to high expression in 3D spheroids. Also other fat cell markers and adipokines (e.g., adiponectin, apelin, LPL) were more strongly expressed in 3D. Strikingly, already on day 2, increased expression of important transcription factors (PPARγ, C/EBPβ, SREBF1) was determined in 3D culture, which represents, at least in part, a likely explanation for the observed 2D/3D-differences at later time points. In 2D, further exogenous stimulation after day 2 was necessary to achieve significant adipogenic differentiation, while the 3D context provided conditions rendering further stimulation unnecessary.

Conclusion. Adipogenesis in the spheroid system proved to be less dependent on external stimulation than in conventional 2D culture. The characterization of the 3D spheroids provided valuable information for their use in adipose tissue engineering as well as in basic research.

Keywords. adipose tissue engineering; adipose-derived stem cells; adipogenesis; spheroids

(1.O11) 5-AMINOSALICYLIC ACID TO SUPPORT ADIPOGENIC DIFFERENTIATION OF ADIPOSE TISSUE DERIVED STEM CELLS IN 2 AND 3-D CULTURES

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Introduction. The aim of this study was to develop a suitable protocol for adipogenic differentiation of rat adipose tissue stem cells (ADSCs) and to investigate its use in different 3-D culture systems. This protocol involved standard supplements for adipogenic induction added for 4 days and supplementation of 5-aminosalicylic acid (5-ASA) with 2% FBS thereafter. Using indomethacin, rosiglitazone and celecoxib as alternative to 5-ASA, we tried to shed light on the mechanisms of 5-ASA. Three different scaffold systems, made from either PLGA or PCL served to find suitable 3-D systems for tissue development.

Materials and Methods. Proliferation: in DMEM high glucose, 10% FBS and 1% PenStrep with 3 ng/ml bFGF, seeding on scaffold systems: centrifugation method and proliferation for another 5 days with bFGF-supplemented medium. Induction: 4 days insulin, dexamethasone, IBMX and indomethacin in proliferation medium without bFGF. Maturation: unsupplemented basal medium (control) or medium containing 0.3 mM 5-ASA. Characterization of adipogenic development on different scaffolds: protein levels, glycerol-3-phosphate-dehydrogenase (GPDH) activity and triglyceride content. Staining of cells: DAPI, Nile Red and osmium tetroxide for light, fluorescence microscopy and SEM imaging after lyophilisation.

Results and Conclusion. The new protocol involving 5-ASA and reduced FBS in 2- and 3-D led to improved adipogenic differentiation compared with continuous supplementation of induction cocktail and control after only 8 days of adipogenic stimulation. Groups receiving 5-ASA or celecoxib differentiated better than groups treated with indomethacin or rosiglitazone, indicating COX-2 involvement in the adipogenic effect. ADSCs attached and proliferated well on all three investigated scaffold types. Differentiation, however, was weak on electrospun PCL fibers compared to PLGA scaffolds and microscaffolds with larger pore sizes. The results show that the new protocol promotes adipogenic differentiation of ADSCs on 3-D carriers in the presence of reduced FBS and 5-ASA. In contrast to standard protocols these conditions can be generated in vivo.

Keywords. bFGF, rat ADSC, polymer scaffolds, Mesalazin, 5-aminosalicylic acid

(1.O12) IN VITRO EVALUATION OF OSTEOCONDUCTIVE STARCH BASED SCAFFOLDS USING A FLOW PERFUSION BIOREACTOR

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Introduction. This works aims at studying the potential of SPCL wet-spun fiber-meshes functionalized with silanol groups as a bioactive matrix enabling highly tailored cellular environments and thus promoting osteogenic differentiation in human adipose stem cells (hASCs). Another point of interest in this work is to understand the influence of a dynamic culture, particularly using a flow perfusion bioreactor, in hASCS cultured onto the functionalized materials.

Materials and Methods. The functionalization of the materials was achieved by a one step methodology using a calcium silicate solution as a coagulation bath for fiber meshes production. After an optimization on the production procedure of the materials, some conditions were selected for biological assays. The influence of the presence of silicium ions in the material, along with a dynamic culturing, on the adhesion, differentiation and proliferation of hASCs was assessed.

Results. The functionalized materials exhibit the capacity to sustain cell proliferation and induce their differentiation into the osteogenic lineage. The formation of mineralization nodules was observed in cells cultured onto the functionalized materials. The culturing under dynamic conditions by using a flow perfusion bioreactor was shown to enhance hASCs proliferation and differentiation and a better distribution of cells within the material.

Conclusion. The promising properties of the functionalized materials along with a simple, economic and reliable production process demonstrate the potential of these materials as candidates for application in bone tissue engineering. The culture of stem cells onto these materials using a flow perfusion bioreactor reveals to be a good strategy to promote osteogenic differentiation.

This work was supported by the European NoE EXPERTISSUES (NMP3-CT-2004-500283) and by the Portuguese Foundation for Science and Technology, FCT, through the projects PTDC/CTM/67560/2006. I. B. Leonor thanks the Portuguese Foundation for Science and Technology (FCT) for providing her a post-doctoral scholarship (SFRH/BPD/26648/2006).

Keywords. Bone tissue engineering, adipose stem cells, silanol groups, instructive materials, wet-spinning, flow perfusion bioreactor.

(1.O13) CYCLIC UNIAXIAL STRAIN UPREGULATES THE SKELETAL MUSCLE-RELATED GENES IN ADIPOSE-DERIVED STEM CELLS

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Introduction. It has been revealed that skeletal muscles have the potential to generate and respond to biomechanical signals and that the mechanical force is one of the important factors that influence proliferation, differentiation, regeneration and homeostasis of skeletal muscle and myoblasts. The aim of our study was to illustrate the role of cyclical strain on myogenic differentiation of adipose-derived stem cells (ASCs).

Materials and Methods. we designed a study within three days with 3 groups: chemical, chemical-mechanical, mechanical on the basis of stimulation of ASCs with chemical factors (on the whole three days) or mechanical strain (just on the second day) and compared the relative expression of myogenic-related genes MyoD, Myogenin and myosin heavy chain 2 (MyHC2) with expression of the same genes in undifferentiated ASCs by Relative gene expression method.

Results. Real-time RT-PCR results demonstrated that uniaxial strain had a significant effect on up-regulation of muscle-related genes in chemical-mechanical group ($P<0.05$) compared to mechanical or chemical groups. Immunocytochemistry also confirmed the myogenic differential effect of cyclic strain on ASCs and showed that this also influenced ASCs morphology and their orientation.

Conclusion. These data suggest that uniaxial cyclic strain could possibly affect the myogenic differentiation of ASCs and cause the muscle-related genes to increase beyond their basal level in ASCs and that the combination of chemical myogenic approach with mechanical signals promote differentiation more than differentiation by chemical approach alone

Keywords. uniaxial cyclic strain, adipose-derived stem cells, skeletal myogenic differentiation

(1.O14) ENHANCED CARTILAGE FORMATION VIA THREE-DIMENSIONAL ENGINEERING OF HUMAN ADIPOSE-DERIVED STROMAL CELLS

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Introduction. Damaged articular cartilage has poor intrinsic regenerative capacity. Autologous chondrocyte transplantation is an effective treatment but involves surgical procedures which may cause further cartilage degeneration. Additionally, in vitro expansion of chondrocytes can result in dedifferentiation and phenotypic property loss. Human adipose-derived stem cells (hADSCs) are an alternative autologous cell source for cartilage regeneration due to their multipotency, relatively easy accessibility and expansion. In this study, we developed an efficient method for in vitro chondrogenic differentiation of hADSCs and in vivo cartilage formation of hADSCs and elucidated the mechanisms of the enhanced in vitro chondrogenesis.

Materials and Methods. In vitro chondrogenesis of hADSCs was promoted by culturing hADSCs in spheroid form in spinner flasks. As a control, hADSCs were cultured in monolayers in tissue culture dishes. Signaling cascades for chondrogenesis of hADSCs cultured with two different methods were examined. To evaluate in vivo cartilage forming ability of the cells, hADSCs cultured either in spheroid form or in monolayers were mixed with fibrin gel and implanted subcutaneously into athymic mice for four weeks.

Results. Polymerase chain reaction (PCR), quantitative real-time (qRT)-PCR, and immunohistochemistry indicated enhanced chondrogenic differentiation of

hADSCs cultured in spheroid forms versus those cultured in monolayers. The enhanced chondrogenesis is likely attributed to mild hypoxia-related cascades and enhanced cell-cell interactions of hADSC spheroids. The in vivo study showed enhanced cartilage formation by implantation of spheroid-cultured hADSCs versus monolayer-cultured hADSCs.

Conclusion. Spheroid culture in three-dimensional bioreactors is advantageous over monolayer culture for in vitro chondrogenic differentiation of hADSCs and subsequent in vivo cartilage formation.

This study was funded by grant (2010-0020352) from the National Research Foundation of Korea

Keywords. human adipose-derived stromal cells, spheroids, three dimensional culture, cartilage formation

(1.O15) RETRO-ASSOCIATED VIRAL GENE TRANSFER OF SOX-TRIO TO HUMAN BONE MARROW DERIVED MESENCHYMAL STEM CELLS IMPROVES CARTILAGE REPAIR

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Objective. The aim of this study was to test the hypotheses that retroviral gene transfer of SOX trio enhances the in vitro chondrogenic differentiation of ASCs, and that SOX trio-co-transduced ASCs promote the healing of osteochondral defects, and arrest the progression of surgically-induced osteoarthritis in a rat model.

Materials and Methods. ASCs isolated from inguinal fat in rats were transduced with SOX trio genes using retrovirus, and further cultured in vitro in pellets for 21 days, then analyzed for gene and protein expression of SOX trio and chondrogenic markers. Sox trio-co-transduced ASCs were implanted on the osteochondral defect created in the patellar groove of the distal femur, and also injected into the knee joints of rats with surgically-induced osteoarthritis. Rats were sacrificed after 8 weeks, and analyzed grossly and microscopically.

Results. After 21 days, ASCs transfected with a single gene of the SOX trio had a 140 to 320-fold greater gene expression of SOX-5, -6, or -9 compared with the control while ASCs co-transfected with SOX trio had 40 to 70-fold greater gene expression. The SOX protein expression paralleled that of gene expression. The GAG content increased approximately 6-fold with SOX trio co-transduction. SOX trio co-transduction significantly increased type II collagen gene and protein expression. SOX trio co-transduction significantly promoted cartilage healing in the in vivo osteochondral defect model, and prevented the progression of degenerative changes in surgically-induced osteoarthritis.

Conclusion. SOX trio co-transduction enhances chondrogenesis from ASCs. SOX trio-co-transduced ASCs promote healing of cartilage defects and arrest the progression of osteoarthritis.

This work was supported by a grant from the Korea Ministry of Education, Science and Technology (Grant No 2010-0000305).

Keywords. retrovirus

(1.O16) THE USE OF STEM CELL CULTURE-CONDITIONED MEDIUM FOR THERAPEUTIC ANGIOGENESIS

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Introduction. Stem cell implantation can be used to induce neovascularization and has been tested as a therapy for ischemia treatment. However, stem cell implantation as a therapy for ischemia treatment may have limitations for clinical applications. Since the methods of stem cell harvest are invasive, it may not be feasible to harvest autologous stem cells from aged patients or patients with cardiovascular risk. Furthermore, poor cell survival after engraftment in ischemic tissue may lower the therapeutic efficacy of stem cells. hADSCs implanted to ischemic tissues support tissue revascularization in large part through secreted angiogenic factors. The goal of this study is to demonstrate that medium collected from human adipose-derived stromal cells (hADSCs) cultured as spheroids can exhibit improved therapeutic efficacy for ischemia treatment.

Materials and Methods. Conditioned medium derived from hADSC monolayer culture (M-CM) or spheroid culture (S-CM), fresh medium (FM), or hADSCs were injected intramuscularly into the gracilis muscle in the medial thigh after mouse hindlimb ischemia modeling.

Results. Due to a mild hypoxic environment formed in hADSC spheroid, spheroid culture was effective to precondition the hADSCs to upregulate hypoxia-inducible factor-1α gene expression following significant enhancement in both angiogenic and anti-apoptotic factor secretion to the culture medium compared to monolayer cultures. S-CM administration to ischemic hindlimbs in mice significantly enhanced neovasclurization, protected muscles from incipient ischemic apoptosis, and improved limb survival as compared to M-CM or FM administration or hADSC implantation.

Conclusions. These data suggest that injection of conditioned medium obtained from hADSC spheroid culture may be more effective therapeutic option for treatment of ischemic diseases than hADSC implantation.

Keywords. angiogenesis, stem cell, conditioned medium

(1.O17) PHARMACOLOGICAL MODULATION OF MESENCHYMAL STEM CELL CHONDROGENESIS BY MARINE POLYSACCHARIDES FOR CARTILAGE TISSUE ENGINEERING

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Mesenchymal stem cells (MSC) are considered as an attractive source of cells for cartilage engineering owing to their availability, capacity of in vitro expansion and multipotency. Differentiation of MSC into chondrocytes is

crucial to successful cartilage regeneration and can be induced by a large variety of biological agents and environmental factors. Glycosaminoglycans (GAGs) are complex carbohydrates that participate in many biological processes through interaction with various proteins including growth factors. We hypothesize that growth factors-induced differentiation of mesenchymal stem cells could be potentiated by marine polysaccharides.

To test our hypothesis MSC were isolated from human adipose tissue obtained by liposuction. Human adipose tissue derived MSC (hATSC) were cultured three dimensionally in pellets in the presence of TGF-□ supplemented chondrogenic medium containing or not two marine polysaccharides analogs of low molecular weight (LMW1 and LMW2, patenting in progress). Chondrogenesis was monitored by the measurement of pellet volume and histological stainings (Alcian blue and hematoxylin) of the pellets. Our data revealed an increase in pellet volume as well as in total collagens and GAG production in the concomitant presence of LMW1 (and not LMW2) and chondrogenic medium. The enhanced hATSC chondrogenesis in response to LMW1 treatment was further demonstrated by the increased expression of COL2A1, ACAN, COMP and SOX9 by real time PCR. In addition, surface plasmon resonance (Biacore) analyses revealed that TGF-β1, but not insulin, binds LMW1 with higher affinity compared to LMW2 polysaccharide. Furthermore LMW1 marine polysaccharide was found to up-regulate the TGF-β dependent phosphorylation of ERK1/2, indicating that LMW1 marine polysaccharide enhanced the MAP kinase signaling activity of TGF-β. These results demonstrate the up-regulation of the TGF-beta-dependent stem cell chondrogenesis by a marine polysaccharide. Whether this data may help monitor and exploit the potential of MSC for cartilage regeneration would be paid further attention.

Keywords. adipose tissue derived stem cells, cartilage tissue engineering, marine polysaccharide, glycosaminoglycan-mimetic

(1.O18) HUMAN ADIPOSE DERIVED STROMAL CELL RESPONSE TO A POLY ε-CAPROLACTONE SCAFFOLD FOR BONE TISSUE ENGINEERING

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Introduction. Tissue engineering represents an interesting challenge to heal several bone lack. The adipose tissue, normally discarded during plastic surgery, has been demonstrated to be an alternative source of stromal cells. The aim of the present study was to evaluate the ability of porous poly(ε-caprolactone) (PCL) scaffold with novel bimodal-micron scale porous architecture (μ-bimodal PCL), to promote and guide the in vitro adhesion, proliferation and 3D colonization of human adipose derived stromal cells(hADSCs).

Materials and Methods. The μ-bimodal PCL scaffold was prepared by the combination of the gas foaming abd selective polymer extraction from co-continous blends techniques. Human ADSCs were enzimatically isolated from fat collected during lipectomy and their characterization was assessed by flow cytometry. Then, hADSCs were expanded and seeded on μ-bimodal PCL scaffolds with an osteogenic medium. Cell adhesion (SEM), proliferation (Pico Green) and viability (Alamar blue), osteoblast differentiation (ALP) and 3D scaffold colonization were assessed at 24h, 1 and 2 weeks. In particular, 3D scaffold colonization was evaluated by using SKYSCAN 1172 microtomographer (μCT).

Results. hADSC resulted positive for CD44, CD73, CD90 and CD105. After 24h, SEM showed that hADSC cell adhered and entirely colonized the seeded surface of PCL scaffold. Cell viability and proliferation increased significantly over experimental time. μCT showed that hADSCs uniformly colonized the entire thickness of the scaffold.

Conclusion. The μ-bimodal PCL/hADSCs interaction study showed the ability of the scaffold to support hADSCs adhesion and proliferation, as well as to promote and guide 3D cell colonization by appropriately designing the microarchitectural features of the scaffold. At the same time, the opportunity of using a novel, non-invasive method as μCT makes easier and more accurate the analysis of the construct in vitro, above all in respect to the cellular distribution.

Keywords. poly(ε-caprolactone), human adipose derived stromal cells

(1.P1) DEVELOPMENT OF A PROTOCOL FOR HUMAN ADIPOSE STEM CELL CULTURE IN CO2 INDEPENDENT MEDIUM IN PERFUSION BIOREACTOR

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Advances in research on stem cells derived from human adipose tissue (hASC) may allow its use for cell therapy and tissue engineering. In such context, it is important to standardize a methodology to culture cells in high quantity. Bioreactors, in which cells are cultured in three-dimensions and may use CO2 independent media, mimic the physiological environment in vitro, allowing the hASC proliferation, differentiation and maintenance. In addition to the cells and the cell culture medium, a suitable biomaterial is critical to the success of bone tissue regeneration. In this study, a sol-gel bioactive glass (BG) was the material of choice, due to its osteoinductive properties. The aim of the study was to evaluate phenotypic stability, proliferation, cell viability and protein secretion by hASC cultured in CO2 independent medium in three-dimensional cell culture in perfusion bioreactor. The hASC was isolated from human lipoaspirate and two-dimensional cell culture was performed in DMEM supplemented with 10% FBS. The cellular adaptation from DMEM to Leibovitz's CO2 independent medium supplemented with 10% FBS (Lei) was gradual, beginning in the first passage with 25%Lei, the second passage with 50%Lei, the third passage with 75%Lei and the fourth passage with 100%Lei. Phenotypic characterization was performed by flow cytometry analysis of the following markers: CD29, CD44, CD73, CD34, CD45, HLA-ABC and HLA-DR. Cell proliferation and viability in BG were evaluated by MTT assay. The undifferentiated state was assessed by Alkaline

Phosphatase Activity assay of cells cultured in two- and three-dimensions in Lei at 7, 14 and 21 days. Two-dimensional comparative tests were performed in DMEM as control. The results suggest that the Lei CO2 independent medium may be a promising model for in vitro expansion of hASC for use in perfusion bioreactor.

The authors gratefully acknowledge the financial support from CNPq and FAPEMIG/Brazil.

Keywords. hASC, Bioactive Glass, Bioreactor, CO2 Independent Medium

(1.P2) PIG MANDIBULAR RECONSTRUCTION BY ADIPOSE-DERIVED STEM CELLS AND FUNCTIONALIZED LASER-SINTERED POROUS PCL SCAFFOLD WITH PLATELET RICH PLASMA: IN-VITRO AND IN-VIVO STUDY

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Introduction. Polycaprolactone (PCL) is a bioresorbable polymer with potential applications for bone and cartilage repair. In this work, the three-dimentional and porous PCL scaffolds were designed and it was fabricated via selective laser sintering (SLS). The aim of this study is to evaluate the osteogenic potential of adipose-derived stem cells (ASCs) in functionalized laser-sintered PCL scaffold with platelet rich plasma (PRP).

Materials and Methods. In the in-vitro study, the laser–sintered PCL scaffold was seeded with ASCs. It was divided into three groups. Group I: PCL/ASCs were cultured in control medium. Group II: PCL/ASCs were cultured in osteogenic medium. Group III: PCL/PRP/ASCs were cultured in osteogenic medium. Alkaline phosphatase activity, RT-PCR of ALP, osteocalcin, RunX II were used to assess the osteogenic ability. SEM and confocal microscope were used to observe the interaction between scaffold and cell. In In-vivo study, the 3 cm porcine mandible defect was created and it was reconstructed with either PCL only or PCL/PRP/ASCs. CT was used to evaluate the bone regeneration 3 months, 6 months after operation. The Young's modulus of both groups was measured and compared with normal bone. H&E stain and IHC stain of osteocalcin, collagen type I were done for confirmation of bone regeneration.

Results. In in-vitro study, alkaline phosphatase activity and RT-PCR all showed the best osteogenic potential in group III(PCL/PRP/ASCs) comparing with other groups. SEM and confocal microscope showed the cells were well attached to PCL in group III. All these data confirms that the PCL combined with PRP was suitable for osteogenic differentiation and attachment of ASCs. In in-vivo study, both groups showed new bone regeneration in PCL scaffold. However, the bone density was less and loose in PCL group and the Young's modulus was only 30% of normal bone. In contrast, the continual and firm bone formation was found in PCL/PRP/ ASCs gorup and the Young's modulus was 90% of normal bone. H&E stain, IHC of osteocalcin, collagen type I all proved the new generation tissue was bone.

Conclusion. In conclusions, modification of the laser-sintered PCL scaffold by PRP enhances the affinity and osteogenic potential of ASCs.

Keywords. selective laser sintering, Polycaprolactone, platelet rich plasma, adipose derived stem cell

(1.P3) INFLUENCE OF SUBSTRATE'S RIGIDITY ON ADIPOSE DERIVED STEM CELLS DIFFERENTIATION

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Introduction. In 2006 intriguing data published in Cell showed the significance of the stiffness of cell support for stem cell differentiation. Since then, a few new reports have revealed the influence of substrate rigidity on cell morphology, motility or viability, however no new data on cell differentiation are available. Since it might have a practical implications for tissue engineering, in this work we analyzed the response of human adipose derived stem cells (HASCs) toward substrate elasticity with particular attention paid to osteogenic differentiation.

Materials and Methods. HASCs from three donors (each population in a separate experiment) were observed in a culture for 14 and 21 days. Inert polyacrylamide gels (PAAM) of two different rigidities (Young Modulus: 2,6kPa and 28,1kPa) served as a support. Cell adhesion was enhanced by coating the gels and control wells (TCP-Tissue Culture Plate) with collagen I. Both cell viability (XTT-assay) and cell number (DNA determined in PicoGreen) were assessed. Differentiation potential was determined by measuring alkaline phosphatase (ALP) activity and expression of ALP and RUNX2, as osteogenic differentiation markers (real-time PCR).

Results. The support had no significant influence on cell number or viability . HASCs differentiation confirmed by RUNX2 and ALP expression was detected/observed in all groups. RUNX2 expression was higher (1,5 fold) on more rigid substrates as compared to the softer ones, either on day 14 or 21 depending on the donor. ALP expression was significantly higher on more rigid gels in all groups both on 14 and 21 day (1,2-1,5 fold). This was accompanied by the enhanced ALP activity on day 14 (1,9-5,3 fold), but not on day 21.

Conclusion. We propose that HASCs, as cells with a high rate of stemness are sensitive to the rigidity of the support. The osteogenic differentiation is more advanced on stiffer substrates.

Keywords. Adipose derived stem cells, elasticity, differentiation

(1.P4) ISOLATION AND CHARACTERIZATION OF MESENCHYMAL STEM CELLS FROM THE FAT LAYER ON THE DENSITY GRADIENT SEPARATED BONE MARROW

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Introduction. Bone marrow (BM) is considered the most reliable source of adults MSCs. From this tissue MSCs can be isolated after density gradient separation (ficoll) and

culturing the mononuclear cell fraction held in the plasma-solution inter-phase at a density between 1.053 and 1.077, which is traditionally considered the only source of progenitor cells (hematopoietic and non-hematopoietic). In this study we presented evidences that MSCs could be also isolated from the very low-density cells of the fat layer, normally discarded.

Material and Methods. BM aspirates were collected from nine volunteers (6 males, 3 females, median age 26 years old, range 6 to 45), after informed consent according to the Hospital Ethic Committee. Samples were separated in different fractions by ficoll density gradient method. MNCs obtained from the plasma-solution inter-phase and the very low density cells of the fat layer were collected, counted and comparatively evaluated in primary cultures, proliferation assays, ex vivo expansions, colony-forming units-fibroblast tests, fluorescence activated cell sorting analysis, and in vitro cell differentiation assays

Results. Cells coming from fat layer exhibited similar proliferation characteristics than cells from the plasma-solution inter-phase. Colony-forming units-fibroblast assays revealed similar efficiency. Proliferation rates of MSCs from both sources were similar and so were the ex-vivo expansions. Immunophenotypical characterization of MSCs showed similar antigens pattern. In vitro MSCs differentiation potential was similar in both source cells.

Conclusion. MSCs could be isolated from the very low-density cells of the fat layer as from the MNCs at plasma-solution inter-phase. MSCs obtained from these cells have similar characters than those obtained from the MNCs at plasma-solution inter-phase. The method represents a simple and cost effective way to increase the MSCs yield from each BM donor. These cells might serve as a complementary source of MSCs to facilitate preclinical and clinical application in tissue engineering and cell therapy.

Keywords. Mesenchymal Stem cells (MSCs), fat floating cells, bone marrow, cell therapy, density gradient method

(1.P5) OSTEOGENIC TISSUE ENGINEERING BY ADIPOSE TISSUE-DERIVED STEM CELLS IN VITRO

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Introduction. Adipose tissue-derived stem cells (ASC) are able to differentiate along the osteogenic lineage, among others. Since adipose tissue is an abundant source of stem cells, tissue engineering approaches based on the utilization of ASC are under development. In this study we have examined in which way osteogenic differentiation of ASC is affected by the supplementation with different osteogenic factors and by 2- and 3-dimensional growth.

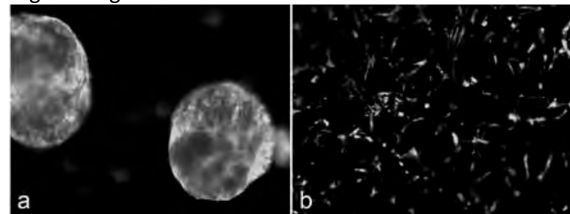
Materials and Methods. ASC cultivation was with DMEM, 10% FCS and antibiotics (basis medium, unstimulated/US). Osteogenic stimulation was with basis medium supplemented with dexamethasone, ascorbic acid, dinatriumglycerol-2-phosphate (OS) and/or BMP2. Cultivation took place on tissue culture polystyrene (TCPS) for 2D or type I collagen scaffolds for 3D evaluation.

Results. Osteogenically stimulated ASC showed an increase in cell number. In contrast, stimulation of ASC with BMP2 led to a reduction. Addition of osteogenic stimuli did not neutralize the effects of BMP2 on cell number. Adhesion of ASC on TCPS and stimulation with BMP2 induced spheroid formation (Fig.1a). On collagen scaffolds, however, ASC developed a spindle-shaped phenotype and no spheroid formation (Fig.1b). As measured by alkaline phosphatase activity and extent of mineralization, collagen scaffolds led to a higher cell number and a higher degree of osteogenic differentiation than TCPS. Fig. 1: BMP2-stimulated ASC on a) TCPS and b) collagen scaffold (vital stain).

Conclusion. Thus, both ways of stimulation, i.e. dexamethasone- and BMP2-based, affect cell proliferation and osteogenic differentiation. However, the way of stimulation greatly changed other parameters of cellular behavior. Growth on collagen scaffolds led to a higher degree of osteogenic differentiation. Since stem cells from adipose tissue are promising candidates for tissue engineering approaches, further studies on the mechanisms and reliability of osteogenic differentiation of ASC are necessary.

This work was supported by the BMBF and the Federal State of Mecklenburg-Vorpommern.

Keywords. adipose tissue-derived stem cells, BMP2, collagen scaffold, osteogenic differentiation, tissue engineering



(1.P6) CHONDROGENIC DIFFERENTIATION OF HUMAN ADIPOSE TISSUE-DERIVED STEM CELLS ON GELATIN-BASED HYDROGELS IN VITRO

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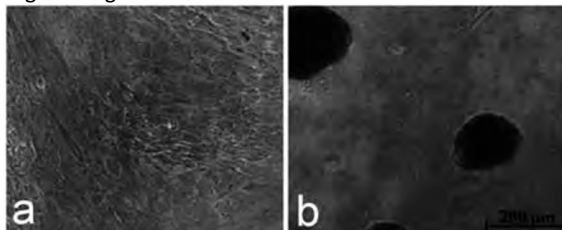
Introduction. Aim of this work was to characterize the chondrogenic potential of human mesenchymal stem cells from adipose tissue (ASC) in dependence on adhesion to tissue culture polystyrene (TCPS) and gelatin type B-based hydrogels. Thus, applicability of ASC for chondrogenic tissue engineering approaches was examined.

Materials and Methods. ASC were seeded in DMEM (10 % FCS) on 2.5 mm thick hydrogel films using methacrylamide-modified gelatin type B (10w/v%) and on TCPS. Chondrogenic stimulation was with ascorbic acid, rhIGF-I, TGF-β1, ITS™, Dexamethasone and antibiotics in serum-free medium. Gene expression of SOX5, SOX9, COLI, and COLII was determined by real time PCR. Proof of glycosaminoglycan synthesis was done by Alcian blue staining.

Results and conclusion. ASC phenotypes differed clearly in dependency on the adhesion substrate: chondrogenically stimulated ASC on TCPS showed a cobblestone-like phenotype (Fig. 1a), whereas chondrogenically stimulated ASC on hydrogels developed spheroidal growth (Fig. 1b). Glycosaminoglycan synthesis, a cartilage characteristic, was detected within the hydrogel-induced spheroids. Chondrogenically stimulated ASC on TCPS, however, were almost negative for glycosaminoglycans. Figure 1: Chondrogenically stimulated ASC on a) TCPS and b) hydrogels. Furthermore, chondrogenic differentiation capacity of ASC was examined by characterisation of cartilage-specific gene expression. Therefore expression of chondrogenesis-regulating factors was quantified. After chondrogenic stimulation SOX5 was generally upregulated compared to unstimulated ASC. The adhesion substrate (i.e. TCPS and hydrogel) did not alter SOX5 gene expression. Expression of COLI was not clearly regulated by chondrogenic differentiation and adhesion substrate, whereas expression of COLII was significantly increased by chondrogenic stimulation of ASC in contact to hydrogels. These results indicate chondrogenic differentiation capacity of ASC, which can clearly be increased by contact to gelatin type B-based hydrogels.

This work was financially supported by the Federal State of Mecklenburg-Vorpommern, the research funding FORUN of the Medical Faculty, University Rostock and the Research Foundation - Flanders.

Keywords. adipose tissue-derived stem cells, gelatin-based hydrogel, chondrogenic differentiation, tissue engineering



(1.P7) COMPARATIVE CHONDROGENIC PROFILE OF RABBIT AND HUMAN ADIPOSE MESSENCHYMAL STEM CELLS

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Introduction. Articular cartilage injuries compromise the quality of life for more than 30 million people per year. Articular cartilage is unable to initiate a spontaneous and efficient repair response when injured. Nowadays, there are three strategies in regenerative medicine for cartilage repair: implantation of chondrocytes (ACI), chondrocytes seeded in a matrix (MACI) or the application of three-dimensional hydrogels containing cells. These strategies use almost exclusively chondrocytes, but in most cases results are not very encouraging because: 1) new cartilage formed is mostly fibrocartilage; and 2) obtaining large amounts of autologous chondrocytes is extremely difficult. The use of mesenchymal stem cells (MSCs) is a promising alternative. To create a new product for articular cartilage regeneration based on pre-differentiated adipose MSCs (AMSCs), it is very important to know the chondrogenic process in the human and in the animal specie selected for the in vivo studies. Here we compare the chondrogenic profile from human and rabbit AMSCs. Objectives: To ascertain that the population of cells from rabbit adipose tissue are MSCs and compare the differentiation stage of hAMSCs with rabbit AMSCs (rAMSCs). To define the optimal time for the differentiation of human AMSCs (hAMSCs) in which these cells not only present the characteristics of the chondral linage, but also mantain their proliferative capacity.

Results. Cells obtained from rabbit adipose tissue are MSCs, as they could be differentiated to bone, cartilage and adipose tissue. Both, hAMSCs and rAMSCs show similar phenotypic and genotypic characteristics of the chondral linage at day four of differentiation. Moreover, in this stage AMSCs are able to proliferate and form colonies.

Conclusion. An optimal expression of chondrogenic markers is obtained after 4 days of differentiation of hAMSCs. Furthermore, in this stage rAMSCs show similar characteristics as hAMSCs, making rabbit a good species to evaluate the effectiveness of the product.

Keywords. Adipose stem cells, rabbit, cartilage regeneration

(1.P8) A COMPARISON BETWEEN HUMAN AND SHEEP ADIPOSE MESENCHYMAL STEM CELLS' PROFILE

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Introduction. Pseudarthrosis or non-unions are severe complications in orthopaedic trauma care and occur in 10% of all fractures. Non-union is a pathological process that happens when the fractured ends of a bone are covered by fibrocartilage and therefore bone consolidation never occurs. The optimal approach is to combine an osteoinductor (cells or growth factors), with an osteoconductor (bone graft substitute). In this sense, we are developing a new autologous product which combines mesenchymal stem cells from adipose tissue (hASC), easily obtained from the patient by liposuction, with a bone graft substitute. The potency of hASC for bone regeneration is well established. However, to progress towards human clinical trials, in vivo experiments are required, being sheep a convenient large-animal model. Hence, the aim of this work is to ascertain that the obtained cells from sheep's adipose tissue are ASC and to draw a comparison of the differentiation potentials and stages between hASC and sheep ASC (sASC).

Materials and Methods. Cells are extracted from adipose tissue of sheep, by surgical procedures, and human volunteers by means of lipoaspiration. The obtained cells populations of both species are assayed for: cell-doubling time, colony forming units' potential (CFUs), immunophenotypical characterization by flow cytometry, and differentiation potential to bone, cartilage and adipose tissue.

Results. Cells obtained from sheep adipose tissue are ASC, since they have the potential to differentiate into the three mesodermal lineages: bone, cartilage and adipose tissue. Despite the phenotypic similarities

between them, the higher proliferative rate that sASC present appears to accelerate the differentiation processes.

Conclusion. The cells obtained from subcutaneous adipose tissue can be considered sASC. Therefore, they could be a good model for efficacy phases of advanced therapy medicinal products containing ASC. However, the differences in proliferative rate and differentiation potential in relation to hASC must be taking into account.

Keywords. Sheep, stem cells, bone regeneration

(1.P9) AUTOLOGOUS ADIPOSE MESENCHYMAL CELLS IN A CAVITARY MANDIBLE DEFECT PROMOTE BONE REPAIR.

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Introduction. The mesenchymal stem cells from subcutaneous adipose tissue (MSCat) are an undifferentiated population which can be easily isolated and expanded in vitro. The MSCat have high potential to differentiate to other lineages. Our objective was demonstrated that MSCat implanted in a defect in rat mandible can be improving the regeneration bone.

Materials and Methods. MSCat were obtained from subcutaneous adipose tissue (N=16) to apply in cell therapy in the Wistar rat model of mandible repair. 106 autologous MSCat fluorchrome PK26 markers were implanted. After 7, 14, 21 and 30 days the bone reparation were studied in histological, immunohistochemical (RunX2, Osteocalcin(OC), TRAP and ED-1) and radiologic analysis.

Results. In a control group the bone reparation was a deficient in all groups of study, reaching 15% to 30 days. In MSCat group the defect was filled in 30% to 30 days this regeneration was evident with a PKH26 marker fluorchrome in repair site. The RunX2 expression was observed maintenance in all times in MSCat group. OC expression was observed only in MSCat group and increase over time. TRAP and ED-1 only was expressed around defect at 7 days in MSCat group.

Conclusion. Mandible defects are susceptible to improve after cell therapy. After mesenchymal cells implantation, the bone regeneration was two folder in the treated vs. control group.

This work has been supported by CAM (S-0505/MAT/0324) and CAM (S-2009/MAT/1472)

Keywords. Cell therapy, Mesenchymal stem cells from adipose tissue, osteogenic regeneration and mandible repair.

(1.P10) HUMAN MESENCHYMAL STEM CELLS CHEMOTAXIS ASSAY UNDER A GRADIENT OF INFLAMMATORY CYTOKINES

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Introduction. The ability of mesenchymal stem cells (MSCs) to repair tissue damage is related to antiapoptotic and trophic effects mediated by MSC-derived soluble factors. MSCs exert immunosuppressive activities by suppressing T- and B-cell proliferation, dampening the generation of mature myeloid dendritic cells, and inhibiting the proliferation, cytokine production and cytotoxic activity of natural killer cells. MSCs express various chemokine receptors supporting chemokine induced migration and display the ability to preferentially home to sites of anatomic lesions. Their immunomodulatory properties, together withtheir tissue-trophic properties, make MSCs good candidates to treat autoimmune diseases, like reumatoid arthritis. Different preclinical models of autoimmune diseases clearly demonstrate the beneficial effects of MSCs on injured tissues by inhibiting immune inflammation and promoting tissue repair.

Materials and Methods. In this study, we have performed a chemotaxis assay adapted for adherent cells, using the Ibidi system “µ-Slide Chemotaxis” in colaboration with the Microscopy and Image Unit of the Instituto Aragonés de Ciencias de la Salud. We have used the Multidimensional Microscopy System with Real Time Control Leica AF6000 XL, which made possible to study in vitro and directly the response and migration of the MSCs under the effect of different cytokines. Two large volume reservoirs are connected by a thin slit, where the MSCs are seeded. The reservoirs contain different chemoattractant concentrations, generating by diffusion a linear and stable concentration profile through the connecting slit. The data have been analyzed with the internet image analysis software Image J, complemented with a migration plugging. We have studied the behaviour of human MSCs isolated from tissue samples collected during surgical operations, which were cultured under a gradient of several cytokines involucrated in inflammatory processes: IL-1β, IL-6 and TNF-α.

Results and Conclusion. We have observed slight differences in the behaviour of the MSCs under the different cytokine gradients, which could help to explain MSCs immunomodulatory properties.

Keywords. mesenchymal stem cells, immunomodulation, inflammation

(1.P11) STUDY OF THE IN VITRO CYTOKINE SECRETION PATTERN OF HUMAN MESENCHYMAL STEM CELLS DERIVED FROM DIFFERENT TISSUES

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Introduction. Cartilage is among the tissues with the highest prevalence of aging-associated pathologies, in part due to the fact that adult articular cartilage has limited regenerative and reparative capacities. Different strategies have been drawn up to deal with this problem, and amongst them the use of mesenchymal stem cells (MSCs) stands out as a good alternative. MSCs are multipotent cells capable of differentiate into several mesoderm lineages, cartilage among them. They have been isolated from different tissues such as bone marrow, adult peripheral blood, umbilical cord blood, synovial liquid and adipose tissue. Besides, MSCs are immuneprivileged and display immunomodulatory capacities, which make MSCs good candidates to treat autoimmune diseases, like reumatoid arthritis. They are also capable of secreting several bioactive molecules, which include cytokines and growth factors. The expression of some adhesion molecules could be important to explain their homing capacity in different organs. The aim of this study was to measure the cytokine concentration in the supernatants of human MSCs cultures that had been derived from different tissues, and to asses whether there was any difference that could be due to their different tissue of origin.

Materials and Methods. Tissue samples were collected from 18 human patients during surgical operations. The samples corresponded to knee Hoffa's fat, subcutaneous fat from hip or knee, bone marrow and synovial liquid. MSCs were harvested by mechanic and enzymatic digestion, and separated by centrifugation. They were cultured in an expansion medium at 37°C under a 5% CO_2 humid atmosphere. The measurement of the different cytokine levels was performed with the xMAP® technology of Luminex® Corporation, using the Milliplex™ MAP kit (Millipore).

Results and Conclusion. Human MSCs, independently of their tissue of origin, secrete mainly IL-6, a pro-inflammatory cytokine, although their secretion does not inhibite the in vitro differentiation of these cells.

Keywords. human mesenchymal stem cells, chondrogenic differentiation, cartilage

(1.P12) IMMUNOMODULATORY PROPERTIES OF HUMAN MESENCHYMAL STEM CELLS ISOLATED FROM DIFFERENT TISSUES

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Introduction. Mesenchymal stem cells (MSCs), or more accurately multipotent mesenchymal stromal cells, are multipotent cells capable of differentiating into several mesoderm lineages, (bone, cartilage, muscle, adipose tissue), and even of transdifferentiating into ectoderm (neurons) and endoderm (lung epitelium) lineages. They represent a useful model in the clinical approaches to a great number of diseases, both in regenerative therapy and in gene therapy. Beside these features, MSCs are immuneprivileged and display immunomodulatory capacities, which together with their tissue-trophic properties, make MSCs good candidates to treat autoimmune disorders. Our group has focused in the study of osteomuscular diseases, reumatoid arthritis among them, for more than nine years.The aim of this study was to evaluate the immunomodulatory capacity of human MSCs harvested from five different tissues: bone marrow, adipose tissue from two different anatomic locations (sucutaneous and intraarticular), synovial liquid and cartilage, and to asses whether the diverse tissue of origin was of any significance for their immunomodulatory properties. We have also investigated the immunomodulatory effects of MSCs both on freshly activated lymphocytes and on long-term activated ones.

Materials and Methods. The different tissue samples were collected from 6 human patients during surgical operations. MSCs were harvested by mechanic and enzymatic digestion, and separated by centrifugation. They were cultured in an expansion medium at 37ºC under a 5% CO_2 humid atmosphere. PBMCs were isolated from blood samples from healthy individuals by density sedimentation on Ficoll-Histopaque gradients. The cocultures were maintained for 4-5 days in 6-well, 12-well or 96-well transwell plates. Lymphocyte proliferation was measured by either MTT assay or flow cytometry (5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-staining).

Results and Conclusion. MSCs differentialy suppressed human PHA-activated-T-cell proliferation, and this inhibition was dependent on the time T-cells were maintained activated before the coculture and on the presence or not in the culture medium of IL-2.

Keywords. mesenchymal stem cells, immunomodulation, chondrogenic differentiation

(1.P13) RAT MODEL FOR ADIPOSE DERIVED STEM CELLS THERAPY

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To avoid transplant rejections from an individual to another, the new tissue engineering field is developing techniques and biological substitutes that re-establish, maintain or improve the damaged tissue function. To achieve this goal, cells, three dimensional biocompatible scaffolds and tissue inductor substances has been investigated to produce the desired tissue in vivo. Adipose derived stem cells (ADSC) have gained in the last years a special relevance in the tissue engineering field because of their plasticity properties.

The aim of this work was to study the behaviour of rat multipotent cells from abdominal and inguinal adipose tissue in different culture conditions. To assess the changes occurred in culture we used optical microscopy, electronic microscopy and flow cytometry.

We observed that cells obtained from rat abdominal and inguinal fat expressed stem cell markers (CD29 and CD73), and had the potential to differentiate in adipocytes, chondrocytes and neurons. The ADSC adhered well to agar, collagen type I and poliglycolic acid, revealing potential for its future use in tissue engineering. We conclude that rat is a good animal model for assessing the adipose derived stem cells potential to differentiate and form tissues in vivo.

(1.P14) AGE-ASSOCIATED IMPAIRMENT OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS ANGIOGENIC PROPERTIES

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Introduction. Tissue regeneration is impaired in aged individuals. Adipose-derived mesenchymal stem cells (ADSCs) are promising source for cell therapy. ADSCs secrete many angiogenic factors and improve vascularization of ischemic tissues. However therapeutic benefit of autologous ADSCs from aged patients could be modest, because of their impaired functions. Here we analysed how donor age affects angiogenic properties of ADSCs.

Materials and Methods. ADSCs were isolated from subcutaneous and pericardial fat obtained from 30 patients during the coronary artery bypass surgery and cultured for 2-3 passages. Expression and secretion of angiogenic factors were measured as well as ability of ADSCs conditioned media to stimulate tube formation by endothelial cell.

Results. ADSCs from "young" (mean age 46,6±3,3 years, n=7) and "elderly" (63,8±7,0 years, n=23) individuals had CD90+/CD73+/CD105+/CD45-/CD31- immunophenotype and percentage of these cells was similar in both groups. mRNA levels of VEGF and PlGF were lower and content of HGF mRNA was higher in cells from elderly patients. In contrast to mRNA, VEGF level was 2,7-fold higher in conditioned media of ADSCs from aged donors. HGF level didn't differ between age groups. Total tube length formed by EA.hy926 cells in the presence of ADSCs conditioned media inversely correlated with donor age (r = -0,64, p=0,008). Blocking of VEGF by neutralizing antibodies inhibited tube formation up to 50%.

Conclusion. ADSC angiogenic properties decline with donor age. This at least partially explains why autologous ADSC from aged patients have an impaired therapeutic potential.

The study was supported by Russian Federal Agency of Science and Innovation (grant #02.527.11.0007).
Keywords. adipose-derived mesenchymal stromal cells, aging, angiogenesis

(1.P15) CALCIUM PHOSPHATES BONE SUBSTITUTES PROMOTED DIFFERENT OSTEOGENIC DIFFERENTIATION PROFILE OF HUMAN ADIPOSE DERIVED STEM CELLS

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Introduction. Human adipose derived stem cells and biomaterials are two fundamentals key in bone tissue engineering and regenerative medicine. This study aims to compare biocompatibility and osteogenic differentiation of human adipose derived stem cells (ADSCs) seeded on different calcium phosphates bone substitutes.

Materials and Methods. 1x105 ADSCs cultured with proliferative (PM) or osteogenic medium (OM) were seeded on 0.5g of Bio-Oss® (Geistlich, Switzerland), Bone Ceramic® (Strauman, Switzerland), Cerasorb® (Curasan, Germany), or KeraOss (Keramat, Spain) granules with protein coating. Cell adhesion and viability were detected by Alamar blue assay at 0,7,14 and 21 days. Cell morphology was observed by SEM. Osteogenic differentiation was evaluated by ALP assay kit and Real-time PCR to quantify gene expression of alkaline phosphatase (ALP), osteonectin (ON) and osteocalcin (OC).

Results. the highest percentage of adherent ADSC (76%) was found in KeraOss with protein coating and lowest (45%) in Bio-Oss. Cell´s number attached to the different scaffolds increased on the time for PM and OM, meanwhile this response was not expressed on BioOss. Different grown profiles were showed for each kind of scaffolds. These results were confirmed by SEM. The results revealed KeraOss granules are able to induce the highest ALP activity of all. On the other hand the Real Time PCR assays showed overexpression of ALP and OC at 14 days on Bone Ceramic and Cerasorb. In addition at 21d ALP, OC and ON were upregulated on KeraOss.

Conclusion. ADSC can attach and grown on all kinds of bone substitutes with exception of Bone Ceramic. These scaffolds induce the osteoblastic differentiation of ADSC in PM however is more intense in OM. This effect increase with protein coating. Depending on the material, the ALP activity and expression level of osteogenic markers changed. These results suggest that the greatest biocompatibility and differentiation is presented on KeraOss and Bone Ceramic granules.

Keywords. ADSC, scaffolds, osteogenic differentiation

(1.P16) SHOULD THE INFLUENCE OF PATHOLOGICAL OBESITY BE CONSIDERED WHEN USING hASCs FOR TISSUE ENGINEERING APPLICATIONS?

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Introduction. Some pathological condition, like obesity, may influence the features of human adipose-derived mesenchymal stem cells (hASCs). Indeed, adipose tissue of obese patients shows a reduced pressure of oxygen, involved in the up-regulation of pro-inflammatory genes that could affect the properties of these cells.

Materials and Methods. We have isolated hASCs from subcutaneous adipose tissue of normal-weight donors (nS-hASCs, n=5, mean age 33±6 years, BMI=24±2) and from pathological obese donors (ObS-hASCs, n=5, mean age 43±10 years, mean BMI=43±5). We have also collected visceral adipose tissue from the obese patients (ObV-hASCs, n=5) in order to evaluate possible

differences in the expression of the cell inflammatory phenotype. We have characterized hASCs clonogenicity, immunophenotype and osteogenic potential. We have also evaluated the effects of hypoxic treatment on obese hASCs cells.

Results. The clonogenic potential of cell populations, is strongly lower in ObS-hASCs than in normal-weight patients (-51%), and it is greater in subcutaneous than in omental tissue among obese patients (+142%). ObS-hASCs show a significantly higher doubling time in comparison to nS-hASCs (+40%); moreover ObV-hASCs doubling time is higher than its corresponding subcutaneous cells (+29%). From the immunophenotipic point of view, the expression of CD54, CD90 and CD166 is significantly reduced in ObS-hASCs respect to normal-weight patients. The osteogenic potential of hASCs is also affected by obesity: indeed, a significant reduction in the alkaline phosphatase activity and calcified extracellular matrix deposition was observed. Preliminary data suggest that both ObS- and OBV-hASCs are responsive to hypoxic treatment resulting in the activation of pro-inflammatory genes.

Conclusion. The pathological obesity negatively affects the self-maintaining and differentiation ability of hASCs, probably due to the inflammatory state related to this conditions. Our data suggest that some pathological condition should be considered before proposing the use of hASCs in tissue engineering applications.

Keywords. adipose stem cells; pathological obesity; differentiative ability

(1.P17) STUDY OF PORCINE ADIPOSE-DERIVED STEM CELLS FOR TISSUE ENGINEERING

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Introduction. Since adipose-derived stem cells (ASCs) may represent a promising approach for osteochondral defects treatment, we have characterized the osteogenic and chondrogenic potential of pASCs (pig-ASCs) in comparison to hASCs (human-ASCs).

Materials and Methods. We have isolated ASCs from caudal pig adipose tissue and from patients undergone aesthetic liposuction under informed consensus. We have analyzed ASCs clonogenicity, proliferation, osteogenic and chondrogenic potential. Moreover, we have also evaluated the osteogenic ability of biocompatible materials.

Results. pASCs proliferate faster than human cells with a doubling time of 54h and 126h, respectively. ASCs of both sources are highly clonogenic with about 15% of colonies formation until the passage 4. On polystirene, both osteogenic differentiated pASCs and hASCs show an increased alkaline phosphatase (ALP) activity of about 100% respect to undifferentiated cells, even if the ALP basal levels were 10-fold higher in hASCs respect to pASCs. The presence of scaffolds seems to significantly increase ALP level both for undifferentiated and differentiated pASCs and hASCs. We have also observed a synergistic effect produced by scaffold plus osteogenic stimuli, supporting the future clinical applications of ASCs bioconstructs. Moreover, both chondrogenic differentiated pASCs and hASCs, aggregated into micromasses, express an abundant amount of GAGs showing a significant increase in comparison to undifferentiated cells.

Conclusion. We show that pASCs and hASCs share common features and possess a similar differentiative ability, supporting the idea that the pre-clinical autologous ASCs reimplantation model in pig might be predictive of the behaviour of ASCs in a future clinical model of regenerative medicine.

Keywords. porcine adipose-derived stem cells, biocompatible scaffols, osteogenic differentiation, chondrogenic differentiation

(1.P18) HUMAN ADIPOSE DERIVED STEM CELLS RETAIN THEIR CHONDROGENIC POTENTIAL DURING EXPANSION WITH HUMAN PLATELET LYSATE

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Introduction. Fetal calf serum (FCS) bears a potential risk for carrying diseases and eliciting immune reactions. Nevertheless, it still represents the gold standard as medium supplement in cell culture.

Materials and Methods. In the present study human platelet lysate (hPL) has been tested as an alternative to FCS for the expansion and subsequent chondrogenic differentiation of human adipose derived stem cells (ASC). ASC were isolated from liposuction material of 8 donors and expanded up to passage 3 with 10% FCS (group 1) or 5% hPL (group 2). Subsequently, three dimensional micromass pellets were created and cultured for 5 weeks in chondrogenic differentiation medium without hPL or FCS but supplemented with 10 ng/mL bFGF and 10 ng/mL TGF-β3. In order to evaluate the effect of hPL on chondrogenesis during cell condensation, micromass pellets of group 2 were additionally treated with 5% hPL within the initial 3 days of micromass pellet culture (group 3).

Results. Growth curves revealed that medium supplementation with hPL strongly increases cell proliferation. Chondrogenic differentiation has been evaluated by qRT-PCR, glycosaminoglycan (GAG) quantification and histological staining. Ten cartilage related markers (COL2A1, COL1A1, SOX9, COL9A2, COL10A1, AGC1, CSPG2, MIA, COMP, CRTL1) were evaluated with qRT-PCR and demonstrated chondrogenic differentiation of both, hPL and FCS expanded ASC. GAG quantification did not reveal significant differences between the three groups, although hPL expanded cells tended to express higher levels of GAG. Histologically, collagen type II and GAGs could also be detected in all groups.

Conclusion. The present study demonstrates that hPL strongly induces proliferation of ASC while retaining the chondrogenic differentiation potential, suggesting that hPL is equal or superior to FCS as supplement for the expansion of ASC particularly with regard to chondrogenic differentiation.

The authors wish to express their thanks to Tamara Jagersberger and Mag. Christa Hackl for excellent technical assistance
Keywords. Human Platelet Lysate, PRP, Cartilage

(1.P19) HUMAN ADIPOSE EXTRACELLULAR MATRIX SUPPORTS CHONDROGENIC DIFFERENTIATION OF ADIPOSE MESENCHYMAL STEM CELLS

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Introduction. Adipose extracellular matrix (ECM) consists of the secreted products of the resident cells of the adipose tissue comprising a three-dimensional ultrastructure and a unique composition that could be useful for a variety of tissue engineering applications. The purpose of this study was to evaluate decellularized adipose tissue as a biologic scaffold for the chondrogenesis of human adipose mesenchymal stem cells (hAMSCs) and in-vitro cartilage-like tissue formation.

Materials and Methods. Adipose tissue of adult patients were collected from elective operations under ethical approval. A novel and practical protocol developed at our laboratory was applied for decellularization. hAMSCs were isolated from the adipose tissue, expanded and characterized immunophenotypically and by their differentiation potential. ECM and hAMSCs were clusterized, cultured in 10% FBS containing DMEM-F12, at 37oC, 5% CO2 and 95% humidity. After a week, the medium was switched to the chondrogenic medium and cultured for 35 days under static and bioreactor conditions. Cell viability, DNA content, formation of the cartilage-like tissue were evaluated at regular intervals, using MTT, picogreen assay, histology and IHC, respectively.

Results. The biochemical and structural properties of the adipose ECM can vary according to the selected protocol. Here, adipose ECM was obtained in a reproducible way and supported chondrogenic differentiation of hAMSCs. Immunophenotypical characterization demonstrated strong positivity for CD 90, CD73, CD105, CD29, CD166, CD44, and was negative for CD34, CD45, CD133. The clusterized ECM and the hAMSCs gained mechanically stability over time, especially in course of the chondrogenic culture process. The hAMSCs seeded inside the ECM scaffold proliferated faster during the initial culture period, and maintained in number during the chondrogenic culture, confirmed by the MTT and picogreen assays. Histology and IHC indicated the formation of a cartilage-like tissue in-vitro.

Conclusions. Results point out the potential of decellularized adipose tissue as a biologic scaffold for cartilage tissue engineering.

Keywords. Adipose extracellular matrix, decellularization, matrix technology, adipose mesenchymal stem cells, chondrogenesis

(1.P20) 2D AND 3D MULTILINEAGE DIFFERENTIATION OF HUMAN ADULT (ADIPOSE TISSUE DERIVED) AND EMBRYONIC STEM CELLS FOR TISSUE ENGINEERING

Declercq H(1), T' Joen V (1), Cornelissen M (1)
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Introduction. Human adipose tissue derived stem cells (AT-MSC) and embryonic stem cells (ESC) are promising alternatives for mesenchymal stem cells (MSC). Abundant amounts of adipose tissue can be obtained and these AT-MSC have multipotent characteristics. ESC have unique dividing capacities and pluripotent characteristics but directed differentiation is necessary. In this study AT-MSC, ESC-MSC and bone marrow (BM)-MSC are compared on their: 1) expansion efficiency and multilineage differentiation capacity.

Materials and Methods. AT-MSC (Cryosave) and BM-MSC (Lonza) were expanded in MesenPRO. ESC-MSC were derived from ESC (H1, VUB01) via different strategies. Adipogenic, chondrogenic and osteogenic differentiation was studied in 2D and 3D cultures. For osteogenic 3D differentiation, MSC are seeded on 3D scaffolds/microcarriers and cultured dynamically for 40 days. The cultures were evaluated by fluorescence microscopy, histology and qRT-PCR.

Results. AT-MSC, BM-MSC and ESC have a population doubling time of respectively 25, 54 and 36 hours. EB formation of ESC is insufficient for large-scale differentiation while predifferentiation of ESC in monolayer culture resulted in a morphologically homogeneous population of MSC-like cells. 2D adipogenic differentiation was apparent by the accumulation of lipid-rich vacuoles and was most obvious for AT-MSC followed by BM-MSC and ESC-MSC. 3D chondrogenic differentiation was achieved in pellet cultures, although the extracellular matrix (ECM) stained less intense than chondrocyte controls. AT-MSC had the highest osteogenic differentiation capacity as demonstrated in 2D and 3D. During 3D osteogenic differentiation on microcarriers, the cell-seeded microcarriers formed clusters after 14-21 days. Histology of the scaffolds/microcarriers revealed fully colonization and a bone-specific ECM formation (figure 1). These data were confirmed with qRT-PCR.

Conclusions. AT-MSC have an excellent adipogenic and osteogenic differentiation capacity in comparison with ESC-MSC. ESC-MSC would offer an alternative source to study cell/biomaterial interactions in vitro. However, the predifferentiation of hESC to hESC-MSC should be optimized to obtain a homogeneous population of MSC.

Keywords. human adipose derived stem cells, human embryonic stem cells, tissue engineering, 3D culture

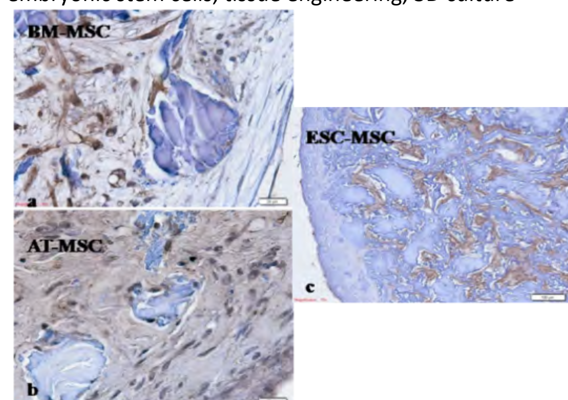

Figure 1. Osteocalcin immunostaining of colonized 3D scaffolds with BM-MSC (a), AT-MSC (b) and ESC-MSC (c) after dynamic culturing for 40 days in osteogenic medium.

(1.P21) TRACHEAL RECONSTRUCTION BY MONOLAYERED MESENCHYMAL STEM CELLS WITH SMALL INTESTINE SUBMUCOSA IN A RABBIT MODEL

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Introduction. There are no proven methods of construction of tracheal defects when end-to-end anastomosis is considered impossible. Trachea replacement using prosthetic or biological substitutes have thus far yielded unsatisfactory results, preventing their clinical application. The failures of these methods have been mainly due to donor sites' restriction, immunologic complications, bacterial infections, and material failure. We aim too investigate the tracheal reconstruction by monolayered autologous mesenchymal stem cells with small intestine submucosa in a rabbit model.

Materials and Methods. Eleven male New Zealand white rabbits were randomly divided into three groups: rabbits with trachea defect without reconstruction (untreated group, n=4), rabbits with trachea defect given small intestine submucosa (SIS) graft (SIS group, n=4), and rabbits with trachea defect that underwent transplantation of monolayered mesenchymal stem cells (MSCs) on SIS (SIS+MSC group, n=4). Histological and endoscopic analyses were performed by hematoxylin-eosin staining (H&E), Prussian blue staining and endoscopy.

Results. Morbidity and mortality in the SIS+MSC group were minimal, compared to untreated group and SIS group. The specimens obtained from untreated group and SIS group showed severe infiltration of inflammation cells and granulocytes into the tracheal lumen at 1 week after operation. In the SIS+MSC group, however, minimal infiltration of inflammation cells and granulocytes was noted. Twelve weeks following the operation, regeneration of pseudostratified squamous epithelium and ciliated columnar epithelium were confirmed by H&E staining with minimal inflammatory infiltration in SIS+MSC group. Moreover, Prussian blue staining clearly demonstrated the presence of labeled MSCs in the regeneration tissue of SIS+MSC group.

Conclusion. Tracheal reconstruction by MSCs with SIS can be used to reconstruct a rabbit tracheal defect with minimal mortality and morbidity, which appears to be a promising therapeutics in the treatment of patients with tracheal defects.

Keywords. Trachea, reconstruction, cell sheet

(1.P22) THE THERAPEUTIC EFFECT OF ADIPOSE-DERIVED STEM CELL AND BDNF-IMMOBILIZED SCAFFOLD IN A RAT MODEL OF CAVERNOUS NERVE INJURY

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Introduction. Post-prostatectomy erectile dysfunction (ED) is a serious side effect for prostate cancer patient, and reduces the patient quality of life. In this study, we investigated the effect of human adipose-derived stem cells (h-ADSCs) and BDNF incorporated Poly-Lactic-Co-Glycolic (PLGA) membrane combined therapy in a rat model of bilateral cavernous nerve (BCN) injury.

Materials and Methods. Sprague-Dawley rats inflicted with BCN crush-injury were used for animal model. Experimental groups were divided 5 groups; normal (N), BCN crush-injury (C), h-ADSC after BCN injury (A), BDNF-PLGA membrane after BCN injury (B), and h-ADSC and BDNF-PLGA membrane after BCN injury (AB). PKH26-labeled h-ADSCs were applied around the injured cavernous nerve, and then BDNF-released PLGA membrane was immediately covered on. Four weeks after operation, erectile function was assessed by detecting intra-cavernous pressure (ICP). Cavernous nerve and corpus cavernosum were collected for histological and molecular examinations.

Results. We found that h-ADSCs engrafted into the cavernous nerve under fluorescent microscopy. In functional study, ICP in group C was decreased compared with N, but ICPs in group A, B and AB were increased compared with group C. In histologic examination, collagen content in corpus cavernosum was increased in group C, but a little changed group A, B and AB. Molecular study showed that the application of h-ADSCs and/or BDNF-PLGA membrane increased cGMP and eNOS expression in corpus cavernosum after BCN injury. The h-ADSCs and/or BDNF-PLGA membrane combined therapy was more effective than each single therapy.

Conclusion. These results suggested that application of h-ADSCs on bilateral cavernous nerve, covered with BDNF-released PLGA membrane can prevent the corpus cavernosum damage after BCN injury. So, this combined approach may provide a novel therapy for post-prostatectomy ED.

Keywords. Adipose-derived stem cell, Brain-derived neurotrophic factor, Cavernous nerve, Erectile dysfunction

(1.P23) REVERSIBLE IMMORTALIZATION OF HUMAN ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS

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Introduction. Background and aims. Adipose tissue-derived mesenchymal stem cells (adMSCs) can be easily harvested from human donors and differentiated into the standard osteogenic, chondrogenic and adipogenic directions, as well as towards a putative endothelial phenotype. However, heterogeneity between donors, dependence of cellular properties on passage number, and limited life span of in vitro adMSC cultures present major hurdles for reproducible experiments. Therefore,

we aimed to establish immortalized adMSC populations with well-characterized properties that can provide a steady supply of homogeneous cells for in vitro work. **Materials and Methods.** The immortalizing genes Bmi-1 and SV40 large T antigen, combined with hTERT, were transduced using Cre-excisable lentiviral vectors into adMSCs of a single donor. Transgene copy number was determined by qPCR relative to RNase P. Expression of all transgenes was verified by immunofluorescence and RT-qPCR, and telomerase activity was measured using TRAPeze assay. Cell surface markers were detected by flow cytometry. Proliferation was assayed using resazurin dye. Osteogenic differentiation was assessed based on alkaline phosphatase immunodetection and enzymatic activity, and sprouting assay for endothelial differentiation was carried out in Matrigel.
Results. Both Bmi-1+hTERT and SV40T+hTERT cell populations have preserved expression of MSC markers, and both have been subcultured for over 30 passages without any sign of senescence. However, the two populations possess clearly distinct properties. While Bmi-1+hTERT is a mixed population with morphology, proliferation and differentiation comparable with the parental adMSC, SV40T+hTERT has quickly become a rapidly proliferating cell line.
Conclusions. Since Bmi-1+hTERT MSCs have maintained close-to-native MSC features, they may be utilized directly in differentiation experiments. SV40T+hTERT, on the other hand, can be efficiently expanded, and may possibly be reverted to a conservative MSC phenotype by subsequent Cre-mediated removal of the immortalizing transgenes. Financial support. This work was supported by the grant TÁMOP-4.2.1 from the Hungarian National Development Agency (NFÜ).
Keywords. adipose tissue-derived mesenchymal stem cells, immortalization

(1.P24) ELECTROPORATION-MEDIATED TRANSFER OF RUNX2 AND OSTERIX GENES TO ENHANCE OSTEOGENESIS OF ADIPOSE STEM CELLS

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Adult stem cells are the promising potential for differentiation into several cell types and predominantly the adipose stem cells (ASCs) obtained from lipoaspirates has the multi-lineage prospective to differentiate into various cell types. Several explorations have shown that ASCs have the potential to differentiate into osteogenic lineages by the transfection of BMP expression vectors. The constraint for the use of BMP expression has low efficiency of its expression in the exogenous in vivo system during osteogenesis. To address these facts several researchers have explored the use of alternative bone specific transcription factors to induce efficient osteogenesis. Transfection of Runx2 and osterix in mesenchymal stem cells leads to the development of osteoblastic cells and bone formation. However the foremost negative aspect of viral transfection methods are immunogenicity and mutagenesis for these reasons much effort has been made to go for advantageous nonviral transfection by electroporation method to transfer the growth factor genes. In the present study, we tested the hypothesis that electroporation-mediated transfer of Runx2 and Osterix genes to provoke in vitro and in vivo osteogenic potential in ASCs.
Acknowledgements. This work was supported by a grant from the Korea Ministry of Education, Science and Technology (Grant No 2010-0000305).
Keywords. gene transfer, ATMSCs, osteogenic differentiation

2. BIOFABRICATION FOR REGENERATIVE MEDICINE APPLICATIONS

Chair: James J. Yoo
Co-chair: Wei Sun
Keynote speaker: James J. Yoo
Organizers: James J. Yoo, Wei Sun
Synopsis: Biofabrication has become an innovative tool for tissue engineering and regenerative medicine. Biofabrication uses cells, biomaterials and macromolecules to create basic building blocks of tissues and organs. This special session will report state-of-the-art research and development of using novel physical, chemical, biological, and/or engineering process for 1) construction of cell assemblies as tissues for regenerative medicine, disease models and drug models; 2) integrated bio-nano fabrication and bio-micro fabrication; 3) cell/tissue printing, patterning and organ printing; 4) cell-integrated biological systems, microfluidic devices, biosensors, and biochips; 5) 3D tissue scaffolds and tissue constructs; 6) Computer-aided biofabrication and tissue engineering; and 7) Protein/biomolecule printing and patterning.

(2.KP) BIOFABRICATION OF TISSUES FOR CLINICAL TRANSLATION

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Advances in regenerative medicine have provided various therapeutic opportunities in the field of medicine. While tissue engineering and regenerative medicine have had initial successes in building a number of tissues clinically, challenges still exist in developing complex tissue systems. One of the challenges that hamper rapid clinical translation is due to the lack of efficient cell delivery methods. Living tissues maintain inherent multi-cellular heterogeneous structures, and rebuilding of such complex tissue structures requires subtle arrangements of different cell types and extracellular matrices at their specific anatomical target sites. Biofabrication using an inkjet printing technology has been proposed as a tool to address this endeavor. In this session novel and versatile methods of building tissue structures using biofabrication technology will be discussed. Development strategies that facilitate a rapid clinical translation will also be discussed.
Keywords. Biofabrication, Bioprinting, Translation

(2.O1) USE OF SILK FIBROIN AS A SUBSTRATUM FOR HUMAN CORNEAL ENDOTHELIUM TRANSPLANTATION

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Introduction. Diseased or damaged corneas are surgically removed and replaced with tissue from deceased donors. Corneal transplantation could be improved by engineering cell layer substitutes. This would overcome the shortage of donors and improve quality. The endothelial layer is vital to corneal clarity and with most transplants this layer alone needs replacing. Our aim is to grow an endothelial layer on a substratum suitable for transplant.

Materials and Methods. Adult human corneal endothelium has low proliferative activity. Most cells will not mitose, but a small proportion can be stimulated to divide by strong mitogens. We have used these along with a free-floating sphere technique in preparing cells with gross normal, ''differentiated'', morphology. To function normally the endothelium is best introduced as a confluent organised monolayer and to achieve this we grew primary cells on silkworm (*Bombyx mori*) (BM) fibroin, prepared as 5 µm thick membranes. Furthermore, to try and improve cell attachment and growth without the need for coatings, we investigated, 1) patterning the fibroin surface and, 2) the use of an alternative fibroin from Antheraea pernyi (AP) silkworms which contains the RGD tripeptide site.

Results. Our BM fibroin membranes are transparent (>96% transmission), strong, and should degrade sufficiently slowly to allow the endothelial cells to establish on the recipient cornea, yet still maintain transparency to retain sight. We achieved cell confluence with normal gross morphology. However, not only was a collagen coating required, but also the membrane was difficult to handle. To improve handling we manufactured 9mm diameter discs with a 1mm supporting ring. AP membranes were difficult to prepare and required a different method.

Conclusion. Silk fibroin can be prepared as a transparent membrane that supports the growth of human corneal endothelium with gross normal morphology. These qualities further the potential application of fibroin for clinical corneal endothelial transplantation.

Keywords. cornea: silk fibroin: endothelium

(2.O2) MODULAR TISSUE FORMATION WITH CONFORMALLY COATED THERMO-RESPONSIVE RIGID MICRO-TEMPLATES

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Generating modular tissue units can be beneficial for applications in tissue engineering, drug discovery, and regenerative medicine. Recently, softlithographically fabricated poly(N-isopropylacrylamide) (PNIPAAm) based microstructures have shown promising results in the release of cell aggregates, though the swelling of molds during temperature changes may cause deformation on cell clusters. In this study, biocompatible, elastic, and gas permeable poly(dimethylsiloxane) (PDMS) was used to fabricate rigid microstructures that were supplied with a conformal coating of PNIPAAm using chemical vapor deposition. At room temperature, conformal PNIPAAm films on PDMS templates swelled to three times their thickness at 37 °C. Combining both the stiffness and the thermo-responsive properties of the resulting microstructures, tissue constructs could be grown to match the dimensions of the microgrooves and furthermore easily retrieved at room temperature using swelling property and hydrophilicity of PNIPAAm at 24 °C (contact angle θ = 30° ± 2) compared to 37 °C (θ = 50° ± 1). Given these results, conformally PNIPAAm coated PDMS microstructures can be integrated with traditional microfabrication techniques and may become a versatile tool for tissue engineering and drug discovery applications.

Keywords. Thermo-responsive templates, modular tissues, chemical vapor deposition, microfabrication

(2.O3) LASER-ASSISTED BIOPRINTING: A TECHNOLOGY FOR DEALING WITH TISSUE COMPLEXITY

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Parallel to scaffold-based approaches, technological advances in the fields of automation, miniaturization and computer-aided design and machining have led to the development of Bioprinting. This later concept has been defined recently as the "the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bio-engineered structures serving in regenerative medicine, pharmacokinetic and basic cell biology studies". As compared to traditional approaches in Tissue Engineering, bioprinting represents a paradigm shift. Indeed, its principle is not more to seed cells onto a biodegradable scaffold but rather to organize the individual elements of the tissue during its fabrication step (before its maturation) through the layer-by-layer deposit (bottom-up) of biologically relevant components.

Besides ink-jet printing and bioplotting by means of pressure-operated mechanical extruders, the Laser-Assisted Bioprinting (LAB) technology has emerged as an alternative method, thereby overcoming some of the limitations of ink-jet and micropen printing devices, namely, the clogging (viscosity, cell agglomeration, ink drying, etc...) of print heads or capillaries used by these printers to achieve micron-scale resolution.

In this context, after describing physical parameters involved in Laser-Assisted Bioprinting, we present its applications for printing nanomaterials and cells, both in vitro and in vivo and we discuss on how this high-throughput, high resolution technique may help in reproducing local cell micro-environment, and hence creating functional tissue engineered 3D constructs. allowing to deal with tissue complexity and heterogeneity.

Keywords. bioprinting, laser

(2.O4) FABRICATING SMALL DIAMETER, BRANCHED VASCULAR SYSTEMS BY COMBINING INKJET PRINTING AND MULTIPHOTON POLYMERIZATION

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Introduction. To date only single *in vitro* engineered tissues are transferred to clinical approaches due to todays inability to fabricate suitable, artifical vascular systems. Combining inkjet printing with high-resolution multiphoton polymerization (MPP) enables us to generate branched, tubular systems with diameters << 1 mm. New synthetic polymers were tailored to match the needs of the technical building process and the elastic properties of blood vessels. The polymers were biofunctionalized to achieve a close coating with endothelial cells (ECs).

Experimental Methods. Based on numerical simulations, branched tubular scaffolds were fabricated by combining inkjet printing and MPP. Precursor polymers, cross linking agent, photo initiators and solvent additives were optimized to yield photo reactive inks with customizd E-moduli. Crosslinked polymers were modified with derivatized heparin and RGD and analyzed by XPS and colorimetric methods. Viability, proliferation, functionality of primary human microvascular ECs on the substrates was determined, using several assays and immunocytological stainings.

Results. A set-up for integrating inkjet printing and MPP has been designed with which branched vessel scaffolds have been fabricated. The diameter of the tubes can range between 20 µm and several millimeters (Figure 1). Material compositions have been developed to achieve E-Moduli of 2-2000 MPa after crosslinking, the lower are similar to natural blood vessels. Suitable after-treatment ensured biocompatibility of the processed polymers, thereafter thio-heparin and RGD have been covalently bound on the surface. On these biofunctionalized substrates an increased adhesion, viability and proliferation of ECs has been determined in comparison with unmodified substrates. EC-typical antigene expression has been observed by immunocytological stainings on all substrates.

Conclusion. The presented combination of rapid prototyping techniques makes it possible to generate small diameter vessel-like systems that can be applied for supplying *in vitro* engineered tissues in a larger scale.

Acknowledgments. We thank the Fraunhofer Gesellschaft for financial support to this project.

Keywords. artfical vessel scaffolds, inkjet printing and multiphoton polymerisation, small diameter and branched

(2.O5) NOVEL APPROACH TO AUTOMATING AND SCALING UP PRODUCTION OF COLLAGEN BASED SCAFFOLDS FOR THERAPY AND SCREENING

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Introduction. Successful translation of research findings into cost effective therapies requires process scale up for production. Often manufacturing issues are overlooked when materials and processes are being developed, resulting in therapies that are difficult or expensive to manufacture reproducibly. This is especially true for cell therapies. Here we describe a new automated system for production of biomimetic cell-containing collagen scaffolds. Our aim is to enable reliable, consistent and cost effective manufacture of cell-based therapies at a commercial scale.

Materials and Methods. Brown et al (1) described a novel approach to collagen engineering, plastic compression, in which water is expelled from cell-seeded hyper hydrated collagen gels. This simple technology allows direct fabrication of strong, biomimetic tissues. Although the manual process is rapid, taking less than 1 hour to make a tissue, it is difficult to achieve good control of process parameters. We have now developed a workstation to automate and control the critical stages of the plastic compression process.

Results. Data will be presented which show that the manual process can be scaled up successfully. Multiple tissues have been made in parallel in a variety of formats; 12, 24 and 96 multi-well plates. 3D cell seeded collagen tissues with different cell types can be made rapidly and reproducibly while retaining good cell viability. The versatility of the system will be demonstrated by reference to properties, such as multi-layering and embossed surface features, that can be engineered into tissues using this technology.

Conclusion. This workstation is an enabling platform technology for making strong, collagen based tissues, and a powerful tool for scientists developing novel tissues for cell therapy or a wide range of research applications. It is useful throughout the development process, supporting process development, biomaterials development and production of tissues for potency assays.

References 1. Brown R.A. et al. (2005) Adv. Funct. Mater. 15(11) 1762-1770

The work was supported in part by funding from the UK Technology Strategy Board

Disclosures. TAP has licensed technology from UCL

Keywords. Translation; Cell therapy; Collagen scaffolds; Scale up

(2.O6) DEVELOPMENT AND IN VITRO DEGRADATION OF PLA/PEG/CaP GLASS BIODEGRADABLE SCAFFOLDS BY RAPID PROTOTYPING

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Introduction. Rapid prototyping allows the development of temporary 3D scaffolds with optimal architecture, providing an adequate support for cell in-growth, differentiation and ultimately tissue regeneration. Particularly, a nozzle-deposition system integrated with pumping technology is a versatile tool that uses a CAD/CAM approach to build complex, reproducible 3D structures. In this study, polylactic acid (PLA) and polyethylene glycol (PEG) were combined with soluble CaP glass particles and processed by RP to obtain fully biodegradable structures with superior mechanical properties and bioactivity. The aim of this work was the development, characterization and in vitro degradation study of biodegradable PLA/PEG and PLA/PEG/CaP glass 3D scaffolds.

Materials and Methods. A blend of 95% Poly(95L/5DL)lactic-acid and 5% PEG (Mw=400) in chloroform (5%w/v) was prepared. In the case of the composite, CaP glass particles (<40um) in the system 44.5P2O5-44.5CaO-6Na2O-5TiO2 were also added (50% w/w). Scaffolds with orthogonal and orthogonal-displaced geometries were fabricated. The in vitro degradation behaviour of the structures was evaluated by immersing the scaffolds in SBF at 37°C for 8 weeks. Differential scanning calorimetry, scanning electron microscopy (SEM), mechanical compression test, micro-computed tomography, and ionic (Ca2+) release were evaluated after different degradation times. Biological evaluation was also carried out.

Results. Well defined structures with 65% porosity were obtained. Initial compression tests showed that both geometry and glass particles affected the scaffolds mechanical properties. Weight loss measurements and SEM images (Fig.1) indicated that scaffolds were slowly degraded loosing up to 7% of their initial weight and increasing their surface microporosity. Nevertheless, mechanical properties slightly decreased preserving the scaffolds stability. Glass particles added an interesting bioactive effect by releasing Ca to the medium. Indeed, the addition of CaP-glass positively affected cell behaviour.

Conclusion. The combination of RP and PLA/PEG/CaPglass turned into promising fully degradable, mechanically stable, bioactive and biocompatible composite scaffolds for TE.

Keywords. biofabrication, rapid prototyping, biomaterials, biodegradable scaffolds, bone, regenerative therapies

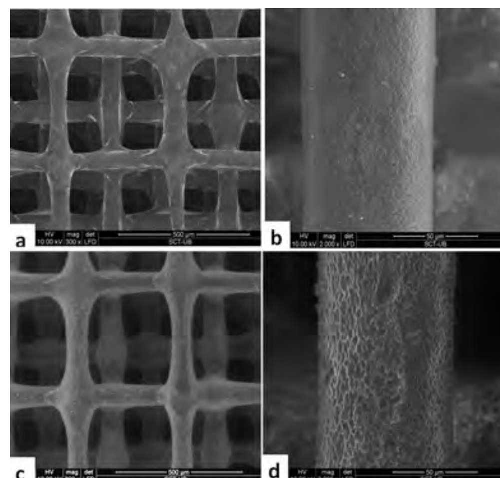

Figure 1. Surface morphology of PLA/PEG scaffold at t=0 weeks (a,b) and t=5 weeks(c,d)

(2.O7) MICROWELL SCAFFOLDS FOR EXTRAHEPATIC ISLET OF LANGERHANS TRANSPLANTATION IN TYPE 1 DIABETES

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Introduction. The conventional therapy for type 1 diabetes is insulin administration. Despite this, some patients are poorly controlled and suffer from hypoglycemia and long-term complications. For these patients, allogeneic islet transplantation into the liver has become an alternative therapy[1]. Patients benefit from this therapy due to near normalization of blood glucose levels without an increased risk of hypoglycemia. However, islet graft function in the liver tends to decline over years indicating that the liver is not an optimal transplantation site[2]. In order to develop alternative transplantation sites with better long-term outcome, we have developed a new microwell scaffold platform.

Materials and Methods. Microwell scaffolds were prepared from dense solution-cast and porous electrospun 4000PEOT30PBT70 block-copolymer films using microthermoforming. Polymer wettability and scaffold topology were assessed by captive bubble contact angle measurements and scanning electron microscopy (SEM), respectively. Furthermore, constructs were characterized for their permeability for the nutrient glucose. To determine the applicability of the constructs for islet transplantation, the morphology and function of human islets after 7 days of culturing were studied by SEM, histological analysis and glucose challenge tests.

Results. We fabricated reproducible dense and porous films, the latter with a fiber-diameter of 1.71±0.42µm. The polymer films were hydrophilic (contact angle <40°) . Diffusion tests revealed that the electrospun scaffolds were permeable for glucose (flux: 0.0018±0.0002 gm-2s-1). Based on SEM and histological analysis there were no indications for islet spreading or outgrowth of islet stromal cells. Function tests revealed that human islets remained responsive to glucose challenge after 7 days of

culturing in the constructs (figure 1). Currently, first in vivo trials are performed.

Conclusion. This study reports on the development of a novel microwell scaffold platform for extrahepatic islet of Langerhans transplantation. Alternative transplantation sites using biomaterial scaffolds may improve islet transplantation outcome.

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[2]E.A.Ryan et al.Diabetes,54,2060-2069(2005)

Keywords. Islet transplantation, Biomaterial, Scaffold, Diabetes

Figure 1 A) Glucose stimulated insulin release of free-floating and scaffold cultured human islets. Islets were subjected to 1 hour sequential incubations in 1.7mM glucose and 16.7mM glucose. Scaffold cultured islets showed a glucose stimulation profile similar to free-floating control islets, B) SEM image of human islets in the construct indicating that scaffold cultured islets preserve their rounded morphology, C) Immunofluorescence revealed insulin (red) and glucagon (green) expression throughout the human islet cultured in the construct (construct is indicated by arrow). Data were obtained after 7 days of culturing.

(2.O8) MICROFLUIDICS FABRICATION OF SELF-ASSEMBLED POLYSACCHARIDE - PEPTIDE MICROCAPSULES FOR CELL THERAPY

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Self-assembling is an appealing methodology for the bottom-up fabrication of new biomaterials that can be used for the controlled growth of cell populations for cell therapies or to promote regenerative processes in vivo. Peptides are excellent structural units to form complex nanostructures that can recreate some of the architectural features of the natural extracellular matrix, as they can self-assemble into fibril nanostructures. We report here a mild cell encapsulation method based on triggering the self-assembly of a multidomain peptide in presence of xanthan gum polysaccharide, which has been investigated in our group as artificial matrix for the encapsulation of chondrocytic cell. The self-assembling peptide K2(QL)6K2 has a central block of glutamine-leucine (QL) repeats, and two flanking positively charged lysine (K) to bind to the negatively charged xanthan. Using a microfluidic device we were able to produce microcapsules with homogenous size (diameter of 300 mm) by forming a water-in-oil multiphase. This technology allows a control over the properties of the microcapsules in terms of size and morphology, being a low stress inducing method suited for cell encapsulation. The properties and performance of xanthan-peptide microcapsules were optimized by changing peptide/polysaccharide ratio and their effects on the microcapsules permeability and mechanical stability were analyzed. Moreover, the effect of microcapsule formulation on viability and proliferation of encapsulated chondrogenic cells were also investigated. The encapsulated ATDC5 cells were metabolically active, showing an increased viability and proliferation over 21 days of in vitro culture demonstrating the long-term stability of the developed microcapsules and their ability to support and enhance the survival of encapsulated cells over prolonged time. Combining self-assembling materials with microfluidics processing proved to be innovative approach to fabricate suitable matrices for cell encapsulation and delivery.

ACM acknowledges to FCT for the financial support (PhD grant SFRH/BD/42161/2007)

Keywords. Peptide self-assembly; Xanthan gum; Microfluidics. Cell encapsulation, Microcapsules

(2.O9) FABRICATION OF A CUSTOMIZED TISSUE ENGINEERING SCAFFOLD FOR BREAST RECONSTRUCTION

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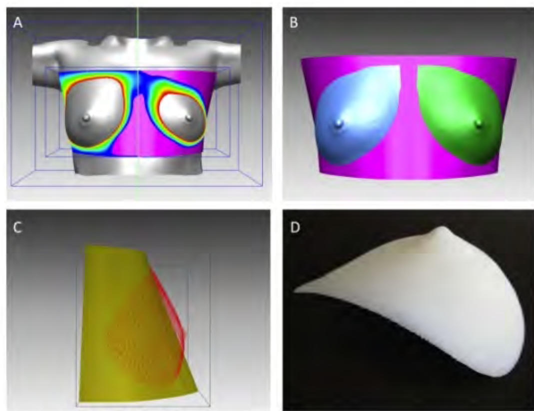
Introduction. Mastectomy can be necessary in breast cancer therapy. To improve the patient's quality of life, plastic surgeons often reconstruct the breast. The state-of–the-art procedure is the transplantation of free fat grafts from the belly to the breast. Disadvantages are long operation times and risk of hematoma, infections or donor site defects. A tissue engineered and vascularized adipose construct could overcome these disadvantages and could mimic the natural breast in respect of shape, ptosis and touch. Tissue engineering scaffolds are needed to shape the breast and support fat formation. Here we demonstrate a method that is close to clinical reality, using CAD/CAM technologies.

Materials and Methods. The body of a young female patient is scanned with a 3D laser scanner from three different angles. These scan images are digitally merged and converted to a 3D model of the patient's body. This 3D model is imported into CAD software. Software algorithms are used to mirror the healthy breast and to adapt this designed breast to the predicted thorax shape, so that the scaffold fits to the recipient area of the removed breast. Furthermore, CAD date are transferred to rapid prototyping commands (STL language) and used to fabricate a full-size breast scaffold with fused deposition modeling.

Results and Conclusion. In conclusion, geometrically complex scaffolds can be manufactured individually and customized with 3D laser scanning, CAD modeling and rapid prototyping.

Keywords. breast reconstruction, customization, clinical setting, rapid prototyping, CAD, CAM



Creation of tailor-made scaffold
Scan data are merged to 3D image of body. Artificial thoracic wall (purple) ist created and used to define boundaries of breasts (A). Breasts are excised along blue-green border. In (B) healthy breast (green) is mirrored (blue). Software algorithm creates grid suface of the breast (C). The geometries of this designed breast are used to manufacture a scaffold via rapid prototyping (D).

(2.O10) 3D-STRUCTURING OF POLY(VINYL ALCOHOL)-BASED PHOTOPOLYMERS

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The fabrication of 3D-scaffolds with defined pore geometries which enable good adhesion of cells is a challenging topic in the field of regenerative medicine. Photopolymers which can be structured by means of Additive Manufacturing Technologies are promising materials for this application. The possibility of structuring these compounds via processes such as microstereolithography (μSLA), Digital Light Processing (DLP) or Two Photon Polymerization (2PP) enables the fabrication of constructs with complex geometries and high resolution mimicking cellular structures of natural materials such as bone.
Beside the considerable irritancy and sometimes toxicity of acrylate-based monomers, the formation of polyacrylic acid through hydrolytic degradation of the polymer is another undesirable aspect of these materials when applied in the biomedical field. Therefore, photopolymers with different polymerizable groups such as vinylesters, vinylcarbonates and vinyl-carbamates which give water-soluble poly(vinyl alcohol) upon hydrolytic degradation, were evaluated. Several monomers were synthesized to examine the properties of these substance classes with focus on cytotoxicity, photoreactivity, mechanical properties and degradation behavior. 3D-parts made of the new materials were implanted into New Zealand White Rabbits to examine the behaviour under physiological conditions. The biocompatibility of these new substances, measured by their cytotoxicity towards osteoblast-like cells, showed better results than for their (meth)acrylate-based counterparts. The photoreactivity was found to be between that of acrylates and methacrylates, mechanical properties were on the same level and degradation characteristics could be tailored over a broad range. The in-vivo studies showed excellent biocompatibility of the materials as well as osteoconductivity due to the layered structure inherent to parts structured with conventional AMTs.
The prepared photopolymers based on poly(vinyl alcohol) show interesting properties for the application in the biomedical field. Under the maintenance of mechanical properties and photoreactivity of conventional photo¬polymerizable monomers based on (meth)acrylates, cytotoxicity and the degradation behaviour could be significantly improved.

Keywords. Additive Manufacturing Technology

(2.O11) BIOFABRICATION OF THREE-DIMENSIONAL COMPLEX CONSTRUCTS VIA MAGNETIC DIRECTED MICROGEL ASSEMBLY

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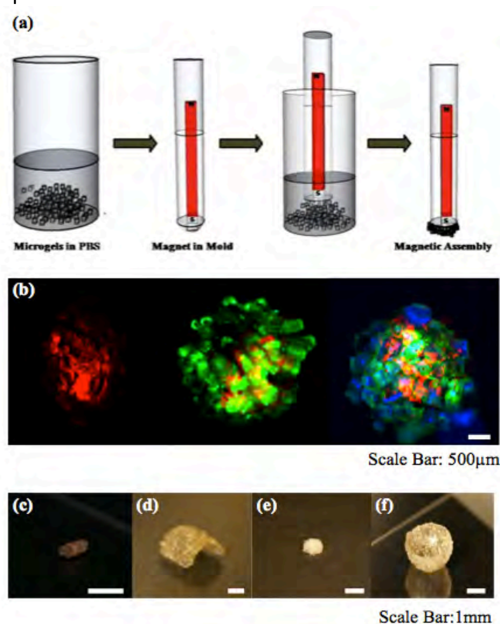
Introduction. Directed assembly of microgels is a promising method for constructing complex three-dimensional (3D) geometries that mimic native tissues. Although several methods have been developed to assemble the microgels, these methods are limited by process complexity, low throughput potential, and the use of organic solvents. A simple directed assembly process with high throughput potential is still an unsatisfied step towards recreating in vivo tissue structures and functions. Here we propose a novel magnetic directed assembly method for fabricating 3D construct using microgels. Magnetic nanoparticles (MNPs) were encapsulated in the microgels and manipulated using externally applied magnets.
Materials and Methods. Microgels (PEG 1000) of different sizes and shapes were fabricated using photolithography. These microgels were fabricated by encapsulating iron (II, III) oxide MNPs within the hydrogels. These M-gels (magnetic nano-particle encapsulated microgels) were attracted with the use of neodymium magnets of 1 tesla power (Figure 1a). They were secondary cross-linked along with 5 μl prepolymer solution for the stability of the structure.
Results. Complex 3D constructs of the microgels were achieved through magnetic directed assembly with precise spatial control. We observed the formation of a 10 mm diameter single layer spheroid within 5 seconds of introducing the magnetic rod. With such time control and varying the concentration of MNPs (0.3%-2%) multi-layer spheroids were fabricated (Figure 1b). We achieved a various complex structures using the flexible templates such as arc, dome, sphere and tubular constructs (Figure 1c-f). The observed M-gel assembly confirm that the gravitational force can be balanced by the magnetic force applied via permanent magnets.
Conclusion. Here we reported a directed assembly method of microgels as building blocks via magnetics into larger constructs that mimic in vivo structures. These results envisage that this method holds the potential to

impact multiple fields including tissue engineering, stem cell technology, regenerative medicine, and pharmacology.

Keywords. Magnetics directed assembly, microgels, complex constructs



(2.O12) TISSUE ENGINEERED CARTILAGE FROM HUMAN BONE MARROW MESENCHYMAL STEM CELLS SEEDED IN PLGA/SOX-TRIO GENE IN VITRO

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Introduction. Articular hyaline cartilage injuries still pose a big challenge to orthopaedic surgeons, because these defects have poor capacity for instisnic repair. Tissue engineered cartilage constructed with a combination mesenchymal stem cells and three-dimensional biomaterial may be a viable therapeutic option. The aim of this study is to investigate the chondrogenic potential of SOX-5, 6 , 9 as chondrogenesis related transcription factors, using SOX-genes conjugated PLGA scaffold.

Methods. Five modifications of porous PLGA scaffolds were tested: 1) PLGA/pEGFP-C1; 2) PLGA/SOX-5; 3) PLGA/SOX-6; 4) PLGA/SOX-9; 5) PLGA/SOX-trio in terms of cell proliferation and chondrogenic potential. Bone marrow mesenchymal cells were seeded on PLGA scaffolds, respectively. After three weeks, cells were analyzed for DNA contents, GAG amount and real time PCR. The rabbits were anesthetized, and the right legs were prepared as described in the previous section. The knee joint was exposed by medial parapatellar incision, and the trochlear groove was exposed by lateral dislocation of the patella. A 3 mm outer diameter trephine drill was used to create osteochondral defects (diameter 7 mm, thickness 2 mm) in the trochlear groove of femur. The animals were divided into three groups: in Group I, the defect was filled with PLGA scaffold ; in Group II, the defect was filled PLGA scaffold seeded with ASCs ; in Group III, the defect was filled SOX-trio incorporated PLGA seeded with ASCs. To prepare the implantation, ASCs (5 x 105 cell in 40µl) were suspended in DMEM/F-12 media and injected inside the scaffold. Five rabbits were allocated to each group. The patella was repositioned and the capsule was repaired with 4-0 nylon sutures. The rabbits were allowed to feed freely in their cage immediately after the operation without a cast. Rabbits were sacrificed after 8 weeks.

Results. When cultured in hASCs seeded in SOX-trio plasmid incorporated scaffolds with chondrogenic medium produced significantly richer ECM than did control vector and single SOX-5,6,9 plasmid incorporated scaffolds. Interestingly, real time PCR analysis demonstrated that hASCs seeded in SOX-trio plasmid incorporated scaffolds showed significantly higer gene expression of type II collagen compared with control vector and single SOX-5,6,9 plasmid incorporated scaffolds .However, after 3 weeks in culture, there was weak expression of type I and X collagen in SOX-trio incorporated scaffolds group, but there was no significantly difference in expression of genes in any other groups. These findings indicate that SOX-trio incorporated scaffolds a higher rate of chondrogenic potential than in any other groups. In an in vivo osteochondral defect model, treatment with PLGA scaffolds and SOX-trio incorporated PLGA scaffolds demonstrated some ability to potentiated cartilage regeneration. SOX-trio treatment led to greater cartilage regeneration than in PLGA scaffols only and ASC with PLGA scaffolds groups. At week 8, macroscopic and histologic assessment demonstrated that treatment with SOX-trio incorporated scaffolds produced good articular cartilage healing with Safranin-O positive hyaline cartilage.

Conclusion. In conclusion, our results suggest that SOX-trio incorporated plasmid DNA loaded scaffolds have higher chondrogenic potential in vitro and induce more cartilage regeneration in an in vivo osteochondral defect model than do control groups. These results further the understanding of the chondrogenic potential of SOX-trio plasmid loaded scaffolds and may contribute to the development of new therapeutic strategies for cartilage repair and regeneration. Finally, we note that the gene expression is occurring within the scaffold microenvironment, and that stimulus to promote cartilage regeneration by the transgene expression must be considered within the context of the microenvironment, which contains architectural, mechanical, chemical, and biological cues. All aspects of the microenvironment created by the scaffold must be considered for its role in promoting tissue formation, and continued development of gene releasing scaffolds holds great promise for numerous applications in regenerative medicine.

Acknowledgement. This work was supported by a grant from the Korea Ministry of Health Welfare (Grant No A080061).

Keywords. plasmid incoporated scaffold

(2.O13) CELL PRINTING FOR 3D TISSUES

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In the new paradigm of tissue science and engineering, living cells and biomolecules are used as basic building blocks for biofabrication of cell-integrated medical

therapeutic products and/or non-medical biological systems with applications found as tissue substitutes, 3D cell and organ biological models, microfluidic biochips and biosensors, and tissue models for study of disease pathogenesis, drug discovery and toxicity testing. This presentation will introduce our recent research in the emerging field of cell printing and report our work on using additive technology for direct cell writing for construction of 3D cell assemble and tissue structures. Presentation topic will include: 1) introduction of direct cell writing process; 2) effect of the process parameters on cell survivability; 3) characterization of biological responses of various cells to the printing process; and 4) applications to the field of tissue science and engineering.

(2.O14) INNOVATIVE THREE-DIMENSIONAL PLATFORM FOR COMBINATORIAL ANALYSIS OF CELL/BIOMATERIALS INTERACTIONS

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Introduction. High Throughput (HT) systems are an uprising area for analysis of biomaterials properties and cell response to substrates. Combinatorial screening allows for the selection of combinations of biomaterials and/or bioactive agents in a preliminary stage of physicochemical characterization or cell behavior assessment. This leads to time and economically effective studies. To have a closer approach to in vivo settings, studies in three-dimensional (3D) conditions must be performed.

An innovative top-down photolithographic approach is here proposed to obtain biochip platforms for material/cell interaction studies using biomimetic polystyrene superhydrophobic surfaces (SHS). Cell encapsulation in alginate-based hydrogels was used for proof of concept.

Materials and Methods. The biochips were prepared by:

• Preparation of polystyrene SHS by a phase-inversion method;

• Generation of superhydrophilic spots by exposure of the SHS to UV/ozone radiation using hollow masks (Fig. 1A);

• Deposition of polymeric solutions mixed with cells, further crosslinked with CaCl2.

Results and Discussion. Superhydrophilic spots with controlled shape (squares) could be fabricated in the SHS. Alginate-based hydrogels could be deposited in the spots, keeping separate due to the wettability contrast between the spots and the rest of the substrate, even after immersion in cell culture medium.Two different cell lines - osteoblast-like (MC3T3) and fibroblast-like (L929) – previously encapsulated in the hydrogel matrices were studied after 24 hours of cell culture. The composition of the hydrogels affected cell response, leading to expected tendencies for the well-known polymer mixtures (shown in Fig. 1B).

The evaluation of cell viability and proliferation was performed by direct methods ("chip-destructive"

Materials and Methods: MTS and DNA quantification) and indirect methods (Calcein and DAPI staining image analysis). The results of both methods were consistent (Fig. 1B).

Conclusions. A biomimetic-inspired 3D biochip allowed for HT cell culture study and result analysis of combinatorial polymeric blends.

Acknowledgments. Mariana Oliveira acknowledges the FCT PhD grant SFRH/BD/71396/2010.
Keywords. High-Throughput; Superhydrophobic Surfaces; Biomaterials; Cell encapsulation

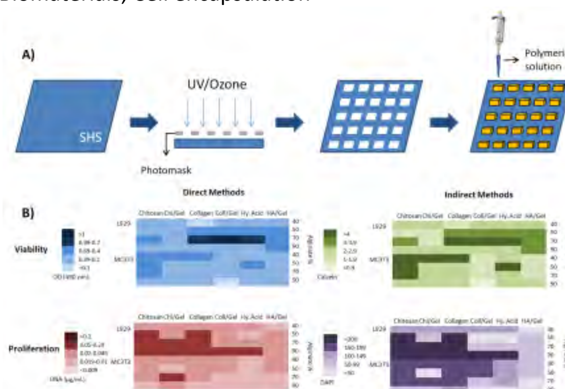

Figure 1 – A) Performance steps for the fabrication of superhydrophilic spots and further deposition of polymeric solution. B) Results of cell viability and proliferation after 24 hours of cell culture, using direct and indirect methods (heatmaps).

(2.O15) ACOUSTICS DIRECTED MICROPARTICLE ASSEMBLY FOR BIOMEDICAL APPLICATIONS

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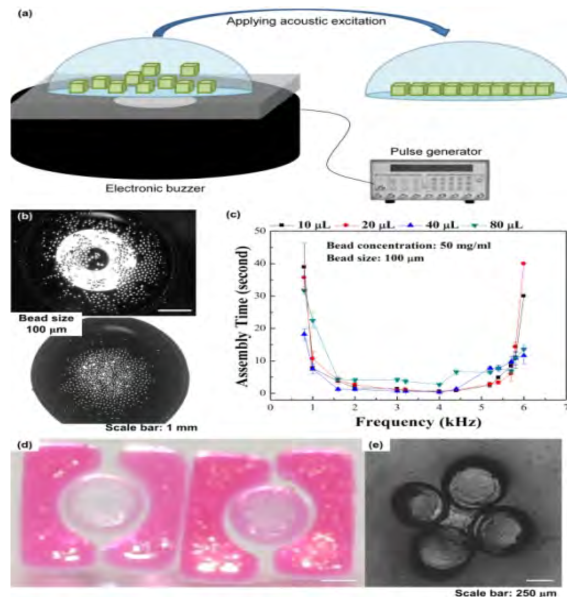
Introduction. Directed assembly of microgels holds great potential for applications in tissue engineering and regenerative medicine. However, there are several limitations associated with the existing techniques (hydrophilic-hydrophobic interactions, surface template) such as complexity of assembly process, involvement of organic solvents. There is still an unmet need for straightforward assembly methods. Acoustic techniques are emerging technologies offering several advantages such as decreased instrumentation complexity and gentler handling of pressure and heat sensitive biological moieties such as cells. However, acoustics have not been used for microgel assembly.

Materials and Methods. In this study we have developed a novel acoustic assembler to assemble microgels, Figure 1a. Microgels (PEG 1000) of different shapes were fabricated using photolithography. The microgels were deposited onto the hydrophobic surface of a petri dish where 40µL of deionized water was added to the group of microgels. The petri dish was placed above a piezo buzzer (Digi-Key, CPE-827) and exposed to acoustic vibrations produced by a pulse/function generator.

Results. To evaluate particle manipulation with our acoustic assembler, we assembled glass microbeads (Figure 1b-c) and microgels with different shapes (Figure 1d-e). After applying acoustic excitation, the microbeads came together at the center of the droplet within 30 sec, Figure 1b. We observed that the microbeads assembly time was dependent on excitation frequency, Figure 1c. During acoustic excitation, we observed that some microgels were immobile due to settling on their untreated surface. It was determined that a frequency sweep provoked mobility in the microgels more so than using a constant frequency, leading to the assembly of orientation specific microgels, Figure 1d-e.

Conclusions. In this study we report an acoustic assembler that utilizes microscale hydrogels as building blocks to create larger constructs via external acoustic fields. This approach has potential to impact multiple fields including tissue engineering, regenerative medicine, and pharmacology.

Keywords. Microparticle assembly, acoustics, microgels



(2.O16) MANDIBULAR RECONSTRUCTION USING AN AXIALLY VASCULARIZED TISSUE ENGINEERED CONSTRUCT

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Introduction. Tissue engineering and Regenerative medicine depend mainly on the so-called extrinsic mode of neovascularization, where the neovascular bed originates from the periphery of the construct. This method is not applicable for large defects in irradiated fields.

Materials and methods. We are introducing a new animal model for mandibular reconstruction using intrinsic axial vascularization by the Arterio-Venous (AV) loop. Cadaveric, mechanical loading, and surgical pilot studies were performed on adult male goats. The cadaveric study aimed at defining the best vascular axis to be used in creating the AV loop in the mandibular region. Mechanical loading studies (3 points bending test) were done to put a base line for further mechanical testing after bone regeneration. A pilot surgical study was done to ensure smooth operative and post operative procedures.

Results. The best vascular axis to reconstruct posterior mandibular defects is the facial artery (average length 32.5±1.9mm, caliber 2.5mm), and facial vein (average length 33.3±1.8mm, caliber 2.6mm). Defects in the anterior half require an additional venous graft. The designed defect significantly affected the mechanical properties of the mandible (P value 0.0204). The animal was able to feed on soft diet from the 3rd postoperative day and returned to normal diet within a week. The mandible did not break during the period of follow up (2 months).

Conclusions. Our model introduces the concept of axial vascularization for mandibular reconstruction after irradiation. This is the first study to introduce the concept of axial vascularization using the AV loop for angiogenesis in the mandibular region. Moreover, this is the first study aiming at axial vascularization at the site of the defect without any need for tissue transfer (in contrast to what was done previously in prefabricated flaps). Qualitative and quantitative data on angiogenesis and osteogenesis is now being further studied by our team.

Keywords. Mandibular reconstruction, Axial vascularization, Bone regeneration, New animal model

(2.P1) ALGINATE FOAMS FOR TISSUE ENGINEERING

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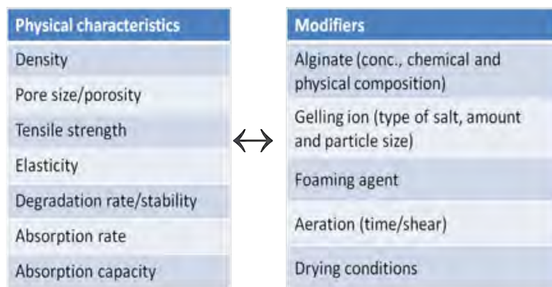
Scaffolds are important tools in the development of applications within tissue engineering and regenerative medicine. Scaffolds made from calcium cross-linked alginate foams are both biocompatible and biodegradable. This study presents alginate foams with controllable physical characteristics. Alginate foams are produced by mechanically agitating a dispersion of an aqueous solution of alginate, plasticizers, foaming agent, gelling agent and slowly hydrolyzing acid. An insoluble gelling ion salt, e.g. $CaCO_3$, is used and Ca^{2+} ions are released as pH is lowered induced by the hydrolysis of D-glucono-delta-lactone. The wet alginate foam is then cast in specific shape using a mold, kept at ambient conditions to complete the gelling reaction, and then dried in an oven at 35-80degC. The integrity of the foams was measured using an SMS Texture Analyzer with tensile grips after the dry foam was re-hydrated in a model physiological solution. Both formulation and process were modified to produce foams with different physical characteristics as shown in the figure. Increasing the particle size of $CaCO_3$ from 4 to 20 µm resulted in increased pore size and decreased foam strength. Increased saturation levels of gelling ions from 25 to 125% led to decreased pore size and increased foam strength and stability. There was a relationship between foam strength and alginate molecular weight. However, at similar molecular weights, stronger foams were formed using a G-rich alginate than an M-rich alginate. Generally, the foams have high pliability, they may be cut into specific shapes and sizes, they are not sticky, they can easily be folded and refolded after hydration, and they can be sutured. The foam will easily dissolve by adding agents that chelate Ca^{2+} such as citrate. Modifications of alginate foam formulations and the production process can be used to construct foams with physical and functional properties tailored for tissue engineering applications.

Keywords. Alginate, scaffold, tissue engineering



(2.P2) THE ADVANTAGE OF COLLAGEN COATING FOR DIFFERENT BIODEGRADABLE MATERIALS USED IN REGENERATION OF THE ABDOMINAL TISSUE

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Introduction. Collagen is proved to be a good biomaterial for use as biomedical implantable device due to its weak antigenecity, excellent biocompatibility, controlable resorbability and ability to integrate with surrounding tissues. It acts as a regenerative template and the collagen which covered implant is progressively degraded and replaced by new cell-synthesized tissue. For implants, the formation of new connective tissue, particularly collagen, plays a key role. The aim of this study was to investigate physical-chemical and morphological characteristics of collagenated meshes and to evaluate in vitro biodegradability and biocompatibility and host tissue response to the prosthetic biomaterials.

Materials and Methods. Different reinforcement meshes for abdominal surgery, made by polypropylene and polyester were impregnated with type I collagen gel, 1.1% (w/w) at 7.2 pH, plasticized with 2% (w/w) glycerin and cross-linked with 0.2% (w/w) glutaraldehyde. Every mesh was immersed into the gel and then free dried at 260C. This step was repeated 5 times and a multilayer integrated membrane was obtained into the knitted structure. These meshes were analyzed by spectroscopic (FT-IR), mechanical and morphological (water absorption, permeability and SEM) analyses. In order to evaluate the collagen matrices the in vitro tests for enzymatic biodegradability and biocompatibility with endothelial cells were also performed. Also, after implantation test, the experimental biomaterials were excised with tissue for histological and scanning electron microscopy evaluation.

Results. The polypropylene and polyester-collagen mesh was very well integrated in the connective tissue, but in the case of polyester mesh was observed the presence of inflammatory elements.

Conclusion. The combination of prosthetic biomaterials with collagen to form various composite meshes will provide a better biointegration of the mesh in the surrounding tissue. Scanning electron microscopy appears as a valuable method in order to establish the biodegradability degree of the biodegradable structures used for abdominal mesh.

Keywords. collagen, abdominal tissue, scanning electron microscopy, biodegradable mesh

(2.P3) A NEW METHOD FOR SELECTIVE OXIDATION OF HYALURONIC ACID – A VERSATILE, NONTOXIC AND CROSSLINKABLE MATERIAL FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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(1) CPN

Introduction. Hyaluronic acid (HA) is a natural linear heteropolysaccharide consisting of D-glucuronic acid and N-acetylglucosamine units, with a molecular weight of 5 - 13000kDa. High concentrations of hyaluronate can be found in skin, vitreous humour, cartilage and the umbilical cord.

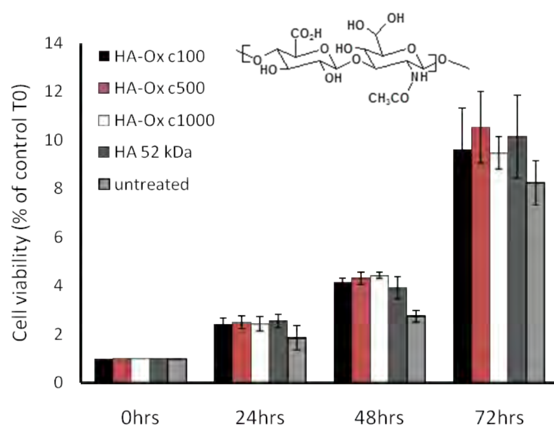
Results. The new type of modification of hyaluronic acid was investigated. The selective oxidation processes leading to the formation of aldehyde moiety in the position 6 of N-acetylglucosamine part of hyaluronic acid dimer were developed and optimised. Two oxidation agents were successfully tested to perform this modification. Dess-Martin periodinane (DMP) in DMSO produced highly substituted derivatives with the degree of substitution (DS) around 50%. Application of DMP caused a significant degradation of polysaccharide resulting the molecular weight around 20kDa (starting material 1MDa). Second system includes 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO)/NaClO in water at lower temperature. Degree of substitution was circa 15%, but no significant degradation was observed (1MDa→ 500kDa). The possibilities to form cross-linked materials were successfully tested using various bis-amino linkers. The structures of products were elucidated by advanced NMR methodologies and by size exclusion chromatography SEC-MALLS.

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to obtain basic information about cell metabolism. The results had shown that the viability of cells after HA-Ox (DS 5-50%, Mw 10-500kDa) treatment was not significantly changed in whole monitored interval (0-72 hours, Fig. 1).

Conclusion. In this study, the synthesis and the influence of HA-Ox on 3T3 fibroblast cell line was examined. HA-Ox did not have any effect on cell viability compared to untreated control and is safety up to 1mg/ml. Crosslinked materials prepared from this precursor are biocompatible and suitable for application in tissue engineering and regenerative medicine.

Acknowledgement: This research project was conducted under financial support provided by the Ministry of Industry and Trade of the Czech Republic.

Keywords. cell viability, oxidation, biomaterial, polysaccharide



(2.P4) IN VIVO BIOPRINTING FOR COMPUTER- AND ROBOTIC-ASSISTED MEDICAL INTERVENTION: PRELIMINARY STUDY IN MICE

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Introduction. Bioprinting technologies have emerged over the last decade to pattern cells and growth factors within 3D engineered structures in vitro.We recently demonstrated the feasibility of using bioprinting in situ and in vivo. This work deals with bioprinting of mesenchymal stem cells and hydroxyapatite nanoparticles into critical size bone calvaria defects of living mice.

Materials and Methods. Critical size bone calvaria defects were performed in fifty-six OF-1 male and in Balb/cJ femelle mice with a 4mm diameter trephine. Hydroxyapatite nanoparticles (n-HA), prepared by wet precipitation, and luciferase-transducted mouse mesenchymal stem cells (D1-Luc cells) were printed directly into calvaria defects using a workstation dedicated to high-throughput Laser-Assisted Bioprinting. Decalcified histology, x-ray microtomography and bioluminescence (Photon Imager-Biospace) were carried out to characterize tissue neoformation over 3 months.

Results. Decalcified histology and x-ray microtomography have shown that in situ bioprinting of nano-hydroxyapatite may favor bone healing. Non-invasive detection, localization and quantification of printed D1-Luc cells using bioluminescence have shown cell survival and proliferation over several weeks.

Conclusion. These preliminary results demonstrate that in vivo bioprinting is possible and that mesenchymal stem cells deposited in situ proliferate. Bioprinting may prove to be helpful in the future for medical robotics and computer-assisted medical interventions.

Keywords. in vivo, bioprinting, bone tissue engineering

(2.P5) USE OF CEMENT IN ANTIBIOTIC IMPREGNATED IN SURGERY ARTHROPLASTY INTERACTIVE

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The surgical cement used to secure the prosthesis in the medullary cavity of the joints is composed of polymethylmethacrylate (PMMA). This polymer allows the attachment of the prosthetic device for penetration into the bone on a metal. The natural wear of the prosthesis occurs inevitably, leading to its replacement after about 10 years of use, through a new procedure (or reintervention) surgery, called interactive arthroplasty. However, the risk of infection foci of infection is also a possible cause of exceptional complication, often requiring a second intervention in order to avoid major problems, along with use of antibiotics postoperatively. The infection may appear early after the intervention (10 to 20%). This study was followed by, first, the effect of adding the antibiotic vancomycin in PMMA reinterventions made during a public hospital in the Brazil, noting the short and medium term evidence of infection. The antimicrobial spectrum of vancomycin is the treatment of gram positive and anaerobic bacteria. Through laboratory tests, he noticed the sensitivity of microbiological patient, realizing that even the additional protocol of 4 to 6 g of antibiotic in the cement, noted the emergence of infections, resulting in replacement of the prosthesis. It was found during reconstruction of PMMA the temperature reached 100°C, causing a loss in biological fixation prosthesis due to cell death in the cement interface, bone, and the degradation of vancomycin, reducing its antimicrobial action. Consequently, using high doses of adjuvant antibiotic for an extended period of 4 weeks. However, in view of the high demand of femoral hip implants and the considerable increase of these failures, we find it essential to intensify the supervision and standardization of all procedures involved in order to optimize the manufacturing process for obtaining a product reliable health care, particularly in the areas of durability and biofunctionality.

Keywords. Arthroplasty, impregnation of antibiotic PMMA

(2.P6) INVESTIGATING THE CONSISTENT SCALED PROCESSING OF HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells (hESCs) are notoriously difficult to maintain in culture. hESC cultures handled inappropriately are often phenotypically and genetically unstable. The analysis of hESC culture quality is complicated by the variation that exists between laboratories within the same hESC lines and between different hESC lines (documented by the ongoing ISCI project - International Stem Cell Characterisation Initiative). There is little clarity regarding the extent to which these differences are intrinsic to the cell lines, the measurement systems or simply the adaptation to different culture conditions or platforms.

The culture of hESCs is further complicated by the gold standard method that produces heterogeneous colonies in undefined culture media using animal components. This state of affairs is not tenable as a platform for regulated therapeutic products where cells of measurable and reproducible purity and potency from a GMP compatible production system are regulatory necessities. If this is ever to be realised, the standardisation of large-scale culture systems capable of achieving consistent cell populations will need to be developed.

This project is driven by the requirements of the project partner, the UK Stem Cell Bank (UKSCB), to achieve reproducible and scalable culture methods for the distribution of stem cells and, builds on the recently published success from Thomas et al(2009, Biotechnology and Bioengineering)demonstrating the capability of a large scale robotic system (CompacT Select) at maintaining both pluripotency and a consistent proliferation rate of hESC lines Hues-7 and Nott-1. The project aims to further characterize the processing of hESCs under different culture conditions by systematically investigating the responses (hESC critical to quality marker profiles) and interactions between several key processing parameters, identified through the creation and analysis of high-detail process maps in an attempt to determine optimal windows of operation for the consistent large scale production of high quality hESCs.

Keywords. Human embryonic stem cells, pluripotentcy, large scale, automation, optimisation, quality, process control

(2.P7) POROUS GELATINE-HYDROXYAPATITE COMPOSITE SCAFFOLDS VIA GAS-IN-LIQUID FOAM TEMPLATING

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Introduction. Gelatin and hydroxyapatite (HAp) sponges because of their biocompatibility and biodegradability have the potential to be used as scaffolds to support osteoblasts and to promote bone regeneration in defective areas. In this work gelatine and HAp composites were fabricated in a foam type via a novel foam templating technique.

Materials and Methods. A dispersion of nano HAp particles in a concentrated solution of gelatine and an appropriate surfactant was foamed using hexafluoroethane as the blowing agent. The foam, once formed, was frozen in liquid nitrogen and then freeze-dried. Subsequently it was cross-linked with a carbodiimide derivative to retain its chemical and thermal integrity. X-ray computed microtomography was used to nondestructively and quantitatively measure the three-dimensional porosity and the morphometric parameters. The samples were scanned with a Skyscan 1072 μ-CT imaging system (Belgium) at 7,32 μm resolution and with following settings: 40 kV and 250 μA. Image reconstruction and analysis were conducted using the software package provided by Skyscan.

Results. All the scaffolds synthesised exhibited an excellent, totally interconnected trabecular morphology. A content of HAp up to 40 % w/w was achieved. Through μ-CT it was shown that HAp particles are distributed homogeneously within the gelatine framework (fig.1). In order to achieve a higher level of HAp content, similar to that of natural bone (~ 70% w/w), the composite scaffold characterised by a HAp content of 40 % w/w was subjected to four cycles of deposition of HAp on the scaffold walls. The final content of HAp as determined by thermogravimetry was very close to 70 % w/w.

Conclusion. The foaming technique described, associated with the deposition procedure permits the preparation of scaffold that fulfil both from a morphological and compositional point of view the main characteristic of trabecular bone and as a consequence are promising as constructs for bone tissue engineering.

Keywords. Micro-computed tomography, biomaterials, bone substitutes, scaffold

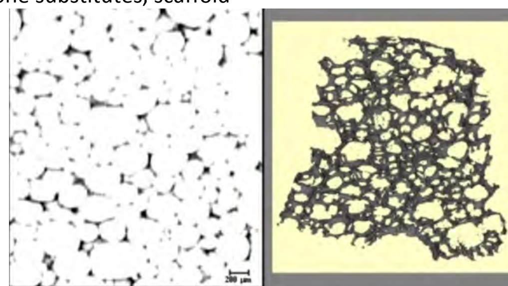

Fig. 1: 2D microtomography slice (left) and 3D reconstruction (right) of a series of 15 slices of a sample.

(2.P8) LAYER-BY-LAYER BIOFABRICATION USING LASER-ASSISTED-BIOPRINTING AND ELECTROSPINNING ENHANCES CELL PROLIFERATION IN VITRO AND IN VIVO

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Introduction. Laser-Assisted-Bioprinting (LAB) is an effective printing technology for patterning cells, biomolecules and biomaterials, and electrospinning may be used to build thin membranes of polymers. The aim of this work was to associate LAB and electrospinning to achieve three-dimensional cellularized materials and to evaluate the influence of layer-by-layer bio-fabrication on MG63 cell proliferation in vitro and in vivo.

Materials and Methods. The LAB setup comprised an infra red laser (Nd:YAG 1064 nm, 30 ns) controlled by scanners, and focused onto glass ribbons coated with a gold absorbing layer (30 nm). Space between ribbon and quartz substrate was 400μm. The Polycaprolacton (PCL) scaffolds (100μm thick) were prepared using a PCL solution (20% w/v in CHCl3) loaded into a syringe and electrospun using a pump and a high voltage generator. MG63 osteoblastic cells transfected with luciferase were cultured in complete medium (IMDM supplemented with 10% FBS). The concentration of cell bio-ink was 50.106 cells/ml, suspended in 1% alginate solution (w/v) and culture medium. The building sequence of the test group comprised three sequential layers of cells and PCL scaffolds stacked. In the control group, a similar amount of cells was printed over three PCL membranes stacked. Then, the materials were cultured in vitro during 3 weeks or implanted 2 months in bone calvarial defects of 20

NOG mice. Follow-up was done using photon imager quantification in vitro and in vivo and histological analyses.

Results. In vitro and in vivo results have shown that layer-by-layer bio-fabrication significantly enhanced cell proliferation. Histological analyses confirmed that the tissues retrieved after sacrifices were thicker in the layer-by-layer group.

Conclusions. We have demonstrated in this model that a layer-by-layer bio-fabrication using LAB and PCL scaffold is an efficient combination to improve cell proliferation in vitro and in vivo.

The authors would like to thank the IFRO, the FRM, the GIS-AMA and the Aquitaine Region for financial support.

Keywords. Layer-by-Layer; Electrospinning; Laser Assisted Bioprinting

(2.P9) IN VITRO ENGINEERING OF A TRACHEAL EPITHELIUM: CO-CULTIVATION OF TRACHEAL EPITHELIAL CELLS AND FIBROBLASTS ON SMALL INTESTINAL SUBMUCOSA SEGMENTS

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Introduction. Surgical correction of large tracheal defects remains to be a tackling problem. Lesions that cannot be treated by an end-to-end anastomosis and need an interponate which often fail to regenerate a functional tracheal epithelium. Here we investigated whether suturable decellularized small intestine submucosa (SIS) may serve as matrix for the in vitro generation of tracheal epithelium.

Materials and Methods. Primary tracheal epithelial cells and fibroblast were harvested from porcine trachea by Protease XIV and Collagenase A digestion, respectively, and cultured in their appropriate culture media. For seeding purposes decellularized SIS, generated from porcine small intestine by decellularization, was clamped in stainless steel frames. Primary isolates of epithelial cells were seeded onto the sub-mucosa side of the SIS after reaching 80% of confluency in culture flasks. Stimulatory effects of tracheal fibroblasts were tested by seeding cells onto the sub-serosa side of the SIS (constructs without fibroblast served as controls). SEM and Histology analysis of constructs were conducted after five days of culture of which three were spent as air liquid interface culture.

Results. SEM examination and Phalloidin stains show a completely covered SIS with orientated respiratory epithelium. Immunohistochemistry against Cytokeratin 14 (basal cell marker), Mucin 5AC (goblet cell marker) and β-Tubulin IV (ciliate cell marker) demonstrated a pseudostratified-like epithelium. The production of glycosaminoglycan and Mucin 5AC was more pronounced after fibroblast co-culture.

Conclusion. Decellularized SIS is suited for culturing tracheal epithelium and may serve as useful matrix for tracheal tissue engineering purposes for the generation of surgical implants.

Keywords. tracheal epithelium, tissue engineering, SIS, air liquid interface culture

(2.P10) BIOFABRICATION OF TISSUE ENGINEERED VASCULAR GRAFTS

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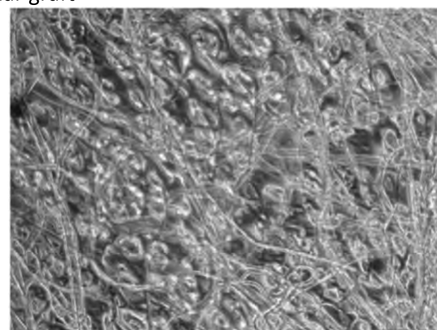
Cardiovascular disease is the largest contributor to mortality in the world claiming nearly 30 percent of all deaths. Tissue engineered scaffolds are essential for small diameter vascular grafts to avoid the fatal risk of thrombosis of the synthetic vascular grafts. In this work, biomimetic gelatine/elastin fibrous scaffolds are proposed, fabricated by electrospinning as tubular constructs. Tissue engineering then takes place in vitro in a bioreactor, in which the tubular scaffold is rotated in a bioreactor surrounded by a smooth muscle cell (SMC)-culture medium suspension, while a suspension of endothelial cells (ECs) flows axially inside the tubular scaffold in a recirculating flow. A novel fluorescence quenching type of sensor has been developed to be embedded at different positions in the scaffold for continuously monitoring the oxygen concentration in the growing tissue. Adherence, growth and proliferation of both types of cells is examined for different scaffold structures and different processing conditions, such as cell concentration, flow rate of the cell-culture medium suspension and rotation speed of the scaffold. The fibrous scaffolds have been crosslinked using glutaraldehyde as a crosslinking agent. Cytotoxicity studies are also carried out to investigate the effect of glutaraldehyde on the cell growth and proliferation.

Keywords. Scaffold, Fluorescent quenching, bioreactor, vascular graft



(2.P11) FABRICATION OF THREE-DIMENSIONAL CELL-LADEN HYDROGEL FOR SOFT TISSUE ENGINEERING

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Three dimensional (3D) scaffolds should be porous to transfer oxygen and nutrient for cell proliferation and differentiation in tissue engineering. Scaffolds have been fabricated using various conventional techniques of salt leaching, freeze drying, fiber bonding, phase separation, and gas expansion. However, they have a limitation of homogeneous cell distribution on the scaffold. Scaffold fabrication techniques need to control 3D pores inside scaffold. In these methods, solid freeform fabrication (SFF) of rapid prototyping (RP) technology has been adopted to 3D scaffold design with controllable and reproducible porosity and well-defined 3D structures for tissue engineering. Especially, soft tissue has a very high

content of water, so scaffolds need a hydrogel material. Hydrogel biomaterials can provide the micro environment to build up by living cells and the extracelluar matrix (ECM) due to their structural similarities to the body tissues, biocompatibility and low toxicity. In this study, we manufactured 3D scaffold plotting system (SPS) to design interconnected scaffold and fabricated interconnected hydrogel scaffold with cells through plotting process and developed the software using the geometrical data obtained from stereolithography (STL) file format for SPS operation. Also, we fabricated cell-laden hydrogel scaffold including gelatin to help cell growth. 2% alginate with cells was plotted under various pressure conditions of SPS system. Cell-laden alginate hydrogel had a regular cell distribution and a good cell viability in vitro test. We confirmed the potential of the 3D hydrogel scaffold for soft tissue engineering application.

Keywords. scaffold, fabrication, hydrogel, soft tissue engineering

(2.P13) DEVELOPMENT OF A NEW TECHNIQUE FOR MUSCLE TISSUE-STEM CELLS CO-CULTURE

Erratico S (1), Belicchi M (2), Razini P (2), Farini A (2), Meregalli M (2), Villa C (3), Torrente Y (2)

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Introduction. Adult stem cells reside in all tissues, where they maintain homeostatic conditions and respond to injuries. These cells are regulated and supported by the surrounding microenvironment, called stem cell "niche", composed by cellular and molecular factors, that interact with and regulate stem cell fate. Key niche components are represented by growth factors, cell-cell interactions and cell-matrix adhesion. Also in muscle tissue niches are present, especially in myofiber basal lamina, where a network of extracellular matrix components and secreted growth factors stimulate muscle stem cell survival, activation and/or proliferation. In order to better understand the influence of muscle tissue secreted factors on stem cells, we developed a new technical approach to perform a muscle tissue-stem cells co-culture; we focused on peripheral blood derived CD133 cells, a population known to possess a myogenic potential.

Materilas and Methods. Muscle tissue sections were cut with a tissue chopper from fresh quadriceps of C57BL mice and inserted in a culture well upon a porous membrane, above cells suspension; cells were isolated through immunomagnetic separation column; immunophenotypic characterization was performed with Cytomics FC500.

Results. The culture system developed creates a physical separation between tissue section and stem cells, allowing soluble factors exchange and preventing tissue-cell contamination; the absence of cell mix was evaluated through cytogenetic analysis of cell karyotype; the reproducibility of the technique has been demonstrated through muscle slice weight monitoring. Preliminary proliferation experiments show an increase in CD133+ stem cells rate of proliferation in presence of muscle tissue; cell immunophenotype monitoring confirmed stemness maintenance.

Conlcusiones. We have developed a reproducible and standardized technique and designed a culture system that guarantees, spatial division of culture environment, no cellular contaminations between culture compartments, in vitro tissue survival, stem cell viability and proliferation, communication between tissue and cells through chemical signals (factors release).

Keywords. stem cells; co-culture; muscle tissue

3. BIOFUNCTIONAL MATERIALS AS EXTRACELLULAR SIGNALS TO PROMOTE TISSUE MORPHOGENESIS

Chair: Elisabeth Engel
Co-chair: Josep A. Planell
Keynote speaker: Abhay Pandit
Organizers: Josep A. Planell, Elisabeth Engel

Synopsis: Regenerative medicine based on tissue engineering needs a step forward in biomaterials design coupled with a search for novel activities and evaluation of their behaviour in biological systems. The body's capacity to regenerate it is not well elucidated but several signals implicated in regeneration have been revealed already. Among all of them, signals connected to adult stem cells mobilization to the injury site and activation of the repair scheme as well as new tissue formation are the most relevant. The ability to direct stem or progenitor cell differentiation via a chemically/naturally synthesized biomaterial, without the need to incorporate growth factors or other molecules that might induce undesirable effects, offers many potential advantages in regenerative medicine. The properties of the own materials are the ones that stimulate cells to produce the appropriate chemokines and growth factors to promote cell activation. This activation can be the mobilization of stem cells out of its niche to go to the injured tissue. At the injured site, cells will produce the molecules to induce tissue repair. For example, ion release can induce this mobilization and call the progenitors to the implant site.

Besides chemical signalling we have to take into account the physical signalling to induce the most appropriate response of the surrounding cells. Surface topography has been demonstrated to have an effect in several biological activities, as cell adhesion, migration, proliferation and differentiation. But we cannot forget the mechanical properties of the biomaterials, as it has been already demonstrated. The surface stiffness plays a definite role in stem cell differentiation, when mimicking the tissue stiffness. Thus, inflammation is also a mechanism in tissue repair. The use of biomaterials that could modulate inflammatory responses to avoid chronic inflammatory responses (characterized by leukocyte adhesion and fibrous encapsulation) but promoting a signalling cascade that will induce tissue formation is also a major issue in tissue engineering. This holistic view will be the next

generation of biomaterials to be applied in advanced therapies to treat diseases related to tissue degeneration. The properties of the biomaterials will conduct the own body repair.

(3.KP) A FUNCTIONALISED SCAFFOLD FOR MODULATION OF INFLAMMATION TO PERMIT STEM CELL SURVIVAL IN MYOCARDIAL INFARCTION

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Cardiovascular disease is the leading cause of death in the developed world and is responsible for approximately 36% of Irish mortality. Myocardial infarction (MI), which is literally the death of cardiac tissue due to lack of oxygenation, accounts for the majority of deaths associated with cardiovascular disease. This death of cardiac tissue leads to a loss of cardiac function as the damaged area becomes a non-contractile scar. Reversal of this process is the main aim of regenerative cardiac strategies such as stem cell transplantation. While initial studies were promising, subsequent clinical trials yielded disappointing results. Stem cell therapy may be limited by the poor survival rate of the cells after implantation into the infarcted heart, which is likely due to the inflammatory response. Thus, anti-inflammatory gene therapy with interleukin-10 (IL-10) was proposed as a method to modulate the inflammatory response after implantation of a collagen scaffold seeded with rat mesenchymal stem cells (rMSCs). IL-10 is considered the most potent anti-inflammatory cytokine produced naturally and has been used in a number of studies to decrease or control inflammation. It was hypothesized that IL-10 gene therapy could be used to increase the retention rate of stem cells in a collagen scaffold when delivered to the ischemic myocardium. The primary objectives were to develop a controlled release scaffold-based gene therapy system suitable for stem cell delivery to the infarcted myocardium. The efficacy of this system was evaluated by assessing stem cell retention, overall cardiac function and the inflammatory response. A crosslinked collagen scaffold was developed and optimised for rMSC culture in vitro. Non-viral plasmid-dendrimer polyplexes were optimized for transfection in both two and three-dimensional culture. When cells were seeded into polyplex loaded scaffolds, relatively high levels of transgene expression were observed for up to three weeks of culture. When the polyplex-loaded scaffolds were implanted in rat skeletal muscle, increased retention of rMSCs was observed. This was associated with decreased inflammation and a change in macrophage phenotype from cytotoxic to regulatory. Similarly, when the polyplex-loaded scaffolds were implanted over the surface of infarcted rat hearts, rMSC retention was increased, the inflammatory and remodelling responses were modulated and, most importantly left ventricular ejection fraction – a measure of cardiac function – was significantly improved. Thus, combining biomaterial, gene and cell therapy improved functional outcomes after rMSC transplantation following MI. This combinatorial strategy can be utilised to provide functional efficacy in disease targets.

(3.O1) EVALUATION AND PREDICTION OF ACUTE INFLAMMATORY CHARACTERISTICS OF IMPLANTABLE SYNTHETIC AND TISSUE-BASED BIOLOGIC MESHES USING A SENSITIVE QUANTITATIVE IN VITRO CHEMILUMINESCENT ASSAY

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Clinical performance and therapeutic outcome of mesh assisted soft tissue augmentation is decided early after implantation as leukocytes interrogate the graft in the first day postoperatively. High degrees of leukocyte activation lead to chronic pain. Reactive oxygen species (ROS) are released by leukocytes when activated. This response can be used as a sensitive measurement of leukocyte activation. The aim of this study was to compare the degree of leukocyte activation of commercially available synthetic and biological meshes.

Materials and Methods. Chemiluminescence assay was performed using modifications to a commercially available kit (Knight Scientific, UK). Whole blood was obtained from 5 different healthy human adults, combined with Adjuvant K, Pholasin and graft, and incubated for 30 minutes with continuous chemiluminescent measurements. Leukocyte stimulants fMLP and PMA were added as controls. Synthetic meshes of varying chemistry (PP, PET, PGA) and knitting patterns and xeno- and allogeneic dermis and small intestinal submucosa (SIS) biological meshes prepared with varying decellualrisation techniques. Statistics were performed using Waller-Duncan post hoc ranking into statistically homogenous subsets (Fig.1).

Results. Chemiluminescence measurements of ROS demonstrated material specific differences in leukocyte activation. Among synthetic meshes, multifilament PGA mesh had significantly higher responses compared to PP and PET meshes ($p<0.05$). Yarn conformation (ie. mono- vs multi-filament) made a greater difference to the leukocyte response than polymer composition. Waller-Duncan post hoc ranking allowed grouping of the materials into statistically similarly ROS stimulating groups.

The biological meshes demonstrated significant differences in leukocyte activation as a function of decellularisation reagent and related tissue origin, the SIS mesh and SDS decellularisation strategies eliciting the greatest stimulation.

Conclusion. The most leukocyte activating synthetic and biological meshes were the Multifilament PGA mesh and the SIS mesh, respectively. In the case of synthetic meshes it was concluded that weave is a greater influence on leukocyte response than polymer chemistry.

Keywords. In Vitro, Leukocyte, Inflammatory Response, Hernia

(3.O2) IMPROVEMENT OF BIOLOGICAL PROPERTIES OF POLYMERIC MATERIALS THROUGH THE BIOFUNCTIONALIZATION WITH ELASTIN-LIKE POLYMERS

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Introduction. In tissue engineering, scaffolds made up of synthetic polymers are usually selected because they fit in

many aspects the requirements of biomedical materials, such as biocompatibility, biodegradability, malleability. However, these synthetic polymers lack bioactivity, i.e. they do not present groups or moieties that guide the interactions between materials and cells, which difficult the implementation of such devices in the biomedical field. In order to improve that aspect, approaches based on the incorporation of active biomolecules on the material surface have been widely investigated. One of the most known and used biomolecule is the RGD peptide sequence, which has been implemented on surfaces in the form of short peptide. This report proposes the incorporation of the RGD sequence through the functionalization of the biodegradable polymeric surface with an elastin-like polymer (ELP) that includes the RGD inside the amino acidic chain. The ELPs are a genetically modified version of the natural elastin. The natural origin gives to the ELP constructs mechanical properties that are not found on short peptides and a more natural cell environment. The report compares the cell response against surfaces of poly(lactic acid) functionalized with ELPs and short peptides. Special emphasis has been put in the comparison of covalent functionalization against the physisorption. Also an ELISA-based assay is proposed for the quantification of peptides on surface.

Materials and Methods. Functionalization of surfaces is obtained through the creation of amide bonds with the EDC/NHS chemistry. Cell response against the different treated surfaces is studied through the quantitatively analysis of the cell adhesion capacities and cell proliferation. For the quantification of grafted molecules, a PEG-biotin molecule is used as an analogue of a peptide molecule in order to draft the design of the assay.

Results and Conclusion. The enhancement of the material biological properties through the ELP functionalization has been proved to be higher than the enhancement obtained with the short peptides. The ELISA-based assay has proved to be able to quantify the amount of biomolecules on surface.

Keywords. biofunctionalization, cell-material interaction, tissue engineering

(3.O3) MOLECULAR MECHANISM INVOLVED IN THE WOUND HEALING EFFECT OF SILK PROTEINS FIBROIN AND SERICIN

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Introduction. Wound healing is a biological process directed to the restoration of tissue that has suffered an injury. An important phase of wound healing is the generation of a basal epithelium able to wholly replace the epidermis of the wound. A broad range of products derived from Fibroin and Sericin is used to stimulate wound healing. However, so far the molecular mechanism of this phenomenon has not been determined. Fibroin is a protein secreted by the silkworm Bombyx mori and has unique properties such as good biocompatibility, lack of immune response and biodegradability. Sericin is the second main silk protein, being the sticky material surrounding Fibroin fibers. The aim of this work is to determine the molecular basis behind wound healing properties of silk using a cell culture model.

Materials and Methods. For this purpose, we assay Fibroin and Sericin in a wound healing scratch assay using Mv1Lu and MDA-MB231 cells. Both proteins stimulate cell migration. Furthermore, treatment with Sericin and Fibroin regulates key factors of the wound healing process upregulating c-jun gene expression and c-Jun protein phosphorylation. Moreover, Fibroin and Sericin stimulates the phosphorylation of SAP/JNK kinase and phosphorylation of ERK 1 and 2. All these experiments were done in the presence of specific inhibitors for some of the cell signalling pathways referred above.

Results and Conclusion. The obtained results revealed that only the inhibitors of SAP/JNK kinase, but not p38, PI3K or ROCK inhibitors prevent cell migration stimulated by Fibroin or Sericin.

Keywords. Fibroin, Sericin, Wound Healing, Silk

(3.O4) DYNAMIC SURFACES TO INFLUENCE STEM CELL DIFFERENTIATION

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Introduction. Stem cell differentiation is governed by a series of complex intracellular signalling pathways. One of the main pathways, the ERK1/2 mitogen activated protein kinase pathway relies on integrin dependent cell-surface interactions to trigger a cascade resulting in changes to gene transcription and expression. The intention of this work is to synthesise a functionalised surface capable of switching from a state that does not actively promote cell adhesion "off" to a state that readily promotes cell adhesion "on" and hence dynamically influence stem cell differentiation. This is achieved using Fmoc (9-Fluorenylmethoxycarbonyl) protected amino acids to build up the desired peptide chain on the substrate (Figure 1). Here the peptide sequence is RGD, a known integrin binding peptide sequence. On demand switching is achievable by enzymatically removing the terminating Fmoc group of a short cleavable peptide which, when attached, acts to prevent cells from interacting with the underlying integrin recognition motif. The long-term aim of this project is to synthesise a surface that can be enzymatically switched by the cells as they run out of room to proliferate and begin to differentiate thus allowing synchronised progression directed by the dynamic material surface.

Method and Materials. Surfaces were synthesised using a method described by Todd et al 2009 [1]. After surface modification, Human mesenchymal stem cells (hMSCs) were cultured directly onto surfaces containing both the "bio-active" RGD and non "bio-active" RGE forms in both the cleaved ("on" state) and non-cleaved ("off") states (Figure 1). After a period of 7 days cell spreading and cell morphology were quantitatively investigated using fluorescence microscopy by staining for actin and vinculin alongside nuclear staining. Focal adhesions were analysed by tracing adhesion outlines and using Image J software to determine numbers and lengths.

Results and Conclusion. For the cells to respond to the "switching on" of RGD groups through enzymatic cleavage of Fmoc, we have derived optimal media conditions. In these conditions, as Fmoc is cleaved, the MSCs rearrange their adhesions increasing numbers of larger adhesions. The formation of large adhesions is important for osteogenesis through support of increased intracellular tension. Future work will focus on balancing MSC proliferation and differentiation through dynamic surface properties [2-3].

With thanks to the EPSRC

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Keywords. Stem Cells, Bioactive Surfaces, RGD, Integrins

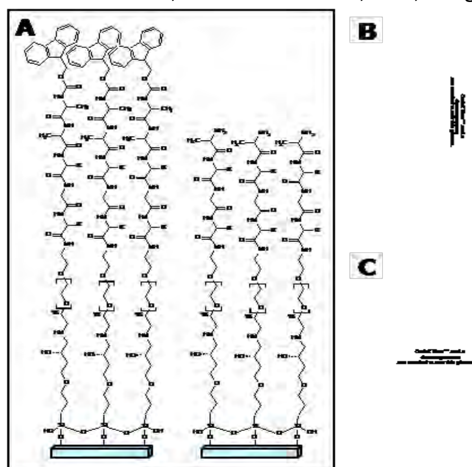

Figure 1: (A) Representation of proposed surface chemistry. When the terminating Fmoc group is in place the RGD sequence is hidden (L). Enzymatic digestion of the cleavable linker with elastase exposes the underlying sequence to the cells (R). R groups are Aspartic acid: CH_2COOH, Glutamic acid: CH_2CH_2COOH and Arginine: $(CH_2)_2C(NH)_2NH_2$. **(B)** Human Mesenchymal stem cell cultured on cleaved surfaces with the "bio-active" sequence RGD and **(C)** Human Mesenchymal stem cell cultured on cleaved surface containing the non "bio-active" sequence RGE. Focal adhesions for both cells are highlighted in pink.

(3.O5) MUSCLE GRAFT OPTIMISATION PRIOR TO IMPLANTATION: SCAFFOLD ARCHITECTURE AND FUNCTIONALISATION INFLUENCE CELL DIFFERENTIATION

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1. Inselspital; Empa; 2.Inselpital

Introduction. Cell therapies and associated paracrine effects for heart regeneration have gained increasing interest. Epicardial implantation of engineered muscle-grafts has been associated with prolonged functional recovery of the ischemic hearts. These observed effects are expected to originate from the cell secretion of cardioprotective, angiogenic or stem cell recruiting factors or by local delivery of these factors via functionalisation of the implanted scaffold. In the present study we performed in vitro studies to investigate the effects of scaffold architecture and surface functionalisation on muscle graft development and related cytokine secretion.

Materials and Methods. Aligned and randomly oriented micron- (3.2±0.8um) or nano- (308±178nm) scaled fibrous polycaprolactone non-wovens were processed by electrospinning. A 15 nm thick oxygen functional hydrocarbon coating was deposited at the surface by an RF plasma process (gas mixture: CO_2:C_2H_4 ratio 6:1; power input: 50 W; process duration: 20 minutes) and characterised by XPS. C2C12 muscle cells were grown on the matrices and analysed for viability, proliferation, orientation and myotube formation. Cell orientation was characterised by a cosine function, where S=1 for aligned and S=0 for randomly oriented cells. Cytokine secretion was assessed using antibody arrays.

Results. The formation of a stable plasma polymer coating resulted in an 8-14% increased oxygen content on the matrix. On all scaffolds, cell viability varied from 40 to 60% relative to TCPS. Architectural cues highly influenced cell orientation. On aligned fibres, cells were highly oriented (S=0.88±0.02) as compared to randomly oriented fibres (S=0.33±0.2). Increased myotube formation was found on CO_2/C_2H_4 coated scaffolds. Graft contractility and cytokine secretion are under evaluation.

Conclusion. We provide evidence that the combined application of architectural and chemical cues is most favourable for advanced muscle development. Fibre alignment and plasma coating induced most pronounced cell differentiation. Ongoing cytokine release identification will further characterise this biograft and its possible promise for cardiac regeneration.

Keywords. electrospinning, plasma coating, muscle differentiation

(3.O6) EFFECT OF LINE PATTERNED CHITOSAN ON CORTICAL NEURAL CELLS

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Introduction. Topographical cues have a direct effect on cell guidance and differentiation. After a lesion in the central nervous system is necessary to promote a regenerative permissive environment that allows replenishment of lost neurons and that guide regenerating axons to their appropriate targets. In this study we considered Chitosan (Ch), a biodegradable biocompatible material carrying good neuronal adhesive properties. We cultured neurons and glial cells on uncoated Ch films and we assessed the effect of line patterns in terms of cell differentiation and orientation by western blot and immunocytochemistry.

Materials and Methods. Patterned Ch films were obtained casting a 2% Ch solution on micro-grooved moulds (2 and 10 µm wide,1 µm deep). 2% Ch films were highly hydrophilic (Contact Angle 34º±3), positively charged (Z potential 15±3mV) and had high water absorption (128±10%.). From the mechanical point of view, Ch films were quite soft and elastic, having the following values: Young Modulus 5.7±1.4 MPa, Elongation at break 56±14% and Tensile Strenght 4.31±0.8 MPa.

Results. Ch films supported good neuronal and glial growth and the presence of micropatterns induced alignment. In the case of neurons, alignment was selective for axons but not for dendrites. Axons on 2µm lines followed single channels, while on 10µm lines, axons form bundles, mimicking their physiological 3D structure. In the case of glial cells, alignment involved the cytoskeleton and not the whole cell shape. Biochemically, flat and micropatterned Ch films promoted a general maturation of glial cells, resulting in an increase of the mature astrocyte marker GFAP and a decrease of the immature astrocyte markers BLBP and Nestin, while they didn't alter the protein expression of neurons.

Conclusion. Uncoated Ch films promoted neurons and glial cells attachment and maturation. The in vivo regenerative ability of Ch scaffolds will be assessed implanting them into the brain of neonatal mice.

Keywords. chitosan, micropattern, nerve regeneration

(3.O7) RESPONSE OF NEURAL CELLS TO DIFFERENT TYPES OF POLYLACTIC ACID

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Introduction. The tissue engineering approach to improve nerve regeneration after an injury is to implant materials that trigger a regenerative response in situ, promoting a favorable environment. This project explores the potential of using Poly L/DL Lactic Acid (PLDLA), an FDA approved biocompatible and biodegradable polymer, as scaffold for nerve tissue engineering. PLDLA 95/5 and 70/30 were used in this study, which contain different proportions of the isomers D and L, have different cristallinity, degradation rate and surface roughness.

Materials and Methods. PLDLA 95/5 and 70/30 films were obtained by solvent casting. Embryonic day 16 neurons (E16) and post natal day 0 (P0) glial cells from mice cerebral cortex were seeded on uncoated PLDLA films, cultured for 5div and analyzed by imunocytochemistry and western blot.

Results. Both neurons and glial cells attached on PLDLA 70/30 and on 95/5, but neurons attached with more affinity on 70/30. In addition, PLDLA70/30 induced a more undifferentiated phenotype of both type of cells. Glial cultures on PLDLA 70/30 expressed high levels of Nestin, BLBP and PH3, markers of proliferating radial glia progenitor cells, while in neuronal cultures increased Pax6 and Tbr2 markers, characteristic of radial glia progenitor cells and neuron restricted progenitors.

Conclusion. This study showed that neurons and glial cells grow on uncoated PLDLA films. PLDLA70/30 was a better substrate than PLDLA95/5 for neural cells growth and promoted an environment rich in progenitor cells. Those results suggest that differences in the proportion of the isomers D and L in the same polymer can induce different responses and that PLDLA70/30 could be a good material for implantation, since it could trigger an in situ regenerative response.

Keywords. brain, scaffold, tissue engineering

(3.O8) USE OF ACELLULAR WHOLE PIG LUNG AS A SCAFFOLD FOR STEM CELL BASED PRODUCTION OF ENGINEERED LUNG TISSUE

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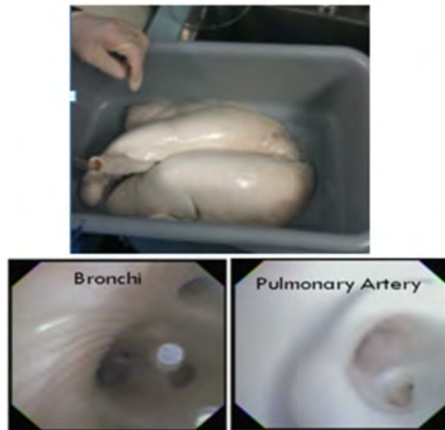
We report here the first attempt to produce and use whole acellular pig lung as a matrix to support development of engineered lung tissue from murine embryonic stem cells (mESC), pig mesenchymal stem cells or human amniotic fluid mesenchymal stem cells. Using a combination of freezing, use of deinonized water and 1% SDS washes administered through both the trachea and the pulmonary artery twice daily for two weeks, four intact pig trachea-lungs were decellularized. Once decellularization was complete we evaluated the effects of our decellularization process on the structural integrity of the lung using two photon microscopy, biochemical assessment of the extracellular matrix and pulmonary function tests (PFTs). Two photon microscopic examinations of trachea and lung tissues showed no cell remnants but some changes in collagen and elastin content as decellularization progressed. Biochemical evaluation of the pig trachea-lung indicated some loss of type IV collagen but retention of elastin and collagen I. PFT measurements of the trachea-lungs showed normal work of breathing, and a non restricted flow pattern. Analysis of the decellularized tissues did not indicate that significant levels of unfragmented DNA remained in the acellular pig trachea-lungs. Although there were some changes in extracellular matrix they were not significant as evidenced by low normal PFTvalues. When repopulated with bone marrow derived mesenchymal stem cells (MSC) or murine embryonic stem cells (mESC) the scaffold supported cell attachment and site specific differentiation. Repopulation of this matrix was similar to what we have the previously described (1) using rat trachea-lung as scaffold. Bone marrow derived pig MSC or mESC cells cultured for 21 days expressed lung cell specific phenotypes such as surfactant protein C, Clara Cell protein 10 and thyroid transcription factor -1.

Keywords. engineered lung, acellular scaffold



(3.O9) SURFACE PATTERNING IN STEM CELL DIFFERENTIATION

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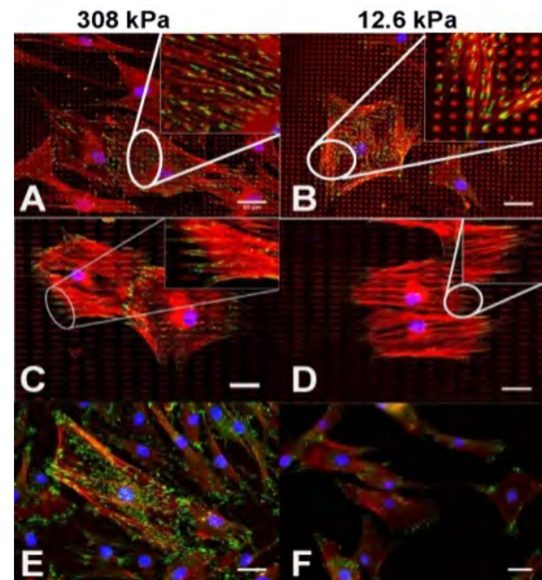
While soluble factors has been the classical method to direct stem cell differentiation, there are growing evidences illustrating the potential of physical cues such as surface properties and matrix stiffness in doing the same. In our work, micro-patterns as big as 20µm lanes to as small as 3µm squares were created on polymer films were created on the surface of polymers to trigger specific human mesenchymal stem cells (hMSCs) differentiation. Stem cells differentiation was characterized by qPCR and immunostaining.Cells cultured on the lane patterns assume highly elongated and spindle shape. Gene expression analysis revealed up-regulation of markers associated to neurognesis and myogenesis while osteogenic markers were specifically down-regulated. However at the functionally relevant level of protein expression, the myogenic lineage is dominant within the time scale studied as determined by the exclusive immuno-detection of cardiac myosin heavy chain for the micropatterned cells. On smaller patterns, the cellular shape change was less defined but the focal adhesions (FAs) showed strong correlation to the patterns. The FAs were regulated into dense and elongated patterns when the micro-patterns were of small square (3.6X3.6 µm) and rectangular (2.5X20 µm) shapes respectively. The synergistic effect of FAs regulation and matrix stiffness was also explored. The results indicated that dense FAs would not induce myogenesis while elongated FAs could promote cytoskeleton alignment and further myogenesis on PDMS substrate with intermediate stiffness of 12.6 kPa at both mRNA and protein level. But on stiff substrate (308 kPa) with or without patterns, the cytoskeleton alignment and myogenesis was not obvious. This work demonstrates for the first time that it is possible to induce hMSCs differentiation by regulating the FAs without any biochemic

We would like to acknowledge Singapore Stem Cell Consortium (SSCC) (Grant no: SSCC/09/017 for financial support.

Keywords. micro-patterning, focal adhesion, cell shape, stem cell differentiation



Immunostaining of hMSCs on PDMS at 7th day of culture. (A) and (B) were S3.6 patterned substrates. (C) and (D) were L20 patterned substrates. (E) and (F) were non-patterned substrates. F-actin (), vinculin (), DAPI labeled nuclear () were overlaid. For the patterned groups (A-D), COLI patterns were labeled with Cy3 (). Scale bars showed 50 µm.

(3.O10) NOVEL PEPTIDE-BASED SCAFFOLDS CARRYING HEPARIN-DERIVED SIGNALS FOR TISSUE REGENERATION

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Extracellular matrix (ECM) is a reservoir of signals for tissue regeneration and repair. These signals can be in different forms like growth factors, glycosaminoglycans (GAGs) or bioactive peptide motifs from structural proteins such as fibronectin or laminin. Although discovery of GAGs (e.g. heparin) goes to century ago, their critical role in regulation of stability and functionality of many growth factors in ECM has been identified in the last two decades. In this manner, designing GAG-mimetic scaffolds for tissue regeneration studies might improve therapeutic efficiency of biomimetic scaffolds, while allowing to get similar physiologial output with lower doses of exogenous growth factors.Taking these into consideration, we designed Heparin-mimetic peptide amphiphile (PA) molecule which can be tuned to form ECM-like gel. Physical characterizations of novel PA scaffolds were performed by using SEM, AFM and rheology, which shows similarity to previously designed PA gels. We identified that novel PA molecule is highly affine to VEGF, which is heparin-binding growth factor and takes critical role in angiogenesis. In vitro angiogenesis data shows that Heparin-mimetic PA matrices induce endothelial cells to form tube-like structures, similar to Matrigel (basement membrane gel). Tube formation is accompanied with increase in expression of angiogenic genes. In vivo studies further strengthened bioactivity of novel PA scaffolds in terms of angiogenesis. PA scaffolds with heparin-mimetic

functionalities shown here are promising candidates for improved regenerative therapies.

Keywords. Angiogenesis, heparan sulphates, peptide amphiphile scaffolds, peptide gel, biomimetic materials

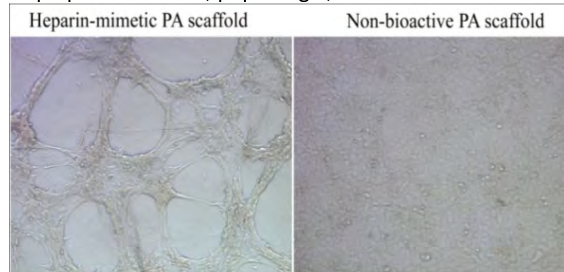

Figure 1. *Heparin-mimetic PA* scaffold induced human umbilical vein endothelial cells (HUVEC) to form vessel-like structures, similar to Matrigel, while non-bioactive PA scaffold didn't show any bioactivity different than tissue culture plate.

(3.O11) AUTOLOGOUS SCAFFOLDS FOR SKELETAL MUSCLE TISSUE ENGINEERING

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Introduction. To use autologous materials for tissue engineering would avoid problems with immune reactions *in vivo*. We have developed a range of macroporous scaffolds based on blood and plasma with a cryogelation technique to be used for skeletal muscle tissue engineering as an alternative treatment of injured or diseased tissues. The most common treatments of damaged skeletal muscular tissue are based on autologous muscle transplantation and transposition, however, these have shown a limited degree of success [1-2].

Methods. Cryogelation of reaction mixtures based on blood or plasma was carried out at -12°C where the reaction took place. The structure and biomechanical properties of the scaffolds were investigated. Myoblasts were seeded on the scaffolds and cultured for 14 days. The cultured myoblasts were evaluated by measuring cell viability, the myogenic phenotype by immunocytochemistry, and the cell morphology was studied in electron microscopy.

Results and discussion. Both types of scaffolds had a macroporous structure with interconnected pores. The blood scaffold was found to have a higher elastic modulus compared to the plasma scaffold, a lower swelling degree and an uneven surface topography (Figure 1A). The cultured myoblasts attached, migrated and proliferated on both types of scaffolds. A typical myogenic morphology was seen in scanning electron microscopy (Figure 1B) and the immunocytochemistry confirmed a myogenic phenotype (Figure 1C).

Conclusions. By using the patient's own blood to create macroporous scaffolds, either from whole blood or plasma, together with the pre-culture of autologous cells offers an easy, cost efficient and safe alternative for successful tissue engineering. We are now investigated these scaffolds *in vivo*.

Acknowledgment. We thank Swedish Research Council (VR) for financial support.

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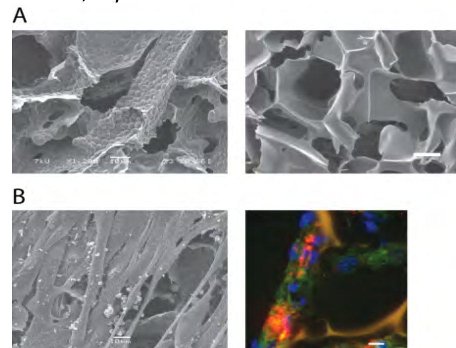
Key words: autologous scaffolds, macroporosity, skeletal muscle tissue, myoblasts



(3.P1) DEVELOPMENT OF A NOVEL FIBRIN BINDING PEPTIDE FOR INCORPORATION INTO BIOMATRICES

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Introduction. Engineered biofunctional scaffolds are becoming an increasingly valuable tool for tissue regeneration. For improved functionality the future generation of biomatrices will need to incorporate various morphogenetic compounds into the 3D matrix to enhance the regenerative response and to encourage cell migration within the injured site. Various methods have been developed to integrate growth factors into the matrix; some form covalent linkages to the biomatrix and others rely upon affinity reagents incorporated within the matrix. To expand upon the technology of integrating growth factors within biomatrices, we used phage display to identify a novel fibrinogen binding peptide for increased retention in fibrin scaffolds.

Material and Methods. Peptide phage display was used to identify a novel fibrinogen binding peptide. A recombinantly produced fibronectin domain genetically fused with the novel fibrinogen binding peptide was produced. Fibrin gels were then formed in the presence of the chimeric fibronectin protein and the release from the gel was observed using ELISAs and western blots.

Results. The peptide has nanomolar affinity for fibrinogen when displayed on the pIII protein of phage as determined by ELISA. The peptide was then fused to a growth factor binding domain of fibronectin and was shown to increase the retention time of the fibronectin domain by more than 10-fold, thereby allowing long-term integration of growth factors into a fibrin matrix. The protein/peptide fusion had mid-nanomolar affinity as determined by biacore measurement.

Conclusion. In this work we identified a novel fibrinogen binding peptide using phage display. Using this peptide we created a variant of a fibronectin domain with the ability to bind fibrinogen within a fibrin clot. This ability to retain a therapeutic protein within a fibrin gel can be used to improve the regenerative properties of fibrin matrixes and enhance wound healing.

Keywords. fibrinogen, fibronection, biofunctionalization

(3.P2) ANALYSIS OF NEURONAL SPONTANEOUS ACTIVITY IN VITRO: A MODEL TO ASSES THE EFFECT OF IMPLANTABLE BIODEGRADABLE MATERIALS

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1. University of Barcelona; 2. Institute for BioEngineering of Catalonia

Implantable biomaterials for CNS regeneration are designed to be biodegradable and often to release bioactive factors (BDNF, VEGF…). The regenerative ability of these scaffolds might be modified by the presence of by-products of degradation that can also have specific bioactive properties. For instance neurons have a high rate of oxidative metabolism and lactic acid is an alternative energy source for them. Thus, lactic acid produced by the degradation of Poly-Lactic Acid (PLA) scaffolds might affect neuronal metabolism and excitability. In this work we analyze the putative effect of these factors by an in vitro approach that measures their effect on the overall spontaneous activity of a neuronal culture. E2dish technology is a novel method for recording spontaneous neuronal electrical activity based on microelectrode arrays. E2dish device uses pair of wells connected by integrated micropipettes (microchannels). The system allows the recording of the activity of neurons whose axons sprout through the existing microchannel between the pair of wells.

Here, we analyze the effect of lactate and BDNF on the spontaneous electrical activity of a neuronal culture. Preliminary results showed that lactate stimulates spontaneous activity in vitro, probably by increasing the formation of synapses in the neuronal cultures, as indicated by increased synaptophysin protein levels in the cultures. On the other hand, BDNF treatment dramatically decreased the spontaneous activity in the neuronal culture. BDNF treated cultures exhibited lower number of burst, in particular lower fast repetitive firing activity. BDNF raises synaptophysin protein levels, a marker for synapses formation, as lactate does. Moreover it also increased the levels of Calbindin, a GABAergic neuron marker. Thus BDNF, in addition to increased synaptogenesis, promotes the maturation of the inhibitory system given us a plausible cause for the lower spontaneous electrical activity in treated cortical neuronal cultures.

Keywords. PLA, BDNF, electrophysiology, synapses

(3.P3) OSTEOBLAST ACTIVITY ON CARBONATED HYDROXYAPATITE DISCS

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1. The University of Manchester; 2. The University of Keele; 3. The University of Bath

Introduction. Hydroxyapatite (HA) is commonly used as a bone substitute and as a scaffold for bone tissue engineering. However HA has certain drawbacks such as limited biodegradability and osteointegration properties. This study investigates another form of HA, carbonated hydroxyapatite (CHA), (which resembles the composition of human bone), to potentially overcome these drawbacks.

Materials and Methods. Experiments to assess the potential of this novel scaffold. CHA discs (4.9 wt% carbonate) in comparison to control HA discs were carried out by seeding discs with MC3T3-E1 osteoblastic cells. Analysis at 4 hours, 7days and 28 days included SEM, Hydroxyproline assay (total collagen), Alamar Blue assay, Live/Dead assay and realtime RT-PCR (collagen I, collagen III and osteocalcin).

Results. Results indicate comparable cell adherence, proliferation and viability of the osteoblast-like cells on the CHA discs in comparison to HA discs. The SEM of the CHA discs showed surface irregularities at 7 days indicating dissolution (whereas the surface morphology of HA remained consistent). Both CHA and HA discs showed their surfaces to be covered by cells with evidence of extracellular matrix production. The total collagen production at 28 days, as evaluated by hydroxyproline assay, did not show any statistically significant difference. Real time PCR revealed an mRNA expression increase of 2.08, 7.62 and 9.86 fold for collagen I a1, collagen III a1 and osteocalcin respectively from cells seeded on the CHA as compared to the HA discs (Figure 1).

Conclusion. In conclusion, the CHA was found to have similar biological response to HA but also has the potential to stimulate local osteoblastic cells to upregulate bone related gene expression.

We would like to acknowledge Karen Walker (SEM, Keele University), Sarah Rathbone (confocal microcopy, Keele University) and Jaisal Patel (Bath University) and BBSRC grant BB/F013892/1 for funding.

Keywords. carbonated hydroxyapatite, MC3T3-E1 cells, proliferation, collagen production, gene expression

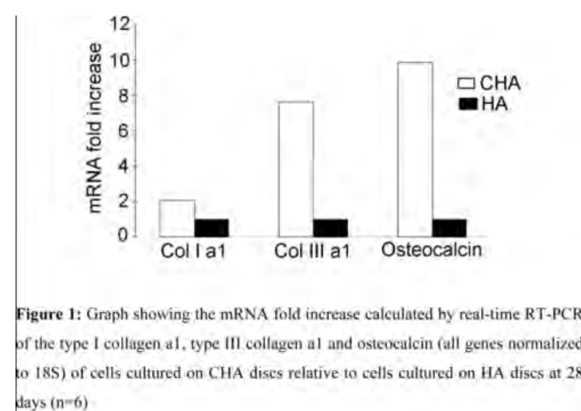

Figure 1: Graph showing the mRNA fold increase calculated by real-time RT-PCR of the type I collagen a1, type III collagen a1 and osteocalcin (all genes normalized to 18S) of cells cultured on CHA discs relative to cells cultured on HA discs at 28 days (n=6)

4. BIOINTERFACIAL ENGINEERING IN REGENERATIVE MEDICINE

Chair: Antonio Peramo
Keynote speaker: Antonio Peramo
Organizer: Antonio Peramo

Synopsis: The interface between tissues and medical implants is prone to infections and, over time, is not conducive to the integration of the implant with the tissue, ending with implant failure. These failures, with higher rates for percutaneous implants due to the permanent disruption of the skin, limit the time and usefulness of the implants and cause significant health care costs and patient morbidity. Seeking solutions for

these problems, the Symposium objective is to introduce this area of research to the regenerative medicine and tissue engineering communities. During the Symposium we will discuss the problems associated with implants, in a broad sense, and then the possible implementation of regenerative techniques applied to the interfaces between tissues and medical implants. Abstracts describing novel technological approaches (ie nanotechnology); implant surface modification; cell delivery; tissue-implant integration (bone, skin or other tissues); osseointegrated prosthesis; dental prosthetics; and other research in the area of cell and tissue engineering and biointerfacial engineering are welcome.

(4.KP) IMPLEMENTING REGENERATIVE MEDICINE AND TISSUE ENGINEERING TECHNIQUES WITH SURGICAL IMPLANTS

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1. University of Michigan

In this talk I will introduce and discuss the use of regenerative techniques applied to the interfaces between tissues and medical implants. This topic itself represents a new area in regenerative medicine and has a high potential to contribute to the current literature and provide solutions in medical implants. The topic is highly relevant to the theme 'Cells and Tissues as Advanced Therapies' because cells and tissues will be used as therapies around the implants. Among the topics for discussion is the concept of dynamically introduce regenerative materials and therapies at the tissue-implant interface. While this concept is valid for all surgical implants, it will be most useful for devices that are implanted for long periods of time or with higher risk of failure, for instance, percutaneous devices.

Keywords. implant, percutaneous, bioengineering, medical implant, biointerface

(4.O1) IN VIVO ENDOTHELIALIZATION OF CARDIOVASCULAR IMPLANTS USING DNA-OLIGONUCLEOTIDES FOR ENHANCED CELL ADHESION

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Current limitations of in vitro tissue engineering include long in vitro culture, accompanied risk of infection and cost intensive infrastructure. Accordingly this study focuses on the development of concepts for in vivo endothelialization. The objective of this study was creation of cell adhesive DNA-oligonucleotide coatings on heart valve and blood vessel surfaces. DNA is an intriguing coating material with non-immunogenic characteristics for easy and rapid chemical fabrication. For synthetic surfaces a coating process with aminoparylene and subsequent DNA-oligonucleotide adsorption was established. In a second approach oligonucleotides were covalently immobilized on decellularized bovine pericardium via an EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) mediated coupling. Immobilization of oligonucleotides proved to be extremely stable. Coupled oligonucleotides withstand shear stress up to 9,3 N/m2, which exceeds physiologic shear stress conditions on heart valve and vessel surfaces. Additionally incubation with human serum up to 96 h showed no oligonucleotide degradation. DNA-oligonucleotides enhanced endothelial cell adhesion under continuous flow conditions significantly. The oligonucleotide coating resulted in a more hydrophilic surface, which facilitated protein adhesion from human blood serum dilutions. This resulted in enhanced cell adhesion. Biocompatibility was investigated by incubation with human blood, granulocytes and thrombocytes and by determination of released thrombogenic and immunogenic factors. Immobilized oligonucleotides revealed low thrombogenicity and good hemocompatibility. Aminoparylene coated surfaces showed no activation of thrombocytes, granulocytes, the coagulation or complement system. Decellularized pericardium however proved to be highly thrombogenic. Crosslinking with EDC reduced the thrombogenic reaction significantly. EDC-crosslinked tissue might open new perspectives as matrix for in vivo tissue engineering.
Surface immobilization of oligonucleotides can facilitate manufacturing of an “off-the-shelf” heart valve or blood vessel for in vivo endothelialization. Additionally, immobilization of oligonucleotides on other types of implants where cell adhesion is desired opens new opportunities for biocompatible coatings enhancing the capability of incorporation in surrounding tissue.

Keywords. In vivo endothelialization, oligonucleotides, hemocompatibility, cell adhesion

(4.O2) A FUNCTIONALLY GRADED SCAFFOLD THAT MIMICS AN ORTHOPAEDIC INTERFACE AND CELLULAR RESPONSE THEREOF

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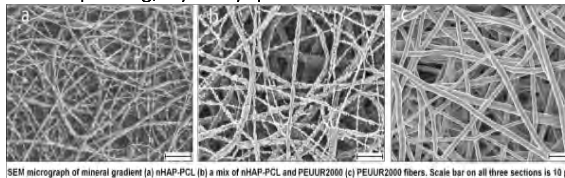
Introduction. A major concern with current scaffolding strategies for the repair of anterior cruciate ligament (ACL) injuries is poor osseointegration and subsequent failure of the scaffold at the ligament-to-bone interface. The natural ligament-to-bone interface consists of gradients in mechanical and biochemical properties that transition from soft unmineralized tissue to stiff mineralized tissue. Therefore, we propose that a functionally graded scaffold that mimics this transition would possess suitable mechanical and chemical properties to ensure spatially guided differentiation of cells towards specific lineages.

Materials and Methods. In this study, a polycaprolactone/nanohydroxyapatite (nHAP-PCL) blend and a poly-(ester urethane) urea elastomer (PEUUR2000) were co-electrospun from offset spinnerets to fabricate graded scaffolds. Scaffolds were then treated with a 5x simulated body fluid to superimpose a mineral gradient atop the existing co-electrospun gradient. The presence of gradients was demonstrated using dye assays and microscopy. X-ray diffraction (XRD) and energy dispersive spectroscopy (EDS) confirmed the presence of hydroxyapatite on the surface of the nano-fibers.

Results. Mechanical testing indicated that the scaffolds possess a gradient in tensile properties. In addition, the failure mechanism of the graded scaffolds was elucidated using real-time imaging in a micro-tensile tester. Finally, MC3T3-E1 osteoprogenitor cells showed an up-regulation of osteogenic markers in a graded fashion along the length of the scaffold.

Conclusion. The study demonstrates that graded scaffolds for orthopaedic applications can be fabricated by employing appropriate polymers and suitable processing techniques, and that these scaffolds can serve as templates to study cell proliferation and differentiation. Ongoing studies include the incorporation of Bone Morphogenic Protein-2 into one set of electrospun fibers in order to achieve a spatially graded release of the protein and subsequent differentiation of bone marrow stromal cells towards an osteoblastic phenotype.

Keywords. Ligament-bone interface, Graded scaffold, Electrospinning, Hydroxyapatite



SEM micrograph of mineral gradient (a) nHAP-PCL (b) a mix of nHAP-PCL and PEUUR2000 (c) PEUUR2000 fibers. Scale bar on all three sections is 10 µ

(4.O3) TAILORING OF SURFACE PROPERTIES AT THE NANOSCALE BY LAYER-BY-LAYER TECHNIQUE

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Introduction. A new generation of coronary stent systems aimed at rapid re-endothelialization and able to protect against thrombus formation and to minimize restenosis is currently needed. The layer-by-layer (LbL) technique is a versatile solvent-free processing allowing the coating of surfaces with uniform ultrathin multilayered films to tailor surface properties and structure at the nanoscale. The aim of the work was the development of a LbL coating with anti-thrombogenic properties and able to support endothelization for vascular tissue engineering and stent coating.

Materials and Methods. Stainless steel plates were supplied by Carbostent Implantable Device (CID). Aminolysed plates by 3-aminopropyltriethoxysilane treatment (APTES; Sigma-Aldrich) were dipped in 0.1% (w/v) heparin (HE; Sigma-Aldrich) aqueous solution for 15 min, subsequently rinsed with water, then dipped into a 0.1% (w/v) poly(diallyl dimethylammonium) chloride (PDDA) aqueous solution for 15 min and dipped again in water. Coatings with 1-11 layers were prepared. Surfaces were characterised by contact angle analysis, FTIR-ATR, SEM, AFM, XPS fluorescence microscopy, colorimetric methods (UV-Vis). In vitro cell tests using endothelial cells and haemocompatibility tests were also performed on coated samples.

Results. Surface characterisation of stainless steel plates after APTES treatment showed the presence of a continuous coating of APTES containing amino groups for further LbL coating. HE/PDDA coating was demonstrated by FTIR-ATR analysis. LbL assembly of HE/PDDA was shown by XPS analysis and a colorimetric method (toluidine blue staining of HE). Endothelial cells were found to attach and proliferate on LbL coated samples. HE was found to contribute predominantly to the good anticoagulation property of the HE/PDDA LbL coating.

Conclusion. A stable HE/PDDA LbL coating was developed on stainless steel plates used as a model for metal stents: the coating was able to promote re-endothelialisation and showed improved anticoagulation properties.

NANOSTENT and ACTIVE projects are acknowledged.

Keywords. endothelization; layer-by-layer; stent; vascular tissue engineering

(4.O4) ADJUSTING THE ORIENTATION OF TROPOELASTIN: TARGETING CELL ADHESION TO SPECIFIC POLYMER SURFACE LOCATIONS

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We recently described how human tropoelastin can direct stem cell behavior (1). This protein is the soluble protein precursor of elastin and has an integrin $\alpha v\beta 3$ binding motif at its C-terminal tip (2). The ability to generate cell patterns on polymer surfaces is critical for the fabrication of biosensors based on living cells, such as fibroblasts, where it is necessary to monitor the status of these cells in closely packed, defined locations (3). Accurate positioning of cells is also a prerequisite for cell based screening (4), cell separation techniques and for the detailed study of cellular biology (5). Recent efforts to pattern human cells on polymer surfaces have typically used aligned microcontact printing, plasma mechanical pattern generation (6), micro lithography, PDMS micro-patterning and microfluidic patterning (7, 8) but these methods are often associated with high cost, involve complex surface chemistry and may not be applicable to retain proteins in preferred orientations (5). There is a paucity of ways to utilize intact ECM molecules to confer biologically relevant cell interactions to the polymer surface. Those methods that do rely on patterned distribution of ECM proteins or protein-derived motifs on a non-adhesive, often PEG-coated, background material; this requires multiple complex chemical steps. We present the use of surface plasma immersion ion implantation polymer modification to both orient and attach tropoelastin to enable the high resolution, patterned distribution of human cells.

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Keywords. tropoelastin, elastin, plasma, interface

(4.O5) COPPER STIMULATES THE OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Introduction. In context with the design of medical implants which both stimulate the regeneration of bone tissue and are suitable to prevent infection due to bacteria, we have been interested in the effects of copper ions on the osteogenic differentiation of human mesenchymal stem cells (MSC). We hypothesized that the release of copper from an implant surface induces bacterial death. However, because of the known physiological role of copper, lower concentration in a later phase after implant incorporation or at greater distances from the implant surface could have a stimulating effect on stem cells.

Materials and Methods. Mineralization of cells as a marker for osteogenic differentiation was measured by calcein bound to extracellular calcium phosphate and visualized by laser scanning microscopy.

Results. The critical concentration of copper ions for the survival of MSC was 0.5 mM. Therefore we studied the effect of copper on the osteogenic differentiation below this concentration. We found that when adding $CuSO_4$ into a medium for osteogenic differentiation, copper ions stimulated the osteogenic differentiation of adherent cells with a maximum at a concentration of 0.3 mM. Copper induced a stronger mineralization when the cells were cultured on cell culture polystyrene than on titanium oxide or titanium surfaces. To see, whether copper implemented into implant materials induces an osteogenic differentiation of MSC, cells were cultured on calcium phosphate surfaces containing copper salts. These surfaces enhanced the osteogenic differentiation of adherent cells compared with copper free surfaces. Concerning possible mechanisms which are involved in the biological response induced by copper, we revealed that copper affected the strength of cell adhesion and the expression of various integrins.

Conclusion. Copper containing implants are suitable to promote bone regeneration by the stimulating effect on the osteogenic differentiation of mesenchymal stem cells.

The work was supported by the government of Mecklenburg-Vorpommern (V230-630-08-TFMV-S-016/F016).

Keywords. copper, implant, mesenchymal stem cell

(4.O6) A NEW GDF-5 MUTANT MEDIATING SUPERIOR TABECULAR AND CORTICAL BONE FORMATION IN A CRITICAL SIZE DEFECT RABBIT MODELL

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Treatment of large bone defects remains a challenge for orthopaedic surgeons. In clinical use for this indication are Bone morphogenetic proteins (BMPs) which are potent agents to induce bone formation. The osteoinductivity of human growth-and-differentiation-factor-5 (GDF-5) is well established, but a reduced amount of ectopic bone is formed compared to other members of the BMP-family like BMP-2. We found previously, that swapping two amino acids in GDF-5 to residues contained in BMP-2 increased osteogenicity of emerging GDF5V453/V456 (mt) and enhanced its ectopic bone formation capacity compared to wildtype GDF-5. Aim of this study was to investigate the potency of GDF-5mt for treatment of critical size bone defect (CSD) in comparison to BMP-2 and GDF-5.

Bone formation in CSD in rabbit radii treated with BMP-2, GDF-5, GDF-5mt or buffer solution was assessed by in vivo µCT scans at 4, 8 and 12 weeks post surgery.

All GDF-5mt treated defects bridged after 4 weeks, while only 6 of 9 BMP-2 treated bones were bridged at 8 weeks. After 12 weeks GDF-5mt increased bone volume compared to BMP-2 and GDF-5 treated animals ($p<0.001$). Bone marrow cavities were remodelled in all GDF-5mt treated animals during 8 weeks, while BMP-2 mediated callus remained spongy at 12 weeks post surgery. Micro morphological parameters in BMP-2, GDF-5 and control defects differed significantly from the GDF-5mt group as well as from contralateral healthy bone. Concomitantly, micro architectural parameters were similar in the GDF-5mt group and healthy bone. GDF-5 wildtype mediated cartilaginous gap formation in 5 of 9 animals - an effect that was not detectable after BMP-2 or GDF-5mt teatment after 8 weeks.

The GDF-5mt showed superior bone formation capacity than GDF-5, and a faster induction and cortical bone formation than BMP-2. GDF-5mt thus represents a promising new growth factor variant promising improved outcome in bone regeneration strategies.

Keywords. Growth factors, GDF-5, BMP-2, GDF-5mt, Rabbit, Bone healing

(4.O7) BIODEGRADABLE DISULFIDE-CATIONIC POLYMER FOR THE GENE THERAPY OF RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA

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Introduction. Dystrophic epidemolysis bullosa (DEB) is a group of inherited diseases characterized by the blistering and scarring of the skin after mild trauma. The most

severe case being transmitted by the autosomal recessive pattern and is known as recessive dystrophic epidermolysis bullosa (RDEB). The overall aim of the project is to demonstrate direct gene delivery of COL7A1 plasmid carrying the correct COL7A1 sequence to human RDEB skin cells using a biodegradable cationic polymer (termed DMA) and a thermoresponsive hydrogel.

Materials and Methods. Polymer synthesis: The polymer was synthesised by deactivation enhanced atom transfer radical polymerisation (DE-ATRP) at 60°C for 6 hours under argon and then characterised by gel permeation chromatography (GPC) and proton nuclear magnetic resonance (NMR). Transfection and cell viability studies: Mouse 3T3 fibroblasts (DMEM, 10% FBS and 1% penicillin/streptomycin) (Sigma Aldrich) and Human primary keratinocytes from RDEB patients (keratinocyte growth medium II, supplement mix and CaCl2) (Promocell) were transfected with DMA/DNA at optimal weight to weight ratios (w/w). Alamar Blue™ (Invitrogen) was used to analyse the cellular metabolic activity. Indirect immunofluorscence: COL7A1 protein expression from RDEB primary human keratinocytes was visualised using polyclonal rabbit primary antibody to human COL7A1 protein and Alexa Flour® goat anti-rabbit secondary antibody. DAPI was used to stain the nucleus.

Results. The polymer showed higher transfection efficiency while maintaining high cell metabolic activity compared to superFect® and LipofectamineTM. Cells that were treated with DMA/COL7A1 polyplexes showed typical patterns of expression of collagen VII (COL7A1) protein compared to untreated cells.

Conclusion. The results suggest direct and long lasting treatment of RDEB using a biodegradable polymeric gene vector has a potential therapeutic application.

DEBRA Ireland and Austria, Heath Research Board (HRB) of Ireland (HRA/2009/121), Science Foundation Ireland (SFI) Principal Investigator and Stokes Lectureship Programmes (10/IN.1/B2981 and 07/EN/E015A), and National University of Ireland, Galway (Scholarship).

Keywords. Gene Therapy

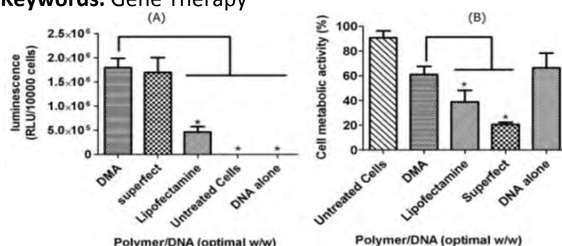

Figure 1. A) DMA transfection efficiency of 3T3 fibroblasts compared to commercial transfection reagents and untreated cells. B) Cell metabolic activity of the same polymer and transfection reagents (n=5, P<0.001) (±SD).

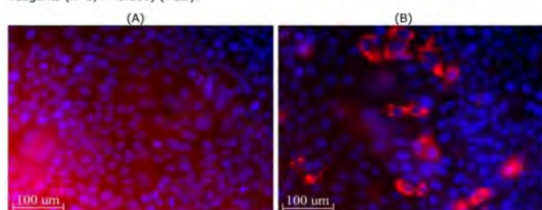

Figure 2. A) Untreated RDEB human primary keratinocytes stained with DAPI and Alexa Flour®. B) Same cells treated with DMA/COL7A1 (optimal w/w) showing expression of COL7A1 protein.

(4.O8) MULTI-SCALE, HIERARCHICALLY POROUS PLLA/POROUS TITANIUM HYBRIDS FOR TRACHEA REPLACEMENT

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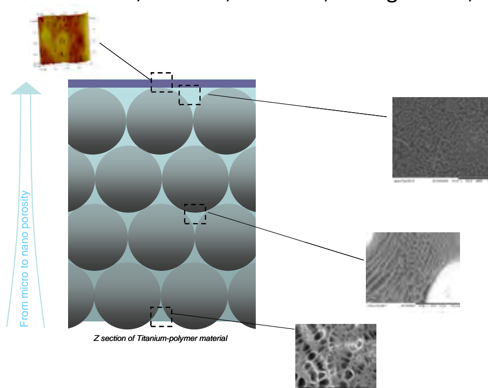
In trachea regeneration, the two most persistent problems are restenosis of the tracheal lumen by migration of cells and lack of epithelialization. For this end, we developed a hierarchically porous (from macropores to nanopores) PLLA/ porous Titanium hybrid scaffold, that can prevent restenosis by fibroblastic cells by size exclusion and that can promote epithelialization by a surface of either nanofibrillar or nanoporous nature (Nanoporous PLLA films or Collagen/Alginate fibrillar polyelectrolyte multilayers). Moreover, since the necessary volume for tissue regeneration would be lower due to the presence of the titanium body, vascularization of epithelium layer would occur faster. This hypothesis was tested invitro by quantifying fibroblast migration through the scaffold and via human respiratory cell culture. Fibroblast movement was significantly impeded by the microporosity gradient and a confluent layer of epithelial cells was obtained.

The hypothesis was further tested in a rabbit model (New Zealand white rabbits) with a 2cm length full reconstruction model with implantation duration of 6 weeks. CRP levels of animals were checked regularly also after removal of the implants and implants were characterized for fibroblast movement and epithelialization, infection and polymer degradation. Results showed that, the porosity gradient effectively prevented clogging of the lumen by the migrating cells and the top film layer was in place after 4 weeks. Epithelial migration was evident but incomplete. Polymer degradation was most prominent at the outer surface were most of the remodeling took place.Fibrovascular tissue development within the pore structures was apparent. For a more in-depth understanding, cytokine composition of the blood of the implanted animals is being investigated now. Our results suggest that, the developed hybrid scaffold can successfully replace tracheal segments. However, for long segments, pre-epithelialization with patients own respiratory epithelium is necessary as the rate of migration is not good enough for full coverage.

Keywords. In-vivo, Trachea, Titanium, Pore gradient, PLLA



(4.P1) CELL ADHESION PROMOTING RGD-SILK

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Introduction. Silk, a biocompatible and biodegradable material with good mechanical properties, is a suitable scaffold material for e.g. bone tissue engineering. By introducing the cell signaling amino acid sequence RGD directly into the primary sequence of a genetically engineered silk we hypothesize that receptor-mediated cell adhesion and spreading will be promoted.

Materials and Methods. A DNA construct coding for silk based on the major ampullate spidroin 1 from the spider Nephila clavipes has previously been assembled (15mer) [1]. We have added DNA coding for the fibronectin derived amino acid sequence VTGRGDSPA both up and downstream of the 15mer gene to create RGD-15mer. The two engineered silks were produced by fed-batch fermentation using a bacterial expression system, purified and cast into films. The films were seeded with DiI stained human mesenchymal stem cells (hMSCs), and cell adhesion was studied with time-lapse microscopy.

Results. hMSCs seeded on the RGD-15mer silk started to polarize and migrate, something that could not be observed on the 15mer silk over the duration of the time-lapse study (2.5 hours). The experiments were performed with serum free medium. Currently osteogenic differentiation on the different materials is being studied.

Conclusions. We have set up a production system for engineered silk materials which we could utilize to produce a silk with integrated RGD sequences. This material showed improved cell adhesion and has the potential to be used as a scaffold material for tissue engineering purposes.

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We thank Professor David Kaplan (Tufts University, Medford, MA) for kindly providing the Nephila clavipes silk (15mer) gene containing plasmid, and Dr. Kristopher Kubow (ETH Zurich) for assistance with the time-lapse studies. This work was supported by the BioEngineering Cluster (ETH Zurich).

Keywords. Silk, RGD, mesenchymal stem cells

(4.P2) NORMAL HUMAN OSTEOBLASTS RESPONSE TO PECVD TIO2 FUNCTIONALIZED PLGA MEMBRANES DESIGNED FOR GUIDED TISSUE REGENERATION (GTR)

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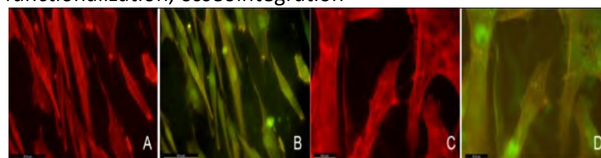
Introduction. The therapeutical approach for reparation of bone defects at the maxillofacial level is now focused to bony tissue reparation, and minimization of connective healing and the recovery time. Guided tissue regeneration (GTR) specifically aims to overcome some limitations of conventional therapy. Aliphatic polyesters -polyglycolic acid, polylactic acid and their bioresorbable copolymer (PLGA)- are arousing a great interest and are approved for the US FDA for certain human clinical use. They can be employed as supporting or stabilizing elements for bioactive materials, i.e.titanium, and their degradation products are removed by natural metabolic pathways. Porous three-dimensional temporary scaffolds play an important role in manipulating cell function and guidance of new organ formation, and their surface chemical composition is a key factor for achieving a durable osseointegration. The establishment, through an "in vitro" study, of the osteoinductive (GTR and mechanotransduction) properties of TiO2 PECVD functionalized and non functionalized PLGA membranes on human osteoblasts.

Material and Methods. Human osteoblasts were grown on TiO2 functionalized and non functionalized PLGA membranes produced in the ICMSE, Seville, Spain. Rhodamine-phalloidine and antivinculin immunolabelled labelled cells were analyzed after 24 and 48 h in culture.

Results. Osteoblasts grown on non functionalized PLGA shown an elongated shape, and distributed in a fascicular pattern, similar to those growing on glass, with small focal contacts all along the cell body (A,B). Osteoblasts cultured on TiO2 PECVD functionalized PLGA surfaces grown and polarized into an organized reticular pattern, with well developed stress fibers oriented to gross focal adhesion points (C,D).

Conclusion. Our results demonstrate that PECVD TiO2 functionalization of PLGA surface induces osteoblasts organization into a reticular pattern that could be more efficient for bone formation in those locations, like maxillofacial bone, that support non oriented and complex mechanical loadings.

Keywords. osteoblasts, guided tissue regeneration, TiO2, functionalization, osseointegration



(4.P3) MEASUREMENTS OF POLY N-ISOPROPYLACRYLAMIDE-CO-BUTYLACRYLATE/3T3 CELLS INTERACTIONS BY ATOMIC FORCE MICROSCOPY

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Poly N-isopropylacrylamide-based (P(NIPAAm)) hydrogels has already been proposed as cell culture support for cell sheet engineering because its thermosensibility associated with a lower critical solution temperature (LCST around 32°C). The hydrophobic/hydrophilic character of P(NIPAAm) hydrogels allows cell growth above LCST and cell release below it, respectively.

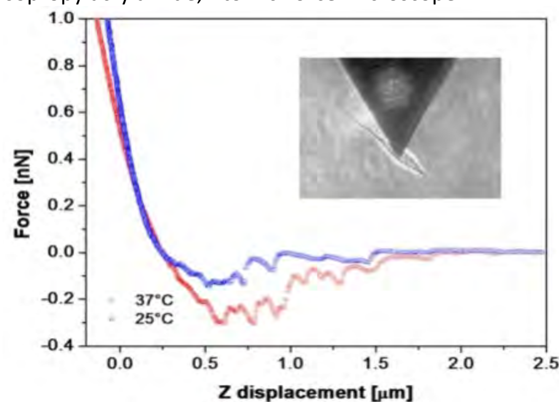
We have observed that poly N-isopropylacrylamide-co-butylacrylate copolymer (P(NIPAAm-co-BA)) is more hydrophobic than homopolymer P(NIPAAm). This characteristic is known to improve cell adhesion, increasing cell-hydrogel interactions through an efficient adhesive proteins adsorption such as fibronectin on the hydrogels. Therefore we propose the use of P(NIPAAm-co-BA) as cell culture support in cell sheet engineering once an adequate balance between hydrophobic/hydrophilic character is provided.

In this work an Atomic Force Microscope (AFM, model-5500, Agilent Technologies) was used to characterize cell-thermosensitive hydrogels interactions at two different temperatures, above and below copolymer LCST. Polystyrene (PS) microbeads was glued to a microcantilever and coated with P(NIPAAm-co-BA) copolymer using a micro-manipulator. Uncoated PS microbeads were the controls. 3T3 Swiss cells were cultured, 24 hours after passage were used in AFM experiments. Maps of force versus distance curves (8 x 8 curves) at 37°C and 25°C were recorded. For each curve (Figure) the PS microbead was brought and kept into contact with a single cell for 10 seconds, afterwards the cantilever was withdrawn and force necessary to microbead detachment from cell was measured. Data were acquired and analyzed using a software developed in LabVIEW (National Instruments, Austin TX).

Maximum adhesion distributions obtained at 25°C and 37°C show a higher adhesion force above the LCST of the P(NIPAAm-co-BA) copolymer, which confirms the dependence of cell-hydrogel interactions with temperature and the possibility of cell release at 25°C. These results support the use of P(NIPAAm-co-BA) copolymer as a cell culture substrate in cell sheet engineering.

Convocatoria Fac-Medicina, Sostenibilidad 2009-2011, Colciencias-doctorados Nacionales-2008

Keywords. cell sheet engineering, Poly N-isopropylacrylamide, Atomic Force Microscope



(4.P4) DEVELOPMENT OF AN IN VITRO 3D MODEL TO SIMULATE THE HUMAN BLOOD-CEREBROSPINAL FLUID (B-CSF) BARRIER

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Introduction. *Neisseria meningitidis* is a strictly human-specific pathogen with the capacity to cause septic shock and meningitids. Therefore, the aim of this study was to construct an endothelial cell barrier in order to develop an in vitro 3D model of a human B-CSF barrier using human brain microvascular endothelial cells (HBMEC). The subarachnoideal space was constituted by a biological vascularized scaffold of collagen I/III (BioVaSc). Culture was performed in static and dynamic conditions.

Materials and Methods. The BioVaSc was processed from porcine jejunum by mechanical, chemical and enzymatic decellularization. Different cell concentrations and culture periods were tested under static conditions. Additionally, a dynamic culture was performed mimicking the bloodstream. The confluence of the endothelial monolayer was verified by measuring the transendothelial electrical resistance (TEER). The static and dynamic studies were also repeated with primary microvascular endothelial cells isolated from human skin. Both cell types were characterized with histological staining against the cluster of differentiation molecule 31 (CD31) and the von-Willebrand-Factor (vWF).

Results. The static culture tests of HBMEC's on the BioVaSc revealed an optimal cell concentration of 2x10^5 cells/cm² BioVaSc and an optimal cultivation period of 2-5 days. With these conditions a cell monolayer was established. However, the monolayer wasn't tight and the cells often grew in untypically multiple layers. In contrast the primary endothelial cells formed a tight monolayer under static conditions. Dynamic culture conditions in a flow chamber resulted in the formation of a tight monolayer of HBMEC's, confirmed by TEER-measurement. The histological staining exposed that the cell line HBMEC in contrast of the primary endothelial cells had lost the endothelial markers CD31 and vWF.

Conclusion. The results of our study show the construction of a tight endothelial cell barrier under dynamic culture conditions. The next steps should be to complete the B-CSF models with meningeoma cells and to infect them with *Neisseria meningitidis*.

Keywords. blood-cerebrosoinal fluid barrier, in vitro 3D model, collagen I and III scaffold, tight HBMEC monolayer

(4.P5) NANOLAYERS OF PEVCD TIO2 SUITABILITY FOR HUMAN OSTEOBLASTS GROWTH FOR TISSUE ENGINEERING

Salido M (1), Terriza A (2), Torres D (3), de la Orden E (1), Barranco A (2), Díaz-Cuenca MA (2), Vilches J (1), González-Elipe AR (2)

1. School of Medicine. University of Cádiz; 2. Instituto de Ciencia de Materiales. Seville (CSIC-Univ. Seville); 3. School of Dentistry. University of Seville, Spain

Introduction. Bone regeneration can be enhanced through implantation of biocompatible scaffolds. The complexity of scaffolds surfaces could positively influence osteoblastic mechanotransduction. Surface chemistry plays an important role in implant fixation and can directly influence osteoblasts adherence, attachment, spreading and metabolism modifying and controlling the osseointegration process. The use of an appropriate template to provide physical support and a local environment is essential for a successful regeneration. With the aim of tailoring suitables surfaces to be tested in vitro, scaffolds activation by Plasma enhanced chemical vapour deposition of Ti appears as an alternative to wet chemical treatments

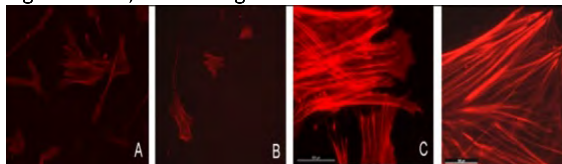
Material and Methods. Human normal osteoblasts were grown on PEVCD TiO_2 functionalized and non functionalized TiO_2 PET samples, produced in the ICMSE (Instituto de Ciencias de Materiales) in Seville, Spain. Rhodamine-phalloidine and antivinculin fluorescent labelled cells were analyzed after 24 and 48 h in culture.

Results. After living osteoblasts examination (phase contrast and DIC microscopy), phenotypical cell changes, like filopodial and lamellopodial emission, mainly

oriented to elongation, alignment and focal adhesions towards the growing surface were observed. Actin cytoskeleton immunolabelling of growing cells revealed a higher polarization and stress fiber development, together with a more defined osteoblast orientation induced by surface, in cells grown on the 100 nm PEVCD TiO_2 functionalized PET samples.(Figure: actin immunolabelling of osteoblasts grown on A: glass; B: PET; C,D:PEVCD TiO_2 PET)

Conclusion. Surface chemistry of the scaffolds plays a key role in osseointegration. In order to render a nanolayer with controlled structure and composition to enhance osteoblasts adherence and differentiation, TiO_2 thin films prepared by plasma enhanced chemical vapour deposition were grown on PET samples.Our present results demonstrate the suitability of PEVCD as an alternative for surface functionalization of polymers that can lead to the development and tailoring of new bioreabsorbable polymeric membranes for bone tissue regeneration.

Keywords. TiO_2 nanolayers, osteoblasts, bone regeneration, osseointegration



5. BIOMATERIALS & ENGINEERED CONSTRUCTS-OUTCOMES IN MEDICINE/EXISTENT SURGERY (BECOMES)

Chair: Amulya K. Saxena
Co-chairs: Richard Ackbar, Herwig Ainoedhofer
Keynote speaker: Amulya K. Saxena
Organizer: Amulya K. Saxena

Synopsis: Biomaterials development resulting from extensive basic research has to be translated in the clinical setting to determine their suitability or their shortcomings in human applications. Translational research will involve the investigation of biomaterials that have been developed under optimal laboratory conditions, but have to be utilized under complex clinical and surgical pathological states. The *Biomaterials & Engineered Constructs- Outcomes in Medicine/ Existent Surgery (BECOMES)* Group focuses on the translation outcomes of biomaterials and generated tissues in clinical and contemporary surgical applications.

The group focuses also on the better understanding of the clinical pathology and relating the difficulties experienced by the clinicians and surgeons in their practise to the tissue engineering community. The group aims to highlight to Researchers in Tissue Engineering the coexistence of conditions (co-morbidities) that will affect or alter the primarily intended functioning of the original biomaterial or engineered tissue. The foremost intention of the group is to expose the Basic Science Research Community in Tissue Engineering with the ground realities in patient pathology and the difficulties experienced by the Medical Practitioners and Surgeons. This exposure is intended to help in the translational research and evaluation and implementation of Tissue Engineering Technologies in Clinical practice.

The symposia intention of the BECOMES Group is 4 fold:

1. *Outcomes of Biomaterials in Contemporary Clinical Applications:* Presentations will be invited from researchers who have applied biomaterials or engineered constructs in the clinical practise. These presentations are intended to showcase the ease or difficulties in the application of these materials in humans. The usage of these materials, their outcomes and their shortcomings will be presented and technical improvements that are desired will be presented to researchers in the area of Tissue Engineering.

2. *Identification of Clinical states demanding Regenerative Medicine:* The second area of presentation will be exploring the clinical states that require biomaterials or engineered tissues. Presentations will be made to expose clinical conditions and the present state of palliative therapies that are offered to the patients. These presentations are intended to expose tissue and clinical states that have not part of the frontline research in tissue engineering, however the demand of tissue is these area is so dire that millions of Euros are being spent in the management of these patients with no optimal solutions in sight.

3. *Focus on Paediatric organ loss:* The focus of the tissue engineering research is mainly on the adult populations and the conditions encountered later in life. There is even a much larger shortage of organs in the newborns, infants and the childhood age group that the tissue engineering research community is not aware about. Paediatric organ shortages are further complicated by donor mismatches (for example if an adult liver donor is found for a newborn who requires a liver transplant- it is almost impossible to fit an adult liver in the child).

Biomaterial research in in-vivo animal models: Presentations will be also done on in-vivo animal models to explain the working of biomaterials or generated tissues in these experimental studies. These presentations will be important for the clinicians and surgeons to understand the development and the present stage of research in animal experiments and the future clinical applications.

(5.KP) TISSUE ENGINEERING FOR CLINICAL SYNDROMES: EXPECT THE UNEXPECTED MICROENVIRONMENT

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1. Experimental Fetal Surgery & Tissue Engineering Unit, Department of Pediatric- & Adolescent Surgery, Medical University of Graz, Austria

Clinical syndromes which affect multiple organs are indirectly a target of research for the Tissue Engineering & Regenerative Medicine groups worldwide. However, while the present research focuses on the development of single organs by interest groups; syndromes that affect multiple organs, present a multimorbid patient, the affection of which changes the microenvironment for the transplanted tissue engineered organ. At present organ development is focused on application of strict protocols for cell isolation, seeding on scaffolds under the “near

perfect" conditions to generate *in-vitro*, *in-situ* and *in-vivo* neotissues. Such neotissues are then envisaged for implantation or transplantation in an individual that offer near to physiological conditions for the neotissue to assimilate, incorporate and integrate. Clinical syndromes affecting major organs soft tissue organs such as the heart, lungs, liver, kidneys and intestine are a focus of this presentation to better demonstrate the ground realities faced in the starting of trials of tissue engineering organs. The impact of these tissues in syndromes is so large that normal tissues within the body are forced to alter their function and structure in such individuals.

Major clinical syndromes can further be divided for better understanding under those affecting the pediatric population to those that can further continue to affect the individual later as an adult. These can be further divided into those affecting the clinical status of the patient versus those that influence states that necessitate surgical corrections. It is important for the Tissue Engineering & Regenerative Medicine community to be aware of these imperfect microenvironments and at some stage work on the ground realities that will determine the success or failures of neotissue implants and transplants. Future work in Tissue Engineering & Regenerative Medicine should focus on these altered microenvironments for successful implementation of this technology in the clinical and surgical patient.

(5.O1) NOVEL BIODEGRADABLE VASCULAR PROSTHESIS: SHORT-TERM RESULTS AFTER CAROTID ARTERY REPLACEMENT IN THE PIG

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1. Geneva University Hospital

Introduction. There is a continuous search for synthetic, shelf-ready, coronary artery bypass grafts. Biodegradable scaffolds, repopulated by recipient's cells regenerating a neo-vessel, can be a suitable option for both adult and pediatric, urgent and elective cardiovascular procedures. We assessed a new biodegradable vascular prosthesis for arterial replacement in the pig.

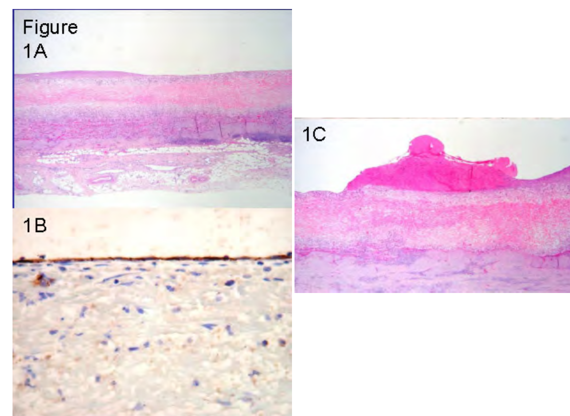
Materials and Methods. Ten anesthetized pigs underwent bilateral carotid artery replacement with biodegradable electrospun Poly(ε-caprolactone) (PCL) nanofibre prostheses (4mm-ID; 5cm-long); or expanded-polytetrafluoroethylene (ePTFE) prostheses serving as control. Peri-operative anticoagulation was achieved with intravenous heparin (double baseline ACT). Post-operatively, until conclusion of the study at 1-month, animals received aspirin daily. Transit

Time Flow (TTF) was measured intra-operatively and at sacrifice. Doppler ultrasound follow-up was performed at 1 and 4 weeks when a selective carotid angiography assessed patency. Graft examination consisted of histology with special stainings, planimetry and SEM.

Results. Surgical handling and haemostasis of the new prostheses were excellent. Patency rate was 78% (7/9) for PCL grafts, compared to 70% (7/10) for ePTFE grafts. TTF and Doppler ultrasound showed no significant changes in flow and velocity or diameter over time in both groups. Both prostheses showed minimal in vivo compliance as compared to native carotid artery. Neoendothelialisation was 79% for PCL (Fig.1:A,B) and 80% for ePTFE grafts. Neointima formation was limited in both grafts. The PCL graft was partially infiltrated from the adventitia by macrophages, myofibroblasts and capillaries with a mild foreign-body reaction and focal thrombus formation (Fig.1:C).

Conclusion. Biodegradable, electrospun PCL grafts showed good surgical properties, no aneurysm formation and similar short-term patency compared to ePTFE grafts. Rapid, good endothelialisation and cell ingrowth confirms the hypothesis of in vivo vascular tissue engineering. Despite good early results long-term follow-up is required before clinical application such as CABG.

Keywords. tissue engineering, scaffolds, animal experiments



(5.O3) ROLE OF SIDE POPULATION CELLS DURING WOUND HEALING IN RAT VOCAL FOLDS

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Introduction. Despite big advances in understanding mechanisms of wound healing in vocal fold injury, it still remains unclear which are the decisive factors that lead to a complete restoration or to scarring. Among several other factors, stem cells are believed to play an important role in vocal fold restoration. Side population (SP) cells are considered to contain high numbers of stem cells and have gained great interest in the tissue engineering community. Aim of the following study was to investigate the recruitment pattern of SP cells in a rat vocal fold injury model.

Materials and methods. Unilateral vocal fold scarring was performed in Sprague Dawley rats. Larynges were harvested 1, 3, 5, 7, 14, 21 and 35 days after initial injury and examined immunohistochemically for the presence of SP cells. This was done in coronal sections of the posterior and anterior macula flava as well as in the mid-portion of the vocal fold investigating the lamina propria.

Results. Number of SP cells peaked significantly after 7 days in the mid-portion of injured vocal folds, with a return to pre-injury levels after 14 days. No increase was detected throughout the observed time in the contra-

lateral side. Number of SP cells increased slightly but not significantly in both anterior and posterior macula flava.
Conclusion. Our findings suggest that SP cells may play an important role in early vocal fold wound healing and may serve as a possible therapeutic target.
Keywords. Side population cells - wound healing vocal folds

(5.O4) NEW POLYMER COATING TO VISUALIZE SURGICAL MESH BY MRI

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Introduction. Magnetic Resonance Imaging (MRI) is widely used for both clinical diagnosis and/or staging of human diseases [1]. Unfortunately, MRI still is a powerless imaging technique for prostheses observation post-operatively [2]. To circumvent this drawback, we synthesised a new MRI visible polymer by grafting a MRI contrast agent on the polymer backbone. Its potential for clinical use was evaluated using in vitro and in vivo experiments.
Materials and Methods. Anionic activation of poly(methyl acrylate) (PMA) chain was performed using lithium diisopropylamide (LDA) [3]. The resulting macropolycarbanion was then reacted with a chelate of a MR contrast agent based on Gadolinium (DTPA-Gd). The in vitro polymer cytotoxicity was investigated using L929 fibroblasts by cells viability and pro-inflammatory response assays. The PMA-DTPA-Gd polymer was coated on commercial polypropylene meshes by spray coating. In vitro MR images were performed on coated meshes embedded in agarose gel. For in vivo visualization, coated meshes were implanted in a Wistar rat's back and MR images were obtained 10 days after implantation using an experimental (7 T) and a clinical (1.5 T) MR apparatus.
Results. MR contrast agent (DTPA-Gd) has been covalently grafted on poly(methyl acrylate) and in vitro cell investigations of the grafted polymer revealed good in vitro cytocompatibility associated to a limited toxicity. After coating, this new polymer allowed to significantly enhance in vitro MR signal of the meshes for a long-term period. After implantation in rat, the coated meshes were unambiguously detectable whatever the location and the morphology was clearly recognizable.
Conclusion. To our knowledge, it is the first non-hydrosoluble MRI visible polymer ever described. This polymer, once coated on an initial MR transparent polypropylene mesh, induces in vivo MR signal enhancement for a long time period and allows a quick MRI localization of the device.

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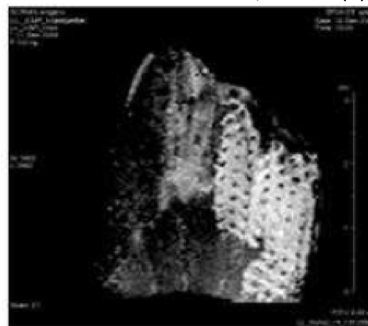

In vivo MR visualization of two meshes coated with poly(methyl acrylate)-DTPA-Gd implanted in a rat

(5.P2) PREPARATION OF ELECTROSPUN POLYCAPROLACTONE (PCL)-SPIRULINA NANOFIBER AS A SCAFFOLD FOR CELL CULTURE

Kim SH (1), Jung SM (1), Shin CH (1), Shin HS (1)
1. INHA UNIVERSITY

Scaffolds are important for pattern of cellular behaviors in tissue engineering. In many scaffold fabrication methods, Electrospinning is a simple technique to make nanofiber mat that is similar to the natural extracellular matrix structure. In recent year, it has been demonstrated that electrospun nanofiber mat comprising synthetic biodegradable polymer such as polycaprolactone(PCL), poly l-lactide acid(PLLA) and poly vinyl alcohol(PVA). Especially, PCL is a semi-crystalline polyester that is a popularly used bio-polymer for tissue engineering. However it has limited cell adhesion, proliferation and differentiation because of their hydrophobic property. . In this research we made PCL nanofiber which contains a blue-green microalgae, spilurina and examined some advanturous specialties for cell culture and tissue engineering. We demonstrated that spirulina-containing PCL nanofibrous scaffolds enhances cell adhesion and proliferation in comparison with PCL nanofiber
This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST). (MEST 2010-0015308)
Keywords. electropinning spirulina polycaprolactone

(5.P3) DIRECTING BIOLOGICAL RESPONSE THROUGH MATERIAL PROPERTIES

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Introduction. Biomaterials are used in tissue engineering to repair, replace or augment healthy tissue. Although mechanical properties derived from the material bulk are well established, attention has turned towards the surface of biomaterials in order to more easily integrate these materials into the body. Cells naturally secrete proteins in order to moderate their environment,

providing a route for many cellular mechanisms including attachment and proliferation. To date, little information on cellular mechanisms in relation to their interaction with biomaterial surfaces has been reported. By understanding such responses will allow for the development of advanced biomaterial coatings, controlling cellular responses with medical devices.

Materials and Methods. Mass spectrometry has been used to evaluate differences in cellularsecretions in relation to a range of surface chemistries. 3T3 fibroblasts were cultured over surfaces presenting OH, COOH, NH2 and CH3 terminal chemistry, prepared using silane self assembled monolayers on glass. Cell culture media was taken at varying time points after cell seeding, being worked up via acetonitrile precipitation and ZipTip desalting procedures and analysed using electrospray mass spectrometry.

Results. Mass spectral differences are found highlighting variation in cell secretions in relation to their interaction with the underlying surface chemistry. Cellular morphology, adhesion and proliferation rates also show varying responses of cells to surface chemistry.

Conclusion. 3T3 fibroblasts have been shown to adhere, proliferate and have distinct morphology depending upon the surface chemistry on which they reside. Differences in secreted proteins were also observed indicating that surface chemistry controls internal cellular processes.

We acknowledge funding from EPSRC DTC programme and the National Endowment for Science, Technology and the Arts (NESTA).

Keywords. Biomaterial, Protein, surface chemistry, cellular reponse, Fibroblast

(5.P4) TISSUE-ENGINEERED HYPERTROPHIC CARTILAGE UNDERGOES ANGIOGENESIS AND OSTEOGENESIS IN CRANIAL DEFECTS

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1. University of Sheffield

Introduction. Tissue-engineered hypertrophic cartilage grafts have significant potential for the repair and reconstruction of large bone defects. Hypertrophic cartilage has the advantage over bone grafts in that it can withstand the low oxygen levels typically found at the site of injury, and it may also induce angiogenesis and osteogenesis. Previous research showed that nasal chondrocytes could form a hypertrophic-like cartilage, but also that this tissue failed to mineralise in vitro.

Objective: To investigate the ability of tissue-engineered hypertrophic-like cartilage to undergo mineralisation to form bone tissue in vivo in a cranial defect in the rat.

Materials and Methods. Rat nasal chondrocytes were cultured for 42 days on poly-glycolic acid (PGA) in standard chondrogenic conditions. 3.5mm circles were cut from the constructs and implanted into cranial defects (3.5mm) of 12 week old Wister rats (n=8) for 4 or 8 weeks. On retrieval, calvaria were fixed in formalin for analysis by µCT and paraffin embedded for histological analysis. All animal experiments were carried out with the relevant regulatory (Home Office) approval.

Results. By week 4, good infiltration of blood vessels was seen throughout the construct and after 8 weeks deposition of bone tissue was observed histologically. Analysis by µCT showed small islands of mineralisation in the constructs after 4 weeks. By 8 weeks bone formation was significant with most of the defect filled with new bone. Relatively little bone formation was seen in empty defects by 8 weeks.

Conclusion. Tissue-engineered hypertrophic-like cartilage grafts underwent angiogenesis and osteogenesis in vivo and promoted healing of calvarial defects.

The work was performed as a part of the EXPERTISSUES Network of Excellence (EC contract: NMP3-CT-2004-500283) and funding was received from the Marie Curie programme (Alea Jacta Est, EC contract MEST-CT-2004-008104).

Keywords. Hypertrophic cartilage; endochondral ossification; tissue-engineering

(5.P5) BIODEGRADATION BEHAVIORS OF SILK FIBROIN MEMBRANE FOR REPAIRING OF TYMPANIC MEMBRANE PERFORATIONS

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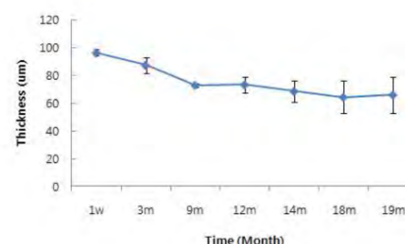
1. Hallym University

Silk fibroin of silkworms has been widely studied as biomaterials. The degradation behavior of silk biomaterials is obviously important for medical applications. But the study about long- term result is few in vivo. In this work, we investigated the degradation behavior of silk fibroin membrane in vitro and in vivo. In vitro assay, we observed degradation of silk membrane in PBS, culture media and enzyme (protease K) solution. In solution with protease K, 80% of silk membranes were degraded within 10 days. Silk membranes presented no cytotoxicity in L929 cells and rat tissue. In order to investigate degradation of silk membrane in vivo, silk membrane implanted subcutaneous in rats and were harvested after surgery until 19 months. SEM, histological analysis of silk membrane explants showed that silk membrane broken in several pieces from 16 months. In conclusion, the results indicated that silk membrane is a good biocompatible and has a long degradation time as biomaterials.

Keywords. silk fibroin, membrane, biodegradation

Fig. Biodegradation of silk membrane *in vivo*.



(5.P6) MICROPARTICLES AGGLOMERATED IN FIBRIN GELS FOR CARTILAGE REGENERATION

Gamboa-Martínez TC (1), García-Cruz DM (2), Carda-Batalla C (3), Gómez-Ribelles JL (1), Gallego-Ferrer G (1)

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Introduction. Recently the production of composite injectable vehicles is a powerful alternative to avoid the drawbacks of implantation of other forms of 3D scaffolds. In this study, we developed a biodegradable composite gel consisting of chitosan microspheres embedded in fibrin gels as potential scaffold for articular cartilage regeneration. The combination of both natural polymers as cellular carrier promotes cell adhesion and allows a 3D arrangement maintaining the chondrocytes in a differentiated phenotype.

Materials and Methods. Chitosan microspheres (Cht MCP) were made by a precipitation process. Chitosan solution was dropped into a gelation solution (sodium hydroxide 0.1M and absolute ethanol at a 70/30 volume ratio) under continuous stirring. Gelled microspheres instantaneously formed, were allowed to cure in the gelation solution for 24 h. Finally, in order to prepare the composites 2 wt. % fibrinogen solution was mixed with microspheres and 1U/ml of thrombin solution. Those gels were allowed to coagulate overnight at 37ºC, the formed composite gel was finally crosslinked with 5 mM genipin solution. Fibrin gel (fbn) without microspheres was used as control. In the biological in vitro experiments human chondrocytes were cultured for up to 4 weeks. Cellular viability was assessed by live/dead cells kit, DNA quantification and MTS assay. Chondrocyte morphology and phenotype were examined by SEM and immunofluorescence staining respectively.

Results. Figure 1 shows the cellular morphology over the fibrin/ Cht MCP. The fibrin fibers covered the nanoporous microspheres forming a smooth surface in the composite and cells adhered firmly to the coated microparticles (a). Chondrocytes remained viable at 14 days of culture (b). A higher number of cells was found in the control samples compared with composite structures. The limited proliferation suggests that cells can maintain better their phenotype avoiding fast dedifferentiation, despite collagen type I expression (c) and actin cytoskeleton development (d) detected in the samples.

Conclusions. Chondrocytes cultured in fibrin gels and in fibrin/chitosan microspheres composite gels are viable but some cells in hybrid materials show characteristics of dedifferentiated chondrocytes.

The authors acknowledge the financial support of the Spanish Ministry through the DPI2010-20399-C04-03 project and the funding of the Instituto de Salud Carlos III and the CIPF for the "Investigación Básica y Traslacional en Medicina Regenerativa".

Keywords. gel, polysaccharide microspheres, fibrin, articular cartilage

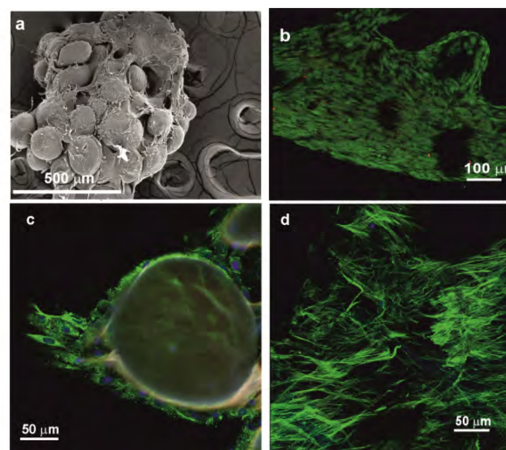

Figure 1 Cross section SEM micrographs of composite gel (a), confocal images of viable (green) and dead (red) chondrocytes (b). Expression of type I collagen in green (c) and actin cytoskeleton (green) (d) after 28 days of culture. Cell nuclei were counterstained with Dapi (blue).

(5.P7) ULTRASTRUCTURE CHARACTERIZATION FOR BONDING EFFICACY OF RESIN TO DENTAL CEMENTUM

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Introduction. Margins of dental adhesive restorations are frequently located at the cementum or cervical outer dentin. The root cementum has high organic content and predominantly consists of cross-linked collagen structure, less hard and more permeable compared with enamel and dentin. New adhesive systems have been developed in an attempt to obtain a reliable bonding to all tooth substrates. The aim of the study was to determine the bonding efficacy of three adhesive systems to human cementum, and to assess the promoted surface roughness.

Materials and Methods. Extracted human canines were used for the present study. Cervical cementum surfaces were ground flat with wet 600-grit silicon carbide paper and bonded. Three adhesive systems were employed: an etch-and-rinse adhesive (Single Bond –SB-), a two-step self-etching (Clearfil SE Bond –CSEB-), and a 1-step self-etching (One-Up Bond F –OUB-) adhesive. After applying the adhesive, resin composite build-ups were constructed and stored in a humid environment for 24 h at 37ºC. Specimens were sectioned into 1 mm2 beams and tested for microtensile bond strength (MTBS). Additional surfaces were conditioned for Atomic Force Microscopy (AFM) analysis; digital images of treated surfaces (5x5 and 15x15 µm) and average surface roughness of the scanned areas were obtained. Data were analyzed by ANOVA and SNK multiple comparisons tests ($p<0.05$).

Results. Means and standard deviations of MTBS (MPa) and roughness (Ra –nm-) values are shown in the table. Letters and numbers show differences in columns.

	5x5 µm (	**15x15 µm**	**MTBS (M**
No treatm	32.77 (11.	74.15 (6.2	--
35% H_3PO_4 et + SB	51.79 (15.	232.96 (35	51.49 (10.
CSEB	36.02 (4.6	62.42 (10.	21.14 (12.
OUB	20.84 (7.2	26.1 (2.7	31.40 (16.

Conclusion. When phosphoric acid treatment was applied, cementum surface roughness increased and a strong demineralization with exposed collagen fibers was observed. The etch-and-rinse adhesive promoted highest bond strength on human cementum surfaces.

CICYT/FEDER MAT2008-02347/MAT, JA-P07-CTS2568 and JA-P08-CTS-3944.

Keywords. dental cementum, roughness, bond strength, adhesive systems

(5.P8) MICROCOMPUTED TOMOGRAPHY ANALYSIS OF BONE REGENERATION AFTER MAXILLARY SINUS AUGMENTATION: A CASE REPORT

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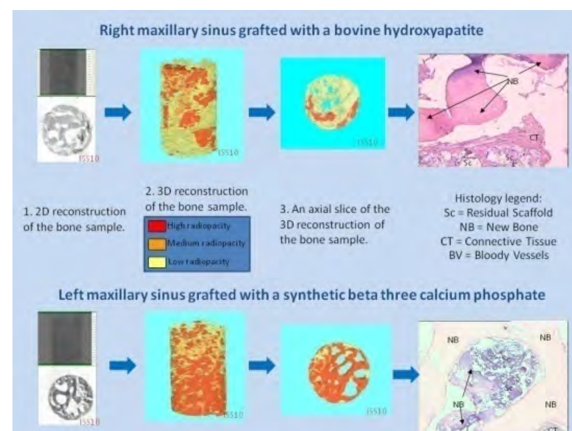
Introduction. In the last few years bone tissue regeneration studies has led to a better knowledge of chemical and structural features of biomaterials. Scaffolds for bone tissue engineering should provide a three-dimensional design and an osteoconductive surface to promote the ingrowth of new bone after implantation into bone defects. The possibility of investigation on their morphometric characteristics allows to evaluate the predictability of regenerative process. X-ray microtomography (microCT) is a miniaturized form of conventional tomography, able to analyze in a non-invasive and non-destructive way the internal structure of small objects, performing three-dimensional images with high spatial resolution (<5 micron pixel size). The aim of this work is to illustrate the possible applications of micro-CT in the analysis of human bone grafted with different scaffolds in order to obtain morphometric parameters and three-dimensional reconstruction by using the SkyScan 1072 scanner.

Methods and Results. We present a case of a patient who needed a bilateral maxillary sinus lift for dental implants placement. One side was grafted with a bovine hydroxyapatite (Endobon, Biomet 3i) while the controlateral sinus received a synthetic beta three calcium phosphate (Cerasorb, Curasan). A bone sample for each side was taken at implant placement surgery (after about six months post sinus augmentation) and was analyzed by microCT. Histologycal examination was also performed to illustrate advantages and disadvantages of microtomography versus traditional microscopy (fig. 1).

Conclusion. Since there is a close relationship between the properties of a bioscaffold and its microstructure, it is necessary to examine it using the highest level of resolution before being able to improve existing materials or to design new products. For a correct analysis, the samples should not have been modified or treated in any way, so the microCT is a non-invasive and non-destructive technique and its appliance gives considerable results in biomaterials' studies and tissue engineering.

Keywords. scaffold, osteoconduction, microcomputed tomography



(5.P9) FUNCTIONALIZATION OF 3D POROUS BONE SUBSTITUTES USING LAYER-BY-LAYER TECHNOLOGY

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Introduction. To avoid donor site morbidity material associated with autologous bone grafting and due to limited availability of bone autograft, the use of cancellous bone allografts (CBA) is an appealing strategy for the therapy of localized bone defects. Functionalization with osteogenically active factors may enhance long-term osseointegration and improve the clinical performance of CBA. The Layer-by-Layer (LbL) method involves the formation of polyelectrolyte multilayer (PEM) films whereby layers of oppositely-charged electrolytes are deposited alternatively. The aim of this study was to establish a protocol for optimized surface coating of 3D porous bone substitute materials applying alternating deposition of hyaluronic acid (HA) and poly-L-lysine (PLL) by the LbL method. The effects of the number of deposition steps on the morphology, proliferation and osteogenic differentiation of mesenchymal stromal cells (MSC) were investigated.

Materials and Methods. Human MSC were cultured in monolayer (2D) and on CBA scaffolds (3D) for up to 14 days. CBA scaffolds and cell culture plates were modified by coating with PLL/HA films (n=12 and n=24). Proliferation was assessed by total DNA quantification. Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) activity. Coating density and cellular distribution were performed by fluorescence microscopy (FM).

Results. Proliferation rate of MSCs with and without the use of PLL-HA films (n=12 or n=24) showed no significant differences after 14 days. By 3D cultivation a decreased proliferation was observed. 2D cultivation of MSCs stimulated osteogenic differentiation as observed by increased cell-specific ALP activity compared with 3D cultivation on day 14. FM demonstrated that polymer films were distributed homogenously throughout the CBA samples and were stable for up to 14 days.

Conclusion. PLL-HA coating with n=24 is suitable for 2D and 3D static cultivation of MSCs on CBA scaffolds.

Further studies will address the evaluation of LbL-mediated growth factor functionalized CBA on proliferation and osteogenic stem cell differentiation.

Keywords. bone substitutes, Layer-by-Layer technology, MSCs

(5.P10) ZINC STABILIZES DENTIN COLLAGEN AFTER ETCHING PROCEDURES.

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Introduction. Partial demineralization of a dentin mineralized collagen by acids may represent a suitable collagen to be remineralized, in the presence of minerals. Demineralized exposed collagen can undergo degradation by endogenous matrix metalloproteinases. Effective inhibitors of matrix metalloproteinases may be included in resin-dentin interfaces to protect the seed crystallite-sparse collagen fibrils, from degradation before they could be re-mineralized.

Materials and Methods. A human dentin beam degradation assay was performed. Dentin beams were obtained and included in: 1) demineralized beams created by 10% phosphoric acid (PA); 2) demineralized beams created by 0.5 M EDTA; 3) immersion of mineralized beams in Clearfil SE Bond primer (SE); and 4) immersion of mineralized beams in Xeno V (XE). Two demineralized dentin (approx. 2 mg) were placed in each incubation media: 1) artificial saliva -AS-; 2) 40 mM chlorhexidine digluconate in AS; 3) 3.33 mg/ml of zinc chloride in AS; 4) doxycycline (1:1) was added to the AS. Dentin beam specimens were incubated in 500 µl of media at 37oC for 24 h or 3 wk. Supernatants were analyzed for the release of collagen degradation product (C-terminal telopeptide of type I collagen -ICTP-) using a radioimmunoassay. Values were analyzed by ANOVA and SNK multiple comparison ($P<0.05$).

Results. Mean ICTP values and multiple comparisons results are in the table. Identical numbers in each row indicate no significant difference. In each column values with identical low case letters indicate no difference between solutions within the same dentin treatment and identical upper case letters indicate no significant difference between treatments within the same solution.

Conclusions. Zinc at high concentrations serves as potent, stable and effective inhibitor of dentin MMPs. MMPs degradation of collagen is strongly reduced in resin infiltrated dentin and zinc addition lowered degradation to values near to those of mineralized dentin.

CICYT/FEDER MAT2008-02347, JA-P07-CTS2568 and JA-P08-CTS-3944.

Keywords. dentin, metalloproteinases, Zinc, inhibitor

Dentin treatment	24 h	3 weeks
PA-treated + AS	70.01 (16.67) 1a C	178.23 (22.51) 2ab C
PA-treated + AS + chlorhexidine	30.82 (8.29) 1b C	200.56 (16.55) 2a C
PA-treated + AS + Zn	16.32 (5.84) 1c C	45.66 (5.98) 2c B
PA-treated + AS + Doxycycline	<0.01 (0.00) 1d A	<0.01 (0.00) 1d A
EDTA-treated + AS	80.46 (8.70) 1a C	160.34(16.32) 2a C
EDTA-treated + AS + chlorhexidine	16.76 (1.40) 1b B	179.88 (14.57) 2b B
EDTA-treated + AS + Zn	10.99 (1.61) 1b B	79.25 (8.06) 2c C
EDTA-treated + AS + Doxycycline	<0.01 (0.00) 1c A	<0.01 (0.00) 1d A
SE-treated + AS	12.24 (1.11) 1a A	49.97 (4.16) 2a A
SE-treated + AS + chlorhexidine	6.24 (0.33) 1b A	30.38 (2.55) 2b A
SE-treated + AS + Zn	1.51 (0.11) 1c A	5.28 (0.49) 2c A
SE-treated + AS + Doxycycline	<0.01 (0.00) 1d A	<0.01 (0.00) 1d A
XE-treated + AS	27.96 (0.91) 1a B	69.32(6.55) 2a B
XE-treated + AS + chlorhexidine	6.64 (0.40) 1b A	32.33(2.14) 2b A
XE-treated + AS + Zn	2.39 (0.28) 1d A	6.33 (0.56) 2d A
XE-treated + AS + Doxycycline	<0.01 (0.00) 1e A	<0.01 (0.00) 1e A

(5.P11) EVALUATION OF CALCIUM PHOSPHATE CERAMICS FABRICATED FROM EXTRACTED HUMAN TEETH FOR TOOTH TISSUE ENGINEERING

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Bioceramic tooth powders were prepared via heat treatment of extracted human teeth using sintering temperatures between 600ºC and 1200ºC, and their properties were investigated for potential tooth tissue engineering. The sintered human tooth powders were characterized using thermal analysis (thermogravimetric analysis (TG) and differential thermal analysis (DTA)), field emission scanning electron microscopy (FE-SEM), X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDX), and Fourier transformed infrared (FTIR) spectroscopy. Additionally, the phase constitutions and chemical homogeneities of the composite samples were examined using a quantitative chemical analysis with inductively coupled plasma (ICP) spectroscopy. The results revealed that the annealing process produced useful hydroxyapatite-based bioceramic biomaterials when annealed above 1000°C. The FTIR spectra and the TG/DTA thermograms of the tooth powders indicated the presence of organic compounds, which were completely removed after annealing at temperatures above 1000°C. The tooth powders annealed between 1000ºC and 1200ºC had good characteristics as bioceramic biomaterials. Furthermore, the biocompatibility of each tooth powder was evaluated using in vitro and in vivo techniques; our results indicate that the prepared human tooth powders have great potential for tooth tissue engineering applications.

Keywords. human tooth powder, calcium phosphate bioceramics, extracted teeth, regenerative medicine, tooth tissue engineering

(5.P12) BONE HEALING USING TISSUE-ENGINEERED CONSTRUCTS FOR LONG BONE CRITICAL SIZED DEFECTS IN SHEEP

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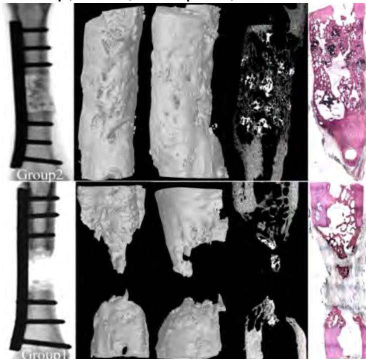
Introduction. In a previous study, our group has shown that standardized biohybrides engineered from Porites (coralline scaffolds) and combined with autologous MSCs, are efficient for bone critical-sized defect repair in sheep. Several authors have emphasized that coupling scaffold resorption and new bone apposition must be obtained for bone healing. Using an ectopic sheep model, we have previously compared the resorption and the bone formation between Porites/MSCs and Acropora/MSCs. This study has demonstrated a less extensive coral resorption as well as a good bone formation in Acropora/MSCs group. The aim of the current study was to validate this promising biohybrid construct in a clinically relevant bone defect.

Materials and Methods. Osteoperiosteal segmental (25mm) defect was created in the left metatarsal bone and the bone marrow was aspirated from the iliac crest of each sheep. Each defect was filled with a poly-methyl-methacrylate (PMMA) spacer. The PMMA spacer was removed 6 weeks later and the defect was filled either with Acropora scaffold (Group1, n=4) or with Acropora scaffold loaded with autologous MSCs isolated from bone marrow (Group2, n=5).

Results. 6 months after implantation, animals were sacrified and each defect was assessed by micro-computed tomography and histology. No bone union was observed in Group1, in contrast to Group2 where bone continuity was observed in 4 sheeps. Acropora resorption rate was higher in Group1 than in Group2 (99.2±0.8% v.s. 95.8±4.7%). The amounts of newly formed bone in defects filled with coral/MSCs were significantly higher from those filled with Acropora alone (677±227 v.s. 1357±471 mm3).

Conclusion. The present study is the first study evaluating Acropora scaffold in an orthotopic model. This study established clearly the benefits of using Acropora scaffold loaded with MSCs for long bone defect in sheep.

Keywords. sheep, coral, Acropora, critical-sized-defect



(5.P13) EVALUATION OF A CORAL BONE SUBTITUTE IN A NEW ORTHOTOPIC LARGE DEFECT IN MICE

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Introduction. Tissue-engineered bone constructs are an appealing strategy to overcome drawback of autograft for the treatment of massive bone defects. In mice, preliminary evaluations of these types of constructs have shown many advantages: low cost, homogeneity of response, functional evaluation of biological processes. However, large diaphyseal bone defect models in mice are sparse and often use bone fixation which does not provide optimal stability and which fills the bone marrow cavity. The aims of this study were to develop a critical-size segmental femoral defect in mice and to evaluate a natural bone substitute in such model.

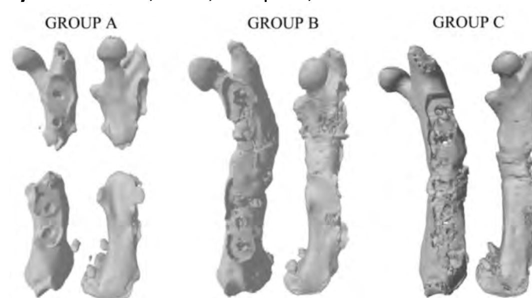
Materials and Methods. NUDE mice were used for this study. A locking plate with 4 screws was applied on the anterior femoral side. A 3-mm mid-diaphyseal ostectomy was subsequently performed using a specific jig. The resulting bone defects tested were as follows: Group A: left empty (n=5); Group B: filled with a massive isograft (n=6); and Group C: filled with massive coral scaffold (Acropora) (n=6). Bone healing was assessed by radiographs and micro-computed-tomography after sacrifice, 9 weeks postoperatively.

Results. Stable bone fixation was maintained throughout the study in all animals.

Bone union did not occur in Group A but were observed in all animals belonging to Group B and C. Bone volumes for Group A and B and volume of new bone formation for Group C inner the defect were 0.78±0.3 mm3; 4.43±0.87 mm3 and 3.65±0.84 mm3, respectively. Results of Groups B and C were similar, and significantly higher than those of Group A.

Conclusion. The present study establishes a novel, reproducible, murine large femoral defect (. Bone bridging occurred in this model when a bone substitute was used. This model allows further studies of the molecular and cellular events that are involved in bone replacement strategies especially with human cells.

Keywords. mice, coral, acropora, critical-sized-defect



(5.P14) ASSESSMENT OF MECHANICAL BEHAVIOUR OF POTENTIAL IMPLANTS MADE OF POLYSILOXANE CONTAINING LAYERED SILICATE NANOPARTICLES

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Introduction. Silicone rubber implants have long been used for local contour corrections such as nasal, chin, and cheek augmentation, because they are water-repellent, heat stable and chemically inert materials. They are also used in the manufacture of medical devices including urological, orthopaedic, ophthalmic and drug delivery devices, such as vaginal rings and transdermal implants. However, most applications require that poly(dimethyl siloxane) (PDMS) be reinforced by solid fillers because the very poor mechanical strength of the unfilled elastomer. Layered-silicate clays are interesting reinforcing agents due to their high aspect ratio and exceptionally stable oxide networks.

Materials and Methods. Condensation type PDMS, grade DMS-S31 (Gelest Inc.), was the elastomer matrix and organic modified montmorillonite (OMMT) under the trade name Cloisite® 20A (Rockwood Clay Additives GmbH) the reinforcement. PDMS/organoclay nanocomposites were prepared using the sonication technique and were characterized by X-ray diffraction (XRD), hardness measurements (Shore A), tensile and tear tests. The solvent uptake of immersed PDMS nanocomposites was also measured at 25oC.

Results. The XRD analysis showed that within the experimental setup of this work, for OMMT concentrations up to 5 phr, intercalated/exfoliation hybrids are formed. The incorporation of montomorillonite significantly improves the tensile properties of PDMS matrix. Increase in tearing strength and hardness of PDMS nanocomposites was also observed in comparison with the unfilled elastomer. The increase of clay content decreases swelling of the elastomer due to an increase in the tortuosity, because of the presence of clay particles, whereas an increase in the crosslinking density can be observed, probably due to physicochemical interactions between the organoclay reinforcement and polysiloxane molecules.

Conclusion. The incorporation of OMMT in silicone rubber significantly improves its performance properties assessed in terms of hardness, as well as tensile and tear strength. This suggests that the studied hybrids would display improved behaviour when used as implants in biomedical applications.

Keywords. polysiloxane elastomers, layered silicate, nanocomposites, implants

(5.P15) TRIPPLE CONFIRMATION FOR BIOACTIVITY OF SYNTHETIC HYDROXYAPATITE (HAp) IN BONY ENVIRONEMENT

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1. Riga Stradina University

Introduction. To evaluate reactogenicity of contacting soft and bone tissue after implantation of synthetic HAp bioceramics in experimental and clinical conditions.

Material and methods. HAp ceramic implants with porosity of ceramics 25% – 30 %, on 14 rabbits were inserted in bone, on 12 animals subperiostaly . After two weeks and three months expression of TGFß and apoptotic cells were evaluated. Late outcomes of maxillary sinus floor elevation with HAp granules and 278 SEMADOS (BEGO) dental implant insertion on 185 patients were evaluated using also cone beam 3D method. In 31 cases biopsy of biomaterial/host tissue hybrid and alveolar bone was done.

Results. Two weeks after intraossal implantation expression of TGFß in bone around HAp implants was in marked number of bone cells while in control side was no expression. After subperiostal implantation in two weeks was no expression of TGFβ either in bone or soft tissue, after three months was rich expression in both bone and periosteum. Apoptotic cells were in moderate number around HAp implants. Radiodensitometry of the elevated sinus floor area showed 98 % after operation, 82% average after 6 months, 76 % after 3 years of loading and 65 % after 5 years. Radiodensity of residual alveolar bone was 55 % after the implantation, 64 % at second stage surgery, 78% after 3 years and 70 % after 5 years. In biopsies after 6 months were bone trabecules, fibrous tissue and granules with degradation by osteoclast – like cells without inflammatory cells.

Conclusion. Release of endogenous growth factor, remodeling of HAp / host hybrid and contacting atrophic bone remineralisation confirm bioactivity of synthetic porous HAp biomaterials in bone environment.

Key words. HAp bioceramics, transforming growth factor β, biopsy, radiodensitometry.

Supported by Latvia State programm's Project „New materials and technologies for substitution of biologic tissue".

(5.P16) RESPIRATORY GATED MICRO COMPUTED TOMOGRAPHY FOR IMPLANTATION SITE IMAGING DURING IN-SITU TISSUE ENGINEERING IN LIVE SMALL ANIMAL MODEL

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1. Medical University of Graz

In our attempts to tissue engineer the esophagus using the in-situ tissue engineering approach, investigations were performed using the Sprague-Dawley rat model using respiratory gated micro CT to observe implanted constructs. Rat esophageal epithelial cells (REEC) were isolated and cultured. The cells were seeded 14 days after isolation on collagen scaffold disks. The cell-scaffold construct were fabricated into tubes using a silicon stent using Polyglytone-6211 monofilament absorbable interrupted sutures and implanted into the rat omentum. Under general anesthesia, a laparotomy was performed and the omentum exposed. During 6 months of in-situ tissue engineering, repetitive imaging of the abdomen was performed using the Inveon® MicroCT equipment (Siemens Medical, Malvern, PA, USA) with the Isoflurane® system and a 125mm detector with a voxel size of 35.55μm. A further advantage of using this device is the respiratory and cardiac gating capability of the rodent being investigated. This is possible through a high-speed shutter that acquires image frames with exposure times as short as 10ms for cardiac and respiratory gated studies with 4 TTL gating ports allowing management of dynamic acquisition. For imaging, the rat was first put into the anesthesia induction chamber, which was connected to the Isoflurane® small animal vaporizer that delivers accurate concentrations under varying conditions of flow rate and temperature, particularly at low flows. For induction the concentration of the anesthetic gas was set on 5%, after which the rat was transferred into the Inveon® MicroCT system. Tubes from the vaporizer were then connected to the anesthesia inputs in the MicroCT device with dedicated rat naso-mouth anesthetic mask placed on the animal.

MicroCT of constructs undergoing in-situ tissue engineering is a non-invasive method for imaging of implanted constructs when a live small animal model.

(5.P17) PCK-26 ANTIGEN EXPRESSION IN ADULT AND FETAL OVINE ESOPHAGEAL EPITHELIAL CELLS IS NOT AN INDICATOR OF END CELL DIFFERENTIATION: IMPLICATIONS FOR ESOPHAGEAL TISSUE ENGINEERING

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Introduction. In esophageal epithelium, cells move from the basal layer towards the lumen demonstrating increased differentiation and reduced proliferation capabilities. The aim of this study was to investigate the expression of cytokeratin in adult ovine esophageal epithelium (AOEE) and fetal ovine esophageal epithelium

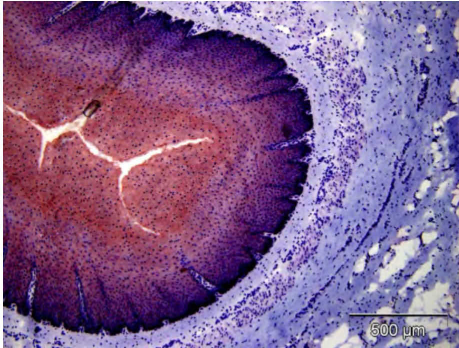
(FOEE) and to determine if cytokeratin expression also represented cells with proliferative potential.

Materials and Methods. Biopsies of ovine esophageal epithelium (OEE) were obtained from fetal lambs in late pregnancy (120 days) and were compared to those from adult sheep. FOEE and AOEE were investigated using pan-cytokeratin antigen (PCK-26) and Proliferating Cell Nuclear Antigen (PCNA) markers. Furthermore, PCK-26 positive esophageal epithelial cells (EEC) were isolated using Fluorescent Activated Cell Sorting (FACS) and analyzed for PCNA expression to estimate their percentage in AOEE.

Results. PCK-26 expression was prominent in AOEE but reduced in FOEE. PCNA expression was found throughout the FOEE, however was limited toward the AOEE basal layer. PCK-26 positive EEC with PCNA expression accounted for 24% of the total cells in AOEE.

Conclusion. PCK-26 antigen is not a marker of differentiation as it also represents cells with high proliferative capability in AOEE. FOEE in late gestation also demonstrate weak PCK-26 antigen expression but a high expression of proliferation.

Keywords. cytokeratin, expression, proliferation



(5.P18) ESOPHAGEAL SMOOTH MUSCLE CELLS DEDIFFERENTIATE WITH LOSS OF A-ACTIN EXPRESSION AFTER 8 WEEKS IN-VITRO CULTURE: IMPLICATIONS ON ESOPHAGUS TISSUE ENGINEERING

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1. Medical University of Graz

Introduction. Esophagus tissue engineering using the hybrid-construct approach which involves assembly of the various esophageal components and amalgamating them to engineer the organ are presently being investigated. The aim of this study was to investigate the dedifferentiation and loss in expression of esophageal smooth muscle after explant expansion in culture.

Materials and Methods. Ovine esophagus smooth muscle cells (OESMC) were sourced from adult biopsies and expanded in culture using the explant technique. The explants were maintained under static in-vitro tissue culture conditions with medium changes performed on every 2nd day. Flow cytometry analysis was performed for α-smooth muscle actin (α-SMA) expression at intervals of 4 weeks for up to 8 weeks.

Results. OESMC reached confluence after 3 weeks in culture and sufficient cells could be obtained after 4 weeks expansion to permit flow cytometry. After 4 weeks 58.5% OESMC exhibited α-SMA which decreased to 28.5% after 6 weeks in culture. After 8 weeks in culture a mere 1.3% OESMC demonstrated α-SMA expression.

Conclusion. OESMC proliferated using the explant technique exhibit a total loss of α-SMA expression after 8 weeks of in-vitro culture. The data obtained from these investigations are crucial for tissue engineering of the esophagus using the hybrid-construct approach.

Keywords. smooth muscle, expression, culture

(5.P19) BIOARTIFICAL TISSUE FOR THE SURGICAL RECONSTRUCTION OF TRACHEAL LESIONS

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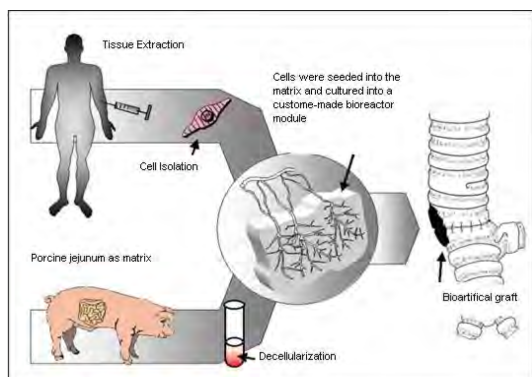
Introduction. Many graft materials and transplantation approaches have been developed to create a clinically applicable tracheal substitute. However, all these substitutes are lacking a vascular system resulting in insufficient oxygen and nutrient supply and ultimately graft necrosis. Here, we present GMP-conform techniques to generate a human, non-immunogenic, tissue equivalent with an innate vascularization that can be used as bioartifical tracheal graft.

Materials and Methods. We improved a well-established protocol to generate a decellularized and DNA-free vascularised scaffold from porcine jejunal segments, which exhibits an arterial and venous pedicle as well as the former luminal structures. Decellularization state, DNA content and endotoxine levels were analyzed by routine histological staining (H&E) and the use assays against gallic acids and a limulus amebocyte lysate assay.Human microvascular endothelial cells were seeded into the capillary system whereas primary fibroblasts and muscle cells were placed into the luminal structure. Culture of the scaffold was performed in a custom-made bioreactor module under dynamic culture conditions corresponding to the human circulation. After 14 days of culture, we performed H&E as well as, immunohistochemical staining (Anti-fibroblast, CD31, vWF, desmin and myoglobin). A vitality assay (MTT) was employed to analyze the reseeding efficiency.

Results. Analyses of the scaffold revealed the complete decellularization with endotoxin values consistent with the prescriptive levels of the European Pharmacopeia. After 14 days of culture within the dynamic bioreactor system, confluent reseeding was observed by the use of vitality assay and histological staining. Furthermore, cellular identify was characterized using defined cellular markers for each cell population.

Conclusion. In summary, we established an efficient GMP-conform process for tracheal graft engineering according to the requirements of the German Drug Act. Currently we perform in vivo experiments to evaluate the risk of vascular thrombosis. After we have received the manufacturing licence, we aim to start clinical trials.

Keywords. bioartificial transplant, Trachea, GMP



(5.P20) LIGHT AND ELECTRON MICROSCOPY CHARACTERIZATION OF A NEW COMPOSITE: COLLAGEN AND ARNICA DERIVED POLYSACCHARIDE-COATED LIPOSOMES

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Introduction. Clinical studies have shown that intra-articular administration of anti-inflammatory drugs encapsulated in liposomes shows prolonged residence in the joint and reduction of inflammation. Moreover, the anti-inflammatory properties of Arnica montana are very useful for the treatment of osteoarthritis and rheumatoid arthritis. The collagen-liposomes conjugates were found to deliver higher levels of active agent over a sustained period of time, in vivo, compared to normal collagenous preparations.

The aim of this study was microscopy characterization of a new composite based on collagen and Arnica montana derived polysaccharides-coated liposomes, in order to use it in the local treatment of rheumatic and inflammatory disorders.

Materials and Methods. Liposomes were prepared using the thin-film hydration method, followed by sonication. Briefly, a mixture of phosphatidylcholine: dioleoylphosphatidylethanolamine: cholesterol: stearylamine (4.125 mg lipids/ml; 4:2:3:1 molar ratio) was dissolved in chloroform/methanol solution (95:5) and a thin, dry film of these lipids was made in a rotary evaporator. The film was hydrated with phosphate buffered saline pH 7.4 containing Arnica montana derived polysaccharides (4 mg/ml). In order to fabricate the composite, a solution of collagen type I (5.3 mg/ml) was mixed with a solution of polysaccharides-coated liposomes, in a ratio of 1:1(v/v). The mixture was gelled at room temperature. Gels were frozen at - 40 0C and freeze-dried 24 h, for obtaining porous composites. Both, gel and porous composites were used for light and ultrastructural studies.

Results. In the presence of collagen type I, polysaccharides-coated liposomes were entrapped within the fibril network. Moreover, collagen fibrils appear to be firmly attached to the liposome surface, suggesting the existence of interaction between collagen and liposome membrane.

Conclusion. Microscopy studies have demonstrated that both, in gel and porous composites, collagen interacts with arnica derived polysaccharides-coated liposomes suggesting their use as intra-articular drug delivery system.

Acknowledgements. This work was supported by Project Cartbiotech, No. 62-059/2008.

Keywords. liposomes, Arnica montana derived polysaccharides, collagen, articular drug delivery

(5.P21) FABRICATION AND CHARACTERIZATION OF THREE DIMENSIONAL SCAFFOLDS OF BIOCERAMIC-POLYMER COMPOSITE VIA MICROSTEREOLITHOGRAPHY TECHNIQUE

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Microstereolithography is a method used for rapid prototyping of polymeric and ceramic components. This technique converts a computer-aided design (CAD) to a three dimensional (3D) model, and enables layer per layer fabrication curing a liquid resin with UV-light or laser source. The aim of this project was to formulate a photocurable polymer reinforced with calcium pyrophosphate (CPP) and fabricate a scaffold for application in tissue engineering. The photopolymer or UV curable ceramic suspension was prepared with an acrylate polyester, multifunctional acrylate monomer with the addition of 50-70wt% of CPP, and photoinitiators. From layer controlled determination, 3 wt% and 0 photoinniators was required to control an effective area of localized photopolymerization, this also depends on the weight fraction of CPP in the suspension. The 3D structure of the photopolymer resin was successfully fabricated using (µSL) apparatus (Envisiontec Perfactory3® Desktop System). The resin were fabricated in 'dumb-bell' form for tensile testing and a rectangular prism shape specifically designed for 4 point bending, and hardness measurement. They were then sintered at high temperature for polymer removal, to obtain a ceramic of the desired porosity. Morphology and CPP content of the sintered polymer was investigated with SEM and XRD, respectively. The addition of CPP coupled with high temperature sintering, had a significant effect on the mechanical properties exhibited by the bioceramic. The density increased to more than 35% and the dimensional shrinkage after sintering were 33%. The success fabrication of novel bioceramic polymer composite with µSL technique offer the possibility of designing complex tissue scaffolds with optimum mechanical properties for specific tissue engineering applications.

Keywords. Biomaterial, microstereolithography, calcium phosphate, tissue engineering

6. BIOMATERIALS AND THE REACTIONS THEY ELICIT IN THE BODY

Chair: Yvonne Bastiaansen-Jenniskens
Co-chair: Yves Bayon
Keynote speaker: Ruud Bank
Organizer: Yvonne Bastiaansen-Jenniskens

Synopsis: Biomaterials are very often used as scaffold for regenerating tissue, either in vitro and to be implanted later, or directly into the defect in vivo. A successful biomaterial will integrate in the body without causing massive inflammation and/or fibrosis. Inflammation is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. When this healing process is out of balance, fibrosis can occur. Fibrosis can be designated as an abnormal healing process characterised by an excessive accumulation of extracellular matrix proteins (in particular collagen). This process alters the extracellular matrix structure and will eventually result in loss of function of the particular tissue.

In this symposium we want to discuss the effects biomaterials have on the behaviour of the cells seeded on or surrounding the biomaterial focussing on reactions related to inflammation, wound healing and fibrotic reactions.

(6.KP) THE FOREIGN BODY REACTION AGAINST GELATIN AND (NON-)CROSSLINKED COLLAGEN DISPLAY MAJOR DIFFERENCES

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Like any other biomaterial, implanted collagen scaffolds induce a series of damage-inflicted processes that include wound healing, inflammation and the foreign body reaction (FBR). Macrophages play a pivotal role in the tissue response. These macrophages interrogate the biomaterial surface, release proteolytic enzymes and/or phagocytose the biomaterial. Under certain circumstances, the macrophages may fuse, to form multinucleated foreign body giant cells. Collagen-based biomaterials can be cross-linked to enhance the stiffness and to dampen the rate of biological degradation. In addition, non-crosslinked (native) collagen as well as denatured collagen, i.e. gelatin, can be used. It is the specific application that determines the choice of the biomaterial. Biomaterial applications must take the tissue response towards biomaterials into account.

Despite the frequent use of collagen-based scaffolds in tissue engineering, remarkably little is known about the nature of the foreign body reaction and the molecular mechanisms that are involved in the breakdown of the scaffolds. In a series of experiments, we observed that the tissue response towards the gelatin disks and the (non-)crosslinked, native collagen disks differs markedly with respect to the number of macrophages, the efficiency of giant cell formation, the size of the giant cells, the influx of neutrophils, and the micro-environment (presence of IL-13 and TIMP-1), the expression of MMPs and cathepsin K, and the expression of the collagen receptors Endo180 and DDR-2. Thus, the physical state of the collagen itself (denatured or native) as well as its chemical nature (type of cross-link) has a dramatic impact on the outcome of the foreign body reaction. We will discuss the observed findings in terms of degradation rates of the scaffolds and the mechanisms involved in this degradation. In addition, we will show that macrophages inside and outside the biomaterial have different phenotypic properties.

Keywords. collagen, macrophages, foreign body reaction, degradation

(6.O1) GENE EXPRESSION PATTERNS IN OSTEOGENIC CELLS TREATED WITH STRONTIUM-SUBSTITUTED BIOACTIVE GLASSES

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Introduction. Bioactive glasses (BG) are used as bone replacements because they bond with living tissue and dissolve upon implantation, releasing ions that stimulate bone formation. Strontium (Sr) ranelate is an anti-osteoporosis drug that works via Sr cations, which stimulate osteoblast differentiation and prevent osteoclast-mediated bone resorption. We have previously shown that BG in which Ca was substituted with Sr, promote osteoblast proliferation and activity and decrease osteoclast activity and resorption. Here, we examine the effects of Sr-substituted BG on gene expression patterns in cultures of human mesenchymal stem cells (hMSC) and primary osteoblasts (hOB).

Methods. BG in which 0, 10 or 100% of Ca was substituted with Sr were produced. Culture media was created by soaking with BG particles to release their active ions. hMSC and hOB from 3 separate donors were treated with BG-treated media for up to 14 days. RNA was isolated and gene expression patterns were analysed by quantitative real-time RT-PCR and whole genome microarray.

Results and Discussion. We demonstrate that genes for bone-specific transcription factors and proteins are upregulated in cultures treated with BG compared to controls treated with basal medium. In osteoporosis patients treated with Sr ranelate, an anabolic effect on bone formation has been observed. Here, we show that osteogenic genes are upregulated to a greater extent in hMSC and hOB treated with Sr-substituted BG compared to standard all Ca BG controls. Taken together, these results suggest that Sr-substituted BG upregulate key genes in bone development, suggesting that it may be possible to reproduce the anabolic effect on the skeleton produced by orally delivered Sr ranelate in a biomaterial that releases Sr locally. More extensive data analysis of microarray results may also reveal insights into the mechanism by which Sr acts on osteogenic cells.

Keywords. Bioactive glass, strontium, bone regeneration.

(6.O2) THE ROLE OF HYDROLYTIC ENZYMES AND REACTIVE OXYGEN SPECIES IN AN IN VITRO MODEL OF MACROPHAGE-MEDIATED DEGRADATION OF POLY(TRIMETHYLENE CARBONATE) NETWORK FILMS.

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Resorbable polymers are used in the human body as drug carriers, as scaffolds for tissue regeneration, and in preparation of degradable implants such as sutures. Macrophages play an important role in the degradation of

these polymers. Enzymes and reactive oxygen species (ROS) were shown to be involved in the degradation process. This research aims at elucidating the involvement of enzymes and/or ROS in the degradation mechanism of gamma irradiated poly(trimethylene carbonate) (PTMC) films.

The roles of enzymes and ROS in degradation were evaluated by culturing murine J774 macrophages on PTMC network films containing inhibitors for specific degradation pathways. The influence of complement on the degradation process was assessed as well. Degradation was quantified by determining mass loss of the PTMC discs. Macrophage activity was measured through cytokine release of Il-6, MCP-1 and MIP-1α as determined with ELISA. Cell coverage was calculated from images obtained with confocal microscopy.

Cholesterol esterase was found to be the main contributor to degradation as assessed by the inhibition of degradation by diethyl umbelliferyl phosphate. The results furthermore demonstrated that acid proteases (inhibited by pepstatin A), serine and cysteine proteases (inhibited by phenylmethyl sulfonyl fluoride) and ROS (indirectly inhibited by apocynin through NADPH oxidase and nitric oxide synthase) contribute less to the degradation of PTMC networks. Activity of macrophages was high both with and without the influence of inhibitors, as indicated by high concentrations of secreted cytokines MCP-1 and MIP-1α. Degradation in media without complement was higher than in media with complement.

The presented macrophage culture model is helpful in reducing the number of animal experiments and provides a useful fast, in vitro model to investigate the mechanism of in vivo degradation of biodegradable polymers.

Keywords. macrophage, model, biodegradation, pTMC, degradable

(6.O3) INNOVATIVE IN-VITRO POLYCULTURE MODEL, AS AN ARTIFICIAL LIVING PERITONEUM, FOR ABDOMINAL MESH EVALUATION

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1. Covidien; 2. Therapol

Introduction. In-vitro assays are unavoidable in the medical device evaluation process, and often represent the first step for biomaterial characterization. However, it is today well accepted that in-vitro cell culture assays are non relevant of real life conditions.

Materials and Methods. In this study, a coculture model involving the cells present in a healthy peritoneum was developed. Human fibroblasts, macrophages, mesothelial cells and/or endothelial cells were seeded in a type I collagen matrix and cocultured up to reach a stable living structure. The model showed baseline cytokine expression which was dramatically increased when a wound was induced on the surface.

Polypropylene (PP), Polyester (PET) and collagen coated polyester (PETc) prostheses, presenting increasing hydrophilicity gradients, were deposited on the coculture model to evaluate the model different reactions when in contact with those materials.

Results and discussion. To generate a wound, scalpel cuts were applied on the coculture model inducing dramatic pro-inflammatory cytokine secretion.

As already observed under single cell culture, the coculture models still showed better cell adhesion and proliferation on prostheses following hydrophilicity gradients (PET and PETc) when compared with hydrophobic prostheses (PP).

Furthermore, the tri-cells models presented a measurable shrinkage (30%* in surface, *$p<0,05$) as reaction to the bare prosthetic materials (PP and PET) while no model shrinkage was measured at all for the collagen coated prostheses (PETc), showing the collagen benefit for the device integration. No shrinkage at all could be measured when endothelial cells were added to the coculture, highlighting physiological contractile reactions.

Conclusion. A new in-vitro complex coculture model was developed as a living peritoneum. This model presented better cell compatibility correlated with surface hydrophilicity gradients and highlighted the collagen positive impact on the in-vivo integration reaction.

Keywords. in-vitro, inflammation, mesh

(6.O4) HYDROPHILIC RESORBABLE AND BIOCOMPATIBLE POLYMER SYSTEMS AS BIOACTIVE COATINGS OF POLYPROPYLENE MESH AND CONTROLLED RELEASE OF ANTIBIOTICS FOR TISSUE INTEGRATION

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Introduction. The reparative process of hernia defects are in general based on the apposition of polypropylene meshes as biostable implants, which guarantees the biomechanical stability of the affected tissue or organ. After more than 30 years of clinical application, it is clear that one critical point is the infection of the tissues or organs in contact with the mesh and the consequences of the infection process reaching statistical values around 20% in a period of 2 or 3 months after implantation, depending on microorganism strain origin of the infection process. In this work we present a novel an excellent results on the application of bioactive and resorbable hydrogel polymers based on copolymers of hydroxyethyl methacrylate HEMA and 2-acrylamide- 2-methyl propanesulfonic acid AMPS, as bioactive coating of lightweight polypropylene PP meshes, and the addition to the polymer system of a well known antibiotic, vancomycin at a concentration of 20 wt-% respect to the coating of the polymer applied.

Materials and Methods. The coating of the PP mesh was obtained by the deeping of the mesh in a solution of 10 % of copolymer with a composition 20 mole-% of AMPS and 80 mole-% of HEMA containing 20% of vancomycin. After drying a homogeneous coating of 2.0 μm was obtained.

The antibacterial activity of the coated meshes was tested by analyzing the inhibition areas of proliferation of agar plates inoculated with Staphylococcus aureus SA or S.epidermidis SE. The bioactivity was analyzed in vitro using fibroblast cultures, and in vivo by implantation of coated meshes in infected areas of the dorsal muscle of

rabbits, and analysis of the prosthesis and surrounding tissue after 14 and 30 days of implantation.

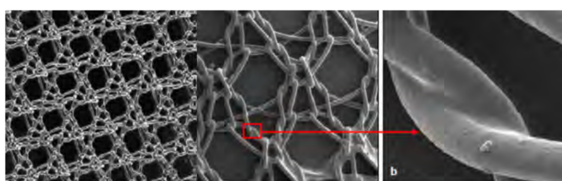
Results. Results of the in vitro test demonstrated the antibacterial activity of the coated meshes after 14 and 30 days, and an excellent correlation with the activity in vivo after implantation during the same period.

The Figure shows the structure and morphology of the mesh with the homogeneous coating of the bioactive polymer containing 0.32 mg/cm2 of vancomycin. This concentration is enough to control the infection without detect vancomycin in the blood flow.

Conclusions

Quantitative evaluation of the release of vancomycin in the animal model, as well the antibacterial activity of the meshes in vitro and in vivo demonstrate that the application of the coating offers excellent opportunities to improve the behavior of PP meshes in clinical applications with a minimum dose of antibiotic applied an release in the point of infection and activity.

Keywords. synthetic polymers, drug delivery, bacteria adhesion



(6.O5) DIFFERENTIATION OF MACROPHAGES INTO PRO- OR ANTI-INFLAMMATORY/ REPAIR SUBTYPE IN CULTURE

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Introduction. Macrophages are key immune cells in the reaction to a biomaterial as they interact with proteins adhered to biomaterials after implantation. To investigate their specific response to biomaterials, it is important to characterize macrophages in detail. Macrophages can be roughly divided into a pro-inflammatory (M1) subtype and an anti-inflammatory/repair (M2) subtype. The goal of our study was to characterize macrophages differentiated towards M1 or M2 phenotype and examine the stability of this differentiation.

Materials and methods. Monocytes were isolated from the blood of healthy donors using Ficoll separation and magnetic cell sorting (MACS) based on magnetic antibodies against CD14. Monocytes were stimulated for one week with cytokines; LPS or IFN-γ for M1-stimulation, dexamethasone or IL-4 for M2-stimulation. After stimulation, cytokines were removed and culture was continued for 7 days. Gene expression for IL-6, TNF-α and IL-10 and morphology were analyzed at 0, 1, 3 and 7 days after removal of the stimuli.

Results. Macrophages stimulated with LPS or IFN-γ (ie M1- stimulated) had an elongated shape and expressed high levels of IL-6 and TNF-α. Macrophages stimulated with dexamethasone or IL-4 (M2-stimulated) had high IL-10 gene expression and these cells had a round morphology. After removal of the stimuli, differences between M1- and M2-stimulated macrophages remain for at least 7 days for all parameters.

Conclusion. Using soluble factors, macrophages can be differentiated into a pro- or anti-inflammatory/repair subtype, as characterized from selected read-out parameters. Based on these parameters, this differentiation appears to be stable for at least 7 days. With this knowledge, differentiated macrophage populations can be further used for the evaluation of the inflammatory properties of implantable biomaterials, in in vitro cell systems.

Keywords. macrophages inflammation biomaterials

(6.O6) TEXTURAL PROPERTIES AND IN VIVO RESPONSE OF CALCIUM PHOSPHATES CEMENTS-BLOOD COMPOSITE

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Introduction. Calcium phosphates cements (CPCs) are used as bone substitutes because of their similarity to the mineral phase of bone. However, they can be considered as fragile materials and they usually present limited osteoconductive properties. So they still are dedicated to fill small bone defects in non-load bearing conditions. The purpose of this study was to investigate how blood addition can interfere with setting processes and final properties of two types of apatitic CPCs, one presenting a shorter setting time (SST) and the other one a longer setting time (LST).

Results and Discussion. α-TCP was transformed into poorly crystalline apatite in all tested samples after 72 hours of incubation in a saline solution. For blood/LST cement, both adhesion properties and time of workability were significantly increased. Compressive strength tests showed a ductile material behavior (fig 1.A). Regarding to blood/SST cement (fig 1.B) only a slight increase of both properties and time of workability was observed. After 12 weeks of implantation we could observe an excellent bone/implant osteocoalescent interface.

Conclusion. This study showed that adding blood can have different effects on CPCs properties assuming that they present different setting features. In vivo, it is known that fibrinogen present in the blood usually polymerize into fibrin within 12 minutes approximately. Our hypothesis is as following: when CPC setting time is longer than 12 minutes, significant modifications, due to the fibrin polymerization, of textural and mechanical properties can be observed. On the other hand, for quicker setting times, those properties are only governed by apatite crystallization which suggests that fibrinogen is not able to polymerize into fibrin in that case. Assuming that biological properties do not seem to be jeopardized by blood addition, it seems we have found a simple way to modulated stiffness and plasticity of apatitic CPCs which could extend applications of these injectable biomaterials.

Keywords. calcium phosphates cements, blood materials interaction, textural properties, in vivo responses

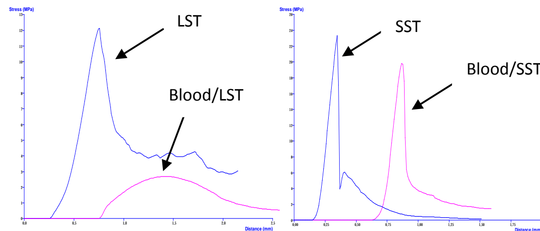

Fig. 1: Comparative compressive strength tests for CPC and blood/CPC (A) LST, (B) SST.

(6.O7) A NOVEL EVALUATION OF THE PERFORMANCE OF SOFT TISSUE REPAIR BIOMATERIALS FOLLOWING INTRAPERITONEAL IMPLANTATION IN HEALTHY AND DIABETIC RATS, BY QUANTITATIVE HISTOPATHOLOGY

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Introduction. New generations of biomaterials are facing the limitations of the ISO standard 10993-6 method of evaluation, focusing mostly on safety parameters. Their additional value comes from an improvement of their performance, eg. by accelerating the wound healing process with earlier cellularization and neoformation of tissue, by reducing the foreign body reaction. Specific histology stainings and methodology were developed for the quantification of wound healing markers, in healthy and diabetic rats.

Materials and methods. An abrasion and a surgical defect were respectively created on the caecum and opposite peritoneal surface of the Sprague Dawley and diabetic Zucker rats. The abdominal wall lesion was intraperitoneally covered with a composite biomaterial combining a textile and a collagen film. The implantation time was 21 days post-operatively. Histopathological evaluations were performed on histological sections stained with:- SHE & Masson's Trichrome, for the semi-quantitative analysis of selected inflammatory and wound healing parameters,- Junqueira (collagen polymorphism) and Feulgen & Rossenbeck stainings (DNA specific) (see Figure) for the quantitative analysis of the extracellular matrix, collagen I / III ratio and the cellularization, by using a customized image analyzer soft ware.

Results/Discussion. The semi-quantitative histopathological analysis showed that the composite biomaterial tended to yield less signs of inflammation and fibroplastic tissue formation, in diabetic vs healthy rats. Differences were more obvious and statistically significant from quantitative histopathology data: much slower formation of extracellular matrix, less mature with predominant collagen III, in diabetic vs healthy rats, as expected from the literature.

Conclusion. Quantitative histology allows simple and, more objective and concise evaluations of the performance of new biomaterials. Its automation should spare time & money and enable its routine use in this perspective.

Keywords. quantitative histology, image analysis, biomaterials, inflammation, wound healing, collagen I & III, cellularization, in vivo model

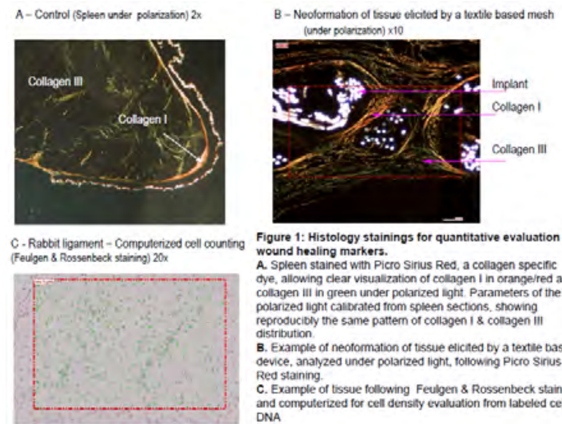

Figure 1: Histology stainings for quantitative evaluation of wound healing markers.
A. Spleen stained with Picro Sirius Red, a collagen specific dye, allowing clear visualization of collagen I in orange/red and collagen III in green under polarized light. Parameters of the polarized light calibrated from spleen sections, showing reproducibly the same pattern of collagen I & collagen III distribution.
B. Example of neoformation of tissue elicited by a textile based device, analyzed under polarized light, following Picro Sirius Red staining.
C. Example of tissue following Feulgen & Rossenbeck staining and computerized for cell density evaluation from labeled cell DNA

(6.O8) CORD BLOOD STEM CELLS EXCEED EMBRYONIC STEM CELLS IN INDUCING ECTOPIC BONE FORMATION IN VIVO

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Introduction. Surgery often leads to massive destruction of the skeleton. Cell-based bone reconstruction therapies promise new therapeutic opportunities for the repair of bone. Umbilical cord blood stem cells (USSC) as well as embryonic stem cells (ESCs) can be differentiated into osteogenic cells and are a potential cell source for bone tissue engineering. The purpose of this in vivo study was to compare these two stem cell lines regarding their ability to promote ectopic bone formation in vivo**.**

Methods. Human umbilical cord blood stem cells and murine ESCs were cultured as monolayer cultures as well as micromasses and seeded on insoluble collagenous bone matrix (ICBM). These constructs were implanted in immundeficient rats. After one week, one, two and three months CT-scans were performed to detect any calcifications. Subsequently, the rats were sacrificed and the implanted constructs were examined histologically.

Results. The radiological examination shows a steep increase of the mineralised tissue in the USSC-groups. This increase can be considered as statistical significant compared to the basic value. Moreover, the volume and the CaHa-Content were about ten times higher than in the ESC-group. In contrast, the volume of the mineralization in the ESC-group increased to a much lower extend during time and the control-group (ICBM without cells) almost shows no alterations during the study. The histological examinations parallel the radiological findings.

Conclusion. Cord blood stem cells in combination with ICBM induce ectopic bone formation in vivo stronger than embryonic stem cells. Thus, this cell population as well as the biomaterial ICBM might be promising components for bone tissue engineering.

Keywords. Cord blood stem cells, embryonic stem cells, in vivo.

(6.O9) INCORPORATION OF INFLAMMATORY SIGNALS IN BIOMATERIALS MODULATES HUMAN NK CELL BEHAVIOR LEADING TO IMPROVED MSC RECRUITMENT

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Introduction. Inflammation is one of the first events taking place upon implantation of a biomaterial. An exacerbated inflammatory response questions biomaterial biocompatibility, but on the other hand inflammation has a central role in regulation of Tissue Regeneration. Therefore, it may be argued that an "ideal" inflammatory response is crucial to achieve efficient tissue repair/regeneration. Natural Killer (NK) cells, being one of the first populations arriving at an injury site, can have an important role in regulating bone repair/regeneration, particularly through interactions with Mesenchymal Stem Cells (MSCs). Here, we studied how biomaterials designed to incorporate inflammatory signals affected human NK cell behaviour and NK – MSC interactions.

Methods. Ultrathin chitosan films were prepared by spincoating, to which the pro-inflammatory molecule Fibrinogen was adsorbed in a monolayer. It was tested how these films affected peripheral blood NK cells and bone marrow MSCs behaviour.

Results and conclusions. Adsorption of Fibrinogen to chitosan films led to a 1.5 fold increase in adhesion of NK cells, which was accompanied by a change in morphology. Freshly isolated NK cells were stimulated by MSC to produce cytokines, but Fibrinogen adsorption did not affect NK cell IFN-gamma secretion. Most importantly, it was found that NK cells are capable of stimulating a 3 fold increase in MSC invasion, a key event taking place in tissue repair, but did not affect expression of the differentiation marker ALP, detected by flow cytometry. Of significant importance, this NK cell-mediated MSC recruitment was modulated by Fibrinogen adsorption. Thus, designing novel biomaterials leading to rational modulation of the inflammatory response is proposed as a possible route in Tissue Regeneration strategies.

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Keywords. Tissue Regeneration, Inflammation, biomaterials, NK cells

(6.O10) HEMOCOMPATIBILITY STUDY OF BACTERIAL CELLULOSE

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Introduction. Vascular grafts must gather various complex attributes, like good mechanical properties, post-implantation healing response without any immunological reaction and no induction of blood coagulation. Over the years, many strategies were developed to modify materials for vascular devices. One strategy involves pre-coating with the tripeptide Arg-Gly-Asp (RGD), which improves endothelialization, thus lowering thrombogenicity. In the present work, the hemocompatibility of native and RGD-modified bacterial cellulose (BC) was studied. Despite being a promising material for vascular replacements, a comprehensive characterization of the BC-blood interaction, namely in the presence of RGD peptide, has not been performed to date.

Methods. Blood from healthy donors was placed in contact with native or recombinant RGD-treated BC and parameters related to a material's hemocompatibility were determined. These included adsorption of plasma proteins, clotting times, whole blood coagulation time, plasma recalcification profiles, platelet adhesion and hemolysis.

Results. The clotting times (aPTT, PT, FT and PRT) and whole blood clotting results demonstrate the good hemocompatibility of BC. A significant amount of plasma protein adsorbed to BC fibres, presenting albumin a higher BC affinity than gamma-globulin or fibrinogen. According to analysis carried out by intrinsic tryptophan fluorescence, BC-adsorbed plasma proteins tested do not undergo major conformational modifications. Although the presence of the adhesion peptide on bare-BC surface increases the platelet adhesion, when the material was cultured with human microvascular endothelial cells a confluent cell layer was readily formed, inhibiting the adhesion of platelets.

Conclusion. Generally, our data demonstrates that both native and RGD-modified BCs may be classified as hemocompatible materials, since they showed to be non-hemolytic and the whole blood coagulation studies show that the results are comparable to those produced by currently available materials for blood replacements.

Acknowledgements. Work funded by FCT project PTDC/EBB-EBI/112170/2009. FKA is supported a CAPES grant. JPS is supported by FCT grant SFRH/BPD/64958/2009.

Keywords. Bacterial cellulose; RGD peptide; Hemocompatibility; Vascular grafts.

(6.O11) IN VITRO AND IN VIVO BIOCOMPATIBILITY EVALUATION OF K-CARRAGEENAN HYDROGELS AIMED AT APLICATIONS IN REGENERATIVE MEDICINE

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Introduction. The development of biomaterials for biomedical applications always requires extensive biological testing to demonstrate the safety of both the material and its degradation components. K-carrageenan is a naturally occurring polysaccharide which forms a hydrogel with potassium ions and the temperature-induced gelation enables its application as an in vitro cell-carrier or as an in vivo injectable system. The aim of this study was to evaluate these novel systems as

biomaterials by in vitro biological screening and by in vivo implantation to assess for the inflammatory response.

Methods. In vitro evaluation: The cytotoxicity of the hydrogels was evaluated under standard tests using the L929 cell. Furthermore, the viability and proliferation of encapsulated human adipose stem cells (hASCs) was determined by fluorescence staining and DNA quantification. In vivo evaluation: discs of k-carrageenan were subcutaneous implanted in a wistar rats for up to 15 days. The materials (agarose-control material and k-carrageenan) were positioned into each pocket. Control animals with empty defect and empty defect injected with lipopolysaccharide were used. After each time period, the biomaterial, surrounding tissue and nearby lymph nodes were explanted and used for histological analysis and molecular biology evaluation.

Results. The cytotoxicity test and biological evaluation of k-carrageenan revealed that this polymer is not cytotoxic and enables long term viability and proliferation of cells in vitro. At implant retrieval there were no macroscopic signs of a considerable inflammatory reaction in any of the animals and no cellular exudates was formed around the implants.

Conclusions. The results indicated that k-carrageenan is a biocompatible material and does not cause a severe host response. These results together with those obtained regarding the properties of k-carragenan investigated under other studies indicate that theses hydrogels may be successfully applied in tissue engineering approaches.

Acknowledgements. The authors gratefully acknowledge Portuguese Foundation for Science and Technology (FCT) for the PhD grants of Popa EG (SFRH/BD/64070/2009), Carvalho P.P. (SFRH/BD/44128/2008), Santo VE (SFRH / BD / 39486 / 2007) and Frias AM (SFRH /BPD/45206/2008).

Keywords. hydrogels, biomaterials, in vitro response, subcutaneous implantation, inflammatory response, cartilage tissue engineering

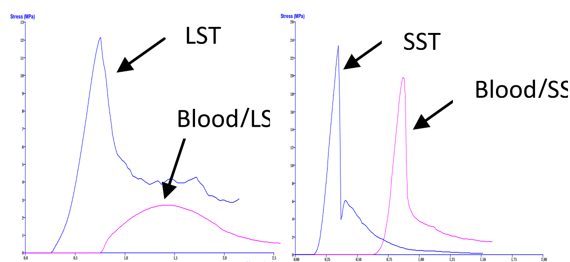

Fig. 1: Comparative compressive strength tests for CPC and blood/CPC (A) LST, (B) SST.

(6.O12) HUMAN HAIR KERATINS FOR TISSUE ENGINEERING

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Introduction. Natural materials, including proteins and polysaccharides, are being widely used as scaffolds for tissue engineering because they provide a bioactive platform for cellular processes and more closely resemble the native in vivo environment. However natural materials, derived mostly from animal sources, present risks of pathogen transfer and may be limited in abundance. This project was initiated to explore the feasibility of transforming human hair, one of the largest sources of bio-waste we produce, into templates that could be used in biomedical applications. Hair is attractive because 1) it is readily available, 2) it is a rich source of keratins which have the ability to self-assemble into a matrix and contain cell adhesion motifs, 3) it offers the possibility to produce autologous scaffolds for clinical applications.

Materials Methods. Keratins were extracted from random human hair samples in reducing conditions, using protocols modified from reported literature. Samples were characterised by Western Blotting to evaluate the profile of keratins obtained. Keratins in solution were subsequently processed into gels, freeze-dried discs or fibrous foams for subsequent feasibility studies. Preliminary cell culture studies were conducted to evaluate in vitro biocompatibility.

Results. In agreement with the literature, we demonstrated that keratins can be effectively extracted from human hair samples. Keratin gels, freeze-dried discs and fibrous foams were successfully fabricated. Scanning electron microscopy images show that 3D, interconnected micro-porous architectures can be produced within the freeze-dried discs and fibrous foams, characteristic of scaffolds suitable for use in tissue engineering. Preliminary cell culture studies show that the keratin templates produced can support cellular attachment and proliferation.

Conclusions. We showed that keratins extracted from human hair have the potential to be processed into various templates. Future studies will focus on using these in specific tissue engineering applications.

Acknowledgements. This work is funded by the National Medical Research Council (NIG09may016).

(6.P1) POLYMER SURFACES COATED WITH HYDROGEL TO IMPROVE BLOOD COMPATIBILITY

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Introduction. The aim of presented research was to develop a method for manufacturing hemocompatible coatings for blood-contacting devices. We present a simple method for fabrication of hydrogel coatings for cardiovascular devices. Polyvinylpyrrolidone (PVP) was chosen as a hydrophilic polymer to produce hydrogel network due to its highly biocompatibility and wide applications in medicine.

Methods. Hydrogel coatings of polyurethane (in a form of discs) were fabricated in a two-step method. First, the PU discs were immersed in a solution containing given amounts of crosslinking agent (EGDMA) and cumene hydroperoxide for 15 minutes at 25°C. After that time, samples were placed in a water solution containing given amounts of PVP, FeCl2 and ascorbic acid for 15 minutes at 25°C. Polymer discs were then washed and dried.

Blood-biomaterial interactions were evaluated using a platelet analyzer (Impact-R, DiaMed). A given volume of a whole-blood sample was dropped onto the characterized surfaces and shear stress was applied to simulate arterial flow conditions. The platelet consumption was calculated as a difference between the initial number of platelets

present in blood sample and the number of platelets after the test.

Results. Presented method is based on free-radical macromolecular polymerization. Cumene hydroperoxide is a source of radicals produced in the redox reaction with Fe2+ ions. Macroradicals recombination leads to PU-PVP grafting, PVP crosslinking and hydrogel formation. The results showed that the platelet consumption decreased from 56% (for unmodified PU) to 10% (for PU grafted with PVP).

Conclusion. Polyurethane grafted with polyvinylopyrrolidone seems to be promising material for cardiovascular applications. Hydrogel coating greatly reduced the level of platelet adhesion and activation.

Acknowledgments. This work has been supported by the Polish Artificial Heart Project and the European Union in the framework of European Social Fund through the Warsaw University of Technology Development Programme.

Keywords. hydrogel; surface modification; blood compatibility

(6.P2) IN VIVO FOREIGN BODY REACTION TO MICROSPHERES COMPOSED OF BIODEGRADABLE HYDROPHILIC MULTI-BLOCK COPOLYMERS

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introduction. Biodegradable hydrophilic multi-block copolymers composed of polyethyleneglycol and Lactide (PEG/LA-MBCP) are considered promising materials for the preparation of controlled release microsphere (MSP) formulations for site-specific or systemic drug delivery. To determine the in vivo biocompatibility of this new class of materials we examined the foreign body reaction (FBR) to subcutaneously administered PEG/LA-MBCP MSPs. Furthermore we studied the FBR in relation to particle size to determine the most optimal size of PEG/LA-MBCP-based MSP for use as injectable drug delivery depots.

Experimental Methods. MSP (particle size 5-200µm) were prepared by a standard W/O single emulsion process. Lyophilized MSP were suspended in sterile water containing 0.4% sodium carboxymethylcellulose and injected subcutaneously on the back of F344 rats. MSP and surrounding tissue were retrieved after 7 days. General histology was evaluated by toluidin blue staining. The FBR was studied by staining for macrophages (ED-1), fibroblasts (FSP-1) and potential wound healing macrophages (ED-1/FSP-1).

Results. A very mild FBR to PEG/LA-MBCP MSP was observed, as indicated by the absence of a fibrous capsule around the MSP. Large (50-200µm) MSP were surrounded by 1-2 cell layers of macrophages. A moderate macrophage infiltration was present between the microspheres and was interspersed with occasional fibroblasts and ED-1+/FSP-1+ macrophages. Small (<µm) MSP were phagocytised while large (30>50-200µm) MSP occasionally elicited giant cell formation.

Conclusion. PEG/LA-MBCP MSP demonstrated excellent in vivo biocompatibility. Small PEG/LA-MBCP MSPs were preferentially phagocytised, while larger MSPs were not. It is concluded that this new class of biodegradable hydrophilic polymers provides a suitable platform for parenteral drug delivery and that microspheres of 20 – 50 micron should preferably be used to minimize the overall foreign body reaction.

Acknowledgements. This research forms part of the Project P3.02 DESIRE of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs.

Keywords. biocompatibility, controlled release, foreign body reaction

(6.P3) ELASTIN MICROSPHERES ARE THROMBORESISTANT BUT NOT IMMUNORESISTANT

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Introduction. Finding an appropriate microsphere that can be systemically administered without causing thrombosis or an immune response is crucial to the success of cardiovascular therapy and diagnosis. Recent work [1,2] has shown that a family of recombinant human elastin-like polypeptides (ELPs) has the potential to protect against thrombotic events. Herein we investigate the effects of platelet and immune cell activation to ELPs (ELP1 and ELP4) that differ by molecular weight and sequence length.

Materials and Methods. Whole blood collected in sodium citrated tubes was tested for platelet activation (CD61 and CD62P) and leukocyte activation (CD62L, CD45 and CD11b) and analyzed using flow cytometry. Physiological blood flow (13.5 dyne/cm2) was simulated using Cellix's microfluidic platform. The biochip channels (400 x 100 µm) were coated with collagen, ELPs and uncoated control channel.

Results. Preliminary results showed that shear stress induced platelet activation (non-coated channel). Blood subjected to collagen-coated channels resulted in a 50% increase of P-selectin expression. Both ELP1 and 4 showed thromboresistant effects (Fig. 1). Initial results of the immunoresistant effects of ELPs indicate that CD11b was not impacted by the presence of the ELPs (data not shown).

Discussion and Conclusions. These preliminary results illustrate that the ELPs have thromboresistant properties. However, the mechanism of this protective effect has to be further studied. Interestingly, the ELP thromboresistant properties appear to be platelet specific, clearly not extending to the leukocyte population.

References. [1] Woodhouse et al. Biomaterials, 25, 4543 (2004). [2] Srokowski et al. J Biomat Sci Polym Ed, (2010) (accepted - JBS3083).

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funding support by the Canadian Institutes of Health Research.

Keywords. elastin, cardiovascular, platelet

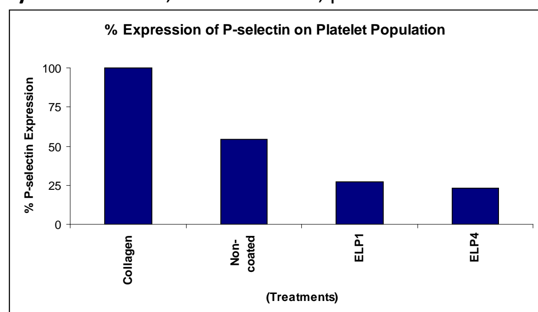

Fig. 1. Expression of CD61/CD62P on blood samples that were subjected to shear stress (13.5 dyne/cm^2) over collagen, ELP1, ELP4 and non-coated channels.

(6.P4) HISTOCHEMICAL EVALUATION OF IN VIVO BIOCOMPATIBILITY OF MODIFIED CARBON FIBRES

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Carbon fibres offer an unusual potential in designing new biomaterials for medical applications. They were attempted to use in the reconstruction of fibrous tissue and for the repair of cartilage and bone defects. The objective of our study was to investigate the biocompatibility of carbon fibres: porous, and coated with hyaluronic acid.

Methods. Three types of carbon fibres: non-modified (CF), porous (CFP), and modified with hyaluronic acid (CF/HA) were prepared from polyacrylonitrile precursor. The in vivo studies were carried out using the rat soft tissues as a model. Equal portions of CF, CFP, and CF/HA carbon fibres were implanted into the skeletal muscle of rats. After 1, 4, 12, and 30 weeks from the surgery, the implants along with their surrounding tissue were excised, frozen in liquid nitrogen and cut in a cryostat microtome. The obtained slides were investigated through histological and histochemical methods to estimate the intensity of inflammation, production of collagen, and metabolic activity of tissues surrounding the implant.

Results. The activity of mitochondrial oxidative enzymes: cytochrome c oxidase and NADH dehydrogenase in the muscle fibres in close proximity to the implants was only slightly lower than in those further away. The presence of a foreign body (i.e. carbon fibres) evoked a prolonged inflammation response (especially around CFP), still intense even in the 30-week series. On the other hand, inflammatory cells helped in the process of regeneration and prevented the formation of a connective fibrous capsule. The fibrous capsule around CFP and CF/HA implants was thin or not present at all - the fibres were in direct contact with the muscle tissue.

Conclusion. The regeneration and enzymic activity of muscle tissue together with the lack of fibrous capsule suggest that the carbon fibres used in our study are biocompatibile and are suitable as scaffolds for tissue engineering.

Keywords. carbon fibres, histochemistry, biocompatibility

(6.P5) NEW MICROSCOPY APPROACH TO EXAMINE THE HOST TISSUE INCORPORATION OF DIFFERENT BIOPROSTHESES USED FOR ABDOMINAL HERNIA REPAIR

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Introduction. Biological prosthetic materials have been added to the materials available for the repair of hernial defects in the abdominal wall. Such materials share the important feature that they are gradually degraded in the host resulting in the formation in its place of a neotissue, which in the long term will completely replace the biomaterial.

We assessed the host tissue incorportation of different bioprostheses, using a new tool that combines immunofluorescence confocal microscopy technique with differential interference contrast (DIC), making it possible to distinguish newly formed collagen from that of the bioprosthesis.

Methods. Partial hernial defects were created in the abdominal wall of rabbits and repaired using crosslinked (Permacol®(Pe),collamend®(Coll)) and non-crosslinked (Surgisis®(SIS)) bioprostheses. Eptfe (Preclude®) was used as control. 14, 30, 90 and 180 days post-implant, specimens were taken for microscopy, immunohistochemistry and qpcr to determine host tissue ingrowth and collagen I/III gene and protein expression.

Results. Eptfe was encapsulated by neoformed tissue while bioprostheses became gradually infiltrated by host tissue. SIS showed better tissue ingrowth and was more rapidly degraded. 14/30 days after placement, the different bioprotheses showed sparse or no immunostaining for collagen I. The levels of this protein increased over time, showing at 90/180 days their staining peak. In the SIS, staining was more discrete and evenly distributed throughout the biomaterial's thickness. At 14 days postimplant, collagen III was highly expressed in the neoformed tissue, and this expression rose at 30 days and continued increasing in the long term. At 14 days, Pe and Coll induced upregulated collagen 1 and 3 gene expression, while SIS only showed increased immature collagen III expression at 90 days.

Conclusions. This new microscopy approach allows monitoring the process of tissue integration and bioprosthesis degradation showing that despite the crosslinked collagen bioprostheses promoting less tissue ingrowth than SIS, they became gradually replaced by good quality host tissue.

This study was supported by a grant from the Fundación Mutua Madrileña 2008 (FMM08), Madrid, Spain.

Keywords. collagen bioprostheses, abdominal hernia repair, tissue integration.

(6.P6) BIOMATERIALS-ASSOCIATED INFECTIONS. INFLUENCE OF PROPHYLACTIC ANTIBIOTICS ON THE COMPETITION BETWEEN BACTERIA AND MAMMALIAN CELLS FOR THE BIOMATERIAL SURFACE

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Introduction. Biomaterials-associated infections represent a major clinical problem. The fate of a biomaterial implant has been described as a 'race for the surface' between microorganisms and tissue cells. Microorganisms are frequently introduced on an implant surface during surgery giving them a head start in the race for the surface. The aim of this study was to assess the influence of one shot of prophylactic antibiotics on the competition between bacteria and mammalian cells in an in vitro model.

Materials and Methods. The model comprised Staphylococcus aureus (ATCC 12600) and U2OS osteoblast-like cells cultured together on PMMA surfaces in a parallel plate flow cell in a modified culture medium (MCM) for up to 72 h. S. aureus were deposited on the surface followed by seeding of U2OS cells. Then flow was started with MCM containing Cephtolin (1xMBC) for 8 h. Subsequently, flow was switched to MCM only for the additional 64 hours. Biofilm growth was assessed using live-dead staining. U2OS cell number and morphology were measured after staining with phalloidin, CLSM and image analysis at 1.5 and 72 h.

Results. In the absence of antibiotics, U2OS cells died within 24 h in the presence of adhering S. aureus. In the presence of Cephtolin, no change in U2OS cell morphology was observed compared to control (U2OS cells without S. aureus). A slow growth of S. aureus biofilm was observed after 8 h of antibiotic treatment. The number of U2OS cells at 72 h was significantly reduced compared to control.

Conclusions. One shot of Cephtolin did not kill all bacteria. The slow growth of S. aureus after incubation with antibiotics is suggestive for decreased metabolic activity. Longer term antibiotic treatment should clinically be considered.

Keywords. infection; biomaterial-associated infection; antibiotic

7. BIOREACTORS TECHNOLOGIES FOR TISSUE ENGINEERING

Chair: Aaron Goldstein
Co-chair: Smadar Cohen
Keynote speaker: Fergal O'Brien
Organizer: Aaron Goldstein

Synopsis: Cell based approaches in regenerative medicine frequently rely on *in vitro* conditioning of cells to permit their proliferation, differentiation, and organization into tissue-like structures with functional properties approaching those of normal tissue. During this period of conditioning, bioreactors can be used to exert external stimuli (e.g., mechanic strain, hydrodynamic pressure and shear, electrical fields) that facilitate matrix deposition and tissue organization, and improve quantitative measures of tissue function.

In this proposed symposium for the TERMIS-EU meeting in 2011, we would include research presentations that describe the development and testing of bioreactors for regeneration of a large range of musculoskeletal and cardiovascular tissues. This includes (but is not limited to) bone, cartilage, tendon/ligament, skeletal muscle, cardiac muscle, blood vessels, and heart valves. Presentations concerning fundamental studies of bioreactor performance, basic studies of cell/tissue development *in vitro*, and translation studies of tissue efficacy *in vivo* would be encouraged. Special emphasis will be placed on presentations describing cutting-edge research, such as bioreactors designed to mimic the dynamic physical/mechanical stimuli that exist *in vivo* and strategies that employ multiple cell types.

(7.KP) COLLAGEN-BASED SCAFFOLDS IN TISSUE ENGINEERING: APPLIED BIOMATERIALS AND CELLULAR RESPONSE TO FLOW PERFUSION

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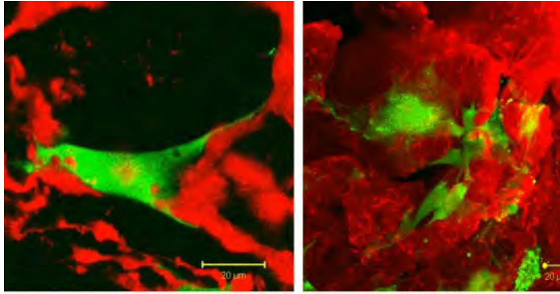
Tissue engineering uses a combination of: (i) biomaterial scaffold (ii) cells and (iii) signalling mechanisms (such as growth factors or mechanical stimuli) to restore the function of damaged or degenerated tissue in vivo or to culture tissue in vitro which can be used for implantation. Recent work in our laboratory has developed a series of collagen-based scaffolds with the optimal composition, pore structure and stiffness to promote bone formation in vitro and healing in vivo. In the cellular area, we are investigating the osteogenic, chondrogenic and angiogenic potential of mesenchymal stem cells on these scaffolds and we have a particular interest in using biophysical stimuli to regulate stem cell differentiation. In this area, we have developed a flow perfusion bioreactor system and have shown that flow perfusion increases the osteogenic potential of cells seeded on the scaffolds and quantified resultant cellular shear stresses using a computational fluid dynamics (CFD) model. Our results demonstrate that mechanism of cellular attachment in the scaffolds is critically important in regulating the optimal biophysical stimuli required to enhance osteogenic potential. We have shown that wall shear stresses required to activate an osteogenic response in calcium phosphate scaffolds with large pores are approximately 40 times higher than in collagen-GAG scaffolds with small pores. Furthermore, the results suggest that levels of cellular deformation are as important as cellular shear stress in regulating differentiation following flow. In scaffolds with smaller pores, cells can bridge the pores, which exposes them to greater deformation, allowing for enhanced osteogenic response at flow rates insufficient to promote a response in scaffolds with larger pores. Using results from the CFD model, we have been able to determine the requisite stimuli required for longer term bioreactor culture and have demonstrated the potential of using the system to improve cell distribution and enhance osteogenesis.

Keywords. collagen-based scaffold, flow perfusion, computational fluid dynamics, cell distribution, osteogenesis



Confocal images showing osteoblast cells (green): (a) bridging and, (b) flatly attached, to struts in a highly porous collagen-GAG scaffold (red). Our results demonstrate that the mechanism of attachment has important consequences for the response of the cells to biophysical stimulus.
(Jungreuthmayer et al. Tissue Eng Part A. 2009 15(5):1141-1149)

(7.O1) MICROBIOREACTORS FOR CARDIAC TISSUE ENGINEERING

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In contractile tissues such as myocardium, functional properties are directly related to cellular orientation and elongation. Thus, tissue engineering of functional cardiac patches critically depends on our understanding of the interaction between multiple guidance cues such as topographical, adhesive and electrical cues.

One of our goals was to determine the interactive effects of contact guidance and electrical field stimulation on elongation and orientation of cardiomyocytes and fibroblasts, major cell populations of the myocardium. We developed a precise microfabricated system, incorporating topographical and electrical cues on a single chip. The cell culture chips were created by hot embossing of polystyrene, with microgrooves and microridges of precisely defined depth, width and periodicity. The two gold electrodes were electrodeposited 1cm apart such that the microgrooves in between were oriented either parallel or perpendicular to the electrodes. Importantly, simultaneous application of biphasic electrical pulses and topographical cues resulted in gap junctions confined mainly at the cell-cell ends rather than the punctuate distribution normally found in neonatal cells. Overall, we observed that i) cardiomyocyte and fibroblast elongation on smooth surfaces was significantly enhanced by electrical field stimulation and ii) topographical cues were a significantly stronger determinant of cardiomyocyte orientation than the electrical field stimulation. The orientation and elongation response of cardiomyocytes was completely abolished by inhibition of actin polymerization and only partially by inhibition of phosphatidyl-inositol 3 kinase pathway.

Our current efforts focus on development of a microarray of cardiac organoids for drug and cell testing, where tissues are created by self-organization of embryonic stem cell derived cardiomyocytes around two microposts. Additionally, to create biological wires of 1-10cm scale capable of propagating electrical impulses, we employ self organization of cardiomyocytes around sutures placed in microbioreactor wells. Thus, the three microbioreactor configurations we developed provide control of cellular microenvironment to enable engineering of functional cardiac organoids.

Keywords. Bioreactor, microenvironment, cardiomyocyte, electrical stimulation

(7.O2) NEW GENERATION BIOREACTOR FOR IN VITRO ENGINEERING OF TUBULAR STRUCTURES

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Introduction. The clinical need to replace tubular organs with functional substitutes, where conventional reconstruction techniques are inadequate, is growing exponentially. Recently, there has been a growing optimism that cell-based tissue engineering methods may provide effective solutions and early promising results have been reported. We have already shown in a clinical setting that our previously developed double-chamber rotating bioreactor allowed multiple cell types to be grown onto a decellularized trachea [1,2]. Here we introduce a second-generation bioreactor for tubular construct engineering with improved functionalities.

Methods. Major aims of the bioreactor design were to: allow proper seeding and culturing of different cell types on both sides of a tubular matrix, promote efficient mass transport within a construct of clinically relevant dimensions and stimulate cells with hydrodynamic stimuli. Modularity, optimization of assembly procedures, control and automation over the entire process were further key requirements.

Results. Our bioreactor combines scaffold pre-tensioning, rotation and luminal perfusion, exposing cells alternatively to liquid and gas phases if half immersed in culture medium. A novel apparatus for the automatic medium exchange was also realised and coupled to the bioreactor, significantly contributing to minimize contamination risks and to protect homeostasis of the culture milieu. The manufactured system was bench-tested under different operating conditions, and preliminary cell culture trials were performed with positive outcome: higher cell survival and much better colonisation throughout the scaffolds thickness with respect to static controls were obtained.

Conclusions. The improved bioreactor is an effective and versatile system that could be used with different scaffolds (Ø, L) to in vitro engineer tubular structures, e.g., trachea and blood vessels. Based on the collected promising results, we have been in further experimental sessions to better investigate its role in driving cell response.

Acknowledgements: Supported by Regione Lombardia and by Harvard Bioscience, Inc. through a sponsored research agreement.

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Keywords. bioreactor, tubular structures, enabling technologies

(7.O3) AUTOMATED, ONLINE, REAL-TIME MONITORING OF CULTURE PARAMETERS IN MULTIPLE INDEPENDENT CHAMBERS OF A PERFUSION BIOREACTOR

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Introduction. Perfusion bioreactors represent a promising possibility for the development of automated, standardized, cost-effective, and safe manufacturing processes of engineered tissue substitutes.

Based on a previously developed perfusion bioreactor for seeding and culture of cell-scaffold constructs (NASA techbrief), in this study we developed and tested an automated device for online, real-time monitoring of critical culture parameters.

Materials and Methods. The bioreactor has been equipped with a motor driven automated sensing system (Fig.1a) and with a customized software (Fig.1b) able to monitor up to 18 independent culture chambers.

Validation tests were performed to monitor pH value within buffers and non pre-equilibrated culture medium without cells, with reference to induced environmental changes monitoring capability.

Moreover, expanded primary human articular chondrocytes were seeded and cultured on collagen (UltraFoam) scaffolds for 7 days in DMEM+10%FBS, with different cell densities. pH and pO2 were optically monitored and ΔpH (difference between pH upstream and downstream the scaffold) was calculated for each chamber. Inoculation of bacteria was performed, so as to simulate possible contamination and detect related changes in pH or pO2.

Results and Discussion. The sensing system was able to detect induced environmental changes due to incubator door opening, medium change and accidental blackout, in non pre-equilibrated DMEM+10%FBS, without cells. Online, real-time parameters monitoring enabled observation of progressive pH drop during cell dynamic culture and of sudden drop both in pH and pO2 due to induced bacterial contamination.

The different cell densities, estimated by DNA and MTT assays at the end of the experiment, could also be discriminated by different ΔpH values, detected by the system, thus giving an index of culture progression, assessable in real-time.

Conclusions. Our perfusion bioreactor, with automated, online monitoring of culture parameters, can represent a step forward towards a reliable device for the safe and automated manufacturing of biological tissues.

Keywords. perfusion bioreactor, online monitoring, automation

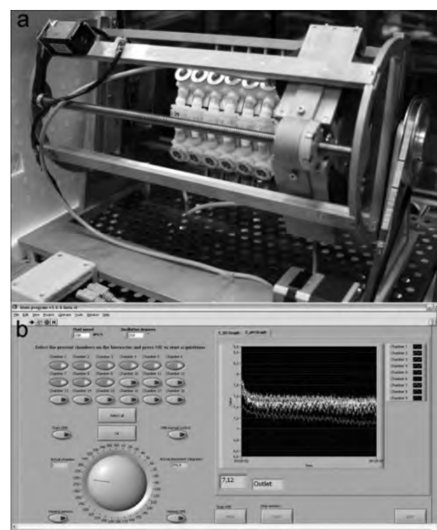

Figure 1: a) The sensorized bioreator system; b) Front panel of the cusutomized software.

(7.O4) MODELING OF FLOW-INDUCED SHEAR STRESS APPLIED ON 3D CELLULAR POROUS SCAFFOLDS

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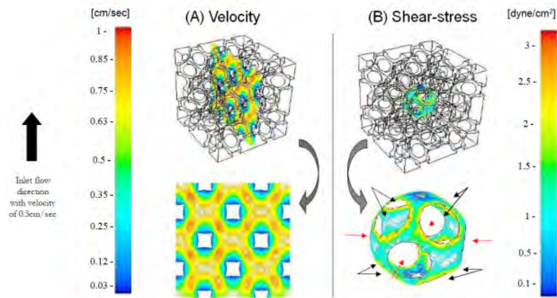
Introduction. Novel tissue engineering bioreactor systems are designed to overcome the size limitations of engineered tissue, which are dictated by oxygen and nutrient diffusion rates. Our bioreactor system employs direct perfusion through porous biopolymer scaffolds, which is meant to simulate physiological interstitial flow conditions. In order to properly estimate the flow-induced shear stress to which the cells are exposed, a computational fluid dynamics (CFD) model was developed. This model takes into account the complex 3D structure of the porous biopolymer scaffold and the growth of the cell layer and calculates the shear stress distribution as a function of the controllable flow rate and culturing time.

Goals. Develop a CFD model to estimate flow-induced shear stress applied on cells seeded on a porous biopolymer scaffold in a direct perfusion bioreactor, as a function of inflow rate and growing tissue layer thickness. The current model was designed to predict high shear stress values within the physiological range naturally sensed by vascular cells (1–10 dyne/cm^2).

Results. Representational maps of velocity and 3D shear stress distribution were obtained for each of the models. Analysis of the calculated wall shear-stress distribution in the acellular scaffold model shows that while the shear stress values positively correlated with increasing inflow velocities, the distribution pattern remained largely unvaried. As is expected in low Reynolds flow (Re<0.02), the flow regime, while convoluted, was absolutely laminar, and mean shear-stress remained proportional to the inlet velocity.

Conclusions. Our model provides an estimation of the dynamic microenvironment to which cells are exposed in our direct perfusion bioreactor. As such, it represents a useful tool for perfusion bioreactor system design, and provides an added level of control over experimental setups.

Keywords. bioreactor, CFD, shear stress



(7.O5) A NEW SEEDING AND CONDITIONING BIOREACTOR FOR HEART VALVE TISSUE ENGINEERING

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Introduction. The purpose of the study was to develop a new seeding and conditioning bioreactor that permits the application of an endoscope for online monitoring and documentation.

Methods. A new system was designed to provide a low pulsatile flow that grants the correct opening and closing of the valve leaflets without high shear stresses. This system consists of three main elements, the actuation unit, the core unit and the monitoring unit. The actuation unit generates an accurately adjustable pulsatile flow in the core unit. The core unit holds the heart valve and ensures a circulating flow through the valve to achieve an opening and closing of the valve leaflets. The monitoring unit fixates an endoscope for a precise monitoring of the valve leaflets.

Results. Bioreactor permitted an effective and sterile valve conditioning and/or seeding. It allowed both recording and documentation of the valve performance under pulsatile flow conditions. Microbiological tests of cell medium after 5 days conditioning revealed no bacterial contamination.

Conclusions. New bioreactor offers a new method that allows colonized cells to adapt to shear stress and to establish a strong extracellular matrix.

Keywords. Bioreactor; Heart valve; Seeding; Conditioning

(7.O6) DESIGN OF A FLOW PERFUSION BIOREACTOR FOR LONGITUDINAL MONITORING OF MINERALIZED EXTRACELLULAR MATRIX GROWTH

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Introduction. Bioreactors are widely applied to create controlled in vitro conditions that mimic the natural environment engineered tissues. Mechanical stress through flow perfusion as well as through improved nutrient transport have been shown to improve cell seeding efficiency, cell proliferation and differentiation into bone-like tissue. Non-destructive micro-computed tomography has been shown to provide qualitative and quantitative 3D data on mineralized extracellular matrix (ECM) development both in end-point measurements as well as in longitudinal monitoring studies. We sought to design bioreactors that combine the two techniques in order to follow the reaction to mechanical stimuli of each sample individually in a controlled environment.

Methods. Custom-made bioreactors were designed to fulfill the demand for sterile conditions during measurements and medium exchange, radio-opacity, and at the same time controllable fluid flow patterns. Mesenchymal stem cells were seeded onto disk-shaped porous silk fibroin scaffolds of 8 mm diameter and 2-3 mm height and cultured under static and dynamic (0.2 ml/min) conditions in osteogenic medium for 7 weeks.

Results. The device offers a cartridge-chamber system that allows investigating into 24 stiff and/or compliant scaffolds in parallel and provided sterility throughout the whole culture time, during micro-CT scanning and media exchange. While the control group showed increasing mineralized ECM, the application of a perfusion flow of 0.2 ml/min resulted in increased cell proliferation without cell differentiation but a better cell distribution throughout the scaffold volume.

Conclusions. Longitudinal monitoring studies over several weeks can be performed with this new bioreactor design without contamination. Optimal dynamic parameter settings still have to be determined to maximize mineralized ECM content. Additionally, this bioreactor may improve current seeding strategies through better distribution of cells throughout the scaffold volume. Acknowledgments: We would like to acknowledge funding from the RMS Foundation, Bettlach, Switzerland. Silk was kindly provided by Trudel Silk Inc., Zürich, Switzerland.

Keywords. flow perfusion, bioreactor, monitoring, stem cells

(7.O7) CYCLIC HYDROSTATIC FORCE APPLIED IN A CUSTOM BIOREACTOR STIMULATES ENHANCED BONE DEVELOPMENT IN THE FOETAL CHICK FEMUR IN VITRO

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Introduction. Hydrostatic force has been suggested as an important stimulus by which osteochondral and progenitor cells sense and respond to mechanical loading in vivo. A model system has here been established to investigate bone development in the chick foetal femur ex vivo in response to low levels of applied cyclic hydrostatic forces using a custom designed bioreactor (Tissue Growth Technologies).

Methods. Femurs isolated from day 11 chick embryos were cultured in vitro in alpha or osteogenic medium. A regime of one hour stimulation per day at 1 Hz, cycling between 0 – 40 PSI (276 MPa) was applied under standard cell culture conditions for 10 out of 16 days. End point analysis was by µCT and Alizarin red assay for matrix mineralisation.

Results. After 16 days femurs in both osteogenic and alpha media that received stimulation were visibly more compact than unstimulated femurs in alpha media. µCT analysis revealed a significant increase in the density and volume of the bone collars in osteogenic media with stimulation over unstimulated controls (fig. 1.). A smaller effect was observed for stimulated femurs in alpha medium. All stimulated femurs displayed increased bone collar density regardless of media type – this was supported by a calcium assay which showed that similar amounts of calcium were present in all stimulated femurs, approximately an 8-fold increase in calcium over unstimulated controls in alpha medium.

Conclusions. Cyclic hydrostatic force stimulates bone collar growth and mineralisation in the chick femur ex vivo. Increased bone formation was observed in both media types, indicating that this type of stimulation can independently stimulate osteogenesis and also act synergistically with soluble factors to enhance bone development in vitro. This stimulation regime could therefore be applied to cell-seeded 3D scaffolds for in vitro conditioning prior to their implantation for applications in osteochondral tissue engineering.

Comments. Fig. 1. µCT of bone collars from chick femurs cultured in alpha or osteogenic media +/- cyclic hydrostatic stimulation.

Keywords. Bioreactor, Osteogenesis, Mechanical Stimulation, Bone

(7.O8) THE EFFECT OF ALTERING FREQUENCY DISTRIBUTION OF MECHANICAL STIMULATION ON MYOCARDIAL-EQUIVALENT TWITCH FORCE

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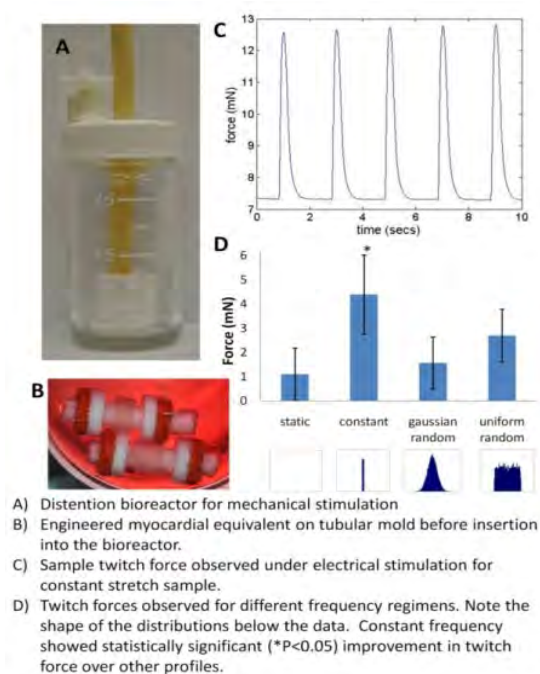
Tissue engineering of myocardium represents a promising approach for the treatment of myocardial infarcts. Previously, we have shown that cell-induced alignment improved cellular communication via increased Cx43 functionality, resulting in an increase in twitch force beyond that of merely aligning the cells. Previous work by other groups in the field has also shown that periodic mechanical stimulation improves the observed twitch force; however, these studies were carried out using constant frequency and amplitude. It has been shown in other tissues that variations in the stretch amplitude can improve matrix deposition and protein synthesis. The induced stretch in myocardium mimics the stretching of the ventricle as it fills with blood. Since blood pressure and heart rate follow a Gaussian distribution, we hypothesize that normally varying the stimulation frequency would improve twitch force compared to uniformly distributed and constant frequency stimulation. To test this, neonatal rat cardiac cells were entrapped in a tubular fibrin gel and cultured in a custom distension bioreactor for 14 days. A computer program controlled the stimulation frequencies according to user-chosen distributions during cell culturing. The constructs were mechanically stimulated with constant frequency (C), Gaussian frequency distribution (G), and uniform random frequency distribution (R). Static culture (S) was used as a control. A preliminary analysis of twitch force data found that C (4.4±1.6mN) constructs improved twitch force over G (1.6±1.1mN), R (2.7±1.1mN), and S (1.1±1.1mN) constructs with statistical significance (P<0.05). Ongoing experiments are being conducted to determine whether this improved function is the result of enhanced cell viability, improved cell communication or increased contraction efficiency. Future experiments include varying the amplitude in accordance with changes in the frequency to evaluate further differences in twitch force, as well as investigating whether mechanical stimulation enhances the function of engineered myocardium created with MSC derived cardiomyocytes.

Keywords. Cyclic Distension, Engineered Heart Tissue, Variable Stretch, Fibrin Gel



A) Distention bioreactor for mechanical stimulation
B) Engineered myocardial equivalent on tubular mold before insertion into the bioreactor.
C) Sample twitch force observed under electrical stimulation for constant stretch sample.
D) Twitch forces observed for different frequency regimens. Note the shape of the distributions below the data. Constant frequency showed statistically significant (*P<0.05) improvement in twitch force over other profiles.

(7.O9) DEVELOPMENT OF AN NOVEL BIDIRECTIONAL CONTINUOUS PERFUSION BIOREACTOR (BCFB), FOR CULTURING CELLS IN 3D SCAFFOLDS

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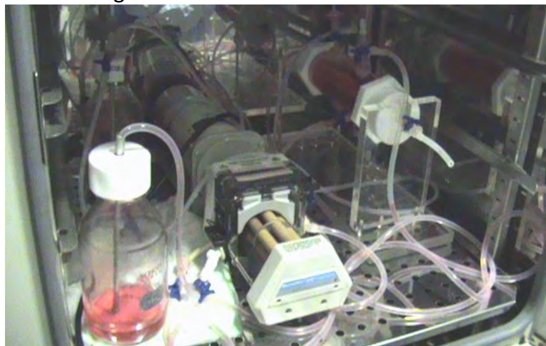
This works presents a new bioreactor, for the culturing of 3D scaffolds aimed at applications in bone tissue engineering. The Bidirectional Continuous Perfusion Bioreactor (BCFB) promotes the mechanical stimulation of cells through the creation of shear forces induced by flow perfusion, using different pressure gradients, controlled by a peristaltic pump. Additionally it provides the possibility of varying both perfusion flow rate/flow direction. The main innovation consists in the possibility of culturing scaffolds of large dimensions, as the control of flow perfusion and pressure gradient in the inside/outside of the scaffold, enables a culture

environment that favours the access to nutrients and removal of metabolic wastes of the cells located in the inner regions. Starch/Polycaprolactone (SPCL) fibbers mesh scaffolds (14 samples with 16mm x 4mm thickness with a concentric hole of 6mm) were seeded with 1x106 goat marrow stromal cells and stacked, completing a 48 mm thick construct. After 14 and 21 days of culture in the bioreactor at a flow rate of 1 ml/min, the samples were collected for DNA/ALP concentration, and SEM. Static cultured constructs were used as controls.
The results showed higher ALP activity levels in dynamic cultures than those obtained under static conditions. However, the number of cells (obtained from DNA amounts) in constructs cultured in the bioreactor showed lower values compared to static cultures, showing that static conditions tend to privilege the metabolic way for cellular proliferation while dynamic conditions tend to privilege the metabolic way for osteogenic differentiation. The lower values of the DNA amount of the constructs in the bioreactor could be explained by shear forces in the constructs, thereby hampering cell proliferation but enhancing cell differentiation. The BCFB can be used for enhancing cellular differentiation and proliferation by applying flow perfusion. Therefore, this bioreactor could be applicable to generate large-sized 3D scaffolds.
Keywords. Bioreactors; Bone Tissue Engineering; 3D scaffolds Large Dimensions



(7.O10) A PERFUSION BIOREACTOR SYSTEM FOR THE DEVELOPMENT OF TISSUE-ENGINEERED BONE CONSTRUCTS

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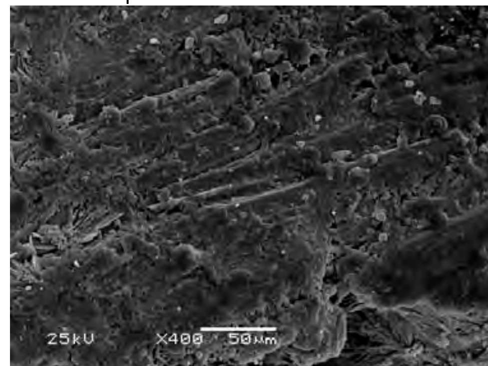
Introduction. The development of tissue engineered bone constructs is of considerable importance to fill defects associated with segmental bone replacement in bone cancer or spinal fusions.
Aim. To culture mesenchymal stem cells (MSCs) on porous and granulated scaffolds using a perfusion bioreactor system (PBRS) and study their proliferation, osteogenic differentiation and distribution compared to statically cultured constructs.
Hypothesis. A PBRS will provide an even distribution of MSCs throughout porous and granulated scaffolds and will enhance MSCs proliferation and osteogenic differentiation compared to statically cultured scaffolds.
Methods. An easily sterilised and assembled PBRS was designed and implemented. The scaffolds were Silicon substituted hydroxyapatite granules (Si-HA) and calcium-phosphate coated Ti6Al4V porous cylinders (CaP-Ti). Ovine MSCs were isolated from bone marrow aspirates, expanded in DMEM with 1%antibiotics-10%fetal calf serum (DMEM+) and characterised by differentiating them down the adipogenic and osteogenic lineages. Seeding studies were conducted for both scaffolds. Seeded scaffolds were either statically cultured in well plates or in the PBRS with a flow rate of 0.75mL/min, both with DMEM+. At days 4, 7 and 14 cell proliferation (AlamarBlue and DNA assays, n=3), osteogenic differentiation (ALP assay, n=3) and cell distribution (histology) were analysed. Constructs were visualised by SEM.
Results. Statistically significant increased cell proliferation ($p \leq 0.05$) was seen in samples cultured under flow perfusion conditions for both scaffolds at all times. ALP activity was significantly higher ($p \leq 0.05$) in the bioreactor constructs at all times points for both scaffolds. Histological analysis revealed a more even cellular distribution in the constructs cultured in the PBRS. The development of a cell layer over time was observed by SEM.
Conclusions. The PBRS used in this study increases cell proliferation and osteogenic differentiation and improves cell distribution throughout the scaffolds. We conclude that the development of constructs for bone tissue-engineering purposes can be achieved by using a PBRS.
Keywords. flow perfusion bone



SEM photo at day 4 of flow perfusion culture of CaP-Ti cylinder where cells have proliferated.

(7.O11) THE IMPORTANCE OF GRADIENTS IN ARTICULAR CARTILAGE

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It is hypothesized that gradients of growth factors (GFs) and GF antagonists exist in articular cartilage and play an important role in the balance between anabolic and catabolic processes. It is believed that such gradients are, at least partially, responsible for the zonal organization of articular cartilage. Despite their importance, current bioreactor designs for articular cartilage tissue engineering have limited options for introducing GF and GF-antagonist gradients. To address this issue we have developed a dual flow bioreactor which can accommodate four articular cartilage cubes (4.5x4.5x3mm) between two medium compartments. The reactor was designed in such a way that it mimics the knee joint as good as possible. The top and bottom compartment are mimicking the synovial fluid and subchondral bone respectively. The bioreactor was

complemented with a plunger that was attached to a compression insert. In this way load can be applied from a vertical position (Figure 1A).

Computational fluid dynamics was used to predict the occurrence of an oxygen gradient, which is shown in figure 1B. The model was then evaluated with a cell line containing a reporter system consisting of a HRE element controlling GFP expression. Medium in the top and bottom compartment were saturated with a different oxygen concentration. Quantification of the GFP expression showed the occurrence of an oxygen gradient (Figure 1C+D).

In conclusion, this unique bioreactor design assists in creating gradients, as shown for oxygen, and it will be used for creating gradients of growth factors and regulatory molecules. The ability to manipulate these gradients can aid in creating an ex vivo environment which may support the engineering of the native structure of articular cartilage.

Keywords. bioreactor, gradient, oxygen

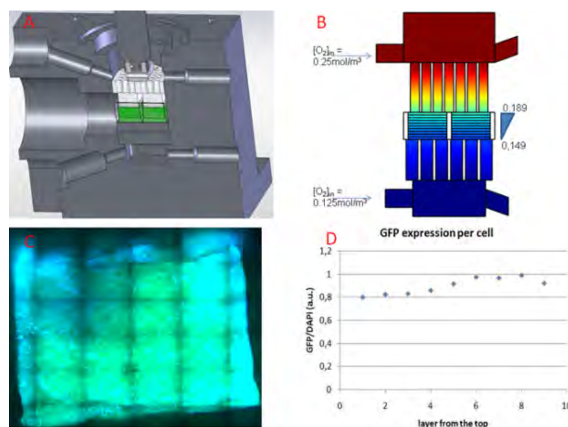

Figure 1: (A) cross section of the bioreactor chamber, (B) computational model of the oxygen gradient within the bioreactor chamber, (C) agarose-embedded cells stained with DAPI and expressing GFP (green) and (D) quantification of different layers in the gel from top to bottom. GFP expression increases when oxygen tension decreases.

(7.O12) NUMERICAL ANALYSIS OF NUTRIENTS TRANSPORT IN CONVECTION-ENHANCED HFMBS FOR LONG BONE TISSUE ENGINEERING

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Introduction. Recent experimental evidence shows that delocalized and distributed nutrients supply and high spontaneous Starling flows in hollow fibre membrane bioreactors (HFMBs) yield cm-scale BMSC aggregates, possibly by relieving nutrients limitations typical of other bioreactors for bone tissue engineering (BTE). The difficult non-intrusive measurement of nutrients and cell concentrations during culture makes mathematical modelling of mass transport, cell growth and metabolic reaction kinetics very attractive: to analyze the effects on cell organization and growth of nutrients transport, cell seeding and bioreactor geometry and operation; and to optimize bioreactor design and operation. Unfortunately, the non-uniform cell distribution observed in culture experiments and high Starling flows render most proposed models inadequate to the purpose. This paper presents mathematical models of HFMBs operated in close shell mode covering the range from diffusion-limited to convection-dominant nutrients transport conditions for both uniform cell distribution and the actual non-uniform cell distribution observed in experiments with BMSCs at different culture times.

Methods. Models are based on a multi-compartment description of HFMBs based on the Krogh cylinder assumption, and on a quasi-steady state analysis of evolution of nutrients and cell concentration profiles. Relevant non-dimensional parameters were identified, and governing momentum and mass transport equations were numerically solved with a finite element commercial code with particular reference to oxygen and glucose. Where possible, parameters assessed from culture experiments were used.

Results and conclusions. Simulation results demonstrate the importance of convective nutrient transport, membrane permeability and packing density in the cell compartment. They also suggest that bioreactor operation should be changed during culture to adapt to the variable nutrients demand of cells in the HFMB shell, as they proliferate and aggregate in 3D structures slowly filling up the shell space and exhibiting a Darcy permeability increasing in time.

Keywords. Nutrient transport; Bone tissue; Hollow fibre membrane bioreactor

(7.O13) VESSEL METABOLISM UNDER MECHANICAL LOAD - IMPLICATIONS FOR VASCULAR TISSUE ENGINEERING

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Introduction. Tissue engineered prostheses like vascular grafts or heart valves are usually generated in perfusion bioreactors which provide mechanical stimuli to condition the constructs. To assess whether conditioning alters nutritional requirements, we investigated the effects of shear forces and luminal pressure in a vessel model.

Methods. Bovine saphenous veins were perfused in mock circulations for 4 days. Group 1 vessels were perfused with M199 at 40ml/min. Group 2 vessels were subjected to increased shear forces (+12% dextran). Group 3 vessels were additionally challenged by increased luminal pressure (+20mm Hg). The corresponding groups 1', 2', and 3' were endothelium-denuded before perfusion. Substrate conversion was calculated from glucose and lactate levels. Blood gases were measured upstream and downstream of the samples. Contractile function and tetrazolium dye reduction were determined before and after perfusion.

Results. Noradrenaline-induced contractions after perfusion were significantly stronger in group 3 vessels and significantly lower in denuded vessels. Tetrazolium dye reduction was attenuated in groups 1'-3'. Glucose was converted stoichiometrically to lactate except groups 3, 1', and 3' which produced more lactate than glucose could supply. Oxygen concentrations were unaltered between vessel inlet and outlet except in group 2.

Conclusions. Vessels did not use oxidative phosphorylation but lactate fermentation to meet their energy needs. Luminal pressure but not increased shear forces alone improved contractile function after perfusion and induced the consumption of substrates other than glucose in an endothelium-independent fashion. Conditioning bioreactors may thus deplete perfusion media of substrates more rapidly and in different patterns compared to static cultures, and may in fact call for media tailored for this purpose, whereas oxygen partial pressures can be adjusted freely to support tissue growth optimally.

Acknowledgements. This study was funded by Deutsche Forschungsgemeinschaft (BI 139/2-1, HA 4380/5-1, and LI 256/68-1).

Keywords. bioreactors; metabolism; pressure; shear forces

(7.O14) DYNAMIC EXPANSION OF HUMAN UMBILICAL CORD CELLS IN A ROTATING BED SYSTEM BIOREACTOR FOR TISSUE ENGINEERING OF HUMAN HEART VALVES

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Introduction. To overcome limitations in static cell culture systems the dynamic expansion of cells could be an important tool for the tissue engineering of human heart valves. Dynamic expansion should provide continuous perfusion of the cells, large numbers of viable pre-conditioned cells after a short time period and controllable environmental conditions; it should also be a reproducible process. For this purpose human umbilical cord myofibroblasts were cultivated and expanded in a rotating bed system bioreactor.

Methods. Myofibroblasts isolated from human umbilical cord arteries (12x106cells) were cultured for 9 days under hypoxic conditions in a bioreactor system which consists of a cylindrical culture vessel with an integrated rotating bed of several polycarbonate slides. Via an integrated control unit several parameters were measured throughout the fabrication process to achieve optimal culture conditions. Perfusion and slow bed rotation minimized mass transfer limitations and therefore supported the cells with sufficient nutrients. Feeding leads to continuous medium exchange in the culture vessel. Tapping for medium samples allowed the amount of nutrients and metabolic waste products i.e. lactate to be controlled. The cells were characterized by a specific surface marker profile using flow cytometric analysis before and after cultivation in the bioreactor system.

Results. Myofibroblasts were successfully expanded by the factor 30. The fast cell growth possessed a large number of viable cells for tissue engineering applications. There was no change in the expression of cell surface markers after cultivation in the bioreactor compared to the expression before.

Conclusion. Expansion of large numbers of viable cells was realized in an easily manageable and controllable bioreactor system in a short period of time, with minimized effort and labor costs. In future applications the dynamic expansion of cells will be an important tool for the tissue engineering of human heart valves.

Keywords. Tissue Engineering, Bioreactors, Dynamic cultivation of cells

(7.O15) A NOVEL CONTROL UNIT TO CULTURE MESENCHYMAL STEM CELLS UNDER CONTROLLED AND REPRODUCIBLE CONDITIONS IN A PERFUSION BIOREACTOR

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Bioreactors are required in Tissue Engineering to ensure controlled and stable conditions for the fabrication of engraftable tissues. This includes the monitoring and regulation of the temperature, pH and pO2, as well as mass transportation of nutrients and waste material. Moreover a bioreactor should mimic the natural environment as accurately as possible. In addition mechanical stimulation during the cultivation performed by a special bioreactor can support the proliferation and differentiation of human mesenchymal stem cells.

Therefore we developed a control unit to guarantee reproducible conditions for bioreactor cultivations in the area of Tissue Engineering. The system consists of a control tower with a GMP conform software, a stirred tank bioreactor (STR), a perfusion bioreactor and a heating cabin. A perfusion bioreactor with 3D-biomaterials has been used for cell culturing to imitate the fluid shear stress in bone tissue. Moreover perfusion bioreactors reduce the limitation of mass transportation, because the media is continuously transported through the 3D-biomaterials. The culture media is preconditioned in the STR due to a combination of air, nitrogen and carbon dioxide prior pumping it through the perfusion bioreactor. Thus the pH and the pO2 value can be adjusted. The temperature of the culture media is regulated by a heating mat below the STR; moreover the perfusion bioreactor is setup in a heating cabin. The glucose and lactate values can be measured offline and if required fresh media can be added into the STR and waste media be removed.

Human mesenchymal stem cells have been cultivated for 3 weeks on 3D-biomaterials in a perfusion bioreactor whereat the media conditions where adjusted by the control unit. The proliferation of the cells has been demonstrated by the consumption of glucose and by the MTT activity test. The pH and the pO2 values have been recorded by the GMP conform software.

Keywords. mesenchymal stem cells, bioreactors, perfusion, control unit

(7.P1) EFFECT OF PERFUSION CULTURE SYSTEM ON IN VITRO OSTEOGENESIS OF HUMAN MESENCHYMAL STEM CELLS SEEDED ON POROUS HYDROXYAPATITE

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Introduction. Dynamic culture yields an excellent homogeneous distribution of cells and matrix, and shear stresses applied by medium stimulate the cells to proliferate and differentiate, ensuring continuous nutrition of cells and removal of waste products (1). The aims of the study were to test the proliferation and differentiation of human Mesenchymal Stem Cells (hMSCs) cultured on porous hydroxyapatite (HA) scaffolds and to compare conventional static culture to dynamic flow perfusion culture.

Materials and Methods. Porous hydroxyapatite (HA) scaffolds were provided by Finceramica Biomedical Solutions [ENGI (SLV002005) ST] (cylindrical form Ø=10mm and H=4 mm with an inner porosity close to 80±5 vol.%). HMSCs were isolated from BM as previously described (2) and seeded on HA scaffolds. The perfusion bioreactor was (Fig. 1) designed and developed by SKE Advanced Therapies S.r.l. The cells/hydroxyapatite construct was perfused for 21 days in osteogenic medium. The flow was monodirectional (100μm/sec). pH culture medium was misured using an optical sensor (Fluorometrix,MA,USA). Cell viability was determined by MTT assay. Calcium content, alkaline phoshatase (ALP) activity and bone extracellular matrix proteins were evaluated as described (3).

Results. MTT assay showed an increase of the living cells in the perfused culture. In agreement with this results, an enhancement of ALP activity, mineralization and bone proteins deposition were observed in the perfused culture.

Conclusions. These results demonstrate the feasibility and benefit of culturing cell/HA constructs in a flow perfusion bioreactor for bone tissue engineering applications.

Acknowledgements. This work was supported by "Project SAL-45" financed by Regione Lombardia and by project financed by FONDAZIONE ALMA MATER TICINENSIS (2010).

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Keywords. Perfusion bioreactor, Human Mesenchymal Stem Cells, Porous hydroxyapatite scaffolds, osteogenic differentiation

(7.P2) THREE-DIMENSIONAL CULTIVATION OF OSTEOBLASTS IN LARGE SCAFFOLD USING RADIAL-FLOW BIOREACTOR

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Introduction. Bioreactors employing different types of in vitro physiological cell stimulation have been developed to obtain three-dimensional cultivation for tissue engineering. The purpose of this study was to determine whether osteoblastic cells proliferated uniformly over a large scaffold with a diameter of 18 mm and height of 10 mm under dynamic cultivation with the radial-flow bioreactor (RFB), and thereby ascertain the potential of this system in the regeneration of jaw bone.

Methods. Mouse osteoblastic cells (MC3T3-E1) were seeded onto type-1 collagen sheets. Cells were then incubated outside the reactor for 6 hours to produce pre-cultured sheets. The 6 pre-cultured sheets were then placed in the RFB to fabricate the large scaffolds. Cells were dynamically cultured for one week at 37℃, pH 7.4, DO 6.86 ppm, and with the culture medium circulating at 3 mL/min. For static cultivation, cells were cultured in the same manner without circulating culture medium. The resulting cell proliferation and cell distribution were analyzed.

Results. After 6 hours of pre-culturing, most of cells were remained in the collagen sheets, and 97% of the cells were still alive and capable of proliferation. This suggests that the pre-culturing system is an effective method for providing viable cells for further dynamic culture. After one week of dynamic cultivation, osteoblastic cells showed uniform proliferation with yielding a large number of cells more than 5 times greater than that obtained with static cultivation.

Conclusions. These results indicate that the RFB is a promising system for three-dimensional cultivation of osteoblastic cells for treating large bone defects by tissue engineering.

Acknowledgements. This research was supported by Oral Health Science Center Grant HRC7 from Tokyo Dental College, and by a “High-Tech Research Center” Project for Private Universities: matching fund subsidy from MEXT of Japan, 2006-2011.

Keywords. radial-flow bioreactor, osteoblasts, large scaffold

(7.P3) A NEW STRETCHING BIOREACTOR FOR DYNAMIC ENGINEERING OF MUSCLE TISSUES

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Objectives. We aim to define in vitro dynamic culture conditions to improve cell density and organisation of engineered muscle construct. We report here our on-going study on the development and validation of a new device for the generation of stretch culture conditions.

Methods. Custom made silicon bulb (produced with a water-soluble wax mold) were covered with electrospun poly-caprolactone (PCL) micron-scaled fiber matrix. Pump controlled volumetric changes induced bulb enlargement and resulted in matrix stretching. Spatial characterisation of the stretch was analysed using a GOM 3D digitizer and GOM ARAMIS software.. C2C12 cells were seeded on the matrix and cultured for 1 week under mechanical stimulation. Static, cyclic (1Hz) and ramp (cycles of 24h stretch /24h rest) stretch with strain conditions were applied. Cellular responses were investigated by scanning electronic microscopy, immunostaining and 3D confocal analysis.

Results. 1: A gradient of surface strains was characterised from the base to the apex of the bulb. When inflated, the apex showed a linear increase in the strain from 4 to 23%. Meanwhile, the increase of strain at the base was limited and ranged from 2% to 8%.

2: Compared to static culture conditions, dynamic culture induced cellular multilayer formation. This effect appeared to be dependant of the applied stretch amplitude. Ramp stretch with low strain (gradient from 3 to 5%) induced a 2-time thickening of the tissue compare

to higher stretch (gradient strain from 6 to 12%). Ramp stretching is associated with randomly oriented cells. In opposite, cyclic strains improved cell orientation.

Conclusion. We provide preliminary evidence that our new device composed of a bulb shape carrier covered with microfibers matrices is promising for structured muscle tissue generation. In addition, stem differentiation and in vitro modelisation of cardiac remodelling are other possible fields of investigation that may benefit from our device.

Keywords. muscle biografts; bulb carrier; electrospinning

(7.P4) HYDROSTATIC PRESSURE IMPROVES BONE CELL MORPHOLOGY AND GENOTYPIC EXPRESSION IN DYNAMIC CULTURE

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The ability to control and influence cell behavior to produce functional tissues is critical in tissue engineering and regenerative medicine. Mirroring both the natural structure and morphology of the native tissue as well as imitating the complex events of the cellular micro-environment is vital for the success of an engineered tissue. Beyond enhancing nutrient diffusion and cell growth, bioreactors are often employed to administer mechanical stimuli to cell cultures with the aim to mimic the stresses observed naturally in vivo. In particular, bone tissue is remarkable in that it has the capacity to adapt its form, i.e. density and internal architecture, in response to mechanical stimulation. Consequently, it has been shown that the application of various dynamic stresses, such as shear and strain, can influence both bone cell genotype and ECM production; however, these complex events linking mechanotransduction to cellular activity are still elusive. To elucidate this phenomena we developed a dynamic culturing method that utilizes hydrostatic compression to stimulate cell substrates. By controlling the frequency, magnitude and even cycle of the applied stress, we aimed to investigate the response of such stimuli on the proliferation, migration and genotypic expression of bone cells.

The poly(D,L-lactic acid) (PDLLA) porous scaffolds utilized were prepared using a salt-leaching method in which scaffolds were tailored to meet specific porosity and pore size requirements. Biological evaluation was carried out using Alamar blue assay for proliferation and visual inspection by confocal laser microscopy (CLSM). RT-PCR was employed to map the cell gene expression during dynamic conditions providing information on matrix production and mineralization, both of which are critically important in the formation of bone tissue.

Preliminary results indicate that cellular activity is enhanced in dynamic culture compared to static controls. This is observed via increased bone genotypic expression as well as bone matrix protein production.

Keywords. Bioreactor, bone tissue engineering, genotypic expression

(7.P5) A USER-FRIENDLY MULTI-CHAMBER PERFUSION PLATFORM: PRELIMINARY TESTS WITH THREE-DIMENSIONAL POROUS PCL SCAFFOLDS

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Introduction. Several flow perfusion bioreactors have been documented for dynamic cell culture within three-dimensional (3D) matrices [1]. Flow perfusion ensures adequate nutrient supply/waste removal within the substrate, and suitable stimuli to the cells, representing an appealing tool to replicate natural tissue microenvironments. In this work, we develop a user-friendly, GMP-compatible, multi-chamber, confined-flow perfusion platform in close collaboration with physicians and biologists, providing a simple and straightforward tool for dynamic cell cultures. Bioreactor testing was carried out using line and primary cells.

Methods. The device (Fig.1A) consists of a six-chamber, stand-alone platform able to manage several independent and simultaneous experiments in controlled culture conditions. Each culture chamber (Fig.1B) consists of a housing, a silicone cartridge that, by virtue of its deformability, acts as a watertight scaffold holder, and a 7-ml medium reservoir coupled with a disposable vented screw cap. The device compact size, the extremely small number of components and the use of bayonet couplings allow a simple, fast, and sterile assembly by the operator. In order to investigate the bioreactor performances, one-way and oscillatory seeding experiments are performed on porous ε-polycaprolactone scaffolds with MC3T3-E1 cells and primary human fibrocytes. Cell adhesion and distribution within the scaffold are adopted as bioreactor performance read-out.

Results. Experimental campaigns with 3D matrices allow us to determine that: i) seeding perfusion rate in the range 0.03-0.1 ml/min improves cell seeding efficacy compared to static seeding, and ii) both one-way (Fig.1C) and oscillatory cell seeding (Fig.1D) result in a uniform distribution of cells within the scaffold.

Conclusions. The developed bioreactor is functional, versatile, and straightforward. The preliminary in vitro tests prove the efficacy of the system in enhancing cell seeding efficiency, opening the way for further studies addressing long term colonization of the scaffold.

Reference. [1] Martin I et al., Trends in Biotechnology; 22 (2004): 80-86

Keywords. Confined low perfusion bioreactor; multi-chamber platform; three-dimensional scaffold; dynamic cells seeding

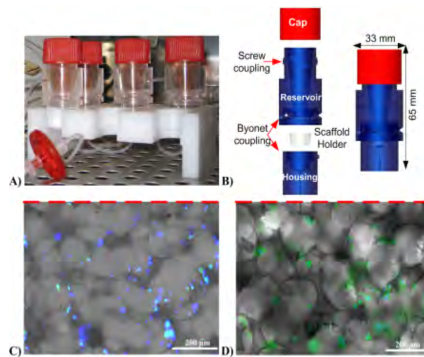

Figure 1: Multichamber bioreactor during culture tests (A) and CAD model of a single culture chamber (exploded and assembled view) (B). Fluorescence images of stained cell nuclei, and actin filaments of longitudinal sections of scaffolds seeded with MC3T3-E1 cells (C) and human fibrocytes (D). The bar is 200 μm and the seeding surface is indicated by a red dashed line.

(7.P6) TIME-COURSE EXPRESSION OF VEGF, FGF-2, AND IL-11 BY HUMAN MESENCHYMAL STEM CELLS UNDER 3D CULTURE IN FLOW PERFUSION BIOREACTOR

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The beneficial effects of delivered mesenchymal stem cells (MSCs) to defect site are related not only to their multipotency but as well to their trophic action. We hypothesized that the release of signaling molecules could be modulated by culture conditions. The objective of this study was to evaluate the time-course expression of angiogenic growth factors vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2) and anti-inflammatory cytokine interleukin-11 (IL-11) by human MSCs under three-dimensional (3D) culture in flow perfusion bioreactor.

Human 105 MSCs (hMSCs) were seeded per coral cube (3x3 mm). The coral-containing hMSCs ("constructs") were cultured in custom-made flow perfusion bioreactor either under static (control) or dynamic flow perfusion culture conditions. At day 0, 7, 14, and 21 the collected constructs and medium underwent analysis to assess gene and protein expression, respectively. Under dynamic conditions VEGF protein level was significantly lower ($p<0.01$); however, the gene expression was significantly lower after 7 days ($p<0.034$) and higher after 14 and 21 days ($p<0.0039$; $p<0.049$, respectively) compared to static condition. FGF-2 was not detected at protein level; however, its gene expression was significantly lower after 7 days ($p<0.00021$) under dynamic over static condition. The IL-11 protein level was significantly increased after 14 and 21 days ($p<0.01$); however, its gene expression was significantly lower after 7 days ($p<0.00067$) and higher after 14 days ($p<0.011$) under dynamic over static condition.

To the best of our knowledge, the present study provides the first evidence about the time-course expression of VEGF, FGF-2, and IL-11 by hMSCs in 3D flow perfusion culture. Moreover, it shows that gene and protein level of studied molecules significantly depend on culture condition applied. A deeper characterization of hMSCs constructs' secretome is needed upon implantation to defect site as the pre-culture period could influence the construct's integration into the host.

Keywords. Stem cells; Bioreactor; Growth factors; Cytokines

(7.P7) A MULTI-LAYER MICROFLUIDIC CHAMBER TO CULTURE UNIFORM-SIZEDCHONDROCYTE PELLETS

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Introduction: In the last decades the biological field has spent a steady effort in reducing experimental systems from a macro- to a micro- or lab-on-a-chip scale [Bauer M et al., *IntegrBiol* 2010]. Only recently, researchers started turning to 3D cell cultures, which better reproduce the *in vivo*-like cell microenvironment [Abbott A, *Nature* 2003]. However, generation and culture of cell aggregates at the microscale still remainschallenging.Main goal of this work was the generation of uniform-sized 3D chondrocyte pellets within a microfluidic-perfused environment.

Methods: A PDMS microfluidic device was developed, consisting of two layers: a top layer containing a straight channel 40mm long provided with two chambers, used for bubble trapping purposes, and a bottom layer, containing a microfluidic chamber for cell collection and pellet formation.After a culture period of 14 days, pellets were analyzed for cell metabolic activity, sGAG and DNA content and the ECM formation was assessed through histology. Control pellets were also obtained and cultured with standard protocols.

Results: Main advantage of the chamber design was the ability to induce pellet generation by means of gravity sedimentation without the need of centrifugation steps.Cells, injected at a concentration of $2x10^{7}$cells/ml, filled the chamber (Fig.1A) and, after 20 hours, aggregated in uniform-sized pellets (Fig.1F). Pellets obtained with the microfluidic system showed a comparable viability to the controls. Moreover, microfluidic pellets were homogeneously populated with spaced cells with interposed matrix.

Conclusions: The methodology described in this work is simple and may be scaled up for culturing large numbers of pellets in a single device. Although this study focused on chondrocytes, the technology described is versatile and should be readily applicable to other cell types in a physiologic-like 3D setting.

Acknowledgments: This project partly was supported by Cariplo Foundation and ProgettoRocca.

Keywords. Microfluidics, Chondrocyte, Pellets, Perfusion

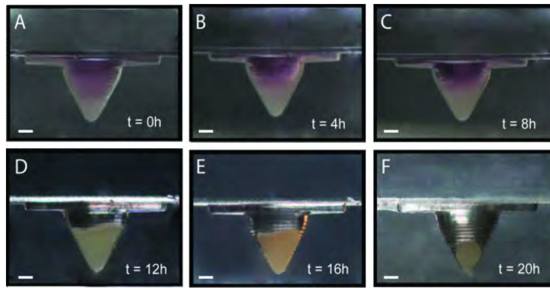

Fig. 1- Lateral view of the culture chamber during the pellet generation. Pictures are taken every 4 hours starting from the end of the cell seeding process (A). After 8 hours (C) cells aggregates and start detaching from the lateral walls. In the inset (F) pellet, with its classical spherical shape, is complete and ready for culture. Scale bar correspond to 500 μm.

8. CARTILAGE

Chair: Pedro Guillén-García
Co-chair: Pedro Hernández-Cortés
Keynote speaker: Pedro Guillén-García
Organizer: BioIbérica
Synopsis: Autologous chondrocyte implantation is a well-established method for the treatment of several chondral defects, although the long-term clinical results of this type of therapy are controversial, and several researchers previously demonstrated that the clinical results of cell therapy using cultured chondrocytes are highly variable in relationship to several factors.
In this symposium, several topics related to the novel therapies applied to the regeneration of the human cartilage will be discussed. Some of the most relevant topics of this symposium are:

- Chondrocytes culture conditions
- Chondrocytes markers
- Bioreactors for chondrocyte culture
- Extracellular matrix in cartilage
- Biomaterials
- Clinical chondrocyte implants (ACI)
- Clinical chondrocytes implants using membranes (MACI)
- Clinical trials in cartilage repair

We expect that all scientists, clinicians and professionals involved in the field of cartilage biology, are invited to submit abstracts to this symposium.

(8.KP) THE TREATMENT WITH AUTOLOGOUS CHONDROCYTES IS NOWADAYS THE ONLY TECHNIQUE THAT REPLICATES THE NORMAL CARTILAGE AFTER A LESION

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Introduction. Articular cartilage damaged has limited potential to heal and if defects are left untreated, they may progress to osteoarthritis. In past decades, research was focused in developing techniques for stimulating cartilage repair and regeneration, in particular cell therapy techniques as autologous chondrocyte implantation (ACI). Another approach is the use of in vitro engineered tissue obtained using cells seeded onto a biocompatible membrane. This procedure is called MACI (Matrix-Induced Autologous Chondrocyte Implantation) and can be combined with arthroscopy. We describe our 8-year experience with MACI, presenting follow-up data from 50 patients.
Material and Methods. We present the results obtained in 150 consecutive patients, evaluated by an in-house validated clinical protocol which included a survey stating the following data: age, sex, location and of the defect, affected limb, number and type of previous surgeries, mobility after MACI implantation and time of sick leave. In 50 cases, a second biopsy was performed in a mean follow-up period of 2 years.
Results. In 126 patients the lesion was located in the knee and in 24 in the ankle. Arthroscopic MACI was carried out in 53 of them while in the remaining 97 an open-fashion procedure was followed. The histological study of the novel tissue formed revealed an architecture of hyaline-like cartilage in all patients, although the number of cells was lower than the normal hyaline cartilage. All the biopsies analyzed expressed the aggrecan, COL I and COL II genes.
Conclusion. The implantation of autologous chondrocytes is a good procedure to treat chondral and osteochondral lesions in the knee and ankle, preserving the integrity of the joints.
Keywords. ACI, MACI, autologous chondrocyte implantation, second look

(8.O1) ANISOTROPIC FIBROUS TISSUE SCAFFOLDS FOR ARTICULAR CARTILAGE REGENERATION

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1. Imperial College London

Introduction. Articular cartilage is a highly organized, fibre-reinforced tissue with a complex extracellular matrix of proteoglycan molecules retained within a fibrillar type II collagen meshwork. The structural arrangement of the collagen fibre network provides the tensile reinforcing elements of cartilage and exhibits unique anisotropic (depth-dependent) organization. The superficial, middle and deep zones of cartilage feature varying collagen II alignment as well as decreasing, depth-dependent tensile properties. Current cartilage tissue engineering solutions fail to mimic this zonal organization; thus the goal of this work was to fabricate anisotropic electrospun constructs that mimic the native fibre organization and tensile properties of articular cartilage.
Methods. Anisotropic electrospun scaffolds were fabricated by electrospinning poly(ε-caprolactone) (PCL) while gradually varying the polymer concentrations (15 or 25 w/v%) and the speed of a rotating mandrel (2000 rpm vs. 100 rpm) to collect either aligned or random fibre networks, respectively. The resulting layered constructs were assessed via electron microscopy, tensile testing, and their ability to support in vitro chondrogenesis of bovine chondrocytes.
Results. Anisotropic constructs were created by sequentially electrospinning different PCL solutions. 3D constructs were generated, featuring variations in fibre morphology, orientation, and tensile properties, mimicking the morphology and mechanical behaviour of articular cartilage (Figure 1). Zonal tensile strength of the anisotropic construct decreased within each layer as indicated in Figure 1: zone B (35 MPa), zone C (7.4 MPa),

and zone D (5.7 MPa). Bovine chondrocytes were able to adhere, proliferate and differentiate on the scaffolds for 5 weeks in vitro on both homogenous and anisotropic constructs with depth-dependent tensile properties (data not shown).

Conclusions. We have fabricated the first anisotropic fibrous construct that mimics collagen fibre arrangement and zonal tensile strength of articular cartilage.

Acknowledgements. The authors acknowledge the Medical Engineering Solutions in Osteoarthritis Centre of Excellence funded by the Wellcome Trust and EPSRC.

Keywords. anisotropic scaffold; electrospinning; zonal organization

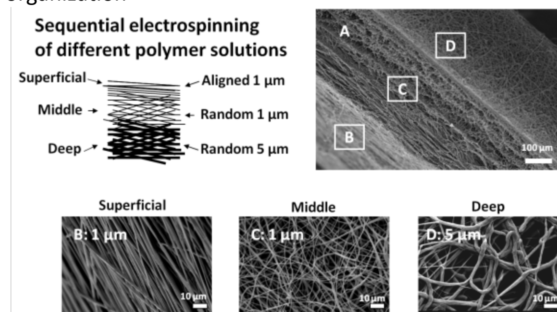

Figure 1: Sequential electrospinning generated a bulk material with different fibre arrangements and morphologies, and depth-dependent tensile properties similar to those of articular cartilage.

(8.O2) INFLUENCE OF CONDITIONED MEDIUM OVER THE CHONDROGENIC DIFFERENTIATION OF ADULT STEM CELLS IN 3D CO-CULTURES WITH ARTICULAR CHONDROCYTES

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1. 3B´s Research Group, University of Minho, Portugal; 2. IBB – Institute for Biotechnology and Bioengineering, Portugal; 3. Department of Biosystems Engineering, University of Minnesota, USA; 4. CMAT, University of Minho, Portugal

Aim. Soluble factors released by chondrocytes have been shown to influence stem cells differentiation onto the chondrogenic lineage. Using conditioned medium obtained from chondrocytes for stimulating stem cells chondrogenic differentiation may be a very interesting alternative for clinical application of these cells. Therefore, we tested the influence of conditioned medium obtained from articular chondrocytes cultures to determine its influence on indirect co-cultures of human bone marrow-derived MSCs (hBMSCs) and human Wharton´s jelly MSCs (hWJSCs) seeded in 3D porous scaffolds.

Method. Indirect co-cultures (using conditioned medium obtained from a culture of human articular chondrocytes) hBMSCs and hWJSCs were established. Cells were isolated from human samples collected at São Marcos hospital, under donors informed consent. Co-cultures were performed in 3D fibrous and porous scaffolds, composed by a blend of 50/50 chitosan and poly (butylene succinate) – CPBS. Co-cultures were maintained during 28 days.

Results. Both types of stem cells were able to undergo chondrogenic differentiation. By the end of the experiment co-cultures showed glycosaminoglycans (GAGs) accumulation and up-regulated expression of cartilage-related gene, for both types of adult MSCs tested. The hWJSCs showed higher chondrogenic differentiation ability when compared to hBMSCs, as denoted by the higher values for GAGs accumulation and cartilage–specific gene expression.

Conclusions. Using conditioned medium obtained from articular chondrocytes induced the chondrogenic differentiation of MSCs and ECM formation. The obtained results showed that this new strategy enables the development of new therapies for cartilage repair.

Keywords. Conditioned media, co-cultures, stem cells, chondrocytes

(8.O3) IN VIVO EVALUATION OF A NOVEL OSTEOCHONDRAL SCAFFOLD FOR OSTEOCHONDRAL DEFECT REPAIR

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1. Royal College of Surgeons in Ireland

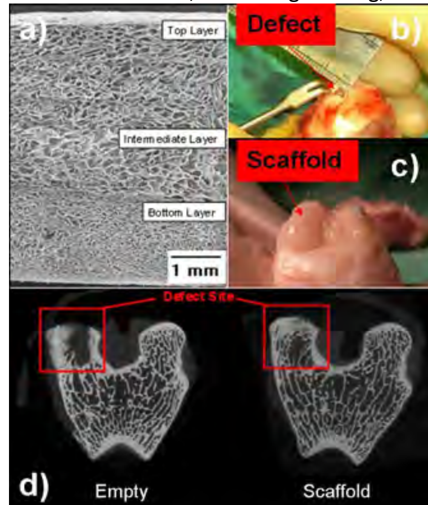
Introduction. Osteochondral tissue has a complex layered structure, organised into cartilage, calcified cartilage and subchondral bone regions. It has poor regenerative capacity and as a result over 15 million people worldwide suffer from knee joint failure each year due to cartilage breakdown (Frost and Sullivan, 2009). Current treatment methods include drilling, microfracture, and osteochondral grafting; however, no treatment has managed to repair large osteochondral defects with long-lasting hyaline cartilage (Klein et al, 2009). The aim of this study was to evaluate the in vivo regenerative potential of ChondroColl, a recently developed, patented multilayer scaffold for osteochondral repair.

Methods. Collagen-based multi-layer scaffolds were fabricated using a novel 'iterative layering' freezedrying technique (WO2010084481). The in vivo performance was evaluated using a rabbit medial femoral condyle model. Scaffolds were implanted into 3mm diameter x 5mm depth critical sized defects. Repair tissue was evaluated 12 weeks post implantation using micro-CT and histological analysis.

Results. Macroscopic analysis at 12 weeks post implantation showed a greater degree of tissue formation in the scaffold group than the empty defect controls. Repair tissue appeared to integrate well with surrounding tissue with no signs of debris or inflammation (Fig. 1c). The International Cartilage Repair Society (ICRS) scoring system indicated the formation of significantly better quality repair tissue in the scaffold implanted group. Micro-CT (Fig. 1d) showed greater repair in the scaffold group than the control, with evidence of subchondral bone repair within the defect and formation of an overlying cartilaginous layer. Histological analysis is currently ongoing.

Conclusions. In vivo analysis of the novel multi-layer scaffold showed that the scaffold enabled successful generation of de novo bone and cartilaginous repair tissue in the defect space. Further histological analysis is on-going to evaluate level of cartilaginous healing.

Acknowledgements. Enterprise Ireland Commercialisation Fund, Proof of Concept (PC/2007/331) and Technology Development Phase (CFTD/2009/0104).
Keywords. Osteochondral, tissue engineering, cartilage



(8.O4) FIBRIN SCAFFOLD WITH GROWTH FACTORS-ENRICHED NANOFIBERS ENHANCED OSTEOCHONDRAL REGENERATION IN MINIATURE PIGS

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Introduction. Nanofibers possess a high surface area which enables adhesion of bioactive substances. The aim of the study was to examine the effect of growth factors-enriched PVA nanofibers on the viability of mesenchymal stem cells (MSC) in vitro and, subsequently, cartilage regeneration in minipigs using fibrin scaffold containing growth factors-enriched nanofibers.

Methods. PVA nanofibers were incubated with basic fibroblast growth factor and insulin, and subsequently seeded with MSC. The cell viability was examined using MTT test after 1, 3, and 7 days. Same scaffold without cells were mixed with Tissucol® and implanted into eight load-bearing osteochondral defects in minipigs. As a control, the defects in the left knees were left untreated. Animals were sacrified 12 weeks after the surgery, and evaluated histologicaly.

Results. The cell viability was significantly higher on modified scaffold compared to pure PVA. In the animal study, the scaffold group showed a regular formation of isogenic lines of chondrocytes near the defect bases and differentiation towards hyaline cartilage. Fibrocartilage was found on the defect surface. The middle and basal zones were predominantly alcian blue positive. Type II collagen was positive in the non-cellular transient zone in the newly formed cartilage and on the border of young isogenic groups. In a control group, fibrocartilage or unorganized fibrous tissue with isogenic groups of chondrocytes was situated at the borders; fibrous tissue accompanied by vascularization was observed on the surface. Alcian blue was positive in the upper part of defects; type II collagen was positive in the newly formed cartilage.

Conclusions. The composite scaffold supported the hyaline cartilage formation, therefore, the scaffold is suitable for cartilage regeneration.

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Keywords. nanofibers, cartilage, growth factors

(8.O5) CELLS FROM SYNOVIAL FLUID: SOURCE OF AUTOLOGOUS CELLS FOR CARTILAGE TISSUE ENGINEERING?

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Introduction. Tissue engineering using mesenchymal stem cells (MSC) for the treatment of cartilage defects appears promising. Among the different sources of MSC used in cartilage engineering such as bone marrow, adipose tissue or synovial membrane, it remains difficult to clearly identify the most clinically relevant source. Recently, the presence of adherent cells in pathological synovial fluid (SF) has been described. Given that SF is easily accessible by simple joint puncture, the aim of this work was to determine whether adherent synovial fluid cells (ASFC) could represent an autologous cells source for future applications in cartilage regeneration.

Materials and Methods. Human ASFC isolated from synovial fluid puncture were characterized for their (i) ability to form colony by CFU-F assay, (ii) surface markers expression by flow cytometry and (iii) multipotency. For adipogenic and osteogenic differentiation, cells were cultured in specific differentiation medium in monolayer for 14 and 28 days respectively. To chondrogenically differentiate ASFC, cells were cultured in specific medium during 28 days in pellets. Cell differentiation was monitored at the level of γmRNA by real time-PCR (ALPL, RUNX2, COL1A1, COL2A1, ACAN, SOX9, COMP, PPAR). Alkaline phosphatase (ALP) activity, histology (oil red O, alizarin red) and immunodetection (type II collagen) were performed.

Results. Our data show that ASFC exhibited proliferation and colony-forming abilities. ASFC also expressed typical stem cell surface markers. Additionally, they were able to differentiate towards the chondro-, osteo- and adipogenic lineages.

Discussion and Conclusions. These results show that ASFC express some of the major MSC characteristics. Wether ASFC could be able to promote cartilage regeneration in adapted animal models should be paid further attention.

Keywords. Synovial fluid cells, cartilage tissue engineering.

(8.O6) THE USE OF FIBROBLASTS FOR THE RECONSTRUCTION OF ANTERIOR CRUCIATE LIGAMENT: RESEARCH ON THE SHEEP ANIMAL MODEL

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1. Clinica CEMTRO; 2. Hospital Carlos III

Summary. The rupture of the anterior cruciate ligament (ACL) is currently treated with a surgical procedure that implies the use of different tendons or ligaments to reconstruct the damaged ACL. Currently, the research on this field is focused in finding a new method to reduce the time of recovery which with these techniques is now of 6-8 months. We have investigated the use of fibroblasts for the reconstruction of broken ACL.

Material & Methods. Ten female sheep with a similar age will be included in this study and were divided into 2 groups.

- Group A: Implanted with 5 million fibroblasts embedded in the membrane
- Group B: Implanted with the membrane without cells

The animals undergone 2 surgeries: one surgery to take an ACL biopsy and the other one to break the ACL and implant the membrane with (Group A) or without (Group B) cells.

After 3 months, the animals will be sacrificed and samples from the ACL regeneration and from healthy areas (control) will be taken. Histological and molecular studies will be performed to compare both treatments between them and with the control.

Results. The architecture of normal ACL was not conserved either in the ACL treated with the membrane with or without cells. However a high number of cells, similar to fibroblast was found in the cell-treated ACL than in those treated only with the collagen membrane, indicating that probably these cells migrated from the membrane to the damaged ACL. RT-PCR studies performed demonstrated that these cells expressed type I collagen, tenascin-C and MMP-13; indicating the fibroblastic origin of the cells.

Conclusion. We think that this novel technique could be a promising tool to treat the ACL rupture and represents a first step in the use of tissue engineering for treatment of the ACL rupture.

Keywords. ACL rupture, biomaterials

(8.O7) TREATMENT OF CHONDRAL DEFECTS WITH AUTOLOGOUS CHONDROCYTES OR MESENCHYMAL CELLS ON TYPE I/III COLLAGEN MEMBRANES IN THE OVINE MODEL

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1. Clinica CEMTRO, Madrid, Spain

Introduction. Autologous chondrocyte implantation (ACI) combined with a periosteal flap, was first performed in the human knee in 1994. In MACI implants, chondrocytes are seeded in a collagen I/III membrane functioning as cell carrier. Some research has been focused in developing techniques based on cell therapy using other cells as mesenchymal cells (MSC).

Materials & Methods. Five 2-3 years-old female sheep were included. A full 10 x 10 mm incision was made in the articular cartilage of the medial femoral condyle. This sample was used as a source o chondrocytes. A second lesion of the same size was done at the trochlea. In this lesion, microperforations were done. A sample of adipose tissue from the Hoffa's fat pad was taken to isolate MSC. One and 5 million of cultured chondrocytes and 5 million MSC, respectively, were seeded on a collagen I/III membrane and then they were implanted. After 12 weeks the animals were sacrificed and tissue samples in the following areas were taken: a) MSC implant area, b) microperforations area, and c) healthy tissue near of perforation area. Histological and molecular studies were carried-out made by hematoxilin-eosin and safranin-O staining. Relative expression of aggrecan and types I and II collagens was determined by real-time polymerase chain-reaction.

Results. The tissue architecture and the expression pattern of proteoglycans was more similar to that observed in normal cartilage in the lesions treated with 5 million chondrocyte followed by 1 million and by MSC and microperforations. These results were supported with the studies of gene expression.

Conclusion. The implantation of 5 million of cultured autologous chondrocytes on I/III collagen membranes seems to give better histological and molecular results than 1 million cells. Microperforations and Hoffa's fat pad derived MSC seem to have no role in the reparation of damaged cartilage.

Keywords. Cartilage repair, ovine model, collagen membrane

(8.O8) TOWARDS IN SITU THERAPY OF OSTEOARTHRITIS: CARTILAGE SPECIFIC CHEMOKINES AND THEIR ROLE IN HUMAN MESENCHYMAL STEM CELL MIGRATION

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Introduction. In situ Tissue Engineering represents a current approach for the regeneration of damaged or diseased joint tissues implying the use of supportive bioscaffolds and bioactive molecules promoting recruitment of mesenchymal stem cells (MSC) and their subsequent differentiation. Several studies have designated chemokines (Ck) as ideal candidates for MSC attraction. Further, it was shown that human articular cartilage secretes Ck that might be potent to attract MSC in vivo. Thus our studies focussed on characterisation of differentially expressed Ck in Osteoarthritis (OA) and healthy cartilage and their ability to induce chemotaxis in MSC.

Methods. Human articular cartilage biopsies were collected from donors with macroscopical and

microscopical signs of OA as well as donors with intact cartilage. RNA was isolated from the biopsies and subjected to whole genome microarray analysis. In addition, cartilage and chondrocyte conditioned supernatants were collected and analysed for their Ck profile using protein arrays. Attraction impact of supernatants on human MSC from healthy and OA donors was examined in 96-well chemotaxis assays.

Results. Among other several new marker genes, microarrays revealed an increased expression of the Ck CXCL2, CXCL3, CXCL14, CCL3 and CCL4. Proteomics confirmed the OA specific secretion of CXCL2-3 and migration assays demonstrated a significantly higher recruitment of MSC by OA cartilage derived supernatants. Conditioned medium from OA chondrocytes displayed increased secretion levels of CXCL1-3, CXCL8 and CCL2. However, no increase in recruitment of MSC was detected here.

Conclusion. Our results show OA cartilage specific gene expression and release of Ck and their potency to recruit MSC from healthy and OA donors. Here, increased levels had either stimulating or inhibiting effects on MSC attraction, displaying involvement of more complex regulations. In conclusion, these are revealing findings towards a Ck guided in situ therapy of OA using MSC.

Keywords. osteoarthritis; chemokines; in situ regeneration; mesenchymal stem cells

(8.O9) A SELF-SETTING HYDROGEL MECHANICALLY REINFORCED WITH A MARINE EXOPOLYSACCHARIDE AS A SCAFFOLD FOR CARTILAGE TISSUE ENGINEERING

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Polysaccharides-based hydrogels have been widely used as 3D scaffolds for cartilage tissue engineering. However none of them showed both mechanical and biological adequate properties. To develop a biomechanically and biologically competent hydrogel for cartilage tissue engineering, a cellulose-based hydrogel (Si-HPMC) was reinforced with a marine exopolysaccharide called GY785. Previously, we have shown that GY785 EPS addition has improved the mechanical properties of the Si-HPMC. Therefore, the aims of the present work were (i) to investigate the ability of this Si-HPMC/GY785 to allow the maintenance and the recovery of a chondrocytic phenotype and (ii) to evaluate the potential of this Si-HPMC/GY785 associated with chondrocytes to form a cartilaginous tissue in vivo.

Primary rabbit articular chondrocytes (RAC) or dedifferentiated RAC were cultured in 3D within Si-HPMC/GY785 for 3 weeks. The chondrocytic phenotype was investigated by real-time PCR (agrecan, type I and II collagen), alcian blue staining (sulphated GAG) and immunostaining (type II collagen). Finally, the ability of Si-HPMC/GY785 to form a cartilaginous tissue was investigated by in vivo transplantation of RAC and equine nasal chondrocytes (EqNC) with Si-HPMC/GY785 subcutaneously in nude mice. After 3 weeks, implants were histologically characterized to determine the presence of sulphated GAG (Alcian blue) and type II collagen (Immunostaining).

Our results showed that primary RAC 3D-cultured within Si-HPMC/GY785 expressed type II collagen and agrecan after 3 weeks. These cells also produced an extracellular matrix containing sulphated GAG and type II collagen. When dedifferentiated RAC were replaced in 3D within Si-HPMC/GY785 the expression of type II collagen and agrecan were recovered and type I collagen expression was decreased. Finally, histological analysis of hybrid constructs transplanted in nude mice revealed the production of sulphated GAG and type II collagen. This study indicates that mechanically GY785 exopolysaccharides reinforced Si-HPMC could appear as a promising hydrogel for cartilage tissue engineering.

Keywords. Cartilage, hydrogel, tissue engineering

(8.O10) IS SELF ASSEMBLY USING PROGENITOR CELLS A BETTER APPROACH TO ENGINEERING FUNCTIONAL CARTILAGE TISSUE THAN HYDROGEL ENCAPSULATION?

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Introduction. Agarose encapsulation and self assembly are two promising methods that have been proposed to engineer functional cartilage tissue. The objective of this study was to compare the functional properties of cartilaginous tissues engineered using Infrapatellar Fat Pad (IFP) derived MSC's using either agarose encapsulation or self assembly.

Methods. Porcine fat pad derived MSC's were encapsulated in agarose, forming cylinders of either 1.5mm or 3mm thickness. Two seeding densities were examined for each thickness of gel (0.88E6 or 4E6 total cells), resulting in four overall gel seeding densities (15E6, 30E6, 68E6, and 136E6 cells/ml). Self assembled constructs were formed by adding either 0.88E6 or 4E6 cells in chemically defined media (CDM) between PDMS O-rings. Constructs were maintained for the first 3 weeks in CDM supplemented with TGF-β3, upon which TGF-β3 supplementation was either withdrawn (TGF-) or maintained (TGF+) for a further 3 weeks.

Results. Matrix accumulation was greater for higher seeding densities (4E6 cells) using both methodologies (Fig.1). Within the low seeding density group we observed greater sGAG accumulation in agarose gels compared to self assembled constructs (TGF+), however at high seeding densities, self assembled constructs (TGF-) were comparable to agarose groups (TGF+). Collagen accumulation was greater in the agarose constructs (TGF+) compared to the corresponding self assembly groups.

Conclusions. In general we observed greater matrix accumulation in agarose constructs compared to self assembly, perhaps indicating it as the more desirable method of the two. However, when normalised to tissue wet weight (data not shown), matrix accumulation was greater in the lighter self assembled constructs, approaching values seen in normal articular cartilage. This suggests that self assembly results in the development of more functional cartilaginous constructs.

Acknowledgements. Funding was provided by IRCSET and an SFI President of Ireland Young Researcher Award (08/Y15/B1336).

Keywords. Self-assembling process; Agarose hydrogel; Functional tissue engineering; mesenchymal stem cells

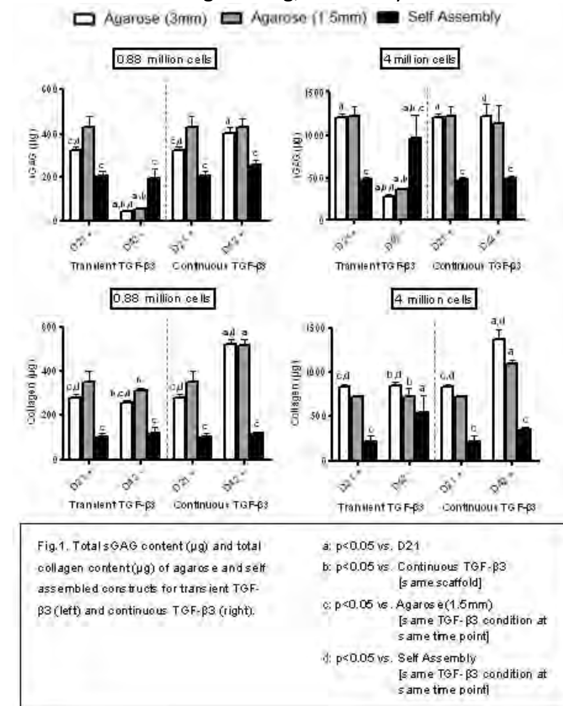

Fig.1. Total sGAG content (µg) and total collagen content (µg) of agarose and self assembled constructs for transient TGF-β3 (left) and continuous TGF-β3 (right).

a: $p<0.05$ vs. D21
b: $p<0.05$ vs. Continuous TGF-β3 [same scaffold]
c: $p<0.05$ vs. Agarose (1.5mm) [same TGF-β3 condition at same time point]
d: $p<0.05$ vs. Self Assembly [same TGF-β3 condition at same time point]

(8.O11) NATURAL CHITIN MATRICES, ISOLATED FROM MARINE SPONGES, AS SUITABLE 3D-SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

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Introduction. Tissue engineering (TE) of articular cartilage is based on a suitable 3D-scaffold. Promising results were reported for synthetic chitosan (chitin-derivative) based scaffolds. Marine sponges of the genius Verongida posses a naturally developed 3D-chitin-skeleton that has been optimized by evolution to support cell seeding and nutrient supply. Aim of this study was to characterise this biomaterial regarding biocompatibility and support of a cartilage-like extracellular matrix (ECM) deposition.

Methods. Chitin scaffolds were isolated from Aplysina cauliformis by repeated extraction of other constituents with acidic acid and NaOH. For in vitro analyses porcine articular chondrocytes were cultured in the scaffolds in chondrogenic medium. For in vivo analyses human chondrocytes were seeded into the scaffolds and implanted subcutaneously into SCID-mice. Samples were analysed for cell vitality and by histological staining. To discriminate between donor and host cells an in-situ-hybridization protocol was developed specifically detecting human and mouse genomic repetitive elements.

Results. Stability and handling of the chitin scaffolds were excellent, no destruction was observed during cell seeding, cultivation, or transplantation. In vitro, primary cells were distributed throughout the scaffold accompanied by high cell vitality (> 80%). After 4-6 weeks cells synthesized a cartilage-like ECM as determined by alcian-blue and type-II-collagen staining. In situ hybridization demonstrated that exclusively implanted human chondrocytes deposited a cartilage-typical ECM and no cells dedifferentiated or evaded into the surrounding fibrous mouse tissue. A small number of murine cells (<5%) were found inside the proteoglycan-rich cartilage matrix which might have invaded the regenerate before deposition of the cartilage-like ECM.

Conclusion. The natural chitin scaffolds represent a promising 3D-matrix for cartilage TE. The structure would particularly be suitable for targeted chemical modifications allowing the specific upgrading with factors supporting cell migration, adhesion, proliferation, or chondrogenic differentiation when replacement of chondrocytes by progenitor cells or in situ cartilage repair strategies are envisaged.

Keywords. marine chitin sponges, cartilage tissue engineering, extracellular matrix, species-specific cell detection

(8.O12) STEM CELL SURFACE MARKER SSEA-4 SELECTS FOR CHONDROPROGENITORS WITH ENHANCED CHONDROGENIC POTENTIAL IN CULTURED HUMAN ARTICULAR CHONDROCYTES

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Introduction. One important challenge for cartilage tissue engineering is to produce a clinically relevant number of cells with consistent chondrogenic potential. In vitro expansion of autologous chondrocytes results in a heterogeneous population of dedifferentiated cells and variable amounts of chondroprogenitors. Identification and isolation of chondroprogenitors could lead to more consistent cartilage formation. We have found that subpopulations of cultured human articular chondrocytes express SSEA-4, a cell surface marker of embryonic and mesenchymal stem cells. In this study, we characterised the proliferation and differentiation potential of human chondrocytes sorted according to SSEA-4 levels.

Methods. Articular cartilage was obtained from three consenting patients undergoing limb amputations. Isolated chondrocytes were expanded and SSEA-4 levels were assessed over several passages by flow cytometry. Cell populations either positive or negative for SSEA-4 were separated at passage 2 by fluorescence-activated cell sorting and either propagated in monolayers for one more week with DNA levels monitored every three days or redifferentiated in pellet cultures over two weeks. In differentiation cultures, pellet sizes were determined and expressions of aggrecan, collagen II and I were quantified by qRT-PCR.

Results. SSEA-4 was not detectable in freshly isolated chondrocytes. However, SSEA-4 levels peaked at 66.7±4.4% positive cells after approximately five population doublings and decreased thereafter. Cultured chondrocytes sorted for SSEA-4 formed 25%±3.1% larger pellets and expressed higher levels of chondrogenic markers during redifferentiation than SSEA-4-negative chondrocytes. However, the latter proliferated slightly

faster (1.12±0.1 days doubling time) in monolayers than SSEA-4 expressing cells (1.29±0.1 days) ($p<0.05$).

Conclusions. Our observations indicate that the stem cell surface antigen SSEA-4 can be used to select for chondroprogenitors with enhanced chondrogenic differentiation capacity in cultured human chondrocytes. Future research will be focussed on the cellular characterisation of purified SSEA-4-positive cells to confirm their superior chondrogenic potential in vivo.

Keywords. cartilage, tissue engineering, surface marker

(8.O13) THE ROLE OF CELLULAR COMMUNICATION IN BONE MARROW DERIVED STROMAL CELL CHONDROGENIC DIFFERENTIATION

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Bone marrow-derived stromal cells (BMSCs) are envisioned as regenerative cells for numerous tissues, including cartilage. Success of BMSC-based therapies, however, relies on a number of methodological improvements, among which is better understanding and control of their differentiation pathways. We investigated here the role of cellular communication (through paracrine signaling and/or cell-cell contact) in the chondrogenic potential of BMSCs.

Bovine BMSCs (n=3 donors) were encapsulated in alginate beads as dispersed cells at 3, 7, and 14 millions cells/ml and as micro-aggregates at 7 millions cells/ml thus creating different paracrine signaling and cell-cell contact conditions. BMSCs were cultured for 21 days under hypoxia (2%O2) and TGFb3 stimulation (10ng/ml). At d0 and d21, cell phenotype was characterized by RT-qPCR (type I and II collagens, sox9, aggrecan, TGFb); produced matrix by histology (Alcian blue staining) and biochemical assays (glycosaminoglycan (GAG) and DNA content); cell morphology by histology (phalloidin staining); and cell viability by live/dead staining.

In all conditions, BMSCs stayed viable and DNA content remained constant up to 21 days. Major chondrogenic markers (type II collagen, aggrecan, sox9) were clearly up-regulated at day 21, with a higher up-regulation for dispersed cells (Figure). Matrix production (GAG/DNA content) increased in time but without significant differences between groups (Figure). Histological analysis is under progress. This study showed that, under TGFb stimulation and in the range of cell concentrations used here, endogenous paracrine signaling does not significantly affect BMSC chondrogenic differentiation, as all dispersed conditions led to the same outcomes. Cell-cell contact (micro-aggregates) has a negative effect on chondrogenic marker expression that is not reflected at the matrix level. Endogenous paracrine signaling and cell-cell contact, however, may have a greater impact on BMSC chondrogenic differentiation under other stimulants such as mechanical loading which may rely on endogenously produced factor or cell-cell communication for amplification of their effects.

Keywords. Bone marrow-derived stromal cells; chondrogenic differentiation; paracrine signaling; cell-cell contact

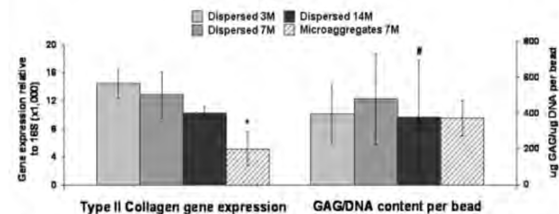

Fig. Type II collagen gene expression and GAG/DNA content at day 21

Values are means ±SD; n=3 donors; *p<0.05 compared to other groups; # data were extrapolated from standard curves. The assay is being repeated to interpolate the data.

(8.O14) PERIOSTEAL FLAP SUBSTITUTE FOR AUTOLOGOUS CHONDROCYTE IMPLANTATION

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Autologous chondrocyte implantation (ACI) is one of the options available to treat osteoarthritis. In this procedure, a periosteal flap is harvested and secured over the defect site to hold the implanted chondrocytes in place. However, the use of the graft is often associated with graft hypertrophy and an increase in subchondral bone density. Hence, a synthetic substitute is highly desirable. In this study, we have a developed a PVA-based membrane to address the problems associated with the use of the periosteal flap. The membrane displayed good mechanical properties, with a Young's modulus of about 1MPa – above the minimum required for hyaline cartilage. Modification of the membrane to present the integrin-binding peptide, RGD, improved initial cell attachment by up to 4-fold, pointing towards improved chondrocyte survival in vivo. In vitro culture of bone marrow-derived human mesenchymal stem cells (hMSCs) revealed that the cells remained attached and viable on the membranes for up to 2 months. Gene expression studies for bone markers, namely collagen type I, RunX2 and bone sialoprotein (BSP), of hMSCs cultured on the membranes showed lower expression as compared to hMSCs cultured on tissue culture plastic, thus lowering the risk of graft hypertrophy. In vivo implantation of the membrane material showed good biocompatibility. These findings demonstrated that the RGD-modified PVA membranes are a potential substitute for the periosteal flap used in ACI, as well as other applications in which the periosteum is required.

Keywords. Biomaterials, Membrane, Cartilage

(8.O15) COMPRESSIVE BIOMECHANICAL PROPERTIES OF A NEW BIO-COLLAGEN SCAFFOLD FOR CARTILAGE TISSUE ENGINEERING

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Introduction. Defects of cartilage in nose and ear are frequent problems caused by trauma or cancer. The need for biomaterials for reconstruction of auricle or nasal septum therefore is enormous. A newly developed bio-collagen scaffold from decellularised porcine cartilage

shows properties more promising than the materials currently in use for tissue engineering applications. The aim of our study was to analyse this novel material in combination with human chondrocytes.

Methods. The proportion of glycosaminoglycans was measured with a DMMB assay, the amount of collagen with a hydroxyprolin assay. To show effects of cellular immigration, scaffolds were seeded with primary human nasal septal chondrocytes up to 42 days in chondrogenic differentiation medium.

Uniaxial confined compression tests were conducted to determine and compare the mechanical properties of native and processed scaffolds (n=12 each). Progress of seeding and immigration of chondrocytes were analysed with histological and immunohistochemical staining.

Results. Due to the decellularisation process the apparent modulus of the scaffolds decreased from 6.5 ± 2.3 MPa to 2.2 ± 1.2 MPa. The DMMB assay showed that the content of glycosaminoglycans was significantly reduced. Relating to the dry weight the proportion of collagen increased, while the fraction of denatured collagen changed from approximately 25 % in native porcine nasal septal cartilage to 50 % in the processed scaffold.

Scaffolds seeded with human septal chondrocytes regained stability. Cells started to produce and incorporate aggrecan into the scaffold in less than 7 days.

Conclusion. Decellularisation and removal of non-collagenous components of extracellular matrix from porcine nasal septal cartilage leads to changes in matrix properties in vitro. Even though the resulting scaffolds maintained their shapes with sufficient mechanical stability and could therefore be suitable for surgical applications. We expect that after seeding with chondrocytes and implantation in vivo the cartilage constructs could retrieve full stability and function.

Keywords. Cartilage reconstruction, biomatrices, human chondrocytes

(8.O16) RECONSTRUCTION OF THE AURICLE WITH THE USE OF BACTERIAL CELLULOSE

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Introduction. Porous bacterial cellulose (BC) is a promising new nano-biomaterial which has shown to possess impressive biomechanical properties and excellent biocompatibility for the use in the field of tissue engineering. BC has already been used as biomedical implant in the field of blood vessels, skin and meniscus replacements. For tissue engineering of an auricle no suitable materials have been found until today. BC seems to be a promising candidate.

Methods. Three-dimensional BC scaffolds are synthesized by the bacterium Gluconacetobacter xylinus. During the fermentation process incorporated paraffin wax beads form interconnected micro-pores. Human chondrocytes isolated from auricular, septal and rib cartilage have been expanded and seeded in different densities onto these scaffolds and cultivated for up to 5 weeks. Adhesion, distribution, proliferation and production of extracellular matrices have been detected with histological and immuno-histological staining methods as well as RT-PCR.

Results. Human chondrocytes adhere at the BC scaffolds and migrate into the interconnected pores where they produce their own cartilage specific extracellular matrix proteins such as collagen II and aggrecan. We found that all cell types are able to retain their differentiated phenotype in this three-dimensional culture system. Furthermore cells proliferate and generate a thick matrix layer on the surface of the BC. Yet, the homogeneous distribution of the chondrocytes in the material is restricted due to uneven interconnectivity of the pores.

Conclusions. The experiments show that human chondrocytes adhere and spread within porous bacterial cellulose while no cytotoxic effects are detectable. BC seems to be a suitable material for the cultivation of human chondrocytes. Continuing experiments for the production of auricular shaped customizable 3D BC scaffolds and the advancement of interconnectivity of the pores are in progress.

Acknowledgement. Supported by the 7th framework programme the EU – Euronanomed - programme EAREG

Keywords. ear cartilage, reconstruction, tissue engineering

(8.O17) CARTILAGE TISSUE REPAIR FROM CLINICAL AND BIOMATERIALS PERSPECTIVE: DECELLULARIZED CARTILAGE AS A NOVEL BIO-MATRIX

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Introduction. Damage or malformation of cartilage structures in the head and neck region are often caused by trauma, tumor resection or congenital defects.

New allogenic and xenogenic collagen bio matrices could be the solution for several problems in reconstruction like multistage surgeries, donor site morbidity, inflammatory reactions or extrusions. These bio implants from natural origin offer a high versatility and good mechanical properties, making them interesting candidates for many biomedical applications.

Materials and Methods. Applying a chemical process, human, porcine and rat nasal septum cartilage samples were completely decellularized and sterilized.

Scaffolds were preincubated for 24 h and seeded with 1x106 primary nasal chondrocytes (PNC). After cell adhesion scaffolds were transferred individually to new wells and cultivated for up to 42 days to examine biomaterials biocompatibility, cytotoxicity and migration behavior of human PNC. Histological as well as immunohistochemical stainings were performed.The vitality of the cells was measured using MTS assay and PI/FDA staining.

Results and Discussion.We performed in vitro allogenic and xenogenic models by seeding processed cartilage bio-matrices from different species with human PNC. Human cells adhere on scaffolds and infiltrate the matrix. Cells occupied empty lacunae independently of the original species of the processed tissue. Two weeks after seeding scaffolds cells synthesized new ECM. At each time point cell population was equal and approximately 1,52x105

cells per scaffold until day 42. The MTS assay showed the intact metabolism of the cells and their vitality. No cytotoxic effects could be detected.

Conclusions. The processed cartilage matrices are completely sterile, free of cells and proteoglycans but are still chondroconductive. No cytotoxic effects caused by the process were detectable. In vitro the biocompatibility between different species could be shown by allogenic and xenogenic models. Therefore the application of processed xenogenic and allogenic cartilage bio-matrices in human applications seems to be possible.

Keywords. Cartilage, tissue engineering, biomatrices, head and neck surgery

(8.O18) TIME COURSE OF JOINT CARTILAGE REGENERATION USING POLY-ETHYL-ACRYLATE SCAFFOLDS IN RABBITS

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Introduction. The aim of this work is to study the time course of articular cartilage regeneration induced by tissue engineering techniques in experimental animals.

Methods. A 3-mm diameter full depth chondral defect was produced in the rabbit knee joint, injurying subchondral bone in order to allow blood to flow into the defect site. A biostable scaffold was laid to fit into the chondral defect. The scaffold were made of a poly(ethyl acrylate-co-hydroxyethyl acrylate) copolymer with 90% of ethyl acrylate monomeric units, containing a well interconnected spherical pores (mean diameter of 90 microns). Scaffolds were allowed to swell with the animal blood that flowed from the defect. Tissue regeneration was studied after 1, 2 weeks, 1, 3 and 12 months with histological techniques.

Results. Regeneration started with initial activation of the chondrocytes located near the edge of the excavated host tissue, where 8-10 chondrocytes where observed in several lacunae, 1 week after implantation. Besides, incipient tissue formation was observed inside scaffold pores, differentiated from mesenchymal cell arriving from subchondral bone. One month after implantation, a well formed layer of tissue was observed over the scaffold, alligned with condylar surface. Thereafter, scaffolds were shifted towards suchondral bone while they were invaded by tissue filling their pores. After 3 months, excellent tissue regeneration was obtained at the cartilage defect site, with a well organized layer of hyaline cartilage at the condylar surface. The pores were filled mostly with cartillaginous tissue in its upper and central parts, and bone tissue adjacent to the subchondral bone.

Conclusions. Synthetic scaffolds induced regeneration of injured joint surface, while they were shifted from the articular surface towards subchondral bone, while were invaded by cells that formed neotissue within their pores.

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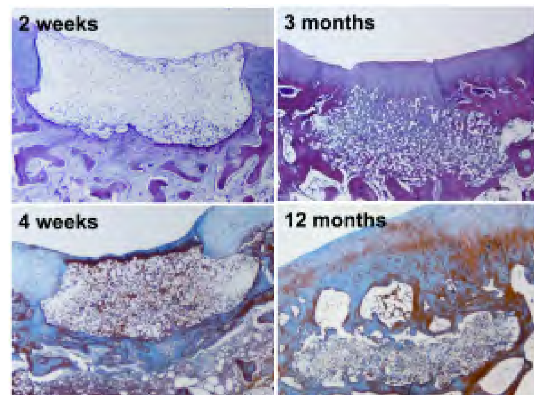
Key words. regeneration, articular cartilage, scaffold



(8.O19) REGULATION OF OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION OF MESENCHYMAL PROGENITOR/STEM CELLS BY IL-1β AND OXYGEN

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Introduction. Because of their regenerative capabilities, Bone Marrow-derived Mesenchymal Stem/Stromal Cells (BM-MSC) are attractive for the repair of osteochondral defects. The milieu of the damaged joint usually contains many inflammatory cytokines, and is characterized by variable oxygen percentages. This work aims at studying the influence of interleukin-1β (IL-1β) and oxygen percentage on the chondrogenic and osteogenic differentiation of human BM-MSC in vitro.

Method. Expanded human BM-MSC (N=5 donors) were cultured with different patho-physiological IL-1β concentrations (0, 50 and 1000pg/ml) and oxygen percentages (19%, 5%, 2%) for 3 weeks in 3D pellets with a defined chondrogenic medium or in monolayer with medium containing osteogenic factors. Bone marrow aspirates were also cultured clonally to assess colony forming unit osteoblast (CFU-O) and fibroblast (CFU-F). Pellets were analyzed for Glycosaminoglycans (GAG) and DNA amount, and by RT-PCR (Collagen II, X). Osteogenic monolayers were analyzed for calcium accumulation and by RT-PCR (Bone-sialoprotein, Osteocalcin, Indian hedgehog) more reproducibly when used at low concentration as evidenced by increased calcium accumulation (2-fold), expression of all the osteogenic genes and CFU-O/CFU-F ratio (1.2-fold), (ii) reduced at low oxygen. ,βResults: Chondrogenic differentiation of BM-MSC was (i) reduced under IL-1β 1000pg/ml as evidenced by reduced amounts of GAG (5-fold) and collagen II and X expression (2 order of magnitude), (ii) moderately but reproducibly increased under IL-1β 50pg/ml, (iii) generally reduced at low oxygen. Osteogenic differentiation was (i) enhanced by IL-1.

Conclusion. The results of this study indicate that the exposure to low doses of Il-1β can enhance both the osteogenic and chondrogenic differentiation potential of BM-MSC in vitro. Controlling the inflammatory environment could enhance the success of therapeutic approaches for traumatic and degenerative osteochondral lesions by resident MSC and as well improve the engineering of implantable tissues.

Acknowledgment. We would like to acknowledge the European Union for financial support (OPHIS; #FP7-NMP-2009-SMALL-3-246373)
Keywords. osteochondral, mesenchymal progenitor/stem cells, IL-1β, oxygen

(8.P1) NOVEL CULTURING TECHNIQUE CREATES CLINICAL SIZED ARTICULAR CARTILAGE CONSTRUCTS

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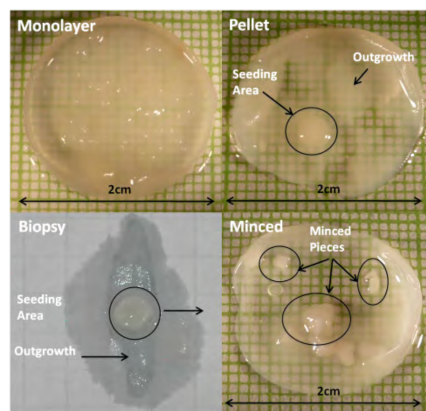
Cartilage tissue engineering requires large cell numbers for construct formation, which is a major limitation. Our previous work demonstrated a continuous flow bioreactor, with NaHCO3 supplemented media to improve cell proliferation and ECM deposition, by creating a near infinite supply of nutrients and by buffering media. Hence, the aim of this study was to use the above technique to produce clinical sized constructs (> 1 cm2) without causing donor site morbidity (~300mg with 2600 cells/mg).

The different seeding techniques used to engineer constructs included: monolayer (20,000 cells or 666 cells/mm2), pellet (200,000 cells), biopsy (5mm diameter constructs) and minced (5mm diameter biopsies cut into smaller pieces). The constructs were cultivated in a continuous flow bioreactor; with 14 mM NaHCO3 supplemented media, at a flow rate of 10 µL/min for 6 weeks, maintained at 37°C, 95% relative humidity and 5% CO2. After 6 weeks of culture the tissue weight, thickness and ECM deposition were determined.

Monolayer constructs outperformed all the other constructs investigated in this study, while minced and biopsy constructs recorded inconsistent data. Monolayer and pellet constructs recorded the following values: thickness: 2069 ± 90 and 1600 ± 47 µm, DNA: 199 ± 33 and 51.6 ± 17 µg/construct, GAG: 8908 ± 1089 and 3428 ± 458 µg/construct, and collagen: 2843 ± 150 and 1495 ± 272 µg/construct, respectively. This significant increase in monolayer ECM accumulation could be due to the combined effect of the bioreactor and NaHCO3 supplemented media. Additionally, the large surface to volume ratio per cell in monolayer compared to the pellet construct (chondrocytes though in larger numbers were tightly packed together) could have provided the cells greater accessibility to nutrients while allowing the chondrocytes to divide, synthesize/accumulate ECM in the monolayer without constraint.

Keywords. sodium bicarbonate; continuous flow bioreactor; seeding techniques; extracellular matrix; articular cartilage; chondrocytes; tissue engineering



Pictorial representation of constructs after 6 weeks of culture in a bioreactor with $NaHCO_3$ supplemented media

(8.P2) CHITOSAN-PVA HYDROGELS AS A SCAFFOLD FOR AURICULAR NEOCARTILAGE FORMATION. MECHANICAL PROPERTIES CHARACTERIZATION

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Introduction. Tissue engineering (TE) of cartilage for reconstructive surgery has proven to be a promising option for the treatment of microtia and other disorders involving cartilage deficiency. The goals of this study were: 1. to determine cell and adhesion viability, and 2. to measure mechanical properties of a biosynthetic hybrid construct. Chitosan (CTS)-Poly (vinyl alcohol) (PVA) films were tested as scaffold for auricular chondrocytes as next step towards the clinical application of TE therapies for pinna reconstruction.

Material and Methods. Auricular cartilage was obtained from New Zealand rabbits. Cartilage was digested mechanically and enzymatically. Biopolymers of CTS-PVA were crosslinked with epichlorohydrin (ECH) to cast films and were seeded with auricular chondrocytes and cultured in standard in vitro conditions. Cell viability onto the polymer was determined by calcein, and morphology characteristics were studied by hematoxylin staining. Environmental Scanning electron microscopy (ESEM) was used to analyze cell adhesion to the polymer. Rabbit's cartilage and Q-PVA-ECH hydrogel were mechanically characterized by uniaxial tension test.

Results. Cells seeded onto Q-PVA-ECH were viable and showed chondral characteristics. Immunohistochemical analysis tested positive for collagen II, aggrecan and elastin. ESEM showed cell adhesion to the polymer. The average ultimate tensile strenght (UTS) for the rabbit cartilage, was 4.7 ± 1.6 MPa. The Young modulus for this material was 45 ± 15 MPa. Based upon mechanical properties characteristics, CTS-PVA-ECH hydrogels mimic the human articular cartilage and it can be considered as mechanically equivalent biomaterial.

Conclusion. Q-PVA-ECH polymer was successfully used to engineer elastic cartilage and may have potential to be used for reconstruction of the external ear.

Acknowledgements. We gratefully thank CONACYT (78798 and 114359) for partial financial support.

Keywords. auricular cartilage, tissue engineering, Mechanical properties, Chitosan-PVA, scaffold

(8.P3) IN VITRO EVALUATION OF COMPOSITE CARBOXYMETHYLCELLULOSE (CMC) AND BICALCIUM PHOSPHATES (BCP) IN ARTICULAR CARTILAGE REPAIR

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The articular cartilage presents a structure anatomic physiological complex with a thin layer viscoelástic tissue aneural, avascular, aliphatic, anisotropic composed of extracellular matrix populated cell discharge of weight which is the ends of bone all joints sinoviais and that allows smooth stable and smooth with minimal friction of the areas of contact. The various strategies used in tissue engineering as support for maintenance, proliferation and differentiation of cells (chondrocytes and others) allowing after trauma and/or diseases repair through the formation of a new architecture (cell, extracellular matrix and irrigation) of cartilage. Hydrogels carboxymethylcellulose (CMC) and hydroxyapatite/beta-phosphate tricálcio (BCP) has been studied as construct for this application by their characteristics and rheologic hydrophilic behavior appropriate macro and micro mechanical this component anatomic. The objective of this work was to evaluate the rheology and the use of conjugate TCP with the gel of carboxymethylcellulose to reestablish the articular cartilage. It was carried out an in vitro evaluation of this biomaterial under sterile conditions with growth factors and culture medium. Several concentrations of this biomaterial were encapsulated by cells of the matrix articulate starting production of the new parent cartilage articular. The in vitro results showed that the hydrogel presents great potential for use in tissue engineering for repair articular cartilage.

Keywords. Biomaterials, Composite, Articular Cartilage

(8.P4) CHITOSAN-POLYVINYL ALCOHOL BASED BIOPOLYMERS FOR AURICULAR NEOCARTILAGE USING AUTOLOGOUS CELLS

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Introduction. Reconstruction of cartilaginous structures of the ear from autogenous tissues continues to be a challenge in reconstructive surgery. In Mexico alone, 1 of 1500 children suffers from microtia (data from 1999) and tissue engineering may provide insight for its treatment. Biomaterials based on chitosan (CTS) and Poly (vinyl alcohol) (PVA) show great potential for the creation of synthetic cartilage. For all the above reasons the goals of this study were (1) to engineer a biosynthetic construct using CTS-PVA blends seeded with auricular cartilage, (2) to study the feasibility of culture and proliferating auricular cartilage in 3D while keeping normal cartilage phenotype and (3) to compare the histology and immunohistochemical composition of engineered constructs.

Materials and methods. Pediatric auricular cartilage was collected as excess tissue from ontological procedures with parent consent. Cartilage was digested and cell cultures were maintained in a monolayer culture. Biopolymers of CTS-PVA were crosslinked with epichlorohydrin (ECH) to cast films and were seeded with auricular chondrocytes and cultured in standard in vitro conditions. Cell viability onto the polymer was determined by methylene blue assay and morphology characteristics were studied by hematoxylin staining. Environmental Scanning electron microscopy (ESEM) was used to analyze cell adhesion to the polymer and immunohistochemistry was performed to evaluate production of cartilage proteins.

Results. Tissue's histological evaluation tested positive to proteoglycans, collagen and elastin. Statistic significance was observed in cell viability and proliferation onto the polymers when compared to a monolayer culture. Cells had normal auricular morphological features and were adhered to the polymer CTS-PVA-ECH analyzed by ESEM. Immunohistochemistry showed constructs were positive to collagen, elastin and aggrecan.

Conclusion. These results demonstrate the feasibility of tissue-engineered cartilage as a potential graft material for microtia treatment.

Acknowledgments. Partial financial support from grants CONACYT 114359 and CONACYT 78798.

Keywords. auricular cartilage, tissue engineering, Chitosan-Polyvinyl alcohol, constructs

(8.P5) THE EFFECTS OF AGAROSE ON CHONDROCYTE DIFFERENTIATION IN A 3D CARTILAGE MODEL

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Introduction. The human articular cartilage is an avascular conective tissue, which presents a low cell-to matrix volume ratio with a highly specialized extracellular matrix (ECM). Articular cartilage degeneration by congenital abnormalities, disease and trauma could have clinical consequences. Fibrin-Agarose (FA) biomaterial has been previously used for the efficient generation of cornea and skin substitutes. However, the influence of this biomaterial on the biological behavior of human hyaline chondrocytes and the ECM proteins that are synthetized in culture are unknown. Here, we describe a fibrin (F) and a fibrin-agarose 0.4% (FA) nanostructured human cartilage substitute and evaluate the sequential changes that take place in the ECM during five weeks of culture.

Materials and methods. Human articular hyaline cartilage biopsies obtained from healthy donors were enzymatically digested with collagenase type II to generate primary cultures of chondrocytes. Then, a nanostructured human articular cartilage substitute was developed in the laboratory using a fibrin and FA-0.4% with condrocytes cultured within. Tissue samples were analyzed after 1, 3 and 5 weeks of culture using

haematoxylin-eosin and alcian blue staining, and Ki-67 and laminin immunohistochemistry.

Results. The histological analysis revealed an increasing number of cells with time in culture in both construct types (F and FA). Alcian Blue staining was progressively positive only in FA constructs, with higher signal after longer times in culture. The inmunohistochemical analysis for Ki-67 and Laminin was positive for both constructs and for all weeks.

Conclusions. These results suggest that both fibrin and fibrin-agarose biomaterials properly allow the progressive prolifferation of the human hyaline chondrocytes cultured within and the synthesis of laminin glycoproteins. However, proteoglycans synthesis was positive only when fibrin-agarose scaffolds were used. For all these reasons, fibrin-agarose scaffolds are recommended for the generation of an artificial human hyaline cartilage.

Keywords. nanostructure, biomaterial, cartilage, proteoglycans, laminin

(8.P6) HUMAN CHONDROCYTES AND MESENCHIMAL STEM CELLS RESPONSE TO A DECELLULARIZED HUMAN DERMA

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Introduction. Biological resurfacing has been advocated as reconstructive treatment and several materials have been proposed including extracellular matrix (ECM). The aim of the study was to evaluate the biological response of two human cell lines to a new decellularized human dermal ECM membrane in comparison with a commercially available human dermis.

Methods. Normal human articular chondrocytes (NHAC-kn) derived from human knee articular cartilage, and human mesenchymal stem cells (hMSC) were seeded in polystyrene wells (TCP) as controls, and on a decellularized human dermis from multi-organ donors (HDM_derm) and GRAFTJACKET® – Maximum Force membrane (GJ) for 7 and 14 days.

Results. NHAC-kn and hMSC proliferation was higher on HDM_derm than it was on GJ at both experimental times. Phenotype expression was maintained on both tested membranes, for NHAC-kn, while hMSC cultures showed significant increases in pro-cathepsin B (108%, $p < 0.005$) and CPII (12%, $p < 0.05$). The synthesis of TGF-β1 was higher in hMSC where significantly higher values were found when cultured on GJ than HDM_derm at both 7 (152%, $p < 0.0005$) and 14 (43%, $p < 0.005$) days with significant increases between the two experimental time for cultures seeded on GJ (237%, $p < 0.005$) and HDM_derm (92%, $p < 0.0005$).

Conclusions. The results obtained showed that HDM_derm seems more suitable than GJ for the differentiation and growth of the NHAC-kn. Further investigations are mandatory to understand better the behaviour of hMSC, above all for their expression towards a chondrogenic phenotype when in contact with HDM_derm. This study represents the first evidence to support the use of a HDM_derm with this new method as a scaffold for soft tissue regeneration with special interest for biological resurfacing.

Keywords. Decellularized human dermal matrix, chondrocytes, mesenchimal stem cells

(8.P7) GLOBAL GENE EXPRESSION ANALYSIS OF MESENCHYMAL STROMAL CELLS FROM OSTEOARTHRITIC DONORS

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Osteoarthritis (OA) is one of the most frequent musculoskeletal disorders and represents the main indication for total joint arthroplasty. However, the exact aetiology of OA remains the focus of ongoing research. Mesenchymal stromal cells (MSCs) can be easily isolated from bone marrow aspirates and provide an excellent source of progenitor cells. Previously differences in proliferation and differentiation of MSCs from osteoarthritic versus healthy donors were reported. To elucidate the role of MSCs in OA aetiology we compared global gene expression of MSCs derived from osteoarthritic versus healthy donors.

MSCs were isolated from bone marrow aspirates of n=13 advanced-stage osteoarthritic and n=15 age-matched healthy donors by density gradient-centrifugation and polystyrene adhesion. After cell expansion until subconfluency total RNA of MSCs at passage 0 were analysed using Affymetrix® GeneChip Human Genome U133 Plus 2.0 Arrays. Raw data were processed by background correction, normalization, and robust multichip analysis followed by statistical analysis using "R" and one-way ANOVA for gender-related or intergroup gene expression differences. Gene ontology (GO) and pathway analyses were performed by use of NetAffx™, DAVID, KEGG, and Babelomics4.

A total of n=690 intergroup differentially regulated genes were identified. Notably the most significantly regulated gene, component of oligomeric golgi complex 5, had recently been reported to be associated with an increased risk of OA. Using GO the functions of genes differentially regulated in OA-MSCs were classified into processes of transport, transcription, protein modification, apoptosis, RNA modification, and cell adhesion. Notch, Wnt and Jak-Stat were identified as the most significantly affected signal transduction pathways by OA in MSCs.

Using global gene expression analysis of MSCs from osteoarthritic versus healthy donors we identified relevant candidate genes and signal transduction pathways. These data support the hypothesis that MSCs play a central role in the aetiology of osteoarthritis and warrant further studies.

Keywords. osteoarthritis, mesenchymal stromal cells, gene expression analysis, microarray

(8.P8) INTEGRATING BIOMIMETIC BIOREACTOR CONDITIONS AND ALGINATE MICROBEADS TO INDUCE FORMATION OF CARTILAGINOUS TISSUE CONSTRUCTS

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Particulate cell supports, especially in the form of microbeads, provide short diffusion distances for efficient mass transfer to cells, as well as possibilities for minimally invasive implantation by injection and environments for uniform cell distribution and extracellular matrix (ECM) regeneration. In addition, interstitial channels create structures enabling development of vasculature between individual particles. We have previously shown that alginate microbeads were suitable supports for immobilization of chondrogenic cells and cartilaginous ECM regeneration in perfusion bioreactors. Bonding of the beads and formation of continuous cartilaginous constructs depended on the cell density, bead size, and medium flowrate. However, since articular cartilage is normally exposed to dynamic loads, in this study we have utilized a novel biomimetic bioreactor integrating dynamic compression together with tissue perfusion applied in physiological regimes in order to enhance regeneration of cartilaginous tissue. Packed beds of alginate microbeads (~900 μm in diameter) with immobilized bovine calf chondrocytes (33x106 cells/ml) were cultivated over 4–6 weeks under dynamic compression (1h on/1h off, frequency 0.4 - 0.6Hz, 10% strain) and medium perfusion (flowrate of 0.28 ml/min corresponding to the superficial velocity of 25μm/s). The bioreactor provided also monitoring of biomechanical properties of the packed beds over the cultivation time (Fig. 1). Compression moduli decreased in the first two weeks of cultivation due to alginate gel weakening but then started to increase as the cells produced ECM. After 4 weeks of cultivation large bonded groups of microbeads were formed. Results of this study can be relevant not only for cartilage tissue engineering but also for controlled studies of particulate cell supports under conditions that imitate physiological in vivo environments as well as for predictions of implant behavior.

Keywords. biomimetics, bioreactor, cartilage tissue engineering, dynamic compression, alginate

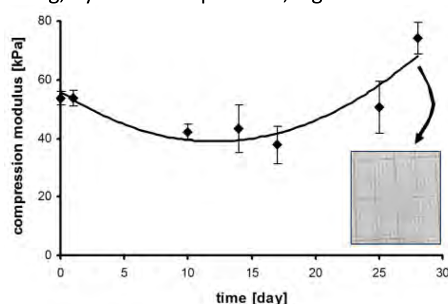

Figure 1. Compression modulus of packed beds of microbeads over 4 weeks of cultivation; insert: merged beads after 4 weeks of cultivation

(8.P9) MIGRATION OF CHONDROCYTES FROM ADULT HUMAN CARTILAGE INTO BIOMIMETIC SCAFFOLDS

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Introduction. Biomimetic scaffolds hold great promise for therapeutic repair of cartilage and bone but still require optimization in terms of their ability to integrate with the host tissue in order to establish an appropriate extracellular matrix (ECM). Adult human cartilage has a limited capacity for repair but there is evidence that chondrocytes can migrate and maintain some chondrogenic phenotype (1). This study investigated the conditions under which chondrocytes migrate out of cartilage and into biomimetic scaffolds in vitro.

Materials and Methods. Articular cartilage explants were cut from femoral condyles and tibial plateux derived from donors undergoing total knee replacement for osteoarthritis (OA). Cultures were maintained for up to 28 days and examined by light microscopy and immunohistochemistry. Explants were also studied using an xCelligence apparatus (Roche) to measure real time cell migration.

Results. Cells migrated to the periphery of cartilage explants after approximately 10 days in culture where they proliferated and deposited an extracellular matrix. The number of migratory cells was related to the location of the original cartilage, cutting method, culture conditions and could be manipulated by the addition of growth factors. Migration of cells and deposition of an ECM into a collagen/glycosaminoglycan scaffold was considerably enhanced by the addition of growth factors.

Discussion. The precise interaction between biomimetic scaffolds and damaged host tissue is seldom investigated prior to in vivo studies. It may be possible to exploit the ability of human OA cartilage as a source of migratory cells to aid the population of biomimetic scaffolds thus enhancing the repair process. Studies are currently in progress to identify and manipulate the phenotype of this migratory cell population.

References. Morales, T. Osteoarthritis Cartilage. 2007 15(8): 861–871

Keywords. Cartilage Repair, Biomimetic Scaffold, Chondrocyte, Migration

(8.P10) COMPARISON OF PLACENTAL AND BONE MARROW-DERIVED STEM CELLS FOR CARTILAGE TISSUE ENGINEERING.

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Introduction. Stem cells offer great potential for regenerative medicine technologies. Embryonic stem cells, in contrast to adult mesenchymal stem cells (MSCs), exhibit pluripotency and can differentiate into many different cell types. A less invasive source of potential foetal-derived stem cells, are the amniotic fluid and placenta.

AIM. The aim was compare human amnion (HAM), amniotic fluid (HAF) and bone marrow-derived MSCs (BM-MSCs) for their chondrogenic potential for cartilage tissue engineering.

Methods. HAM, HAF and BM-MSCs were seeded dynamically onto PGA scaffolds after the expansion of the cell numbers in monolayer culture. The resultant constructs were cultured for 4 weeks in classical

chondrogenic media (DMEM containing 1mg/ml BSA, insulin/transferrin/selenium, 10-7M dexamethasone, 25µM ascorbic acid, TGFß). At 2 and 4 weeks, constructs were taken for analysis of gene expression by real-time PCR (AGC1, COL2A1, COL9A2, COL10A, SOX9, MIA, CRTL1, CSPG2 and COMP). After 4 weeks, proteoglycans (detected as glycosaminoglycans (GAG) were measured using dimethylmethylene blue and frozen sections taken for histochemical and immunochemical analysis of the extracellular matrix.

Results. BM-MSCs accumulated the most extracellular matrix containing collagen II and proteoglycan. BM-MSCs showed the highest expression of markers for hyaline cartilage (AGC1, COL2A1, COL9A2, SOX9, CTLR1, MIA) and accumulation of GAG. Expression of collagen I was similar for BM-MSCs and HAF cells and lower in HAMs. BM-MSCs also showed the highest expression of the hypertrophic marker, collagen X. Neither collagens II, X nor aggrecan was expressed by HAM cells.

Conclusions. Under the conditions used, BM-MSCs were the more appropriate stem cell type for cartilage tissue engineering but displayed a tendency to hypertrophy.

Acknowledgements. This collaborative research would not have been possible without funding from the European Commission which established the EXPERTISSUES Network of Excellence [reference 500283]. A.M. Frias is recipient of a post-doctoral scholarship from Fundação para a Ciência e Tecnologia (SFRH/BPD/45206/2008).

Keywords. stem cells, placental, bone marrow, cartilage tissue engineering

(8.P11) MORPHOLOGICAL AND MOLECULAR ANALYSIS OF THE INTERACTIONS AMONG BONE, CARTILAGE AND BIOMATERIALS BY MICROSCOPY NMR

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Introduction: The deeper understanding of detailed interactions among bone, cartilage and biomaterials will allow obtaining essential information for improving the design of most biological compatible materials. Microscopy Nuclear Magnetic Resonance (MNMR) is a non-destructive technique that can provide morphological description of the biological sample and, at high magnetic fields, molecular information. Morphological and molecular initial results in fresh and fixed rabbit's knee bone samples by NMRM will be discussed. A preliminary NMRM approach for a biomaterial/bone knee sample will be presented.

Methods: New Zealand rabbit's knee samples, fresh or fixed and decalcified have been studied by MNMR at 14T. Different MRI microscopy pulse sequences and distinct MR parameters have been tested. MNMR morphological 2D and 3D images by using MSME, MDEFT, FLASH and M-MSME sequences with different in plane and section resolution have been obtained. Likewise, PRESS single voxel spectra at short echo time have been acquired. SE3D, GE3D and SPI3D MNMR images have been acquired for a pilot biomaterial/bone knee sample included in PMM.

Results: An example of morphological images by MNMR in fresh and fixed rabbit's knee is shown. Particularly significant is the adequate resolution between bone and cartilage achieved by MDEFT technique. M_MSME images show important constitutive regions of the bone part. In addition, differences can be observed in the cartilage and bone structures between both samples. The metabolic profiles of the fresh sample are different at distinct regions. Different parts of the bone and cartilage can be observed in the biomaterial/bone knee sample by MNMR images.

Conclusions: MNMR can provide morphological and molecular information complementary to histology and CT. Some structural differences have been observed between fresh and fixed samples by MNMR. MNMR, as non-invasive technique, can be applied in non-manipulated biological samples and its results can be translated to MRI in vivo applications.

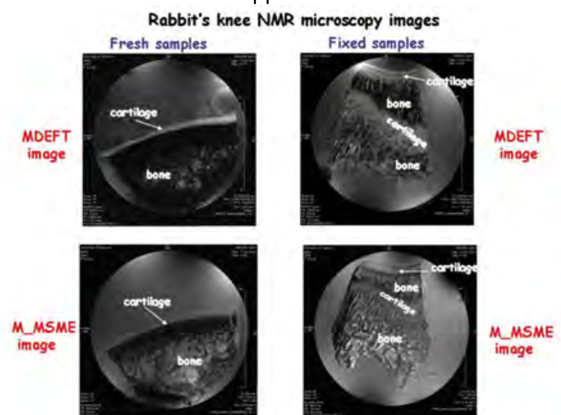

Figure 1. NMR microscopy images (MDEFT and M_MSME) for two different knee's rabbit samples: - left part: fresh sample; - right part: decalcified and fixed sample.

(8.P12) LOW OXYGEN TENSION IS NOT BENEFICIAL FOR THE NEOCARTILAGE FORMATION IN SCAFFOLD-FREE PRIMARY CHONDROCYTE CULTURES

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Introduction. Articular cartilage is an avascular tissue that lives at low oxygen (O2) environment in vivo. The present study was aimed to investigate whether 5% O2 could be beneficial for the neocartilage formation when bovine primary chondrocytes were cultured in type II collagen-coated membrane insert, and whether hyaluronan (HA) or glucosamine sulfate (GS) would enhance the extracellular matrix production of the neocartilage when the cells are cultured in insert at 5% oxygen tension.

Methods. Primary chondrocytes isolated from articular cartilage of bovine femoral condyles were seeded into insert at the cell density of 6 million, and cultured in DMEM supplemented with 10% FBS and antibiotics (control), or GS or HA at 5% or 20% O2 atmosphere for 2, 4, and 6 weeks. The samples were then collected for histological staining of PGs and type II collagen, qRT-PCR of aggrecan and procollagen α1(II) mRNA expressions, and PG content quantification.

Results. Neocartilage produced at 20% O2 appeared larger than at 5% O2, and it was larger and more

homogenous in GS-treated cultures than in other cultures at 20% O2. Histological staining showed that more PGs, type II collagen and better native cartilage structure was produced at 20% compared to 5% O2. The thickness of neocartilage increased following culture period. Quantitative RT-PCR showed that aggrecan and procollagen α1(II) mRNA expressions were significantly higher at 20% O2, as well as PG content. However, no significant difference in gene expression and PG content found between control and GS- or HA-treated culture either at 20% or 5% O2.

Conclusions. We conclude that, in contrast to monolayer cultures, engineered-cartilage from scaffold-free primary chondrocytes at 20% O2 produced better extracellular matrix production than at 5% O2. The PGs mainly consisted of large ones. However, exogenous GS or HA was not beneficial for the ECM production in scaffold-free cultures.

Keywords. 5% oxygen, scaffold-free, neocartilage

(8.P13) METHOD FOR SCAFFOLDING TO CARTILAGE TISSUE SUBSTITUTION A TRACHEAL APPROACH

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The present paper present the early results about of different methods for obtain a xenograft for a tracheal tissue cartilage substitution, was used four method to wash the trachea segment from porcine using a enzymatic detergent, and partial enzyme degradation to remove cell material own of the extracellular matrix and avoid the immune reaction, was done the characterization by Scanning Electron Microscopy (SEM) and histology to evaluate the cell removing and morphological change in the extracellular matrix, the samples were further characterized by thermal techniques like Thermo Gravimetric Analysis (TGA) and Differential Scanning Calorimetric (DSC), furthermore the cell viability was measured by cell culture and the biological response were evaluated by implantation in New Zealand Rabbits. The first results shown that the treatment with enzyme degradation is the most effective to remove the cellular material and avoid the immune reaction.

Keywords. Cartilage, Scaffold, Trachea

(8.P14) EVALUATION OF HYALURONIC ACID-CHITOSAN SPONGES FOR CARTILAGE TISSUE ENGINEERING: IN-VITRO AND IN-VIVO STUDIES

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Introduction. The aim of this study was to develop a scaffold system based on hyaluronic acid (HA) and chitosan (C) that could support the proliferation and chondrogenic differentiation of bone marrow mesenchymal cells (BM-MSCs) for cartilage tissue engineering.

Methods. HA-C sponges were prepared by forming a polyelectrolyte complex precipitate from aqueous blends of HA and C. The final product was attained after lyophilization and crosslinking. SEM and FTIR were used to evaluate morphology and chemical composition, respectively. The swelling ratio and in-vitro degradation rate of the sponge were determined. In-vitro cell culture experiments were performed using rat BM-MSCs and rat articular chondrocytes (ACs; as control), under chondrogenic conditions (+TGF-β1). Cell distribution and morphology were followed. Cell viability and proliferation were determined by the MTT assay; total GAGs synthesis were determined as well. Cellular sponges were subcutaneously transplanted into Wistar rats and explanted cellular grafts were evaluated by histology.

Results. SEM demonstrated that HA-C sponges had a highly porous structure composed of interconnecting pores providing a suitable environment for cell migration. FTIR spectra of the HA-C sponge showed the expected peaks of HA and C indicating the presence of both polymers within the sponge. HA-C sponge was mechanically stable under the culture conditions for the duration of in-vitro studies. The seeded BM-MSCs and ACs adhered and proliferated inside sponges as indicated by the MTT test. Total sGAG secreted by the differentiated BM-MSCs were comparable with that of ACs. Chondrogenic differentiation of BM-MSCs was confirmed by histology. In-vivo studies revealed that both cellular and acellular sponges showed a mild level of tissue reaction. Histology also confirmed the homogenous distribution of the cells and cartilage-like tissue formation inside the HA-C sponges.

Conclusions. These data seem to indicate that HA-C sponges provide a suitable 3D-scaffold environment for cartilage tissue engineering.

Keywords. hyaluronic acid, chitosan, mesenchymal stem cells, chondrogenesis

(8.P15) PLATELET-RICH PLASMA HAS ANTI-CATABOLIC PROPERTIES IN OSTEOARTHRITIC CHONDROCYTES

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Introduction. Platelet-rich plasma (PRP) has recently been postulated as a treatment for osteoarthritis (OA). Although anabolic effects of PRP on chondrocytes are well documented, no reports are known addressing anti-catabolic responses. Since OA is characterized by a catabolic joint environment, we studied whether PRP exerted anti-catabolic effects on primary human osteoarthritic chondrocytes.

Methods. PRP was prepared from whole blood from three healthy donors. Human OA chondrocytes from six donors were cultured in alginate beads in the presence of IL-1β to mimic an osteoarthritic environment. Medium was supplemented with 0%, 1% or 10% PRP releasate

([PRPr] the active releasate of PRP). After 48 hours, gene expression of collagen type II (COL2), aggrecan (AGCN), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4, ADAMTS-5, matrix metalloproteinase (MMP)-13 and cyclo-oxygenase (COX)-2 was analyzed. Additionally, glycosaminoglycan (GAG) content, nitric oxide (NO) production and nuclear factor kappa B (NF-κB) activation were studied.

Results. IL-1β diminished expression of the anabolic genes COL2 and AGCN in chondrocytes, while it increased expression of the catabolic ADAMTS-4, MMP-13 and COX-2 ($P<0.03$ for all genes). PRPr diminished IL-1β induced inhibition of COL2 ($P=0.003$) and AGCN ($P=0.001$) gene expression. PRPr also reduced IL-1β induced increase of ADAMTS-4 ($P=0.001$) and COX-2 ($P=0.004$) gene expression. ADAMTS-5 gene expression and GAG content were not influenced by IL1-b nor PRPr. MMP13 gene expression and NO production were upregulated by IL-1β but not affected by PRPr. Finally, PRPr reduced IL-1β induced NF-κB activation to control levels containing no IL-1β ($P<0.001$).

Conclusions. PRPr diminished multiple catabolic IL-1β effects in human osteoarthritic chondrocytes. PRPr exerted anti-catabolic effects on genes regulating extracellular matrix formation, as well as inflammation, in human chondrocytes. Moreover, PRPr decreased NF-κB activation, a major pathway involved in the pathogenesis of OA. These results encourage further use and study of PRP as a treatment for OA.

Keywords. platelet-rich plasma; PRP; cartilage; chondrocyte; osteoarthritis; anti-catabolic

(8.P16) CO-CULTURE OF MONOCYTES AND MESENCHYMAL STEM CELLS UNDER HYPOXIC CONDITIONS

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Introduction. The overarching goal of therapeutic cartilage repair is to adopt a biomaterial to deliver cells and growth factors into the articular cartilage defect to facilitate the healing response. Adult cartilage is an avascular tissue; thus an injury is not followed by an influx of monocytes. Mesenchymal stem cells (MSCs) stand out as an ideal progenitor cell source for cartilage tissue engineering. This study describes the development of osteochondral graft consisting of primary monocytes and MSCs on a collagen biomaterial scaffold.

Materials and Methods. A novel collagen-GAG scaffold (Chondromimetic®, Tigenix) composed of type I collagen and chondroitin sulphate provided a 3D microenvironment for the osteochondral graft. Ovine MSCs were a gift from Mesoblast (Australia) and primary ovine monocytes were prepared by centrifugation of fresh, heparinized whole blood using Lymphoprep™. MSCs were plated either in the scaffold or as micromasses on tissue culture plastic. After 24 hours monocytes were added in various concentrations. Cultures were maintained for up to 21 days both in normoxic and hypoxic cell culture conditions.

Results. Ovine MSCs maintained their characteristics in the presence of monocytes. After 2 weeks in culture, the extra cellular matrix production (ECM) was increased by the addition of monocytes. Hypoxic culture conditions were found to have a positive effect on the monocyte activity. The collagen-GAG biomaterial scaffold supported the cell attachment and proliferation towards chondrogenic lineage.

Discussion. The molecular interaction between monocytes and MSCs has yet to be established but is has been thought to involve paracrine factors. The results of this study suggest that each cell type, monocyte and MSC, may contribute independently of each other in supporting the osteochondral graft properties. Further studies are underway to evaluate whether the positive effects of monocytes are due to an adherent progenitor cell population in the circulating monocytes.

Keywords. cartilage repair, MSC, hypoxia

(8.P17) IN VITRO AND IN VIVO EVALUATION OF HA/CARBON FABRICS SCAFFOLD FOR CARTILAGE REPAIR

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Introduction. Carbon fibers have been widely investigated as cellular growth supports in cartilage tissue engineering. However, long duration of the process of cartilage restoration limits the applicability of CFs implants in the treatment of cartilage tissue defects. Hyaluronic acid plays a key role in cartilage tissue development, repair and function. In the present study we focused on the in vitro and in vivo evaluation of two types of carbon nonwoven fabrics: hyaluronan modified, and non-modified carbon nonwovens.

Experimental Methods. Hyaluronic acid sodium salt (HA) was purchased from CPN spol s.r.o. (Czech Republic). Carbon nonwoven fabrics (CNFs) were prepared from polyacrylonitrile precursor via two-stage process: stabilization (150-280°C) and carbonization (1000°C, argon). Hyaluronic acid (HA) was immobilized onto the surface of macroporous carbon nonwoven fabrics. The cytotoxicity of the samples (CNF and CNF_HA) were determined in the culture of human lung adenocarcinoma cell line A549. MG-63 cells (European Collection of Cell Cultures, Salisbury, UK) were used for testing the cell-material interaction in vitro. The knee cartilage of rabbits was used as a model tissue for in vivo studies.

Results And Discussion. Direct contact of the cell line A549 did not show any cytotoxicity effect neither in CNF nor in CNF_HA. Adhesion of MG-63 cells was better on CNF_HA composites than on unmodified carbon fabrics. MG-63 cells adhered well to carbon fibers showing an elongated shape. Knee defects treated with CNF_HA were repaired in different degree with hyaline-like cartilage tissue, granulation tissue and bone. Numerous capillaries

present in regenerating tissue allow to expect the proper reconstruction of chondral and bone tissues.

Conclusion. Modified carbon nonwovens used in our study are a promising scaffold which allows cells to grow within it and to form new cartilage and bone.

Acknowledgments. Work supported by Polish Ministry of Science and Higher Education (Iuventus Plus: IP2010034270).

Keywords. scaffolds, fibers, cartilage, hyaluronic acid

(8.P18) DESIGN OF EXPERIMENTS AND RESPONSE SURFACE MODELLING FOR OPTIMISATION OF DEFINED CHONDROGENIC MEDIUM

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Introduction. The golden standard of defined media for cartilage differentiation in cartilage tissue engineering was originally optimised for Mesenchymal Stem Cells (MSCs) 12 years ago by Johnstone et al[1]. It was thereafter applied to human chondrocytes by Tallheden et al [2] and others. The medium has, however, never been optimised for chondrocytes. In this abstract we show the usefulness of computer based design of experiments (DoE) and response surface modelling (RSM) for optimisation of a defined medium for tissue engineering of articular cartilage with human chondrocytes.

Methods. Surplus chondrocytes from two patients (mean age 18±2 yrs) undergoing autologous chondrocyte implantation (ACI) were expanded in DMEM/F12 and 10% human serum. The cells we cultured for two weeks in pellet mass culture in 17 different medium formulations designed in Modde 8.0 (Umetrics AB, Sweden), with 6 variables (TGFb1, Dexamethasone, Human serum albumin, ITS+, Ascorbic acid, Glucose). The pellet size was assessed and RNA was extracted after two weeks of culture. Expression of matrix components were assessed with qPCR. The results were displayed in response surfaces (fig 1).

Results. TGFbeta1, dexamethasone and glucose showed to be significant factors for pellet size and matrix components expression. Optimized medium for chondrocyte differentiation was 13 ng/ml TGFbeta1 (+30%), 50 nM dexamethasone (-50%) and 5.5 g/L glucose (+22%), compared to the golden standard ($p<0.009$).

Conclusion. The golden standard culture media in cartilage tissue engineering needs to be revised for optimal culture processes. Computer based DoE and RSM are powerful tools for this and other culture optimization purposes.

References

1. Johnstone et al, Exp Cell Res. 1998;238(1):265-72 l

2. Tallheden et al, Osteoarthritis Cartilage. 2004;12(7):525-35.

Keywords. Cartilage, Design of Experiments, Response Surface Modelling, Medium Optimisation

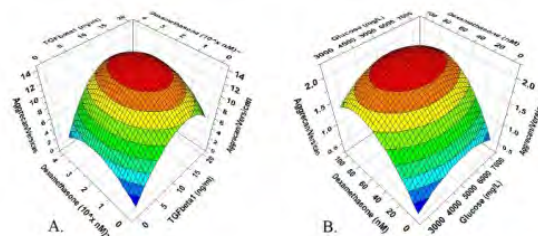

Figure 1. Response surfaces for qPCR gene expression for Aggrecan/Versican ratio for factors a) Dexamethasone and TGFbeta1 b) Dexamethasone and Glucose

(8.P19) HUMAN OSTEOARTICULAR COMPLEXES: CHARACTERIZATION AND EVOLUTION OF PRESEEDED CHONDROCYTE BIOMATERIALS/SCAFFOLDS USING A NUDE MICE MODEL

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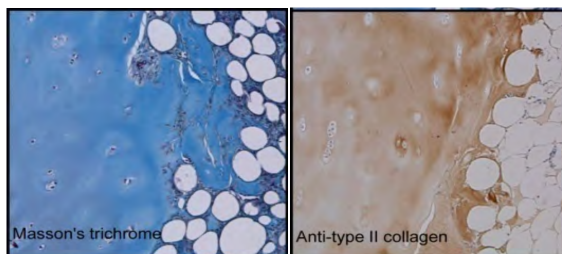
Introduction. The aim of the present work is to study the response of human chondrocytes seeded in a 3D construct consisting in biomaterial microspheres embedded in a fibrin clot, a model that provides the cells with a human osteochondral environment while implanted in nude mice.

Methods. Osteochondral cylinders (1-cm diameter) were obtained from healthy areas of the tibial plateau of patients undergoing knee replacement surgery. A centered 3-mm cavity was drilled on the articular surface, which was filled with microspheres of a biomaterial preseeded with human chondrocytes, and mixed with fibrin. Three polymers were tested: chitosan with or without hialuronic acid and polycaprolactone. The cylinders were subcutaneously implanted on the back of nude mice. Animals were sacrificed after 1, 2 and 4 weeks of surgery, and cylinders were removed and processed for histological and immunohistochemical (type-I and -II collagens, and human nuclear-antigen detection) analysis.

Results. Most of the microspheres remained in the cavity 4 weeks after implantation of the osteochondral cylinder. Histological analysis showed an inflammatory response, assessed by neutrophil infiltration, when chitosan or chitosan-hyaluronic acid were used as biomaterials. No signs of inflammation were observed when polycaprolactone was used; furthermore, Masson's trichrome stain showed the deposition of new cartilage-like extracellular matrix in these samples, which was confirmed by immunodetection of type-II collagen.

Conclusions. From the three polymers tested, fibrin-glued polycaprolactone spheres showed a better biocompatibility. This biomaterial favored the synthesis of cartilage-like extracellular matrix. The human or murine origin of the cells responsible of the new extracellular matrix is under study. These results indicate the feasibility of polycaprolactone spheres injection for the restoration of the articular cartilage.

Acknowledgements. Parly supported by Spanish MCINN grants MAT2007-66759-C03-01-03 and MAT2010-21611-C03-01-03.
Keywords. Human osteochondral complex; chondrocyte; scaffold



(8.P20) HYDROSTATIC PRESSURE AND TGF-β3 INTERACT TO REGULATE CHONDROGENESIS OF JOINT TISSUE DERIVED MESENCHYMAL STEM CELLS

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Introduction. Hydrostatic pressure (HP) is a key component of the joint mechanical environment and has been shown to enhance chondrogenesis of chondrocytes (CC) and mesenchymal stem cells (MSCs). The objective of this study was to investigate the interaction between HP and TGF-β3 in regulating chondrogenic differentiation of joint derived MSCs.

Methods. MSCs were harvested from synovial membrane (SM) and infrapatellar fat pad (FP) of the femoro-patellar joint, centrifuged to form pellets and subjected to 10 MPa of HP at 1Hz for 4 hours (14 days), controls were maintained in free swelling (FS) conditions. Pellets were cultured with different concentrations of recombinant human TGF-β3 (0, 1 and 10 ng/ml). Samples were analysed biochemically (Glycosaminoglycan (GAG) and collagen content) and histologically.

Results. SM and FP derived MSCs underwent robust chondrogenesis in FS conditions when supplemented with 10 ng/ml TGF-β3, HP having no effect at this TGF-β3 concentration. In contrast at 1 ng/ml TGF-β3, HP significantly increased GAG accumulation. The same trend was observed for collagen accumulation. Chondrogenesis was not observed in the pellets cultured in the absence of TGF-β3 in either the FS or HP groups. HP appeared to influence the organization of the neo-tissue, as evidenced with the appearance of a core region in the FS pellets compared to more homogeneous organization in the HP group (Fig.1).

Conclusion. HP alone did not induce chondrogenesis, but when applied with low concentration of TGF-β3, it acted to promote chondrogenesis for FP and SM MSCs. At high magnitudes of TGF-β3, HP had no additional synergistic effect on chondrogenesis, suggesting an upper limit on the stimulatory effects these two chondrogenic cues can have. Future studies will investigate the influence of HP on the development and organization of cartilaginous tissues engineered using joint tissue derived MSCs.

Acknowledgements. Supported by Science Foundation Ireland [SFI/08/YI5/B1336]
Keywords. MSC, chondrogenesis, hydrostatic pressure, TGF-β3

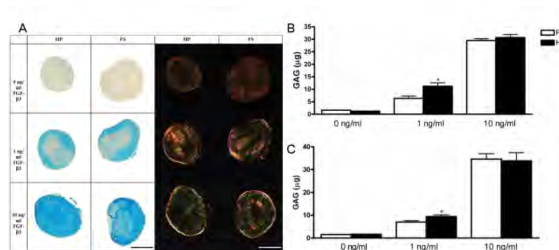

Figure 1: A Histology results for synovial membrane mesenchymal stem cells. Alcian blue (stains GAG, left columns) and picro-sirius red (stains collagen, right columns) in pellets at day 14. Scale bar 500µm. **B** GAG content in synovial membrane pellets at day 14. **C** GAG content in fat pad pellets at day 14. Mean ± SD. * significance vs FS group. p<0.01. FS: Free swelling. HP: Hydrostatic pressure.

(8.P21) POTENTIAL GENE SPECIFIC MARKERS TO PROOF THE PURITY OF HUMAN ARTICULAR CHONDROCYTES IN MONOLAYER CULTURE

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Introduction. Due to the new EC regulations, No 1394/2007 on Advanced Therapy Medical Products, there is a need to establish markers to proof the purity of human articular chondrocytes in monolayer culture before treatment. The most likely contaminants in the cultures are synoviocytes due to synovial overgrowth of the biopsy area. The aim of the study was to (i) study chondrogenic differentiation of synoviocytes and to (ii) identify genes that could specifically determine purity regarding synoviocyte contamination.

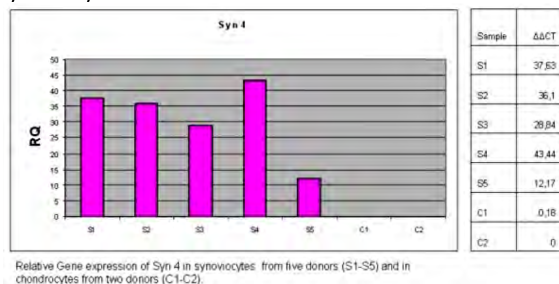
Methods. Synoviocytes were isolated from human tissues (n=5) and the cells were expanded in monolayer culture (ML) followed by RNA preparation or seeding to a hyaluronan scaffold (HYAFF 11, Fidia Advanced Polymers) subsequently cultured for 14 days in a modified differentiation media. The scaffolds were analyzed regarding handling property, morphology and histology. Messenger RNA from ML cultures was subjected to gene expression analysis using oligonucleotide microarray (Affymetrix). Expression data was compared to previous microarray data from human chondrocytes in ML (n=5). Candidate genes selected from the microarray analysis were confirmed by real-time PCR.

Results. When comparing monolayer chondrocytes with synoviocytes no differences were observed microscopically and synoviocyte seeded scaffolds showed similar handling characteristics as chondrocyte seeded scaffolds. However, the histology results showed slightly higher matrix production in the chondrocyte seeded scaffolds. The gene expression comparison identified a distinct set of 4 genes (designated Syn 1- 4) that were barely detected in chondrocytes but highly expressed in synoviocytes.

Conclusions. Although the articular cartilage and the synovium tissue can be easily identified in biopsies, it is impossible to exclude potential contaminating synoviocytes in the subsequent culture process. We here demonstrate that three of the identified candidate genes

have the potential for identifying chondrocyte purity regarding synoviocyte contamination.

Keywords. Purity, gene marker, chondrocytes, synoviocytes



Sample	ΔΔCT
S1	37,63
S2	36,1
S3	28,84
S4	43,44
S5	12,17
C1	0,18
C2	0

Relative Gene expression of Syn 4 in synoviocytes from five donors (S1-S5) and in chondrocytes from two donors (C1-C2).

(8.P22) MICROENCAPSULATION OF CHONDROCYTES IN THE ALGINATE –CHITOSAN-ALGINATE SYSYTEM

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Introduction. Microencapsulations of chondrocytes in the alginate-chitosan-alginate (ACA) microcapsules, fibers and sheets were performed. Both chitosan and alginate have chemical composition and properties similar to the cartilage, what makes them the material of choice for cartilage cells growth. Presented work provides the detailed examination of the impact of the microencapsulation methods on the growth and viability of chondrocytes. Potential application of the presented work is the reconstruction of damaged joint cartilage.

Methods. The microcapsules, fibers or sheets were obtained by three steps method. Alginate with cell was gelled in calcium chloride solution, than it was coated with chitosan and then again in the alginate solution of ten times lower concentration then in the first phase. Obtained forms were stored in the medium at 36 degrees. The influences of alginate concentration, gelation time, type of chitosan, the form of microcapsule and cell concentration on cells growth and viability were investigated.

Results. The process of encapsulation does not destroy the chondrocytes, the chosen method does not contain harmful stages and creates conditions for their growth. Application of encapsulated cells is slightly restricted by the smaller growth rate as compared with that of free cells. The growth and viability of chondrocytes are significantly influenced by the size and the shape of produced microcapsules but not by the type of chitosan applied.

Conclusion. Considered method does not have any limiting stage; all components which build the membrane are highly biocompatible and capable for immunoisolation. Microcapsules coating make them mechanically strong, the glucosamine compounds, which are the products of enzymatic hydrolyzis exist naturally in cartilage, and the alginate capability to reduced dedifferentiation provides synthesis of appropriate type of collagen. Thanks to all these advantages ACA system appears to be a promising method for reconstruction of damage cartilage.

Keywords. chondrocytes, cartilage, alginate, chitosan

(8.P23) HYALINE CARTILAGE REGENERATION BY COMBINED THERAPY OF MICROFRACTURE AND LONG-TERM BMP-2 DELIVERY

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Introduction. Microfracture of cartilage induces migration of bone marrow-derived mesenchymal stem cells (BMMSCs) to cartilage defect sites. However, this treatment often results in fibrocartilage regeneration. Growth factors such as bone morphogenetic protein (BMP)-2 induce the differentiation of BMMSCs into chondrocytes, which can be used for hyaline cartilage regeneration. Here, we tested the hypothesis that long-term delivery of BMP-2 to cartilage defects subjected to microfracture would result in regeneration of high quality hyaline-like cartilage, as opposed to short-term delivery of BMP-2 or no BMP-2 delivery.

Methods. Rabbit articular cartilage defects were treated with microfracture combined with one of the following: no treatment, fibrin, short-term delivery of BMP-2, Heparin-conjugated fibrin (HCF), or long-term delivery of BMP-2. HCF and normal fibrin were used as carriers for the long and short-term delivery of BMP-2, respectively. Eight weeks after treatment, the cartilage regeneration was evaluated by morphometrical analysis, histological analysis, GAG contents analysis, and real-time polymerase chain reaction(RT-PCR).

Results. Histological analysis revealed that the long-term delivery of BMP-2 group (microfracture + HCF + BMP-2) showed the most staining with alcian blue. A biochemical assay, RT-PCR assay, and western blot analysis all revealed that the long-term delivery of BMP-2 group had the highest GAG content as well as the highest expression level of collagen type II.

Conclusion. The long-term delivery of BMP-2 to cartilage defects subjected to microfracture resulted in regeneration of hyaline-like cartilage, as opposed to short-term delivery or no BMP-2 delivery. This method could be more convenient for hyaline cartilage regeneration than autologous chondrocyte implantation due to its less invasive nature and lack of cell implantation. Since BMP-2 and microfracture are currently in use clinically, this approach would be highly feasible.

Acknowledgements. This study was supported by a grant (No. 2010-0020352) from the National Research Foundation of Korea

Keywords. Bone morphogenetic protein-2; Cartilage regeneration; Heparin-conjugated fibrin; Microfracture

(8.P24) MESENCHYMAL STEM CELLS EXERT PARACRINE EFFECTS ON OSTEOARTHRITIC CARTILAGE AND SYNOVIUM.

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Introduction. Osteoarthritis (OA) is characterized by an imbalance of anabolic and catabolic processes in synovial joints, resulting in progressive cartilage damage. Mesenchymal stem cells (MSCs) have recently been discovered to have immunomodulatory capacities by secreting several anti-inflammatory cytokines and growth factors. MSCs are promising candidates for OA therapies, although applied cells do not seem to actively participate in formation of new cartilage. We studied the paracrine effects of MSCs on OA cartilage and synovium explants in vitro.

Methods. To stimulate primary human MSCs to secrete immunomodulatory factors, they were cultured in medium containing 10% FCS with additional TNFa and IFNg (50 ng/ml each). After 24 hours medium was collected and designated “conditioned medium”. Human cartilage and synovium explants were cultured for 48 hours in conditioned medium or in unconditioned control media with or without TNFa and IFNg (50ng/ml). Explants were studied for expression of genes regulating inflammation and extracellular matrix degradation.

Results. Cartilage: IFNg en TNFa upregulated ADAMTS-4 and IL-1RA in cartilage explants, while ADAMTS-5 and MMP-13 were unaffected. Conditioned medium by MSCs further upregulated IL-1RA and downregulated ADAMTS-5 gene expression, whereas ADAMTS-4 and MMP-13 remained unchanged.

Synovium: IFNg and TNFa upregulated TNFa, IL-1b, IL-1RA and SOCS1 in synovium. Conditioned medium downregulated the cytokine-induced IL-1b expression and further upregulated IL-1RA and SOCS1. MMP-13 gene expression was not affected by IFNg and TNFa stimulation or conditioned medium.

Conclusions. Conditioned medium containing factors secreted by MSCs caused anti-catabolic and multiple anti-inflammatory responses in our cartilage and synovium explants. This indicates that MSCs have beneficial paracrine effects on the metabolism of osteoarthritic cartilage and synovium. These results offer a possible working mechanism for MSCs to modulate the osteoarthritis process, and encourage further use and study of MSCs as a treatment for OA.

Keywords. Osteoarthritis, MSC, mesenchymal stem cell, paracrine, cartilage

(8.P25) THE ANTI-OSTEOARTHRITIS DRUG, PENTOSAN POLYSULFATE, STIMULATES BONE MARROW DERIVED STRO 3+ MESENCHYMAL PRECURSOR CELL PROLIFERATION, CHONDROGENIC DIFFERENTIATION AND REDUCES APOPTOSIS WHEN CULTURED IN POROUS COLLAGEN SCAFFOLDS

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Introduction. Our previous studies have shown that Pentosan Polysulfate (PPS) stimulated Mesenchymal Precursor Cells (MPC) chondrogenic differentiation in micromass cultures. In this study we examine the ability of PPS to induce MPC chondrogenesis, proliferation but reduce apoptosis when seeded in commercial collagen scaffolds.

Methods and Materials. MPC cells (70,000) were injected into commercially available collagen sponges (Gelfoam or OsseoFit) (2x6mm discs) then cultured in DMEM (+10% FBS) supplemented with 0.0 - 20.0 ug/ml PPS for up to 21 days. In some cultures TGF-Beta-3 (0 - 20ng/mL) was include in the absence or presence of PPS. Cell apoptosis, viability and proliferation were monitored by Tunnel/DAPTI /WST8 and 3H-Thymidine assays. Proteoglycan (PG) synthesis was quantified by incorporation of 35-S into sulphated glycosaminoglycans normalised for cell number (DNA). MPC gene expression was followed over days 7, 14 and 21 using real-time PCR (RT-PCR).

Results. Bioassays showed that MPC viability and proliferation was stimulated and apoptosis decreased by PPS over 21 days. DNA synthesis was maximal with 2.5 ug/mL PPS ($p < 0.03$) on day 10. Proteoglycan biosynthesis was maximal on day 10 (82% > control, $p = 0.0005$) with 2.5 ug/mL PPS, while 100% > control was observed on day 14 at both 2.5 and 5.0 ug/mL PPS ($p < 0.0001$). TGF-Beta-3 induced maximal PG synthesis by MPC at 10ng/mL (400%, $p = 0.00001$) but this effect was enhanced synergistically to 650% in the presence of 5.0 ug/mL PPS ($p = 0.00001$). RT-PCR confirmed increased expression of SOX-9, Aggrecan and type II collagen genes at PPS concentrations of 2.5 - 10 ug/mL.

Conclusions. These studies confirmed that human MPC cultured in collagen sponges in the presence of PPS undergo proliferation and chondrogenic differentiation. These data support the notion that PPS in combination with MPC can be used for the repair of cartilage osteochondral defects.

Keywords. Mesenchymal stem cells, chondrogenesis, pentosan polysulfate, collagen scaffolds

(8.P26) PLATELET-DERIVED GROWTH FACTOR-AA IS A POTENTIAL CHEMOATTRACTANT FOR MIGRATION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IN ARTICULAR CARTILAGE INJURED ATHYMIC RATS

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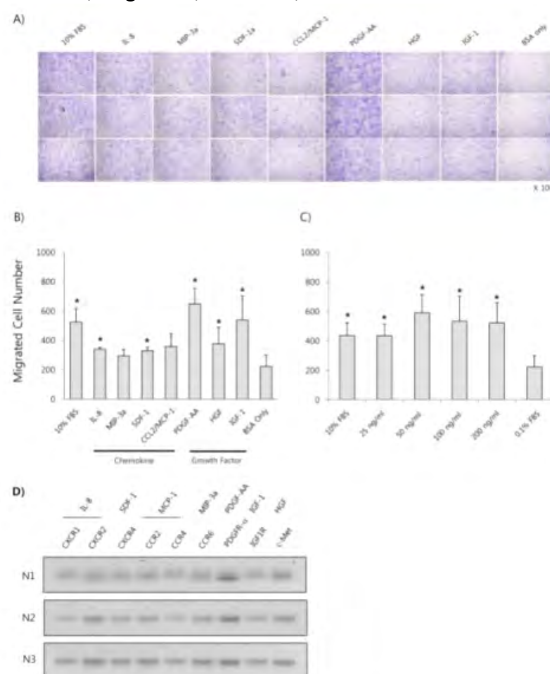
Cell motility is controlled by extracellular matrix substrates and by secreted molecules such as chemokines and growth factors. Cell migration in response to specific external signals is termed chemotaxis. The understanding of cell chemotactic mechanisms may lead to the novel therapeutic strategies for tissue regeneration. The purpose of this study is to investigate that molecules such as chemokines and growth factors may direct BMSC migration. For this, we used Boyden chamber assays to select which chemokines or growth factors are able to induce migration of human BMSC. BMSCs significantly responded to several chemokines (IL-8, MIP-3a, SDF-1 and CCL2/MCP-1) and growth factors (PDGF-AA, HGF and IGF-1). The most potent chemotactic effect was observed with PDGF-AA compared to other chemoattractants. The optimal response was observed at 50 ng/ml of PDGF-AA in BMSC. The migration of human BMSCs reached peak

values at 593±123. Further the study was confirmed by the expressions of chemoattractant receptors in BMMSCs. It was found that the expression of CXCR2, PDGFR-α, and c-Met were increased in serum-free conditions, compared to the expressions of other receptors. Interestingly, the expression level of PDGFR-α was significantly increased than that of other chemoattractant receptors. This study revealed that the migration effect of BMSCs by PDGF-AA may be related to the steady expression of PDGF receptor alpha in serum-free conditions and futher need to explore the receptor signalling mechaism of PDGF in the chemotaxis of BMSC. We also investigated that the selected migration factor may effectively direct BMSCs in vivo nude rat model through migration factor conjugated fibrin gel scaffolds. In conclusion, this study demonstrates the ability of human bone marrow-derived mesenchymal stem cells to migrate in response to PDGF-AA, and probability of cartilage repair in nude rat model. We suggest that PDGF-AA conjugated fibrin gels are useful biomaterials for injured-articular cartilage regeneration.

Keywords. human bone marrow-derived mesenchymal stem cells, Migration, PDGF-AA, in vivo nude rat model



(8.P27) OPTIMIZATION OF AN AGAROSE SANDWICH MODEL FOR CARTILAGE TISSUE ENGINEERING

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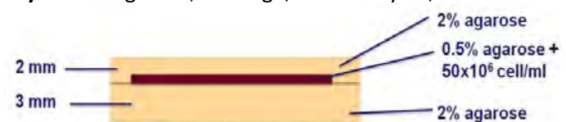
Introduction. The chondrocyte-seeded agarose model is a well-established in vitro system used in cartilage tissue engineering. Previously, we have shown that reducing agarose concentration results in increased and more uniform matrix production. Besides, it is known that mechanical loading is an essential trigger for stimulation of cartilage growth. However, direct mechanical loading of low-concentration agarose constructs is impossible, because initially these are weak and brittle. Seeding cells in a low-concentration agarose layer in between stiffer agarose layers ('agarose sandwich') would enable to apply mechanical loading to low-concentration agarose constructs. In this study we explore the feasibility of using such a sandwich model.

Methods. A 4 mm layer of 3% agarose was allowed to gel in a 12-wells plate. Afterwards, a ± 500 µm layer of 0.5% agarose containing 50x106 bovine chondrocytes per ml was added, and covered with another 2 mm layer of 3% agarose (Fig 1). As a control 0.5% agarose discs containing 50x106 chondrocytes per ml were used. All constructs were cultured in a) medium containing FBS (n=6 per group) or b) serum-free medium with 10 ng/ml TGF-β3 (n = 6 per group) for 32 days. All constructs were analyzed for viability, biochemical content and matrix distribution.

Results. No significant differences in viability, matrix content and distribution were observed between standard agarose discs and the agarose sandwich model. Discs and sandwiches cultured in presence of TGF-β3 contained significantly more proteoglycan and collagen compared to those cultured in FBS-medium, and matrix distribution was more homogeneous.

Conclusions. These results demonstrate that the agarose sandwich model is suitable for use in cartilage tissue engineering studies. The layered system did not limit tissue development due to for instance an effect on diffusion of matrix or nutrients. We will proceed to use this model system for application of mechanical loading to low-concentration agarose constructs.

Keywords. Agarose, cartilage, chondrocytes, TGF-B3



Schematic representation of the agarose sandwich model

9. CELL TRACTION: THE PROS AND CONS IN VALVULAR AND VASCULAR TISSUE ENGINEERING

Chair: Anita Driessen-Mol
Co-chair: Stefan Jockenhoevel
Keynote speaker: Stefan Jockenhoevel
Organizers: Anita Driessen-Mol, Stefan Jockenhoevel

Synopsis: Valvular and vascular tissue engineering rely on extracellular matrix production by cells seeded into a degrading scaffold material. The seeded cells adapt a myofibroblast phenotype, characterized by synthetic as well as contractile activity, and naturally exert traction forces to their surroundings. In nature, these surroundings are capable of withstanding these forces by the hemodynamic environment the tissue is in (e.g. pressure), the degree of constraint of the tissue (e.g. blood vessels are constrained in axial direction), and the extracellular matrix properties (both composition and mechanical behaviour).

Tissue engineering has made us realize how delicate this balance in nature is. Cell traction on the one hand is shown beneficial for tissue maturation and alignment in engineered tissues, while on the other hand is causing loss of shape. Unbalance in the extracellular matrix properties, hemodynamics and cell traction in engineered

heart valves was demonstrated to result in leaflet shrinkage, a problem commonly observed in animal studies, and resulted in regurgitation of the valve and loss of function. This symposium offers a platform to discuss the pros and cons of cell traction in valvular and vascular tissue engineering. We hope to share insights on its fundamentals and to work towards fine-tuning of this delicate balance between cell traction, extracellular matrix properties and hemodynamics towards functional valvular and vascular tissue engineering.

(9.KP) CELL TRACTION: THE PROS AND CONS IN VALVULAR AND VASCULAR TISSUE ENGINEERING

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Valvular and vascular tissue engineering rely on extracellular matrix production by cells seeded into a degrading scaffold material. The seeded cells adapt a myofibroblast phenotype, characterized by synthetic as well as contractile activity, and naturally exert traction forces to their surroundings. In nature, these surroundings are capable of withstanding these forces by the hemodynamic environment the tissue is in (e.g. pressure), the degree of constraint of the tissue (e.g. blood vessels are constrained in axial direction), and the extracellular matrix properties (both composition and mechanical behaviour). Tissue engineering has made us realize how delicate this balance in nature is. Cell traction on the one hand is shown beneficial for tissue maturation and alignment in engineered tissues, while on the other hand is causing loss of shape.

The keynote lecture will give an overview on the physiological and pathophysiological mechanisms of tissue shrinkage in general and specifically the relevance of these factors on cardiovascular tissue engineering. Furthermore the three major factors of tissue engineering (1) cells, (2) scaffolds and (3) stimuli will be analyzed with regard to their influence on tissue retraction.

(9.O1) ENDOGENOUS TISSUE CONTRACTILITY SPATIALLY REGULATES THE VEGF SIGNALING AND ANGIOGENESIS IN SELF-ORGANIZING MICROFABRICATED TISSUES

Rivron N (1), Vrij E (2), Rouwkema J (2), Truckenmuller R (2), Le Gac S (2), Van den Berg A (2), Van Blitterswik C (2)
1. University of Twente, Hubrecht Institute; 2. University of Twente

Endogenous physical forces can drive the organization of tissues (1-2). The underlying mechanisms are currently based on cell surface mechanics (3) or mechanotransduction (4) and are thus separated from known conserved mechanisms including the formation of morphogen gradients. Here using an array of autonomously contracting and deforming, 3D, microfabricated, tissues, we show that tissue geometry and endogenous contractility spatially regulates the Vascular Endothelial Growth Factor (VEGF) signaling and the local formation of vascular patterns. The microfabricated tissues stereotypically and heterogeneously changed shape, compacted and formed robust patterns of vascular structures in regions of high deformation. This emergence correlated with the local over-expression of the receptor VEGFR2 and with the formation of a tissue-scale gradient of VEGF. We propose that endogenous tissue contractility and deformation is a morphogenetic regulator of angiogenesis, a finding which should stimulate new therapeutic strategies for vascular diseases and regenerative medicine.

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Keywords. vascular pattern, endogenous contractility, VEGF signaling

(9.O2) THE POTENTIAL OF PROLONGED TISSUE CULTURE TO REDUCE STRESS GENERATION AND RETRACTION IN ENGINEERED HEART VALVE TISSUES.

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1. Eindhoven University of Technology

Introduction. Tissue engineered heart valves develop a good tissue architecture, induced by traction forces, when cultured constrained. However, during culture cell traction causes tissue compaction, resulting in leaflet flattening. At time of implantation, the leaflets have to be separated and cell traction causes leaflet retraction. To get insight into these mechanisms and to develop solutions, we have developed an in vitro model system to quantify and correlate stress generation, compaction, retraction and tissue quality during a prolonged culture period of 8 weeks.

Methods. PGA/P4HB strips were seeded with vascular-derived cells and cultured for 4, 6 and 8 weeks (n=5 per time point). Compaction in width was measured during culture, while stress generation and retraction in length were measured after culture when constraints were released. Further, the amount of DNA, GAG, collagen and collagen cross-links was assessed.

Results. Compaction started after 2 weeks and continued up to 66.2±1.7% at week 4, after which width remained constant (fig 1A). Stress generation reduced from 11.8±0.9 kPa at week 4 to 2.4±0.4 kPa at respectively week 8 (fig 1B). Tissue retraction reduced from 44.0±3.7% at week 4 to 26.1±2.2% at week 8 (fig 1C). The reduced stress generation over time correlated with the reduced retraction. The amount of DNA, collagen and collagen cross-links was constant at all time points. The amount of GAGs was increased at week 6 and 8 compared to week 4 and correlated to the reduced stress generation.

Conclusion. In summary, increasing culture time resulted in decreased stress generation and retraction, likely as a result of the increased amount of GAGs. These results demonstrate the potential of prolonged tissue culture in developing functional, non-retracting, TE heart valves.

Acknowledgement. The authors gratefully acknowledge the support of the Smart Mix Program of the Netherlands

Ministry of Economic Affairs and Education, Culture and Science.
Keywords. compaction, retraction, stress generation, heart valve tissue

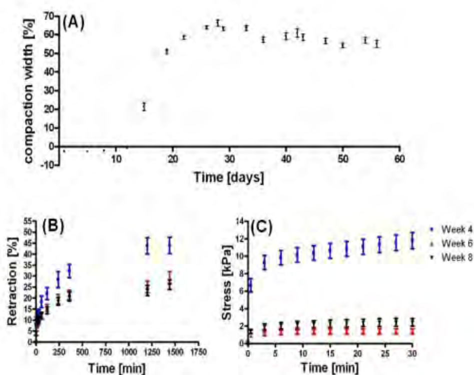

Figure 1: Compaction during culture (A), retraction (B) and stress generation after culture (C) at week 4, 6 and 8.

(9.O3) EFFECT OF CROSSLINKING OF FREEZE-DRIED AND CRITICAL POINT DRIED COLLAGEN SCAFFOLDS ON PHYSICAL PROPERTIES AND CELL FUNCTION: RELEVANCE FOR HEART VALVE TISSUE ENGINEERING

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Introduction. Suitable cell-scaffold constructs for tissue engineering a heart valve should be mechanically strong and compatible with cells, allowing them to grow, secrete appropriate extracellular matrix components (such as collagen, elastic fibres and proteoglycans/glycosaminoglycans) and not promote calcification. Collagen based scaffolds have several desirable characteristics. However, optimal processing methods have not been established.

Methods. We have evaluated the cellular compatibility and physical properties (thermal stability, resistance to enzymatic degradation, Young's modulus, pore size and permeability) of 4 different collagen scaffolds. Scaffolds were manufactured using freeze drying (FD) or critical point drying methods (CPD) and either physically crosslinked with dehydrothermal treatment (DHT) or chemically crosslinked with 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS). Cell compatibility was studied using a dynamic seeding process with human mesenchymal stem cells (MSCs) and following cell proliferation and cell phenotype.

Results. Chemical crosslinking proved to increase scaffold resistance and decrease its permeability better than physical crosslinking. All scaffolds were compatible with MSCs as judged by proliferation of the cells and their ability to produce extracellular matrix and not to differentiate towards osteogenic, chondrogenic or endothelial lineages. FD scaffolds with EDC/NHS crosslinking were the only scaffolds able to withstand pressures up to 80 mmHg and showed the highest Young's modulus compared to the other scaffolds.

Conclusions. Our results suggest that FD EDC/NHS scaffolds are good candidates for heart valve tissue engineering applications.

Keywords. Collagen scaffold, heart valve tissue engineering, Extracellular matrix, Mesenchymal stem cell

(9.O4) EFFECT OF MECHANICAL CONDITIONING ON CELL-MEDIATED TISSUE CONTRACTION IN FIBRIN-BASED TISSUE ENGINEERED HEART VALVES

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Introduction. Functional valvular tissue engineering aims at developing living tissue by seeding or embedding cells into degradable scaffolds which are mechanically conditioned in bioreactors to reach functionality prior to implantation. The presence of cells is responsible for production of extracellular matrix and reorganization of fibers. However, it also causes contraction of the tissue and consequent changes of its 3D geometry, which in the case of heart valves results in insufficiency as commonly observed in vivo and in vitro. We applied different conditioning protocols to fibrin-based 3 leaflet-heart valves in bioreactors and evaluated their effects on leaflet retraction by ultrasound.

Methods. The fibrin gel valves were produced using a previously reported moulding technique by mixing a fibrinogen solution (20mg/ml) with TBS containing ovine carotid artery-derived cells, CaCl2 and thrombin. The cell concentration was 10X106/ml of the final volume.

After moulding two different conditioning strategies were followed in custom made bioreactors: 1) dynamic conditioning and 2) static conditioning on the mould followed by dynamic conditioning. Ultrasound images were taken to evaluate leaflet configuration and function.

Results. The heart valves conditioned only dynamically were able to open and close in a satisfactory way at the very beginning of the protocol but showed clear shrinkage of the leaflets in the following days till no leaflet could be detected anymore (Fig.1a). The valves receiving static and dynamic conditioning showed a much reduced leaflet retraction and good functionality (Fig.1b).

Conclusions. Optimization of the conditioning protocol is one crucial step towards the development of functional tissue engineered heart valves. The capability to drive and actively modulate the production of the extra cellular matrix is particularly important to reach adequate functionality.

Acknowledgements. The authors thank the Fördergemeinschaft Deutsche Kinderherzzentren e.V. for financial support.
Keywords. fibrin, heart valve, mechanical stimulation, tissue contraction

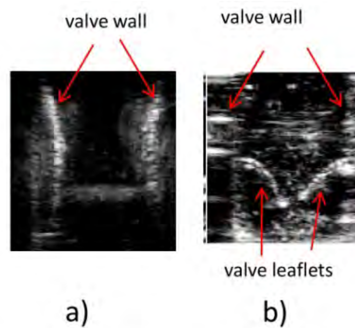

Fig1: Ultrasound images of tissue engineered heart valves conditioned a) only dynamically and b) statically and dynamically

(9.O5) VESSEL DERIVED STEM CELLS CONTRIBUTE TO ENDOCHONDRAL OSSIFICATION OF ATHEROSCLEROTIC PLAQUE

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Introduction. Pericytes, although traditionally considered as supporting cells, have recently been proposed to have a more active role in the repair and pathogenesis of various vascular diseases. There is growing body of research work indicating that the vessel wall contains a number of progenitor cell niches that remain as yet completely defined. In this study, we hypothesized that a pericyte-like stem cell population, termed vessel derived stem cells or VSCs with chondrogenic and osteogenic potential exists in the vessel wall and in presence of the inflammatory cytokines seen in atherosclerotic environment, contributes, along with the circulating mesenchymal stem cells to the calcification of atherosclerotic plaque which occurs through the endochondral pathway.

Methods. VSCs from aortae of ApoE-/- mice and background C57BL/6 mice were isolated and characterized for cell surface markers by flow cytometry and immunocytochemistry. MSCs from bone marrow of these mice were also isolated and characterized. Chondrogenic potential of these cells was investigated in presence or absence of inflammatory cytokines such as IL-6 and IFN-γ. Real time PCR was performed to analyze the up- or down-regulation of key factors in chondrogenic pathway.

Results and Discussion. VSCs were strongly positive for Sca-1, CD44 and negative for CD31 and CD34. Immunocytochemistry for specific pericyte marker 3G5 revealed that a sub-population of VSCs expressed 3G5 (Figure 1). Differentiation assays demonstrated the ability of the cells to differentiate into bone and cartilage. VSCs had significantly higher GAG/DNA ratio than MSCs indicating increased chondrogenesis. That both MSCs and VSCs from the ApoE-/- atherosclerotic mice generate a more mature hypertrophic chondrocyte than cells from the C57BL/6 mice is interesting and suggests that the atherosclerotic environment may modulate the stem cell phenotype. Col-type II and aggrecan expression and effect of oxidized LDL will be investigated to further test this hypothesis.

Acknowledgments. This work is funded by the Irish Research Council for Science Engineering and Technology
Keywords. Atherosclerosis, Stem cells, Chondrogenesis, Endochondral ossification

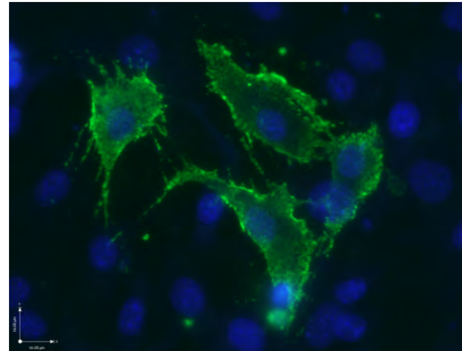

Figure 1. 3G5 staining.

(9.O6) CLAY-GELS CAN LOCALIZE VEGF AND INDUCE ANGIOGENESIS IN VITRO AND IN VIVO

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Introduction. Hydrogels offer considerable potential as tissue engineering matrices, however their essential hydrophilicity presents challenges for the retention, in space and time, of bioactive molecules. Certain clays are known for their ability to adsorb biological molecules due to the large and highly charged specific surface area of the nano/micro-sized particles. We show the potential of a synthetic smectite clay suspension to self-organise, encapsulate viable cells and localise exogenously applied angiogenic factors inducing an angiogenic response in vitro and in vivo.

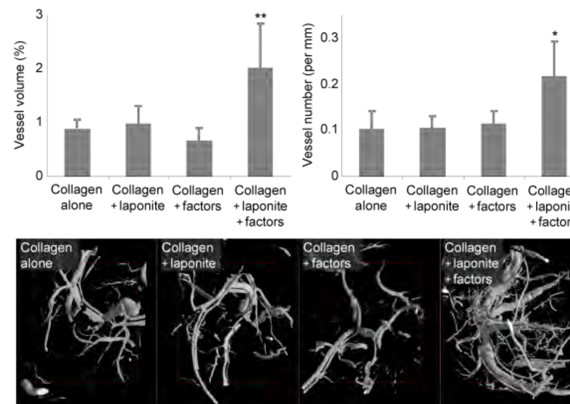
Methods. Suspensions of laponite, a synthetic smectite, were added drop-wise to cell culture media containing model protein (albumin, lysozyme) or vegf165. Protein diffusion and uptake by laponite capsules was assessed by assaying supernatant using bradford assays and elisas, or confocal analysis via flouroprobe labeling. In vitro angiogenic induction by laponite-bound vegf was assessed using the human umbilical vein endothelial cell (huvec) tubule-formation assay. For in vivo characterization, laponite encapsulated collagen scaffolds were incubated in vegf media for 2hrs before implantation in a murine femoral defect model. Neo-angiogenesis was quantified via micro-ct.

Results. Upon addition to physiological saline, free-flowing laponite suspensions self-organized into stiff gels allowing encapsulation of cells, matrix proteins and growth factors. While negligible diffusion of protein out of laponite capsules was observed over 14 days, rapid and extensive uptake and binding of protein by laponite capsules was observed. To test the bioactivity of laponite-bound protein, the effect of laponite-bound vegf on huvec tubule-formation was assayed. Laponite films exposed to vegf for two hours before washing yielded equivalent tubule-organization to positive controls. In

vivo studies revealed significantly enhanced vascularisation compared to controls (fig 1).

Conclusion. This work describes a novel clay-gel based strategy for the delivery and application of growth factors without the need for complex chemical modifications thus offering significant potential for the delivery of regenerative microenvironments.

Keywords. hydrogels, growth factor delivery, angiogenesis, clays



(9.O7) ADVANTAGES OF DENUDED HUMAN UMBILICAL VEIN (HUV) OVER DECELLULARIZED HUV AS SCAFFOLD FOR VASCULAR TISSUE ENGINEERING

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Objective. To compare the utility of endothelium-denuded and completely decellularized human umbilical veins (HUV) as scaffolds for tissue-engineered small-caliber vessel grafts.

Methods. HUV were endothelium-denuded by luminal dehydration (60 ml/min carbogen) or decellularized using (1) a detergent mixture (Triton X-100, sodium deoxycholate, IGEPAL-CA630, 0.025% each), (2) peroxyacetic acid (0.1 %), or (3) alternating washes with 3M NaCl and distilled water, each followed by nuclease treatment and extensive washing. Scaffold compositions were analyzed by histology and immunohistology. Failure stresses were determined in a tensile testing rig. Calcein AM stained HUVEC were seeded on the scaffolds at densities of 5E5 cells/cm2.

Results. Denudation removed endothelial cells without damaging other wall components or decreasing tetrazolium dye reduction. Decellularization caused an almost complete loss of H&E-stainable material. Remnants of degenerate nuclei were removed by nuclease treatment. In contrast to denudation, decellularization caused a loss of laminin and fibronectin staining, as well as fragmentation of elastic fibers. Failure stresses were not decreased by denudation or by chemical treatments, but by nuclease treatment and were extrapolated to burst pressures of 2160 mm Hg (native), 1880 mm Hg (denuded), and 1580 mm Hg (decellularized). Static HUVEC seeding resulted in a confluent neoendothelium on denuded vessels after 3 days culture. Decellularized vessels showed incomplete coverage on day 1 and a loss of viable cells until day 3. Seeding of denuded HUV in a perfusion bioreactor resulted in a flow-resistant neoendothelium.

Discussion. Denuded HUV maintain a metabolically active smooth muscle layer and provide a superior surface for endothelial cell seeding compared to decellularized HUV. This may be attributed to the preservation of intact basement membranes. Therefore denuded HUV are to be preferred to decellularized HUV for vascular tissue engineering.

Acknowledgements. This study was funded by Deutsche Forschungsgemeinschaft (BI 139/2-1, HA 4380/5-1, and LI 256/68-1).

Keywords. endothelium; decellularization; scaffold; umbilical vein

(9.P1) INFLUENCE OF ADDITIONAL NORMOBARIC HYPOXIA ON EXERCISE INDUCED HEMATOPOIETIC STEM CELL RELEASE

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Introduction. The release of adult hematopoietic stem cells (HSCs) was shown to improve regeneration processes in the human body. Recent data suggest that an elevated level of HSCs in the peripheral blood supports tissue renewal and patients recovery. Both physical exercise and normobaric hypoxia may act as triggers for HSC mobilization. The aim of our study was to investigate the effect of normobaric hypoxia and physical exercise on the release of HSCs from the bone marrow into the circulation.

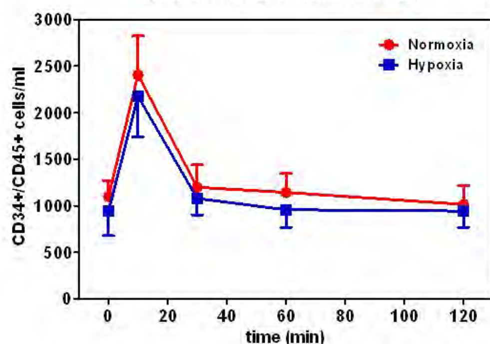
Methods. Six healthy male subjects (26,5 +/- 5,1 yrs) underwent a standardized incremental exercise test (40 W+20 W/min) under either normoxic (FiO2 ~ 0.21) or hypoxic conditions (FiO2 < 0.15, equals 3.500 m, 3 h exposure) within a time span of at least one week. Blood was drawn from the cubal vein before and 10, 30, 60 and 120 min after the exercise. The number of HSCs in the peripheral blood was analyzed by means of flow cytometry (CD 34/ CD 45 positive cells). Standard markers of exercise performance (Pmax, VO2max, Lamax, HRmax) were obtained.

Results. The physical challenge of the incremental test showed a significant increase of HSC release under normoxic as well as hypoxic conditions (repeated measures ANOVA using Fisher's LSD, $p < 0.05$; Fig.1) after 10 min of recovery. There was not any significant difference detectable between normoxia and hypoxia regarding the HSC level ($p > 0.05$).

Conclusions. The results of this study indicate that the HSC release to the peripheral blood is induced by intensive exercise under both hypoxic and normoxic

conditions, but there was no greater effect on circulating HSC numbers by addional hypoxia. We may suggest that the short term hypoxic exposure of 3 h at approx. 3.500 m simulated altitude does not have any (additional) effect on HSC mobilization from the bone marrow.

Keywords. hematopoietic stem cells, exercise, hypoxia, facs analysis



(9.P2) HYBRID POLYMERIC IPN SCAFFOLDS FOR CARDIAC HEART VALVE TISSUE ENGINEERING

Martínez-Crespiera S (1), Fernández N (1), Herrero M (1), González S (1), Rodríguez C (1), Saint-Pierre G (1)

1. PERA

Introduction. Despite the efforts made in order to improve the mechanical and biological performance of the current clinical available mechanical and bioprosthetic heart valves, the ideal prosthesis has not been yet developed. Tissue engineering appears as a promising alternative to overcome the main drawbacks of the existent prosthesis (non-obstructive, non-thrombogenic, biocompatible and long-term lasting). However, due to the technical difficulties of an efficient heart valve prosthesis design, to date no tissue engineered heart valve has demonstrated to be successful at clinical level. Bioscent project (see acknowledgment) aims at developing interpenetrating network (IPN) of natural and synthetic polymers to provide a novel generation of scaffold for heart valve tissue engineering.

Materials and Methods. IPN of natural (sodium alginate, chitosan) and synthetic polymers (PVA) are prepared. Solvent casting is used to produce the polymeric blends.

Results. Solvent casting of the IPN systems offers homogeneous, flexible and biocompatible scaffolds. The mechanical properties have been validated with a developed aortic valve FEM (Finite Element Method) model. Through this model it has been demonstrated how these IPN scaffolds are suitable materials that enable the development of a tissue engineering autologous heart valve compliant with the behaviour of the native valve. Moreover it has been proved that mechanical properties are not affected by the presence of calcium ions.

Conclusions. In the present work hybrid IPN for heart valve scaffolds are presented. These systems are biocompatible and present suitable mechanical properties for the tissue engineering of the heart valves.

Acknowledgments. The present work is carried out in the scope of BIOSCENT. Project full title: “BIOactive highly porous and injectable Scaffolds controlling stem cell recruitment, proliferation and differentiation and enabling angiogenesis for Cardiovascular Engineered Tissues” funded by European Commission FP7 Program under Grant agreement no.: ID214539.

Disclosures. The present results are property of the Bioscent Consortium.

Keywords. interpenetrating network (IPN), scaffold, polyvinyl alcohol (PVA), sodium alginate (SA), solvent casting

(9.P3) DEVELOPMENT OF NOVEL TISSUE ENGINEERED SMALL DIAMETER VASCULAR GRAFT USING TRIMER PEPTIDE

Narita Y, (1), Kuwabara F (1), Yamawaki-Ogata A (1), Kanie K (2), Kato R (2), Satake M (3), Kaneko H (3), Honda H (2), Ueda Y (1)

1. Department of Cardiac Surgery, Nagoya University Graduate School of Medicine; 2 Department of Biotechnology, Nagoya University Graduate School of Engineering; 3. Technology Innovation Center, Teijin Limited

Introduction. Both rapid endothelialization and the prevention of intimal hyperplasia are essential to improve the patency of small-diameter vascular grafts (SDVGs). Using the peptide array-based screening system, we identified the peptide “Cysteine-Alanine-Glycine (CAG),” which has a high affinity for endothelial cells and a low adhesive property for smooth muscle cells. It is known that thrombosis contribute early stage occlusion, and intimal hyperplasia contributes to late stage occlusion of the SDVG. Meanwhile, thrombosis is caused by luck of endothelium, and intimal hyperplasia is caused by excessive synthesis of extracellular matrix from dedifferentiated smooth muscle cells. In this study, we report an in vivo analysis of the novel SDVGs that were constructed with a biodegradable polymer (poly-ε-caprolactone) containing CAG peptide in rat.

Methods. The novel tissue engineered (TE)-SDVG, which measured 0.7 mm in diameter and 7 mm in length, was fabricated using the electrospinning technique. The carotid arterial replacement was performed on Sprague-Dawley rats using the SDVGs with (group CAG) or without CAG (group C). Histological and biochemical assessments were performed at 1, 2 and 6 weeks after implantation.

Results. The ratio of endothelialization was significantly higher in group CAG compared to group C (CAG vs C: 64.4±20.0% vs 42.1±8.9% at 1 week p=0.02, 98.2±2.3% vs 72.7±12.9% at 2 weeks p=0.001, and 97.4±4.6% vs 76.7±5.4% at 6 weeks, p<0.001). Additionally, Western blot analysis showed that the intensity of the endothelial nitric oxide synthase at 1 week of group CAG was significantly higher than that of group C (CAG vs C: 1.20±0.37 vs 0.34±0.16, p=0.01), and that α-smooth muscle actin at 6 weeks in group CAG was significantly lower than that of group C (CAG vs C: 0.89±0.06 vs 1.25±0.22, p=0.04).

Conclusions. Our developed TE-SDVG with CAG promoted rapid endothelialization and potential to inhibition of intimal hyperplasia.

Keywords. small diameter vascular graft, electrospinning, peptide

10. CELL VIABILITY AND TISSUE BANKING

Chair: Blanca Miranda
Co-chair: Antonio Fernández-Montoya
Keynote speaker: Alice Warley
Organizers: Blanca Miranda, Salvador Oyonarte
Synopsis: Construction of artificial tissues and organs by tissue engineering is one of the fields of medical research that has experienced major progress in recent years. In this regard, and to ensure the appropriate function of the developed organs, an accurate evaluation and quality control of the constructed tissues and organs is very important, especially if these are generated and stored in Tissue Banks for clinical uses.
Evaluation of the suitability of the developed tissues and organs has to be carried out at different levels and using different techniques. In the first place, the researcher must evaluate the viability of the primary cell cultures that will be used to generate the tissue constructs, since only viable cells are suitable for clinical use. In this regard, the development of extremely sensitive techniques like the electron probe X-ray microanalysis allows the scientist to not only evaluate the viability of the cells in the culture, but also to predict the short and long-term behaviour of these cells. On the other hand, evaluation of the constructed tissue substitutes have to ensure that both the structure and the function of the constructs is adequate and that these tissues and organs are similar to the normal, native tissues that the researcher pretends to reproduce in vitro. In this milieu, long-term storage of bioengineered tissues in tissue banks is highly dependent on the use of several cryoprotection agents. However, most preservation protocols are associated to certain degree of loss of cell viability or structural tissue damage. For that reason, evaluation of cell viability and tissue structure is especially important for tissues stored in tissue banks.
All kind of works focused on the evaluation of cell viability of cells and tissues generated by tissue engineering and on tissue banking are welcome to this symposium, including methods and techniques based, among others, on:
- Dye exclusion tests.
- Intracellular components release.
- Metabolic and functional tests.
- Electron-probe X-ray microanalysis.
- Colony formation assays.
- In vitro and in vivo analyses.
- Gene expression analyses.
- Cryoprotection.
- Tissue storage.
- Vitrification.
- Tissue banking.

(10.KP) X-RAY MICROANALYSIS IN THE STUDY OF CELL VIABILITY

Warley A (1)
1. CUI, King's College London

For cell culture studies and for tissue engineering the ability to assess cell viability is important. Whereas traditional dye exclusion methods are routinely used in tissue culture, they are difficult to adapt for tissue engineering and also have the added disadvantage that they monitor rather than predict cell death. The ratio of K/Na has proved to be a reliable indicator both of cell viability and of cell vitality, and distinguishes between apoptotic and necrotic cell death pathways. Here I will review the use of X-ray microanalysis, an electron microscopy technique that allows the detection of element content in cells and tissues provided that suitable preparation procedures have been followed, for the study of cell viability/vitality in cell cultures, and suggest how this technique might be adapted to study viability in tissue constructs.

(10.O1) MATERIALS CHARACTERISATION AND MESENCHYMAL STEM CELL RESPONSE ON PLCL MATERIALS

Barron V (1), Rooney N(2), Barry F (1), Murphy M (1)
1. NUI, Galway; 2. Proxy Biomedical Ltd

To date, a range of biomaterials have been employed to create scaffolds for a great variety of tissue engineering applications. Previous research has shown that the biological response of cells on tissue-engineered scaffolds depends on the surface topography, chemical composition and mechanical properties of the construct. As a consequence, it is important to develop a deep understanding of the materials properties and cell response. To this end. two poly(l-lactide--caprolactone) (PLCL) materials with the same chemical composition but different surface topographies were investigated. As expected, there was no difference in the chemical composition of the two PLCL materials with characteristic peaks at 1750cm-1 for C=O, 1180–1080cm-1 for C-O-C and 1041cm-1 for CH3 functional groups. With respect to polymer morphology, both materials exhibited a glass transition temperature (Tg) of 17°C, with no crystalline melt peaks, indicative of an amorphous blend. As seen previously [1], the mechanical properties were altered as a result of the surface features (Figure 1), with values of 1.39MPa and 2.02MPa recorded for the pitted and porous materials, respectively. To investigate cell response, human mesenchymal stem cells (MSC) were seeded at a density of 20,000 cells/cm2 and maintained for 24 hours at 37°C in a humidified atmosphere of 5% CO2 at 37˚C. PLCL strips, 1/10 of the total area of the well, were placed directly on the cells and incubated for an additional 24 hours. Using a Guava Cytosoft cell sorter, it was determined that there was statistical difference in the cell viability of cells grown in the presence of the PLCL materials when compared to the controls on TCP. In summary, the data presented herein gives a deeper insight into the materials properties and the cell response of two PLCL materials and as such is the first step in characterizing these PLCL substrates as delivery vehicles for MSC.

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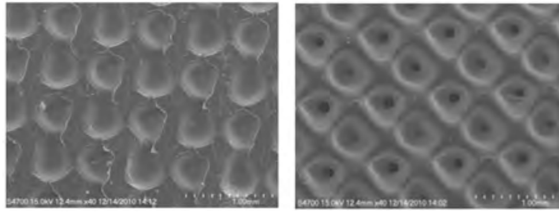
Acknowledgments. The authors would like to thank the Science Foundation Ireland (09/SRC/B1794) for providing financial support to this project.
Keywords. PLCL scaffolds, materials characterisation, stem cell response



(10.O2) MEASURING CELL VIABILITY IN 3D SCAFFOLDS USING CONFOCAL MICROSCOPY

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Introduction. Cell viability (CV) is an important parameter to evaluate the effect of environmental conditions on cell behavior, yet current assays are rather invasive. Recently, we demonstrated that two-photon microscopy could accurately assess CV in situ in three-dimensional (3D) scaffolds without staining based on differences in auto-fluorescence emission spectra of live and dead cells. However, two-photon microscopy requires more specialized equipment. Therefore, the objective of this study was to evaluate confocal microscopy as a non-invasive tool to assess CV, in a similar fashion in 3D collagen gels.

Methods. Mixtures of live and dead C2C12 myoblasts (0%, 25%, 50%, 75%, and 100% live cells) were prepared and CV was determined using the trypan blue (TB) assay. Cell seeded collagen gels (CSCG, n=5/cell mixture) were produced by mixing collagen solution with the live/dead cell mixtures (3.5x106 cells/CSCG). After polymerization, two consecutive confocal microscopy images (λ_{exc}=458nm) of the CSCG were acquired through bandpass filters of 475-525nm and 560-615nm, respectively (n=30 images/CSCG). An intensity ratio per imaged cell was calculated as averaged intensity from image 1/averaged intensity from image 2. Receiver operating characteristic (ROC) analysis was performed to calculate a threshold ratio for cell differentiation.

Results. Ratios of 100% live and dead cells were significantly different and a threshold ratio of 0.68 was determined (Fig.1A). Applying this threshold, no significant differences between the TB assay and confocal microscopy were found in measuring CV (Fig.1B). Nevertheless, CV values acquired with confocal microscopy showed no significant differences between 0% and 25%, and 75% and 100% targeted CVs, whereas all TB viability groups were significantly different.

Conclusion. The results demonstrate that auto-fluorescence intensity as measured by confocal microscopy can be used to assess CV in 3D scaffolds. However, it appears to be less sensitive to constructs with mostly alive or dead cells.

Funding. EU-FP7 consortium Genodisc

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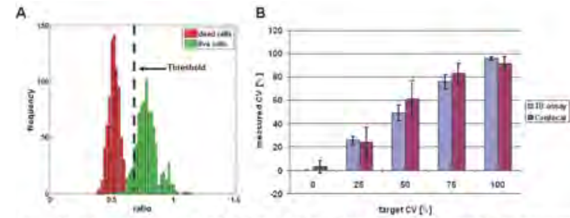

Figure 1: A) Intensity ratios of the CSCG with 0% and 100% CV. B) Measured CV as evaluated using TB assay and confocal microscopy. Values are mean +/- standard deviations, n=5 CSCG. All TB assay groups were significantly different from each other. The confocal microscopy groups were significantly different from each other, except for 0% and 25%, and 75% and 100% CV, respectively (one-way ANOVA, P<0.01).

(10.O3) EFFECTS OF CRYOPRESERVATION ON PERIPHERAL BLOOD MONONUCLEAR CELLS AND ENDOTHELIAL PROGENITOR CELLS

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Introduction. Regenerative medicine has become an appealing therapeutic method; however, stem and progenitor cells are not always freshly available. Cryopreservation offers a way to freeze the cells as they are generated, for storage and transport until required for therapy. Nevertheless, the effects of cryopreservation on the cells, in this case endothelial progenitor cells (EPCs) shown to be involved in neovascularization, have not been extensively studied.

Methods. Peripheral blood mononuclear cells (PBMCs) were extracted from healthy donors (n=6) using density gradient centrifugation. The freshly isolated cells were either analyzed or frozen with liquid nitrogen in media containing 6% plasma serum and 5% dimethyl sulfoxide. After being frozen for 1 day (early) or 28 days (late), the PBMCs were thawed and analyzed or cultured on fibronectin with endothelial basal media for 4 days to generate EPCs. Analysis of the cells consisted of flow cytometry, for viability and various progenitor and stem cell surface markers, as well as functional assays for the adhesion and migration potential.

Results. The viability of PBMCs decreased after cryopreservation ($p<0.01$). CD34 and VEGFR2 expression increased both at early and late thaws ($p<0.05$), whereas the adhesion marker L-selectin was decreased ($p<0.05$), and endothelial marker CD31 was unchanged. EPC viability decreased both at early and late time points ($p<0.1$). There was no significant difference in markers CD31, CD34, VEGFR2 and L-selectin in EPCs derived from cryopreserved PBMC samples, but uptake of low-density-lipoprotein was increased after both 1 and 28 days of cryopreservation ($p<0.05$). Adhesion and migration properties of PBMCs and EPCs were unaffected by cryopreservation.

Conclusion. Cryopreservation of PBMCs decreased viability, but did not affect migrative or adhesive functions. PBMCs were affected phenotypically, with changes in CD34, VEGFR2 and L-selectin expression. Overall, it appears that the more therapeutic EPCs tolerate cryopreservation better than the heterogeneous PBMC population.

Keywords. Endothelial Progenitor Cells, Cryopreservation, Cell Viability

(10.O4) ESTABLISHMENT OF AN INDIVIDUAL HUMAN VASCULAR CELL BANK CONSISTING OF UMBILICAL CORD CELLS FOR THE TISSUE ENGINEERING OF VASCULAR CONSTRUCTS UNDER GOOD MANUFACTURING PRACTICE (GMP) CONDITIONS

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Introduction. Fabricated tissue-engineered vascular constructs could provide an alternative to conventional vascular replacements. One of the bases for tissue engineering of vascular constructs is an adequate cell source. Cells from the human umbilical cord can be directly isolated and cryopreserved until needed. Currently no cell bank for human vascular cells is available. Therefore, the establishment of a human vascular cell bank conforming to GMP conditions, including important quality controls such as cell viability, cell growth and marker expression, is desirable.

Methods. A fundamental step was to adapt conventional research and development agents to agents conforming to GMP for the cell isolation, cultivation and cryopreservation process. Vascular cells were isolated, cryopreserved and recultured subsequently. Cell viability, growth potential and the expression of cell-specific markers from fresh and cryopreserved cells were studied over several passages using Trypan blue staining, flow cytometry analysis and immunofluorescence staining.

Results. Viability tests of directly thawed and recultured cells demonstrated an increase of viability with rising passage number and rapid adaptation to viabilities of fresh cells. Growth potential of cryopreserved, recultured cells was similar to that of fresh cultivated cells with regard to the entire cultivation period. Furthermore, a specific surface marker profile for vascular cells was successfully established using FACS analysis. Fresh cultivated and cryopreserved myofibroblasts were positive for the cellular markers alpha- smooth muscle actin, CD105, CD90, CD73, CD146 and HUVEC expressed CD31, CD146, CD105 and CD144. Additionally immunofluorescence staining using the same markers was performed.

Conclusion. Adaptation of cell isolation, cell cultivation and cryopreservation procedures to GMP conditions was successful. For potential future applications standard operating procedures (SOPs) and a validation process have to be developed to make the establishment of an individual human vascular cell bank feasible.

Keywords. cell bank, vascular umbilical cord cells, quality controls, good manufacturing practice (GMP).

(10.O5) GROWTH ARREST OF HUMAN MSC HAS DIFFERENT EFFECTS ON OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION

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Mesenchymal stem cells are a promising cell source for tissue regeneration. During embryonal development proliferation and differentiation are tightly linked because proliferation is necessary in order to produce enough cells for the differentiation step. We here asked whether proliferation is an absolute requirement for successful differentiation or if MSC can still differentiate after growth arrest into osteogenic and chondrogenic lineage in vitro.

Human MSC (n=5) were isolated from bone marrow and expanded up to passage 3. In vitro chondrogenesis was induced in a high density pellet culture system for 6 weeks and newly synthesized DNA in spheroids was marked with BrdU at different time points. Additionally spheroids were treated with Mitomycin C (20µm) before and during differentiation. Success of differentiation (proteoglycan-, collagen type II-deposition) and BrdU-labelling were detected histologically. Osteogenic differentiation of treated and untreated MSC was induced for three weeks. Mineral deposition and Alkaline-Phosphatase activity were quantified. The BrdU-labelling showed strong proliferation at day 1 of chondrogenic induction which peaked again between day 14 and 21 in areas becoming positive for collagen type II deposition. Mitomycin blocked MSC proliferation while metabolic activity was maintained.

Mitomycin-induced growth arrest of MSC before start of induction or at distinct time points during the first 2 weeks of chondrogenic induction prevented proteoglycan- and collagen type II-depostion according to histology. Mitomycin treatment at later time points was harmless. In contrast osteogenic parameters were apparently not affected by growth arrest.

Proliferation in the early phase of differenation is a requirement for successful chondrogenic differenation of MSC in vitro but not for osteogenic differentiation.

Keywords. MSC, proliferation, differentiation

(10.P1) GLUCOSE REQUIREMENT FOR IN VITRO AN IN VIVO SURVIVAL OF MESENCHYMAL STEM CELLS UPON IMPLANTATION

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The use of human mesenchymal stem cells (hMSCs) has emerged as a potential new treatment of a variety of diseases but has generated marginally successful results. Actually, a consistent finding of most studies is the massive death of transplanted cells. The underlying reasons for the observed limited cell viability are not yet fully understood but in vivo massive death of the transplanted cells after engraftment into tissue-constructs is a major and serious problem. A possible explanation for the aforementioned limited cell survival upon implantation is that MSCs encounter an ischemic (with low oxygen tension and nutrient depletion) environment. In this study, we challenge the current paradigm that gives a pivotal role to oxygen on hMSCs massive cell death and hypothesize that exogenous glucose and not only oxygen supply is required for survival of hMSCs upon transplantation. To this aim, hMSCs were exposed in vitro to sustain near anoxia-hypoxic environment and the influence of exogenous glucose on cell viability and functionality was assessed. Results obtained showed that hMSCs were able to survive

21 days under sustained anoxia without serum providing that they were cultured in the presence of glucose. These results established that glucose depletion but not sustained anoxia affected cell survival. Moreover, hMSCs when cultured 21 days under anoxia in the presence of glucose, kept their stemness and ability to differentiate into osteoblasts and adipocytes. To further investigate the role of glucose, MSCs were seeded onto scaffold composites supplement or not with glucose and their ability to enhance MSC survival was evaluated in an ectopic mouse model. Results showed a striking increase of cell viability in tissue construct supplement with glucose. At day 14, a seven-fold increase in cell number was observed in tissue constructs supplemented with glucose when compared to the one of control tissue constructs.

(10.P2) PLATELET-RICH CONCENTRATE IS PROTECTIVE AGAINST FLUOROQUINOLONE - INDUCED EXTRACELLULAR MATRIX CHANGES IN HUMAN TENOCYTES

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Introduction. Previous research has already been carried out on the damaging effect fluoroquinalones have on tendon cell viability (Zargar N. 2011). It has been shown that platelet-rich concentrate (PRC) protects against this cell death. The use of PRC as an adjunct in tendon repair research is growing in popularity. The aim of this work is to investigate the potential role of PRC in mitigating the effects of fluoroquinolone therapy, notably Ciprofloxacin, by studying extracellular matrix synthesis and maintenance in a tendon cell culture model.

Methods. PRC was extracted from fresh human whole blood via centrifugation, was immediately clotted and left in medium overnight to release all biological factors. Human tenocytes were treated over a 10 day period with Ciprofloxacin with/out 10% PRC. The amount of collagen and glycosaminoglycan's (GAG) in the cell layer and medium was measured using both Sircol™ soluble collagen and dimethylmethylene blue assays respectively.

Results. Preliminary results show that from as early as 24 hours, up to at least 3 days, there is a significant reduction of collagen in the cell layer in the presence of Ciprofloxacin. This reduction is reversed by the addition of 10% PRC. There are no significant changes in GAG content in the cell layer, however the amount of GAG released into the medium is reduced by Ciprofloxacin. Addition of 10% PRC partially restored control levels.

Discussion and Conclusions. This study suggests that Ciprofloxacin either directly or indirectly causes disruption of the ECM, potentially explaining the increased risk of Achilles rupture (Sode J. 2007). Addition of PRC appears to reverse this effect. Detailed mechanisms-of-action are unknown, therefore future investigations will focus on gene expression changes of all individual collagen types, decorin, and versican. This work emphasises the potential for wider use of PRC in protecting against environments damaging for tendon cells and tissue.

Acknowledgements. This work was funded by Joint Action.

Keywords. Tendon; Extracellular Matrix; Ciprofloxacin; Platelet-rich concentrate

(10.P3) HGF-PRODUCING MESENCHYMAL STROMAL CELLS SUPPORT CHRONIC LYMPHOCYTIC LEUKEMIC B CELLS SURVIVAL

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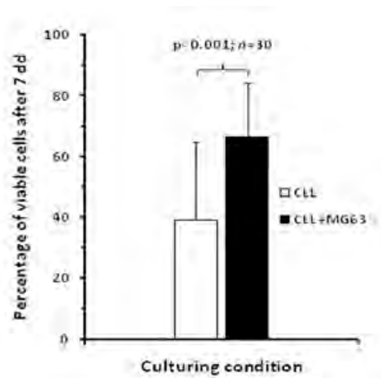
Introduction. The longevity of chronic lymphocytic leukemic B cells (CLL) in vivo contributes to leukemia expansion and relapse. Cell survival capacity is lost in vitro, evidencing the role of cellular interactions and microenvironmental factors in CLL viability. Bone marrow (BM) encompasses several cell types among which bone marrow stromal cells (BMSC), capable to differentiate along several lineages. Little is known on the effects of undifferentiated/differentiated BMSC on CLL, nor which BM-resident cells contribute to CLL progenitors maintenance. We thus used BMSC and other cells of mesenchymal origin to investigate the mechanisms of CLL survival.

Methods. Co-cultures of mesenchymal-derived stromal cells were performed with B cells from thirty leukemic patients; CLL viability was assessed by annexin V/propidium iodide flow cytometry analysis. Transwell cultures and conditioned medium from the same cell types were then tested to ascertain if viability could depend upon released factors. Gene expression profiles of differently supportive mesenchymal cells were used to identify the soluble factors potentially involved in CLL survival; signal-transduction and RNA interference assays were then undertaken to verify this assumption.

Results. Co-cultures or conditioned medium of human BMSC, osteoblasts-like MG63 cells or trabecular-bone derived osteoblasts prolonged survival of CLL cells, while chondrocytes or endothelial cells did not. Gene expression analysis suggested a possible role of hepatocyte growth factor (HGF) in CLL viability. Real-time RT-PCR analysis demonstrated that HGF was produced only by CLL-sustaining mesenchymal cells and that CLL expressed c-MET, the HGF receptor. HGF addition to CLL cultures enhanced CLL viability and induced Tyr705-STAT3 phosphorylation; both were inhibited by siRNA-mediated HGF knockdown, as well as by inhibitors of STAT3 phosphorylation.

Conclusion. At the BM level, HGF contributes to apoptosis resistance of CLL through the activation of c-MET/STAT3 axis. These results can be related to chronic lymphocytic leukemia progression, suggesting new possible therapeutic targets for the disease.

Keywords. chronic lymphocytic leukemia, mesenchymal stem cells, growth factors, survival



(10.P4) RESPONSE OF HUMAN OSTEOBLASTS AND MESENCHYMAL STEM CELLS TO CRYOGELS BASED ON THE SYSTEM 2-(DIMETHYLAMINO) ETHYL METHACRYLATE / (2-HYDROXYETHYL) METHACRYLATE αE/TRICALCIUM PHOSPHATE

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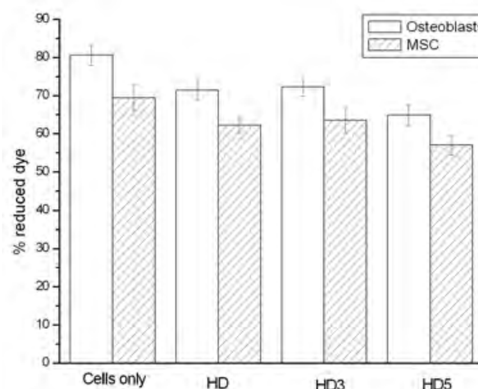
Introduction. Cryopolymerization is a clean processing technique that produces highly hydrophilic and elastic porous materials. The potential of this method in the production of scaffolds for calcified tissue engineering has been recently studied. The aim of this work is to study the response of different cell types to the manufactured cryogels.

Methods. Cryogels were prepared by free radical copolymerization of the monomers (2-hydroxyethyl) methacrylate (HEMA) and 2-(dimethylamino)ethyl methacrylate (DMAEMA), α-tricalcium phosphate, and N,N,N´,N´-tetramethylethylene diamine as activator, at -20ºC. Specimens with different monomer/water ratio (5-40), ceramic content (0-20%) and crosslinker concentration (0-2%) were prepared. Biocompatibility was tested with human osteoblasts and MSCs isolated from bone marrow stroma, then expanded until 90% confluent and cultured on the different cryogels for 7, 14 and 21 days. Cell viability was assessed, through the alamar blue assay. Cell distribution and morphology were determined by histological techniques. Expression of type-I collagen and alkaline phosphatase (ALP) were analyzed by immunohistochemistry.

Results. DMAEMA/HEMA ratios up to 25/75 were studied. Greater porosity (75%) and pore size (1 mm) was obtained for a 75/25 monomer ratio. 5% ceramic loaded specimens produced an increase in the elastic modulus of the specimens, from 1125 to 1161 Pa, for a 75/25 specimen while not affecting significantly the porosity of the specimens. After 96h, both cell types had adhered and proliferated on the materials' surface (Fig.1). Material colonisation could be observed along the 21 days of culture, inferring their biocompatible profile. Expression of type-I collagen could be detected whilst ALP expression appeared to be correlated with α-TCP content.

As summary, results indicate that the materials tested are biocompatible, showing vital cells adhering to the materials, proliferating and giving evidence of early expression of biochemical markers of osteoblastic phenotype.

Acknowledgements. Authors would like to acknowledge CIBER-BBN, MAT2010-18155, CICYT- Spain and fellowship CNPQ, Brazil.
Keywords. MSC; ceramic; cryogels



(10.P5) PHOTODYNAMIC RESPONSE OF PIGMENT CELLS IN ZEBRAFISH LARVAE INCUBATED WITH PHOTOSENSITIVE AGENTS

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Introduction. In vitro experimental systems treated with fluorescent organelle probes and photosensitizers, a characteristic redistribution of fluorescence in cell structure occurs after light irradiation. In vivo experimental system the reversible changes in pigmentation brought on by prolonged exposure to either light or dark environments have reveled that this occurs through relocalization of pigment organelles within cell, changes in cell morphology and apoptosis. These phenomena have promoted investigated on the etiology of it, maybe mediated, among other way, through the pigment cell itself.

Objective: The aim of this work was to examine the photodynamic response of pigment cells in wild-type zebrafish larvae from 3 dpf incubated with photosensitive agents (PSs).

Material and Methods: Acridine orange 10-4 M, methylene blue and toluidine blue 10-5 M were used. Zebrafish larvae, from 3 dpf were partially immobilized and placed on a LED array constructed for irradiation. It is composed of a matrix of 4 light sources with emission peak at 636 nm. The larvae were exposed during 5, 10 and 15 min. Embryo survival, melanophores cell morphology with fluorescence microscopy was analyses subcellular localization of PSs and statistical analysis.

Results: Photodynamic treatment resulted in a light dose-dependent diminution of larvae survival. Pigmented melanophores extend from the level of the hindbrain to about the middle of the yolk ball. The cells showed the typical stellate morphology of melanophores. After irradiation the melanophores changes from initial stellate appearance to punctuate form. Conspicuous changes in the fluorescence pattern were observed.

Discussion: The photodynamic response of pigment cells resulted in dramatic morphological changes probably linked to a redistribution of melanosomes within cells or changes in cell shape.

Keywords. relocalization of photosensitizers

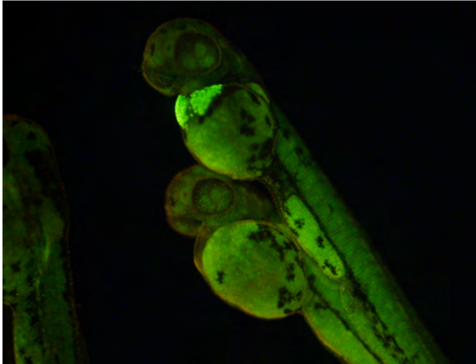

Figure: Cells of the hatching gland present on the pericardium over the anterior yolk sac revelated with acridine orange.

(10.P6) STABILITY OF MESENCHYMAL STEM CELLS FROM HUMAN CHIN BONE MARROW AFTER EXPANSION PROCESS

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Mesenchymal stem cells (MSCs) offer great therapeutic potential in regenerative medicine because of its features: readily available from several sources, high in vitro proliferation, immunomodulating capacity and injury site migration among others. However, due to their low percentage within different sources (0.1 - 0.0001%), it is necessary to subject them to expansion processes that can lead to genomic instability, cellular dysfunction and malignancies development after transplantation. Human chin bone marrow is a potential new source for MSCs isolation, however, expansion for an extended period is required, and is necessary to assess their stability after this process. In this study we analyzed morphological changes and nuclear chromatin by cytogenetic and comet assay of human chin bone MSCs samples expanded for approximately 11 generations. Preliminary results did not show chromosomal instability neither significant clastogenic effect (absence of single strand breaks in DNA) in analyzed samples. Nevertheless, morphological changes, increased population doubling time, decreased ability to form colonies, differential condensation of nuclear chromatin in interphase nuclei and nuclear malformations after expanding by 11 generations were observed in three samples. Although preliminary studies showed that MSCs isolated from chin can be expanded, further studies are necessary to design and to ensure safe cell therapies.

Keywords. Cellular therapies, Cell stability, Chromosomal abnormalities

(10.P7) STATUS OF CELL TRANSPLANTATION IN IRAN

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In recent years, like many other countries, modern cell therapy has been started in Iran. Autologous Schwann cell transplantation for spinal cord injury, Mesenchymal stem cell transplantation for multiple sclerosis, cirrhosis, diabetes and myocardial regeneration, and Hematopoietic fetal stem cell transplantation for diabetes, cirrhosis and multiple sclerosis are prominent examples of current clinical trials. These trials are generally regulated by scientific and ethical committees of medical universities but two of them have been registered in ministry of health (as national project). Lack of national regulation and defined standards is the main safety concern for cell therapy projects and each cell bank has its own quality policy and referred standards (like cGMP, cGTP and GLP). Recently Food and Drug Organization (Ministry of Health) has started working on a plan to regulate and harmonize cell and tissue banking activity in Iran. In the end of 2010 the draft of national standard for cell and tissue banking was announced that can be a basic reference for cell therapy center as a minimal safety standard. In order to achieve the higher level of safety the authors recommend that a more specialized national standard for cell therapy should be publishing by the government. We also believe that implementation of some general quality management system based on the ISO 9001 and ISO13485 can improve safety of cell based products.

Keywords. Cell therapy, Iran, ISO, GMP

(10.P8) CO-CULTURE OF OSTEOBLASTIC AND ENDOTHELIAL CELLS IN THE PRESENCE OF BISPHOSPHONATES

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Introduction. Long-term therapies with Bisphosphonates (BPs) can cause osteonecrosis of the jaws, a condition characterized by tissue dehiscence, chronic bone devitalization, hypocellularity and lytic radiographic features, being usually refractory to therapy. Among the diverse and complex mechanisms of action recently suggested for BPs, the anti-angiogenic properties appears to be a relevant feature of their pharmacological profile. Due to the intimate relationship between angiogenesis and osteogenesis during bone formation events, the aim of this study was to analyze the effect of representative BPs – alendronate and zoledronate, two widely used BPs in therapeutics - in the behavior of a co-culture system of human osteoblastic and endothelial cells.

Methods. MG63 osteoblast-like cells (103 cell/cm2) and human dermal microvascular endothelial cells (104 cell/cm2) were cultured isolated or co-cultured in endothelium medium, in the presence of 10-12 to 10-6 M Alendronate or Zolendronate. Cultures were maintained for 14 days and characterized for cell viability/proliferation (MTT assay), pattern of cell growth (CLSM) and gene expression of osteoblastic and endothelial markers (RT-PCR; Collagen type1, ALP, BMP-2, OPG, M-CSF, CD31, VE-Cadherin and vWF).

Results. In control conditions (absence of BPs), co-cultures of osteoblast and endothelial cells maintained the viability/proliferation and presented a characteristic pattern of cell growth, i.e. the formation of cell clusters of

endothelial cells surrounded by osteoblast cells, and inductium and/or earlier expression of osteoblastic and endothelial markers. The presence of Alendronate or Zolendronate did not affect cell viability/proliferation but caused decreased gene expression of endothelial associated markers in monocultures and co-cultures.

Conclusion. The inhibitory effects of BPs in endothelial cells might play a role in the deleterious effects of BPs in the bone tissue.

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Keywords. Bisphosphonates, osteoblast cells, endothelial cells, co-culture

(10.P9) ISOLECTIN OF PHYTOHEMAGGLUTININ-INDUCED APOPTOTIC PATHWAY IN LUNG CANCER CELLS

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Apoptosis is a physiological mechanism required for maintaining cell numbers and removing unnecessary cells. Deregulation of apoptosis will result in many diseases including cancer. Lung cancer is the leading cause of cancer-related death all over the world. In prior research reports of cancer therapy, phytohemagglutinin (PHA), the lectin extracted from red kidney beans, demonstrated the ability to inhibit the growth of human cancer cells. However, one of its isoforms, erythroagglutinating (PHA-E) has yet to be evaluated on its anti-cancer effects against lung cancer cells A-549.

First, we used MTT assay and G6PD release assay to evaluate cell viability and cytotoxicity on A-549 cells. Next, PHA-E was used to induce apoptosis in order to determine the possible signal transduction pathway, as measured by flow cytometry assays, fluorescent stains and western blot analysis.

The results showed that PHA-E treatment caused a dose-dependent increase of cell growth inhibition and cytotoxicity on A-549 cells. In Annexin V/PI and TUNEL/PI assay, we found that the rate of apoptotic cells was raised as the concentration of PHA-E increased. In addition, cell morphological changes, chromatin condensation and fragmentation, were observed by DAPI/TUNEL stain after treatment with PHA-E. Treatment of A-549 cells with PHA-E resulted in enhancing the release of cytochrome c, which thus activated an increase in protein levels of caspase-9 and caspase-3, up-regulation of Bax and Bad, down-regulation of Bcl-2 and phosphorylated Bad, and finally the inhibition of epidermal growth factor receptor and its downstream signal pathway PI3K/Akt and MEK/ERK.

In conclusion, PHA-E can induce growth inhibition and cytotoxicity of lung cancer cells, which is mediated through activation of the mitochondria apoptosis pathway. These results suggest that PHA-E can be developed into a new therapeutic treatment that can be applied as an effective anti-lung cancer drug in the near future.

Keywords. Apoptosis; Lung cancer; Phytohemagglutinin Erythroagglutinating

(10.P10) CORTICOIDS ALLEVIATE RSV-INDUCED LOSS OF CILIATED CELLS AND ENHANCED MUC5AC IN DIFFERENTIATED HUMAN BRONCHIAL EPITHELIAL CELLS (D-HBE)

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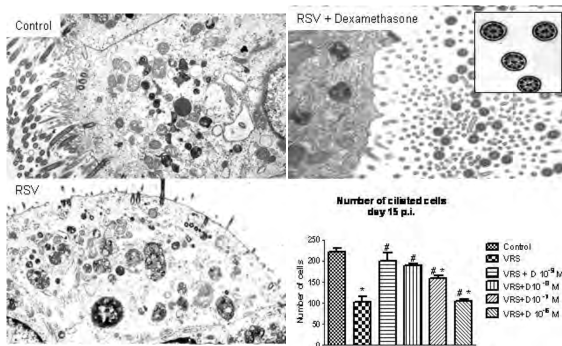
Introduction: Respiratory syncytial virus (RSV) may cause COPD exacerbations. Anti-inflammatory compounds reduce the risk of COPD exacerbations in clinical studies. This study investigated whether in vitro dexamethasone influences the interaction of RSV with ciliated D-HBE. **Methods**: D-HBE from standard air-liquid interface culture were infected with RSV (MOI 0.3 PFU/cell) in the presence of dexamethasone 1 µM or vehicle. After 10 days the following was measured (i) number of ciliated cells based on assessment of cilia activity by high-speed video microscopy, (ii) cilia markers FOXJ1 and DNAI2 (mRNA), (iii) MUCAC (mRNA) and (iv) ultra-structure of cilia by electron microscopy. Results are given as mean ± SEM from 10 (number of ciliated cells), 3 (mRNA analyses) and 1 (TEM) independent experiments.

Results: At day 10 following RSV infection the number of ciliated cells declined from 300 ± 5 / field to 68.2 ± 20.5 / field. This was partially prevented by dexamethasone (194.8 ± 14.7 / field; $p<0.05$ vs RSV). His results are in line with TEM analysis of cilia ultrastructure. In line, RSV reduced FOXJ1 and DNAI2 transcripts to 53.7 ± 0.1 % and 19.2 ± 0.2 % of control, respectively. Dexamethasone 1 µM fully prevented the loss in DNAI2 and partially restored expression of FOXJ1 to 74.5 ± 0.2 % of control ($p<0.05$ vs RSV). In parallel, a 4.87 ± 0.3-fold rise in MUC5AC mRNA secondary to RSV was abolished in the additional presence of dexamethasone.

Conclusion: In differentiated human bronchial epithelial cells RSV caused a loss of ciliated cells and associated markers while MUC5AC expression was increased. Dexamethasone 1 µM reversed these effects.

Acknowledgements: This work is supported by grants of the local government of Valencia (Conselleria de Sanitat), the Spanish Ministry of Science and innovation (SAF2008-03113) and the Health Institute Carlos III (CIBERES, CB06/06/0027).

Keywords. Cilia, airway epithelial cells, ALI, COPD, tobacco smoke, corticoids, inflammation



(10.P11) PLATELET RICH PLASMA PROTECTS TENOCYTES FROM DRUG-INDUCED SENESCENCE AND DEATH

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1. University of Oxford

Introduction. Tendon disorders are frequent and cause month-long disabilities due to poor healing mechanisms. The underlying causes of tendon diseases are not fully understood and effective treatments are limited.

Certain drugs such as dexamethasone and ciprofloxacin interfere with innate healing processes and are thought to predispose tendons to rupture, presenting clinically relevant tools with which to investigate damage mechanisms in tendon. However, both drugs are highly effective in treatment of inflammatory and infectious conditions, therefore new strategies to minimize their adverse effects are of strong interest. Platelet rich plasma (PRP), a rich autologous source of growth factors, has been used to enhance tendon healing. This study investigated the effects of both drugs on parameters of human tenocyte viability, senescence and death. Secondly, the possible use of PRP to mitigate negative effects of both drugs was tested.

Materials and Methods. Centrifuged PRP, from fresh human whole blood, was immediately clotted and left in medium overnight to release biological factors. Human hamstring tenocytes were exposed to ciprofloxacin and dexamethasone with / without PRP. Alamar Blue, β-galactosidase assay and live / dead stain were used to measure respectively viability, senescence and death in tenocytes.

Results. The viability of tenocytes treated with ciprofloxacin decreased dose-dependently, with no induced senescence but increased cell death. Dexamethasone reduced viable cell number without overt cell death but the number of senescent cells increased up to 50%. After co-treatment with 10% PRP viable cell number increased significantly in both conditions and dexamethasone-induced senescence was reduced to 8%.

Conclusion. We demonstrated that ciprofloxacin and dexamethasone have differing adverse effects on human tenocytes, ciprofloxacin inducing cell death while dexamethasone primarily induces senescence. Since it is necessary to continue using dexamethasone and ciprofloxacin therapeutically, our results suggest that co-injection of PRP could block side-effects of these drugs and promote healing in tendons.

Keywords. Platelet Rich Plasma, Dexamethasone, Ciprofloxacin, tenocyte

(10.P12) EX VIVO CYTOTOXIC EFFECTS OF ANTI-GLAUCOMA PROSTAGLANDIN ANALOGUES ON HUMAN CONJUNCTIVAL CELLS

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Background. Glaucoma is a leading cause of blindness in the world. Elevated intraocular pressure is the most important risk factor in its pathogenesis and most of the clinicians choose medical therapy with prostaglandin analogues (PGs) as first option of treatment. However, cytotoxic effects of these drugs on the human conjunctiva are not well known. In this work, we have evaluated the cytotoxic effects of several PGs ophthalmic solutions using an ex vivo cell culture model.

Methods. Primary cell cultures of human conjunctival fibroblasts were stablished from biopsies of healthy patients. The cells were isolated by enzymatic digestion. These cells were maintained using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. We investigated the cytotoxic effects of sequential dilutions bimatoprost, tafluprost, travoprost and latanoprost on human conjunctival cell cultures by using WST-1 method, a non-radioactive colorimetric quantitative assay that measures mitochondrial enzyme activity, which is directly proportional to the number of viable cells. We evaluated each PGs for 5, 30 and 60 minutes at 6 different concentrations.

Results. The WST-1 assay suggested that the four PGs showed a significantly higher level of cytotoxicity in higher concetrations. Tafluprost showed a less toxic profile at the three times of exposition while the latanoprost seems to be most harmful, although this differences decrease when the drug was pure.

Conclusions. The WST-1 is a reliable technique for assessing citotoxity in conjunctival cells. In our study, all drugs showed significant levels of cytotoxicity at higher concentrations, although tafluprost seems to be less toxic at these times. This fact could likely be related to the fact that tafluprost does not have benzalkonium chloride on solution. Further studies are needed to clarify the role of this preservative in cell death.

(10.P13) CELL VIABILITY QUALITY CONTROL OF DENTAL PULP STEM CELLS FOR TISSUE ENGINEERING

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1. Tissue Engineering Group, Dept. Histology, University of Granada, Spain

Introduction. Dental pulp stem cells (DPSCs) have been recently reported as a potential reservoir of cells with high differentiation and transdifferentiation capabilities that allows the construction of new tissues in regards to the therapeutical needings. In this context, the identification of DPSCs´s cell viability profile could be promising for tissue engineering protocols. The aim of

this study was to determine the viability patterns of (DPSC) in order to establish the ideal passage for the use in Tissue Engineering.

Methods. Dental pulp stem cells (DPSCs) were isolated from two extracted human third molars by enzymatic digestion. Primary cell cultures were maintained under standard cell culture conditions and trypsinized along 10 sequential passages. Firstly, for cell viability study trypan blue and LIVE/DEAD® assays were performed at the 10 passages. Secondly, WST-1 cell proliferation assay were also carried out in all study samples. Statistical analysis was done by U Mann-Whitney and Kruskal Wallis test.

Results. DPSCs were viable > 85% during all the study although there were differences on cell viability, detected by each of the assays carried out, between different DPSCs subcultures. From primoculture to fourth passage, viability results were wavering due to an adaptation stage to the *in vitro* conditions. At fifth passage viability increases from 91,27% to 97,53% (p = 0,0003), maintaining an slight upward trend to eighth passage: 96,72% at sixth passage (p = 0,798). At seventh subculture, viability showed an slight decrease to 95,03% (p = 0, 020) that was offset at the next passage, reaching the top-point of viability: 96,97% (p = 0,015). From here, viability seems to keep constant until the end of the study.

Conclusions. After four subcultures cells were adapted to *in vitro* environment. DPSCs at eighth passage showed the highest viability, suggesting the ideal conditions for use in Tissue Engineering.

11. CELL-BASED THERAPIES AT BED-SIDE

Chair: Dimitrios I. Zeugolis
Co-chair: Yury Rochev
Keynote speaker: Masayuki Yamato
Organizer: Dimitrios I. Zeugolis
Synopsis: Injuries and degenerative diseases constitute a bottleneck in medical and surgical practice. As the human population ages and life expectancy increases, injuries and degenerative conditions will continue to rise putting a financial strain on healthcare. It is therefore imperative to develop functional tissue regeneration strategies. Natural or synthetic scaffold-based therapeutic approaches are traditionally used to improve regeneration and functional recovery. However, advancements in molecular and cell biology have allowed the use of cell-based therapies for tissue engineering and regenerative medicine applications. The driven hypothesis of this venerable concept is that replacement, repair and restoration of function can be accomplished best using cells that will create their own host-specific extracellular matrix. Indeed, cells are *professional matrix makers* and assemble into large aggregates together with ligands, growth factors and other matrix components with a precision and stoichiometric efficiency that is still unmatched by man-made devices, recombinant technologies derived components or chemical compounds. Cell-based injectable systems and cell-sheets derived from autologous primary cell isolates; from established cell lines; and from a variety of stem cells have been used for numerous clinical targets, including cornea, skin, blood vessel, cartilage, lung, cardiac patch, oesophagus and periodontal applications. This symposium aims to highlight clinical applications of scaffold-free cell-based therapies and discuss key advancements and current hurdles that still prohibit the widely adaptation of this technology in Tissue Engineering and Regenerative Medicine.

(11.KP) CELL SHEET ENGINEERING FOR REGENERATIVE MEDICINE: ITS CURRENT STATUS OF CLINICAL APPLICATIONS AND SUPPORTING TECHNOLOGIES

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We have developed a novel strategy for regenerative medicine to recover tissue functions by using temperature-responsive cell culture surfaces on which temperature-responsive polymer is covalently grafted by electron beam irradiation or other chemical reactions. These surfaces achieve temperature-responsive cell adhesion and detachment with no need for proteolytic enzyme such as trypsin and dispase. To overcome the limits of conventional tissue engineering methods such as the use of single-cell suspension injection and the use of biodegradable polymer scaffolds, we have applied transplantable cell sheets fabricated with temperature-responsive culture surfaces for cell delivery. Only by reducing temperature around room temperature, all the cells are harvested from the dish as a single contiguous cell sheet. Since these cell sheets retain extracellular matrix deposited during culture below them, integration to tissue or other cell sheets is observed immediately after the transplantation. Here, we show the pipelines and current status of clinical applications of regenerative medicine using cell sheet engineering. Skin and corneal defects have been treated with transplantable cell sheets fabricated on the surfaces. In bilateral cases, patients' own oral mucosal epithelial cells are utilized as the cell source, since both eyes are damaged and no epithelial stem cells are obtained from the patients. Now, we have performed the clinical trial under EMEA (European Medicines Agency) of the corneal regenerative medicine in Europe. We expect that we will obtain the approval in 2011. Severe heart failure was also treated with cell sheets fabricated from patient's own skeletal myoblasts. Esophageal defects after endoscopic tumor dissection have been treated by cell sheet engineering. In these cases, we also utilize patients' own oral mucosal epithelial cells as the cell source. We expect further improvements of stimuli-responsive culture surfaces will realize the reconstruction of more complex tissues to potentially treat a wide range of diseases.

(11.O1) ENDOTHELIAL CELLS POTENTIATE CELL SHEETS OSTEOGENIC ABILITY

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Introduction. Bone Tissue Engineering strategies based on the use of scaffolds and osteogenic cells present drawbacks such as cell necrosis at the bulk of the scaffold related to poor vascularization of the constructs. Cell sheet (CS) engineering has been proposed as a successful scaffold-free alternative for the regeneration of several tissues. The use of this technology is herein proposed for bone regeneration by combining osteogenic CSs and endothelial cells.

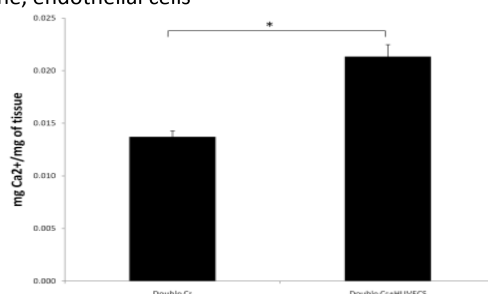
Materials and Methods. Osteogenic CSs were created by differentiating male rat bone marrow cells (rBMSC) in thermo-responsive culture dishes in osteogenic medium. Human umbilical vein endothelial cells (HUVECs) were seeded on the rBMSCs to create co-cultured CSs. The CSs were recovered by lowering the temperature; the osteogenic CSs were stacked on top of either a co-cultured or a similar osteogenic CS and transplanted to female nude mice. Implants were recovered after 7 days and characterized by hematoxilin&eosin (H&E) and alizarin red (AR) stainings, immunohistochemistry for osterix, osteopontin, SRY (to identify transplanted male rat cells) and CD31, and calcium quantification.

Results. H&E and AR stainings showed mineralized tissue formation in the implants both with and without HUVECs. Osterix and SRY immunostaining demonstrated the presence of host and donor osteogenic cells at the mineralization site. HUVECs contribution to neo-vascularization was confirmed by human CD31 identification. Furthermore, calcium quantification results (figure 1) showed a higher degree of mineralized tissue after the transplantation of the constructs with HUVECs.

Conclusions. This work confirmed the potential of transplanted osteogenic cell sheets for bone regeneration as well as the advantage of promoting cross-talk between osteogenic and endothelial cells for improved new tissue formation. The proposed approach avoids the constraints of scaffold use while successfully addressing the important issue of implant vascularization.

Acknowledgements. PhD grant SFRH/BD/44893/2008 to R.P. Pirraco by the Portuguese Foundation for Science and Technology is acknowledged.

Keywords. Cell sheet engineering, tissue engineering, bone, endothelial cells



(11.O2) MODULATION OF THE IN VITRO MICROENVIRONMENT USING MACROMOLECULAR CROWDING

Satyam A (1), Joshi L (2), Raghunath M (3), Pandit A (1), Zeugolis D (1)

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Introduction. In vitro, cells are customarily cultured in highly diluted aqueous conditions which are disgustingly disparate from the macromolecularly crowded microenvironment they have been derived from. As a consequence, cells lose in vitro their phenotype, functionality and therapeutic potential. Recent reports show that macromolecular crowding (MMC) - the addition of macromolecules to culture media, not only enhances the deposition of extracellular matrix, but also preserves cell phenotype. Here, we analysed the influence of various crowding molecules on in vitro deposition of extracellular matrix from human lung and skin fibroblasts under variable serum concentrations.

Methods. Human primary fibroblasts (e.g. lung, skin) were cultured under MMC (e.g. 100µg/ml dextran sulphate; 37.5mg/ml Ficoll™70 and 25mg/ml Ficoll™400; and 100 µl/ml sepharose-CL) and various serum concentrations (0.0 to 10%). The influence of various crowders on cell morphology and metabolic activity was evaluated using phase-contrast microscopy and alamarBlue® assay respectively at day 2, 4 and 6. The deposition of extracellular matrix proteins was analysed by SDS-PAGE and immunocytochemistry for collagen type-I and fibronectin.

Results. Phase-contrast microscopy (Figure-1A) revealed that the fibroblasts maintained their spindle-shaped morphology independent of macromolecular crowder present or the serum concentration up to 6-days in culture. AlamarBlue® analysis demonstrated that cell metabolic activity was not affected independent of the macromolecular crowder present or the serum concentration even up to 6-days in culture ($p>0.05$) (not shown). Densitometric analysis (Figure-1B) of SDS-PAGE demonstrated that MMC significantly increase collagen-I deposition ($p<0.0001$) at all tested serum concentrations. Immunocytochemistry (Figure-1C) further confirmed the enhanced deposition of collagen-I and its co-localisation with fibronectin in presence of macromolecular crowders.

Conclusions. Modulation of the in vitro microenvironment with macromolecular crowding not only maintains cell-viability and morphology, but also enhances extracellular matrix deposition even under low or even zero serum supplementation.

Acknowledgments. Science Foundation Ireland (Grant-09/RFP/ENM2483) and SFI-ETS-Walton award for financial support.

Keywords. Macromolecular Crowding, Collagen Type I Deposition, Human Skin and Lung Fibroblasts, Serum Concentration

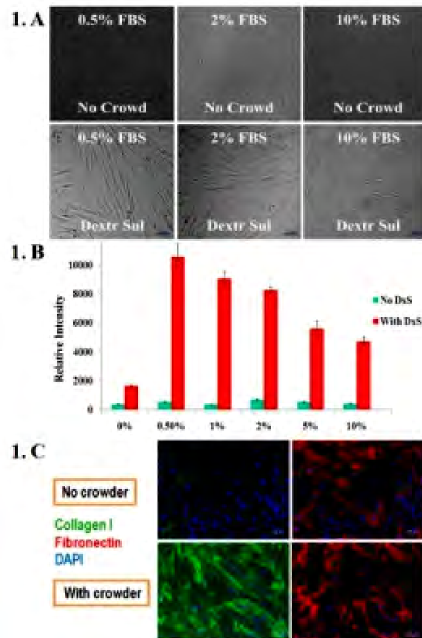

Figure 1A: Phase-contrast microscopy of fibroblasts under crowded (with dextran suphate) and non-crowded conditions at different FBS concentration. **Figure 1B:** Densitometric analysis of SDS-PAGE gels for collagen I deposition **Figure 1C:** Immunocytochemistry for Collagen I (green) and Fibronectin (red). Nuclei were counterstained with DAPI (blue).

(11.O3) CONTROLLED VEGF EXPRESSION ENSURES SAFE ANGIOGENESIS AND FUNCTIONAL IMPROVEMENT IN A MODEL OF MYOCARDIAL INFARCTION

Melly L (1,2), Marsano A (1), Helmirch U (1), Heberer M (1), Eckstein F (2), Carrel T (3), Cook S (1,3), Giraud-Flück MN (1,3), Tevaearai H (1,3), Banfi A (1,3)

1. Cell and Gene Therapy, Basel University Hospital; 2. Cardiac Surgery, Basel University Hospital; 3. Department of Cardiovascular Surgery, Inselspital, Bern University Hospital

Introduction. VEGF can induce normal or aberrant angiogenesis depending exclusively on the amount secreted in the microenvironment. To make this concept clinically applicable, we developed a FACS-based technique to rapidly purify transduced progenitors that homogeneously express a specific VEGF level from a heterogeneous primary population. Here we aim at inducing safe and efficient angiogenesis in the heart by cell-based expression of controlled VEGF levels.

Method and Results. Human adipose-tissue stem cells (ASC) were transduced with retroviral vectors expressing either rat VEGF linked to the FACS-quantifiable surface marker CD8, or CD8 alone (CD8) as control. VEGF-expressing cells were then FACS-purified to generate populations producing either a specific (SPEC) or heterogeneous (ALL) VEGF levels. In a non-ischemic study, 107 cells of each treatment group (CD8, SPEC, ALL) were injected into the myocardium of nude rats. After 4 weeks, vessel density was increased 2-3 fold by both VEGF-producing groups. However, ALL cells caused the development of numerous aberrant angioma-like structures, while SPEC cells induced only normal and stable angiogenesis (Figure 1). To determine the safety and functional efficacy in cardiac ischemia, 70 nude rats underwent myocardial infarction. Two weeks later, animals received at the infarction border either 107 cells of one of the 3 treatment groups or PBS. Four weeks post-treatment, the ejection fraction was significantly worsened by treatment with either ALL VEGF (-13.4%) or control (CD8) cells (-8.8%) as well as the PBS group (-8.0%) compared to SPEC VEGF cells (+1.7%). Initial histology results confirm the induction of aberrant structures in the ALL group, which were completely prevented by SPEC cells similarly to the non-ischemic tissue.

Conclusions. Controlled VEGF delivery by FACS-purified ASC is effective to reliably induce only normal vascular growth in the myocardium and is a promising novel strategy to achieve safe and therapeutic angiogenesis to treat cardiac ischemia.

Keywords. Angiogenesis, FACS, myocardium, VEGF

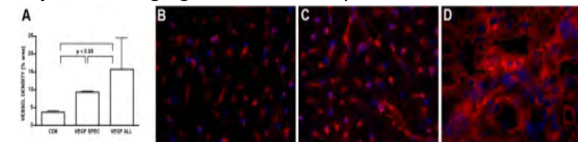

Figure 1. **A** Vessel density quantified as the percentage of CD31-positive structures (as a marker for endothelium) per 20x field (mean±SD). **B-D** Immunoflurorescence staining for CD31 (red) and DAPI (blue, staining nuclei) in the 3 conditions : control (B), VEGF SPEC (C) and VEGF ALL (D).

(11.O4) HUMAN UMBILICAL CORD PERIVASCULAR STEM CELLS (HUCPVCS) AND THEIR CONDITIONED MEDIA INCREASE PROLIFERATION, SURVIVAL AND DIFFERENTIATION IN THE DENTATE GYRUS OF ADULT RAT HIPPOCAMPUS

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Recently, it was shown in vitro that the conditioned media (CM) of human umbilical cord perivascular cells (HUCPVCs) are able to modulate the survival, viability and proliferation of neural precursors, neurons and glial cells. However, in vivo studies, particularly in the brain regions where neurogenesis occurs, were still missing. Therefore, the main aim of this work was to analyse the effect of HUCPVCs and their CM on the proliferation, survival and differentiation of dentate gyrus (DG) resident cells in the adult rat hippocampus. HUCPVCs were isolated from the perivascular region of the human umbilical cord and their CM was collected 24 hours after conditioning. Animals were sacrificed 1, 4 and 12 weeks after injections of HUCPVCs or their CM in the DG for immunohistochemical characterization of the above referred parameters. Results revealed that the effects of HUCPVCs and their CM in the DG resident cells had different trends. Concerning the animals injected with HUCPVCs, we observed an increase on the proliferation (Ki-67 and BrdU positive cells) both at one week and one month after. Moreover in this group it was also possible to observe that a small percentage of HUCPVCs were co-localizing with GFAP, which indicates a possible differentiation of these cells towards astrocytes. On the other hand, in the animals injected with CM, the effect caused in the DG was more evident for astrocytes (GFAP+) and neuronal (MAP-2+) cell densities. Finally, the CM were also able to induce

the differentiation of resident neural precursor towards the neuronal and astrocytic lineages. With this work it was possible to show, for the first time, that the HUCPVCs secretome is able to modulate the in vivo induction of cellular proliferation, survival and differentiation. This observation opens up a good perspective for the application of HUCPVCs and their CM in regenerative medicine approach.

Keywords. Secretome, Umbilical cord, Stem cells, Neural differentiation

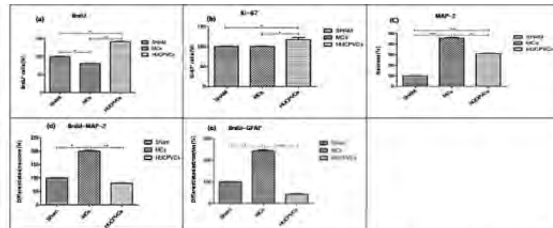

Figure 1. Graphs showing percentage of: (a, b) cell proliferation (BrdU+ / Ki-67+); (c) neurons (MAP-2+); (d) differentiated neurons (BrdU-MAP-2+) at a seven days and (e) differentiated astrocytes at three months in the DG (These results were normalized for Sham group: mean ± sd, * p<0,05, ** p<0,01, *** p<0,001).

(11.P1) AXON FORMATION IN THE EMBRYONIC STEM CELL-DERIVED MOTONEURON

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Developing neural cell must form a highly organized architecture to properly receive and transmit nerve signals. Neural formation from embryonic stem (ES) cells provides a novel system for studying axonogenesis, which are orchestrated by polarity-regulating molecules. Here the ES-derived motoneurons, identified by HB9 promoter-driven green fluorescent protein (GFP) expression, showed characteristics of motoneuron-specific gene expression. In the majority of motoneurons, one of the bilateral neurites developed into an axon that featured with axonal markers, including Tau1, vesicle acetylcholine transporter and synaptophysin. Interestingly, one-third of the motoneurons developed bi-axonal processes but no multiple axonal GFP cell was found. The neuronal polarity-regulating proteins, including the phosphorylated AKT and ERK, were compartmentalized into both of the bilateral axonal tips. Importantly, this aberrant axon morphology was still present after the engraftment of GFP$^+$ neurons into the spinal cord, suggesting that even a mature neural environment fails to provide a proper niche to guide normal axon formation. These findings underscore the necessity for evaluating the morphogenesis and functionality of neurons before the clinical trials using ES or somatic stem cells.

Keywords. Motoneuron, embryonic stem cells

12. CHARACTERIZATION OF TISSUE MECHANICS

Chair: Guillermo Rus
Keynote speaker: Quentin Grimal
Organizer: Guillermo Rus
Synopsis: The rational principles of solid mechanics are an exciting framework to understand, monitor and control functional tissue engineering and quality at all scales form cell to organ. Tissue mechanics understanding can also be extended to diagnose pathologies that manifest by tissue consistency changes, such as pulmonary and coronary arterial walls, tumours or osteoporosis, just to mention a few, but also for therapeutic uses: for instance, as a means to alter the pharmacokinetics and drug permeability through cell membranes, ranging from transdermal drug delivery to gene therapy. Addressing tissue biomechanics requires a concerted, collaborative effort between engineers, physicists and clinicians.

A particularly active research area is currently growing on exploring various physical principles to quantify mechanical properties of tissue. They can be classified as invasive, like indentation, tensile or compression testing, or non-invasive and on-line technologies, like ultrasound, high-frequency ultrasound, vibroacoustography, X-ray or MRI-based elastography, among others. Understanding the complex mechanical laws that govern soft tissues is also a fundamental challenge. They show non-linear, hysteretic, viscoelastic and in some cases also viscoplastic behaviour, in addition to following a heterogeneous and anisotropic pattern.

This symposium covers the multiple disciplines that the characterization of tissue mechanics requires: mathematical models, reconstruction algorithms, inverse problems, sensor engineering, physiology, histology or biochemistry, just to begin the list. The scientific challenge relies on searching expertise and control ranging all the way from the ground research on the challenging physics interaction with tissue to the applied development of tissue engineered materials, and understanding in-depth from the micromechanical scale of the tissue to the organ-level physics.

(12.KP) ULTRASONIC ASSESSMENT OF BONE MECHANICAL PROPERTIES: BOTTOM-UP APPROACH FROM THE TISSUE SCALE TO THE ORGAN SCALE

Grimal Q (1), Laugier P (1)

1. CNRS-UPMC

Introduction. One remarkable property of ultrasonic waves (UW) is the scalability. Meaning, the spatial resolution of measurements is scalable to the wavelength. In particular, UW are advantageous to investigate hierarchically organized materials such as mineralized tissues. Samples can be investigated with frequencies from 0.5MHz to assess overall mechanical properties (e.g. hip strength) to 1GHz to probe intrinsic elastic properties at the tissue level (down to micrometer scale). This will be illustrated with recent studies.

Methods. Ten human femurs were involved in an in vitro study. The cortical shell of the proximal femur was assessed as a whole in through transmission at 0.5MHz and the UW characteristics compared to the failure load. In addition the intrinsic anisotropic elastic properties at the millimeter scale were determined from contact measurements at 2MHz on parallelepiped samples. Impedance images of their surfaces were obtained from 50 MHz acoustic microscopy, from which porosity and mineralized matrix rigidity were derived.

Results. UW propagation time was found to be predictive of femur strength (R2=0.79). This is because UW reflects

both geometry and elasticity of the cortical shell. The latter was found to be essentially determined by the vascular porosity ($R2=[0.73 – 0.84]$). The mineralized matrix properties were almost constant. We also evidenced that the anisotropic elasticity can be successfully modeled with popular continuum mechanics homogenization schemes.

Conclusions. With different ultrasound measurements, we have reached a clear understanding of the relationships between the composition (porosity, mineral) of cortical bone and elastic properties. This will be useful for the assessment of mineralized tissues of unknown composition. The confounding factor of sample shape (e.g. complex shape at the femoral neck) can be overcome by a thorough analysis UW with simulation tools. The developed methods and models are also applied to other mineralized tissues including growing and healing tissues.

Keywords. Mineralized tissues, ulrasound characterization, elasticity, osteoporosis, bone

(12.O1) BIAXIAL MECHANICAL PROPERTIES OF THE AORTIC VALVE: EFFECT OF THE HYALURONIC ACID

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1. Imperial College London

Hyaluronic acid (HA) is an important component of the glycosaminoglycans (GAGs) that are present in valve leaflets. At present, little is known about the contribution of the individual GAGs to the mechanical function of the valve. To understand this, HA was selectively removed from porcine aortic valves (AV) and the mechanical properties of the valve cusps assessed.

Fresh right coronary (RC), left coronary (LC) and non-coronary (NC) AV cusps were dissected from adult pig hearts sourced from a local abattoir. Each leaflet was radially cut in two halves. In the RC group one half was treated enzymatically to remove hyaluronic acid (HA) while other half was left untreated as a control. In the LC and NC groups both halves were treated using the control buffer. Each specimen was cut into 5mm strips and mounted on a BOSE electromechanical tensile testing machine. A peak level of load equal to 1.1 N/cm was applied. Each strip was first preconditioned to this level with a frequency of 0.1Hz for 20 cycles. Stiffness and percent relaxation were analysed. Alcian Blue/Sirius Red staining was used to evaluate how efficient the enzyme treatment was. Tinctorial staining showed that most of the sulphated GAGs were removed from the RC cusp after 24 hours' enzymatic incubation. Removal of HA increaseed the percentage decay of force during relaxation test (17 % vs 22% control vs HA respectively), however this effect was not statistically significant ($P=0.07$). The stiffness of the valves was not affected by removal of HA (0.19 vs 0.17 N/mm control vs HA respectively, $p=0.57$). The results from the LC and NC groups showed no difference in mechanical behaviour between the two sides of each cusp (stiffness and % decay were 0.22 N/mm and 25% respectively) showing there was no difference in control group between the 2 halves of the cusp.

These data suggest that HA does not contribute to the reported effects of GAGs on the mechanical properties of the AV. The identities of the GAGs that affect the mechanical stiffness of the valve require further investigations.

Keywords. Hyaluronic acid, stress relaxation

(12.O2) ULTRASONIC MONITORING AND PARAMETERS IDENTIFICATION OF SIMULATED TISSUE CULTURE

Rus G (1), Bochud N (1), Rodríguez JM (1), Alaminos M (1), Campos A (1)

1. Universidad de Granada

Introduction. A monitoring Petri dish is tested for real-time measurement of mechanical properties of thin layers of tissue culture. To verify the sensitivity, a transformation process is monitored during approximately an hour, and validated numerically.

Methods. A layer of phantom gel of about 100 [µm] thickness is cultured on a Petri dish. The gel suffers consistency changes during a period in the order of magnitude of one hour. Simultaneously, an evaporation process is also expected. For this task, the device was excited by high-frequency ultrasonic burst waves at a central frequency of 20 MHz, a duration of one cycle and an amplitude that amounts to 5 Vpp. The signal was registered during a period of 5 [µs] and a sampling rate of 400 [MHz]. The forward problem simulation of the experimental system is proposed using a semi-analytical model of the ultrasonic wave interactions within the Petri dish and gel based on the transfer matrix formalism. This modelling includes dispersion effects associated with relaxation processes that occur during the propagating of the ultrasonic wave. An inverse problem (IP) is proposed for determining the sensitivity of the mechanical properties of the gel regarding the time evolution of the transformation process.

Results. This propagation model, combined with the inversion algorithm, allow to determine the time evolution of the mechanical properties of the gel, such as the stiffness and the attenuation coefficient, and thus to interpret the transformation procedure.

Conclusions. The feasibility of the proposed reconstruction procedure using genetic algorithm to quantify consistency changes from a single measurement is evaluated. This framework open a number of questions to be answered in ongoing works, such as the extension of the forward modelling to nonlinear constitutive laws.

Acknowledgements. The authors want to thank the following institutions: SAS, Junta de Andalucía (PI-0308), and MICINN (DPI2010-17065), for funding.

Keywords. Inverse problem, non-destructive evaluation, ultrasonics, tissular mechanics

(12.O3) LOW-INTENSITY ULTRASOUND FOR STIMULATION OF TISSUE CULTURE

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1. Universidad de Granada

Introduction. The propagation of mechanical waves and interaction generated with tissular microstructure has not been addressed enough to characterize both physical principle of diagnosis and treatment. Recent results evidence these aspect: Ultrasound (US) technology has been used in biotechnology for improving of cell viability via its ability to increase mass transport, and also in the

context of cartilage and bone regeneration or tissue engineering, where it increased cellular activity. In accordance with them, a stimulation ultrasound wave device is proposed. The ultrasonic wave energy, frequency and shape is estimated to be compatible to those used in previous references by analyzing the signal from a receiver.

Experimental methodology. Layout of design and methodology has been developed based on simulation-optimization of a high energy and low energy transductor, using Finite Elements Methodology (FEM). A robust algorithm to reconstruct mechanical parameters from measured signals was applied. Equipment allows to generate a variable frequency-energy-shape excitation ultrasonic signal. The transmitted signal was generated as a 10-cycle burst composed by a 50 500 [kHz] sine of variable amplitude with a repetition rate of 10 [ms]. This signal interacts with the culture and the interaction is captured by the ultrasonic receiver. The received signal is amplified, digitized with a high resolution A/D converter, and digitally processed off-line in a computer, using MATLAB.

Results. If a transmitted signal of frequency f = 50 [kHz] at amplitude Dt = 10 [V] yields a registered signal of amplitude Dr = 0.5 [mV], the stress at the tissue to be stimulated is estimated to be of the order of 368 [Pa]. Similar magnitudes were observed in the range between f = 20 − 500 [kHz], with a monotonically increasing trend.

Conclusions. Scientific and strategic strength lie on ultrasounds interaction with tissue, and applied engineering in physical devices, to face up a deep understanding at a micromechanical scale of tissue and physical organ level. Establishing a link between regenerative medicine and a possible contribution during clinical surgery.

Acknowledgements. The authors want to thank the following institutions: SAS, Junta de Andalucía (PI-0308), and MICINN (DPI2010-17065), for funding.

Keywords. Inverse problem, low-intensity ultrasounds, non-destructive testing, tissular mechanics

(12.O4) EXPERIMENTAL CHARACTERIZATION AND CONSTITUTIVE MODELING OF THE MECHANICAL BEHAVIOR OF THE HUMAN TRACHEA

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Introduction. Cartilage and smooth muscle constitute the main structural components of the human trachea; their mechanical properties affect the flow in the trachea and contribute to the biological function of the respiratory system. The aim of this work is to find out the mechanical passive response of the principal constituents of the human trachea under static tensile conditions and to propose constitutive models to describe their behavior.

Methods. Histological analyses to characterize the tissues and mechanical tests have been made on three human trachea specimens obtained from autopsies. Uniaxial tensile tests on cartilaginous rings and smooth muscle were performed. Cartilage was considered an elastic material and its Young Modulus and Poisson Coefficient were determined fitting the experimental curves using a Neo-Hookean model. The smooth muscle was proved to behave as a reinforced hyperelastic material with two families of fibers, and its nonlinearity was investigated using the Holzapfel strain-energy density function for two families of fibers to fit the experimental data obtained from longitudinal and transversal cuts. FE-simulations were made using the experimental results to check the influence of a tracheal implant on swallowing.

Results. For cartilage, fitting the experimental curves to an elastic model, a Young modulus of 3.33 MPa and Nu= 0.49 were obtained. For smooth muscle, several parameters of the Holzapfel function were found out C10=0.877 KPa, k1=0.154 KPa, k2=34.157, k3=0.347KPa and k4=13.889 demonstrated that the tracheal muscle was stiffer in the longitudinal direction. The FEM results permitted to estimate the consequences of a Dumon stent implantation in the stress state of the trachea during swallowing.

Conclusions. The better understanding of how these tissues mechanically behave is essential for a correct modeling of the human trachea, a better simulation of its response under different loading conditions, and the development of strategies for the design of new endotracheal prostheses.

Keywords. Tracheal cartilage, smooth muscle, tensile tests

(12.O5) MYOFIBROBLAST AND CARDIOMYOCYTE INTERACTIONS STUDIED IN A MODEL SYSTEM

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Interactions between myofibroblasts and cardiomyocytes are important to understanding the long-term consequences of cardiac fibrosis and myocardial infarction, but are difficult to quantify in natural tissue. We therefore study these in an idealized model system known as engineered heart tissues (EHTs), assembled from embryonic cardiomyocytes and containing defined fractions of myofibroblasts randomly distributed throughout the tissue.

EHTs are assembled by suspending ~106 cells obtained from 10-12 day chicken embryos in 1 ml of ~1mg/ml type I rat tail collagen. Over several days of incubation the primary fibroblasts convert to myofibroblasts that compress and stiffen the collagen. Within 4-7 days the cardiomyocytes, which begin contracting independently, establish gap junctions and begin to beat coherently. Then the EHT twitch force is readily measurable with an isometric force transducer, and the spread of electrical excitation can be measured using optical mapping techniques. The fraction of cardiomyocytes can be varied from ~5% to ~ 95%.

Central questions are how myofibroblasts and cardiomocytes are coupled electrically in EHTs, and how overgrowth of tissues by proliferative myofibroblasts affects mechanical function. We present here progress towards answering these questions.

Keywords. Cardiac fibrosis, myofibroblasts, engineered heart tissue, model systems

(12.P1) STRUCTURAL AND FUNCTIONAL CHANGES IN RABBIT CAROTID ARTERIES AFTER EXERCISE TRAINING

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Introduction. The response of the endothelium to training exercise depends on a number of factors that include the training program duration, and the size and anatomical location of the artery.

Objectives. To evaluate whether physical training produces histological and functional changes in rabbit carotid artery.

Methods. Eleven rabbits were exercised for 6 weeks following a protocol on treadmill and another twelve rabbits were stabulated during the same period. After exercise program, the rabbits were anaesthetized and killed, and the carotid arteries were dissected, fixed and included in paraffin blocks with horizontal and transversal orientation. Hematoxylin and eosin microphotographs were digitized and analyzed using Photoshop and Image Proplus software. The number of the muscular layers and the thickness of the vascular structures were measured. To study the vascular function, arterial segments (3 mm long) were mounted for isometric recording of tension in organ baths containing Krebs-Henseleit solution

Results. The number of vascular smooth muscle cell layers were similar in control and trained animals (9 to 10) but a thinning of the media layer was observed in trained animals (77±8μ vs 65±10μ, p<0.001). Potassium chloride (5-120 mM) induced a concentration-dependent contraction that was lower in arteries from trained rabbits (EC50 values: 27±2 mM for control group vs 42±4mM for training group, n=10; p<0.001). Sodium nitroprusside, an endothelium-independent relaxant (10-9 to 10-6M) produced concentration-dependent relaxation that was higher in arteries from trained rabbits (EC50 values: 2.7x10-8M for control group vs 1.3x10-8M for training group, n=10; p<0.05) while acetylcholine, an endothelium-dependent relaxant, (10-9 to 3x10-6M) produced concentration-dependent relaxation that was lower in arteries from trained rabbits (EC50 values: 3.7x10-8M for control group vs 7.1x 10-8M for training group, n=10; p<0.05).

Conclusion. Exercise training decreases smooth muscle thickness, increases basal production of NO in the smooth muscle cells and decreases NO release from the endothelium.

Keywords. Rabbit carotid artery, exercise training, histological changes, smooth muscle

(12.P2) SPECIFICITY OF PIEZOELECTRIC TISSUE STIFFNESS SENSOR: MODELING

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Introduction. Reliable quantification of the stiffness modulus of soft tissue is an open issue with relevance for the diagnostic of pathologies that appear as drastic changes in the consistency of the tissue, such as tumors. The reconstruction of such parameters from non destructive testing based on ultrasonic transmission of pulses and model-based solution of the identification inverse problem is proposed as a novel technique with high potential for the direct relationship and sensitivity of the propagation of those mechanical waves to the mechanical stiffness of the tissue, which defines the ultimate criterion for diagnosis.

Methods. A model-based Inverse Problem is applied to reconstruct the values of the linear stiffness constants that best fit the experimental measurements. Two inputs need to be introduced: the parametrization, responsible for which parameters of the model control the characterization of the sought model and the experimental measurements. The latter ones were obtained by performing a finite-element simulation. The experimental measurements are simulated by a finite-element model that includes the whole implied boundary and transducer layers effects. A gaussian noise with zero mean and standard deviation is added to the simulated measurements, considering several signal-to-noise ratios. Thus, the complete wave interactions within the specimen are described.

Results. The model-based inverse problem that governs the theory of elasticity has demonstrated feasibility to to reconstruct the stiffness modulus odd soft tissue.

Conclusions. This work allows (i) to validate to which extent a one-dimensional linear-elastic model of wave propagation is consistent to identify the full complexity of a simulated experiment based on the multidimensional modeling of wave propagation within soft tissue; and (ii) to extract practical parameters for final tissue quality assessment.

Acknowledgements. The authors want to thank the following institutions: SAS, Junta de Andalucía (PI-0308), MICINN (DPI2010-17065) and AECID (A/027182/09), for funding.

Keywords. Inverse problem, ultrasonics, non-destructive testing, tissular mechanics

(12.P3) AN STOCHASTIC-INVERSE APPROACH TO MODEL THE EVOLUTION OF THE MECHANICAL PROPERTIES OF A TISSUE CULTURE

Chiachio J (1), Chiachio M (1), Rus G (1)

1. UGR

A stochastic framework is proposed to model the evolution of the mechanical properties of a simulated tissue culture by means of discrete-time non-stationary Markov chains. Even under controlled laboratory conditions a Markov-type evolution of the tissue mechanical properties is expected, under the hypothesis that the future of the process depends only upon its present state, and not upon its past states. A unitary time-transformation concept by means of monotonic cubic Hermite splines is introduced to take into account the nostationarity of the process. An inverse-problem based procedure is proposed to find the optimal stochastic model parameters together with the time transformation parameters by minimizing a cost function that quantifies the mismatch between experimental and numerically predicted distribution functions. The validity of the proposed methodology is discussed in relation to real time experimental data. A monitoring Petri dish with

a 20 [MHz] ultrasonic transmitter and receiver is specifically designed and stochastic mechanical data of a culture process is taken. This approach has been tested successfully in materials with a complex stochastic evolution such as composites materials. As further work, ultrasonic transmission signals are examined to be used as raw experimental data within a Bayesian Inverse Problem framework. In this way the accuracy of the method is expected to improve due to the use of the redundant data contained within the signals.

Keywords. Tissue culture, Markov chains, inverse problem, nonstationarity, ultrasounds

13. COMMERCIALIZING CELL THERAPIES. TRAGEDY, TUMULT AND TRIUMPH

Chair: Brian Newsom
Keynote speakers: Eduardo Bravo, Gil Beyen
Organizer: Brian Newsom
Synopsis: Taking a cell therapy from development to commercialization has proven to be a rocky road. Along this road we see many that have fallen to the wayside and there are many more that will no doubt end up with that fate. There are some, however, that have overcome the hurdles (the regulation, the funding, the clinical proof) to bring their therapies to late stage trials and commercialization. We will hear of the trials and tribulations of those that have paved the way to success in the area of Tissue Engineering and from those on the brink of success.

Bringing a cell therapy to the market takes a lot more that interesting or even useful science. It takes a lot of dedicated people, exceptional funding (and therefore fundraising skills), good clinical practice, attention to detail in manufacturing, quality and logistics & and strong case for being able to generate revenues at the end of a 10+ year development cycle. To date only 4 companies have managed to finish this trek, and only one under the new European ATMPs. We will hear from this company as well as another company that may join them as the second member of this elite group to find out how they achieved this feat and what wisdom they can pass along to those currently developing cellular therapies.

(13.KP1) CELLERIX: EXPERIENCES AND LESSONS IN CELL THERAPY

Bravo E (1)
1. Cellerix

Stem cell therapy is regarded as one of the most promising biopharmaceutical approaches currently in development. However, the unique challenges of bringing a “living medicine” to the market has required the industry to address a diverse range of aspects in multiple areas, including but not limited to:
- Advanced therapies’ regulation being put in place as R&D advances
- Past IP makes it difficult to ensure efficacious protection of new developments
- Challenges to be overcome in production, logistics and later on commercialization
- Difficult financing environment (association of cell therapy with gene therapy, lack of success cases, etc)

Cellerix is focused on the development of expanded adult stem cells from adipose tissue (eASCs) for the treatment of immune mediated inflammatory indications. The Company began development in a niche indication with an autologous product to take advantage of the clearer regulation and faster route to market. Almost seven years after inception, Cellerix is now starting a phase I/II trial in the blockbuster rheumatoid arthritis indication with IV infusion of allogeneic stem cells.

This fast advance has been possible thanks to various strategic decisions taken by the Company that include:
- The early adoption of the clinical trial route vs. taking advantage of unclear regulation that exempted certain stem cell treatments from clinical trials
- The close communication with regulatory agencies on development plans and protocols
- The investment in the development of a platform rather that a specific product

Cellerix’ platform builds upon a well characterized stem cell population with a common preclinical, CMC and manufacturing package. This strategy has been instrumental in growing the Company’s pipeline and creating value, as it has allowed Cellerix to capitalize on past work performed by building upon first generation products irrespective of their clinical trial results for the development of second generation treatments.

Keywords. Adipose Derived Stem Cells, AMTP, Inflammatory indication

(13.KP2) CHONDROCELECT: FIRST COMMERCIAL EXPERIENCE WITH AN ATMP

Beyen G (1)
1. Tigenix

ChondroCelect is the first Advanced Therapy Medicinal Product (ATMP) centrally approved under the new European ATMP regulation. ChondroCelect is an autologous cell therapy product consisting of in vitro expanded autologous chondrocytes, and is indicated for the repair of damaged cartilage of the knee. This medicinal product is currently being reviewed in several European key countries by the national Health Agencies responsible for pricing and reimbursement. As ChondroCelect is the first ATMP undergoing this evaluation, a wealth of experience in this matter is being gained. Selected case studies in different European countries will be presented.

Keywords. Tissue Engineering, cell therapy, ATMP, cartilage, ChondroCelect

(13.O1) ORGANOGENESIS INC.: THE ROAD TO COMMERCIALISATION

MacKay G (1)
1. Organogenesis Inc.

Organogenesis Inc. was an early pioneer in regenerative medicine. The company incorporated in 1985 to develop cell therapies originally developed at MIT. A key milestone was achieved in 1998 when Organogenesis received the first FDA approval for a living, allogeneic, human cell-based product. Apligraf® has now treated hundreds of thousands of patients and is now a standard-of-care option for chronic wounds in the USA. In fact, an

Apligraf is applied to a patient Monday to Friday every hundred seconds.

Organogenesis Inc. had difficulty transitioning from a research based company to one with solid commercial skills. There were little or no models to follow and several initial approaches failed. Through this learning, a company has emerged with the unique skill sets to take living technology from applied research, through scale-up, to full commercialisation to medical clinics in multiple countries.

The goal of this presentation is to highlight some of the choices, approaches and eventual successes addressed during the path to business success. These involve tough R&D decisions, process approaches, automation investments, regulatory and sales and marketing build up. Having built up a material level of revenue, profit, infrastructure and a skilled team, the presentation will finish with a view of what's next.

Keywords. Regenerative medicine, Apligraf

(13.O2) CHONDROGENIC BUT NOT OSTEOGENIC DIFFERENTIATION OF BONE MARROW DERIVED STRO-3+ MESENCHYMAL PROGENITOR CELLS IN THE OVINE CERVICAL SPINE

Ghosh P (1), Goldschlager T (2), Zannettino A (3), Gronthos S (3), Itescu S (1), Jenkin G (4)

1. Mesoblast Ltd.; 2. Monash medical Centre; 3. Hansen Institute; 4. Richie Centre, MIMR

Introduction. The objective of this animal study was to show that adult allogeneic Stro- 3+Mesenchymal Precursor Cells (MPC) formulated with Pentosan Polysulfate (PPS) and embedded in biodegradable collagen scaffolds would produce hyaline cartilage (HC) but not bone.

Methods and Materials. Eighteen ewes were subjected to C3/4 and C4/5 anterior cervical discectomy, followed by the implantation of interbody cages packed with collagen sponges with and without MPC. Group A (N = 6) contained sponge alone; Group B (N = 6) sponge +1 million MPCs; Group C (N = 6) sponge +1 million MPCs + 10ug PPS. Radiographs of the cervical spine were taken 1, 2 and 3 months postoperatively. All animals were sacrificed at 3 months, spines removed and scanned by CT. For histological studies the C3/4 and C4/5 motion segments encompassing the cages were isolated. After decalcification and paraffin embedding, sagital sections were cut through the cages and stained with H&E and Alcian Blue. Using the ICRS scoring system the histological sections were examined by a blinded observer to assess HC and bone deposition.

Results. CT analysis demonstrated the presence of new bone within 75% of the cages of Group A and 92% of Group B. In equivalent regions of Group C cages containing MPC+PPS, only 8% of the levels showed evidence of new bone formation ($p = 0.0009$ versus Group A and $p = 0.0001$ versus Group B). Histological scoring confirmed that there was significantly more HC and less bone deposited within the cages of the PPS+MPC (Group C) compared with both Group A ($p = 0.003$) and the Group B ($p = 0.017$).

Conclusions. This is the first in-vivo study to demonstrate the feasibility of using formulations of MPC + PPS to produce hyaline cartilage within a biological environment normally conducive to the production of new bone (spinal fusion).

Keywords. Mesenchymal stem cells, chondrogenic differentiation, new discs, pentosan polysulfatedisc, extracellular matrix, repair

(13.O3) SUCCESSFUL REGULATORY STRATEGIES FOR COMMERCIALISING ADVANCED THERAPIES

Zwart I (1), Blakie R (1)

1. ERA Consulting

The European legislation concerning Advanced Therapy Medicinal Products (ATMPs) has recently been changed in an attempt to harmonise the regulatory requirements for the development of ATMPs within the EU and improve patient access to such products. Despite this attempt to simplify the regulatory environment, only one advanced therapy has been granted a marketing authorisation in the EU to date. This lack of approval of cell-based medicinal products is due to the failure of many companies to negotiate the maze of EU legislation and overcome the regulatory hurdles that still stand in the way of the commercialisation of advanced therapies. In addition, a lack of regulatory foresight for products previously classified as transplants means that many products that were under development prior to the enforcement of the ATMP Regulation do not meet the standards now required of an advanced therapy.

This session will therefore outline the current EU regulatory framework for advanced therapy medicinal products and assess the recent regulatory experience with cell-based medicinal products in light of the ATMP Regulation. The key to the success of a product is the development of a regulatory strategy early on, alongside interaction with the regulatory authorities in the EU during product development. Fortunately, many common pitfalls that have led to the delay or failure to obtain marketing authorisation for a product can frequently be overcome by increasing awareness of the current regulatory climate and maximising the use of the regulatory incentives available for advanced therapies.

Keywords. EU regulation, Marketing authorisation, Commercialisation

(13.O4) LEGAL CHALLENGES FOR ATMP DEVELOPMENT

Stevens H (1), Verbeken G (1), Verlinden M (1), Huys I (1)

1. K.U.Leuven

Introduction. Innovative breakthroughs in medicinal products for advanced cell or gene based therapies (ATMPs) offer hope for unmet or unsatisfied medical needs. Certain cell based products are already successfully applied in therapeutic context while gene based clinical trials offer potential for a long-term treatment of certain monogenetic diseases. However, the number of legislative rules and guidelines increases, as well as the cross-border challenges in multidisciplinary studies. The complexity, plasticity and fragility of cell and gene based products impede the legislator to present an exact definition for these products. It is the exact definition that is needed in order (1) to satisfy the criteria of quality, safety and efficacy in the Medicinal Product legislation, as well as (2) to safeguard adequate legal protection through the patent system.

Methods. Several patents on gene and stem cell inventions were analyzed using in-house developed patent landscaping methods in order to perform claim analysis and typology. The findings were put into the light of the recent legal evolutions in the EU and US with respect to cell and gene patenting, substantiated by influencing case law and doctrine.
Results. Some possible legal mechanisms are proposed as a solution for the legal uncertainties within the domain of cell and gene therapies.
Conclusion. The suggestions may offer new insights for ATMP development.
Keywords. ATMP - patents - regulation

14. COMPUTATIONAL MODELING IN TISSUE ENGINEERING

Chair: José Manuel García-Aznar
Co-chairs: Hans van Oosterwyck, Georg N. Duda
Keynote speaker: Georg N. Duda
Organizers: José Manuel García-Aznar, Hans van Oosterwyck
Synopsis: Computational modelling is a useful tool for research in tissue engineering that, in combination with experiments, can increase our quantitative understanding of understanding of underlying mechanisms, as well as for the development of new technologies. Models allow analyzing the influence of multiple factors that are relevant in tissue engineering: coupling of many different biochemical and biophysical factors at different temporal and spatial scales (from the whole organ to the cellular level). The development of this kind of models is also a challenge from a computational point of view, involving multiphysics and multiscale analysis in evolving tissues and tissue constructs.
Specific topics in this symposium could be:
• Computational modelling of cell and tissue dynamics, relevant for tissue engineering
• Computational models to quantify mass transport in tissue engineering constructs
• Use of computational techniques to optimise scaffold design
The symposium wants to demonstrate that, by combining computational analysis with (in vitro or in vivo) experiments, new possibilities are being created both in terms of fundamental understanding as well as applications.

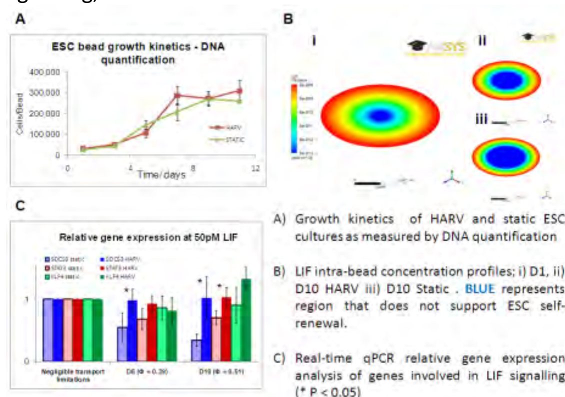
(14.KP) MECHANO-BIOLOGY OF ENDOCHONDRAL OSSIFICATION – EMPLOYING COMPUTATIONAL MODELING TO GAIN UNDERSTANDING OF THE UNDERLYING MECHANO-REGULATION OF TISSUE REGENERATION
Duda GN (1)
1. Charité - Universitätsmedizin Berlin, Julius Wolff Institut and Center for Musculoskeletal Surgery, Germany
Using the example of bone healing, the power of computational approaches to unravel mechano-biological regulation principles will be demonstrated. Limitations of such approaches and opportunities shall be presented and discussed using comparisons of computer simulation with histology and material characterization over a period of regeneration. Bone healing provides an ideal model to investigate the influence of mechanics on the biological processes during musculoskeletal tissue regeneration. Previously, decreased fixation stability was found to prolong the chondral phase of healing suggesting endochondral ossification in particular to be mechano-sensitive. The aim of our analyses was to investigate potential mechanisms regulating ossification processes during bone healing. The finite element method was used to estimate the local stresses and strains in the callus initially and at 2 and 3 weeks post-osteotomy. The local stresses and strains were then correlated with the corresponding histological patterns of tissue formation. Initially, strains and pressures in regions of initial bone formation were determined to be low, regardless of the fixation stability. At 3 weeks however, high tensile strains were estimated on the surface of the hard callus and coincided with regions of cartilage formation, implying a potential role for these strains in regulating the chondral phase of bone healing. Possible explanations for the influence of fixation stability on the processes of ossification during bone healing are provided.

(14.O1) AN EXPERIMENTALLY VALIDATED CYTOKINE TRANSPORT/BINDING KINETICS MODEL FOR MODEL-BASED ESC BIOPROCESS DESIGN
Yeo D (1), Torii R (1), Kiparissides A (1), Xu XY (1), Mantalaris A (1)
1. Imperial College London
Embryonic stem cells (ESCs) are suitable for tissue engineering applications due to their unlimited expansion and differentiation potential. A bottleneck towards implementation in clinical settings is their efficient direction towards the intended cell type. Previously, we established that sub-optimal nutrient/metabolite culture conditions result in spontaneous differentiation of ESCs. Herein, we develop a cytokine transport/binding kinetics model and address the concentration gradients within our 3D ESCs culture systems.
Leukemic inhibitory factor (LIF ~20kDa), is indispensable for the self-renewal of undifferentiated murine ESC (mESC). It binds with its receptor at a rate (KD) of 1 pM but does not pluripotency below 0.5 pM concentration. MESCs were encapsulated (2.5×106 cells/ml) within alginate-gelatin hydrogels (beads) and cultured in either static or rotating wall vessel (HARV bioreactor) fed-batch culture systems. Following a 10 day culture, both systems reached similar cell densities of 20-fold expansion. We estimate the Thiele modulus (Φ) to increase from 0.11 to 0.51, reducing ligand binding activity by 13%. CFX simulations of LIF concentration show a concurrent reduction of bead volume able to support pluripotency (<0.3 LIFR occupancy). Our model also demonstrates that improved LIF transfer in HARV bioreactors lead to a 2.5x volume reduction in comparison to static. LIF activates JAK-STAT3 signalling, which integrates with mESC pluripotency networks via KLF4 triggering its inhibitor SOCS3. Relative gene expression analysis shows SOCS3, STAT3 to be significantly lowered on day 10 in static compared to HARV cultures corroborating our predictions.

We present a model to elucidate growth factor interaction within our 3D systems. Our model adapts to fit other ESC-relevant soluble factors such as FGF4, NODAL and BMP4 improving model fidelity. Finally, we demonstrate that the sub-optimal delivery of growth factors leads to reduced cardiomyogenesis owing to premature ESC differentiation.

Acknowledgements. The authors acknowledge support from the Department of Trade and industry (UK).

Keywords. Cytokine transport/binding-kinetics model, 3D, embryonic stem cell, bioprocessing, LIF-Jak-Stat3 signalling, thiele modulus



A) Growth kinetics of HARV and static ESC cultures as measured by DNA quantification

B) LIF intra-bead concentration profiles; i) D1, ii) D10 HARV iii) D10 Static . BLUE represents region that does not support ESC self-renewal.

C) Real-time qPCR relative gene expression analysis of genes involved in LIF signalling (* P < 0.05)

(14.O2) MATHEMATICAL MODELING OF CANCER SPHEROIDS IN BIOENGINEERED 3D MICROENVIRONMENTS AND TREATMENT WITH AN ANTI-CANCER DRUG

Loessner D (1), Rizzi S (2), Byrne H (3), Flegg J (4), McElwain S (4), Clements JA (1), Hutmacher DW (2)

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Introduction. A critical step in the dissemination of ovarian cancer cells is the formation of multicellular spheroids from cells shed from the primary tumor. These cells then spread further into the peritoneum, attaching to the mesothelial cell layer and invading into the underlying extracellular matrix to grow secondary tumors which is clearly the critical step leading to poor outcome. The objectives of this study were to establish bioengineered three-dimensional (3D) microenvironments for culturing ovarian cancer cells biomimetically in vitro and simultaneously to develop computational models describing the growth of multicellular spheroids in these bioengineered matrices.

Methods. Cancer cells derived from human epithelial ovarian carcinoma were embedded within biomimetic hydrogels of varying stiffness and cultured for up to 4 weeks. Immunohistochemistry was used to quantify the dependence of cell proliferation and apoptosis on matrix stiffness, long-term culture and treatment with the anti-cancer drug paclitaxel.

Results. Two computational models were developed. In the first model, each spheroid was modeled as an incompressible porous medium, whereas in the second model the concept of morphoelasticity was introduced to incorporate details of the bioengineered tumor microenvironment stresses and strains. Each model was formulated as a free boundary problem. Functional forms for cell proliferation and apoptosis motivated by the experimental work were applied and predictions of both models compared with the experimental data sets.

Conclusions. This work aimed to establish whether it is possible to discriminate between two alternative models of solid tumor growth on the basis of cell biological data with respect to spheroid size, cell proliferation and cell death. Both models simulated how the growth of cancer spheroids was influenced by mechanical and biochemical stimuli including matrix stiffness, culture time and anti-cancer treatment. Our mathematical models provide new perspectives on future experiments and have informed the design of new 3D studies of multicellular cancer spheroids.

Keywords. 3D microenvironment, cancer spheroids, incompressible porous medium model, morphoelastic model

(14.O3) FLUID MECHANICS MODELLING OF PERFUSED CONSTRUCTS IN BONE TISSUE ENGINEERING

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One of the key issues in generating functional tissue in bioreactors is to quantify and optimize the hydrodynamic mechanical microenvironment in the vicinity of the cells within porous scaffolds. Theoretical multiphysical and multiscale analysis related to momentum and mass transfer phenomena through porous media could be proposed as an interesting tool to improve culture technique and bioreactor design [1].

In this context, using a commercial code (Femlab® 3.1; Comsol), a computation model was developed to solve coupled fluid dynamics and transport equations at the microscale of the porous implant. The major characteristics of the complex channels (pore size around one hundred microns, tortuosity larger than two) have been taken into account by designing a sufficiently simplified three-dimensional representative geometry. Inside this element, the structure of the local flow field, its related shear stresses distribution and nutrients transport effects have been analyzed using dimensionless fundamental parameters.

It was shown that, as expected for such a low Reynolds flow mimicking experimental conditions, the velocity field structure roughly reproduces the features of the substrate microarchitecture. Nevertheless, an unexpected secondary flow due to the tortuous pathway is also observed, leading to streamlines helicity and vortical structure of the overall flow field. Thus, at the pore scale and for sufficiently high flow rates, the associated convective effect in the transverse direction and the

diffusive effect become comparable. This may contribute to a significant increase in the nutriment transport process from the centre of the pore towards the cells at its periphery. Moreover, a concomitant non unidirectional and inhomogeneous repartition of viscous stresses is obtained near the channel surface (around 1 mPa for typical experimental conditions).

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Keywords. Porous Media Microfluidics Transport Phenomena

(14.O4) MESENCHYMAL STEM CELL AGEING: AN INDIVIDUAL CELL-BASED MODELING APPROACH

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Introduction. Clones of mesenchymal stem cells (MSCs) from the same donor often differ in their in vitro properties. This kind of heterogeneity has been suggested to originate from an individual decline in MSC function called ‘stem cell ageing’. For therapeutic applications of MSCs understanding the impact of in vitro culture on this heterogeneity is crucial.

Methods and Model. Single cell-derived clones were generated from bone marrow-derived MSC. Their expansion was quantified and lineage/senescence markers were assessed. Expanded clones were subsequently applied in differentiation assays.

Our mathematical approach builds on an individual cell-based model of MSC organization. MSC differentiation is assumed to be a stochastic process for each individual cell with its dynamics determined by the environment. In parallel cell-cell interactions and proliferation were explicitly considered to impact the spatio-temporal organization of the populations.

‘Ageing’ is introduced by the assumption that each cell division increases the amplitude of stem cell state fluctuations, de-stabilising these states in the progeny.

Results. We found that single-cell derived clones of MSC show largely distinct in vitro properties regarding expansion and both, spontaneous and induced differentiation. While fast expanding clones did undergo efficient induced chondrogenic and osteogenic differentiation, slow expanding clones lack this potential. Interestingly, spontaneous differentiation was increased in slow compared to fast expanding clones. Co-culture of different clones is not associated with a growth benefit.

Our model consistently describes these experimental findings. We demonstrate that in vitro expansion itself is sufficient to explain the observed clonal heterogeneity and suggest further experiments to confirm our model predictions. First qualitative modelling results on in vivo ageing are confirmed by CFU-F of rat MSC.

Conclusion. Our model explains the observed heterogeneity by an ageing process and suggests that in vitro and in vivo ageing rely on the same mechanisms.

Keywords. Mesenchymal Stem Cells, Stem Cell Heterogeneity, Cell Plasticity, Ageing

(14.O5) PREDICTION OF OSTEOGENIC DIFFERENTIATION STATUS OF MESENCHYMAL STEM CELLS BASED ON IMAGE ANALYSIS COMBINED WITH BIOINFORMATICS

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For the industrialization of regenerative medicine, the technology for providing both higher safety assurance and efficient cell processing is strongly required. However, conventional and traditional experimental techniques were considered to be inappropriate for the continuous quality check in regenerative medicine. Since cells produced for therapy are limited and promised to be pure without any testing regents. In this aspect, image analysis is one of the few methodologies that could estimate the final condition of implanting cells after cell processing. There had been reports of such non-invasive cell evaluation strategies based on cell images. However, most of the image analysis has focused on few cell morphologies intentionally selected by experts, and there were no scientific reason to select such parameter.

In our research, we introduced bioinformatic analysis strategy in the image analysis of culturing cells to select the best combination for predicting cell quality. By successful combination strategy with the fully-automatic cell culture and monitoring system BioStationCT (Nikon Instruments Inc.), we succeeded in establishing a computer model for quantifying and predicting the osteogenic differentiation status of human mesenchymal stem cells. We analyzed time-lapse phase contrast images of more than 8,000 images to extract the morphologic features and mobility features of three individual cell lots, and examined to predict the ALP activity and calcium deposition rate of the future (ALP activity in two weeks later, or calcium deposition in three weeks later). From the image analysis combined with the regression model analysis, we found that both biologically defined osteogenic differentiation rates could be effectively predicted with high accuracy by the time-lapse image information of cells. We here propose the practical applicability of our image analysis scheme for the non-invasive cell quality analysis for the future industrialization of regenerative medicine.

Keywords. MSC, Osteogenesis, Prediction, Image analysis, Bioinformatics

(14.O6) A COUPLED AGENTS-TRANSPORT MODELLING FRAMEWORK AS A DESIGN TOOL FOR BIOREACTORS

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Bioreactors serve as tools for the ex vivo development of functional tissues and as culture model systems shedding light on fundamental dynamic mechanisms of cell function. Despite the technology advances, bioreactors are still, to a great extent, utilised as black-boxes where trial and error eventually leads to the desirable cellular outcome. With the advent of computational techniques, investigators have tried to recapitulate the dynamics of tissue growth inside a bioreactor but with limited success – mainly due to inherent assumptions and restrictions of the modelling platforms tried.

In this study, a multi-paradigm modelling framework combining and coupling fully an agent-based approach with computational transport phenomena is presented, aiming to serve as a design tool for the construction of bioreactors. The impact of factors such as volume, cell density, flow velocity, shear stress, mass transfer and others, on cell behaviour can be analysed before the actual construction of a design prototype.

To demonstrate the impact of bioreactor geometry and initial conditions on tissue growth, and vice versa, a series of test cases are simulated in virtuo. Three virtual bioreactors are constructed and seeded with varying densities of virtual cells. The virtual cells were considered as entities governed by a set of simple rules that are capable of displaying migration, division, proliferation, chemotaxis and apoptosis. The rules governing the virtual cells involve constants as well as variables; the latter emerging from aspects of the computation simulating mass transfer inside the bioreactors.

We conclude that bioreactor geometry and initial conditions as well as the nature of evolving cellular behaviour has a cumulative impact on the dynamics of the overall tissue development and that the modelling framework presented here can be used as a concept selection tool during the bioreactor design process to choose, given the desired cell phenotype, optimal specifications.

Keywords. Bioreactors, computational modelling, tissue engineering, agent-based modeling

(14.O7) A POPULATION BALANCE MODEL TO INVESTIGATE THE KINETICS OF IN VITRO CELL PROLIFERATION

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The goal of this work it to develop a novel mathematical model helpful to investigate the kinetics of in vitro proliferation of adherent cells. The proposed model is based on a Population Balance (PB) approach that allows to describe cell cycle progression through the different phases experienced by all cell of the entire population during their own life. Specifically, the proposed model has been developed as a multi-staged 2-D PB, by considering a different sub-population of cells for any single phase of the cell cycle (G1, G0, S, and G2/M). These sub-populations are discriminated through cellular volume and DNA content, that both increase during the mitotic cycle. The adopted mathematical expressions of the transition rates between two subsequent phases and the temporal increase of cell volume and DNA content are thoroughly analysed and discussed with respect to those ones available in the literature. Specifically, the corresponding uncertainties and pitfalls are pointed out, by also taking into account the difficulties and the limitations involved in the quantitative measurements currently practicable for these biological systems.

To this aim, a series of numerical simulations related to the in vitro proliferation kinetics of adherent cells is presented. First the complex task of assigning a specific value to all the parameters of the proposed model is addressed, by also highlighting the difficulties arising when performing proper comparisons with experimental data. Then, a parametric sensitivity analysis is performed, thus identifying the more relevant parameters from a kinetics perspective.

Keywords. Adherent cells, proliferation, population balance, modeling

(14.O8) COMPUTATIONAL SIMULATION OF MECHANOELECTRIC INTERACTIONS BETWEEN MYOFIBROBLASTS AND CARDIOMYOCYTES IN A TISSUE MODEL

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Myofibroblasts are central to the wound healing process, serving to repair and contract wound surfaces. Under conditions of hypertension and following myocardial infarction cardiac fibroblasts convert from their quiescent state to this larger and contractile phenotype that can lead to a pathologic condition, fibrosis, involving the formation of excess fibrous connective tissue. In both cases, the interactions of myofibroblasts with cardiomyocytes and their ramifications for tissue function are uncertain. To address this, we have implemented an integrated suite of computational models of mechanical and electrical interactions of the two types of cells, in parallel with an idealized extracellular matrix, and are working to validate and refine predictions through experiments on a model system known as engineered heart tissues (EHTs).

The computational model is formulated at the cellular level taking into account individual cardiomyocyte and myofibroblasts to yield the pattern of impulse spread as modulated by the presence of myofibroblasts acting either as insulators or resistors. The excitatory impulse activates the contraction of individual viscoelastic cells that are mechanically linked to other cells and the extracellular matrix (ECM). Three classes of models are linked in these simulations: electrophysiologic models, models of the contractile response of individual cardiomyocytes as a function of their internal non-bound calcium levels, and models linking these cellular responses to the overall mechanics of an EHT.

The modeling objective is to predict the effects of myofibroblasts on electrical and mechanical functioning of EHT specimens. The typical simulation predicts twitch forces and patterns of electrical depolarization of an EHT with defined composition that is held isometrically and paced electrically. We will present results that shed light on how myofibroblasts can both improve and attenuate the active mechanical function of EHTs.

Keywords. Cardiac fibrosis, myofibroblasts, engineered heart tissue, model systems, electrophysiological model

(14.O9) SCAFFOLD DESIGN FOR BONE TISSUE ENGINEERING

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Introduction. In Bone Tissue Engineering, the scaffolds' functions are to promote cell proliferation, diffusion of oxygen, nutrients, waste products and to ensure the required mechanical properties. Therefore, scaffolds

should have enough strength but also be highly permeable.

The objective of this study is to develop a computational tool for scaffold design, optimizing its performance with respect to these requirements.

Methods. First, a computational analysis of scaffold permeability was performed, applying homogenization methods to Darcy Law, in order to obtain the equivalent homogenized permeability coefficients. The analysis was done for nine models with cylindrical pores in the three directions (different pore sizes and porosity degrees), designed using custom IDL programs. Then, three examples of each model were built using Solid Free Form techniques and tested experimentally. Finally, based on previous study, a scaffold topology optimization model was developed using a multicriteria formulation.

Results. The comparative permeability study shows that the computational values were not completely identical to the experimental ones. Nevertheless, the relations between permeability, porosity and pore size were similar in both cases, supporting the use of this mathematical approach for scaffold design optimization.

With the topology optimization tool based on homogenization methods, it was possible to obtain structures with interconnectivity in all the directions by maximizing permeability; structures presenting a material distribution such that the mechanical function is optimized by maximizing elasticity; and compromising solutions between both criteria when using the multicriteria formulation.

Conclusions. The computational approach assumed in this work can be extremely useful in scaffold design for Bone Tissue Engineering. It has demonstrated its capability to provide solutions of microstructures able to promote diffusion without compromising the mechanical properties, allowing the scaffold to promote the growth of new bone even in bearing load situations.

Acknowledgements. This work was supported by FCT, project PTDC/EME-PME/104498/2008 and PhD scholarship SFRH/BD/46575/2008.

Keywords. Bone Tissue Engineering, Homogenization, Scaffold Design Optimization

(14.O10) A 3D MULTIPHYSIC MODEL FOR THE PREDICTION OF ENGINEERED TISSUE GROWTH IN PERFUSED BIOREACTORS

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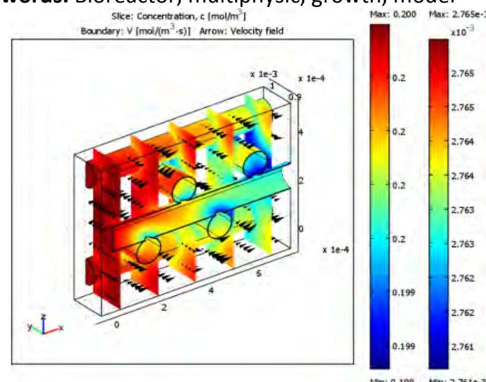
An essential step toward the obtainment of functional tissue in vitro is to control its growth process. This depends on various space- and time-varying biophysical variables of the cell environment, primarily mass transport and mechanical variables, all involved in the cell's biological response. In the aim to obtain a quantitative law for tissue growth in function of such variables, we have developed an advanced growth model of cartilaginous tissue, featuring a mini-bioreactor system, allowing local and non-destructive assays on the cellular constructs, interfaced to a multiphysic model of tissue growth.

The mini-bioreactor hosts 3D cellular constructs, 400 microns in thickness, seeded with chondrocyte cells and cultured under interstitial perfusion of the culture medium. Time-lapse fluorescence microscopy is used to estimate local cell and extra-cellular matrix densities, within specific locations of the scaffold, over the time course of culture. The biomass growth around the scaffold fibres is modelled imposing moving boundary conditions at the biomass surface interfaced with the flowing medium. The boundary movement is modelled as a function of the local oxygen concentration and fluid shear stresses, calculated at the boundary itself.

Several aspects of the non homogeneous tissue growth seen in vitro could be quantified with this growth model. For example, the decrease in tissue growth during the course of the culture, either along the flow direction, due to progressive depletion of oxygen from the flow (See Figure), or in areas of higher tissue volume fraction, due to the inhibition effect of non physiological fluid-induced shears.

Acknowledgements. This research is funded by the grants: 'Biosensors and Artificial Bio-systems'- Italian Institute of Technology (IIT-Genoa); '5x1000-2009-HMED: Computational Models for Heterogeneous Media'- Politecnico di Milano; '3D Microstructuring and Functionalization of Polymeric Materials for Scaffolds in Regenerative Medicine'- Cariplo Foundation (Milano).

Keywords. Bioreactor, multiphysic, growth, model



(14.O11) MODELLING MECHANOSENSING IN CELL-MATERIAL INTERACTION: IMPLICATIONS FOR TISSUE ENGINEERING

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Introduction. Cells sense the mechanical environment by pulling on the extracellular matrix (ECM). Understanding of how mechanical environment is able to guide cell function (proliferation, migration and differentiation) is fundamental for multiple tissue engineering applications.

The main purpose of this work is to explore through computer modelling how cells interact with their surroundings.

Methods. We construct a phenomenological model that incorporates the main mechanical components of the cell when it is interacting with the material:

• The active part of the cell corresponding to the contractile acto-myosin system is simulated following a Hill force-elongation relationship.

• The actin filaments are the main component that bear tension and work in conjunction with the acto-myosin system.

• The passive component of the rest of the cell is due to the contribution of the microtubules and the cell membrane linked to the external ECM through focal adhesions and transmembrane integrins that are simulated as rigid unions.
To evaluate the predictive potential of this model we have computed different mechanical properties of the material and with different geometrical configurations of the substrate (planar and curved).
Results and Conclusions. After the analysis of these simulations, predicted results are in concordance with different experimental measurements:
• Tensional forces generated in the cell increase with the stiffness of the material in which the cell is adhered.
• External forces modify the orientation and the forces generated by the cell.
• Substrate curvature regulates the stress distribution in the cell and may guide the cell polarization in the direction of minimal curvature.
Therefore, the mechanical properties of ECM scaffolds and its local geometry are basic parameters to mimic a local favourable environment for tissue regeneration.
Acknowledgements. The authors gratefully acknowledge the research support of the Instituto Aragonés de Ciencias de la Salud through the research project PIPAMER10/015
Keywords. Finite Element Modelling, cell mechanics, mechanosensing, durotaxis, tensotaxis, contact guidance

(14.O12) MODELING AND FABRICATION OF FUNCTIONALLY GRADIENT VARIATIONAL PORE IN HOLLOWED SCAFFOLDS WITH CONTINUOUS PATH PLAN
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Introduction. In this paper a novel continuous toolpath planning methodology has been proposed to control the internal scaffold architecture with hollow feature for tissue engineering.
Methods. Functionally gradient variational pore architecture has been achieved with the desired pore size and porosity by combining two consecutive slices generated from the 3d model. Porosity architecture in this paper is built in stacks of two consecutive layers: (i) ruling line based zigzag pattern and (ii) concentric spiral like pattern. Modeling of the first layer with equal area sub-regions from ruling line represent the zigzag pattern ensures the biological and mechanical requirement and the consecutive circular pattern layer mainly enforces the desired porosity of the scaffold. A continuous and interconnected optimized tool-path has been generated as an input for the solid free form fabrication process.
Results. Three-dimensional layers formed by the proposed tool path plan vary the pore size and hence the porosity based on the required biological and mechanical properties. The proposed methodology has been implemented in this work and illustrative example has been provided in figure 1. Also a comparison result has been performed between proposed design and conventional Cartesian coordinate scaffolds which shows the proposed method reduces design error significantly. Moreover, sample examples are fabricated layer-by-layer using a micro-nozzle biomaterial deposition system and shown in figure 1.
Conclusions. The proposed methodology generates interconnected and controlled pore size with desired accuracy along the scaffold architecture resulting variational porosity and a continuous deposition path planning appropriate for SFF processes which might address multiple desired properties in the scaffold such as better structural integrity, improved oxygen diffusion during cell regeneration, cell differentiation and guided tissue regeneration.
Keywords. Continuous deposition path, scaffold architecture, variational pore size, solid free-form fabrication

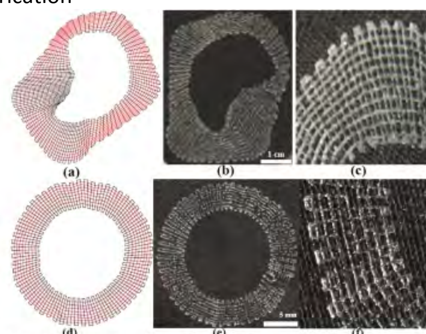

Figure 1. Two consecutive slices and internal architecture with designed constant porosity and fabrication with micro-nozzle (200 μm) hydrogel deposition (a-c) Femur with 85% porosity (d-f) Aorta with 64% porosity.

(14.O13) COMPUTATIONAL FLUID DYNAMICS AS A DESIGNING AND TROUBLESHOOTING TOOL FOR MULTIPHASE BIOREACTORS: CASE STUDY IN AIRLIFT BIOREACTORS
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Computational fluid dynamics (CFD) has been adopted as a designing or troubleshooting tool in bioprocess especially for stem cell application in which the process characteristics are inaccessible due to the contamination concern. The only way to transfer from a lab-scale to a bench-scale is using a bioreactor. Moreover, different types of cells need different types of bioreactors to achieve their functionality. Hence, the airlift bioreactor has been recently used as a device to differentiate embryonic stem cells into type II pneumocytes in the lung. The airlift bioreactor provides a physiological environment, which theoretically has been known to simulate the gas-exchange interface encountered in the lung alveoli. Airlift bioreactors require a low power input and provide a low shear environment with good mixing. Herein, the hydrodynamics (gas holdup, superficial liquid velocity, and shear rate) and mass transfer (kLa, the volumetric mass transfer coefficient) features of different airlift designs were determined by CFD. The simulations were based on a three-dimensional (3D) transient model, Eulerian-Eulerian approach, and two-phase liquid/gas model with all phases being treated as laminar flow. The superficial gas velocity was varied from 0.001 m/s to 0.02 m/s. The O_2 transfer both simulated at normoxia (21% O_2) and hypoxia (2% O_2). The simulation results indicated that the hydrodynamics were corresponded to the data found in the literatures and the gas holdup were agreed

with our experiment validation. The CFD results also suggested that in which range of superficial gas velocity (ug) that we can operate without any fluctuation in term of the hydrodynamics. In addition, the airlift bioreactor is suitable for shear sensitive cells with high mass transfer rate, e.g. kLa, = 180 hr-1 at ug= 0.01 m/s and normoxia condition. The results from these simulations have been initially utilised as a promising hypothesis to design an airlift bioreactor for the scalable and automatable culture in multiphase bioreactors.

Keywords. Computational fluid dynamics (CFD), airlift bioreactor, embryonic stem cells

(14.O14) MECHANICAL PROPERTIES AND FUNCTION OF TISSUE-ENGINEERED CARTILAGE DEPEND ON THE RATE OF COLLAGEN AND PROTEOGLYCANS SYNTHESIS

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Introduction. During cartilage tissue engineering (TE), the synthesis of proteoglycans (PG's) is faster than that of collagen. In the present study we hypothesize that this difference in synthesis rates may be unfavorable to the development of the implant mechanical properties. The rationale is that fibers, which are synthesized early during culture, resist swelling of PG's and are pre-strained as a consequence. Fibers synthesized at late stages do not limit swelling and are not pre-strained. Here, we explore numerically the effect of the relative synthesis rates of collagen and PG's, for post-implantation tissue strains during loading.

Methods. A fibril-reinforced poro-viscoelastic swelling model was used in an axisymmetric finite element model of medial tibia cartilage (properties: see [1]), containing a TE implant with ½ matrix stiffness, ¾ of the PG's and ¼ collagen content of the native tissue [2] (ABAQUSv6.9 (RI, USA); Fig. 1.a). Three cases were compared in which all, half or one-third of the fibers were synthesized early, and the remainder was synthesized late. Fibers strains before and after implantation under 568.75 N (gait load) were evaluated.

Results. Pre-implantation average fiber strain increased from 4% when all collagen fibers were synthesized early (Fig. 1.b.top) to 5% and 7% when half (Fig. 1.b.middle) and one-third (Fig. 1.b.bottom) of the fibers were synthesized early. This resulted in excessive collagen strains of 10% and 13% for the two later cases under loading (Fig. 1.c).

Conclusion. The faster synthesis rate of proteoglycans (PG's) compared to that of collagen during cartilage tissue engineering is predicted to result in excessive fibers strain post-implantation. Such excessive strain may induce implant failure.

Acknowledgment. Funding from the Dutch Technology Foundation STW (VIDI-07970) is acknowledged.

Keywords. Cartilage Tissue Engineering, Implant, Synthesis Rate, Collagen

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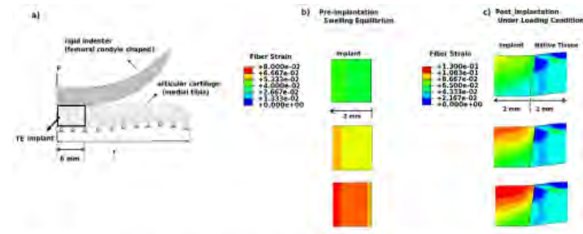

Figure 1. Axisymmetric model of knee cartilage with central TE implant. Fiber strains at end of culture (a),or post-implantation under joint loading (b), when all (top), half (middle) or one-third (bottom) of the collagen is synthesized early.

(14.O15) A BOOLEAN NETWORK APPROACH TO DEVELOPMENTAL ENGINEERING

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1. ULG; 2. K.U.Leuven

Introduction. Developmental engineering (DE) proposes a biomimetic approach to replace empirical TE by using developmental pathways to increase robustness, consistency and quality of stem cell-derived TE products. In order to be able to direct and observe a bone TE process in vitro the different regulators of the in vivo ossification need to be determined. Given the complexity and high interdependency of the signalling pathways in endochondral ossification a Boolean approach is taken to model the developmental process.

Methods. In this study, a large-scale literature-based Boolean model of the regulatory network governing endochondral ossification was developed. The model is implemented in GINsim (Gene Interaction Network simulation), a program geared towards Boolean modelling.

Results. The network is able to sequentially capture the different stable states (resting, proliferating and hypertrophic) the chondrocytes go through as they progress through the growth plate, which are identical to the cell states of chondrocytes during endochondral ossification. The prehypertrophic state was predicted to be an unstable state at the transition between proliferation and hypertrophy, similar to experimental observations. In a first corroboration step, the effect of mutations in various signalling pathways of the growth plate network was investigated. The model was able to successfully predict the changes in the growth plate structure for all simulated cases.

Discussion. These first corroboration results indicate that the proposed growth plate network provides a comprehensive and coherent description of chondrocyte behaviour and cell state in endochondral ossification. This Boolean model will allow integrating multiple external signals and determine their effect on the cell state, providing a rationale to guide an in vitro TE process.

Acknowledgments. This work was supported by the Special Research Fund of the University of Liège (FRS.D-10/20).

Keywords. Boolean, gene network, developmental engineering

(14.O16) HOW INTEGRINS MAY MODULATE THE MECHANOTRANSDUCTION BETWEEN HYDROGEL MATRIX AND THE CHONDROCYTES IN CARTILAGE TISSUE ENGINEERING

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Introduction. Mechanical stimulation enhances matrix synthesis in cartilage tissue engineering. Because this effect is mediated by integrins [1], it is thought to depend on cell-matrix interaction. Here, we explore how attachment between a chondrocyte and its pericellular matrix (PCM), embedded in agarose, may influence the distribution of strains during axial compression.

Methods. An axisymetric biphasic multi-scale finite element model was used. Boundary conditions of the micro-scale model of a chondrocyte with a PCM embedded in agarose (Figure1.a) were derived from a simulation of 5% unconfined compression at the macro-scale [2]. The two conditions simulated at the micro-scale were: frictionless contact and tied contact between the chondrocyte and its pericellular matrix.

Results. The results showed that cell-matrix interaction may considerably change the micromechanical environment in and around a chondrocyte. With cell-matrix attachment, the intracellular strain becomes more homogeneous and peak strains are reduced, compared to frictionless contact (Fig 1b,c: top vs. bottom). Obviously, the actual magnitude and distribution of strains inside the cell depends on the organization of the cytoskeleton and other organelles, as well as on the structure of the matrix. Regardless, the effect that cell-matrix interaction is important for the strain field experienced by the chondrocytes likely persists.

Conclusion. Our findings suggest that blocking integrin binding may influence the physical signals transmitted from the PCM to the cells. Tensile and compressive strains are known to fluence chondrocyte activity[3], and excessive strain reduces cell metabolism[4]. Therefore, we speculate that alteration of intracellular physical signals due to blocking integrin attachment may explain, in part, the experimental observation that blocking integrins modulates the effect of mechanical loading in chondrocyte-seeded agarose cultures[1].

Acknowledgments. Funding from the Dutch Technology Foundation STW (VIDI-07970) is acknowledged.

Keywords. Computational Modelling, Mechanotransduction, Integrin, Cartilage tissue engineering

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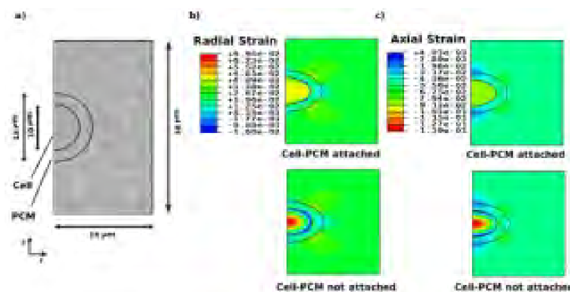

Figure 1. (a) Axisymmetric finite element model of a chondrocyte, (b,c) Computed radial and axial strain with (top) or without (bottom) cell-matrix binding.

(14.O17) A COUPLED CHEMO-MECHANO-BIOLOGICAL MODEL FOR BONE ADAPTATION

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1. Czech Technical University in Prague; 2. University of Zaragoza; 3. Institute of Themomechanics, Prague

Introduction. We believe that modelling of processes in biology requires an interdisciplinary approach. This needs to comprise biochemistry and often mechanics, or physics in general. There were two quite different approaches to modelling bone remodelling - Spanish group has developed a detailed mechanical description including the influence of damage and the Czech team has developed a model base on biochemical knowledge of the process control together with mechano-chemical coupling. The main objective was to combine models for bone remodelling into a new one that would take advantages from both different approaches.

Methods and results. The new model posess a constitutive relation that takes mineral content, damage, and porosity into account. Further, complex influence of damage is included: fatigue damage growth and repair, how mineral content affects fatigue, and how RANKL-RANK-OPG pathway is directly influenced by damage. The mechanical stimuli is of dynamic origin which is accordance with the current knowledge. Bone volume fraction (Vb) is a function of mechanical stimulus (dynamic loading) daily strain history, further it is a function of biochemical constituents: RANKL, RANK, OPG, Estradiol, PTH, NO, and damage affects RANKL concentration and in turn Vb.

Conclusions. Martin proposes inhibition of bone remodelling by loading which exactly corresponds to behaviour of bone formation index in this model. Further, the model estimates ash fraction, mineral volume fraction (calcium content) and bone volume fraction in bone which should help to understand the relationship between bone mechanics and biology that leads to osteoporosis.

Acknowledgements. This research has been supported by the Czech Science Foundation project no. 106/08/0557 and by the "Programa Europa XXI de estancias de investigación-CAI".

Keywords. Multidisciplinary modeling, bone remodeling, bone biochemistry, damage

(14.O18) COMPUTER SIMULATION OF MANUFACTURE AND DEGRADATION OF SCAFFOLDS

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In recent years, biomedical research has been developing new strategies to release drugs into the human body in a controlled manner. One of these strategies is based on the degradation of polymeric fibres that contain drugs inside them. As the fibres degrade, the drug is released in a controlled way into the surrounding environment. The drug release rate changes depending on, among other factors, the geometry of the initial microstructure of the scaffold, the drug distribution inside the fibres and the type of polymer used.

The aim of the present work is to parametrically generate valid 3D models, by which the degradation can be simulated depending on different scaffold architectures. A specific algorithm was developed for the generation of initial electrospun microstructures with cylindrical geometry. In the model, the fibre trajectory was defined as a polyline. The resulting 3D microstructures are then discretised into small homogeneous cubic elements (voxels). In addition, a different algorithm was developed for the simulation of the surface degradation process. The model is based on a Monte Carlo method, according to which the degradation probability of a given voxel is related to the number of solid surrounding neighbours, thus relating fibre degradation to its surface curvature. The inclusion of drugs inside the fibres will also allow the model to predict the average drug release rate.

The validation of the proposed model against empirical measurements will result in a more effective scaffold design, taking advantage of extended in-silico optimisation of the design parameters before starting time-consuming empirical experiments.

Keywords. Computer simulation, scaffold design, degradation

(14.O19) MODELLING OF NUTRIENT MASS TRANSFER AND CELL TRANSFER AND PROLIFERATION IN ENGINEERED VASCULAR TISSUE AND SCAFFOLD OPTIMISATION

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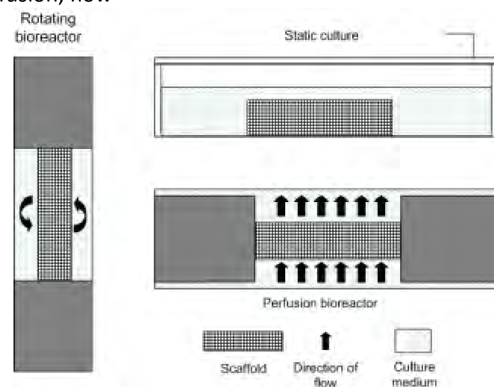
1. University of Surrey; 2. National Physics Laboratory

The tissue engineering of vascular grafts is a complex multidisciplinary science that involves the growth of smooth muscle and endothelial cells in a supporting scaffold in vitro in the presence of an appropriate culture medium containing the necessary nutrients such as oxygen and glucose, and other substances including growth factors, antibiotics, etc. The biofabrication process is usually developed in an ad hoc manner to determine the optimum scaffold and processing conditions that will ensure the adhesion of cells, their homogeneous incorporation in the scaffold, their survival and their further growth and functioning to produce extracellular matrix (ECM); this can be both expensive and time consuming, hence there is huge interest in the development of comprehensive process models. This work presents a mathematical model including flow of the cell-culture medium suspension through the porous scaffold, mass transfer of various nutrients and also of cells with convection and diffusion terms, nutrient consumption, cell adherence and cell motion, cell growth and death. Various flow conditions are considered for different types of bioreactors, static, rotating, and perfusion bioreactors. Furthermore, a growing tissue with a dynamically changing structure is considered starting from the scaffold design and proceeding with local adjacent cell layers continuously growing into the pore spaces. The model is validated with experimental data of smooth muscle cells growing into an electrospun and crosslinked gelatine scaffold for which predictions are compared to actual data for oxygen and cell concentration gradients. Furthermore, results are presented from computer simulations for different scaffold parameters (porosity, pore size, fibre diameter) and processing conditions, and suggestions are made for the optimisation of scaffold design and biofabrication.

Keywords. Scaffold, vascular, mathematical modelling, diffusion, flow



(14.O20) A NEW CONSTITUTIVE MODEL TO DESCRIBE COLLAGEN REMODELING IN TISSUE ENGINEERING APPLICATIONS

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1. Trinity College Dublin

Introduction. Extracellular matrix remodeling is ubiquitous in biological tissues and their engineered counterparts. The collagen network can remodel its orientation and stress-free configuration related to the transition stretch above which the uncrimped fiber begins to bear load. Remodeling of collagen crimp has been shown to be involved in long bone growth, contracture, scar pathologies and collagen gel compaction among others. It can be cell mediated or occur via cell-independent mechanisms. The objective of this study is to develop a new continuum model to describe collagen remodeling in terms of stress-free configurations and angular orientations of collagenous tissues.

Methods. The deformation gradient is multiplicatively decomposed into a remodeling tensor and an elastic part. The resulting intermediate configuration locally describes the stress-free state of the collagen network and allows the definition of appropriate deformation and structure tensors. Evolution equations are defined for the remodeling tensor and the mechano-regulated angular fiber reorientation.

The model is applied to fibrin cruciform and collagen gel compaction, cartilage tissue engineering and remodeling of periosteum held at fixed lengths.

Results. The model successfully predicted the compaction of collagen gels and the associated anisotropy that occurs

within such constructs along with their developing shape. The simulations of periosteum adaptation captured the temporal changes in force-deformation behavior. Dynamic compression was predicted to influence the developing mechanical properties of tissue engineered cartilaginous constructs by affecting the collagen organization.

Conclusion. Understanding how mechanical signals regulate shape, organization and mechanical properties of engineered soft collagenous tissues can be greatly facilitated by computational models. The presented framework allows in silico investigation of a large variety of collagen remodeling related phenomena in both tissue engineering and regenerative medicine. Critically the model successfully captures mechano-regulated structural aspects such as orientation and natural configuration. Extension is directed towards the regulation of ECM constituent concentrations.

Acknowledgments. IRCSET, SFI

Keywords. Collagen, remodeling, hydrogel, fibrin

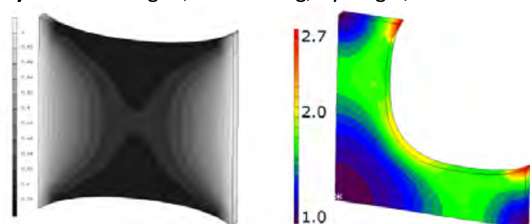

Figure 1: Construct shapes for clamped collagen gel (left) and fibrin cruciform (right). Contours show the transition stretch (left) and the degree of anisotropy (right).

(14.P1) ENHANCING EMBRYONIC STEM CELL EXPANSION BY THE COMBINATION OF PERFUSION FEEDING AND BIOPROCESS MODEL DESIGN

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Embryonic stem cells (ESC) are suitable candidates for regenerative medicine due to their high proliferative and differentiation potential. A bottleneck to their usage is the formation of differentiation by-products such as teratomas which necessitates the implementation of efficiently directed culture protocols. Cell culture variables have been shown to strongly affect ESC pluripotency levels. We investigated the effects of metabolic stress (levels of nutrients and metabolites sub-optimal to ESC metabolism) to ascertain their impact on ESC expansion.

Murine ESCs were expanded in batch cultures and a multi-scale bioprocess model was developed to analyze their cellular kinetics, basic metabolism and gene expression. We observed growth kinetics typical of batch cultures and showed that ESCs differentiated even in the presence of sufficient growth factors. Our mathematical model predicts the emergence of a differentiated population due to persistent exposure to inhibitory levels of metabolites during the latter stages of the culture. Thereafter, perfusion feeding operation eliminated this metabolic stress enabling the maintenance of a 16-fold total expansion from seeding density. The different metabolic characteristics for perfusion feeding necessitates changing 6 in 29 model parameters. We observe the expression of pluripotency - related genes in conjunction with a decrease in differentiation levels was unaccounted for by growth factor availability. Furthermore, our mathematical model also predicted the preferential propagation of ESCs in a naive state at the expense of differentiated cells in the metabolically favourable conditions.

We contend that the metabolic well-being of ESC cultures supersedes the effects of growth factors in determining pluripotency levels based on the contrasting behaviour we observed in perfusion and batch feeding cultures. Furthermore, the use of model-based design of bioprocesses provides insights into ESC pluripotency and metabolism, thereby facilitating the development of optimized culture protocols.

Keywords. embryonic stem cells, mathematical modelling, stem cell bioprocess, perfusion feeding, pluripotency, tissue engineering

(14.P2) RAPID MANUFACTURING OF THREE-DIMENSIONAL RESORBABLE SCAFFOLDS FOR THE TISSUE ENGINEERING OF HUMAN HEART VALVES

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1. German Heart Institute Berlin; 2. TU Berlin

Introduction. In the past 3 years rapid prototyping has been further developed into rapid manufacturing, a process allowing not only the establishment of real 3-D models as prototypes but also the fabrication of 3-D objects. Rapid manufacturing provides the novel opportunity to generate real 3-D objects with product quality directly from computer-based manufacturing processes and to establish absolutely novel applications. Using appropriate rapid manufacturing processes custom-made human heart valve scaffolds should be fabricated and additionally seeded with vascular cells from human umbilical cords.

Methods. Protocols were established to collect 3-D data from healthy heart valves using computed tomography or magnetic resonance imaging. After model generation a standardized segmentation and reconstruction logarithm was generated and biocompatible casting molds for selective laser sintering were constructed with the latest visualization technologies. Subsequently, the optimal parameters of biocompatible materials and the associated production technique were developed and established. Scaffolds produced were analyzed for capability of cell seeding and quality controls were performed using a contactless stripe-light scanner.

Results. Using custom-designed segmentation and reconstruction software 3-D data of a heart valve from a healthy male were processed to generate a 3-D model. A 3-D printer for selective laser sintering was used to fabricate the heart valve scaffold consisting of a flexible synthetic material. Recently, different resorbable polymeric granules and powders based on polyglycolic acid (PGA) and polylactide acid (PLA) have been analyzed for the fabrication of heart valve scaffolds using the rapid manufacturing process.

Conclusion. In general, direct 3-D printing of human heart valve scaffolds using specific software and laser sintering systems is feasible. With regard to the future clinical application of tissue engineered human heart valves,

rapid manufacturing provides the crucial step from a model to a suitable product.

Keywords. Rapid manufacturing, heart valves, resorbable scaffolds, tissue engineering

(14.P3) MULTI-SCALE FINITE ELEMENT STUDY BASED ON IN VIVO DATA TO EVALUATE BIOMECHANICAL STIMULUS IN BONE SCAFFOLD

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1. Laboratory of Biomechanical Orthopedics-EPFL

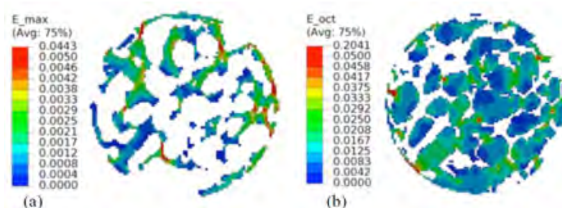
A micro-FE analysis was developed allowing us to evaluate the mechanical stimulation in a bone scaffold inserted in a rat condyle when external load is applied to the leg of the rat. The developed model corresponds to an in vivo study.

Both distal femoral condyles of Wistar rats were operated and a PLA based scaffold was implanted inside the hole. Three days after the surgery, the loading (10 N at 4 Hz for 5 minutes) of the right knees started. The bone formation was quantified using a SkyScan 1076 in vivo micro-CT scanner. A rat femur geometry was imported in ABAQUS for numerical analysis. The strain of the scaffold was then calculated and was used as boundary conditions for the micro-FE model of the scaffold. A scaffold similar in size and architecture to those implanted was scanned. An in-house Matlab script was used to convert the thresholded images into 8-noded cubic finite element mesh for ABAQUS. It was assumed that all pores are filled by granulation tissue. The strain values were computed for each element of granular tissue and the octahedral shear strain was calculated as the mechanical stimulus.

The 10 N load applied on the rat femoral condyle resulted in average largest principal strain of 620 µε in the scaffold. The average maximum principal strain in scaffold tissue was 0.14±0.11% (Fig. 1a). The average octahedral strain in granulation tissue was 1.2±0.8% (Fig. 1b).

In summary, we used a multi-scale finite element modeling to estimate the biomechanical stimulus in our rat distal femur model which resulted in enhanced bone formation inside scaffold. We found out that strain as high as 1.2% in the granulation tissue is osteogenic. This is of practical use when designing scaffolds for bone tissue engineering in load-bearing situations.

Keywords. In vivo test, µCT, µFEM, bone



(14.P4) AN INTEGRATIVE MODEL BASED APPROACH TO OPTIMIZE CALCIUM PHOSPHATE SCAFFOLD-STEM CELL COMBINATIONS

Carlier A (1), Chai YC (1), Moesen M (1), Schrooten J (1), Van Oosterwyck H (1), Geris L (1)

1. K.U.Leuven

Introduction. Experimental evidence indicates a key role for calcium ions (Ca2+) in mesenchymal stem cell (MSC)-driven bone formation in calcium phosphate (CaP) scaffolds. This study aims to develop a computational model of MSC-driven bone formation in CaP scaffolds with an emphasis on the role of Ca2+.

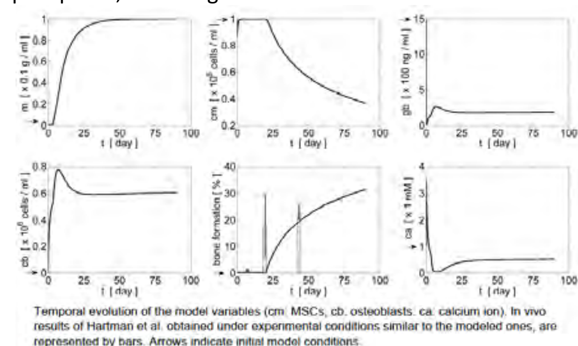
Methods. The mathematical model describes the temporal evolution of the densities of MSCs, osteoblasts, osteoid, mineralized bone and the concentrations of Ca2+ and a generic, osteogenic growth factor by means of differential equations (1D). The model parameters were derived from in-house in vitro experimental data and literature. The set of non-linear delay differential equations was solved using Matlab (The MathWorks, Inc.) for several biologically relevant initial conditions and compared to published in vivo data.

Results. The mathematical model predicted 31% bone formation at 90 days post implantation, which agreed well with experimental data. The model also predicted the absence of bone formation in the case of insufficient cell seeding or scaffold decalcification. Moreover, the model shows that a low initial MSC density requires a low calcium release rate, while a high initial MSC density requires a high calcium release rate in order to maximize the amount of bone formation. Furthermore, this optimization window is narrow for low initial MSC concentrations.

Conclusion. A mathematical model of the effect of Ca2+ on cellular activities and MSC-driven bone formation was developed and verified by means of in vivo data. The results obtained in this study suggest that this model can be used as a tool to design and optimize CaP scaffolds in tissue engineering applications. In the future the model could be refined with additional cell and tissue types, allowing for an in silico triage of cell-customized biomaterials.

Acknowledgements. Aurélie Carlier is a PhD fellow of the Research Foundation Flanders (FWO-Vlaanderen). This work is part of Prometheus.

Keywords. Bone tissue engineering , calcium, calcium phosphate, modeling



Temporal evolution of the model variables (cm: MSCs, cb: osteoblasts, ca: calcium ion). In vivo results of Hartman et al. obtained under experimental conditions similar to the modeled ones, are represented by bars. Arrows indicate initial model conditions.

(14.P5) A COMPUTATIONAL SOLID AND FLUID MECHANICAL ANALYSIS OF CAD- VERSUS MICRO-CT-BASED MODELS OF REGULAR Ti6Al4V SCAFFOLDS FOR BONE TISSUE ENGINEERING

Truscello S (1), Kerckhofs G (2), Moesen M (2), Torcasio A (1), Van Bael S (1), Van Lenthe GH (1, 3), Schrooten J (2), Van Oosterwyck H (1)

1. Division of Biomechanics and Engineering Design, K.U.Leuven; 2. Department of Metallurgy and Materials Engineering, K.U.Leuven; 3. Institute for Biomechanics, ETH Zurich

Introduction. Osteogenic cell behaviour can be influenced by both the local strain distribution (under mechanical

loading) and the fluid flow inside bone tissue engineering (TE) scaffolds. In addition, fluid flow enhances the transport of nutrients and soluble factors in general. Thus, characterization of these properties is key in TE scaffold evaluation. As the solid and fluid mechanical properties of produced TE scaffolds may differ from the design values due to additive manufacturing production constraints, this study evaluated the importance of this potential difference using finite element analysis (FEA) and computational fluid dynamics (CFD) analysis on (i) a computer-aided design (CAD) unit cell model and (ii) a 3D micro-CT-based model. Simulation results were also compared to experimental measurements.

Methods. A selective laser melted Ti6Al4V scaffold was used as a test case. Micro-CT images were generated (12.5 µm voxel size) without mechanical loading for FEA and CFD analysis and (ii) at different discrete loading steps, using in-situ compression, for experimental local strain mapping. The apparent stiffness and compressive strength were determined experimentally using compression tests and a dedicated set-up was used to evaluate experimentally the permeability. The CAD-based and micro-CT-based computed apparent stiffness, local strain distribution, permeability and wall shear stress (WSS) distribution were compared.

Results. A good agreement was found between simulated (CAD-based, micro-CT-based) and measured permeability and apparent stiffness values. The difference between the average WSS as predicted by the micro-CT and CAD-based CFD model was 13% (Fig. 1). Surface inhomogeneities inherent to the production process were captured in the micro-CT-based but not in the CAD-based model. However, they did not influence the local properties significantly.

Conclusions. For the determination of both the global and local solid and fluid mechanical properties, within the investigated dimensional scale window, CAD-based modelling performs as well as micro-CT-based modelling and has lower computational requirements.

This work is part of the Prometheus, the Division of Skeletal Tissue Engineering of K.U.Leuven.

Keywords. Bone tissue engineering, computational fluid dynamics, finite element analysis, regular scaffolds

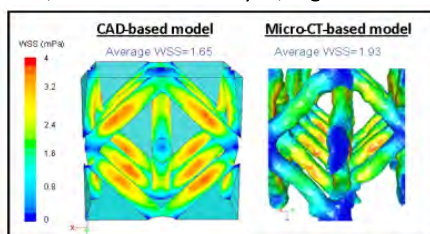


Fig 1: Wall shear stress contours map resulting from the unit cell CAD based model (left) and the micro-CT based model (right)

(14.P6) THE INFLUENCE OF THE ELECTRON BEAM ON NANOLAYERS ANALYSIS

Miculescu F (1), Jepu I (2), Posornicu C (2), Lungu CP (2), Miculescu M (1), Stancu M (3), Bojin D (1)

1. Politehnica University of Bucharest; 2. National Institute for Laser, Plasma and Radiation Physics, Bucharest-Magurele; 3. Petroleum-Gas University of Ploiesti

Introduction. SEM is one of the most popular tools used for the thin films' characterization. In the field of SEM, the use of simulation programs for the electron beam-sample interactions enables the visualization of the interaction volumes between accelerated electron beams and samples.

Methods. The analyzing programs lies the possibility of the planning and interpretation of the imaging (SEM) and microanalytical Results. In this study we used CASINO® software. for investigation of the effect of electron beam energy on the penetration depth using SEM / EDS analysis of Cu-Ni-Cu-Fe-Ta multilayer structures with different thicknesses deposited by Thermionic Vacuum Arc onto Si wafers.

Results. The influence of electron beam accelerating voltage, ranging from 5 to 30 kV, on multilayer structures with total thickness of 38 nm and 1220 nm prepared by TVA, has been studied. When nanolayers are analyzed, it is recommended the usage of different acceleration voltages, in order to excite at least the K lines of the low elements and L lines of the heavy elements. At low energy of the incidence electrons, the absorption effect is less important, because the interaction volume is closer to the surface.

Conclusions. At a higher beam energy, the electron beam can penetrate deeper and an intense signal of Si substrate can be detected. The metal layer thickness is in an almost linear relationship with the energy required for electron beam penetration. Based on the experimental results and mathematical models applied in Cu-Ni-Cu-Fe-Ta multilayers study, relations between the detected signal intensity function of the incidence electron beam acceleration voltage were established. The simulation results are in good agreement with experimental results.

Acknowledgements. Authors recognise financial support from the European Social Fund through POSDRU/89/1.5/S/54785 project: "Postdoctoral Program for Advanced Research in the field of nanomaterials".

Keywords. nanolayers, SEM/EDS, volume of interaction, computer simulation

(14.P7) SIMULATIONS OF CELL SEEDING USING PARTICLES CODE AND RAPID PROTOTYPING SCAFFOLD

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1. Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain

The control of cell seeding is critical for the development of functional tissue engineering products. This study presents a novel methodology to predict the cell distribution after seeding. The optimum experimental time, concentration of cells, scaffold microstructure and hydrodynamic environment are the principal parameters that can be controlled in this model. In addition, the model is capable to determine the specific position of cells on the scaffold wall after cell seeding. The simulation was validated against in vitro experimental under perfusion conditions. Based on rapid prototyping scaffold fabricated by stereolithography, a scaffold with different pore size in the radial direction of the cylindrical samples was modelled. Human articular chondrocytes (HAC) were suspended in culture medium, and seeded under oscillating perfusion fluid flow.

An Eulerian-Lagrangian model was used to simulate the multiphase phenomenon (cells and culture medium) and cell adhesion conditions were applied. The cell adhered

after seven cycles in the simulation for a central section was compared with the threshold z-stack confocal images for cell-seeded in the *in vitro* experiment (Figure 1). A similar distribution was obtained showing the clustering of cells in the central part of scaffold and poor adhesion on the periphery. This relation is attributed to the distribution of pores in radial direction. Although the model shows some very good similarity with the experimental results, the model remains very simple. In particular effort must be put into a more precise simulation of cell attachment and separation as a function of wall shear stress and of biological affinity with the biomaterial surface. Nonetheless in this study we find that the pattern of transportation of cells is a determining factor in the final cell distribution and that it is strongly dependent on the distribution of available surface area.

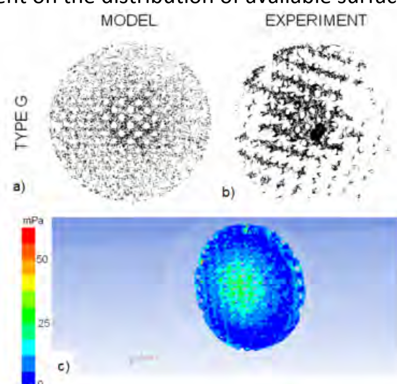

Figure 1: a) Distributions in particles attached through the models in 1mm thickness and b) threshold z-stack confocal images (500 μm thickness) of cell-seeded on experiment c) The shear stress distribution is shown in a cross section of the scaffold.

15. ENGINEERED HYDROGELS (AND STEM CELLS) FOR TISSUE REGENERATION

Chair: Manuela Gomes
Co-chairs: Rui L. Reis, Ali Khademhosseini
Keynote speaker: Ali Khademhosseini
Organizer: Manuela Gomes
Synopsis: The continuous technological developments in the areas of micro and nanofabrication has allowed for a finer control over the architecture of scaffolds for Tissue Engineering applications. However, being micro and nanotechnologies such young scientific areas in the field of TE, it is expectable that their development is still in an early stage. Its state of development is still mostly limited to the top-down approach in which small products are created with the help of large devices. Nonetheless, several researchers have been studying the combination of top-down and bottom up approaches for the development of microgel units (top-down), which are then assembled (bottom-up) to generate a tissue construct. These microengineered hydrogels constitute a very interesting approach for obtaining 3D tissue like structures, enabling the possibility to control materials properties such as adhesiveness, stiffness, cell signaling potential, size and shape. Additionally these microengineered hydrogels may be designed to incorporate biomolecules, enabling the additional function as drug or gene carrier systems. Several polymers have been proposed for building such structures, including natural origin polymers.
An additional important issue in any tissue engineering approach is the need for an appropriate stem cell source, but independently of the selected source, these will always require an appropriate 3D environment, in a great extent dictated by the 3D scaffold, to proliferate and differentiate in the desired phenotype. The physical microenvironment can be tailored through the fabrication of microengineered structures that aim to mimic the micro/nanoscale environment of tissues. These highly organized and cooperative micro/nanoscale building blocks can assemble in a controlled way to ultimately build functional tissue substitutes. This syposium is expected to provide an overview of recent work on the development microengineered hydrogels with the ability to direct stem cells behavior through nano/micro design features combined with the controlled release of biological molecules and hence obtaining highly functional tissue engineered substitutes.

(15.KP) MICROENGINEERED HYDROGELS FOR STEM CELL BIOENGINEERING AND TISSUE REGENERATION

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1. Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA; 2. Harvard-MIT Division of Health Sciences and Technology, MIT, Cambridge, MA; 3. Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA

Micro- and nanoscale technologies are emerging as powerful tools for controlling the interaction between cells and their surroundings for biological studies, tissue engineering, and cell-based screening. In addition, hydrogel biomaterials have been increasingly used in various tissue engineering applications since they provide cells with a hydrated 3D microenvironment that mimics the native extracellular matrix. In our lab we have developed various approaches to merge microscale techniques with hydrogel biomaterials for directing stem cell differentiation and generating complex 3D tissues. In this talk, I will outline our work in controlling the cell-microenvironment interactions by using patterned hydrogels to direct the differentiation of stem cells. In addition, I will describe the fabrication and the use of microscale hydrogels for tissue engineering by using a 'bottom-up' and a 'top-down' approach. Top-down approaches for fabricating complex engineered tissues involve the use of miniaturization techniques to control cell-cell interactions or to recreate biomimetic microvascular networks within mesoscale hydrogels. Our group has also pioneered bottom-up approaches to generate tissues by the assembly of shape-controlled cell-laden microgels (i.e. tissue building blocks), that resemble functional tissue units. In this approach, microgels were fabricated and seeded with different cell types and induced to self assemble to generate 3D tissue structures with controlled microarchitecture and cell-cell interactions.

(15.O1) FABRICATION OF HYDROGEL FIBER BUNDLES FROM ASSEMBLY OF POLYELECTROLYTES

Coutinho DF (1), Sant S (2), Shakiba M (3), Gomes ME (1), Neves NM (1), Reis RL (1), Khademhosseini A (2)

1. 3B's Research Group; 2. Harvard-MIT; 3. University of Toronto

In many natural tissues, fibrils align in parallel and closely pack into three-dimensional (3D) hierarchical bundles of fibers. These fibers provide tensile strength to the various tissues such as heart, brain, bone or skin. Given their importance in tissue function, the engineering of these hierarchical features into materials is therefore of biomedical relevance. Numerous strategies for the development of a synthetic fiber bundle have been proposed, such as electrospinning or extrusion of polymers into aqueous solutions. However, most of the existing techniques fail to replicate simultaneously, the hierarchical architecture of these tissues and the microenvironmental physical and chemical cues. Thus, the aim of this work was to engineer hydrogel fibers that both mimic the natural architecture of the fiber bundles and enable the encapsulation of cells. Fiber bundles were fabricated by polyionic complexation between cationic chitosan (CHT) and anionic methacrylated gellan gum (MeGG) that occurred in a polydimethyl siloxane (PDMS) channel. The fibers were then collected and stabilized by photocrosslinking the MeGG. The resulting architecture of the fiber bundles was studied with atomic force and scanning electron microscopy. Each bundle was approximately 100 µm in diameter and contained small fibers that were 1-5 µm in diameter. Confocal microscopy of the hydrogel fiber bundles engineered with FITC-labeled CHT showed homogenous distribution of CHT throughout the fiber bundles. Their stability was maintained in phosphate buffered saline over a period of one week. A closer system to biological matrices was achieved by covalently incorporating the adhesive motif RGD in the MeGG backbone. Furthermore, encapsulated cardiac fibroblasts adhered to and spread along the fibril direction. This system combines polyelectrolyte complexation and fluidics technology to engineer hydrogel fibers that closely mimic the natural architecture of fiber bundles and may be beneficial for various tissue engineering and regenerative medicine applications.

Keywords. Hydrogel, microfibers, cell encapsulation, tissue engineering

(15.O2) ENGINEERED STARPEG-HEPARIN HYDROGELS ARE EFFECTIVE MULTI FACTOR DELIVERY MATRICES TO PROMOTE ANGIOGENESIS

Freudenberg U (1), Zieris A (1), Chwalek K (1), Prokoph S (1), Levental KR (1), Welzel PB (1), Werner C (1)

1. Leibniz Institute of Polymer Research, Dresden, Germany Technische Universität Dresden, Center for Regenerative Therapies Dresden, Dresden, Germany

Introduction. Effective vascularization is a prerequisite for the success of various different tissue engineering concepts. While short time delivery of various growth factors has been shown to boost angiogenic response the therapeutic more relevant long time delivery of signal molecules from biomaterials is still a major challenge. To address this issue we present here a novel biomimetic material in which the high affinity of the polysaccharide heparin was utilized to design a highly efficient release matrix for several cytokines.

Materials and Methods. Modular StarPEG heparin gels were synthesized, characterized and subsequently the uptake and release of signal molecules (VEGF, FGF-2) were studied applying Enzyme-linked immunosorbent and radiolabeling techniques. Pro angiogeneic response was studied in vitro using human umbilical vein endothelial cells (HUVECs) and in vivo using a chicken embryo chorioallantoic membrane (CAM) assay.

Results. As the utilized gels contain high quantities of heparin, loading and subsequent release of both cytokines occurred independently from each other and could be tuned to customized release profiles. The combined delivery of FGF-2 and VEGF through these matrices resulted in pro-angiogenic effects in vitro (study of cell adhesion, survival/proliferation, morphology and migration) and in vivo (quantification of CAM vascularization) being clearly superior over those of the administration of single factors.

Conclusions. This study demonstrated that modular starPEG-heparin hydrogels could be successfully utilized for the combined immobilization of large quantities of FGF-2 and VEGF and permitted an independent, tunable delivery of both growth factors. In in vitro and in vivo experiments combined FGF-2 and VEGF delivery exerted superior effects on cell behavior and the angiogenic response when compared with the provision of single cytokines. As such, the starPEG-heparin hydrogels performed outstandingly as an effective cytokine delivery matrix, allowing for the application in multi-factor settings essential for effective regenerative processes.

Acknowledgments. The work was supported by grants from the European Commission Seventh Framework Programme in the project Angioscaff (NMP-LA-2008-214402), the European KidStem network, and DFG grants (WE 2539/7-1 and EXC CRTD).

Keywords. Biohybrid hydrogel, heparin, growth factor release, HUVECS, VEGF, FGF-2

(15.O3) IMMOBILIZATION OF BIOMOLECULES ON HYDROGEL SURFACES WITH DIFFERENT STIFFNESSES FOR THE MODULATION OF (ADULT) STEM-CELL FATE

Zouani OF (1), Kalisky J (1), Ibarboure E (2), Labrugère C (3), Mehdi A (4), Durrieu MC (1)

1. INSERM; 2. LCPO ; 3. ICMCB; 4. CMOS

Introduction. Microenvironment elasticity and extreme surface conditions appear important in stem cell lineage specification (1). Here we propose a protocol for the immobilization of different biomolecules that contain N termini groups corresponding to a wide range of surface matrix elasticity. We describe the synthesis of a co-polymer of acrylamid and acrylic acid with different elasticities ranging from 0.5 to 70 kPa and then the covalent attachment of biomolecules directly without spacers. This stiffness range is considered important for stem cell fate (1). Cell behavior can be achieved in the presence of adhesion or induction-promoting biomolecules.The approach should be a suitable method for the study of stem cell differentiation in different lineages with multifactor variation. Thus, generation of

materials to direct stem cell fate holds potential for tissue engineering.

Materials and Methods. Poly(acrylamyde-co-acrylic acid)/polyacrylamide hydrogel was prepared. Elastic modulus was measured with Dynamic Mechanical Analysis (DMA). Peptide immobilization was performed following the procedure described previously (2). Functionalized hydrogels were characterized with X-ray photoelectron spectroscopy and high resolution micro-imager. For this study, human mesenchymal stem cells (from LONZA) were used.

Results. Three parameters were studied on the different microenvironments of functionalized surfaces. Fist, the change in cell shape was observed. Then we evaluated the osteogenesis gene markers. Finally, cells were stained with lineage-specific labeled antibodies: neurogenesis with anti- β3 tubulin and osteogenesis with anti-runx2. An example of direct effect on the fate of adult stem cells is the sensitivity of these cells to their neuronal differentiation in contact with a soft matrix (0.1-1 kPa). Whether this takes place or not depends on the biomolecule grafted onto the surface of this matrix. We grafted RGD peptide or BMP-2 mimetic peptide (3) on these soft matrices (3.21kPa) and we observed that stem cells have become different after 96h of culture.

Conclusion

The results of this study suggest that “precommitting” stem cells to a specific lineage via in vitro matrix conditions is multi-factorial.

Acknowledgments. This work was supported in part by the “Région Aquitaine” as well as the “Agence Nationale pour la Recherche” (ANR) and Advanced Materials in Aquitaine (GIS).

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Keywords. hydrogel, surface modification, stem cells, differentiation

(15.O4) HYDROGEL-BASED MICROFLUIDICS FOR TISSUE ENGINEERING

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Introduction. One of the major limitations in tissue engineering is the lack of proper vascularization. Nowadays skin and cartilage grafts are successfully used in-vivo mainly thanks to their low requirement for nutrients and oxygen that can be met by the host's vascularization. However this approach fails when applied to complex and massive tissues. The formation of new blood vessels is indeed a slow phenomenon and the deficiency of oxygen and nutrients supply rapidly cause widespread cell death in the graft's core. With the aim to overcome this hindrance we developed an innovative technique based on sacrificial elements. In this approach fluidics channels are deeply embedded within the hydrogel scaffold in order to favor biomimetic synthetic vasculature generation.

Methods. The sacrificial structure of polysaccharides is fabricated by injection molding. Murine fibroblasts (NIH-3T3) are encapsulated into a liquid hydrogel matrix (PEGDA-RGDS) and cast around the sacrificial structure, suspended in a mold. After the UV hydrogel crosslinking, the sacrificial template is dissolved in PBS forming interconnected channels inside the cell-laden hydrogel. The construct is incubated and perfused with culture medium (DMEM) for three days. Live/Dead assay is performed for cell viability analysis. HUVEC cells are then cultured in microchannels and CD31 fluorescence staining is performed.

Results. In the core sections of cell-laden hydrogel, cultured in static condition, the cell viability decreased with time, achieving cell death after 72 hours of in vitro culture. In perfused microfluidic hydrogel, cell viability is significantly higher than in static control (Fig. 1). CD31 staining confirmed rudimental endothelial tubule formation.

Conclusion. This technique allows a high diffusion rate of nutrients and oxygen throughout the hydrogel scaffold. Further developments are required to generate a “biomimetic synthetic vasculature”, mostly combining prefabricated vessels with controlled blood vessel–recruiting growth factors to induce growth of functional vascular network.

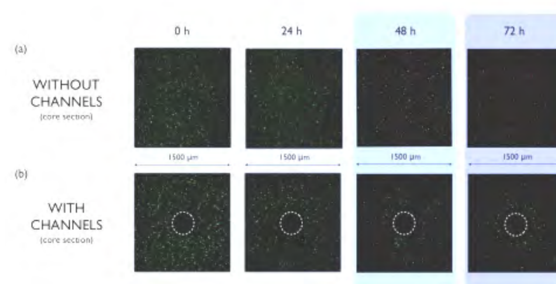

Figure 1. Confocal micrographs of the central section of the scaffold without (a) and with (b) culture medium perfusion. In green, living cells; in red, dead cells. The dashed circle indicates the perfusion channel

(15.O5) PRODUCTION OF ENGINEERIZED ALGINATE BASED MICROCAPSULES FOR CELL IMMUNOISOLATION CONTAINING EXTRACELLULAR MATRIX COMPONENTS

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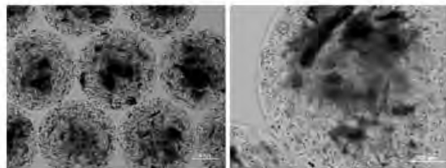
This paper reports the production of alginate microcapsules, with highly controlled morphological and dimensional properties, intended for cell encapsulation and tissue engineering applications. In an attempt to reconstitute the cell environment in a immunoisolating device for cell immobilization, we entrapped a powder form of ECM, isolated and purified from urinary bladder (Urinary Bladder Matrix, UMB), together with living cells (primary cells), in alginate based microcapsules. The aim was to demonstrate that UBM powder can produce an

optimal substrate for in vitro culture, possibly ameliorating the viability and functions of the co-entrapped cells. In particular, the combined use of alginate and urinary bladder matrix resulted in a synergic activity of both materials. On one side, the engineerized microcapsules offer the mechanical and material properties of alginate, which can be, in addition, varied through "on demand" gelling procedures. On the other side, UBM provides an array of bioactive functions that ameliorate the viability and functions of the co-entrapped cells. Taking these features into consideration, alginate microcapsules were applied to primary cells encapsulation as potentially immunoprotective barrier material and extracellular matrix (UBM) was immobilized, into the alginate microcapsules, to promote cells survival and function within the encapsulation microenvironment. We demonstrated that the incorporation of UBM powder does not alter significantly the morphological and dimensional characteristics of the microparticles (see Fig 1) and the presence of the co-entrapped UBM promote cell viability and function. In conclusion, the engineerized microcapsules, here presented, may represent a novel approach to enhance immunological acceptance and to implement viability of the entrapped cells for tissue engineering applications.

Keywords. Hydrogel, urinary bladder, microcapsules



(15.O6) FORMATION OF HARVESTABLE CELL AGGREGATES IN RESPONSIVE HYDROGEL MICROWELLS FOR HIGH-THROUGHPUT SYSTEMS

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Fabrication of cell aggregates and their high-throughput analysis are potentially useful for stem-cell engineering, tissue engineering, and drug discovery applications. High-throughput systems require stable aggregate formation to prevent deformation in cell clusters under flow and agitations and their retrieval for further use and analysis. Soft lithographically fabricated hydrogel based microwell structures were proven to be useful tools for aggregate formation and integration in high-throughput systems. Previously, glass bottomed poly(ethylene glycol) microwell arrays were offered to stably form the cell aggregates, though it was hard to harvest the aggregates from these templates without using digestive enzymes or physical forces which can potentially deform cell clusters. In this study, we fabricated glass bottom thermo-responsive microwells to generate cell aggregates on adhesive substrates and harvest them from microwells by utilizing the temperature dependent swelling property of responsive hydrogel. Temperature mediated swelling of the responsive polymer regulated microwell shapes applying mechanical forces on cell clusters which subsequently allowed their ejection from microwells. Given their ability to stably form aggregates and facilitate their further retrieval, these thermo-responsive microwell arrays can be potentially useful for stem cell biology, modular tissue engineering, and drug discovery and be applicable in high-throughput screening systems.

Keywords. Cell aggregates, responsive hydrogels, microwell arrays, high-throughput systems

(15.O7) ENGINEERING A MSC SEEDED FIBRIN HYDROGEL CONTAINING TGF-BETA 1 LOADED GELATIN MICROSPHERES FOR CARTILAGE REPAIR

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Introduction. Articular cartilage has a limited capacity for repair. Tissue engineering using mesenchymal stem cells (MSC)seeded within hydrogels has been promoted as a potential solution to repair cartilage defects. A central challenge with such an approach is creating an appropriate biochemical environment to allow MSCs to undergo chrondrogenic differentiation following implantation. The objective of this study is to develop a MSC seeded fibrin hydrogel containing TGF-β1 loaded gelatin microspheres to allow a controlled release of growth factor over a prolonged period.

Methods. Microspheres of diameter 50-100 µm were manufactured and loaded with TGF-β1. MSCs derived from porcine infrapatellar fat pad (IFP) were used for this study. Fibrin was prepared by mixing thrombin and fibrinogen to give a final fibrin concentration of 50mg/ml. The microspheres and cells were suspended throughout the fibrin hydrogel prior to gelation. The release of TGF-β1 over a 21 day culture period was measured using an ELISA. Chondrogenesis was examined by measuring sGAG production using Alcian blue staining and a DMMB assay and collagen production using picro-sirius red staining and a hydroxyproline assay.

Results. A sustained release of TGF-β1 over the 21 day culture period was observed (Fig.1a). Release tended to be lower in hydrogels seeded with cells than those without cells. GAG and collagen accumulation was significantly higher after 21 days in hydrogels containing TGF-β1 compared to hydrogels with TGF-β1 free microspheres (Fig. 1b).

Conclusions. It has been demonstrated that TGF-β1 loaded gelatin microspheres embedded in a fibrin hydrogel enabled the controlled release of growth factors capable of inducing chondrogenesis of IFP derived MSCs and hence promoting the release and accumulation of cartilaginous extracellular matrix components within the hydrogel. We believe these advanced hydrogel systems have the potential to be used for cartilage defect repair.

Acknowledgements. Funding provided by the European Research Council.

Keywords. Hydrogel, stem cells, cartilage, growth factors

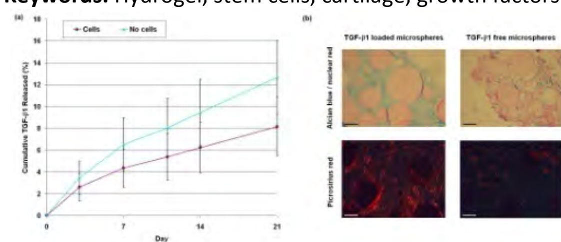

Figure 1: (a) Culumlative release of TGF-beta 1 as a percentage of the total TGF present in the microspheres in hydrogels with and without cells; (b) sections of hydrogels with TGF loaded and TGF free microspheres stained with alcian blue for GAG and picrosirius red viewed under polarized light for collagen (scalebar = 50µm)

(15.O8) EVALUATION OF POLYELECTROLYTE BASED SCAFFOLDS FOR MSCs HEART THERAPY

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Introduction. The aim of this work is to engineer biocompatible materials to improve the efficiency of cell therapy in the treatment of myocardial ischemia. Injection in the damaged organ of mesenchymal stem cells (MSCs) is already used as a therapeutic strategy subsequently to infarction and reperfusion. Unfortunately the therapeutic benefits are limited by early cell death in the first three days after graft. A tailored scaffold, able to improve cell survival and efficiency by providing to MSCs a biomimetic and protective environment would be of great interest to encapsulate and localize transplanted cells near the injury site, promote their viability and paracrine activity. Among all, the porosity of the scaffold appears as a key parameter to control MCSs survival and fate post implantation. Scaffolds three-dimensional structure and mechanical resistance may also play a major role in cell attachment and commitment. In this context, generating 3-D patches based on polyelectrolyte complexes (PEC) seems to be promising.

Materials and Methods. Ultrapur alginate and medium Mw chitosan were used to generate patch scaffolds of 10mm diameter and 2mm thickness. Interaction between polymers chains was studied by confocal microscopy. Matrices were characterized in terms of microstructure, porosity, swelling and mechanical properties. Scaffolds with acceptable physico-chemical properties were then loaded with human MSCs and tested in vitro. hMSCs viability, functionality and attachment were evaluated.

Results. Scaffolds exhibiting various Chitosan / Alginate ratios were prepared. Whatever A/C ratio, scaffolds exhibiting an interconnected porosity (average pore size 100 µm), with maintained biocompatibility, were obtained. Results clearly showed that chitosan addition improved scaffolds adhesivity and mechanical properties. Moreover, MSCs cytoskeleton organization revealed better cell attachment.

Conclusion. This study demonstrates the interest of using PEC to generate porous scaffolds for mesenchymal stem cell delivery on ischemic myocardium. In vivo tests are currently under investigation in our laboratory.

Keywords. Polymers, mesenchymal stem cells, myocardial infarction

(15.O9) OPTIMISING MICROGEL NICHES TO INFLUENCE MESENCHYMAL STEM CELL DIFFERENTIATION

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Introduction. Low back pain is associated with degeneration of the intervertebral disc (IVD) and affects the quality of life in our society. Cell therapy of the IVD is limited by the lack of appropriate cell sources, thus appropriate strategies for the differentiation of stem cells to nucleus pulposus (NP) cells-like phenotype have to be found. In the native IVD, NP cells are found sparsely in spherical microenviroments of coll II and proteoglycans that are known to influence the differentiation of stem cells. It is hypothesized that, spherical niche-like structures composed of type II collagen (coll II) - hyaluronan (HA) will mimic the NP microenvironment and promote the differentiation of adipose derived stem cells (ADSCs) to an NP cell-like phenotype. The specific objective of the study is to create the optimal microenvironment to promote the differentiation of ADSCs by varying coll II/HA concentration, cell density and amount of crosslinking.

Material and methods. Microgels were created by mixing coll II in different concentrations with HA at a ratio of 9:1 respectively. Cells (ADSCs or NP) were encapsulated within the hydrogels varying their density (105-107/mL). Different concentrations of a (ethylene glycol)-based crosslinker were mixed to the solution with coll II/cross-linker ratios (1:1, 1:2, 1:4). The hydrogels were then deposited on a hydrophobic surface to create a spherical shape and incubated for 1h at 37°C. The hydrogels were maintained in culture for 14 days before assessment of cell viability, GAGs synthesis and gene expression.

Results. The viability of both NP cells and ADSCs is maintained after encapsulation. The characterization of NP cells revealed high GAGs and coll II expression. These results show that the niche-like structure of microgels and their composition are able to maintain the NP cells' phenotype, and therefore this is a promising strategy for the induction of NP-like differentiation of ADSCs.

Acknowledgement. European Commission under the DISC REGENERATION project (NMP3-LA-2008-213904).

Keywords. Intervertebral disc, Adipose derived stem cells, Cell delivery, niches

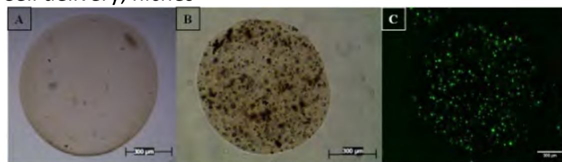

Figure 1:a) Spherical microgel fabricated by crosslinking of type II collagen/hyaluronan; b) ADSCs encapsulated in a spherical microgel; c) calcein dye shows the viability of ADSCs encapsulated in spherical microgel.

(15.O10) ROLE OF GLYOXALASE 1 IN DEFECTIVE ISCHEMIA-INDUCED NEOVASCULARIZATION IN DIABETES

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Introduction. Vascular dysfunction caused by diabetes leads to tissue ischemia and impaired wound healing. This study examines possible links between the diabetic condition, the defect in circulating progenitor cells (CPCs) and the lack of angiogenesis. In diabetes, methylglyoxal accumulation reduces the hypoxia-inducible factor 1-dependent expression of angiogenic genes, thereby inhibiting neovascularization. We hypothesize that this defective neovascularization can be reversed by increasing the activity of glyoxalase-1 (GLO1), which metabolizes methylglyoxal.

Methods. Bone marrow (BM) cells were extracted from control mice (C57/BL6) and mice that overexpress human GLO1 (hGLO+), and transplanted into irradiated control mice (+streptozotocin-induced diabetes). Hindlimb ischemia was induced, CPC mobilization was analyzed by flow cytometry, perfusion was analyzed by laser Doppler, and immunohistochemistry and cytokine arrays of tissues were performed.

Results. Compared to baseline, the number of mobilized angiogenic CXCR4+ CPCs increased 2.4-fold in mice with hGLO1+ BM cells at 1-day post-ischemia, versus a 1.1-fold change in control mice ($p<0.05$). Total mobilization of CPCs (2.3-fold increase) was greater in mice with hGLO1+ BM cells by day 7 compared to controls (0.9-fold; $p=0.04$). The tissue level of vascular endothelial growth factor was 1.33-fold greater in mice with hGLO+ BM cells vs. control diabetic mice. Vascular density and incorporation of CPCs into vasculature was greater in mice with hGLO1+ BM cells compared to wild-type mice, as determined by staining for von Willebrand factor (endothelial cells) and α-smooth muscle actin (arterioles). In addition, reduced perfusion (ischemic/non-ischemic ratio) was unchanged in control mice after 2 weeks (49±4%), but was restored in mice with hGLO+ BM cells (84±13%; $p=0.02$).

Conclusion. This evidence suggests that GLO1 is a potential target to restore CPC function and neovascularization in diabetes.

Keywords. Glyoxalase-1, diabetes, hindlimb ischemia, neovascularization

(15.O11) MULTI-MATERIAL PRINTING FOR HETEROGENEOUS TISSUE SCAFFOLDS

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Introduction. Alginates have been widely applied as hydrogel synthetic extracellular matrices (ECMs) in wound care due to their gelatin property during in contact with body fluid. Due to short biological half life, potential carcinogenesis risk and lack of tissue selectivity, release kinetics of proteins and growth factors needs to be controlled temporarily and spatially. This research aims to develop heterogeneous wound scaffolds with localized control of release kintics of active materials. Pressure assisted multi-chamber single nozzle deposition system is used to fabricate wound scaffolds with multi-material.

Materials and Methods. Sodium alginate from brown algae and calcium chloride were purchased from Sigma-Aldrich. Nozzle tips for dispensing systems were purchased from EFD. 3%- 4.5% (w/v) alginate solutions with different concentration and colors were prepared and loaded into Chamber A and Chamber B in the fabrication unit respectively. Solutions were deposited through multi-chamber single nozzle dispensing system with 250 μm nozzle tip. Calcium chloride solution with 0.6% (w/v) DI water then dispensed onto printed alginate structure through another nozzle for crosslinking purpose.

Results. Wound image of a pressure ulcer from [5] is processed in Image J software (See Fig. 1(a)). The wound geometry is then inputted into feature-based 3D blending process to generate heterogeneous wound scaffolds with uniform regions (in Fig. 1(b)). In Fig. 1(c), a concentration profile is shown as a continuous function increasing from 3% to 4.5% assumed to follow tissue engineering and wound healing needs. Finally, heterogeneous scaffold shown in Fig. 1(d) is printed with four regions.

Discussion and Conclusions. Heterogeneous wound scaffolds with varying material concentration is designed and fabricated in a way that wound healing process can be synchronized with release kinetics of any loaded proteins.

Acknowledgement. This research is supported partially by DoD, U.S. Army Medical Research Grant #: W81XWH-05-1-0401.

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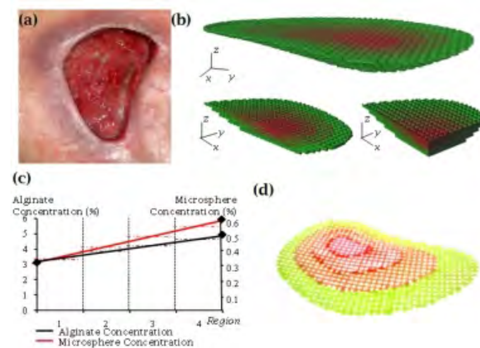
Keywords. Scaffold printing, alginate, heterogenous scaffold



(15.O12) CONTROLLED RELEASE OF STROMAL CELL-DERIVED FACTOR-1 FOR ENHANCED PROGENITOR CELL RESPONSES IN ISCHEMIA

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Introduction. Following an ischemic event, the body releases stromal cell-derived factor-1 (SDF-1) in an effort to recruit CXCR4+ circulating progenitor cells (CPCs) to injured sites; but this is insufficient for effective repair. The current study aims to enhance this endogenous response by injecting a collagen matrix with SDF-1 releasing microspheres into ischemic muscle.

Methods. Alginate microspheres (+/- SDF-1) were created using a spray gun/air compressor. Matrix was created by blending collagen I and chondroitin sulfate on ice, and cross-linking with EDC/NHS. CPCs were isolated from healthy human donors, and cultured in the presence of blank or SDF-1-loaded microspheres. Rheology and SDF-1 release was assessed for matrices +/- SDF-1 microspheres. Femoral arteries of mice were ligated, and animals received intramuscular injections of: PBS, matrix, or SDF-1-matrix. CPCs and hindlimb perfusion were assessed over 2 weeks. After sacrifice, hindlimbs were assessed for arterioles, CXCR4+ CPC engraftment, and cytokine profiles.

Results. Adding microspheres increased matrix viscosity by 17%, and prolonged SDF-1 release from approximately 1 to 10 days. SDF-1 microspheres were bioactive; 2.3- and 3.2-fold more CPCs were adhesive and migrative in their presence, respectively, compared to blank microspheres. From days 1-14 post-ligation, SDF-1-matrix treatment increased flk+ CPCs; and earlier and later time points saw respective increases in CXCR4+ and c-kit+ CPCs. At 14

days, matrix and SDF-1-matrix treatments restored hindlimb perfusion, and SDF-1 treatment increased arteriole size by 2 -fold. Matrix and SDF-1-matrix treatments recruited 2.5- and 4.5-fold more CXCR4+ cells, respectively, compared to PBS. SDF-1-matrix treatment also reduced inflammatory cytokines IL-1α, MIP-3α, and increased angiogenic cytokines IGF-1, bFGF. For all results, $p<0.05$.

Conclusions. We have exploited the SDF-1 axis to increase CPC activity following an ischemic event. Injection of a matrix with SDF-1 microspheres allows for controlled SDF-1 release, recruitment of CPCs, increased vascularity, and restoration of perfusion.

Keywords. Angiogenesis, Circulating Progenitor Cell, Ischemia, Stromal Cell-Derived Factor-1

(15.O13) MULTIGRADIENT HYDROGELS TO DECODE EXTRINSIC REGULATION OF HEMATOPOIETIC STEM CELL FATE

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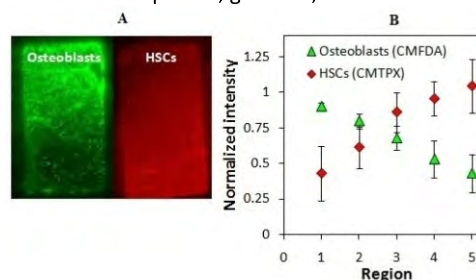
Introduction. Hematopoietic stem cells (HSCs) are responsible for the generation of all blood and immune cells of the body. HSCs are primarily found in specific microenvironments (niches) within the bone marrow. The HSC niche, composed of other cell types, the ECM and soluble biomolecules, is thought to provide extrinsic signals that influence HSC fate decisions. However, little is known about the mechanisms that underlie niche regulation. Here, we develop novel 3D biomaterial systems that mimic aspects of the complex niche microenvironment in order to systematically assess the influence of cell-cell interactions on HSC fate.

Methods. We create multiple opposing gradients of cells and/or hydrogel biomaterials in a novel multi-gradient microfluidic chamber (~180 uL volume) to encapsulate HSCs and Osteoblasts (a putative niche cell) in a collagen hydrogel. The HSCs are isolated as Lin-c-kit+Sca-1+ from murine bone marrow. Discrete sections within this chamber contain defined ratios of HSC:niche cells that can be isolated to probe HSC biology using tools such as surface antigen expression, MTS assay, gene expression and functional assays.

Results. We have successfully created opposing gradients of fluorescent microbeads (1 µm dia. FluoSpheres, Invitrogen), osteoblasts and HSCs:osteoblasts (Figure A) in distinct collagen suspensions (1 – 2.5 mg/mL). We have used fluorescent image scanning as well as flow cytometry and imaging of discrete regions to quantify the resultant gradients (Figure B). We hypothesize that co-modulating the local niche cell and hydrogel densities while applying known biomolecules of the HSC signaling cascades will enable us to understand and quantify direct vs. indirect (paracrine signaling) interactions between niche cells and HSCs.

Conclusion. This project will develop transformative, high throughput tools to systematically explore the significance of cell-based cues on HSC fate and provide significant new insight into the relationship between extrinsic cues and internal signaling cascades regulating HSC biology.

Keywords. Hematopoietic, gradient, biomaterial



(15.O14) IN VIVO EVALUATION OF ANGIOGENIC FACTORS IN A COLLAGEN-CHITOSAN MATRIX AS A POTENTIAL ISLET TRANSPLANT SITE

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Introduction. Islet transplantation for the treatment of type I diabetes often fails due to a lack of proper blood supply to support islet survival at the transplant site. Endothelial progenitor cells (EPCs) promote angiogenesis while collagen matrices can promote the homing of functional EPCs. Adding chitosan to these collagen matrices improves matrix stability and enhances their ability to stimulate angiogenesis in vitro. In vivo data suggests that collagen-chitosan matrices better stimulate vascular growth;1 however, the mechanism(s) responsible, such as the expression of pro-angiogenic growth factors were not evaluated. In the current study, collagen and collagen-chitosan matrices +/- EPCs were tested for their ability to promote pro-angiogenic cytokines in vivo and viability of islets cultured in vitro.

Methods. Human peripheral blood mononuclear cells were seeded onto fibronectin-coated tissue culture polystyrene for 4d to select for EPCs. Collagen and 10:1 collagen:chitosan matrices +/- EPCs were subcutaneously implanted into the backs of nude mice (n=4). After 14d, the explants were analyzed using RayBiotech® Mouse (Angiogenesis) Cytokine arrays. Neonatal pig islets were harvested from the pancreas and cultured in islet media, collagen or collagen-chitosan matrices for up to 7d.

Results. Both matrices promoted cell infiltration. Twenty-one pro-angiogenic cytokines were significantly stimulated in the collagen-chitosan matrix compared to collagen matrices (+/- EPCs; $p<0.05$) including VEGF which has been shown to be important for promoting islet vascularization and function post-transplantation.2 Pro-angiogenic factors monocyte chemotactic protein-5, eotaxin and keratinocyte chemoattractant were also stimulated. In vitro, neonatal islets in the collagen-chitosan matrix showed similar responses to controls.

Conclusion. The collagen-chitosan matrix promotes production/retention of pro-angiogenic cytokines compared to collagen-only matrix, which may contribute to the increased vascularization observed in vivo using these matrices. Therefore, the collagen-chitosan matrix warrants further evaluation in islet transplantation models.

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Keywords. Collagen, chitosan, EPC, islet, diabetes

(15.O15) MICRO-ENGINEERING VASCULAR-LIKE STRUCTURES BY MEANS OF ELECTROCHEMICAL CELL PRINTING IN PHOTO-CROSSLINKABLE HYDROGELS

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Introduction. A key challenge in engineering functional tissues in vitro is reproducing the complex in vivo 3D micro-architecture, in particular the controlled generation of vascular networks. In this work we propose and characterize a new tissue micro-engineering approach, relying on the combination of electrical cell printing and hydrogel photo-patterning techniques, to fabricate vascular-like structures.

Materials and Methods. Human umbilical vein endothelial cells (HUVECs) were seeded on gold substrates previously modified with self-assembled monolayers (SAM) of an RGD containing oligopeptide via electrically cleavable gold-thiolate bonds. Cell transfer to photo-crosslinkable gelatin methacrylate (GelMA) hydrogels was then investigated, w or w/o electrical potential application, at 16/72 h of culture. Vascular-like structures were generated by culturing HUVECs on SAM-modified gold rods and transferring the resulting tubular monolayers to the inner surfaces of micro-channels in plain or cell laden GelMA hydrogels.

Results. Cell transfer efficiency to GelMA at 16 h of culture, both w/ and w/o electrical potential application, was close to 100%. At 72 h of culture, cell transfer w/o potential application was decreased to ~30%, whereas electrical potential application improved it to ~86% and allowed to maintain VE-Cadherin cell-cell interactions in transferred HUVEC monolayers .

With this approach 600-µm-diameter capillary-like structures were fabricated in hydrogels, which remained persistent in perfusion culture for at least 15 d. As a first step toward more complex and in vivo-like structures an additional cell laden hydrogel layer was photo-patterned around the HUVEC channel mimicking the vascular smooth muscle cell layers.

Conclusions. Our results show that coupling electrical cell printing and photo-patternable GelMA hydrogels is a fast and reliable method with great potential for devising micro-engineered endothelialized 3D vasculature for regenerative medicine or in vitro drug screening applications.

Acknowledgments. Authors acknowledge support provided by the NIH, NSF and by the Progetto Rocca.

Keywords. Oligopeptide self-assembled monolayers, methacrylated gelatin tissue engineering

(15.O16) INNOVATIVE CROSSLINKED GELATIN HYDROGELS AS KEY SCAFFOLDS FOR ADIPOSE STEM CELLS DIFFERENTIATION

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Introduction. Gelatin-based hydrogels may find application in drug delivery, wound dressing and as scaffolds for tissue regeneration. The aim of this work was to design hydrogels in which gelatin was covalently crosslinked with a synthetic component, so to conjugate the ability of gelatin to promote cellular adhesion with an adequate mechanical stability.

Materials and Methods. Gelatin A (from bovine skin, Sigma) was crosslinked by Michael-type addition with methylene-bis-acrylamide (MBA). The efficiency of the reactions was evaluated with FTIR spectroscopy. Swelling and weight loss were studied in distilled water and phosphate buffered saline (PBS, pH = 7.4) at 37°C. Mechanical properties of the swollen samples were assessed in an unconfined cyclic frequency sweep and stress relaxation-recovery compression tests. Cytotoxicity was evaluated in vitro by an indirect contact test with L929 fibroblasts and cell viability of adipose mesenchimal stem cells (provided by Istituto Nazionale dei Tumori, Milano, I) cultured onto the hydrogels for 7 days was assessed by optical microscopy and MTT assay and their differentiation into adipocytes was examined by oil red O staining.

Results and Discussion. All the synthesized hydrogels were stable in distilled water and PBS up to 35 days. The hydrogels exhibited E' values in the range 2.5 – 23 kPa. Stress relaxation-recovery tests evidenced the predominance of elastic behavior on the viscous one. No release of cytotoxic degradation products was detected up to 7 days with L929 cells. Oil red O staining showed a differentiation of ASCs in adipocytes onto all the prepared hydrogels (Fig.1).

Keywords. Gelatin hydrogels, mechanical properties, adipose tissue regeneration

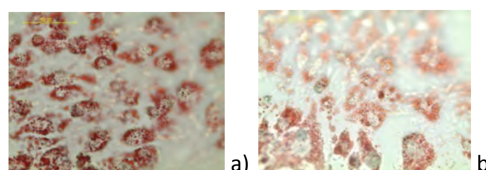

Figure 1 – Oil red O staining of ASCs differentiated into adipocytes onto two gelatin/MBA hydrogels

(15.O17) SELF-GENERATED CONSUMPTION GRADIENTS BY STEM CELLS IN 3D DETERMINE ANGIOGENIC SIGNALLING

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The specific O2 environment in which cells reside affects their phenotype and signalling. Stem cells in the bone marrow cavity typically reside in O2 tensions of between 1-5%. Most in vitro cell culture is done in O2 tensions of around 21%, which can translate as oxidative stress to a cell. By culturing cells within 3D, we have previously reported O2 consumption gradients from the surface to

the core, ranging from between 18-3%1. We have now tested the effect of O2 gradients (3-18%) generated by stem cells within these tissue models and its effect on generating the angiogenic signalling cascade in distinct locations with 3D constructs exposed to specific O2 levels. We found that vascular endothelial growth factor (VEGF) expression was up regulated earlier in core cells (3% O2) (significantly increased by day 4) compared to surface cells (18%O2), where no significant increase in VEGF was measured up to 6 days (figure 1). This pattern was also similar for Hypoxia-Inducible Factor Iα (HIF-Iα) and Platelet derived growth factor (PDGF), both critical factors in the angiogenic cascade. Low O2 did not have an effect on cell viability tested up to 6 days. We therefore conclude that Stem cells when, exposed to 3-5% O2 optimally up-regulated the three angiogenic factors tested within 4 days, whilst still maintaining cell viability. In stem cell based therapies, where angiogenic response is critical (e.g. non-union fractures where stem cell therapies are being tested and delayed fracture healing where angiogenic response is poor) using hypoxia to generate these angiogenic cascade signals in vitro prior to in vivo implantation has potential therapeutic benefits.

References: 1. Cheema, U. Brown, R.A. Alp, B. MacRobert, A.J. (2008). 'Spatially defined oxygen gradients and VEGF expression in an engineered 3D cell model.' Cell. Mol. Life Sci. 65(1): 177-186

Keywords. Stem Cells, Collagen type I scaffolds

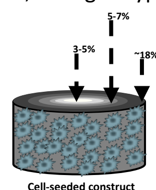

Figure 1. O_2 gradient formation in spatially distinct regions o seeded construct, with VEGF signalling over 6 days.

(15.P1) STUDY OF BIOMATERIAL-HEPATOCYTE CONSTRUCTS: A FUNDAMENTAL BASIS FOR THE DEVELOPMENT OF BIO-ARTIFICIAL LIVER DEVICES

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Introduction. Artificial liver support systems are urgently needed as bridge to liver transplantation or regeneration. The search for a bio-artificial system is hampered by the difficulty to keep hepatocytes fully functional and differentiated, once isolated from the liver. The answer to this problem might lay in the biomaterial that is used for cultivation or encapsulation of the cells. Our goal is to compare different biomaterials, modifications and cell-packing methods, and to select a material and set of conditions that is biocompatible and allows hepatocytes to maintain full differentiation.

Methods. HepG2 cells are encapsulated in different biomaterials and viability is followed over time via standardized viability assays such as MTT assay, calceïne/propidium iodide staining. In case of acceptable viability, hepatocyte phenotype is checked via immunohistochemistry stainings, collimetric assays and ELISA techniques. Based on the results, the biomaterials are evaluated and adapted if necessary.

Results and conclusions. Different equivalents and w/v% of metacrylate-modified gelatin were, and are currently, tested. Until now, the gelatin-metacrylate (1 eq., 10 w/v%) shows the best results for the encapsulation of HepG2 cells with a viability of about 70 %, compared to the control culture. The encapsulated cells still show storage of glycogen, expression of HNF4α and albumin but, in general, less than the control culture. Currently we are investigating whether additional modifications with galactose could improve the phenotype. When encapsulated in bismetacrylate-modified pluronic F127, calceïne/PI staining of the HepG2 cells suggests problems with membrane integrity, while mainly death cells were seen when cells were encapsulated in bisacrylate-modified pluronic F127. When cells were encapsulated in pluronic-ALA-L , mainly living cells were observed. The current results show that small changes to biomaterial parameters have a great influence on the cell behavior and that it might be possible to find conditions where the cells maintain full differentiation.

Keywords. Gelatin, pluronic, hepatocytes

(15.P2) TRABECULAR TITANIUM™ COMBINED WITH A CELLULOSE-BASED HYDROGEL AND HUMAN BONE MARROW STROMAL CELLS: AN INNOVATIVE STRATEGY TO IMPROVE IMPLANT OSTEOINTEGRATION

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Introduction. Titanium is widely used for several medical implants but the interface between implant and bone remains the weakest point during the initial healing period. To accelerate osteointegration we propose to use an amidated carboxymethylcellulose hydrogel (CMCA) seeded with bone marrow stromal cells (BMSCs) combined with Trabecular Titanium™, a material characterized by an innovative multiplanar hexagonal cell structure imitating trabecular bone. To validate this composite bioconstruct we determined BMSCs viability and osteogenic differentiation on CMCA and on Trabecular Titanium™ disks unloaded (TT) or loaded with CMCA (TT+CMCA, Fig.1a).

Methods. Human BMSCs were harvested from 9 donors (59±2 years), under written consent. BMSCs were expanded, seeded on CMCA, TT and TT+CMCA (7.5x10^5 cells/sample) and cultured in control (CTRL) or osteogenic (OSTEO) medium. After 7, 14 and 21 days on biomaterials, viability and proliferation of BMSCs was determined and alkaline phosphatase activity (ALP) was measured to evaluate osteogenic differentiation.

Results. CMCA well supported BMSCs growth during the whole culture period, with a significantly higher viability in OSTEO BMSCs compared to CTRL BMSCs up to 14 days of culture. After 14 and 21 days on CMCA, OSTEO BMSCs showed higher levels of ALP compared to CTRL BMSCs, demonstrating that culture on CMCA allowed cells to maintain their osteogenic potential. OSTEO BMSCs were able to grow on TT and TT+CMCA, maintaining a high viability for the entire culture. The presence of CMCA ameliorated the osteo-conductive properties of TT as

shown by increased ALP levels (+61%) after 21 days of culture for BMSCs on TT+CMCA compared to BMSCs on TT (Fig.1b).

Conclusions. Based on these results, the use of a composite construct as TT+CMCA represents a promising approach to deliver and retain autologous BMSCs at the implant site and to accelerate osteointegration.

Acknowledgements. This work was supported by Italian Ministry of Health (Project RF-IOG-2007-647233).

Keywords. Titanium, Stem cells, Hydrogel, Bone.

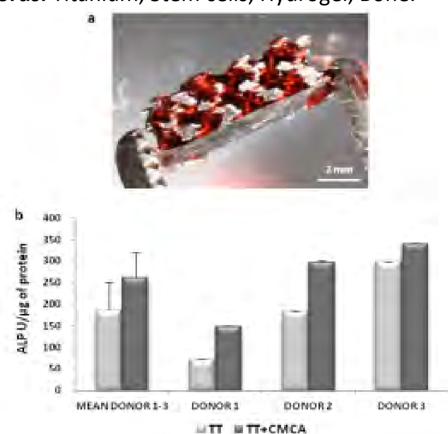

Figure 1: (a) Section of TT+CMCA colored with azorubine (CMCA stained in red); (b) ALP activity in OSTEO BMSCs cultured for 21 days on TT and TT+CMCA (ALP Units/µg of protein).

(15.P3) POTENTIAL OF A NEW TYPE OF LOW MOLECULAR WEIGHT HYDROGEL FOR IN VITRO CULTURE AND IN VIVO IMPLANTATION OF HUMAN ADULT STEM CELLS

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A new thermosensitive hydrogel based on low molecular weight Glycosyl-nucleoside-lipid (GNF) Units, has been recently synthesized via double and simple click chemistry approaches. This hydrogel is characterized by a self-assembly of amphiphile monomers which constitute a network of nanofibers in an aqueous environment. This hydrogel appears as a potential scaffold for cell culture and tissue engineering applications. Indeed, the incorporation of a fluorocarboned moiety as the hydrophobic part of the GNF has been shown to improve the cytocompatibility of this hydrogel. We investigated the possibility to use GNF hydrogels as a scaffold for adipose tissue stem cells (ADSCs) for in vitro culture and in vivo implantation. ADSCs are pluripotent mesenchymal stem cells isolated from adipose tissue and capable of differentiating into several cell types.

In vitro and in vivo assays showed the high stability of the GNF gel. Indeed, when implanted subcutaneously, GNF hydrogel was still present after four weeks. GNF hydrogel was then tested as culture support for human ADSCs. Cytotoxicity assays showed a good cytocompatibility. When isolated ADSCs were seeded either on the surface or within the GNF hydrogels, they were unable to adhere and spread normally, resulting in cell death after a few days. However, ADSCs cultured as spheroids trapped in the GNF gel demonstrated a long term survival of cells and stability of the spheroids. When ADSCs spheroids encapsulated in GNF hydrogels were implanted subcutaneously in mice, cells were shown to survive and to remain as clusters after at least one month. In contrast, spheroids implanted without gel were far less stable. In addition, the hydrogel was colonized by host cells and features of inflammation were observed.

Together, these data are encouraging to propose GNF hydrogels as a scaffold for tissue engineering and regenerative medicine applications.

Keywords. Stem cells, thermosensitive hydrogel

(15.P4) INFLUENCE OF BIOFUNCTIONALIZED PEPMHA HYDROGELS ON MESENCHYMAL STEM CELL CHONDROGENIC DIFFERENTIATION

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Introduction. The process of chondrogenesis within a healing articular cartilage lesion can be enhanced by regenerative signals provided to the site of articular cartilage repair. Several growth factors, such as the family of transforming growth factors-βs (TGF-βs), have shown to play an important role in the growth and differentiation of articular cartilage as well as in the chondrogenic differentiation of mesenchymal stem cells (MSCs). Semi-interpenetrated networks (SIPNs) of poly[2-ethyl(2-pyrrolidone) methacrylate] (PEPM) and hyaluronic acid (HA)- PEPMHA, are biocompatible and can be potential candidates as delivery vehicles for bioactive proteins. The presence of HA within this system could provide a favourable niche for MSCs chondrogenesis, as these cells express CD44, one of the primary receptors for HA. We investigated the use of PEPMHA biofunctionalized with TGF-β3 in the induction of MSCs chondrogenesis.

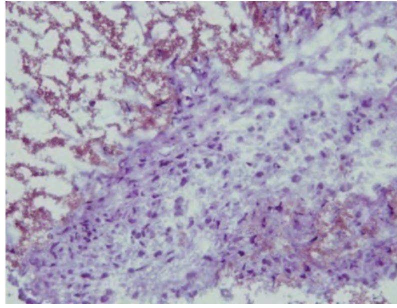
Methods. PEPMHA SIPNs were produced, using triethylene glycol dimethacrylate as crosslinker and K2S2O8 as initiator. Transforming growth factor-β3 was directly loaded into the PEPMHA hydrogels. Effects of growth factor release from the PEPMHA polymeric systems on the differentiation of the mesenchymal stem cells isolated from bone marrow stroma, previously expanded, were studied for different periods of time. The cell distribution, morphology, differentiation and extracellular matrix components deposition were determined by histological and immunohistochemical stainings.

Results. Highly swellable PEPMHA hydrogels present a porous structure that can be intimately related to their swelling character, endearing themselves as highly diffusible systems. After 21 days in culture, MSCs were able to proliferate and secrete an extracellular matrix incorporating type-II collagen and proteoglycans.

Conclusion. Biofunctionalization of PEPMHA hydrogels with TGF-β3 may constitute a valid alternative for pursuing future cartilage tissue engineering strategies by enabling the modulation of a chondrogenic differentiation process.

Acknowledgements. The authors would like to acknowledge CIBER-BBN network and MICINN-PLAN E, for providing financial support and BIOIBERICA for HA supply.

Keywords. Hyaluronic Acid, Bone Marrow derived mesenchymal stem cells, growth factors release



(15.P5) HYPOXIA IS INFLUENCED BY CYCLIC MECHANICAL LOADING IN EARLY FRACTURE HEALING – AN IN VITRO ANALYSIS

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Introduction. Mechanical loading after fracture has an influence on the healing outcome. The fracture damages the vascular system and impairs the oxygen supply in the fracture hematoma. With the lack of oxygen a hypoxic environment develops which may lead to a delayed healing. The aim of this study was to analyze the influence of cyclic mechanical loading on the oxygen tension with an in vitro setup.

Methods. Human mesenchymal stem cells were embedded in fibrin hydrogel constructs mimicking the fracture hematoma and placed in a bioreactor system. Cyclic loading, adjusted to the one experienced during a patients gait, were applied to an in vitro culture system. The hypoxic volume inside the constructs was visualized by immune histological staining based on a hypoxic marker applied to the culture medium. Additionally, oxygen tension was quantified by an optic-chemical fiber-sensor placed in the center of the cell construct.

Results. It was observed that mechanical stimulation has a positive effect on the oxygen concentration in the fibrin construct. Cyclic compressed constructs qualitatively exhibited in histological staining a smaller hypoxic region compared to not stimulated controls. This observation was confirmed by quantitative data recorded with an oxygen sensor. The oxygen tension increased in the construct with increasing duration of cyclic compression. Once stimulation was stopped, the oxygen tension decreased to the value of the unloaded constructs.

Conclusions. In the early fracture healing phase, hypoxia develops due to rupture of blood supply. Hypoxia is important for triggering angiogenesis, however, prolonged hypoxia might delay regeneration processes. Diffusion-based oxygen transport is limited. However, our investigations showed that an appropriate mechanical loading is able to actively transport oxygen into non-vascularized tissues and expand the supplied region.

Acknowledgements. We acknowledge financial support by Synthes GmbH.

Keywords. Oxygen tension, fibrin hydrogel, mechanical stimulation

(15.P6) BONE DEVELOPMENT WITH HUMAN HEMATOPOIETIC CELLS SEEDED ONTO A NATURAL POLYMER

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Introduction. Reconstruction of craniofacial bone defects caused by trauma and ablative oncologic procedures, or by congenital anomalies, is a frequent surgical challenge.

Objetive. This study evaluated the feasibility of creating tissue-engineered bone using an osteogenic unit with human stem cells (HSC) and demineralized bone seeded onto a dermis acellular as a scaffold.

Material and Methods. For the experimental group, critical bone defects were made surgically in the skull of athymic mice and repaired with the osteogenic unit in contact with the dura mater. The control group did not receive the osteogenic unit. Subjects were sacrificed at three months after implantation, the neotissue was morphologically and histologically evaluated using descriptive histology. Also, immunohistochemical analysis was performed to determine the presence of alkaline phosphatase, collagen I, osteopontin and osteocalcin. Mineral analysis was done using edax in order to evaluate the presence of the bone main minerals.

Results. In the control group no obvious bone formation was found. The tissue layer that covered the repaired defect in the experimental group with the osteogenic unit showed new bone tissue when analyzed with descriptive histology. There was a significant increase in the expression of phosphatase alkaline, collagen I, osteocalcin and osteopontin in the experimental group. Likewise, the amount of calcium, magnesium and phosphorous was statistically significant in animals implanted with the osteogenic unit, compared with the control group.

Conclusion. The grafts obtained in vivo through tissue engineering using adult stem cells seeded onto dermis acelullar, and demineralized bone, showed osteogenic properties with potential for many clinical applications.

Acknowledgments. This study was partially supported by a grant of CONACYT sectoriales 114359.

Keywords. Bone, tissue engineering, craniofacial defects, scaffold

(15.P7) GALACTOSYLATED CELLULOSIC SPONGE ACCELERATES HEPATOCYTE REPOLARIZATION

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Hepatocytes lose their differentiated functions rapidly upon isolation from their native niche in liver. One way to solve this problem is by achieving rapid hepatocyte repolarization in vitro. Culturing hepatocyte as 3D spheroid is one approach to induce hepatocyte repolarization due to increase in cell-cell contact. However, many current platforms are still taking time to repolarize (between 48-72 hours). Here we decipher our method by using hydrogel-based sponge conjugated with

galactose, which is known as hepatocyte chemical cues in forming spheroids, to rapidly aggregate hepatocyte upon seeding thus accelerating functional hepatocyte polarity formation.

Cellulosic sponges were synthesized from hydroxypropyl cellulose (MW 10,000), which was side chain-modified with allyl isocyanate and conjugated with β-galactose. The obtained product was then dissolved in water and gamma irradiated to crosslink the sponges.

Hepatocytes seeded in the sponge were monitored in terms of their aggregation process, polarity formation and cellular morphology.

Rat hepatocytes seeded in cellulosic sponge reorganize themselves to form 3D spheroids within 24 hours (figure 1b). Hepatocyte aggregation itself was observed as fast as 7 hours postseeding (figure 1a), while repolarization started at 16 hours postseeding as indicated by accumulation of fluorescein diacetate dyes in the regions between two neighboring cells (which is known to be bile canaliculi, figure 1d-f). The accumulation of the dyes at 48 hours shows stronger signal, revealing more functioning bile canaliculi. SEM images of hepatocyte spheroids after 24 hours postseeding decipher tightness of spheroids surface and inability to distinctively present the single cell boundary (figure 1c). This phenomena show the robustness of our cellulosic sponge to help in inducing hepatocye spheroids formations and restoring the hepatocyte repolarization quickly upon seeding. Other hydrogel to culture hepatocytes show improved hepatocyte differentiated functions maintenance but not readily induce rapid repolarization, which is important factor in preserving the differentiated functions.

Keywords. Hepatocyte polarity, scaffold, hydrogel

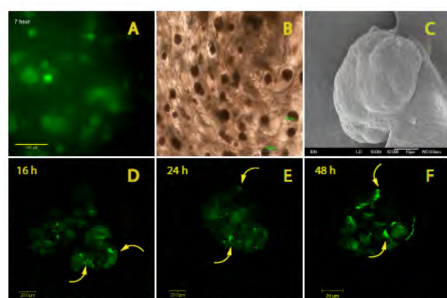

Figure 1. a) hepatocyte aggregations at 7 hours postseeding, b) hepatocyte spheroids formed 24 hours postseeding, c) SEM images of hepatocyte spheroids 24 hours postseeding, d-f) fluorescein diacetate excretion staining at 16, 24 and 48 hours postseeding

(15.P8) CONTROLLED RELEASE BY BIODEGRADABLE HYDROGELS (PLGA-PEG-PLGA TRIBLOCK COPOLYMER) ENHANCES THE BONE FORMATION OF BONE MORPHOGENETIC PROTEIN-2 ON CRITICAL-SIZE BONE DEFECT

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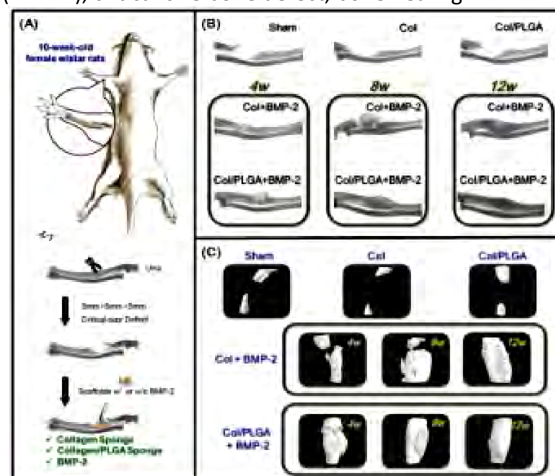
Background and Purpose. PLGA-PEG-PLGA triblock copolymer is a kind of thermal-sensitive hydrogel. In this study, we used this triblock copolymer as a drug carrier for control release. The recombinant human bone morphogenetic protein-2 (rhBMP-2), which was manufactured from Industrial Technology Research Institute, plays an important role in bone formation. The aim of this study is to prove that rhBMP-2 wrapped up in PLGA-PEG-PLGA hydrogel and trapped in type I collagen sponges have clinical potential on critical-size defect model.

Methods. 45 Wistar rats were assigned into five groups (n=9), and a 5 mm × 5 mm × 5 mm segmental bone defect in the ulnar shafts were created by surgery. The two experimental groups of type I collagen and type I collagen / PLGA-PEG-PLGA scaffolds received local injection of 8 μg rhBMP-2. The negative control groups received type I collagen and type I collagen / PLGA-PEG-PLGA scaffolds soaked with PBS only. Radiographic evaluation and histological stains were performed on bone healing and bone formation.

Main Results. The X-ray data indicated that rhBMP-2 directly guiding bone formation at the defect site. Through the micro-CT analysis, the data showed the percentage of bone volume (BV/TV) significantly increased in the experimental group, which implanted type Ⅰ collagen / PLGA-PEG-PLGA scaffolds / rhBMP-2 scaffolds at 4, 8 and 12 weeks.

Conclusions. Through this study, we demonstrated that the efficacy of type Ⅰ collagen / PLGA-PEG-PLGA scaffolds combining with rhBMP-2 has better osteoinductive effect and can promote fracture healing on radiographs and histological stains of an experimental critical-sized bone defect model. The results indicated that type Ⅰ collagen / PLGA-PEG-PLGA scaffolds / rhBMP-2 implants would provide the possible clinical applications in orthopedic surgery and regenerative medicine.

Keywords. PLGA-PEG-PLGA, bone morphogenic protein-2 (BMP-2), critical-size bone defect, bone healing



(15.P9) THERMORESPONSIVE POLY(N-VINYLCAPROLACTAM)-g-COLLAGEN: SYNTHESIS, CHARACTERIZATION, IN VITRO CYTOTOXICITY AND IN VIVO BIOCOMPATIBILITY EVALUATION

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Introduction. Thermoresponsive polymers have a variety of applications in medicine and biotechnology. They are characterized by sudden reversible phase separation with

an on-off switch using the transition between the extended and coiled forms of the polymer brushes in response to a certain temperature range. Poly(N-vinylcaprolactam) (PNVCL) is a non-toxic synthetic polymer with a LCST of ~32oC. Here, we synthesized PNVCL-g-collagen and PNVCL-g-chitosan, and evaluated some of the physical, chemical and biological properties of these conjugates.

Methods. Firstly, PNVCL-COOH was synthesized by free radical polymerization. Thereafter PNVCL-COOH was conjugated with the natural biopolymers using EDC and NHS. Phase transition temperatures were determined by measuring the optical transmittance at 480 nm over the temperature range of 20-50oC. The structures were characterized by FTIR and DSC. The swelling kinetics of the thermoresponsive biopolymers were determined. Cell attachment and growth on the conjugates was evaluated by MTT using rat bone marrow mesenchymal stem cell cultures (BM-MSCs). Finally, the histocompatibility of the thermoresponsive polymers was evaluated in the subcutaneous rat model.

Results. Formation of the copolymers were confirmed by FTIR. Both of the copolymers exhibited a temperature-dependent transition. The LCST values of the PNVCL-g-collagen and PNVCL-g-chitosan copolymers were found to be 35oC and 31oC, respectively. Water uptake experiments indicated that PNVCL-g-collagen was more hydrophilic than PNVCL-g-chitosan. MTT findings demonstrated that the copolymers supported the attachment and growth of the cells, and were basically not toxic to BM-MSC cultures. In-vivo studies were in line with the in-vitro studies, thus the histological analysis did not show any significant signs of inflammation and the conjugates were quite well tolerated by the subjects.

Conclusions. The water-soluble and non-toxic thermoresponsive polymer, PNVCL-g-collagen had a LCST value (35oC) closer to the physiological temperature. This feature may have an advantage in manipulating mammalian cell cultures that are more sensitive to temperature fluctuations

Keywords. Thermoresponsive polymers, poly(N-vinylcaprolactam)-g-collagen, cell sheet engineering, polymer brushes.

(15.P10) OSTEOBLASTIC DIFFERENTIATION OF BONE MARROW STROMAL CELLS WITH GFOGER PEPTIDE ON FIBRIN SCAFFOLDS

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Implants of scaffolds of several biomaterials like calcium phosphate slurries have been broadly used on diverse therapeutic protocols for severe bone tissue damage, acting not only as a mechanical support but promoting a short time cell migration process of resident cells form neighborhood healthy bone tissue into the implant, by other side it has been demonstrated that the use of collagen mimetic peptides whit sequences as GFOGER which act as integrins receptors enhance migration and proliferation. However osteoblastic differentiation do not occur over this kind of scaffolds. In this study we use an hydrogel of fibrin in combination with GFOGER peptide to induce osteoblastic differentiation on bone marrow stromal cells (BMSC).

Mice bone marrow were collected from tibia and femur of five individuals by experiment, cell suspension was pre cleared by 100 um filtration, seeded on DMEM/SFB 10% and cultured overnight permitting the adhesion of BMSC to the plate, myeloid and blood cells were discarded and adherent cells harvested and transferred into a fibrin solution supplemented with GFOGER peptide, finally fibrin solution was solidified with calcium chloride and the resultant hydrogel was cultured during 24 days on DMEM/dexamethasone/ascorbic acid. After culture period hydrogels were processed for transmission and scanning electron microscopy and paraffin histology.

After 14 days BMSC seeded on fibrin hydrogel with GFOGER shown a rounded morphology and their extra cellular matrix resembles osteocyte lacunae, also mineralization of extracellular matrix it was demonstrated by the histology stain of von Kossa. Finally both 14 and 24 day of culture on fibrin-GFOGER, BMSC derived osteoblastic-like cell were positive by immunohistochemistry for osteopontin, and pro-collagen type I.

Acknowledgegments. TH Raquel Guerrero Alquicira, M. en C. Patricia Bizarro Nevares, CONACYT 50396-M, DGAPA PAPIIT IN213510 e IN214109-3, Posgrado en Ciencias Medicas Odontológicas.

Keywords. Fibrin, BMSC

(15.P11) α-HELICAL PEPTIDE HYDROGEL MATRICES FOR 3D CELL CULTURE AND TISSUE ENGINEERING

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Introduction. Tissue engineering (TE) promises to regenerate a patient's own tissue via the delivery of scaffolds cells and biomolecules to the defect site. Recently, self-assembling peptide hydrogels have shown promise as TE scaffolds for such proposes. Potentially, these systems offer reduced complexity, allow iterative redesign and, ultimately permit recombinant production. The work presented here investigates an α-helical dual-peptide system (hSAFs) for the 3D cell culture of a range of different cell types, and evaluates the effect of covalently tethered RGD motifs on cell adhesion and migration [1].

Methods. Peptides were synthesised using standard solid-phase 9-fluorenyl-methoxycarbonyl chemistry, purified by reverse-phase HPLC, and confirmed by mass spectrometry. CD Spectra were recorded between 190 and 260 nm using a Jasco J-810 circular dichroism spectrometer. Fibres were imaged using negative stain transmission electron microscopy. For gelation experiments, 1 mM of each hSAF peptide was mixed on ice for 5 min then incubated for 25 min at room temperature, and overnight at 37 oC. PC12 cells were cultured for 14 days.

Results. The hSAF system comprises two peptides with coiled-coil repeats, which direct the assembly of a heterodimeric interface and leave exposed surfaces to promote weak hydrophobic interactions between fibrils. These design features were tested and corroborated

using a combination of CD spectroscopy, electron microscopy and rheology. The resulting hSAF hydrogels support the growth and differentiation of PC12 cells.

Conclusion. We present a new self-assembling peptide system, which forms hydrogels and has promise as scaffolds for 3D cell culture and TE applications.

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Keywords. Self-assembly, peptide, hydrogel

(15.P12) MODULATION OF INFLAMMATION TO ENHANCE BONE REGENERATION BY DUAL DELIVERY OF ANTI-IL-6 DRUG AND BONE MORPHOGENETIC PROTEIN-2 WITH GELATIN HYDROGELS

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Introduction. Inflammation is a body response necessary to start the natural process of tissue regeneration. However, excessive inflammation responses sometimes result in the suppression of regeneration process. In this study, bone regeneration in the conditions of pro-inflammatory cytokines (e.g. IL-6 and TNF-α) suppression was investigated to evaluate effect of inflammation modulation on the process of tissue regeneration.

Methods. Triptolide of an anti IL-6 drug was encapsulated in L-lactic acid-grafted gelatin micelles to allow it to solubilize in water. Gelatin with the water-solubilized anti-IL-6 drug in micelle was crosslinked by glutaraldehyde to obtain gelatin hydrogels incorporating anti-IL-6 drugs. After the subcutaneous implantation in C57BL/6 mice, the number of inflammatory cells and the expression of pro-inflammatory cytokine genes were evaluated by flow cytometry and real-time polymerase chain reaction (RT-PCR), respectively. The gelatin hydrogels incorporating anti-IL-6 drug combined with bone morphogenetic protein-2 (BMP-2) were implanted into the ulna critical-sized defects of Wistar rats to examine bone regeneration.

Results. The number of macrophages and neutrophils infiltrated around the gelatin hydrogels incorporating anti-IL-6 drug was significantly reduced comparing with that of the drug-free gelatin hydrogels. The implantation of gelatin hydrogels incorporating anti-IL-6 drug significantly down-regulated the expression of IL-6 and TNF-α genes, although the level was enhanced at higher drug doses. The combinational application of anti-IL-6 drug and BMP-2 in gelatin hydrogels resulted in an enhanced bone regeneration in the bone defect 4 weeks post-operatively. However, when the dose of drug and BMP-2 increased, no bone regeneration was observed.

Conclusions. An anti-IL-6 drug, Triptolide, showed a dose-dependent effect on the modulation of inflammation and bone regeneration. The gelatin hydrogels incorporating an optimal combination of anti-IL-6 drug and BMP-2 efficiently enhanced bone regeneration.

Keywords. Inflammation, anti-IL-6, BMP-2, bone regeneration

(15.P13) THE SIMULTANEOUS ENCAPSULATION OF HUMAN ADIPOSE DERIVED STEM CELLS AND TRANSFORMING GROWTH FACTOR β1 IN CARRAGEENAN HYDROGELS AS A NEW SYSTEM FOR CARTILAGE TISSUE ENGINEERING

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Introduction. The combination of hydrogels with stem cells and growth factors (GFs) became a promising approach to promote cartilage regeneration in the recent years. Previous studies have shown the ability of human adipose derived stem cells (hASCs) to differentiate towards a chondrogenic lineage when entrapped in a three-dimensional (3D) environment while Transforming Growth Factor-β1 (TGF-β1) is one of the main GFs influencing chondrogenic differentiation. Therefore, the combination of both components encapsulated in a temperature-responsive hydrogel is proposed in this study as a new system for cartilage tissue engineering (TE).

Methods. Carrageenan hydrogels (2 and 2.5% (w/v)) were prepared by ionic gelation with potassium chloride. In a preliminary study, ATDC5 cell line was encapsulated to analyze the biomaterial cytotoxicity and the influence of polymer concentration in cell viability and proliferation. Furthermore, the encapsulation of hASCs was performed together with the entrapment of TGF-β1 in the hydrogels networks. The cells and TGF-β1 were quickly mixed with the polymer solution around 40ºC and allowed to cool down until gelation occurred. Afterwards, the constructs were cultured in vitro up to two weeks in chondrogenic and basal mediums. The gels encapsulating only hASCs and cultured in chondrogenic medium were used as controls. The constructs were then characterized for cell viability, proliferation, histology and immunohistochemistry for cartilage specific markers.

Results. We demonstrated that κ-carrageenan is suitable biomaterial for cell and GF encapsulation as cells remain viable in culture for both ATDC5 and hASCs. The culture of the constructs for 14 days in different conditions revealed specific cartilage extracellular matrix molecules expression by hASCs.

Conclusions. The incorporation of TGF-β1 within the carrageenan-based hydrogel enhances the chondrogenic differentiation of hASCs. These findings suggest a new system for cartilage TE, which is even more promising for future applications as an injectable system due to its thermoresponsive behavior.

Acknowledgments. European NoE EXPERTISSUES (NMP3-CT-2004-500283), European FP7 Project Find&Bind (NMP4-SL-2009-229292), FCT(PTDC/FIS/68517/2006, PTDC/QUI/69263/2006, BD/39486/2007), Hosp. da Prelada.

Keywords. Adipose derived stem cells, Transforming Growth Factor, hydrogel, cartilage tissue engineering

(15.P14) ISOLATION OF HUMAN MESENCHYMAL STROMAL CELLS ON HYDROGEL SCAFFOLDS WITH VARYING STIFFNESS AND LIGAND FUNCTIONALISATION

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Bone marrow-derived multipotent mesenchymal stromal cells (MSCs) are able to self-renew and differentiate into a variety of cell types of mesodermal as well as into non-mesodermal origin making them attractive for studies in regenerative medicine. Current isolation protocols generate rather heterogeneous populations using percoll density gradient centrifugation of bone marrow aspirates and subsequent adhesion and expansion on tissue culture plastic. Since recent studies assigned material properties such as elasticity and ligand presentation an important role in MSC differentiation in vitro, we aim at establishing a new expansion strategy to identify and harvest distinct subpopulations of MSCs.

Layers of biohybrid heparin and multi-armed poly(ethylene glycol) hydrogels are utilized to adjust stiffness and adhesion ligand presentation in defined ways. The hydrogel layers were produced in thicknesses of about 50 µm and with degrees of crosslinking resulting in elastic moduli between 2 and 42 kPa. Adhesion peptides consisting of amino acid sequences found in the extracellular matrix components fibronectin, collagen, and laminin were covalently conjugated to the heparin units of the gels. The results demonstrate that the compared variants of the gel materials can effectively modulate the adhesion, proliferation, and differentiation of MSCs. Based on that, current studies concern the application of the hydrogel surfaces to isolate distinct MSC subpopulations out of primary density gradients of bone marrow aspirates. The MSCs expanded on hydrogel surfaces are thoroughly characterized using flow cytometry and clonal as well as differentiation assays. Taken together, a set of gradually adjusted biohybrid hydrogel materials is used as layered cell culture carrier system to isolate, maintain, and expand human MSCs offering valuable options for the more targeted application of the marrow-derived cells in regenerative therapies.

Keywords. Biohybrid starPEG/Heparin hydrogel, MSC

(15.P15) ESTABLISHMENT OF AN IN VITRO MODEL WITH HUMAN MESENCHYMAL STEM CELLS AND MICROVASCULAR ENDOTHELIAL CELLS FOR MENISCAL REPAIR

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Introduction. To date, tissue engineering constructs for meniscus regeneration failed, due to their limited integration capacities. A vascularised cell-based meniscus graft should overcome these limitations. Therefore, human mesenchymal stem cells (hMSCs) were embedded in three-dimensional (3D) collagen matrices in vitro to evaluate the optimal scaffold with homogenous cell distribution. Additionally, a co-culture-system of hMSCs and human microvascular endothelial cells (hmvECs) was established to predict the interactions between both cell types.

Methods. HMSCs isolated from bone marrow were seeded with various cell concentrations onto 5 different collagen matrices, fibrous as well as hydrogels. With histological stainings (HE and live-dead-staining) the cell distribution and viability in the matrix were visualized. To characterize hMSCs in the construct immunhistological analysis was performed. To build-up the co-culture-system, first investigations were made by seeding hMSCs and hmvECs in hanging inserts. The cell proliferation was compared to the normal tissue culture surface using a WST-1 assay. Further both cell types were cultured in different mixtures of the cell-type-specific media to evaluate the optimal medium composition.

Results. A homogeneous cell distribution was attained by seeding 500,000 cells/ml collagen-I-hydrogel as well as on 50 mm2 collagen-I-electrospun matrix. The cells were viable, even inside the matrix. The functionality of hMSCs was demonstrated by the synthesis of collagen-I. All other tested matrices showed an hMSC monolayer on the outer edge of the construct but no cells inside the scaffold. The co-culture-system was established with hMSCs on the insert membrane and hmvECs on the tissue culture surface because of the limited proliferation of hmvECs on the insert membrane. As culture medium a mixture 10:1 of endothelial to hMSC medium showed only minor impact on cellular behaviour for both cell types.

Conclusions. Further investigations have to show whether a co-culture of hMSCs and hmvECs in 3D constructs has an influence on the differentiation of the cells.

Keywords. Vascularised meniscus tissue, in vitro 3D model, stem cells, scaffold

(15.P16) ENHANCED SKIN WOUND HEALING BY A SUSTAINED RELEASE OF GROWTH FACTORS IN PLATELET-RICH PLASMA

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Introduction. Platelet-rich plasma (PRP) contains growth factors that can promote tissue regeneration. Previously, we have shown that heparin-conjugated fibrin (HCF) can exert a sustained release of growth factors that have affinity to heparin. Here, we hypothesized that treatment of skin wound with a mixture of PRP and HCF would exert a sustained release of several growth factors contained in the PRP and promote the skin wound healing.

Methods. PRP was prepared by centrifuging whole blood at 2,400 rpm for 10 min and subsequently 3,500 rpm for 15 min. Full-thickness (2.0 x 2.0 cm) wounds were created on the dorsum of athymic mice. HCF mixed with PRP and thrombin was applied at the wound sites. No treatment, application of PRP with thrombin, PRP with fibrinogen and thrombin served as controls. Skin regeneration was evaluated by histological and immunohistochemical analyses.

Results. The release of fibroblast growth factor 2 (FGF2), platelet-derived growth factor-BB (PDGF-BB), and vascular endothelial growth factor (VEGF) contained in PRP from HCF was sustained for a longer period than that from either PRP only, C-PRP, or a mixture of F-PRP in

vitro. At 12 days after injury, PRP with HCF group showed complete epithelialization of the wound compared to the other groups. The macroscopic wound sizes of PRP with HCF group were statistically smaller than the other groups at 12 days. The HCF with PRP groups showed excellent epithelial maturation.

Conclusion. The enhanced skin regeneration in HCF-PRP group may be at least partially due to enhanced angiogenesis in the wound beds. This method could be useful for skin wound treatment.

This work was supported by the Korea Health 21 R&D project, Ministry of Health and Welfare (A100443).

Keywords. Growth factors; Heparin-conjugated fibrin; Platelet-rich plasma; Skin wound healing

(15.P17) LOCAL TRANSFORMING GROWTH FACTOR-BETA DELIVERY IN FIBRIN HYDROGEL: RELEASE KINETICS AND EFFECTS ON HUMAN MESENCHYMAL STEM CELL CHONDROGENESIS

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Introduction. Structural extracellular matrix molecules gain increasing attention as scaffolds for cartilage tissue engineering due to their natural role as a growth factor repository. We recently observed that a collagen type I/III matrix (Col-I/III), human recombinant TGF-β protein, and fibrin glue (FG) combined to a biphasic construct provided sufficient long-term TGF-β support to drive in vitro chondrogenesis of human mesenchymal stem cells (MSC) for 6 weeks. Here we ask whether FG and Col-I/III can both retain TGF-β, describe the influence of cells on TGF-β release and compare the quality of chondrogenic differentiation of human MSCs between soluble versus local TGF-β supply.

Methods. Release of growth factor from mono- and biphasic scaffolds augmented with increasing amounts of TGF-β was analysed over 7 days and chondrogenesis was assessed over 42 days.

Results. Low TGF-β release rates from Col-I/III as opposed to higher release from FG indicated that both molecules retained TGF-β, with Col-I/III being the superior storage component. Embedding of cells significantly reduced the cumulative TGF-β release but fibrin-entrapped TGF-β remained bioactive and supported MSC chondrogenesis similar to standard scaffold-free MSC pellets supplied with soluble TGF-β. FG plus soluble TGF-β allowed significantly more proteoglycan and collagen type II deposition per construct than FG plus local TGF-β and pellet controls. However, less collagen type X relative to collagen type II and no MMP-13 was induced at local TGF-β supply suggesting a reduced hypertrophy during chondrogenesis.

Conclusions. Local growth factor application, thus, opens an interesting new perspective to modulate differentiation routes between more stable as opposed to transient cartilage.

Keywords. human mesenchymal stem cells, chondrogenesis, fibrin hydrogel, transforming growth factor beta, collagen type X

(15.P19) NON-COVALENTLY CROSS-LINKED HYDROGELS FOR APPLICATIONS IN REGENERATIVE MEDICINE

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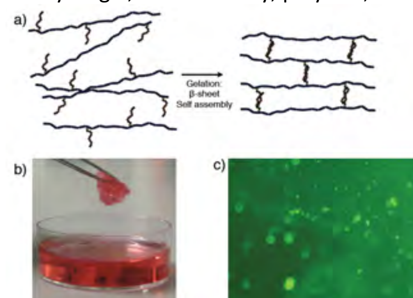
Introduction. Numerous approaches have been used to create three-dimensional scaffolds for tissue engineering, including electrospun polymers, polymer hydrogels and self-assembled peptides. While each of these approaches is promising, they can be hindered by things such as initiator toxicity for polymeric hydrogels and degradation of mechanical properties at low strains for self-assembled materials. Designing an injectable material that combines the strain resistance of polymeric materials but can be gelled without initiators would be an important advance in the field of biomaterials. We have designed a novel system that consists the biopolymer poly (γ-glutamic acid) (γ-PGA) functionalized with self-assembling peptide groups that are designed to act as non-covalent cross-links, as depicted in Figure 1a.

Methods. The β-sheet peptides of interest had N-terminal cysteines that were covalently coupled to poly (γ-glutamic acid) (γ-PGA) functionalized with maleimides. The polymer-peptide composite was dissolved in water at a physiological pH. A549 lung carcinoma cells were seeded into the peptide-γ-PGA gels and covered with media. Cell viability was assessed using a LIVE/DEAD stain containing calcein AM and ethidium bromide, respectively.

Results. We have successfully modified γ-PGA with a self-assembling peptide using thiol-maleimide chemistry. These peptide-polymer composites form a hydrogel with just a few percent of the carboxylic acids functionalized with the β-sheet peptide. The hydrogel was capable of being stretched and manipulated with tweezers, as shown in Figure 1b. Figure 1c shows that cells encapsulated in the hydrogel showed good viability in the scaffold after 18 hours. Cell differentiation assays with mesenchymal stem cells are ongoing.

Conclusions. Non-covalent cross-linking of biopolymers is a promising method for engineering enzymatically biodegradable hydrogels for applications in regenerative medicine. This allows for minimally invasive injectable therapies and functionalization of the scaffold with orthogonal chemistries, such as click-chemistry, allows for multiple bioactive groups to be incorporated on a single scaffold.

Keywords. Hydrogel, self-assembly, polymer, stem cells



(15.P20) SKELETAL IN SITU TRANSDIFFERENTIATION OF ASCS IN A 3D CULTURE SYSTEM

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Introduction. Generation of skeletal tissues by tissue engineering should use a single and accessible cell source and an adequate tree-dimensional scaffold biomaterial. In this work, we have developed a novel model of 3D bone and cartilage tissue substitutes by means of transdifferentiation of ASCs using fibrin-agarose hydrogels.

Methods. ASCs primary cultures were obtained from human biopsies of subcutaneous adipose tissues. After ASCs cultures reached subconfluence, the cells were seeded in a three-dimensional scaffold which consisted of human fibrin and agarose type VII using DMEM as basal culture medium. To generate bone and cartilage-like tissues, constructs were induced to the osteogenic and chondrogenic lineages for 21 days. Then, samples were obtained after 24 hours; 7, 14 and 21 days and processed for histological and immunofluorescence analyses to verify the transdifferentiation process using alizarin red S and alcian blue stains for histochemestry and alkaline phosphatase and collagen type II antibodies for immunofluorescence correspondingly.

Results. After 14 days of induction bioengineered tissues induced to both, the osteogenic and the chondrogenic lineages showed an incipient osteogenic and chondrogenic differentiation as determined by alizarin red S and Alcian blue staining. After 21 days of induction the synthesis of both the calcic and mucopolysaccharides materials increased. Immunofluorescence revealed a high alkaline phosphatase activity after 14 and 21 days of osteoinduction whereas collagen type II showed an incipient signal after 7 days and increased after 14 and 21 days.

Conclusions. Fibrin-agarose 3D culture model could support the efficient transdifferentiation capability of ASCs, suggesting that the generation of three-dimensional human tissue substitutes using fibrin-agarose scaffolds is a feasible technique in the laboratory and could be used in regenerative medicine.

Acknowledgments. This work was supported by CTS-115 Tissue Engineering Group.

Keywords. Engineered skeletal, tridimensional scaffold, pluripotent

16. ENGINEERING A FUNCTIONAL TENDON

Chair: Dimitrios I Zeugolis
Co-chair: Oded Shoseyov
Keynote speaker: Wei Liu
Organizers: Dimitrios I. Zeugolis, Oded Shoseyov

Synopsis: Advances in medical care have greatly improved survival rate and life expectancy following trauma or degenerative conditions of the musculoskeletal system, leading to an ever-increasing need for functional tissue substitutes to improve quality of life. Tendon and ligament injuries constitute the most common musculoskeletal disorders that clinicians address daily. Indeed, from over 33 million musculoskeletal injuries per year in United States alone; almost 50% of them are tendon and ligament related with approximately 95,000 new cases per year. As tendons have limited regeneration capacity, suitable substitutes are required for regeneration and functional recovery. Surgical repairs are still suboptimal due to fibrous adhesions or failure arising from the mechanical demands placed on imperfect integrative healing at tendon-tendon or tendon-bone interfaces. Therefore, to develop strategies for functional tendon regeneration is of paramount importance. However, in order to be able to imitate nature, we need to understand how the tissue forms and behaves *in vivo* under normal and pathophysiological conditions. Upon this knowledge, we can develop strategies that will encourage scaffold interaction with extracellular matrix components, growth factors, cells and cell surface receptors. We will also be able to identify bioactive and therapeutic molecules that should be incorporated into the 3D construct that will positively interact with the host and promote functional regeneration. This symposium will discuss current tissue engineering strategies that improve tendon regeneration and functional recovery by using recent advancements in material optimisation and scaffold functionalisation through incorporation of biophysical cues and biochemical and biological signals.

(16.KP) DERMAL FIBROBLAST BASED TENDON ENGINEERING: FROM BASIC RESEARCH TO PRE-CLINICAL TRIALS

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One of the major challenges in tendon engineering is the selection of proper cell source. In our group, we explored the possibility of using dermal fibroblasts as the cell source for tendon engineering. At the cellular level, the fibroblasts were forced into an elongated morphology and then subjected to a unilateral mechanical stretch. The results demonstrated that dermal fibroblasts could actually be transdifferentiated into tenocytes by expressing tenogenic markers such as tenomodulin, tenacin, deocrine, collagen VI. Interestingly, the tenogenic transdifferentiation became most prominent only when the mechanical force was applied parallel to the long axis of the elongated cells. At the tissue level, it was found that human fibroblasts could form better tissue structure when they were seeded on a parallel aligned polymer fiber scaffold than on randomly arranged fibers. Furthermore, during in vitro culture, the mechanical loading resulted in better engineered tendon tissue than non-loaded tissues including stronger mechanical strength, better tissue structure, and thicker collagen fibrils. Quantitative analysis revealed no difference in above mentioned characters between human dermal fibroblast and tenocyte engineered tendons, indicating the importance of mechanical loading in transforming fibroblasts to tenocytes and tendon tissue formation. To further translate the finding to pre-clinical study, a complex scaffold with reinforced mechanical strength was fabricated and used for in vitro tendon engineering along with dermal fibroblasts. The results showed that the tensile strength of the in vitro engineered tendon could reach about 50 Newtons. Then the tendon graft was implanted in vivo for repairing the flexor tendon

defect in a monkey model. After 6 months, the repair flexor tendon could regain its major function.

Keywords. Dermal fibroblast, tendon engineering, in vitro, transdifferentiation

(16.O1) TENOMODULIN PROMOTES THE TENOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Aim. Tendon tissue engineering provides a promising approach for tendon defects. While the mechanism of development and maturation for engineered tendon remains unknown, due to lack of specific tendon related markers. Recently tenomodulin (TNMD) was demonstrated to be a relative specific tendon marker. However, the effect of TNMD on the tenocyte or mesenchymal stem cells (MSCs) and its' possible application in tendon tissue engineering are unexplored. This study employed gene transfection to investigate the effects of TNMD on the tendon precursor cells (TT-D6) and MSCs (C3H10T1/2).

Method. The in vitro cultured TT-D6 cells and C3H10T1/2 cells were stable transfected with pCAGGS-TNMD using the Fugene HD. After confirmation of successful transfection, the morphology, the proliferation and the expression of some tendon related genes were analyzed. At the same time, the multilineage differentiation of C3H10T1/2 cells after transfection was also tested.

Results. Successful overexpression of TNMD was confirmed by RT-PCR, quantitative PCR and immunofluorescent staining. No obvious differences in morphology were seen in both normal TT-D6 cells and C3H10T1/2 cells after transfection. While faster proliferative ability was shown in both cells. For TT-D6 cells transfected with pCAGGS-TNMD, the expression of collagen1, collagen3, collagen6, biglycan and decorin were up-regulated significantly. The expression of scleraxis, COL1, COL3, and decorin were increased dramatically, while the expression of COL6 and biglycan were not influenced obviously in C3H10T1/2 cells. Differentiation test indicated that TNMD could inhibit the adipogenic differentiation, the chondrogenic differentiation and AKP activity of C3H10T1/2 cells.

Conclusions. These findings demonstrate that TNMD can enhance the expression of the extracellular matrix of the tendon precursor cells and promote the the tenogenic differentiation of mesenchymal stem cells, which may indicate the possible application of TNMD in tendon tissue engineering and tendon repair.

Keywords. Tenomodulin tendon transfection

(16.O2) CURRENT CLINICAL OPINION OF ANTERIOR CRUCIATE LIGAMENT TISSUE ENGINEERING

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Introduction. Donor site morbidity, poor graft site integration (causing slippage) and incorrect mechanical performance are all common problems with grafts currently used for repairing the anterior cruciate ligament (ACL). A tissue engineered (TE) ligament has potential to overcome these problems. We have obtained input from clinicans who currently treat these injuries to deal with any potential design short-comings associated with TE ACLs before they arise.

Methods. An online questionnaire was created relating to ACL tissue engineering. The questionnaire was peer reviewed and approved by local research ethics committee (project number 09/H1204/64, approved 15/10/09). Between July and October 2010, three hundred orthopaedic surgeons specialising in ligament and tendon repair in the UK were contacted by email and invited to participate. From this e-mailed input request, eighty surgeons responded.

Results. 86% of surgeons would consider using a TE ACL if it were an option (provided it showed biological & mechanical success) if it significantly improved the patient satisfaction (63%) and shortened surgical time (62%), and would be prepared to wait 4-30 weeks for it to be created. 42% were either concerned or very concerned (25%) about its successful integration into the bone. 76% of surgeons felt that using a TE ACL would be more appropriate than a patellar tendon, hamstring or quadriceps autograft if it could be engineered to be an exact match to the native tissue. 62% thought using a TE ligament would take less surgical time & felt surgical time needs to be reduced by 10-30 minutes to be considered to be a significant improvement.

Conclusions. Overall it appears that most surgeons would be prepared to use a TE ligament. Future research needs to concentrate on integration of the TE ACL into the patients' bone. This information confirms a demand for tissue engineered ACL's and highlights important areas for improvement.

Keywords. Survey, anterior cruciate ligament, tissue engineered construct, implantation

(16.O3) IMPLANTATION OF ELECTROSPUN POLY(ε-CAPROLACTONE) 3D SCAFFOLDS IN ACHILLES TENDONS

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Introduction. Tendons are susceptible to wear and tear and even spontaneous rupture. There remains an unmet clinical need for the development of a medical device capable of regenerating damaged tendons. We have fabricated an electrospun, synthetic, biodegradable scaffold with proven biocompatibility and appropriate interface when grafted in vivo.

Methods. Electrospun fibres were fabricated using poly(ε-caprolactone) (Mn 80,000) dissolved in Acetone (concentration 10%w/v) and parameters: voltage - 20kV, flow rate - 0.05ml/min, distance to collector – 15cm. 2D fibre mats were manipulated into 3D fibrous scaffolds. Scaffold biocompatibility with tenocytes was assessed in vitro over 14 days by cell proliferation and topographical cues. In a pilot animal study, partial removal of the Achilles tendons' of mice was performed to create a critical defect, which was grafted with a single electrospun 3D scaffold. Mice were monitored for 21 days and assessment of the scaffold-tissue interface was determined by variable-pressure Scanning Electron Microscopy.

Results. Tenocytes adhered and proliferated along the longitudinal axis of scaffold fibres, demonstrating that fibres provided adequate topographical cues to guide cell orientation. This was further confirmed by comparison to cells cultured on randomly orientated electrospun fibres. In all mice receiving scaffold grafts, normal ambulation was observed within 48hrs post-operatively. Initially there was good interaction at the tissue-scaffold interface (Figure), and by day 21 post-implantation the scaffold had been fully integrated into the tendon, suggesting new tissue formation. All mice survived the time period investigated.

Conclusion. This study has demonstrated the biocompatibility and successful implantation of 3D electrospun scaffolds into the Achilles tendons' of mice. On-going work performing long-term in vivo assessment and histomorphometric analysis will advance this technology towards our ultimate goal of clinical tendon regeneration.

Acknowledgements. The Authors wish to acknowledge RegeNer8 and the UMIP Premier Fund for funding this research.

Keywords. Electrospinning, polycaprolactone, tendon, nanofibres

(16.O4) GEL SPINNING OF ALIGNED HUMAN RECOMBINANT COLLAGEN FIBERS BY INJECTION OF NEMATIC LIQUID CRYSTALLINE DOPE

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Collagen is the most abundant structural protein in mammals. It is a key component in load bearing tissues (Ligaments and bones), giving them tensile strength and resilience. The unique mechanical properties of these tissues are dependent on the highly ordered and hierarchical organization of the collagen fibers.

There are significant advantages for the use of collagen based scaffolds for tissue repair because of its biocompatibility and biodegradability, but so far there was only a limited success in creating structures that would have the required strength. This is partly attributed to a lesser degree of order achieved so far in artificial collagen fibers.

Above a threshold concentration collagen solutions become liquid crystalline, and that property plays a key role in the formation of certain tissue structures, such as the dogfish egg capsule. In this work, we utilize collagens unique liquid crystalline and self assembly properties to create ordered scaffolds for tissue replacement.

Liquid crystalline human recombinant collagen dope, in acidic pH, was injected through a 30 ga. needle that exerts strong shear force on it. A nematic order was induced in the outer layer of collagen rod-like molecules, which then spreads through the injected material as determined by polarized light microscopy and cross-section SEM analysis. The fibers were extruded into a coagulation bath that induces fibrillogenesis, a sol-gel transition. A neatly ordered array of aligned collagen fibrils is with the characteristic D-banding obtained, as determined by high resolution SEM and AFM (figure 1).

Crosslinking can be performed by a plurality of methods, to improve the fibers mechanical properties or reduce swelling and biodegradation.

The fibers thus obtained display good mechanical performance, including a UTS similar to natural rat tail tendon (350 MPa), and may find applications in regenerative medicine in general and tendon repair in particular.

Keywords. Recombinant Human Collagen, Tendon Repair, Liquid Crystall, Gel Spinning

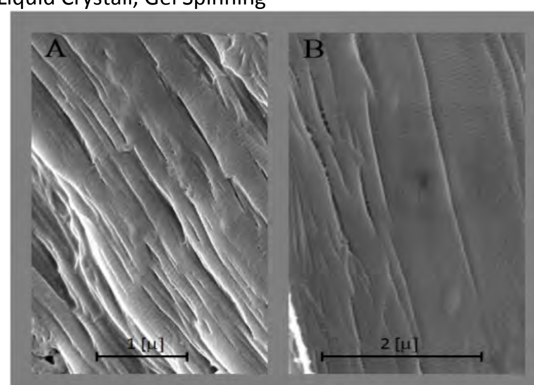

Figure 1: Scanning Electron Micrograph of the mesophase aligned fibers, after drying and gold sputter coating. A – array of aligned fibers, with diameter between 100 and 300[nm]. B – a D-banded surface of fused collagen fibrils.

(16.O5) EVALUATION OF CELLULAR FUNCTIONS AT THE NANO-BIO-INTERFACE

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Introduction: Cell-substrate interactions at the nano-bio interface are becoming increasingly important in our understanding of a range of physiological processes. Indeed, nano-textured biomaterials have been shown to favourably promote cell attachment, migration and differentiation, since they closely imitate the in vivo niche. However, to facilitate clinical translation of such technologies, it is important to comprehend the influence of nano-topography on the cellular and molecular level, and to use this knowledge to design the next generation of nano-biomaterials. Herein, the influence of nano-topography, induced by different scaffold conformations, on cellular function was studied.

Methods: PLGA nano-textured scaffolds were produced using solvent casting, electro-spinning, laser and nano-imprinting lithography. Human primary (WI38 lung fibroblast) and immortalised (SAOS2 Osteosarcoma) cells were seeded on the fabricated scaffolds for 2 to 14 days. Subsequently, the influence of surface topography on cell

behaviour (e.g. morphology, attachment, alignment, migration, phenotype maintenance) was evaluated.

Results: Scanning electron micrographs show the various conformations of scaffolds used in this study (Figure-1.1). Rhodamine-phalloidin and DAPI staining (Figure-1.2) demonstrate that only aligned electro-spun nano-fibrous mats (Figure-1.2.c) facilitated cell attachment and alignment in the direction of the nano-fibrous substrate. The metabolic activity of SAOS2 was significantly lower ($p<0.05$) on electro-spun mats than on tissue culture plastic at day 10 and 14 (Figure-1.3). Similarly to aligned electro-spun nano-fibrous mats, nano-imprinted scaffolds facilitated cell attachment and alignment and exhibited a decrease in metabolic activity of WI38 fibroblasts at day 5 and 7 (data not shown).

Conclusions: Aligned electro-spun mats and nano-imprinted films provide a conductive environment for cell attachment and orientation, whilst decreasing metabolic activity. Studies are underway to understand the effect of nano-topography at the molecular level to decipher the effect on gene expression.

Acknowledgments. This work was supported by Enterprise Ireland, CCAN (Project No. CCIRP-2007-CCAN-0509) and by the Irish Government under the NDP 2007-2013.

Keywords. Nanotopography, Electro-spinning, Nano-imprinting; Cell Behaviour; Gene Expression

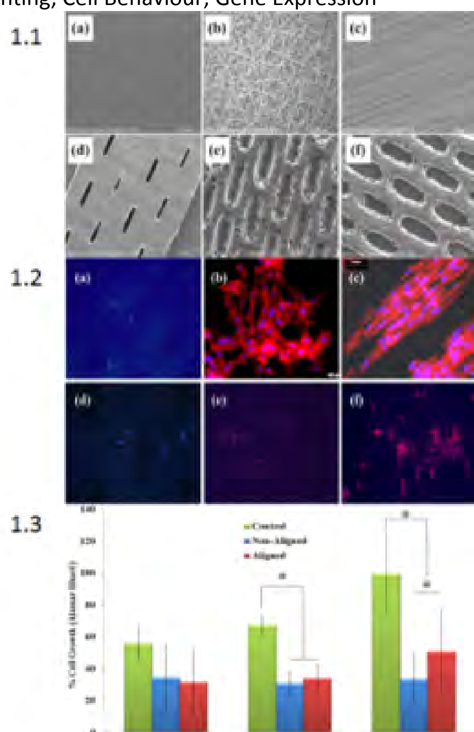


Figure 1.1 shows scanning electron micrographs of (a) solvent casted films; (b) non-aligned electro-spun nano-fibrous mats; and (c) aligned electro-spun nano-fibrous mats. (d), (e) and (f) represent laser lithography treated (a), (b) and (c) samples respectively. **Figure 1.2:** Immunocytochemistry images illustrating the cellular attachment and orientation on the scaffolds that were viewed in figure 1.1. **Figure 1.3:** Cell metabolic activity assay for osteosarcoma cells (SAOS2) on electrospun mats. Control: Tissue Culture Plastic.

(16.P1) POLY(3-HYDROXYBUTYRATE-CO-3-HYDROXYHEXANOATE) (PHBHHX) SCAFFOLDS FOR TENDON REPAIR IN THE RAT MODEL

Lomas AJ (1), Webb WR (1), Zeng G (2), Forsyth NR (1), El Haj AJ (1), Chen GQ (2)

1. Keele University, UK; 2. Tsinghua University China

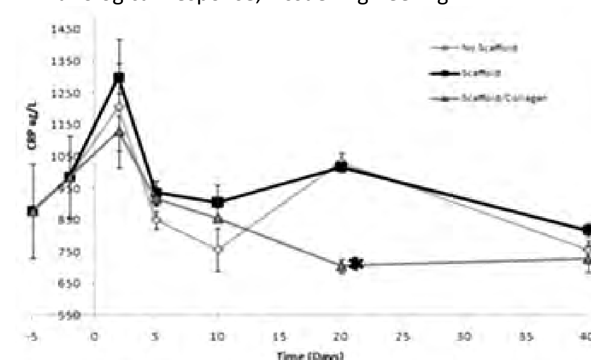
Introduction. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) was investigated for possible application in repairing damaged tendon, with a range of in vitro and in vivo experiments utilised to design and test suitable scaffold designs.

Methods. Scaffolds consisting of fibre reinforced porous tubes were prepared using particle leaching and an extrusion method. Mechanical testing demonstrated that PHBHHx scaffolds could be produced that had comparable mechanical properties to natural Rat Achilles tendon. Sprague–Dawley (SD) rats were split into 3 experimental groups; no construct/control, PHBHHx scaffold, and PHBHHx scaffold/collagen hybrids. The in vivo functionality of scaffolds was explored in a surgically induced Achilles tendon defect model, with polymer breakdown products and C-Reactive protein blood plasma concentrations measured throughout the experiment. Mechanical testing and histological analysis was performed after animal sacrifice at day 40.

Results. Mechanical tests demonstrated that the PHBHHx scaffolds had comparable mechanical properties to natural tendon, with maximal loads of 23.73 ± 1.08N, compared to 17.35 ± 1.76N in undamaged rat Achilles tendon. Restoration of movement and mechanical loading was restored in scaffold-recipient rats at an earlier time than those without scaffold, with almost complete motion returning 10 days post surgery compared to 20 days in the control. In vitro mechanical testing of day 40 tendons demonstrated that the repair induced in the scaffold/collagen model was comparable to undamaged tendon (18.02 ± 7.45N vs. 17.35 ± 1.78N) and integration was observed. Histological analysis of the damaged area found evidence for tenocyte invasion coupled with tissue remodelling. No significant secondary immune response to PHBHHx was observed over time with blood C-reactive protein levels remaining at control cell levels throughout. In addition, measurement of B-hydroxybutyrate (a degradation product of PHBHHx) blood concentration demonstrated no correlation to immune response.

Conclusions. PHBHHx collagen hybrids have been successfully used as a material for tendon tissue engineering in vivo.

Keywords. Polyhydroxyalkanoates, Tendon, Immunological Response, Tissue Engineering



17. ENGINEERING BIOMIMETIC SCAFFOLDS FOR IN VITRO STUDIES AND REGENERATIVE THERAPIES

Chair: Helena S. Azevedo
Co-chair: Alvaro Mata
Keynote speaker: Matthias Lutolf
Organizer: Helena S. Azevedo, Alvaro Mata
Synopsis: Recent advances in biomaterials research have enabled engineering of scaffolds that reproduce the biological, physical and biochemical complexity of the natural extracellular matrix (ECM) environment. Within regenerative medicine, the role of the scaffold is essential and continues to increase primarily because of our growing ability to create materials that can mimic the natural ECM and elicit specific biological responses. The opportunity to create scenarios that are bioactive and biomimetic in the laboratory offers very attractive opportunities for many *in vitro* applications. For example, scaffolds that facilitate the execution of systematic studies to elucidate the molecular mechanisms underlying physiological and pathological processes, deconstruction of complex biological processes or extracellular environments, and analysis of specific signals or mechanisms that affect cell-cell or cell-ECM interactions. Biomimetic structures that better recreate *in vitro* the complex natural *in vivo* environment have tremendous implications in the design of novel therapies, drugs, tissue engineering or regenerative medicine scaffolds, or biomaterials while decreasing the need for *in vivo* testing.
State-of-the-art scaffolds for such *in vitro* applications vary widely from highly porous biodegradable supports, bioactive substrates or smart drug-eluding materials, to self-assembling hydrogels, precise microfabricated structures, or lab-on-hip devices. Therefore, this symposium will focus on work that features bioengineering approaches to develop novel scaffolds (defined as substrates, structures, or matrices), designed to recreate the hierarchical complexity of tissues, to study cell behaviour *in vitro*, learn fundamental processes for future regenerative therapies, or aid the design or effectiveness of regenerative therapies such as expanding cell populations for cell therapies or facilitating new drug discoveries. The symposium will cover the following topics:
- Scaffolds that facilitate the deconstruction of biological processes or cellular microenvironments to study cell behaviors and learn basic embryogenesis, homeostasis, or regeneration mechanisms.
- Scaffolds that facilitate the controlled growth of cell populations for cell therapies.
- Scaffolds that facilitate the recreation of environments for drug discovery.

(17.KP1) DESIGNING SMART BIOMATERIALS TO INSTRUCT STEM CELL FATE
Lutolf MP (1)
1. Laboratory of Stem Cell Bioengineering, EPF Lausanne, Switzerland
Proper tissue maintenance and regeneration relies on intricate spatial and temporal control of biochemical and biophysical microenvironmental cues, instructing stem cells to acquire particular fates, for example remaining quiescent or undergoing self-renewal divisions. Despite rapid progress in the identification of relevant niche proteins and signaling pathways using powerful in vivo models, to date, many adult stem cell populations cannot be efficiently cultured in vitro without rapidly differentiating. To address this challenge, we and others have been developing biomaterial-based approaches to display and deliver stem cell regulatory signals in a precise and near-physiological fashion, serving as powerful artificial microenvironments to study and manipulate stem cell fate both in culture and in vivo. In this talk I will highlight recent efforts in my laboratory to develop microarrayed artificial niches based on a combination of biomolecular hydrogel and microfabrication/robotic technologies. These platforms allow key biochemical characteristics of stem cell niches to be mimicked and the physiological complexity deconstructed into a smaller, experimentally amenable number of distinct signaling interactions. The systematic deconstruction of a stem cell niche may serve as a broadly applicable paradigm for defining and reconstructing artificial niches to accelerate the transition of stem cell biology to the clinic.

(17.KP2) SOFT NANOSTRUCTURE BIOMATERIALS PROMOTE DEVELOPMENT OF FUNCTIONAL TISSUE ENGINEERING PLATFORMS
Semino CE (1,2)
1. Bioengineering Department, Institut Químic de Sarrià, Universitat Ramon Llull, Barcelona, Spain; 2. Translational Centre for Regenerative Medicine, Leipzig University, Leipzig, Germany
In our laboratory we have been studied several *in vitro* 3D-tissue systems using nanostructured materials such as self-assembling peptides scaffolds and natural collagen gels with the main aim of understanding the basic biological and biophysical parameters that affect processes like cell differentiation and function. In my presentation I will like to review our main results from experiments using cell lines, tissue and organ derived cells, including functional mature cells and stem cells, from species such as human, rat and mouse. I will mainly focus on liver stem cell differentiation, mature hepatocyte phenotype maintenance as well as embryonic and adult fibroblast culture and multi-potent lineage commitment. The use of soft nanostructured materials has a clear promising future for applications in tissue engineering and regenerative therapy.

(17.O1) DIFFERENTIATION OF PRE-OSTEOBLAST CELLS ON POLY(ETHYLENE TEREPHTHALATE) GRAFTED WITH RGD AND/OR BMPS MIMETIC PEPTIDES
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Introduction. Some BMPs such as BMP-2, BMP-7 and BMP-9 play a major role in the bone and cartilage

formation (1). After having designed a mimetic peptide of these growth factors, we immobilized these peptides as well as a peptide of adhesion (RGD) on polyethylene terephthalate (PET) surfaces and we evaluated the state of differentiation of pre-osteoblastic cells. The behavior of these cells on various functionalized surfaces highlighted the activity of the mimetic peptides immobilized on surfaces. The induced cells (observed in the case of surfaces grafted with BMPs mimetic peptides) were characterized on several levels. These induced cells take a different morphology compared to the cells in a state of proliferation or in a state of extracellular matrix (ECM) production.

Materials and methods. Mimetic peptides design: The FATCAT (2) program was used as a tool to search for the BMP-2 protein homologues. The structural alignment was performed with STAMP (3) and then optimized with ViTO (4). The interactions between the three BMPs and the receptor II were determined according to the experimental data (5) and also by analyzing the crystallographic structure of the BMPs in complex with its receptor II.

Mimetic peptides grafting: PET was grafted in two subsequent steps (6).

Cell culture: For our study MC3T3-E1 cells, a clonal pre-osteoblast-like cell line, were cultured in a αMEM supplemented with 10% serum. The messenger RNA was quantified with the Syber green intercalating agent at different time points. For cells observations, scanning electron microscope (SEM Hitachi S2500) was used.

Results. The detailed study of the interaction between the BMPs and their receptors and the following analysis of the selected peptides' folding contributed to the design of three mimetic peptides. We clearly observed an overexpression of Runx2 in surfaces that have the mimetic peptide of the BMP-2, BMP-7 and BMP-9. In the case of surfaces grafted with mimetic peptide of BMPs a formed ECM was observed. Finally, we observed a much more significant mineralization on surfaces with BMPs mimetic peptide than on surfaces oxidized or with RGD after 5 days.

Conclusion. In our study we propose to mimic the function of certain BMPs by using small peptides. We show synergy between two pathways due to the surfaces bifunctionalized with two mimetic peptides.

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Keywords. Surface functionalization, osteoinduction, BMPs mimetic peptide, biomimetic material

(17.O2) EFFECT OF SUBSTRATE STIFFNESS AND FIBRONECTIN ACTIVITY ON HMSC DIFFERENTIATION

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1. Universidad Politécnica de Valencia. Spain; 2. University of Southampton. UK; 3. University of Glasgow. UK

Cell differentiation can be triggered by the properties and composition of the extracellular matrix (ECM). Whilst we know that biomaterials can influence stem cell differentiation, there is some debate in the literature as to which material properties trigger specific differentiations. We hypothesize that the reported differences may be a consequence of the organization of ECM proteins adsorbed on the surface. This study correlates fibronectin (FN) adsorption to human mesenchymal stem cell (hMSCs) differentiation, on a family of substrates with tailored stiffness and minute variations in surface chemistry.

Polymer substrates which consist of a vinyl backbone chain with side groups - $COO(CH_2)_xCH_3$ (x=0, 1, and 3) were prepared. Surfaces were characterized (AFM, water contact angle, elastic modulus). FN was adsorbed from a solution of concentration 20 µg/mL. The amount of adsorbed FN was quantified by western-blotting, and its distribution on the material surface was directly observed with AFM. hMSCs were cultured on the FN-coated substrates in serum-free conditions. Focal adhesions, actin cytoskeleton formation, and the expression of key transcription factors in the differentiation to bone and cartilage lineages (such as RUNX2, phospho-RUNX2, and SOX9) as well as other non-collagenous proteins present in bone ECM (osteopontin, osteocalcin) were quantified on the different surfaces by immunofluorescence followed by image analysis.

Substrates with decreasing stiffness were obtained by subtle variations in material chemistry, i.e. by sequentially adding methyl groups in the side group of a vinyl chain. The same FN density was obtained on every substrate, but the supramolecular organisation of the protein at the material interface was different for x=0 than the other surfaces. This allows one to investigate the effect of substrate stiffness on cell differentiation after differences in FN activity have been ruled out. hMSC differentiation to cartilage and bone lineages depended of the interplay between substrate stiffness and FN activity.

Keywords. Material properties, protein adsorption, stem cells differentiation

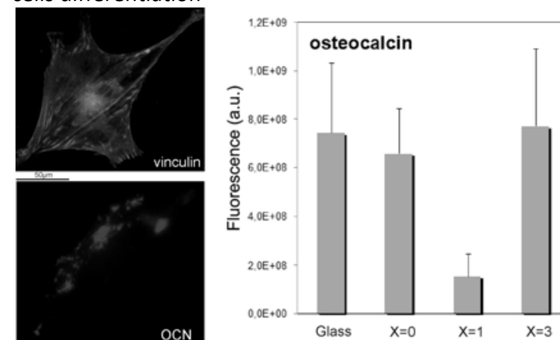

Figure. hMSCs on substrates with controlled stiffness obtained by minute variations in polymer chemistry as explained in the text ($COO(CH_2)_xCH_3$,with x=0, 1, and 3). Cell adhesion was studied via vinculin immunostaining. Differentiation to cartilage and bone lineages was quantified via immunoluorescence for the expression of characteristic proteins. The graph shows osteocalcin levels on the different substrates.

(17.O3) SELF-ASSEMBLING BIOMIMETIC MATRICES: OPPORTUNITIES FOR RESEARCH AND THERAPIES IN SKIN REGENERATION

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Extracellular matrix (ECM) plays a key role in wound healing as ECM components are known to have the ability to regulate cellular processes, such as adhesion, growth and migration in the different phases of the healing process. Therefore, bioactive matrices that can mimic multiple aspects of native cellular environments (biochemical and physical signals) would be of great benefit in skin regeneration strategies. Towards this challenge, we report here the development and characterization of bioactive membranes that result from the instant self-assembly between peptide amphiphiles and the glycosaminoglycan hyaluronic acid (HA), a major component of skin ECM. To foster cell adhesion and proliferation on the self-assembling membranes, the fibronectin-derived RGDS epitope was incorporated into the peptide structure. Due to their ability to recapitulate biochemical signals of skin tissue niche, these molecules offer many unique advantages as starting materials for skin regeneration applications. Degradation studies showed that these matrices are susceptible to enzymatic degradation by hyaluronidase (HAase). In the presence of HAase at physiological concentration the matrices degrade gradually over time, which may present an advantage over other systems, since slow degradation will induce the migration of cells. The matrix degradation also provides space that is essential for new tissue formation. When grown on membranes without the cell recognition epitope RGDS, human dermal fibroblasts showed lower adhesion to the matrices when compared to the ones containing RGDS. We expect that the proposed biodegradable hybrid matrices could offer significant potential in skin regeneration strategies, as a bioactive supportive matrix for promoting wound healing, and also as model systems for fundamental mechanistic studies in wound remodeling.
Acknowledgesments. Daniela S. Ferreira acknowledges the financial support received from Fundação para a Ciência e a Tecnologia (PhD scholarship SFRH/BD/44977/2008
Keywords. Skin regeneration, biomimetic membranes, hyaluronan, self-assembly, RGDS, cell adhesion

(17.O4) EFFECT OF 3D-MICRO-ENVIRONMENT ON MICE CELLS' GENE EXPRESSION

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1. Tissue Engineering Laboratory, Bioengineering Department, Institut Químic de Sarrià, Universidad Ramón Llull, Barcelona, Spain
Nowadays, the development of biomaterials that substitute natural cell environments is one of the main objectives of tissue engineering. Since cells in vivo are in tridimensional (3D) environments, it is important to reproduce this condition in vitro for being more adequate than traditional two-dimensional (2D) cultures. In the present work we evaluated the influence of a non-instructive soft nanofiber scaffold (RAD16-I self-assembling peptide scaffold) on mouse embryonic fibroblasts (MEFs). We found that when MEFs were cultured using this methodology they dedifferentiate into a primitive progenitor with mesodermal commitment. Initially, it was observed the spontaneous up-regulation of a subset of chondrogenic markers, the transcription factor Sox9 and two main components of the chondrocytes extracellular matrix, Collagen II and cartilage specific proteoglycans. Then, we also found that the expression of the organizer gene noggin (present during embryogenesis) was also up-regulated very early in our system only under low matrix stiffness (G' ~ 100 Pa). This could indicate that the 3D-construct is undergoing a process that recreates some aspects of a vestigial cellular self-organized system during development. Thus, presenting the subset of early gene programs to progress autonomously into a default cartilaginous commitment. Interestingly, none of these processes occurred to MEFs cultured on 2D. These results suggest that 3D-environments are more adequate for natural cell development than the 2D ones, indicating that self-assembling peptides are promising materials to be used in cartilage repair.
Keywords. 3D-microenvironments, Differentiation, Chondrogenesis

(17.O5) MATRIX ENGINEERING TO LOCALLY CONTROL CELL FUNCTION AND FORMATION OF ARTIFICIAL TISSUES

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1. Eidgenössische Technische Hochschule (ETH) Zürich and University Hospital Zürich. Switzerland; 2. University Hospital Zürich. Switzerland; 3. École Polytechnique Fédérale de Lausanne (EPFL). Switzerland; 4. Eidgenössische Technische Hochschule (ETH) Zürich. Switzerland
Introduction. Naturally derived matrices such as collagen or fibrin have been widely used for 3D cell culture. One major limitation of these hydrogel materials is their limited flexibility in engineering applications. We describe completely synthetic matrices that are modularly designed and can be specifically tailored towards biological applications. These materials are based on biologically inert star-shaped Poly(ethylene glycol). In order to obtain biological functionality the materials can be made sensitive towards proteolytic digestion and decorated with specific integrin ligand domains or covalently immobilized morphogenetic cues that might direct cell behaviour2.
Methods. PEG precursors that are decorated with factor XIII (FXIII) substrate sequences TG (NQEQVSPL) or Lys (FKG) are reacted in presence of growth factors or cells by the addition of FXIIIa. The hydrogels were formed in presence of cells, growth factors and integrin ligand RGD by casting them into defined layers and by printing. The building blocks were formed in consecutive steps to reach a controlled three dimensional organisation of materials and cells.
Results. We show that by matrix engineering migration of encapsulated cells can be enabled or prohibited. In consequence the cellular distribution and movement in vitro and in vivo can be influenced by matrix design. As our matrices allow the local presentation of growth factors, they can not only be used to control the formation of cellular structures, but also provide them

with instructive cues. We show the 3D arrangement of cells, matrices and growth factors by means of layer-by-layer assembly or printing. Examples of how cell behaviour can be controlled locally and how this translates to tissue-like constructs will be presented.

Discussion. We believe that our novel materials platform can serve to study fundamental cellular processes in a 3D setting and to generate tissue mimicking structures.

Acknowledgements. This work was supported by CCMX and SNF grant CR32I3_125426/1

Keywords. Artificial extracellular matrices; instructive microenvironments; artificial tissue-like models; printing; layer-by-layer

(17.O6) INFLUENCE OF CELL DENSITY ON VIABILITY AND GROWTH OF HUMAN PERIOSTEUM DERIVED CELLS IN POLYETHYLENE GLYCOL HYDROGELS

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Introduction. Providing cells with a biomimetic 3D culture environment is key in tissue engineering. However, determining the initial cell seeding density remains a pragmatic issue that can determine cell responses and eventually matrix formation during in vitro culture. This study explored if viability, metabolic activity and growth of human periosteum derived cells (hPDCs) alters when seeded at different densities in biomimetic poly(ethylene glycol) (PEG) hydrogels.

Methods. After expansion, hPDCs were encapsulated in 30 µl MMP-degradable PEG with RGD motifs (QGel) at densities of 1 million cells/ml, 2 million cells/ml and 5 million cells/ml. The gels were transferred to 24-well plates and cultured in growth medium (DMEM + 10% FBS) for one week. Cell viability was analysed using LIVE/DEAD staining (Invitrogen). Metabolic activity was monitored with Alamar Blue (Invitrogen) and changes in cell number were quantified by measuring DNA content (Invitrogen).

Results. After encapsulation, predominantly viable cells were found homogeneously distributed throughout the gel for each cell density. However, at day 7 viability reduced with increasing cell density. While 77% of the cells were viable in gels with initial cell density of 1 million cells/ml, only 41% were alive with the highest initial cell density. DNA content did not change significantly after one week, except for gels with 5 million cells/ml. In these gels, a reduction in DNA content of 40% was measured. On the other hand, Alamar Blue assay indicated a significant increase in total cellular activity in gels with 1 million cells/ml (219% increase at day 8 as compared to day 1).

Conclusions. Significant differences were found between hPDCs that were 3D cultured in PEG hydrogels at different seeding densities. These results emphasise that besides a suitable biomaterial, an optimised initial cell density is required to obtain the desired biological response.

Keywords. Human periosteum derived cells, cell density, polyethylene glycol, biomimetic

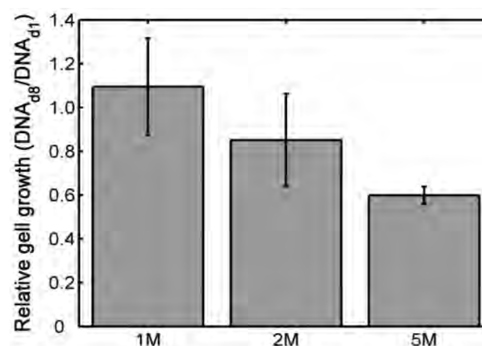

Figure. Relative cell growth at day 8 (n=3).

(17.O7) BIOACTIVE COMPOSITE SCAFFOLDS MIMIC BONE TISSUE

Gentile P (1), Mattioli-Belmonte M (2), Baino F (3), Tonda-Turo C (1), Chiono V (1), Vitale-Brovarone C (3), Ciardelli G (1)

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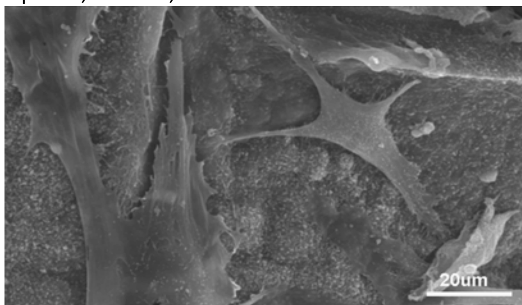
Introduction. Composites based on apatites and natural polymers have received increasing attention in bone tissue engineering due to their ability to preserve the structural and biological functions of the damaged hard tissues in a biomimetic way. In this work porous scaffolds, containing bioactive glass (CEL2) stimulating the biomineralization and chitosan/gelatin (CH-G) blends supporting cell adhesion and proliferation, were developed.

Methods. A 3% (wt/v) CH-G solution (1:2 weight ratio) was dissolved in 0.5M acetic acid at 40°C. CEL2 was added to the polymeric solution (POL) to obtain CEL2/POL composites with various weight ratios between the components: 0/100; 40/60; 70/30 (wt/wt), coded as CEL2/POL_0/100; CEL2/POL_40/60; CEL2/POL_70/30. Genipin was added at defined weight percentage (2.5% wt/wt). Each mixture was kept at 50°C under stirring, poured into Petri dishes and freeze-dried at -20°C for 24h. The obtained scaffolds were characterized for their morphology, water stability, bioactivity in Simulated Body Fluid (SBF), mechanical and biological behaviour using periosteal progenitor cells (PCs)

Results. The increase of CEL2 amount stabilized the composites in water solutions, as shown by swelling tests. CEL2/POL samples showed interconnected pores having an average diameter ranging from 120±5µm for CEL2/POL_0/100 to 95±5 µm for CEL2/POL_70/30. The compressive modulus increased by increasing CEL2 amount up to 2.6± 0.3MPa for CEL2/POL_70/30. The SBF tests showed the high bioactivity of the scaffolds containing CEL2. MTT viability test using PCs showed the biocompatibility of scaffolds. Scaffold composition affected cell morphology (Fig 1).

Conclusions. Composite porous scaffolds containing CEL2 showed an interconnected porosity, bioactivity and suitable mechanical properties. Moreover, their ability to sustain PCs growth strengthened the hypothesis of periosteum as stem cell source for osteo-chondral tissue regeneration based on in situ cell recruitment.

Acknowledgements. This work was supported by ACTIVE (Regional Project, Regione Piemonte) and FIRB projects (RBAP10MLK7).
Keywords. Biomimetic, bioactivity, bone repair, composite, scaffold, stem cells



(17.O8) THE EFFECT OF NANOFIBER TOPOGRAPHY ON CELLULAR BEHAVIORS OF PRIMARY RAT ASTROCYTES IN VITRO

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1. Nanyang Technological University. Singapore; 2. DUKE-NUS Graduate Medical School. Singapore; 3. University of Science and Technology of China

Astrocytes play an important role in the regeneration of the central nervous system (CNS). In particular, glial scar formation by reactive astrocytes after nerve injuries serves as a major hindrance to axonal regeneration. Unfortunately, detailed understanding on astrocyte behavior and interaction with microenvironmental signals remain limited. By investigating astrocyte responses towards topographical cues, we aim to gain insights to scaffold design for CNS regeneration. Poly(caprolactone-co-ethyle ethylene phosphate) (PCLEEP) nanofibers with uniform diameter of 655 ± 11 nm were fabricated by electrospinning and PCLEEP films were prepared by solvent-casting as a two-dimensional (2D) control for primary rat astrocyte culture. Cell proliferation rate as measured by EdU assay indicated significantly lower EdU incorporation rate on nanofibers than on films (18.9% vs. 40.2%, $p < 0.05$). Meanwhile, TUNEL assay demonstrated a higher apoptosis rate on fibers than on films (11.9% vs. 7.1%, $p < 0.05$). Astrocytes on nanofibers adopted a smaller cell area and exhibited an elongated shape as compared to the fully stretched morphology on films, as revealed by GFAP immunostaining. However, the insignificant change in vimentin and GFAP expression levels as shown by western blotting implied a quiescent state of astrocytes despite morphological changes in respond to nanofibers. In our work, the astrocyte responses including proliferation, apoptosis, cell morphology and phenotypic changes towards nanofibrous topography compared to the flat 2D surfaces was demonstrated. The suppressed growth and enhanced apoptosis of astrocytes on nanofiber topography suggest that electrospun nanofiber may serve as a potential bio-functional scaffold for CNS regeneration.

Keywords. Astrocyte, nerve regeneration, nanofiber, topography, electrospinning

(17.O9) FUNCTIONALLY GRADIENT COLLAGEN/NANO-HYDROXYAPATITE OSTEOCHONDRAL SCAFFOLDS

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Introduction. This paper reports a strategy for the fabrication of functionally gradient collagen/nano-hydroxyapatite (HA) composite osteochondral scaffolds to provide an appropriate physical environment for hMSCs to migration and differentiation.

Methods. A modified in situ nano-HA precipitation integrated centrifugation method was used. The resultant composite plugs were cut into 1 mm think sections further examinations with respect to its composition, structure and crystalline. In vitro performance was evaluated using hMSCs.

Results. The XPS analysis demonstrated that the resultant composite scaffold has a continuous composition gradient. It could be categorised into four zones: (1) superfacial zone consists of pure collagen for cartilaginous tissue formation; (2) HA-deplete zone with less than 10% HA content; (3) middle region with HA content in the range of 10% ~ 50%; (4) distal region with rich HA content for bone tissue formation. Superfacial layer exhibited a higher average pore size of 210 mm and porosity of 80%; HA-deplete layer exhibited a pore size of 160 mm and a porosity of 52%; while as the middle section of the scaffold shown a pore size and porosity of 123mm and 45%, respectively. The distal region has dense structure with small pore size in the range of 10 µm ~ 85 µm, and porosity at about 33%.
Histological examination on 4 weeks in vitro cultured specimens revealed there were patches of bone-like tissue formed around HA particles at the HA-rich regions; while patches of cartilage-like tissue were observed with superficial regions and HA-deplete region.

Conclusions. Such gradient composite scaffold may be an appropriate substrate that facilitates formation of tissue for regions of tissue attached to each other, where each region differs in terms of its resident cell type and composite, and could lead to a better understanding of the cellular requirements for co-culture of tissues.

Keywords. Osteochondral tissue engineering, scaffold, collagen, hydroxyapatite

(17.O10) CROSSLINKED GELATIN NANOFIBRE SCAFFOLDS FOR PERIPHERAL NERVE TISSUE ENGINEERING

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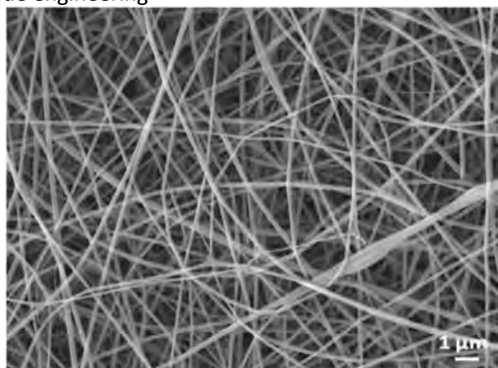
Introduction. Fibrous matrices mimic the complex biological structure of the extracellular matrix and provide the mechanical support to allow the cells of the damaged tissue to adhere, proliferate and migrate properly, forming three-dimensional tissue structures. In this work, electrospinning was used to prepare γ-glycidoxypropyltrimethoxysilane (GPTMS) crosslinked gelatin (GL) nanofibrous scaffolds (GL/GPTMS_NF). The GL based nanofibrous scaffolds were found to be suitable matrices for cell attachment and proliferation and promising materials for peripheral nerve regeneration.

Methods. GL was dissolved in demineralised water at 50°C. Then, GPTMS was added to GL solutions with

various concentrations (the amount of GPTMS was calculated respect to the molar concentration of amino groups of hydroxylysine, lysine and arginine residues to obtain a ratio of 2/1 between the amino groups and the GPTMS molecules) and then solutions were left stirring one hour before spinning.
The electrospinning process parameters and solution concentration were optimized to obtain GL/GPTMS_NF, which were characterized for their morphology, porosity and water stability. The cellular response using neonatal olfactory bulb ensheathing cells (NOBECs) was evaluated on GL/GPTMS_NF.
Results. The electrospinning parameters (solution concentration, applied voltage, needle-collector distance, solution flow rate, temperature) were fixed respectively at 15% wt./vol., 30kV, 15cm, 10µl/min, 50°C to obtain fibres with 356±59 nm size (figure 1). NOBECs adhered and proliferated on the fibrous scaffolds and were found to align into the fibre direction. Moreover, no apoptotic cells were found on the fibrous matrices using a DeadEnd Fluorimetric Tunel System.
Conclusions. Process parameters for the preparation of GL/GPTMS_NF from aqueous solutions were optimised. Nanofibers were found to support the in vitro adhesion, survival and proliferation of glial-like cells.
Acknowledgements. This work was supported by MOVAG (Compagnia San Paolo) and ACTIVE (Regional Project, Regione Piemonte) projects.
Keywords. Electrospinning, gelatin, peripheral nerve tissue engineering



(17.O11) AFM INSIGHTS ON FIBRONECTIN BEHAVIOR AT THE CELL-MATERIAL INTERFACE

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Introduction. The initial cellular events that take place at the biomaterials interface mimic to a certain extent the natural adhesive interaction of cells with the extracellular matrix (ECM). In fact, cells cannot interact directly with foreign materials, but they attach to the adsorbed layer of proteins. Among the ECM proteins, the importance of fibronectin (FN) as a mediator of cell adhesion to a substrate was early recognized. Atomic force microscopy (AFM) is a powerful tool widely used to analyze biological molecules, in particular, protein adsorption on material surfaces, mostly in air environments. Nevertheless, the systematic investigation of the cell-material interface has been scarcely addressed in the literature by AFM. This work investigates fibronectin behavior at the cell-material interface at the nanoscale making use of AFM. Additionally, it approaches conditions in which ECM proteins and cells are found in physiological fluids in vivo or in culture medium in vitro, working in liquid environment.
Methods. Polymers with well-characterized physicochemical properties were used: Poly(L-lactide) acid,PLLA, Poly(methyl acrylate),PMA, and poly(ethyl acrylate),PEA. Thin films were prepared by spin-casting from different polymer solutions on glass coverslips. FN adsorption on the surfaces was performed from solutions of different concentrations (5 and 20µg/ml). Osteoblast-like cells and fibroblasts were cultured on the previously FN-coated substrates in serum-free conditions. Initial FN conformation on the different surfaces as well as the cellular reorganization of the FN layer was directly observed by AFM.
Results and conclusions. Different FN conformations were obtained for each material. Non-connected FN aggregates are observed on PLLA and PMA, whereas PEA is able to induce the formation of a protein network –FN fibrillogenesis- establishing FN-FN interactions. FN reorganization was studied visualizing the FN near and far from cells, in order to compare the effect of cells on adsorbed FN layer.
Acknowledgments. This work was supported by MAT2009-14440-C02-01
Keywords. AFM, cell-protein-material-interaction, protein conformation, FN reorganization

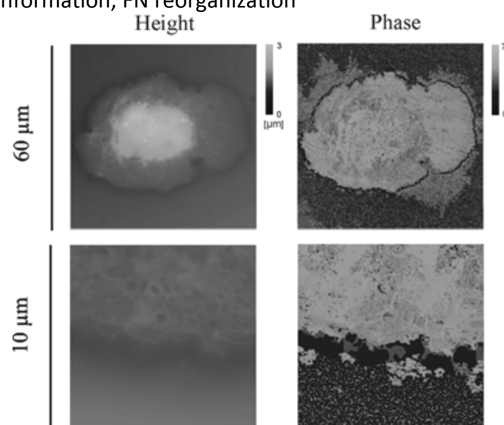

Figure. AFM imaging of cell-mediated FN reorganization

(17.O13) BIOCOMPATIBILITY EVALUATION OF DIFFERENT BIO-INSPIRED SiC AND ITS UTILITY AS IMPLANTABLE DEVICES FOR PREVENTING STAPHYLOCOCCUS AUREUS INFECTIONS

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Bio-inspired silicon carbide (BioSiC) is a new ceramic material obtained from natural resources with excellent mechanical properties, suitable for bone implants. Its microstructure has cell-friendly porosity which also allows

the inclusion of therapeutic molecules adequate for prophylaxis and treatment of device-related infections due to bacterial adhesion and subsequent bio-film formation at the implantation site. VEGF can be also included in order to induce angiogenesis, key process in tissue engineering.

Disks (ø 6mm x 2mm) of BioSiC obtained from different woods: pine (Pinus pinnaster), sapelli (Entandrophragma cylindricum) and oak (Quercus rubra) were produced. The in vitro biocompatibility was tested with a human osteoblast cell line (SAOS-2). Cell proliferation on BioSiC disks unloaded and loaded with vancomycin (1,257 mg), were compared at different times after seeding using SEM and also quantified by a MTT assay. The vancomycin release kinetics were obtained by placing the dried drug-loaded disks in vials with 3ml of phosphate buffer (PBS) at 37ºC. The antimicrobiological activity was carried out against Staphylococcus aureus. Disks were loaded with VEGF, cultured with Human Mesenchymal cells and Smooth Muscle cells.

Differences in the BioSiC porosity cause also variations in the release kinetics. All BioSiCs are characterized by a rapid delivery during the first 2 h, after which the release rate decreases.µAccording to the SEM micrographs, the cells were able to adhere and grow on the BioSiC surfaces. After 15 days all the surface was covered with cells which also grow inside the pores. Differences in microstructure lead variations in cell response. The highest proliferation was obtained on BioSiC from oak possibly as a result of the presence of big pores (100

The results validate BioSiC as potential Easy-to-obtain scaffolds for tissue engineering.

Keywords. BioSiC, microstructure, cell biocompatibility, device-related infections

(17.O14) DYNAMIC CULTURE OF ENDOTHELIAL CELLS ON NEW BIOFUNCTIONALIZED 3D-PRINTABLE POLYMERS FOR SMALL DIAMETER GRAFTS

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Introduction. Effective vascularization is the central demand in tissue engineering. Therefore we developed artificial three-dimensional (3D) blood vessels, which could be dynamically cultivated to supply surrounding scaffolds in vitro. New printable polymers were synthesized, with biomimetic molecules functionalized and in cell culture experiments analyzed for their applicability with endothelial cells. The small diameter vessels were seeded with cells and dynamically cultivated in a new developed bioreactor system.

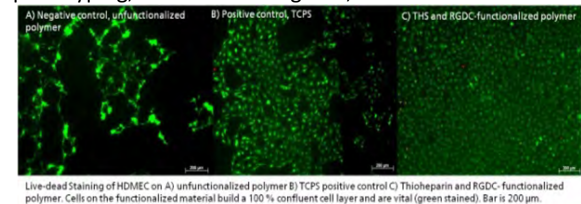
Materials and Methods. Different types of alpha, omega-hydroxyoligoethers adapted to the needs of rapid prototyping process have been synthesized and characterized. The surfaces were biofunctionalized with covalently immobilized thioheparin and the adhesion peptide RGDC. The amount and stability of the surface modification molecules were quantified by XPS, toluidine-blue assay and ELISA. Adhesion, morphology, proliferation and functionality of primary human endothelial cells (EC) seeded on the functionalized substrates were characterized by viability assays and immunohistochemistry. Concurrent with ongoing cell-material-interaction studies, a bioreactor was developed for the dynamic culture of ECs in the printed artificial blood vessels.

Results. The synthesis of suitable polymers for rapid prototyping processes with remaining functional groups was successful. Biofunctionalization of the polymer was shown by the increase in surface sulphate content after thioheparin immobilization by XPS and photometrical with toluidin-blue. Seeded endothelial cells on the functionalized surfaces showed a higher viability and confluence as the cells on control substrates. All cells could be characterized using specific EC markers. Furthermore we designed a bioreactor system for culture experiments and performed cell experiments under dynamic flow conditions.

Conclusion. Promising biomaterial research is affected by the integration of biology, chemistry and engineering. In our interdisciplinary approach, we developed new 3D-printable polymers. The materials were successfully biofunctionalized and cell experiments showed an increased adhesion of EC on the biomaterial surface.

Acknowledgements. We thank the Fraunhofer-Gesellschaft and the Landesgraduiertenförderung Baden-Württemberg for funding the project.

Keywords. Biofunctionalization, in vitro, tissue engineering, vascularization, heparin, RGD, rapid prototyping, small diameter grafts, bioreactor



Live-dead Staining of HDMEC on A) unfunctionalized polymer B) TCPS positive control C) Thioheparin and RGDC- functionalized polymer. Cells on the functionalized material build a 100 % confluent cell layer and are vital (green stained). Bar is 200 µm.

(17.O15) CHARACTERISTICS OF A BIODEGRADABLE POROUS PHB/PCL NERVE GUIDE CONDUIT BIOFUNCTIONALIZED WITH STAR-PEG HEPARIN HYDROGEL

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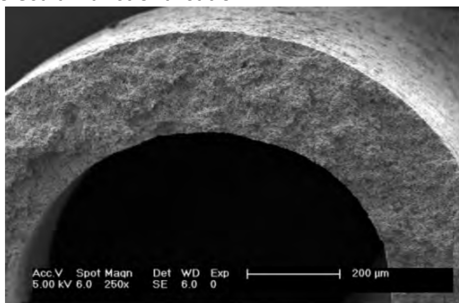
Alternatively for autologous nerve grafts, artificial nerve guidance channels are eligible candidates since dimensionally mismatch, limited supply and second surgery can be avoided. The basic strategy of axonal regeneration by tubulisation is the usage of a hollow structure that is bridging the gap between the two stumps of a severed nerve. Ideally, the nerve ends are inserted into a hollow porous tube, which is functionalized with biomolecular and/or structural cues, promoting the oriented growth of axons and ensuring the transport of nutrients and metabolites over a wide distance. A bioabsorbable material is considered advantageous, since the material will be degraded and metabolized with time. Thus, contusion of the

regenerated nerve and a second surgery site can be prevented.
The latest results towards the fabrication, modification and characterisation of a nerve guidance channel which addresses all of the required properties are presented. Poly(3-hydroxybutyrate) (PHB) is of great relevance for medical applications due to its natural origin and biodegradability. However, PHB is an inherently brittle material. Blended with poly-ε-caprolactone (PCL), biodegradable, mechanically stable and bendable hollow fibers can be fabricated in various dimensions by means of extrusion or melt spinning. The desired porosity can be adjusted by particular leaching of a sacrificial component, for instance polyvinylpyrrolidone. Finally, a well chosen biomolecular functionalization based on a star-polyethylene-glycol heparin hydrogel layer results into a tunable bio-hybrid structure. The hydrogel layer allows for a specific load and release rate of neurotrophins that are necessary for a particular stimulation of the axonal regeneration process. This bio-hybrid structure is expected to be advantageous in comparison to conventional models since it provides desirable mechanical, structural and biomolecular characteristics and is regarded to be beneficial as transplant in regenerative medical therapy as well as scaffold for in vitro studies.
Keywords. Biodegradable scaffold, nerve regeneration, biomolecular functionalisation



(17.O16) HYBRID COMPOSITE SCAFFOLD CONSISTED OF POLYCAPROLACTONE MICROSTRUTS AND ELECTROSPUN COLLAGEN-NANOFIBERS

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In recent years, based on CAD/CAM technology, solid free-form fabrication (SFF) technologies allow to design by computer both the microscopic and the macroscopic shape of scaffolds. The use of computer-based technology to easily fabricate the scaffolds for tissue engineering is advantageous because it facilitates the production of complex computer designed architectures. Unfortunately, the use of fabricated scaffolds, however, is challenging since applicable materials are limited to synthetic biopolymers, and the pore structure can be too large compared to various cells. Those provided low biophysical and biocompatible properties to the scaffold. To overcome these problems, we proposed a hybrid technology, which combines a melt-plotting system (one of SFFs) with electrospinning processes, to produce a hierarchical 3D structure consisting of micro-sized polycaprolactone (PCL) strands and collagen nanofibers. To improve the cellular behavior on the scaffold, we adapted collagen nanofibers in PCL strands since the collagen is the major constructional element of extracellular matrix (ECM) and outstanding biocompatibility and biodegradability. To complete one layer, the perpendicular PCL strands were plotted first, and the upper stage connected to an electrospinning apparatus was moved automatically to the one-layered strands; then the collagen nanofibers (the diameter of collagen fiber is 300–700 nm) were electrospun on top. The layer-by-layer structure of PCL and collagen was approximately 20 × 20 × 1.5 mm3. To evaluate the efficiency of cell attachment, proliferation, and differentiation within the hierarchical scaffolds, we cultured osteoblasts (MG63) for regeneration of bone. The hierarchical scaffold exhibited various positive qualities. In particular, since the collagen is main component of ECM, the interactions between the cells and hierarchical scaffolds containing collagen were much more positive than those between the cells and conventional 3D PCL scaffolds.
Keywords. Scaffold, Polycaprolactone, collagen

(17.O17) MODULATION OF 3D-CULTURED hMSC BEHAVIOUR THROUGH CHANGES IN MATRIX PHYSICOCHEMICAL PROPERTIES

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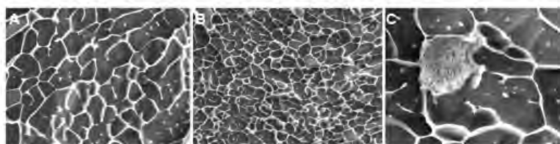
Introduction. Gel-like microenvironments based on cell-instructive biomaterials are becoming increasingly relevant not only for regenerative medicine applications, but also as 3D cell culture models. We previously reported the modification of RGD-alginate hydrogels with protease-sensitive domains, and showed that altering the matrix bio-functionality had a dramatic effect on 3D-cultured hMSCs. In this study, we looked at the possibility of modulating cell behaviour only by changing the matrix physicochemical properties, and compared 2D vs 3D cell response.
Materials and Methods. RGD-alginates with a range of polymer concentrations (0.5-2.5wt%), but constant peptide density were prepared. In situ crosslinking was promoted by adding CaCO3/GDL. Hydrogels microstructure (CryoSEM) and mechanical properties (DMA) were characterized. hMSCs were combined with gel-precursor solutions before crosslinking to establish 3D-cultures; or seeded in spin-coated films (2D). Cell behaviour was analysed and matrices (cell-laden, cell-free) were periodically imaged.
Results. Matrices with different microstructures and pliability were prepared by changing alginate concentration. Hydrogels presented loose (0.5wt%) or dense meshes (2.5wt%), and stiffness increased with polymer concentration. 2D-cultured hMSCs spread (F-actin), and proliferated (3H-thymidine) in all formulations; but 3D cell response was highly dependent on matrix properties. Cells maintained high viability (live/dead assay) and metabolic activity (resazurin), but reduced cell spreading and growth, particularly at higher polymer concentrations, due to the biophysical hindrance imposed by the matrix. In looser hydrogels, cells were able to pull the hydrogel and migrate towards the core, forming dense aggregates. The diameter of those matrices decreased concomitantly.

Conclusions. When cultured under 3D-conditions, cells become physically constrained by the polymeric network that interferes with several cellular functions, in particular with spreading, migration and proliferation, as shown here. The matrix effect is highly dependent on its physicochemical properties and has to be taken into consideration when drawing conclusions from 3D-culture studies.

Acknowledgements. INL for PhD scholarship, FCT for funding (PTDC/SAU-BEB/101235/2008 and FCOMP-01-0124-FEDER-010915).

Keywords. Hydrogel, Stem-cells, 3D-cultures

Fig 1. CryoSEM structural analysis of alginate hydrogels prepared with (A) 0.5wt% and (B) 2wt% of polymer dry-mass. (C) Image of a cell inside a 2wt% alginate matrix.



(17.O18) ELASTIC BIODEGRADABLE FIBRE-MESH SCAFFOLDS COATED WITH BIOMIMETIC CALCIUM PHOSPHATE (CAP) LAYERS FOR PROTEIN DELIVERY

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Introduction. Several studies suggest the possibility of optimizing scaffold elasticity to control cell behaviour. In the recapitulation of cellular microenvironments, scaffolds may play an important role in providing a platform to influence the perception and response of cells to substrate mechanics. We present a fibre-mesh scaffold with elastic properties and drug delivery functions with potential in tissue engineering applications and also as 3D cell culture platform for in vitro studies.

Methods. Fibre mesh scaffolds were produced by wet-spinning using a starch/poly-ethylene-vinyl alcohol blend. The compressive mechanical properties of the scaffold were evaluated and their morphology analysed by scanning electron microscopy. The scaffolds were coated with CaP layers using a biomimetic methodology. To investigate the carrier potential of these coatings for the delivery of multiple proteins, fluorescently labelled proteins were incorporated at different stages of the coating formation. The protein distribution within the coating was visualized by confocal laser scanning microscopy and their release profile determined. Metabolic activity and proliferation of osteoblasts (SaOs-2) seeded onto uncoated and coated scaffolds were assessed by MTS assay and DNA quantification, respectively.

Results. We were able to fabricate a highly porous and degradable 3D structure with elastic behaviour in the wet state. Protein incorporated in the outer coating layers is released faster, whereas the protein present in the inner layers shows a more sustained release, thus showing the carrier potential of the hybrid scaffolds for the controlled release of proteins that can regulate the function of seeded cells. MTS and DNA assays proved that the cells seeded on the scaffolds remain viable with an increased metabolic activity and proliferation rate.

Conclusions. The structural and degradable properties and positive cellular response suggest that the developed fibre-meshes may be good candidates as tissue engineering scaffolds.

Acknowledgements. M. Susano thanks the Portuguese Foundation for Science and Technology for providing her a grant PTDC/CTM/67560/2006.

Keywords. Biodegradable scaffolds, elastic behaviour, biomimetic CaP coating, protein delivery

(17.O19) RESORBABLE CALCIUM PHOSPHATE SCAFFOLDS FOR BONE REPAIR AND REGENERATION

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Introduction. Development of tissue-engineered scaffolds for bone repair and regeneration which deliver results equivalent to autografts remains a clinical imperative. Hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) are widely used due to their excellent biocompatibility and osteoconductivity. However, differences in their respective resorption rates present difficulties in achieving optimal tissue formation. Developing a biphasic HA:β-TCP scaffold could potentially deliver the optimum resorption rate. The aim of this study was to understand the material factors influencing the rate of resorption of biphasic scaffolds.

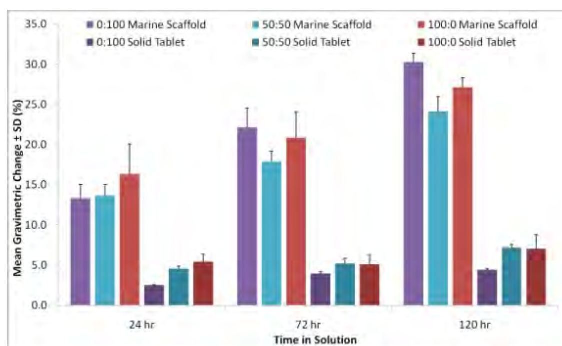
Methods. Porous scaffolds of five HA:β-TCP ratios (0:100, 25:75, 50:50, 75:25, 100:0wt%) were fabricated using a sponge replication technique with natural marine sponge (Spongia agaricina) as the precursor. Solid HA:β-TCP tablets were also produced for comparison. An in-vitro model (pH4 buffer solution, 37degC) was used to simulate osteoclast-like resorption. Samples were removed from solution at intervals of 1, 6, 24, 48, 72, 96 and 120h. Gravimetric and dimensional analysis was conducted. Micro-computed tomography scans were conducted to determine changes in scaffold architecture as a function of time in solution.

Results. Gravimetric analysis (Figure1) showed an increase (R^2=0.8056-0.9469) in mass loss over time. A significant difference (p-value<0.001) was observed for the porous scaffolds when compared to the solid tablets; due to the greater exposed surface area of the scaffolds. Increasing the β-TCP content in the ceramic slurry resulted in a proportional increase (R^2=0.2153-0.7070) in mass loss.

Conclusion. Scaffold resorption rate can be controlled by combining HA and β-TCP in scaffold fabrication; and establishing an appropriate mix-ratio could optimise resorption in line with bone remodelling. The experimental data will be used to validate a dissolution algorithm for computational mechano-biology simulations of tissue differentiation, thereby helping maximise scaffold properties and, potentially, reduce the need for 'trial and error' research.

Acknowledgements. This work is part-funded by Science Foundation Ireland (North-South Supplement Grant).

Keywords. Calcium phosphate, Dissolution, Scaffolds



(17.O20) BIOINSPIRED NANOCOMPOSITES OF RESILIN AND CELLULOSE WHISKERS FOR TISSUE ENGINEERING APPLICATIONS

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Introduction. Resilin is an elastomeric rubber-like protein secreted by insects to specialized cuticle regions, in areas where high resilience is required. Resilin binds to the cuticle polysaccharide chitin via a chitin binding domain and is further polymerized through oxidation of the tyrosine residues resulting in the formation of dityrosine bridges and assembly of a high-performance protein-carbohydrate composite material combining the elasticity of resilin and toughness of chitin. Inspired by the remarkable mechanical properties of insect cuticles we hypothesized that novel composites of cellulose and resilin, cross linked via non toxic resilin polymerization chemistry will enable the fabrication of implants with suitable mechanical properties for orthopaedic load bearing implants.

Materials and Methods. Recombinant resilin fused to cellulose binding domain (CBD) was expressed and purified from E. coli. Cellulose Whiskers (CW) were prepared by H_2SO_4 hydrolysis of micro crystalline cellulose followed by sonication resulting in honey like liquid crystal suspensions. CW suspensions were subsequently cast into aluminium molds and lyophilized resulting in highly porous sponges. Resilin-CBD solutions were embedded into the sponges and polymerized by ruthenium-bis -pyridinium (Fig. 1B) or the Fe/H_2O_2 photo-Fenton system (Fig. 1C, 1D) that we have recently developed for this protein.

Results. Composite sponges of resilin and CW resulted in dramatic alteration in mechanical behaviour including high elasticity and no plastic deformation/high resilience following repeated cycles of mechanical stress (Fig. 1B). In addition we report the successful polymerization of resilin by photo-Fenton reaction resulting in elastic, rubber-like, hydrogels (Fig. 1C,1D).

Discussion and Conclusions. Tendons, ligaments and spine related diseases are among the most common health problems in adult populations. In spite of impressive advances in regeneration, these tissues require biomaterial scaffolds that can handle dynamic loading. Photo Fenton polymerized resilin-CBD and cellulose nano-composite systems may offer the biomechanical properties of resilience, toughness and the durability necessary for load bearing orthopaedic implants. We are currently investigating the combination of plant derived human collagen with resilin composites to support cell proliferation and neo-tissue formation.

Keywords. Resilin, tissue engineering, composites

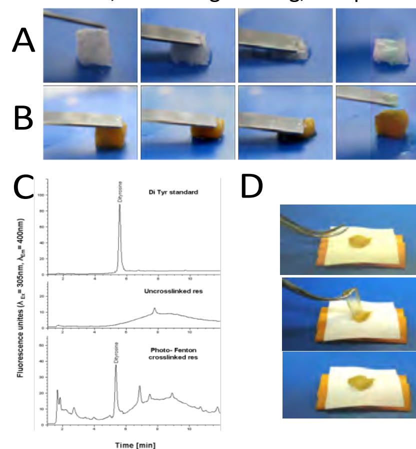

Fig. 1. **A;** Pure low resilience CW sponge **B;** Highly elastic composite resilin- CBD/CW sponge. **C;** Acid hydrolysis products of photo-Fenton polymerized resilin samples separated on a C-18 reverse phase column with fluorescence analysis for di-tyrosine detection **D;** Novel photo-Fenton polymerized elastic resilin

(17.O21) DEVELOPMENT OF BIOACTIVE MEMBRANE SCAFFOLDS FOR TISSUE ENGINEERING

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Introduction. The use of thin bioactive membrane scaffolds in tissue engineering and regenerative medicine could have many applications in vivo, directly replacing or stimulating tissues, and in vitro, facilitating well-controlled studies of cell-cell communication or nutrient permeability. In this work we report on the combination of a top-down/bottom-up approach to develop thin self-supporting bioactive membranes based on Elastin-Like Polymers (ELPs).

Materials and Methods. ELPs containing the cell adhesive epitope arginine-glycine-aspartic acid-serine (RGDS) were synthesized using standard recombinant protein production techniques and cross-linked with 1,6-hexamethylene-diisocyanate (HMDI). The ELP membranes were fabricated by a drop-casting/evaporation technique using a spin-coater to precisely control membrane thickness. Membranes were fabricated with a variety of topographical patterns on either one or both sides, uniform and well-defined through-holes, and exhibiting multi-layers. Membrane swelling and stiffness were characterized by atomic force microscopy (AFM), nanoindentation tests, and scanning electron microscopy (SEM). The membrane biocompatibility and bioactivity

were assessed by in vitro culture using rat mesenchymal stem cells (rMSCs).

Results. Membranes were reproducibly fabricated with thicknesses varying between 500nm–100µm depending on the fabrication conditions, exhibited sufficient structural integrity to be handled and sutured, and served as in vitro cell culture substrates. Membranes were also fabricated comprising topographical features with heights ranging between 500nm and up to 10µm. Optical, inumuno-fluorescence, and scanning electron microscopy demonstrated that rMSCs adhered on the ELP membranes, exhibiting a spread morphology and well-defined actin cytoskeleton.

Discussion and Conclusions. We have developed a variety of fabrication techniques based on micro and nanotechnologies to create thin self-supporting membranes that comprise bioactive epitopes and a variety of topographical, morphological, and structural components that could be fine-tuned to stimulate specific biological processes. These structures could potentially serve as thin bioactive, biomimetic, multifunctional, and biodegradable scaffolds for a variety of applications in tissue engineering and regenerative medicine.

Keywords. Membrane scaffolds, microfabrication, bioactivity, elastin-like polymers

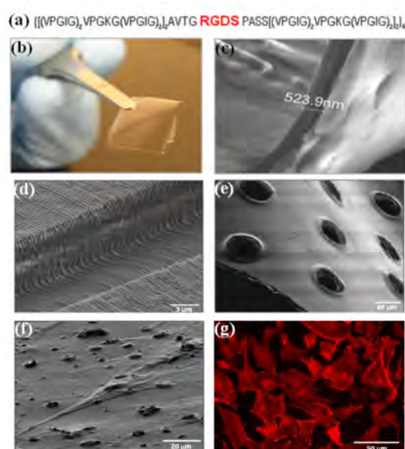

Figure 1. (a) ELP sequence used in the development of self-supporting (b,c) smooth membranes or (d) topographically patterned ,(e) porous membranes that (f,g) support growth of rMSCs.

(17.O22) MULTILAYER VASCULAR GRAFTS BASED ON COLLAGEN-MIMETIC HYDROGELS

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Introduction. The urgent clinical need for small-caliber vascular prostheses has prompted investigation of biomimetic grafts with properties that more closely match native blood vessels. To this end, we have established a novel biomaterial platform based on a collagen-mimetic protein derived from group A Streptococcus, Scl2.28. Scl2 has the triple helical structure of collagen, but unlike collagen, Scl2 is a non-thrombogenic protein that can be modified to have selective cell adhesion. We have developed the methodology to incorporate Scl2 proteins into a poly(ethylene glycol) (PEG) based hydrogel matrix. These bioactive hydrogels facilitate optimization of bioactivity and mechanical properties which offers unique control over the endothelialization of the graft. However, scaffold properties that promote endothelialization may not be consistent with the mechanical properties necessary to withstand physiological loading. To address this issue, we have reinforced Scl2/PEG hydrogels with an electrospun polyurethane mesh. This multilayer vascular graft design decouples requisite mechanical properties from endotheliazation processes and permits optimization of both design goals.

Methods. Polyurethane chemistries and electrospinning parameters were varied to optimize compliance, burst pressure, and suture retention of composite grafts. Platelet adhesion under flow of whole blood was evaluated to determine graft thrombogenicity. Additionally, endothelial cell (EC) adhesion and migration was evaluated in response to changes in Scl2 concentration and identity in the hydrogel layer.

Results. Constructs were developed with biomechanical properties comparable to human saphenous veins in current clinical use (Table 1). Platelet adhesion was statistically less than collagen-coated tissue culture polystyrene and equivalent to PEG hydrogels. EC adhesion on the Scl2/PEG hydrogels was comparable to collagen-based hydrogels.

Conclusions. Our multilayer design can achieve a non-thrombogenic intimal layer that promotes EC adhesion and migration while providing mechanical properties of current autologous grafts. These results demonstrate the great potential of these vascular grafts as an off-the-shelf graft for small diameter arterial prostheses.

Keywords. Collagen-Mimetic Proteins, PEG hydrogels, Vascular graft

Property	Multilayer Graft	Saphenous Vein
Burst Pressure	1404 ± 40 mmHg	1680 ± 307 mmHg
Compliance	5.2 ± 0.5 mmHg-1X10-4	4.7 mmHg-1X10-4
Suture Retention	306 ± 21 gf	196 ± 2 gf

Table 1. Biomechanical properties of multilayer vascular grafts are comparable to saphenous vein allografts currently used in bypass surgeries.

(17.O24) INTEGRATION OF MULTIPLE CELL-MATRIX INTERACTIONS INTO ALGINATE SCAFFOLDS FOR PROMOTING CARDIAC TISSUE REGENERATION

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Background and aims. Engineering a functional cardiac tissue in vitro is one of the most challenging tasks for tissue engineers. In this research, we aimed to reconstruct the microenvironment promoting cardiac tissue regeneration by presenting multiple cell-matrix interactions, in a similar manner to their presentation by the extra-cellular matrix (ECM) in vivo. Thus, two fibronectin-derived peptides, (RGD and heparin binding peptide (HBP), were bound to alginate scaffold, mimicking the specific interactions of ECM with integrin and syndecan on cell membrane, respectively

Methods and Results. The peptides (GGGGRGDY, GGGGSPPRRARVTY or their combination) were covalently-attached to alginate via the carbodiimide

chemistry, creating an amide bond between the peptide terminal amine group and the alginate carboxylic group. High efficiency of peptide attachment and uniform distribution in the scaffold were confirmed by using fluorescently-tagged peptides. Peptide binding did not have an effect on scaffold internal morphology (e.g., porosity by Scanning Electron Microscope) or matrix stiffness. The HBP/RGD-modified scaffold was more favorable compared to that with single peptide- or unmodified alginate scaffolds, as reflected by the increased AKT phosphorylation, indicating to the activation of adhesion-dependant pathway and pro-survival signaling. Furthermore, already by day 7, well-developed myofibers with distinguished striation were observed in HBP/RGD scaffolds . In the RGD-attached scaffold, sporadic islands of striation were seen, but no developed myofibers. In contrast, the HBP-modified and unmodified scaffolds had no such as effect on cardiac reorganization. Finally, alpha-actinin, Connexin-43 and N-Cadherin expression profiles presented better tissue maturation and regeneration of a functional cardiac muscle tissue within the scaffolds with the multiple functional cues

Conclusions. Our data establish the potential use of HBP/RGD alginate scaffolds as a better ECM-mimicking microenvironment for inducing regeneration of functional cardiac tissue, in vitro.

Keywords. RGD, heparin binding peptide, alginate scaffold, cardiac tissue regeneration

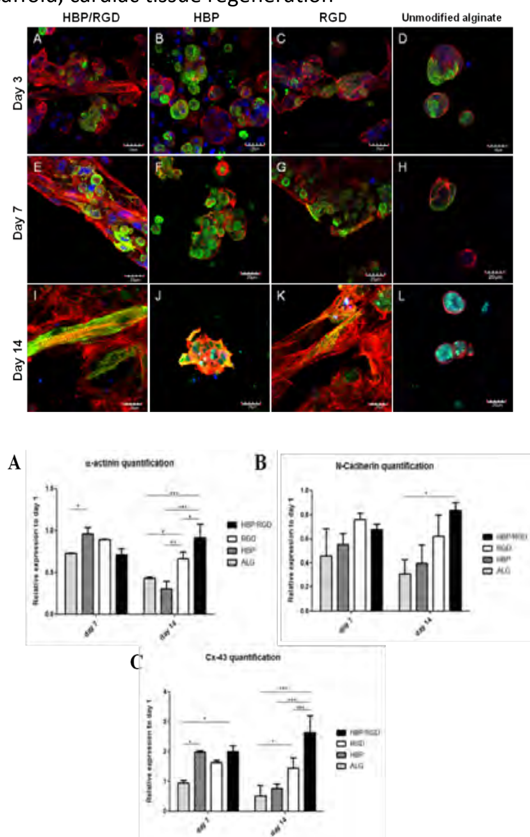

Figure 1. (a) Confocal images of cardiac constructs in HBP/RGD, HBP-, RGD-modified and unmodified scaffolds at different times during cultivation. Cardiac cells are stained for F-actin(red), sarcomeric alpha-actinin (green) and nuclei (blue). (b) Expression of representative cardiomyocyte proteins by Western Blot. The relative folds of increase in the contractile protein a-sarcomeric actinin (A), the cell-cell adhesion molecule N-Cadherin of the intercalated disc (B) and the gap junction protein Connexin-43 (C).Asterisks denote significant difference (2-way ANOVA), when *p<0.05, **p<0.01 and ***p<0.005.

(17.O25) CELL HARVESTING FROM ELASTIN-LIKE RECOMBINAMERS GRAFFTED SURFACES

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This work describes an efficient method for removing single cells or cell sheet which maintaining intact cell-cell and cell-extracellular matrix (ECM) interactions without necessity of using mechanical, physical or enzymatic methods. For this aim bioactive elastin like-recombinamers (ELRs) were covalently grafted to glass cover slides or polystyrene creating bioactive thermo-responsive surfaces by Click Chemistry.

The ELRs are a class of proteinaceous materials which exhibit smart behaviour, bioactivity and unmatched biocompatibility.The smart behaviour is due to transition temperature which permit us obtain thermo-responsive surfaces by grafted these polymers.Their biosynthesis by means of recombinant DNA technologies provides tailored polymers with an absolute control of the architecture, lack of randomness in amino-acid sequence, stereochemistry, and exact molecular weight.Moreover, is possible to build complex amino-acid sequences including different and specific functionalities as adhesion cellular sequences or to modify the transition temperature by a single change in their amino-acid sequences. Each step of the modification of the polymer and surfaces (glass and polystyrene) before reaction as well as the polymer functionalized surfaces were characterized by water contact angle measurements, amino-acid analyses, mass spectroscopy, infrared spectroscopic analysis, TOF-SIMS, XPS and AFM.

Thus, we obtain bioactive thermo-responsive surfaces to achieve detachment single cells or cell sheet without use mechanical, physical or enzymatic methods which can damage the interaction between cell-cell and cell-ECM. For this aimis necessary a single decrease on the environmental temperature. We also check if these single cells or the cell sheet detached are viablethroughout colorimetric methods and flowcytometry. They are able to adhesion, grow and proliferate to new polystyrene tissue culture surface after temperature treatment.Moreover, it is possibly to combining different bioactive sequence polymers more or less specific to create particular cellular areas onto surface building cell sheet that mimicking natural tissue.

Keywords. Elastin-like Recombinamers, Click Chemistry, Cell-Harvesting

(17.O26) BIORESORPTION BEHAVIOUR OF ALGA-HA BONE GRAFT SUBSTITUTES

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One area for clear improvements in synthetic grafts is their in situ bioresorption performance. Clinicians would prefer the graft to resorb completely to minimise the risk of failure from infection. From an engineer's prospective,

resorption should be in synchrony with new bone formation. Too slow a resorption rate, and new bone formation will be impeded, whereas too fast a rate will cause mechanical instability at the defect site. The bioresorption profile of synthetic calcium phosphate (CaP) bone grafts depends on the physicochemical properties of the material involved and the biological environment: some highly crystalline hydroxyapatite (HA) bone grafts, for example, have been reported to remain in situ for up to five years. This study investigated the bioresorption profile of a semi-crystalline algal-HA (QUB HA) and a highly crystalline algal-HA (manufactured by methods analogous to those used to produce the commercially available Algipore). The materials were placed in vitro in both a cell free and a cellular environment, in their original granular form and formed into discs. For cell-free dissolution the materials were placed in buffer solutions at pH7.4 and pH4.0 with agitation at 37°C. Dissolution behaviour was monitored using inductively coupled plasma-mass spectroscopy (ICP-MS) to quantify Ca, P and Mg ions dissolution and mass loss over 28 days. An osteoclast assay using RAW 264.7 cell line expanded in sRANKL was performed on the materials at 6 and 12 days. ICP and SEM quantifying resorptive pit formation were used as an outcome measure. The results showed that processing conditions affect the rate of dissolution/bioresorption with respect to time and both decreased with increased crystallinity and porosity.

Keywords. Algae, Microporous, Calcium Phosphate, Bioresorption

(17.O27) IN VITRO BEHAVIOUR OF MESENCHYMAL STEM CELLS ON A CONDUCTIVE ELECTROSPUN SILK FIBROIN NANOFIBER SCAFFOLD COATED WITH POLYPYRROLE FOR BIOMEDICAL APPLICATIONS

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Introduction. The possibilities of scaffolds composed of nanofibers of silk fibroin (SF) could be greatly enhanced by conferring them electro conductive functionalities. Here we present the generation of a hybrid material made of SF coated with polypyrrole (PPy-SF), studying its biocompatibility as scaffold for proliferation of primary human mesenchymal stem cells.

Methods. The silk fibroin mesh was obtained by electro spinning of a 17% (w/v) SF solution (in HFIP). After the annealing with methanol the meshes were coated with polypyrrole. The characterizations of physical, structural and mechanical properties were developed using SEM, FTIR spectroscopy, and a mechanical tester. Electrochemical experiments were also performed.

MSCs were obtained by direct aspiration of iliac crest from volunteer donors. The cells were isolated by gradient ficoll using a SEPAX™ System device and cultivated in DMEM supplemented with 10% FCS and penicillin/streptomycin. Pieces of mats were seeded (2.0 x 104 cells/cm2) into 24 wells cell culture plates and proliferation was measured by MTT staining at 1, 7, 14 and 21 days.

Results. The average diameter of SF-PPY coated fibers was 2630 nm (ranging from 472 to 8670 nm). FTIR spectroscopy indicates that the conjugated polymer has some interactions with the peptide linkage affecting to SF macromolecular chains.

Cells showed an excellent adhesion on the materials tested just 72h after the seeding and a slight growing tendency was observed.

Conclusions. Our results show the ability of electrospun silk matrices to support MSCs attachment, spreading and growth in vitro. We added an original variable, using conducting polymers (PPY) adsorbed to SF fibers in order to increase the electric conductivity of the mats with its possible additional benefits due to the relevance of electric fields in cell function and spatial disposition.

Acknowledgements. This work was supported by INIA and by RETICS RD/0010/2012 grants from the ISCIII.

Keywords. Fibroin, polypyrrole, nanofibers, MSCs

(17.O28) ASSEMBLY OF PLATELET-LYSATE LOADED CHITOSAN-CHONDROITIN SULFATE NANOPARTICLES AS NEW THREE-DIMENSIONAL HYDROGEL CONSTRUCT FOR ENTRAPMENT OF HUMAN ADIPOSE DERIVED STEM CELLS FOR CARTILAGE TISSUE ENGINEERING

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Introduction. Platelet lysates (PL) are an outstanding autologous source of growth factors (GFs) that can play an enhancement role over the proliferation and differentiation ability of mesenchymal stem cells. Natural based chitosan/chondroitin sulfate nanoparticles (CH/CS NPs) were developed with the ultimate goal of encapsulating bioactive agents to promote and enhance cartilage regeneration. Previous studies performed in our group reported the successful incorporation of PL in these NPs, which were then released in a controlled manner in two and three dimensional (2D and 3D) in vitro cultures of human adipose derived stem cells (hASCs), enhancing the proliferation rate of hASCs while they are differentiating into the chondrogenic phenotype. The CH/CS complex mimics the extracellular matrix (ECM) interactions and when used at determined concentrations, the PL-loaded NPs can assemble in simple and quick mode and form a 3D stable hydrogel while in suspension with hASCs, following a mild centrifugation. The cells are then entrapped in this enriched 3D environment, recreating the ECM in cartilage.

Methods. The PL-loaded hydrogels were cultured in vitro in chondrogenic and basal mediums up to 28 days and were characterized for cell viability, proliferation, glycosaminoglycans production, histology, immunohistochemistry and gene expression (by polymerase chain reaction) for cartilage regeneration. hASCs pellets and empty NPs hydrogels were used as controls.

Results. The presence of PLs influences the biological response of the entrapped cells, stimulating their

viability, proliferation and production of a cartilage ECM throughout the culture time. It was also possible to detect an enhancement of gene expression for chondrogenic markers, indicating the positive role of GFs release from PL on the differentiation ability of hASCs.

Conclusions. The assembly of PL-loaded NPs in combination with hASCs enabled the development of an innovative and effective 3D system with multiple functionality and thus with high potential for application in cartilage tissue regeneration.

Acknowledgments. FCT for the PhD grants of Santo VE and Popa EG (SFRH/BD/39486/2007 and SFRH/BD/64070/2009), IPS, Hospital da Prelada. EXPERTISSUES (NMP3-CT-2004-500283), Find&Bind (NMP4-SL-2009-229292).

Keywords. Adipose derived stem cells; platelet lysate; hydrogel; cartilage tissue engineering

(17.O29) ABILITY OF A MARINE SPONGE-DERIVED POROUS HA SCAFFOLD TO SUPPORT BONE CELL GROWTH AND DIFFERENTIATION

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Bone tissue engineering may provide an alternative to autograft use for particular clinical applications, however scaffold optimisation is still required to maximize bone ingrowth. In designing scaffolds, pore size, distribution and interconnectivity may affect bone cell attachment, proliferation and differentiation and there is evidence that cells prefer a degree of non-uniformity and a structure that closely resembles that of natural bone. The aim of this study was to compare scaffolds derived from a porous marine sponge (Spongia agaricina) with those derived from synthetic polyurethane foam.

Hydroxyapatite scaffolds of 1cm3 were prepared via ceramic infiltration of marine sponge and a polyurethane (PU) foam. Porosity, pore size distribution and pore interconnectivity were measured. For biocompatibility studies, human foetal osteoblasts were seeded at 1x105 cells/scaffold for up to 14 days. Cytotoxicity, cell number, morphology and differentiation were investigated. PU-derived scaffolds had 84-91% porosity with pore sizes ranging from 50µm-1000µm (average 577µm) and 99.99% pore interconnectivity. In comparison marine sponge-derived scaffolds had 56-61% porosity with pore sizes ranging from 0-500 µm (average 349µm) and 99.9% pore interconnectivity. hFOB studies showed that more cells were found on marine sponge-derived scaffolds at d4, d7 and d14 than on the PU scaffold but there was no difference in cell differentiation as measured by alkaline phosphatase activity and expression of cbfa-1, collagen I and osteocalcin. XRD and ICP showed that more Ca and Si ions were released from the marine-derived scaffold.

Three dimensional porous constructs have been manufactured that support cell attachment, proliferation and differentiation but significantly more cells were seen on marine-derived scaffolds. This could be due both to the chemistry and pore architecture of the scaffolds with optimum mechanical stimulus of the cells derived from the pore characteristics, in addition to a biological stimulus from increased dissolution of Ca and Si ions.

Keywords. Marine Sponge, Biomimetic, Scaffold, in vitro

(17.O30) DESIGN OF INDUCTIVE SCAFFOLD FOR THE OSTEOCHONDRAL DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS (HMSC)

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Challenge and Goals. HMSC differentiation depends on the environment wherein the cells reside, especially on the spatio-temporal presentation of the differentiation-inductive factors. We aim to reconstruct the microenvironment promoting the osteochondral differentiation of MSCs, by presenting the chondro-inductive Transforming Growth Factor-beta1 (TGF-beta1), and the osteo-inductive Bone Morphogenetic Protein-4 (BMP-4) in a similar manner to their presentation by the extracellular matrix.

Methods and Results. TGF-beta1 or BMP-4 were individually bound to two alginate-sulfate-containing macroporous alginate scaffolds, subsequently to be combined into a bilayered osteochondral inducing system. The affinity binding to alginate sulfate resulted in a sustained factor release for 7 days, in contrast to the burst release of these factors from unmodified scaffolds. The factors retained their bioactivity, as revealed by the enhanced collagen deposition in fibroblasts culture. HMSCs, seeded in these scaffolds, showed prolonged and elevated phosphorylation levels of Smad2 and ERK1/2, for up to 14 days, indicating the long-term activity of the affinity-bound factors. Masson's trichrome staining and immuno-staining of 14 days-old cell constructs demonstrated substantial deposition of collagen and collagen type II, in the TGF-beta1/affinity-bound layer and the cells in this layer presented round morphology of committed chondrocytes. In the BMP-4/affinity-bound layer, elevated levels of alkaline phosphatase (ALP) and increased mineralized bone matrix deposition after 3 weeks indicated their osteogenic differentiation. The ALP activity in the bilayered system was significantly greater compared to the activity in the only BMP4-loaded scaffolds, suggesting a mutual effect of the factors and their spatial arrangement within the system on MSC differentiation.

Conclusions. These data indicate the potential use of the affinity-binding alginate scaffolds combined with spatial presentation of TGF-beta1 and BMP-4 for guided differentiation of hMSCs; allowing the reconstruction of the microenvironment for osteochondral tissue formation.

Keywords. Mesenchymal stem cells, osteochondral, microenvironement, affinity-binding, scaffold

(17.O31) FABRICATION AND CHARACTERISATION OF ELECTROSPUN, TUBULAR, AXIALLY ORIENTED FIBRILAR GELATIN SCAFFOLDS FOR VASCULAR TISSUE ENGINEERING

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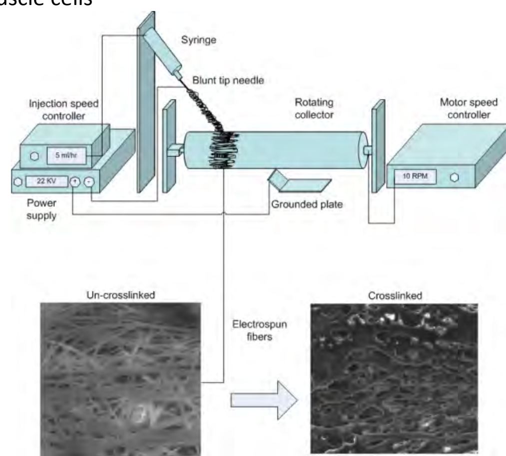
Electrospinning of polymer solutions or melts to develop fibrous constructs that can be used as cell scaffolds is a promising fabrication technique for tissue engineering due to a number of reasons, including controlled fibre diameter to low nanoscale that can be sometimes useful

for cell adherence, incorporation of nanoparticles or a second phase to mimic the natural extra cellular matrix (ECM) as for collagen/elastin vascular grafts, the ability to control porosity, pore size and fibre orientation with relative ease coupled with the cost effectiveness of the process. Gelatin has proved to be an advantageous material to use in tissue engineering due to its favourable interaction with the cells, its chemical and hierarchical physical structure being similar to the collagen structure and its low cost. However gelatin lacks the mechanical strength required for the tissue engineering process leading to the necessity of crosslinking. The following work discusses the fabrication of electrospun, tubular, fibrous, gelatin scaffolds with axial fibre orientation manufactured with a variety of structural features, i.e. different fibre diameter, fibre volume fraction, degree of crosslinking, pore size and porosity. Furthermore, tubular fibrous scaffolds with graded structure and porosity from the outer layer to the lumen have also been fabricated. Smooth muscle cells (SMCs) and endothelial cells (ECs) are then seeded on the scaffolds and monitored for adherence, mass transfer into the scaffold, growth and growth of cells layer surrounding each fibre. The cell proliferation across the depth of the scaffold is examined by staining. The results show a direct correlation between the physical properties of the crosslinked scaffolds and the transfer rate and proliferation rate of the cells. Finally the cytotoxicity of the glutaraldehyde, used as a crosslinking agent, is also examined to determine its effect on the cells.

Keywords. Scaffold, electrospinning, gelatin, smooth muscle cells



(17.P1) 3D CELL GROWTH IN ALGINATE FOAMS

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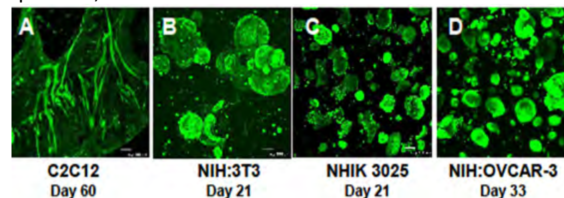
Growing cells in a 3-dimensional (3D) matrix instead of traditional 2D cultures can approximate cell architecture and cell-cell contact found in tissues, organs and tumors. NovaMatrix-3D™ is an alginate-based cell culture system comprising an alginate foam matrix and an alginate immobilizing solution. In principle, cells are first suspended in a sodium alginate solution then the cell suspension is applied to calcium alginate foams. In situ gelation occurs when calcium ions are donated from the foam cross-linking the added alginate, effectively entrapping the cells within the pores throughout the foam. The utility of this new cell culture system is shown using NHIK 3025 (cervix carcinoma), MCF7 (breast adenocarcinoma), ZR-75-1 (breast ductal carcinoma), C2C12 (myoblast), NIH:3T3 (fibroblast) and NIH:OVCAR-3 (ovarian adenocarcinoma). Cell localization within the foam was visualized using confocal microscopy to identify fluorescently labeled cells (CellTrace™ CFSE). Cells were immobilized with or without RDG-coupled alginate to investigate the importance of the presence of cell attachment peptides within the alginate. For some of the cell lines, cell proliferation and multicellular spheroid formation was independent of the presence of RGD. Cell proliferation was measured by counting cells after de-gelling the foam using sodium citrate. Spheroids could also be removed intact by de-gelling the alginate matrix and further processed for histological staining. One example shows the selective staining of apoptotic cells within the spheroid. Use of alginate foams with concomitant in situ immobilization of cells results in a 3D cell culture model with the potential to approximate cell proliferation and architecture within tissues or tumors. The technology enables biomimetic approaches by varying e.g. matrix elasticity, gelling ions, attachment peptides and foam degradation making NovaMatrix-3D™ a versatile cell culture system. Portions of this work have been funded with support from the METOXIA project no. 222741 under the 7th Research Framework Programme of the European Union.

Keywords. 3D cell culture, alginate, multicellular spheroid, scaffold



(17.P2) 3D PLLA SCAFFOLDS BY DIRECTIONAL THERMALLY INDUCED PHASE SEPARATION (TIPS): ARCHITECTURAL TUNING AND BIOLOGICAL VALIDATION

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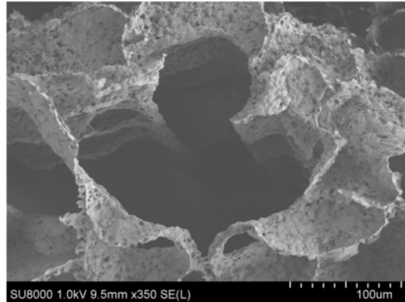
The role ascribed to the scaffold architectural organization, as dictated by tissue engineering paradigms, is foremost. As an artificial endoskeleton, the scaffold is requested to provide optimal frameworks for the seeded cells to organize into a functional tissue. Directional thermally induced phase separation (dTIPS) is a versatile, cost-effective technique for fabricating highly porous scaffolds from different materials, having fully tailorable porosity, and strongly anisotropic pore architectures. Consequently, dTIPS scaffolds represent an ideal support for the growth of biological tissues that exhibit gradient morphology, such as bone, tendons, ligaments, nerves, liver, pancreas, and in particular blood vessels. The reconstruction of vascular grafts is in fact a prerequisite when the growth of thick tissues is needed.

In the present work, we investigated the effect of the process parameters, such as cooling temperature, (-30°C ≤ Tc ≤ +5°C), cooling time (2.5 h ≤ tc ≤ 32 h), and polymer concentration (1.5 to 6.5 wt%), on the pore microstructure of poly(L-lactic acid) 3-D scaffolds made by dTIPS. The scaffolds exhibited highly ordered dendritic domains, having overall porosities up to 95%, and interconnectivity over 98%. By controlling the cooling regime and polymer concentration we were able to tune the pore diameter from few tenths of micrometers up to 260 µm, while keeping the peculiar pore hierarchy unaltered, accompanied by a decrease in scaffold compression modulus from 8 to 1 MPa. Moreover, the biological validation assessed after 7 DIV of mesenchymal stem cells culturing, evidenced massive scaffold colonization.

In summary, the possibility to scale up and down the pore architecture in dTIPS scaffold by one order of magnitude by simple adjustments of the process parameters, may allow creating a gradient porosity for in-growth of complex tissues at any length scale (e.g., from macro to micro blood vessels) in one single construct.

Keywords. PLLA scaffold, Thermally Induced Phase Separation, mesenchymal stem cells, vascular tissue



(17.P3) INFLUENCE OF SCAFFOLD ANISOTROPY ON TENOGENIC MESENCHYMAL STEM CELL DIFFERENTIATION - ALIGNED COLLAGEN I NANOFIBRE SCAFFOLDS FOR POTENTIAL ROTATOR CUFF REPAIR

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Introduction. The utilisation of cell-seeded, biomimetic scaffolds that reflect the high anisotropy (collagen I fibre alignment) of native rotator cuff tissue might be promising for the reconstruction of substantial defects of the rotator cuff. Collagen I electrospinning was modified using a rotating target to obtain nanofibre scaffolds (NFS) with a different degree of anisotropy to investigate the influence of fibre alignment on the behaviour of bone marrow derived mesenchymal stem cells (MSC).

Material and Methods: Collagen I was isolated and purified from rat-tail tendon. Collagen I was electrospun onto a rotating mandrel at various speed (0,3 -10m/s). The resulting NFS were characterised by scanning electron microscopy (SEM) and mechanical testing. Collagen I-NFS were seeded with bone marrow derived MSCs (2 x 105/scaffold) and cultured under static conditions in serum supplemented (10% FCS) medium for up to 21 days. Cell orientation, tenogenic marker gene expression (RT-PCR) and histological appearance were evaluated at defined time points.

Results. Increased linear translation of the rotating mandrel led to a higher fibre alignment (>90% at 10 m/s) and increases tensile properties of the scaffolds. MSC seeded on aligned NFS showed a high degree of cell axis orientation parallel to the fibre alignment (>90% at 10 m/s) in contrast to a random orientation on non-aligned NFS (0,3 m/s). RT-PCR revealed higher expression of tenogenic marker genes (Scleraxis, Elastin, Col I) in aligned vs. non-aligned scaffolds.

Conclusion. The consideration of ultrastructural aspects of the target tissue is a crucial parameter in the process of scaffold design for MSC-based tissue engineering approaches. High anisotropy of collagen I-NFS supported mechanical properties, MSC orientation and tenogenic marker gene expression. Thus, underlining the superior potential of aligned collagen I-NFS in MSC-based approaches for the reconstruction of tenogenic tissues.

Keywords. Electrospinning, anisotropy, stem cell, tenogenic differentiation, nanofiber

(17.P4) A COLLAGEN MATRIX ACTIVATES THE ERK PATHWAY AND IMPROVES THE SURVIVAL AND FUNCTION OF ENDOTHELIAL PROGENITOR CELLS

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Introduction. Biomaterials are being developed to augment the efficacy of endothelial progenitor cell (EPC) therapy. EPC transplantation with a collagen matrix was previously shown to be superior to EPCs alone for restoring function to ischemic tissue. This study explored a possible mechanism through which the matrix may confer improved EPC therapy, specifically investigating activation of the ERK pathway, which is involved in the transduction of external signals to normalize intracellular activities.

Methods. Human EPCs were cultured on fibronectin (control) or a collagen/chondroitin sulfate-C matrix, cross-linked with glutaraldehyde. Cell lysates were probed for ERK using Western blotting. Flow cytometry was performed to assess cultures for progenitor cells (CD34, CD133), for endothelial cells (CD31, CD144); and for proliferation (EdU). Migration and adhesion of cells, with or without ERK inhibitor (PD98059), were assessed. Finally, cells were exposed to serum deprivation, and viability was assessed using 7-AAD staining.

Results. Increased ERK1 (1.4-fold) and ERK2 (1.1-fold) phosphorylation was observed in matrix-cultured cells ($p \leq 0.05$), indicative of greater ERK activity. Proliferation of CD133+ and CD133+CD34+ cells was increased on the matrix compared to fibronectin (by 2.9- and 1.6-fold, respectively; $p \leq 0.02$). Adhesion potential was greater on collagen (4.0-fold; $p=0.02$), and 40% ($p=0.02$) more matrix-cultured cells were observed to migrate. When ERK inhibitor was applied, the differences between treatments in adhesion and migration were abrogated. After serum deprivation, there were 3.8-fold ($p=0.07$) more viable CD34+ cells and 7.8-fold ($p=0.02$) more viable CD133+ cells on collagen matrix.

Conclusion. A collagen matrix confers pro-survival and proliferative signals for progenitor cells, and enhances cell adhesion and migration capacity, mediated by the up-regulation of ERK. The use of collagen matrices is promising for enhancing cell-based regenerative therapies.

Keywords. Endothelial Progenitor Cell; ERK; Cell culture; Cell therapy

(17.P5) RECOMBINANT SPIDER SILK PROTEINS FOR BIOMEDICAL APPLICATIONS

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Spider silk is made up of unique proteins, spidroins, with a tripartite composition; an N-terminal non-repetitive domain, a highly repetitive central part composed of ~100 poly-Ala/Gly-rich co-segments, and a C-terminal non-repetitive domain. Recent data on the N- and C-terminal domains indicate that they have different specific functions in the formation of spider silk fibres. Miniaturized spidroins have been designed by combining the terminal domains with a limited number of repetitive segments and produced recombinantly. Such miniaturized spidroins have been found to recapitulate the properties of native spidroins to a surprisingly large extent, provided that they are produced and isolated in a manner that retain water solubility until fibre formation is triggered. Moreover, recombinant spidroins can be genetically modified to incorporate specific cell binding motifs or improve mechanical strength. Herein, we investigate some steps towards the realization of the potential of recombinant spider silk for biomaterial applications.

Miniature spidroins that include the C-terminal domain can form macroscopic fibres within hours. When the N-terminal domain also is included, immediate self-assembly is observed at pH values below 6.4 (as observed in the spinning duct of the spider), while the protein can be stored for days in soluble form above pH 7 (as observed in the gland of the spider). These properties can be used in the development of a controlled polymerization process. Generally, the self-assembly process of these miniature spidroins seems robust, as also modified variants, e.g. those with incorporated cell binding motifs, can be processed into various formats, such as free standing films, porous foams, capsules and 3D meshes. These results, together with the facts that the silk matrices are of non-animal origin, mechanically robust, easily sterilized, biodegradable and well tolerated in vivo, hold promise not only for in vitro cell culturing, but also for tissue engineering applications.

Keywords. Silk scaffold self-assembly biomimetic

(17.P6) A NOVEL DESIGN OF AN ARTIFICIAL ISLET-CARRIER; THE EVALUATION OF ISLET SUPPORT, ADHERENCE AND FUNCTION

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Introduction. Transplantation of the islets of Langerhans is one promising treatment for diabetes. Unfortunately currently available procedures suffer from low efficacy due to loss of function and survival of the pancreatic cells. The low success rates are incompletely understood but prior to transplantation, during islet isolation, the environment surrounding the cells is disrupted. Therefore establishment of an environment optimized for islets is necessary for the design of a possible artificial, islet-carrier for transplantation. In order to do so, a highly versatile biomaterial is needed as a scaffold.

Experimental methods. Recombinant spider silk, 4RepCT is a strong and highly versatile material that can acquire various forms e.g. three-dimensional fiber meshes, foams or films1,2.

This newly generated synthetic variant of spider proteins; both the wild type and variants modified by incorporation of different intergrin and laminin related cell-binding motifs (e.g RGD, IKVAV and YIGSR) was used to define an environment for pancreatic islet adherence, islet function and survival after isolation.

Isolated human and mouse pancreatic islets were cultured up to 5 days either plated onto wells coated with the 4RepCT protein in the various forms or without the protein (control islets).

Results and discussion. The islet adherence to the 4RepCT various forms showed that both human and mouse islets do adhere with an increased number to the foam structure. There is also a preference for adherence onto the foam with RGD cell-binding motif. The islets plated on the 4RepCT were functionally active demonstrating insulin release both under basal glucose concentration and its' stimulation with increase in concentration of glucose.

Conclusion. The properties of 4RepCT can be used as scaffolds mimicking the natural cell environment thus providing support for the islets of Langerhans after isolation.

Acknowledgements. The authors would like to thank Vinnova and Barndiabetesfonden for providing financial support to this project".

Keywords. Recombinant spider silk, Islets of Langerhans

(17.P7) COMPOSITE SCAFFOLDS FOR VASCULAR TISSUE ENGINEERING

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Introduction. Extracellular matrix (ECM) influences cellular response by interacting with cellular adhesion molecules, growth regulators, binding proteins1,2,3. Our idea is to realize scaffolds composed of synthetic polymer integrated with lyophilized decellularized aortic matrix (DAM) which should introduce specific attachment sites for cell proliferation.

Methods. Bovine DAM was obtained with the detergent-enzymatic method of Meezan4. A 1:1 (w/w) mixture of DAM homogenate and polyvinyl alcohol (PVA) aqueous solution was used to realize small-diameter vascular scaffolds by low temperature treatments. After scaffolds analysis by SEM, their citocompatibility was evaluated by seeding endothelial cells. Cell presence was evaluated by

DAPI, H&E stainings and Movat's pentachrome technique. DAM has been also analyzed by proteomic methods.

Results. Composite scaffolds were realized using a mixture of PVA and lyophilized DAM. Proteomic analysis evidenced ECM proteins like collagen I and VI. The three-dimensional structure of the scaffolds has been evaluated by SEM analysis. After in vitro seeding, with human endothelial cells, scaffold sections have been stained with DAPI and H&E to confirm the presence of cells and with Movat's pentachromic to stain typical ECM proteins. Cells proliferated only on constructs conditioned with DAM matrix, evidencing its specific role on cell attachment.

Conclusions. The presence of ECM regions in DAM based/PVA scaffolds created specific attachments sites for cell growth. Further analysis will be necessary to evaluate the mechanical behaviour of the PVA scaffolds after cell colonization. Once optimized all the conditions for in vitro cell growth, we will try to implant in vivo these vascular constructs.

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Keywords. Scaffolds, vascular graft, extracellular matrix, tissue engineering

(17.P8) DEVELOPMENT OF BIOACTIVE PCL MATRICES FOR TISSUE ENGINEERING OF LIGAMENT

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Introduction. Ligament tissue engineering needs appropriate source cells and growth matrix to support cell proliferation and collagen synthesis. To control cell response, new porous poly(ε-caprolactone) (PCL) scaffolds were modified by grafting bioactive polymers, poly(sodium styrene sulfonate) (PolyNass), that can induce difference in fibroblast morphology and cell activity during in vitro assay1. In the present work, cell behaviour was first estimated onto 2D-PCL films and thereafter into 3D cross-linked polymer scaffolds.

Methods. PCL films were manufactured by spin-coating. Porous cross-linked PCL scaffolds were obtained using a particulate-leaching process and paraffin beads as porogen agents. For both films and scaffolds, surfaces were functionalized through radical polymerization of Poly(NaSS) after sample ozonation. Evidence of grafting was provided by a toluidin blue colorimetric method and X-ray photoelectron spectroscopy (Pr. David Castner, NESAC/BIO, Seattle, USA). The porosimetry of porous scaffold was analyzed by scanning electron microscopy. Biological assays were carried out using McCoy cell line.

Discussion and Conclusion. Porosity in the range of 75-80% was obtained for cross-linked PCL scaffolds in agreement with the amount of porogen incorporated. Spherical macropores were obtained with a remarkable interconnection. Toluidine blue assay suggests an homogeneous grafting of bioactive polymer on surface samples. Cell response on grafted or non-grafted samples indicates absence of toxicity. First results are encouraging and further in vitro investigations have to be done.

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Keywords. Tissue engineering ligaments porous scaffolds

(17.P9) FABRICATION OF 3D CHITOSAN SCAFFOLDS USING AN INVERSE PLOTTING METHOD

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To create regenerated damaged tissues, cells are attached and cultured onto a scaffold that is ultimately implanted at the injured area of the functioning tissue, so that the scaffold should be biocompatible and biodegradable material. In two-dimensional scaffold, cells are restricted to spread and attach to flat surface, so that biophysical properties of the scaffold, which should provide a spatial effect, may not be applied in the implanted body. However, three-dimensional (3D) scaffolds provide physical signals to guide cell colonization as well as chemical signals of cell-binding sites to support cell attachment and proliferation. To achieve the ideal spatial architecture of the scaffold, solid free-form fabrications (SFFs) have been introduced to construct scaffolds in a layer-by-layer manner. Since the SFFs can provide scaffolds with complex internal structure, which cannot difficult with conventional fabricating methods, the techniques are unique methods for designing scaffolds. Generally, chitosan scaffolds have been fabricated as porous structures by freeze-drying process and electrospinning process. However, more work for fabricating the chitosan scaffold should be required due to difficult control of pore size and low pore interconnectivity. To overcome these structural problems of chitosan scaffold, we adapted a combined technology of inverse plotting method with a sacrificing mold and freeze-drying method. Using this method, we can acquire a highly porous and stably pore-interconnected structured 3D chitosan scaffold. To observe the feasibility as a scaffold, we cultured MG63 cells in the scaffold and the results were compared with a conventionally designed spongy type scaffold.

Keywords. Chitosan, 3D scaffold, Bone

(17.P10) SCAFFOLDS TAILORED FOR BONE TISSUE REGENERATION: EFFECT OF BIOCERAMIC FILLER CONTENT ON ELECTROSPUN MEMBRANE PROPERTIES

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Introduction. A large number of composite scaffolds have been trailed for the tissue engineering applications, however, a search for an optimal scaffold properties and fabrication conditions is one of the key directions of tissue engineering. The incorporation of nanofillers into polymer matrix enhanced mechanical properties, and improved osteoblast responses. More favourable cell responses are typically associated with the chemistry,

topography and mechanical properties of the scaffolds, which are tailored by inorganic fillers. Our aim was to fabricate electrospun membranes modified with different ceramic fillers and assess a function of the filler chemistry on bioactivity. The scaffolds are intended for bone applications.

Experimental methods. Membranes were electrospun from polymer/ceramic solutions. Polymer matrix: PLDL (PURAC), PCL (Sigma-Aldrich). Ceramic fillers: n-HAp (AGH-Poland), TCP (Plasma-Biotal). The solutions were spun at a working distance of 20cm, driving force of 30kV. The solution flow rate was 15ml/h. The membranes complex structure and their chemistry were characterised using SEM, FTIR, and WAXD. The mechanical properties were assessed on the basis of tensile tests. Biomimetic growth of the apatite on the surface of biomaterials after incubation in SBF was confirmed by SEM, EDX, WAXD and FTIR.

Results and discussion. SEM after 7 days of incubation in SBF revealed dense and uniform apatite layers, with typical for apatite globular structure. Differences in ability to apatite grains forming were observed between samples with different fillers. The occurance of apatite layer was detected in FTIR spectra after only 3 days of incubation in SBF for PLDL/n-HAp samples, while for PLDL samples a very weak FTIR bands associated with HAp appeared after seven days.

Conclusion. These studies demonstrated that the incorporation of ceramic filler into electrospun membranes improved bioactivity, which was found to be related to the chemistry of the filler.

Acknowledgments. Polish Ministry of Science and Higher Education (project: N N507550938).

Keywords. Scaffolds, bone tissue, hydroxyapatite, TCP, PLA, PCL

(17.P11) THE BIOMIMETIC POLYLACTIDE/ BETA-TRICALCIUM PHOSPHATE SCAFFOLD AS BONE GRAFT FOR TISSUE ENGINEERING

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In this study, we took polylactide (PLA) filaments to form 3-dimensional braids by using 16-spindle braid machine and enabling its structure to possess the even holes and the tunnels, and then we stuffed the fabric into the beta-tricalcium phosphate (β-TCP) tube to adjust its mechanical stress, which is similar to the human bone's structure. In vivo results indicate that polylactide/ beta-tricalcium phosphate scaffold can promote contact osteogenesis.

Keywords. Polylactide, braid, tricalcium phosphate, bone graft

(17.P12) INFLUENCE OF TCP CONTENT ON CHITOSAN AGGLOMERATED SCAFFOLD PROPERTIES

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Introduction. In hereby presented work a technique for bone scaffold preparation is described. The method is based on the agglomeration of chitosan/composite microspheres. Authors present the fabrication process and essential properties of the materials obtained.

Methods. In the first step microspheres (CH, CH_5%TCP and CH_10%TCP) were extruded in the drop forming rate from chitosan solution into precipitation bath. When completely dried they were subjected to agglomeration in presence of acetic acid, then subsequently neutralized, washed and finally dried. The influence of TCP content on physical and biological properties concerning HBDCs culture was evaluated herein.

Results. The presented technique allows generating porous materials with controllable shape, pore size distribution and their interconnectivity. It was established that microspheres extruded from CH solution only were much smaller than those containing additionally TCP and the diameters were enclosing in the range of 600 – 1000µm. As far as CH/TCP microgranules it was found that the diameters were mostly over 1000µm (1000-1450). Young modulus established on the basis of stress-strain curves was similar for all of the materials and equaled about 250 MPa. On the other hand we found that compression strength decreased with increasing TCP concentration. Our preliminary study concerning HBDC culture did not show a clear influence of TCP concentration on the viability of the cells, but XTT measured after 48h revealed values of the viability enclosing in the range of 60 and 85% when compared with control sample.

Conclusions. In contrast to many methods for porous materials manufacturing, the presented technique permits to fabricate scaffolds with well-developed surface for cell attachment. Mechanical properties were found to be similar to natural bones. Satisfactory viability of HBDCs after 48h of a direct contact with the investigated material in culture is promising. Further detailed studies on the interaction between the chitosan scaffolds and cells are planned.

Keywords. Chitosan, agglomeration, in vitro

(17.P13) MECHANICAL STIMULATION OF FIBROBLASTS IN MICRO-CHANNELED NANO-CELLULOSE SCAFFOLDS ENHANCES PRODUCTION OF ORIENTED COLLAGEN FIBERS

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Introduction. Current meniscal repairing methods do not repair all the meniscal tears, especially those occurring in the avascular region. Even though meniscal transplantation offers the best results for radial tears, complex tears and degenerative tissue, current research shows that degeneration of the articular cartilage still

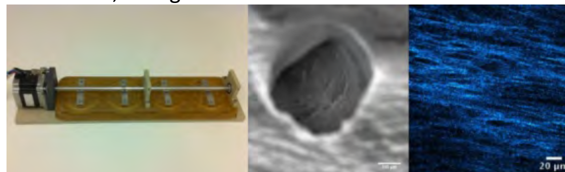
occurs. Tissue engineering of fibrocartilage is a promising solution to restore the function of the joints. Our aim is to mimic the ultrastructure of fibrocartilage and implement it in a nano-cellulose matrix. The strength of fibrocartilage is attributed to the high content and alignment of collagen fibers. Therefore, it is of extreme importance to control the orientation of the cells and their extra-cellular matrix.

Methods. In this study, we have developed a novel scaffold concept based on nano-cellulose (NC) produced by bacteria (Gluconacetobacter xylinus) and perforated by micro-channels to mimic the ultrastructure of the outer portion of the meniscus. The scaffolds with micro-channels (~350 µm diameter) were prepared and seeded with 3T6 fibroblasts. A compression bioreactor was designed and constructed to evaluate the effects of mechanical stimulation on collagen production. Dynamic compression was applied to the NC scaffold/cell constructs at a frequency of 0.1 Hz and compression strain of 5%. A static culture was used as control. The laser-based nonlinear microscopy techniques second harmonic generation (SHG) and coherent anti-stokes raman scattering (CARS) were used to visualize collagen fibers and cell arrangement, respectively.

Results. Results from SHG, CARS and brightfield microscopy showed that the micro-channels facilitate the alignment of the cells and collagen fibers. Furthermore, collagen production is enhanced by mechanical stimulation.

Conclusions. These results show that it is possible to engineer a composite biomaterial consisting of a nano-cellulose matrix reinforced with oriented collagen fibers and having potential to be used for development of a knee meniscus implant.

Keywords. Nano-cellulose, micro-channels, mechanical stimulation, collagen fibers



(17.P14) DEVELOPING A INJECTABLE BIOFUNCTIONAL, BIOMIMETIC HYDROGEL SCAFFOLD FOR REGENERATIVE MEDICINE APPLICATIONS

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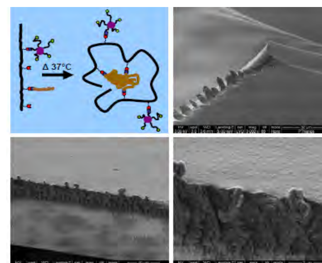
Multifunctional, biomimetic hydrogels presenting unique physical and biochemical signals that enhance and orchestrate a variety of biological processes during tissue regeneration would potentially be a great material platform for new therapies and in vitro studies.

Thus, bottom-up design scheme employing a nano-functional platform based on multifunctional dendrimers and a thermo-responsive hyaluronan hydrogel are reported. The dendrimers are branched polyethylene glycol nano-structures with five end-groups that can bear azide functions and peptides to interact with alkyne groups (e.g. Huisgen 1,3-dipolar cycloaddition (CuAAC)) and specific cell-surface receptors thereby inducing desired cellular responses.[1] Hyaluronic acid (HA) is a major component of the extracellular matrix in connective tissues, synovial fluids, and as a provisional matrix in developing organs. Furthermore, the thermo-responsive HA composition prepared by the CuAAC of propargylamide substituted HA with azido functional poly(N-isopropylacrylamide) has been recently described and explored as a biodegradable scaffold in regenerative medicine therapies.[2]

The project goal was to prepare dendrimers bearing RGDS and azide functionalities that could be grafted via CuAAC onto the thermo-responsive HA compositions. Dendrimers with 4 RGDS peptide and 1 azide, 4 RDSG scramble peptide and 1 azide were grafted at a peptide concentration of 0.005mM/ml. After physicochemical characterizations of the biomaterials, the behavior of human mesenchymal stromal cells (hMSCs) seeded onto the biomimetic gels were studied in vitro for 1 week. Alamar Blue, Trypan Blue assays and histology was performed. Preliminary data indicated that the dendrimers were successfully grafted onto the HA (1H NMR). Also, the gelling and mechanical properties of the thermo-responsive HA compositions were influenced by the presence of the hydrophilic dendrimers. No significant differences were observed in the hMSC viability seeded on the HA gel containing the RGDS and scramble peptides dendrimers after 7 days, indicating that both biological and mechanical cues are important when developing a 3-D biomimetic matrix.

Keywords. Hydrogel, hyaluronan, dendrimer, peptide, stem cells



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(17.P15) TITANIUM SUBSTRATES COATED WITH CALCIUM PHOSPHATE BY BIOMIMETIC METHOD

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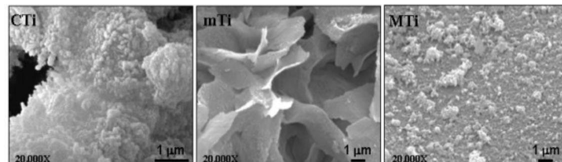
Titanium (Ti) implants have been coated with calcium phosphate (CaP) in order to improve their osseointegration at the implant-bone interface, due to the high biocompatibility of the mineral. This work aims to study a biomimetic method for coating different Ti substrates. It was used as substrates, micro (mTi) and macroporous (MTi) titanium ASTM/grade 2 samples, produced by powder metallurgy, with 19.56% and 61,38% porosity, respectively, and commercially Ti ASTM/grade 2 dense sheet (CTi), with 2.8 µm medium roughness. The

samples were pre-treated for surface bioativation using a 1M NaOH solution followed by heat-treating at 200ºC in air. Then they were immersed for 21 days in a simplified solution (SS) at 37°C, based on $CaCl2.2H2O$ and $Na2HPO4.2H2O$ salts. Phase characterization of the CaP coatings was achieved by low angle X-ray diffractometry (XRD) and Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR-ATR). The CaP microstructure was identified by scanning electron microscopy (SEM).

CaP precipitation with globular (MTi and CTi samples) or plate-like (mTi sample) morphologies was observed by SEM. The coatings XRD diffractograms showed different CaP phases precipitated on the Ti substrates with hydroxyapatite (HA) characteristic peaks for all samples, octacalcium phosphate (OCP) for mTi sample and carbonate apatite (CAp) for CTi and mTi samples. The identified CaP phases were confirmed by FTI-ATR analyses, which results were quite similar. Spectra from mTi, MTi and CTi samples presented OCP and CAp absorption bands. MTi and CTi samples also presented HA absorption bands. The results demonstrated that the biomimetic method used in this work successfully precipitated bioactive CaP coatings onto the Ti samples with different substrate types. However, adjustments in the methodology will be necessary in order to obtain continuous coating in shorter immersion times in SS.

Acknowledgements: CNPq, FAPERJ and Iberoamerican Network BioFab-CYTED and LNLS/Campinas-SP/Brazil for financial support.

Keywords. Calcium phosphate, Titanium, coating, biomimetic



(17.P16) HYDROXYAPATITE COATING ON POLYMER SCAFFOLD USING POLYDOPAMINE FOR BONE REGENERATION APPLICATIONS

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Introduction. Biodegradable polymer/ceramic composite scaffolds for bone regeneration applications are advantageous over either biodegradable polymer or ceramic alone. This study describes a simple and fast method to coat polymer scaffolds with hydroxyapatite (HA). Dopamine is a peptide sequence found in mussel adhesive protein. It was investigated whether polydopamine (DOPA)-coated polymer scaffolds can be coated with HA nanoparticles.

Materials and Methods. Polyglycolic acid (PGA) meshes were coated with HA by immersing the scaffolds in a 2-(N-morpho1ino)ethanesulfonic acid buffer solution containing polydopamine (2 mg/ml) and HA nanoparticles (20 and 5 mg/ml) for various periods of time. HA coating on scaffolds were examined by selective staining of ceramic particles, scanning electron microscopy, attenuated total reflectance Fourier transformed-infrared spectroscopy, X-ray photoelectron spectroscopy, and energy-dispersive spectroscopy. To evaluate bone formation efficacy of scaffolds in vivo, PGA scaffolds, DOPA-coated PGA (DOPA-PGA) scaffolds, and HA/DOPA-coated PGA (HA-DOPA-PGA) scaffolds were implanted to critical size defects in mouse skulls for 8 weeks.

Results. Various analyses showed that DOPA coating can efficiently induce HA nanoparticle adsorption on PGA mesh surfaces. Substantial HA coating on PGA scaffolds was achieved within 24 hours of incubation. Soft X-ray radiography, microcomputed tomography and histological analyses showed that bone regeneration in vivo was more extensive on HA-DOPA-PGA scaffolds compared to the other scaffolds.

Conclusion. DOPA offers an efficient and simple method for HA coating on polymer scaffolds. HA-polymer composite scaffolds fabricated with this method exhibited enhanced bone formation efficacy as compared to the polymer scaffolds.

Acknowledgement. This study was supported by a grand (A101539) from the Korean Health 21 R&D Project, ministry of Health and Welfare, Republic of Korea.

Keywords. Bone regeneration, hydroxyapatite composite, polydopamine

(17.P17) SYNTHETIC MATRIX-MIMETIC POLYPEPTIDE CONSTRUCTS ENHANCE ATTACHMENT OF MESENCHYMAL CELLS TO DIVERSE SCAFFOLD SURFACES

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For both bone tissue regeneration and implantation, efficiency of cell attachment to the scaffold or implant surface is critical to success. However, several widely used surgical and implant materials have limited ability to promote cell adhesion. Failure of cells, either engrafted or host, to adhere to the surface may impede regeneration or lead to implant loosening. In the present work, we have tested the ability of synthetic polypeptide constructs to improve attachment of various mesenchymal cells (human mesenchymal stem cells [MSCs] isolated from adipose tissue or differentiated from embryonic stem cells, as well as MSC-like human foreskin fibroblasts) to diverse surfaces such as non-tissue-culture plastic, titanium, bone substitute of bovine origin (Bio-Oss®, Geistlich Biomaterials), and a surgical mesh (TIGR™, Novus Scientific). The polypeptide constructs consisted of a polylysine backbone decorated with matrix-mimetic oligopeptide motifs attached to spacer arms. Surfaces were functionalized by simple physical adsorption of the polypeptide conjugates. Attachment, survival, and differentiation of cells was followed up to 8-21 days by fluorescence and phase contrast microscopy, viability assays, as well as fluorescent and Alizarin red staining. Our observations confirmed that the polypeptide

conjugates increased the affinity of surfaces to cells with efficiency comparable to that of fibronectin. As these synthetic polypeptide conjugates can be manufactured in a reproducible and cost-efficient manner, can be lyophilized and stored indefinitely, are easily reconstituted, and once applied to a surface remain inert under normal conditions, they may provide a reasonable alternative to recombinant protein-based surface treatment. This work was supported by the Hungarian National Office for Research and Technology (NKTH, BIO_SURF).

Keywords. Extracellular matrix-mimetic peptide, bone tissue regeneration, mesenchymal cell

(17.P18) GENERATION OF BIOARTIFICIAL HEART TISSUE BY COMBINING 3D GEL BASED CONSTRUCT WITH DECELLULARIZED MATRIX

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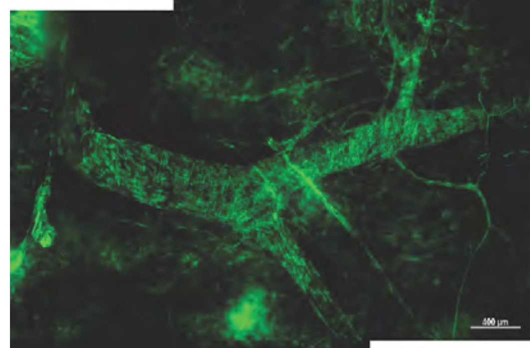
Introduction. A central problem in generating bio-artificial cardiac constructs (BCC) in vitro is the efficient supply of 3-dimensional tissues with nutrients and oxygen. In order to create a functional, suturable implant, we combined a gel based cardiac construct, with decellularized porcine small intestinal submucosa (SIS) and analyzed the reorganisation of seeded cells.

Methods. Isolated rat neonatal heart cells were mixed with collagen I, and Matrigel and casted onto SIS, which were pre-seeded with a monolayer of cells from the same preparation either with or without supplementation of 7% GFP labelled rat endothelial (RHE) cells. During the cultivation period (14 d) BCC were functionally investigated in respect to frequency and direction of contractions. Histological and immunohistological stains were conducted to observe cellular organisation of BCCs.

Results. All BCC contracted spontaneously and rhythmically, as one unit, in the direction of collagen fibres within the SIS, with an average rate of 200 beats per minute. Cells within the constructs appeared in aligned manner, and cardiomyocytes were elongated and well organized. A dense CD31 positive, 3D network of endothelial cells through the whole construct could be observed after 7 days. GFP labelled RHE cells were found not only along the monolayer between SIS and the gel construct, but also upwards growing through the gel construct up to the top, and also downwards into the SIS. Moreover, after 14 days, pre-existing decellularized vessel structures of the SIS were re-populated to a high degree (Fig 1).

Conclusion. A 3D tubular-like network built by endothelial cells, being a cellular component of neonatal rat heart isolates in a solid bio-artificial cardiac construct, may offer a connecting system for the vascularization of this tissue upon implantation. Thus, it might be an important precondition for the survival of thicker myocardial replacement constructs.

Keywords. Small-intestinal submucosa; bio-artificial cardiac construct; vascularization; endothelial cells



Re-populated vessel structures of the SIS with stably transfected GFP-tagged RHE cells following 14 days of cultivation

(17.P19) PERFUSION-BASED 3D MICROTUMOR CULTURE PLATFORM FOR CANCER CELL CULTURE AND ANTI-CANCER DRUG TESTING

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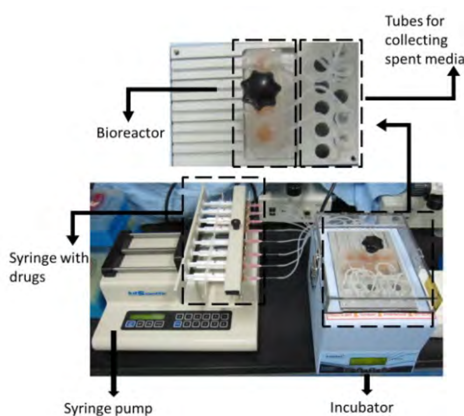
Many cell lines have been successfully cultured in different three dimensional models in vitro. But 3D culture has the limitation of nutrition and oxygenation perfusion, which cannot be achieved by simple diffusion. 3D dynamic culture model, as a simulation of vascular system, can significantly improve the cell viability in vivo. In this study, a multiple parallel perfusion-based bioreactor (TissueFlex®) was employed to study the differences between static culture and perfusion culture on cell activities and drug responds. Two commercial available anti-cancer drugs (Paclitaxel and Cisplatin) were tested on DLD1 and NCI/ADR cell lines in a monolayer and three dimensional formats. Perfusion culture system is believed to provide stable and physiological environment by continually supplying culture medium and removing waste medium. Cells show higher growth rate and higher cell activity in perfusion culture than static. And for drug treatments, cells shows significant different toxic responds under perfusion and static culture for monolayer and 3D culture. Cells cultured in perfusion system are more sensitive to drug dose-response and show lower growth inhibition, which indicates the importance of providing suitable system to testing cellular responds to drugs.

Keywords. Cancer cells; 3D culture; perfusion culture; toxicity testing



(17.P20) HUMAN OSTEOBLAST-LIKE CELLS ON BORON-DOPED NANOCRYSTALLINE DIAMOND THIN FILMS

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Introduction. Nanocrystalline diamond (NCD) is a promising material for various biotechnologies, including construction of biosensors, detection, separation and purification of biomolecules, and surface coating of bone implants. NCD films can be rendered to be electrically conductive by doping with boron, which may increase their attractiveness for cell colonization.

Methods. Nanocrystalline diamond (NCD) films were deposited on silicon substrates by a microwave plasma-enhanced CVD process and doped with 133, 1000 and 6700 ppm of boron in the gas phase. The films were seeded with human osteoblast-like MG 63 cells and their adhesion, growth and osteogenic differentiation were investigated. The adsorption of collagen I, an important component of bone extracellular matrix, was also studied by confocal laser scanning microscopy, two photon microscopy and second harmonic generation microscopy imaging.

Results. The electrical resistivity of the films decreased from >10 MΩ (non-doped films) to 55, 0.6, and 0.3 kΩ (doped films with 133, 1000 and 6700 ppm of B, respectively). The increase in the number of MG 63 cells in 7-day-old cultures on NCD films was most apparent on NCD doped with 133 and 1000 ppm of B (152,500 ± 13,900 and 152,200 ± 10,400 cells/cm2, respectively, compared to 112,900 ± 9,700 cells/cm2 on non-doped NCD films). On NCD films with 6700 ppm of B, the cells contained the highest concentration of focal adhesion protein vinculin, measured per mg of protein. Similarly the concentration of osteocalcin, an important marker of osteogenic cell differentiation, increased with increasing level of B doping. Boron doping also positively influenced adsorption of collagen I and its production by cells.

Conclusions. Our results suggest that the potential of NCD films for bone tissue regeneration can be further enhanced by boron-doping.

Acknowledgements. Supported by the Acad. Sci. CR (grants No. KAN400480701, IAAX00100902), and the Grant Agency of the CR (grant No. P108/11/0794, LG 06063).

Keywords. Carbon nanoparticles, nanotechnology, electrical conductivity, bone tissue engineering

(17.P21) TRANSFERASE-CATALYZED BIOMIMETIC HYDROGELS FOR TISSUE ENGINEERING

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Synthetic hydrogels are key elements in emerging strategies for tissue engineering and cell biology. However, the current shortage of highly specific and biocompatible methods to form and functionalize these materials hampers their wider use in pharmaceutical and medical applications. To this end, we envision that enzymatic cross-linking schemes could be an essential and still underexplored option for biomaterials development.

We present an application of phosphopantetheinyl transferase (PPTase) for covalent cross-linking of poly(ethylene glycol) (PEG)- based hydrogels. PPTase is an enzyme that plays a key role in the biosynthesis of many natural products and has been employed as a biotechnological tool for site-specific protein modification. PPTase performs a highly specific transfer of phosphopantethyinyl residue of Coenzyme A (CoA) into the active site of specific carrier proteins (CPs). In our recently developed PPTase-based hydrogel system, cross-linking occurs between CoA-functionalized multi-arm PEG macromer and a genetically engineered CP dimer.[1] Importantly, here we have explored the possibility of replacing CP by a small synthetic peptide analog. Chemically synthesized short CP was tested as a dimer or was conjugated to multi-arm PEG, which offers the opportunity for further optimization and modulation of gel network architecture. In this study, the physiochemical properties of hydrogels produced by these different approaches are compared. Furthermore, we show that using this enzymatic scheme, site-specific modification of PPTase-hydrogels is possible, as demonstrated by covalent incorporation of integrin-binding cell adhesion ligand in 2D as well as 3D cellular assays.

In conclusion, PPTase-based hydrogels represent a novel class of functional and bioactive materials which offer the possibility of tuning physiochemical properties through a rapid, highly specific cell-friendly cross-linking reaction. Furthermore, the completely synthetic design of this material is a key feature which may be relevant in clinical settings. Consequently, we envision a wealth of useful applications of this new gel system in cell biology and tissue engineering.

Reference. 1. Mosiewicz, K. A.; Johnsson, K.; Lutolf, M. P., Journal of the American Chemical Society 2010, 132, (17), 5972.

18. ESB - TERMIS SYMPOSIUM: BIOMECHANICS IN TISSUE ENGINEERING

Chair: Damien Lacroix
Co-chair: Dominique Pioletti
Keynote speaker: Manuela Teresa Raimondi
Organizer: European Society of Biomechanics
Synopsis: Biomechanics plays a major role in the development of tissue engineering approaches as it has been recognized that mechanical stimuli acting directly on cells affect gene expression. Therefore, throughout the development of tissue engineering as a discipline of bioengineering, the progress made in the design and construction of bioreactors, and the morewidespread use of bioreactors have allowed to understand better the interactions between biomaterial scaffolds, cells and mechanical stimuli, and have allowed to develop more functional scaffolds for different applications of regenerative medicine. More recently, progress has been made in the development of in silico techniques that enable to simulate the different biological processes occurring in tissue engineering such as cell seeding, cell proliferation and cell differentiation. These techniques not only bring a better understanding in the mechanobiological processes underlying tissue engineering but also provide tools to optimize the bioreactor conditions for the development of functional scaffolds and therefore avoid the experimental 'trial and error' approach.
In this symposium new advances in the biomechanics of tissue engineering will be presented. Contents of the presentations in this symposium with a focus on mechanical stimuli can include:
- Design of new bioreactors
- Mechanical loading on scaffolds
- Effect of mechanical stimuli in scaffolds in bioreactors
- In vivo mechanical stimulation of tissue regeneration
- Simulation of nutrient transport in bioreactors
- Simulation of mechanical stimuli in bioreactors
- Simulation of in vivo tissue growth and regeneration

(18.KP) MECHANOBIOLOGY OF CARTILAGE TISSUE ENGINEERING

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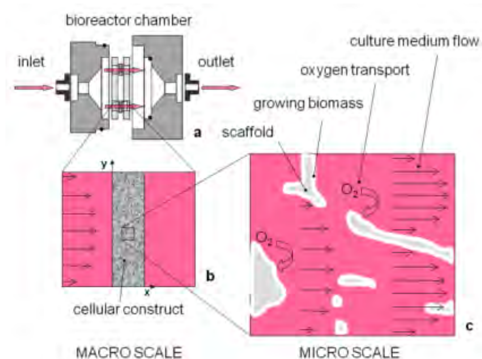
To engineer a cartilaginous tissue in vitro, the basic idea is to expand a cell population, to seed the cells on a biomaterial, and to culture the construct until its maturation into a functional tissue. An essential step toward the obtainment of functional cartilage is to control its growth process. This process depends on various space- and time-varying biophysical variables of the cell environment, primarily mass transport variables and mechanical variables, all involved in the cell's biological response.
In the general aim to obtain a quantitative law for tissue growth, in function of the above mentioned variables, we have developed several growth models, in which the cellular constructs are subjected to a flow of culture medium and/or to cyclic pressurization on a macroscopic scale, and computational modelling is used to quantify variables of the biophysical field induced on the cells on a microscopic scale.
Using this technique, we have quantified specific aspects allowing to control the culture conditions. For perfusion alone, we estimated the relationship between the global production of matrix proteins by the cells, and the level of fluid-induced shear exerted on the cells. For perfusion combined to cyclic pressurization, we estimated the relationship between the local level of oxygen tension sensed by the cells, and the local up-regulation of hyaline matrix protein production, in response to pressurization.
Our recent developments include a more advanced growth model, featuring a mini-bioreactor system, allowing local and non-destructive assays on the cellular constructs, to be interfaced to a multiphysic model of tissue growth, in which the known dependences are non-linearly coupled.
Acknowledgements. This research is funded by the grants: 'Biosensors and Artificial Bio-systems'- Italian Institute of Technology (IIT-Genoa); '5x1000-2009-HMED: Computational Models for Heterogeneous Media'- Politecnico di Milano; '3D Microstructuring and Functionalization of Polymeric Materials for Scaffolds in Regenerative Medicine'- Cariplo Foundation (Milano).
Keywords. Biomechanics, mechanobiology, regeneration, model



(18.O1) BIOMECHANICAL CONCEPTS TO DESIGN PERFUSION BIOREACTOR FOR ENGINEERING BONE

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One challenging task in engineering bone tissue with bioreactor is to maintain an adequate balance between high supply of medium and sufficiently low fluid shear stresses applied to cells. This trade-off can be achieved in designing a system based on the concepts in fluid dynamics of porous media. Therefore, we designed a new perfusion bioreactor, for the culture of bone constructs of clinically-relevant size, using flow in fluidized bed [1].
Natural coral, a microporous and biocompatible material, was used as three-dimensional scaffolds. This bioreactor provided a stable environment of the cells in terms of mechanical and physicochemical properties. The chamber contains around 150 constructs (cell-seeded in cubic samples of 9 mm3 volume) imbedded in the flowing cell

culture medium. Such constructs are settled with randomized localization and orientation leading to a complex design of the scaffold structure. The overall substrate contained in the perfusion bioreactor can then be roughly considered as a porous medium presenting a large spectrum of m to 1 mm and an overall porosity greaterμpore dimensions, from 100 than 50 %. Accounting for the value of the applied perfusion mean m/s) and the architectural characteristics of theμvelocity (about 102 substrate, an approached evaluation of the applied shear stress would be around 1 mPa. These values are commonly advanced in case of noticeable mechanotransduction effects of cells embedded within three-dimensional substrates without risk of cell detachment. Bone constructs engineered in this system resulted in significantly high cell proliferation and homogenous cell distribution. Furthermore, these bone constructs were shown to be osteogenic when transplanted subcutaneously in sheep. This techniques thus appears to be particularly relevant to the production of bioengineered bone with clinically-relevant volume.

[1] B. David & al., A perfusion bioreactor for engineering bone constructs: An in vitro and in vivo study, Tissue Engineering C (2010)

Keywords. Perfusion Bioreactor, Coral Scaffold, In Vitro Study

(18.O2) THE INFLUENCE OF HYDROSTATIC PRESSURE ON THE CHONDROGENESIS OF MESENCHYMAL STEM CELLS EMBEDDED IN EITHER AGAROSE OR FIBRIN HYDROGELS

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Introduction. Mechanical loads have been shown to play an important role in the differentiation of mesenchymal stem cells (MSCs). Hydrostatic pressure (HP) specifically has been shown to affect extracellular matrix (ECM) synthesis in vitro. Cell attachment is directly affected by the scaffold substrate and plays a key role in differentiation. The objective of this study was to examine the interplay of cell attachment and hydrostatic pressure on the chondrogenesis of MSCs.

Methods. MSCs were harvested from porcine bone marrow and seeded into hydrogels that either permitted (fibrin) or prevented (agarose) cellular attachment. The hydrogels were subjected to 10 MPa of hydrostatic pressure for 4 h/d at a frequency of 1 Hz for 5 days per week. Scaffolds were cultured in a chemically defined chondrogenic media, and cultured with different concentrations of human TGF-β3. Samples were biochemically analyzed and observed with confocal microscopy.

Results. Confocal microscopy demonstrated that cells seeded in fibrin attained a spread, flattened morphology, while cells in agarose retained a round, spherical morphology (Fig. 1A). Fibrin hydrogels permitted MSC proliferation, while cell death occurred in the agarose hydrogels. HP significantly decreased the proliferation of MSCs in fibrin cultured in 1 ng/ml TGF-β3 (Fig. 1B). Collagen accumulation was greater in fibrin hydrogels subjected to HP in 10 ng/ml TGF-β3 (Fig. 1B). HP had no influence on matrix accumulation in agarose hydrogels.

Conclusions. This study demonstrated that HP effects cellular proliferation and matrix accumulation in fibrin hydrogels, but has no effect on proliferation in agarose constructs. Fibrin better supported cell viability and accumulation of collagen relative to agarose. These results demonstrate that cell-matrix interactions regulate MSC response to HP.

Acknowledgements. Funded by a Naughton Fellowship and SFI PIYRA [SFI/08/YI5/B1336].

Keywords. Hydrostatic Pressure, Cell-Matrix Interactions, Mesenchymal Stem Cells

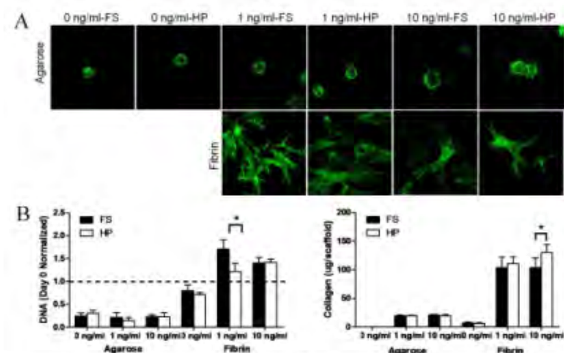

Figure 1 (A) Rhodamine phalloidin staining of actin cytoskeleton. (B) DNA and collagen content of agarose (A) and fibrin (F) samples after culture in 1 or 10 ng TGF-β3 and subjected to free swelling (FS) or cyclic hydrostatic pressure (HP). DNA content normalized to day 0 samples. Significance marked by asterisk, p< 0.05

(18.O3) A NOVEL BIOREACTOR FOR THE SYSTEMATIC DEVELOPMENT OF FUNCTIONAL 3D SCAFFOLDS FOR IN SITU CARDIOVASCULAR TISSUE ENGINEERING

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Introduction. State-of-the-art cardiovascular tissue engineering (TE) strategies are increasingly directed towards an in situ TE approach. This approach is based on using unseeded, 'smart' instructive scaffolds as replacement grafts, promoting endogenous cell recruitment and subsequent remodeling [1]. Clearly, the interactions between the scaffold and circulating cells under physiologic hemodynamic conditions play a pivotal role in this process and determine the optimal scaffold design. The aim of the current study is to develop an in vitro model system for the systematic development of such functional 3D scaffolds.

Methods. The model system consists of a custom-made cross-flow chamber (CfC) that houses 3D scaffolds (Fig. 1A). The CfC is incorporated into a flow setup designed to drive a cell suspension along the scaffold with physiologic wall shear stresses (0.1-8 Nm-2) and perfusion pressures (80-100 mmHg). Performance of the CfC was assessed with computational fluid dynamics and validated experimentally with fluorescent microbead (Ø10 µm) tracing studies. For proof-of-principle, human peripheral blood mononuclear cells (hPBMC) were isolated and labeled with Cell Tracker Green (CTG). The hPBMC were driven along a 3D electrospun scaffold under physiological flow conditions and infiltration of CTG-labeled cells into the scaffold was analyzed.

Results. Computational predictions demonstrate a fully developed flow in the region of interest, with a homogenous wall shear stress distribution (Fig. 1B,C). Consistently, microbeads followed a straight trajectory without turbulations (Fig. 1D). Furthermore, achievable

levels of shear stress and perfusion pressure are within the physiological range and are independently controllable. Additionally, hPBMC infiltration and adhesion could be monitored in real-time with confocal microscopy during the cell studies. Studies on the effect of scaffold architecture on cell recruitment under physiologic hemodynamic conditions are ongoing.

Conclusion. Our model system provides an ideal screening platform for the development and systematic evaluation of functional 3D scaffolds for in situ cardiovascular TE.

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Keywords. Cell-scaffold interaction hemodynamics bioreactor

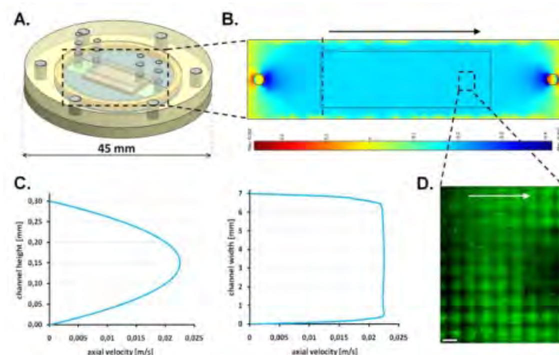

Figure 1. Design of the cross-flow chamber (A) with predicted shear stress distribution at the scaffold surface (B). For steady-state flow, the flow at the scaffold is fully developed with a parabolic profile in height and minimal wall effects in transverse direction (C). Snapshot of fluorescent microbeads in flow (scale = 500 µm) (D).

(18.O4) EARLY STAGE rMSC DIFFERENTIATION CAN BE INDUCED BY FLUID FLOW IN THE ABSENCE OF OSTEOGENICALLY SUPPLEMENTED MEDIA

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1. Royal College of Surgeons in Ireland

Previous work in our laboratory has shown that mechanical stimuli (fluid flow) can regulate osteoblast osteogenesis when cultured on collagen-based scaffolds in the presence of osteogenic supplements [1],[2],[3],[4]. The current focus of our work aims to determine if mesenchymal stem cell (MSC) differentiation towards an osteogenic lineage is similarly regulated by flow. Furthermore, through de-coupling the effects of physical and chemical stimuli, we seek to provide furthered understanding regarding the individual contributions of mechanically and chemically activated signalling pathways on rMSC gene expression during initial stages of differentiation. Rat MSC cell-seeded collagen-GAG scaffolds, cultured in growth medium (no osteogenic supplements), were exposed to oscillatory or steady flow regimes for 49hr and compared to static controls.

Flow significantly decreased levels of SOX9 and PPARgamma gene expression, transcription factors associated with chondrogenic and adipogenic differentiation, whilst maintaining levels of RunX2 (pro-osteogenic). Alkaline phosphatase (ALP) and integrin alpha 1 (ITGA1) gene expression were down-regulated, whilst osteopontin (OPN) and collagen type-1-alpha-1(Col1A1) levels were maintained (Figure 1). Down regulation of ALP and ITGA1 suggests cells are exiting a proliferative state and entering differentiation, which is supported by the transcription factor gene expression data, with cells appearing to commit to an osteogenic lineage. Changes in gene expression levels of later stage osteogenic related proteins were not observed at this early stage of differentiation.

This work highlights flow can play a significant role in directing early stage rMSC osteogenic differentiation. Further investigation is required to determine if flow alone can direct cells into a mature lineage phenotype. Ongoing studies culturing rMSC in the presence of osteogenic supplements will endeavour to prize apart the roles of chemical and mechanical stimuli on gene expression during rMSC differentiation.

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Keywords. Mesenchymal Stem Cell; Perfusion Bioreactor; Osteogenic Gene Expression; Collagen-based scaffolds

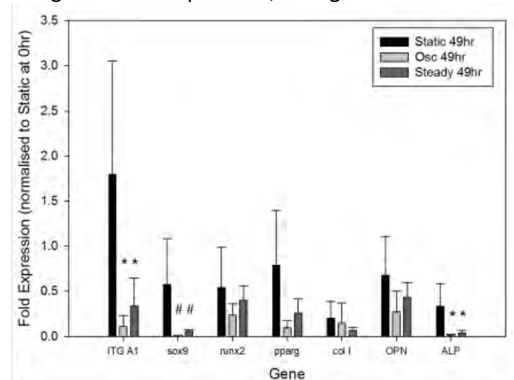

Figure 1: Changes in gene expression levels for rMSC seeded collagen-GAG scaffolds after 49 hours of either static or dynamic (steady or oscillatory) culture conditions. Values are normalised to static control at 0hr. Error bars represent the standard deviations (n=3/4).

(18.O5) THE EFFECTS OF FLOW-PERFUSION ON HYPERTROPHIC DIFFERENTIATION OF ENDOCHONDRAL BONE CONSTRUCTS

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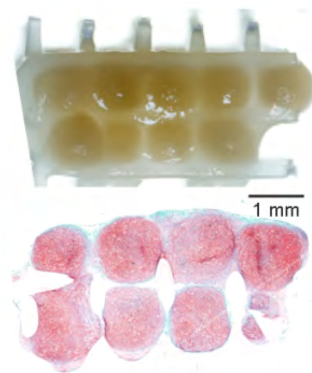
Endochondral bone tissue engineering is an attractive strategy to circumvent vascularization issues as it involves a cartilaginous transition tissue that is naturally avascular. Attractive cells for this purpose are the bone marrow-derived multipotent stromal cells (MSCs). There are strong indications that mechanical cues can control cellular differentiation. In particular, mild shear stresses were shown to enhance hypertrophy in e.g. chondrocytes (Wong et al., Bone 33, 2003). Therefore, we hypothesized that the endochondral process of chondrogenic MSCs can be enhanced by imposing appropriate mechanical cues, such as flow-perfusion-induced shear stress.

Human MSCs were isolated, expanded and centrifuged to form spherical aggregates. These were subsequently mounted into 3D-printed porous, polycaprolactone (PCL) scaffolds in basic chondrogenic differentiation medium (Figure). The hybrid constructs were then allowed to form

a cartilaginous matrix for 18 days, before they were transferred to a custom-built flow-perfusion system. Controls were maintained under static culture conditions. After 3 days, samples were harvested for RT-PCR of chondrogenic marker genes, COL2A1 and SOX9 and hypertrophic markers COL10A1 and BGLAP (osteocalcin). Additionally, samples from both groups were processed for histology and Western blot analysis of collagen type X at day 28. Deposition of proteoglycans (Figure; in red) and collagen type II are indicative of the formation of a cartilaginous matrix, while collagen type X and matrix mineralization indicate hypertrophic differentiation of the newly formed tissue.

Feasibility of this novel hybrid construct assembly method from cell aggregates and printed polymeric scaffolds was shown. Additionally, the progression of differentiation of the MSCs following mild shear stress stimulation in these hybrid constructs can be evidenced by detection of chondrogenic and hypertrophic markers. Demonstrating that mechanical stimulation affects hypertrophic differentiation is relevant for both the maintenance of engineered cartilage and for inducing endochondral bone formation.

Keywords. Mechanical, shear, MSC, hypertrophy, endochondral



(18.O6) DAMPING PROPERTIES OF THE NUCLEUS PULPOSUS

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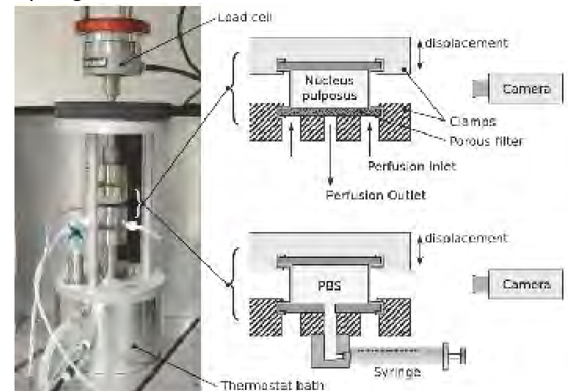
Questions persist in the investigation of the viscoelastic behavior of the nucleus pulposus (NP) of the intervertebral disc. In particular, the damping properties of the NP under physiological large deformations are still to be addressed. Bovine coccygeal NP tissues have been harvested and encapuslated into a deformable and permeable device. The encapsulation device is composed of a medical grade 40 µm pore size sintered steel filter, an nonporous rigid disc, and a 100 µm thin polydimethylsiloxane. The proposed approach allowed us to monitor the water content of the samples during mechanical tests which of primary importance in the dissipation evaluation process (Figure 1). The specific damping capacity of the NP in large compressive deformations (12.5%) and for frequencies ranging between 0.01 and 10 [Hz] was assessed using a paired statistical study. Damping ranged between 18 and 33% with a minima at 0.1 [Hz]. Because the NP can show both fluid and solid behaviors, the specific damping capacity used here was defined by dividing the energy loss (hysteresis) by the work input. It represents the proportion of energy that is dissipated into heat. This energetic approach is particularly convenient to study nonlinear viscoelastic materials such as biologic soft tissues. In summary, in the present study, we introduce a reliable method to address the damping properties of hydrogels under large and physiological deformations, and to investigate the damping properties of the coccygeal bovine nucleus pulposus in order to provide data for the design of nucleus replacement devices.

Keywords. Soft tissue, dissipation, nucleus pulposus, hydrogel



(18.O7) INVESTIGATING THE POTENTIAL OF HIGH FREQUENCY LOW MAGNITUDE (HFLM) LOADING INTERVENTIONS FOR TENDON REPAIR USING A NOVEL IN-VITRO LOADING SYSTEM

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Introduction. Mechanical stimulation has been postulated as an essential factor in maintaining tendon health, and there are indications it may be beneficial for promoting tendon repair. Several in-vitro studies have examined the effects of mechanical stress on healthy tendons by using loading frequencies of 0.01-3Hz since such loading frequencies may occur during physical exercise. More recently, studies have shown evidence for the special effects of using high frequency low magnitude (HFLM), loading regimes in promoting bone health and counteracting bone disease. In this study, a novel in-vitro loading system (IVLS) has been developed with the aim of investigating the potential of HFLM stimulation for tendon repair.

Materials and Methods. Tendon fascicles from male Sprague Dawley rat tails (4-6months) were cultured in centrifuge tubes (DMEM 10%FCS). Fascicles were incubated under conditions of no load, static load, or cyclic load, using a custom IVLS . Cyclic loading of specimens was achieved using a pulsed electromagnetic field to perturb a magnet suspended from the fascicle. The load magnitude and frequency applied onto fascicles was measured using a sensitive load cell and software analysis. Live-dead staining was used to examine tissue viability after 0 (fresh), 1, 4, and 7 days. Tensile testing and a Glycosaminoglycan assay were performed to measure biomechanical and extracellular matrix alterations.

Results and Discussion. Preliminary results revealed that the developed IVLS can sustain tissue viability for a minimum of 7days subsequent to static and HFLM loading interventions. The load frequency applied was confirmed to be 20Hz and peak loads varied between 0.15-0.25N. Furthermore, by day 4, fascicles cultured under static load showed significantly higher Modulus and Glycosaminoglycan content(figure 1)compared with load deprived specimens (2 fold difference). These results demonstrate the capability of the developed system for investigating the potential of HFLM loading interventions in promoting tendon repair.

Keywords. Tendon, mechanobiology, in-vitro loading system, repair

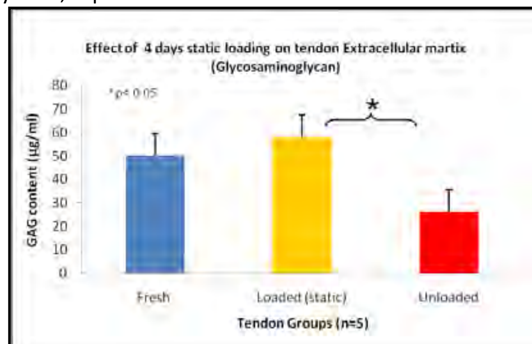

Figure 1: The Glycosaminoglycan content for fresh tendon fascicles, and cultured tendon fascicles after 4days of unloading, and static loading

(18.O8) STRAIN INDUCED REMODELLING OF POTENTIAL SCAFFOLDS FOR TISSUE ENGINEERED BLOOD VESSELS

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Introduction. The advantages of applying dynamic culture conditions to cell-seeded scaffolds are well established in terms of tissue maturation, extracellular matrix formation, and enhanced mechanical properties [1]. Bacterial cellulose (BC) has been investigated as a potential tissue engineered blood vessel scaffold [2]. This study determines the biological response of cell-seeded BC to dynamic culture conditions with the aim to extend the study to repopulated decellularised porcine coronary artery (PCA).

Methods. 15x15mm sections of BC were adhered to Bioflex® culture plate membranes. Bovine aortic smooth muscle cells (BASMC) were statically cultured on the BC sections at a concentration of 300,000 cells/cm2. Following 72 hours of culture a mean cyclic uniaxial strain of 6.5% with 3% amplitude was applied by a Flexercell® FX-4000TM for 120 hours in humidified air with 5% CO_2 at 37°C. Cell infiltration was determined by hematoxylin and eosin (H&E) staining and smooth muscle α-actin was used to examine cell phenotype.

Results. H&E staining of cyclically strained BC showed BASMC infiltration of 34%, which shows enhanced infiltration compared to static controls (10%), see Fig.1. The cells maintained their smooth muscle phenotype.

Conclusions. Following the successful remodelling of cell-seeded BC under cyclic strain this study can be extended to other scaffolds. Decellularised PCA has the capability for cell attachment as demonstrated by static culture of BASMC [3]. Cell culture experiments will be performed to characterise the proliferation, migration and infiltration of repopulated cells in decellularised PCA under dynamic test conditions to determine the viability of the scaffold as a potential tissue engineered blood vessel.

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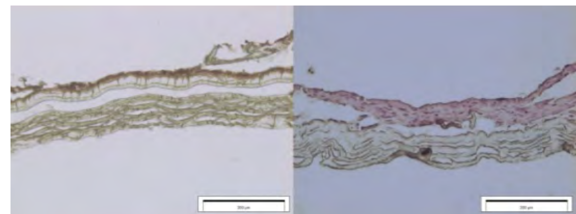
Acknowledgments. Funded by Science Foundation Ireland Research Frontiers Grant (08/RFP/ENM1378).

Keywords. Scaffolds, Cell Seeding, Mechanical Stimulation



(18.O9) CREATING A MECHANICALLY FUNCTIONAL DESIGN FOR PARTIAL MENISCUS REPLACEMENT

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Introduction. The most important meniscus property is its ability to withstand compressive, tensile and shear stresses. Its main functions are shock absorption, load transmission and joint stability. When the tissue is damaged, repair can be attempted by tissue engineering. The aim of this study was to construct collagen-based foams and investigate the influence of crosslinking conditions on the properties of this potential scaffold.

Materials and Methods. Collagen type I (COLL I) was used to prepare the foams (2 %, w/v). Both physical (dehydrothermal, DHT) and chemical (genipin, GP, and EDC/NHS) crosslinkings were applied. Compression (5 mm/min), tension and shear (0.5 mm/min) tests were performed onto dry foams.

Results and Discussion. Foams were crosslinked physically, chemically or in combination. When the samples were crosslinked with DHT the mechanical properties were higher than those of UXL ones. However, DHT in combination with GP or EDC/NHS, resulted in much higher (5 to 6-fold) compressive and tensile properties. In the case of shear testing, crosslinkage did not, however, significantly improve the shear properties (Fig. 1). Meniscus regeneration is known to occur in about 6 months and the degradation rate of the material used for engineering a tissue should be comparable with that of the tissue formation. Even though the highest mechanical properties were obtained with foams crosslinked with EDC/NHS; however, a 3 month degradation test revealed that these constructs are not

stable since almost 80 % of the foam was lost. Therefore, double crosslinked (DHT+EDC/NHS) foams were more appropriate. In all, compressive and shear moduli (585 kPa and 160 kPa) were above that of natural tissue (150 kPa) whereas tensile properties (2.5-4 MPa) were below the target value (100-150 MPa). As it is, this construct is suitable for partial meniscus replacements.

Conclusion. Foams suitable for partial meniscus replacement were produced in this study.

Keywords. Meniscus Tissue Engineering; Mechanical Properties

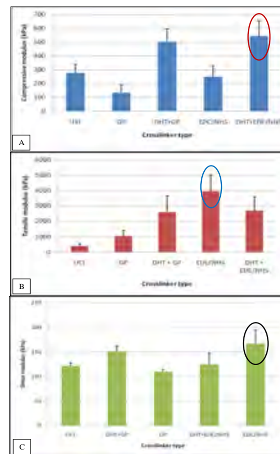

Figure 1. Mechanical properties of COLL I-based foams. (A) Compressive (B) tensile and (C) shear properties.

(18.O11) COMPUTER AIDED CUSTOMIZED CREATION OF SCAFFOLDS

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Introduction. The design of scaffolds and search for the optimal pore shape and size is a topic of ongoing research within the tissue engineering community. In a 2009 review paper Hollister1 finds i) the need for a more complete understanding of scaffold material and design requirements and ii) the need to better integrate computational design techniques with manufacturing methods as two of the six main reasons why the penetration of new scaffolding materials and structures from research laboratories to the clinic has been extremely limited.

Methods. This paper presents a method to obtain fully customized 3D computer scaffold designs starting from patient specific scan data. The resulting scaffolds are ready to be produced via rapid manufacturing techniques. This method is then illustrated on a mouse bone scaffold coming from micro-CT scan data using Mimics Innovation Suite software. From the virtual design a 3D printed scaffold is created in polycaprolactone using a fused deposition modelling technique.

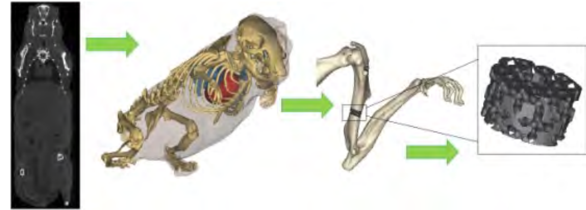
Results and discussion. From patient specific data a high quality 3D triangle mesh model is calculated. From this model the anatomy to be replaced by a scaffold is selected and virtually separated.

A porous unit cell which can be designed by the user, also represented by a triangle mesh, is patterned into a geometry which envelopes the separated anatomy from above. A virtual cutting operation on triangle mesh level between the separated anatomy and the patterned grid results in a customized scaffold structure.

Conclusions. The ability to create customized scaffolds with an unlimited freedom in unit cell structure can increase the speed of research and understanding of the influence of scaffold pore size and shape on cell differentiation and cell growth rate.

References. 1 Hollister S.J. Scaffold Design and Manufacturing: From Concept to Clinic. Advanced Materials 21 (32-33), 3330-42, 2009.

Keywords. Scaffold, design, custom, personalized



(18.P1) COLLAGEN-BASED SCAFFOLDS AS CARRIERS FOR HYPOXIA-MIMICKING BIOACTIVE GLASSES FOR ORTHOPAEDIC TISSUE REGENERATION

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2. Dept of Materials, Imperial College London, UK

A significant problem with tissue engineered constructs is the lack of vasculature and ability to fully integrate with the host tissue which poses one of the biggest challenges in regenerative medicine. The Hypoxia Inducible Factor (HIF-1a) pathway is activated under hypoxic conditions and results in the production of pro-vasculogenic genes such as Vascular Endothelial Growth Factor (VEGF). In our laboratory, we have developed a series of collagen-based scaffolds for tissue repair. The aim of this project was to use these scaffolds as carriers for novel cobalt releasing bioactive glasses which have shown strong potential as hypoxia-mimicking materials by activating the HIF-1a pathway. In this work, a bioactive glass suspension was added to collagen and collagen-glycosaminoglycan (CG) slurries and subsequently lyophilized. A series of variables were examined including bioactive glass particle size (100 or 38 um diameter) and concentration (0.1, 0.2 & 0.5 mL) as well as different constant cooling lyophilization rates (1oC/min and 4oC/min). We found that a slower cooling rate (1oC/min) produced a more homogenous pore structure compared to the faster cooling rate of 4oC/min (Fig.1). Uniaxial compression testing revealed that the inclusion of bioactive glass significantly improved the mechanical properties of both the collagen only and CG scaffolds, and that the compressive moduli increased with increasing concentration of bioactive glass added. We also found that there was no significant effect of particle size on the resultant properties. While porosity decreased with increasing amounts of bioactive glass, all composites still maintained high degrees of porosity above 97%. In conclusion, we have successfully combined cobalt bioactive glasses with collagen-based scaffolds. Through ongoing research focusing on the assessment of the cobalt bioactive glasses' ability to induce in vitro angio- and osteogenesis, we propose that these composite scaffolds will have demonstrated potential as pro-angiogenic scaffolds for tissue repair.

Keywords. Collagen, Scaffold, Bioactive Glass, Hypoxia Inducible Pathway

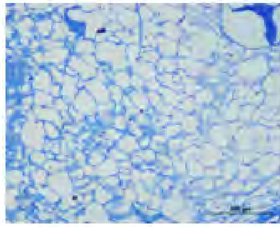

Fig. 1 Toludine blue-stained scaffold section revealing the homogenous pore structure of the collagen-bioactive glass composite. Scale bar is 500 um.

(18.P2) EVALUATION OF MECHANICAL PROPERTIES DURING PLLA SCAFFOLD DEGRADATION

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Introduction. The application of different biodegradable polymeric materials with three-dimensional structure to facilitate the adhesion, diffusion and proliferation of cells for cartilage regeneration has been widely studied (1). A well designed scaffold should reduce, ideally, their mechanical properties in the same rate as the tissue is growing. A mechanical and microstructural characterization of the scaffold degradation is important to evaluate its future mechanobiological behavior. The present work shows experimental parameters of PLLA scaffold degradation (hydrolysis) under static conditions.

Materials and Methods. Scaffolds were immersed in PBS for 6 months and preserved at 37ºC. PBS was replaced every week. To characterize the mechanical properties of the scaffold, uniaxial static tests like Unconfined (UC) and Confined compression (CC) have been performed. The Young Modulus (ES) and the Aggregate Modulus (HA) are respectively calculated from the slope of the best linear fit of the stress-strain graph. Poisson's ratio (v) can be directly deduced from ES and HA. Interconnected porosity is an important variable in the mechanical characterization of the scaffold. The microtomography (Micro-CT) allows us to define the porous size, percentage of pore structure and also to perform FE models. A permeability test is carried out to determine how much interconnected the porous are.

Results. A significant increase in the porous size took place in the first time point of our study (1,5 months). A constant increase in porous size was observed for the whole time of the study. An exponential increase in permeability was detected during the degradation of the PLLA scaffold. No significant changes were observed in the results obtained in the unconfined compression test, as previously described in literature, but, a significant decrease in the aggregate modulus was observed after 1,5 months.

Conclusion. Taking into account that PLLA scaffolds are going to be located into the cartilage in a confined way, it is important to test their mechanical properties under confined tests. In this case, significant differences in the aggregate modulus were detected although no changes had been previously reported for unconfined tests.

Keywords. Scaffold, PLLA, degradation, mechanical properties

(18.P3) EVALUATION OF THE BIOMECHANICAL PROPERTIES OF ARTIFICIAL SCAFFOLDS MADE OF 0.1% FIBRIN-AGAROSE FOR TISSUE ENGINEERING APPLICATIONS

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Introduction: Scaffolds made of fibrin-agarose are characterized by high resistance, firmness and elasticity, as fibrin is among the most resilient proteins in the natural world; these scaffolds have shown to be successful biomaterials in several biomedical applications, including cornea, skin and oral-mucosa implants, because of the great biocompatibility. The objective of this work was to generate scaffolds made of fibrin-agarose at 0.1% for the evaluation of their mechanical properties, to define the potential of this biomaterial for novel biomedical applications.

Methods: Mechanical tests were performed on the samples: Young's modulus, stress and strain values were determined using a tensile testing method. Due to the lack of standardization for this kind of mechanical test on biological materials, a new Standard Operating Procedure for the performed experiments was developed. Different imaging methods were used to evaluate the micro-level network structure of the biomaterial, and the reasons behind its biomechanical properties.

Results: The mechanical experiments showed values of tensile stress at fracture of 0.03 MPa, Young's modulus of 0.02 MPa and tensile strain deformation at fracture of 120%. The imaging methods showed the alignment of the micro-fibers of the network when tensile stress is applied, whose behavior explains the elastic properties of the biomaterial.

Conclusions: According to these results, scaffolds made of fibrin-agarose at 0.1% have great elasticity properties. The mechanical behavior of this biomaterial makes it interesting for an eventual future development of innovative scaffolds made of fibrin-agarose, with structure and mechanical properties with great potential for the production of novel kinds of biomedical applications.

Supported by grant P10-CTS-6060 from Junta de Andalucia, Spain.

Keywords: mechanical properties, fibrin, agarose, scaffold

19. EUROSTEC: PROGRESS AND FUTURE ASPECTS OF SOFT TISSUE ENGINEERING FOR CHILDREN

Chair: W. Feitz

Co-chair: P. Geutjes
Keynote speaker: E. Oosterwijk
Organizer: W. Feitz
Synopsis: In this symposium new overall aspects of the interactions and developments will be presented by the different participating institutes (www.eurostec.eu). A keynote lecture will be given on new scientific developments and training methods for researchers in the field of TERM. EuroSTEC is an Integrated Project (IP) on 'Soft tissue engineering for congenital birth defects in children: from 'biomatrix - cell interaction - model system' to clinical trials', funded by the European Commission under the Sixth Framework Programme (FP6). The project brings together 15 partner organizations (10 research institutes and 5 companies) from 9 European countries.
Modern tissue engineering approaches are used to develop new treatments for children with structural disorders present at birth, such as spina bifida, urogenital defects, gastroschisis, diaphragmatic hernia and esophageal atresia. A translational route through in vitro and animal experiments will lead to future clinical trials. Ethical and regulatory issues are addressed with a dialogue with society, including patient's associations. Different new aspects have been studied such as micro-computed tomographical imaging of soft biological materials using contrast techniques, human skin substitutes, oesophagus tissue engineering, skin defects in a fetal sheep model, and fetal mesenchymal stem cells and new urogenital treatment options for tubular reconstructions. Clinical trials with a main focus on fetal intervention in case of congenital diaphragmatic hernia. What do experts in the field think about the ethical aspects of soft tissue engineering for congenital birth defects in children. The EuroSTEC symposium will include recent developments, training aspects in the field of TERM as well as a selection of new scientific highlights in the field of soft tissue engineering.

(19.KP) multiTERM: TRAINING MULTIDISCIPLINARY SCIENTISTS FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE, A MARIE CURIE INITIAL TRAINING NETWORK. KEYNOTE PRESENTATION

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Tissue engineering and regenerative medicine (TERM) is a multidisciplinary field where scientists need to cut across traditional fields of study. They need to understand completely different aspects - ranging from material choice, cell biology, to clinical translation - to successfully design and clinically implement engineered tissue. Unfortunately, such scientists are scarce, because such TERM-specific interdisciplinary training is missing. To fill the gap that currently exists, the EC has funded MultiTERM, is a training network to provide early stage researchers with individual and centralized training in key elements of TERM: biomaterials, cell biology, bioreactors, animal modeling, clinical and industrial translation. Materials and implants for tissue engineering as well as state-of-the-art novel visualisation procedures to monitor the behaviour of the implanted tissues are developed by MultiTERM participants. Here the idea behind MultiTERM will discussed as well as results of the various studies performed within MultiTERM.

Keywords. multidisciplinary training

(19.O1) HUMAN ECCRINE SWEAT GLAND CELLS CAN RECONSTITUTE A STRATIFIED EPIDERMIS

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Eccrine sweat glands are generally considered to be a possible epidermal stem cell source. Here we compared the multilayered epithelia formed by epidermal keratinocytes and those formed by eccrine sweat gland cells. We demonstrated both in vitro and in vivo the capability of human eccrine sweat gland cells to form a stratified interfollicular epidermis substitute on collagen hydrogels. This is substantiated by the following findings: (1) a stratified epidermis consisting of 10-12 cell layers is formed by sweat gland cells; (2) a distinct stratum corneum develops and is maintained after transplantation onto immuno-incompetent rats; (3) proteins such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and transglutaminases I and III match with the pattern of the normal human skin; (4) junctional complexes and hemidesmosomes are readily and regularly established; (5) cell proliferation in the basal layer reaches homeostatic levels; (6) the sweat gland-derived epidermis is anchored by hemidesmosomes within a well-developed basal lamina; and (7) palmo-plantar or mucosal markers are not expressed in the sweat gland-derived epidermis. These data suggest that human eccrine sweat glands are an additional source of keratinocytes that can generate a stratified epidermis. Our findings raise the question of the extent to which the human skin is repaired and/or permanently renewed by eccrine sweat gland cells.

Keywords. tissue engineering, skin, human dermo-epidermal skin substitute

(19.O2) DEVELOPMENT OF A NEW IN SITU PIG BLADDER MODEL USING TISSUE ENGINEERING TECHNIQUES

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Introduction. Experimental research on the urinary bladder often requires the use of laboratory animals. For urological research, the pig bladder is the best translational model. However, because of practical and financial reasons, small animal models, e.g. rats or rabbits are often selected. With the current tissue engineering techniques it is possible to keep tissues viable under in vitro conditions. The aim of this study is to develop an in situ bladder model that can be used to explore physiology and regeneration of this organ.

Methods. Bladder mucosa was mechanically isolated from freshly dissected abattoir pig bladders. Twenty sterile punches (0.5 mm Ø) were taken. First, the biopsies were cultured on 3 different substrates (i.e. type I collagen scaffold, PET membrane and metal raster) and cultured for 3 weeks. Secondly, five different culture media were tested (KSFM®, SMCM®, DMEM®, RPMI®, Epilife®). Biopsies were evaluated after different time points (0, 2d, 1wk, 3wk, 6wk) using standard HE, scanning electron microscopy (SEM) and immunohistochemical staining, i.e. apoptosis (TUNEL), proliferation (Ki67) and cell type (CK's, αSMA, Desmin and Vimentin).
Results. Only on the type I collagen scaffolds the mucosa remained viable for more than 3 weeks. Although smooth muscle cells and myofibroblast were also found in the scaffolds, the outgrowth consisted mainly out of urothelial cells. Urothelial cells proliferated and covered the cutting edges within 2 days. Of the 5 media used, 3 (SMC, DMEM, RPMI) were able to sustain the mucosa in good condition with normal morphology, proliferation (Ki67), and hardly any apoptosis (TUNEL-assay) for at least 1 week (see figure).
Conclusions. Bladder mucosa cultured on type I collagen scaffolds under optimal circumstances, can be used as a biological experimental model for the bladder. This new in situ bladder model is a possible alternative for currently used laboratory animal models.
Keywords. In situ, pig, bladder, model

(19.O3) IN VIVO IMPLANTATION OF HIGH-DENSITY COLLAGEN GEL TUBES FOR URETHRAL REPAIR IN A RABBIT MODEL

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Urethra birth defects or injuries can impair proper bladder voiding thus necessitating a surgical repair. Such repair is usually undertaken using preputial skin or buccal mucosa as donor tissue for the graft, which can lead to long-term complications. Therefore tissue engineering is regarded as an alternative to produce these grafts. We had previously developed high-density collagen gel tubes that are suitable for urinary tract tissue engineering and can be produced, ready seeded with cells, within hours. Our approach here was to use these constructs in vivo as grafts for urethral repair in a rabbit model. All animals underwent a bladder tissue biopsy by laparotomy one month before the urethra surgery. During the urethra surgery, high-density collagen gel tubes were implanted in male New Zealand white rabbits after the creation of a 1cm-long urethral defect and anastomosed with fibrin glue to the remaining urethra segments. A total of 16 animals were split into four groups, two implanted with constructs seeded with autologous smooth muscle cells isolated form the bladder biopsy and the two other groups with acellular constructs. Animals were submitted to urodymanic measurements and sacrificed 1 or 3 months after the urethra surgery depending on the groups.
Results are expected to show a good recovery of the urodynamics and a good tissue regeneration assessed by histology. This in vivo study should confirm that these high-density collagen gel tubes, which have the potential to drastically shorten the production time of cell-seeded tissue-engineered urinary tract grafts, are suitable for urinary tract regeneration.
Keywords. in vivo, urethra, tissue engineering, collagen, smooth muscle cells

(19.O4) BLADDER AUGMENTATION USING MULTIPLE SCAFFOLDS IN ONE BLADDER AND GROWTH FACTORS IN A PORCINE MODEL

Roelofs LAJ (1), Geutjes PJ (1), de Gier RPE (1), Farag F (1), Tiemessen TM (1), Oosterwijk E (1), Versteeg EMM (1), Daamen WF (1), van Kuppevelt TH (1), Kortmann BBM (1), Feitz WFJ (1)
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Purpose. Tissue engineering aims to develop alternatives for the current technique of bladder augmentation. When using a large acellular scaffold central necrosis may occur due to the lack of cell ingrowth and blood vessel development. In order to overcome this problem we studied the concept of implanting multiple scaffolds in one bladder instead of 1 large construct. Furthermore, we studied the use of growth factors to enhance cell growth in the scaffold.
Methods. Three different scaffolds of 3 cm Ø were investigated: 1) crosslinked type I collagen scaffold (Col-X) 2) Col-X incorporated with heparin (Col-X-Hep) 3) Col-X-Hep with 3 growth factors (VEGF, FGF-2 and EGF) (Col-X-Hep-GF), which we compared to a 'sham-operated' group. In total 13 pigs were operated. Three pigs were operated in each group and 3 scaffolds were implanted, or 3 lesions were sutured without implant (Sham group). Urodynamics were performed before operation. After 3 months functional (cystogram and urodynamics) and histological evaluation (HE, CK7, vimentin, α-sma, desmin, smoothelin) was performed on the bladders.
Results. Twelve of 13 operated pigs fulfilled the entire experiment, one pig died because of urine leakage and peritonitis. Survival rate was 92%. In all animals the cystograms were normal. Urodynamic studies did not show differences in compliance or capacity between all groups, due to the very high compliance and capacity of porcine bladders. Histological evaluation revealed a normal urothelial layer and good neovascularisation in all groups. Smooth muscle ingrowth was enhanced in the Col-X-Hep-GF group. No signs of central maldevelopment were seen. The scaffolds were almost fully degraded, some remnants were visible in the Col-X-Hep group.
Conclusions. We showed the feasibility of implanting multiple scaffolds in one bladder in order to improve its capacity. Incorporation of heparin with growth factors improved ingrowth of muscle cells.
Keywords. bladder augmentation collagen growth factors

(19.O5) LARGE DIAMETER TUBULAR CONSTRUCTS FOR TISSUE ENGINEERING: SCAFFOLD PREPARATION, CHARACTERIZATION AND CYTOCOMPATIBILITY

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Introduction. Tissue engineering can be used for treatment of birth defects e.g. esophageal atresia or in reconstructive surgery e.g. urinary diversion, by developing large diameter tubular constructs which have biological function and suitable mechanical characteristics. In this study different tubular constructs were developed, characterized and evaluated for cytocompatibility.

Methods. Large tubular scaffolds (Ø 15 mm) were prepared from highly purified bovine type I collagen with and without commercially available synthetic polymer mesh (Vypro-II mesh, Ethicon, Inc.), frozen in defined moulds, lyophilized and carbodiimide-crosslinked. These constructs were characterized by scanning electron microscopy (SEM), standard histology (H&E), immunofluorescent (IF) staining, TNBS assay (to assess the degree of crosslinking), and tensile strength analysis. Characterized scaffolds were seeded with primary porcine epithelial cells, cultured for 1 week under static or bioreactor conditions (Bose-ElectroForce®) and analyzed by SEM, H&E, and IF staining.

Results. Two types of constructs were prepared with distinct differences (Fig.1a-d). SEM and H&E showed a highly porous network with polymer towards the outside. Incorporation of the polymer into the collagen scaffold significantly increased the tensile strength from 0.25±0.04 N/mm to 1.25±0.19 N/mm. Cells were evenly distributed in the lumen (Fig.1e,f) and were positive for cytokeratin 5, indicating epithelial phenotype.

Conclusions. In this study we successfully prepared large tubular constructs consisting of type I collagen and synthetic polymer mesh. The incorporation of the polymer significantly increased the tensile strength of the construct. Culturing under bioreactor conditions allowed for homogeneous coverage of epithelial cells in the lumen. From mechanical and cytocompatibility results we conclude that large tubular collagen-polymer constructs may be a suitable candidate scaffold for treating hollow tubular organ defects. This study demonstrates the feasibility of producing constructs for tubular tissue engineering, which may lead to new approaches in (pediatric) surgery.

Acknowledgements. This Project was financially supported by the EuroSTEC program (LSHB-CT-2006-037409).

Keywords. Tubular; Polymer; Collagen; In Vitro

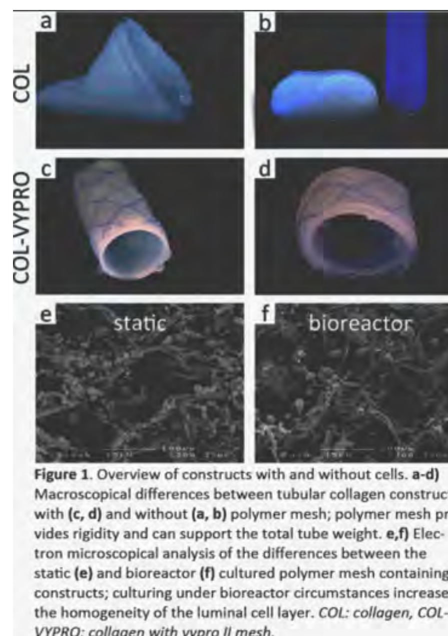

Figure 1. Overview of constructs with and without cells. **a-d)** Macroscopical differences between tubular collagen constructs with **(c, d)** and without **(a, b)** polymer mesh; polymer mesh provides rigidity and can support the total tube weight. **e,f)** Electron microscopical analysis of the differences between the static **(e)** and bioreactor **(f)** cultured polymer mesh containing constructs; culturing under bioreactor circumstances increases the homogeneity of the luminal cell layer. *COL: collagen, COL-VYPRO: collagen with vypro II mesh.*

(19.O6) ESOPHAGUS TISSUE ENGINEERING: IN-SITU GENERATION OF RUDIMENTARY ESOPHAGEAL CONDUIT USING THE FETAL MODEL

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Background. Esophagus replacement using the present surgical techniques is associated with significant morbidity. Tissue engineering of the esophagus may provide the solution for esophageal loss. In our attempts to engineer the esophagus, this study aimed to investigate the feasibility of generating vascularized in-situ esophageal conduits using the fetal model.

Methods. Esophageal biopsies were obtained from ovine fetus (80-120 days of gestation) and esophageal organoid units (EOU) were proliferated. The EOU were seeded on to bovine collagen sheets pre-seeded with fibroblasts. After 2 weeks of maintaining the constructs in-vitro, the constructs were tubularized on stents to create a tube resembling the esophagus and implanted into the omentum for in-situ tissue engineering. The edges of the omentum were sutured using non-absorbable suture material. The implanted constructs were retrieved after 4 weeks after birth.

Results. The omental wrap provided vascular growth within and around the constructs as they were integrated along the outer surface area of the scaffold. After removal of the stents, the engineered conduit revealed a structure similar to the esophagus. Histological investigations demonstrated esophageal epithelium organization into patches on the luminal side and vascular ingrowths on the conduits' outer perimeter.

Conclusion. Our study demonstrated the feasibility of using the fetal ovine model for esophagus tissue engineering. Seeding of EOU on fibroblast pre-seeded collagen scaffolds and formation of a rudimentary conduit resembling esophageal morphology after in-situ omental implantation. Vascular coverage and in-growth in the periphery of the construct could also be demonstrated. These findings hold future promise for the engineering of the esophagus with improved micro-architecture.

Keywords. Esophagus tissue engineering

(19.O7) EVALUATION OF LARGE TUBULAR CONSTRUCTS FOR URINARY DIVERSION IN PIGS

Geutjes PJ (1), Roelofs LAJ (1), Hoogenkamp HH (2), Walraven M (1), Kortmann BBM (1), de Gier RPE (1), Farag FF (1), Tiemessen DM (1), Oosterwijk E (1), Daamen WF (2), van Kuppevelt TH (2), Feitz WFJ (1)

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Introduction: Invasive bladder cancer usually requires radical cystectomy followed by creation of a urostomy. Intestinal tissue is used to create such a urinary diversion, but complications occur in 30-80% of the patients (e.g. infection, urinary stones, urine blockages and metabolic disorders). Tissue engineering may be the technical platform to develop alternatives for urological surgery. The aim of this study is to evaluate large tubular collagen-polymer constructs (Fig. 1A) for urinary diversion *in vivo*.

Methods: From all female pigs (n=10), bladder biopsies were taken and urothelial cells were isolated and expanded. After one month, the animals received an acellular construct (n=4), or a cell seeded construct (n=6). To create the urostomy, the right ureter was attached to the tubular construct with an end-to-side anastomosis. The construct was positioned in the retroperitoneal space (to induce blood vessel ingrowth) and fixed to the fascia and skin (Fig. 1B). The other ureter was left intact to enable normal voiding. After one month video urodynamics were performed, and the animal was sacrificed for further macroscopic and immunohistological evaluation.

Results: Survival rate was 80% (with one related and one unrelated death). After one month, the collagen was resorbed and a retroperitoneal tunnel was formed which could withstand 40 cm H_2O water pressure. Although the tunnel functioned as a urostomy, two animals had retroperitoneal leakage and stenosis was observed in all animals (Fig 1C). Immunohistochemistry showed neovascularization, a moderate immune response and formation of a neo-epithelial like layer in the lumen of the construct. No major differences were observed between cellular and acellular constructs.

Conclusions: The tissue engineered retroperitoneal tunnel functioned, in most cases, as a urostomy. Therefore, these large tubular scaffolds may be an alternative for intestinal tissue in urostomy surgery, but improvements are needed to reduce (skin) contractions and fibroblast deposition and improve clinical applicability.

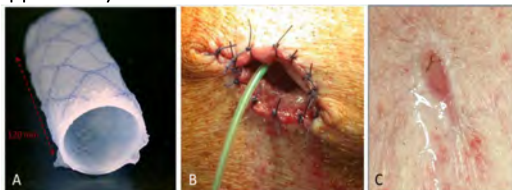

Figure. Evaluation of large tubular construct for urinary diversion: large collagen-polymer construct (A), urostomy just after operation (B), urostomy one month after operation (C).

(19.P1) IN VITRO CHARACTERIZATION OF HUMAN AND PORCINE UROTHELIAL CELLS

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Introduction. An estimate of 400 million people worldwide suffers from bladder diseases. In the case of congenital anomalies and acquired diseases when replacement surgery is necessary, the current treatment plans are not optimal. Tissue-engineered constructs will be a clinical option. The aim with tissue-engineered constructs is that it should function throughout a patient's lifetime. Since, stem cells are by definition cells able to sustain tissue homeostasis and wound healing, they are the optimal cells to recruit or seed within the constructs.

Method. Porcine and human urothelial cells were isolated and cultured on irradiated 3T3-J2 fibroblasts. Growth capacity and differentiation capacity of the cultured cells was evaluated.

Results. We successfully cultured porcine and human urothelial cells for 17 weeks resp. 9 weeks. Differentiation of urothelial cells into superficial cells, as evaluated by uroplakin-3 expression, was sparse but present in both porcine and human cultured urothelial cells.

By clonal analysis of porcine urothelial cells, we observed that the porcine bladder epithelium contains different types of colony forming cells that can be further characterized thanks to the isolation of pure clonal populations.

Conclusion. We have started to build an argument that we can isolate urothelial progenitor/stem cells, but a crucial next step for the isolated cells will be to implant the clonal cells in vivo.

Keywords. Human, Porcine, Urothelium, Progenitor/stem cells

(19.P2) CLINICAL TESTING OF ADVANCED THERAPY MEDICINAL PRODUCTS

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Introduction. Before December 30, 2008, tissue engineered products fell between two legislative categories, which prompted the creation of a new class of medicinal products: Advanced Therapy Medicinal Products or ATMPs, of which tissue engineered products are a sub-category. Clinical testing is essential in the development of any new medical technology. In evidence-based medicine, the randomized controlled trial (RCT) is still the gold standard. Recently, however, there

has been some debate in the literature on whether the RCT is the best method to research treatment with ATMPs.
A clinical application of an ATMP, currently being researched in the EuroSTEC project, is the use of tissue engineered constructs in children with congenital urological defects. Ethically speaking, application of an ATMP in this patient group creates a very specific situation through a combination of several factors. Firstly, we are dealing with a young child with (in the best possible scenario) an entire life ahead of them in which both positive and negative effects of treatment can occur. Secondly, the characteristics of the ATMP itself: a dynamic product with a substantial degree of variability, that interacts with the body through an irreversible process. Combining these features with the prerequisites of an RCT (for example finding a suitable gold standard treatment for the comparator arm), leads us to question whether testing an ATMP in an RCT would be possible.
Objective. In this paper we wish to investigate whether, given that the RCT is the gold standard in evidence-based medicine, the RCT is also the most appropriate method to research treatment of children with a congenital urological defect with ATMPs. To this purpose, we will conduct a literature study, complemented with expert interviews.
Acknowledgments. The research for this contribution was funded by the European Commission (EuroSTEC: EU contract LSHB-CT-2006-037409).
Keywords. Ethics, clinical trials, ATMP

20. EXTRACELLULAR MATRIX: FROM DEVELOPMENT BIOLOGY AT TISSUE ENGINEERING

Chair: Sebastián San Martín
Co-chairs: Telma Zorn, Ornella Parolini
Keynote speaker: Telma Zorn
Organizer: Sebastián San Martín
Synopsis: During the morphogenesis and development of organs, a coordinated process of proliferation and differentiation of cells are requires. In this context, adequate relationships with the extracellular matrix (ECM) components are essential for embryo since the fecundation, placentation and during the organogenesis in mammals. The ECM comprises a variety of versatile proteins and polysaccharides arranged in a cell surface-associated network. The ECM is required for many specialised cell functions and consists of various combinations of molecules, such as collagens, proteoglycans and glycoproteins, which form either long fibres or porous sheets, binding to cell surface receptors and to other ECM components. The extracellular matrix molecules have several function related with the promotion of an adhesive substrate to the different types of cells, provide structure, present growth factor to their receptor, sequesters and stores growth factors, sense and transducer mechanical signal. The role of the ECM and its interaction with cells in these natural process, provide the basic principles of material sciences that could be applied to address mimic and explain the interaction of the cells in artificial construct for tissue engineering.
It is well known that bioengineered tissues should emulate the cellular and molecular structure of the native organ, and the structure and level of differentiation of the artificial constructs should be equivalent to those of the tissues to be replaced. Thus, quality control of substitutes developed by tissue engineering should verify that the bioengineered tissues reproduce the structural patterns of differentiation and gene expression of the native tissue. Taking into account the importance of the ECM to the developmental process, organization and function of several tissues, the structure and composition of the ECM of the bioengineered tissues developed in the laboratory should be evaluated in vitro and in vivo models. In this Symposium, some examples will be present that showed as the ECM is necessary for normal development of mammals during the pregnancy and artificial construct develop by tissue engineering.

(20.KP) EXTRACELLULAR MATRIX REMODELLING DURING DECIDUALIZATION IN RODENTS

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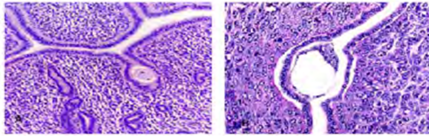
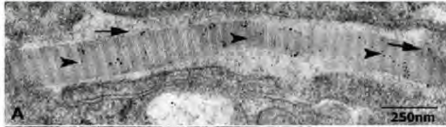
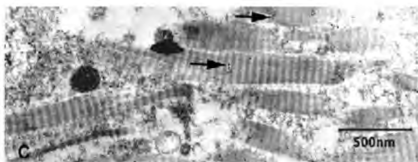
Since the early stages of pregnancy the uterus is deeply modified to acquire a favorable microenvironment to implant and embryo by a process called decidualization. Importantly, each uterine compartment is specifically modified and express characteristic set of molecules, which will play a role in the interaction between the embryo and maternal tissues. In response to the embryo implantation, in human and rodents, the endometrial fibroblasts acquire an epitheliod phenotype forming the decidual cells. Those cells originate a new and provisional organ during pregnancy, the decidua.
Decidualization comprises cell proliferation, cell growth, and the establishment of extensive intercellular junction between decidual cells , that promote a deep reduction of the extracellular spaces in the decidualized regions. Consequently, the extracellular matrix (ECM) is under extraordinary remodeling. The first morphological signal of ECM remodeling in the mouse endometrium was observed on the second day of pregnancy when collagen-containing phagossomes were seen in the cytoplasm of the endometrial fibroblast. Moreover, the mouse decidua is characterized by the presence of very thick collagen fibrils with irregular profile. These thick collagen fibrils are close related with decidual transformation since they are exclusively found in decidualized regions. Recently, thin serial section and double immunogold labeling demonstrated that these thick fibrils are formed by lateral aggregation of previously existent thin fibrils formed at least by collagens types I, III and a homotrimeric form of collagen type V.
Proteoglycans are also affected by decidualization. Gold electron microscopy showed that the biglycan is associated with thick collagen fibrils whereas decorin is associated exclusively with thin fibrils. Finally, the estrous cycle modulates proteoglycans expression in the mouse uterus suggesting a role of the ovarian hormones in the synthesis and /or degradation of these molecules.

Keywords. Collagen, decidua, implantation, proteoglycans, extracellular matrix



A: Day five of pregnancy showing a morula inside of an uterine crypt; B: Day five of pregnancy showing an implanted blastocyst. Compare the arrangements of the endometrial stroma.





Double immunogold labeling (5 and 10 nm gold particles): (A) Thick collagen fibril of decidua shows double immunoreaction for collagen type I and III. The arrows indicate type I collagen (gold particles 10 nm) and the arrowheads indicate type III collagen (gold particles 5 nm); (B) thick collagen labeled by antibody against type I collagen (gold particles 5 nm) indicated by arrowheads and labeled by type V collagen (gold particles 10 nm) (arrows); (C) thick collagen fibrils are immunoreactive to type III collagen (gold particles 5 nm) (arrowheads) and to type V collagen (gold particles of 10 nm) (arrows).

(20.O1) UPREGULATED EXTRACELLULAR MATRIX COMPONENTS DURING JAW PERIOSTEAL CELL OSTEOGENESIS

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Objectives. The extracellular matrix and its components have an amazing impact on cell fates like adhesion, migration and proliferation. However, there is only a little knowledge of the interplay of osteogenesis-related components within the extracellular matrix. We analyzed the expression patterns of different ECM components like collagens and the inhibitors of metalloproteinases during osteogenesis using jaw periosteal cells (JPC) in order to gain more understanding of basic processes.

Methods. Gene and protein expression was analyzed at three different time points of osteogenesis – 5, 10 and 20 days after induction and the cells were divided into 1. untreated jaw periosteal cells, 2. osteoblast differentiating media treated cells and 3. osteoblast differentiating media containing BMP-2 treated cells. The mineralization capacity of jaw periosteal cells was verified performing alizarin red. Furthermore, the alkaline phosphatase activity was detected.

Results. The gene expression pattern on 2D cultured OB treated JPC that posess mineralization capacity and enhanced alkaline phosphatase activity showed a strongly increased induction of collagen type VII, VIII and XI in comparison to collagen type I, where the basal levels of untreated cells are quite high. Furthermore, it seems that type I, VIII and XI levels are not affected by BMP-2. The strong elevation of collagen type XI was also detected in 3D cultured cells growing within polylactic acid scaffolds. Matrix turnover components like TIMP-4 and COMP were also strongly upregulated during JPC osteogenesis in 2D cultured cells, whereas in 3D culture, COMP levels were not enhanced.

Conclusions. We were able to identify genes that are related to the in vitro osteogenesis of jaw periosteum-derived cells. These data and basic knowledge help us to understand the process of osteogenesis in detail and to optimize conditions for tissue engineering applications in oral- and maxillofacial surgery using jaw periosteal cells as a suitable stem cell source.

Keywords. Extracellular matrix components, osteogenesis

(20.O2) MATRIX METALLOPROTEASE-MEDIATED CAPILLARY TUBE FORMATION IN COCULTURE OF HUMAN BONE MARROW STROMAL CELLS AND HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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1. INSERM 577, Bordeaux and University Victor Segalen Bordeaux 2

Introduction. Angiogenesis is essential to tissue reconstitution and currently represents one of the major challenges in tissue engineering. Our previous studies showed that the coculture of Human Bone Marrow Stromal Cells (HBMSCs) and Human Umbilical Vein Endothelial Cells (HUVECs) could induce capillary tube formation, which has attracted our much interests and the roles of different molecules in the formation of capillary tubes have been investigated. Based on the studies of communications between HBMSCs and HUVECs, the current study aimed to investigate the communication between matrix and cells, focusing on the roles of matrix metalloproteases (MMPs) for the formation of capillary tubes.

Methods. Cells were monocultured or cocultured in an Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 1% (v/v) FBS. Supernatant of the cultures were collected and cell extract were maintained at 14 hours and 24 hours for further analysis. Zymographic techniques, quantitative real time polymerase chain reaction, western blot, as well as functional studies against urokinase plasminogen activator (uPA) were applied to measure the enzymatic activities, expression and functionality of the MMPs.

Results. Results show that the activities of MMP-1 and MMP-2 decreased at 24 hours and there are significant upregulation in the enzymatic activities and expression of MMP-2 in cocultured cells than in monocultured cells. For MMP-1, its expression was significantly increased but its enzymatic activities were hardly to be detected. Expression of TIMP-1 and TIMP-2 were clearly upregulated at 24 hours. Function studies showed that the neutralization of uPA significantly downregulated the expression of MMP-1 and MMP-2.

Conclusion. During the capillary tube formation in coculture of HBMSCs and HUVECs, MMP-2 seems to play a more important role than MMP-1. In addition, TIMP-1 and TIMP-2 acted in accordance with MMP-2. uPA has an important effect on the regulation of MMP-2 and MMP-1. These matrix metalloprotease activities in a coculture of

endothelial and osteoprogenitor cells could contribute to formation a prevascular network for bone vascularized tissue strategies.

Keywords. Matrix Metalloprotease, angiogenesis, Cocultures, Tissue engineering

(20.O3) DEVELOPMENT OF NOVEL TYPE FIBRINOGEN/PLDLA NANOFIBERS FOR CONTROL ENDOTHELIAL CELLS BEHAVIOR

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We are currently interested how cells respond to the spatially organized signals from the extracellular matrix (ECM). Electrospinning is a technique capable of producing nanofibers (NFs) with dimensions similar to those of the fibrilar components of ECM. Electrospun NFs can be further designed in respect to their organization and cell binding properties. Previously, we have successfully spun NFs from fibrinogen (FBG) - a multifunctional protein involved in various physiological and pathological conditions, including the coagulation cascade and the provisional ECM formation. We further showed that these FBG NFs are well recognized by the endothelial cells but represent poor mechanical properties. Here we report on the development of a novel type of hybrid, FBG - poly-L,D-lactic acid (PLA) NFs with improved biomechanical properties. Indeed, the cell culture experiments showed that the endothelial cells interact very well with these NFs, representing better alignment along the oriented fibres in comparison to pure FBG ones. The well developed focal adhesion complexes and actin cytoskeleton of adhering cells confirm this view. Conversely, the pure FBG NFs tend to fuse each other and thus promote cell spreading on more than one fibres. Thus, the composite PLA/FBG NFs represent optimal properties, combining the good cell recognition of FBG with the advanced mechanical characteristics of PLA, which characterizes them as promising material for tissue engineering application.

Keywords. Extracellular matrix, nanofibers, endothelial cells, cellular behavior

(20.O4) INFLUENCE OF PORES GEOMETRY AND ARCHITECTURE ON COLLAGEN 3D ASSEMBLING

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The main goal of bone tissue engineering is to guide the healing process and to address bone reconstruction by using scaffolds designed as a temporary extra cellular matrix (ECM) able to trigger normal cell function. Recent studies have shown that cells are sensitive to chemistry, stiffness, surface properties and morphological matrix parameters. Starting from the new approach to consider the ECM organization to evaluate the goodness of different scaffolds, the aims of the work was to estimate how different scaffold morphologies influence the early stages of bone development in particular collagen production and assembling.

Poly(D,L-lactic acid) was used to produce 3D salt-leached and microfabricated scaffolds. Biological testing was carried out using MG63 cell line. Cell proliferation, growth, distribution and morphological evaluations were assessed by Scanning Electron Microscopy and Confocal Laser Microscopy (CLM) imaging. Collagen production and fibers assemblage were analyzed using CLM after DirectRed80 and immunohistochemical stainings.

The results showed that in both scaffolds pores were well interconnected but with different shapes, distribution and size ranges. Cells were able to invade pores as long as a deeper migration of cells occurred with incubation time. Furthermore an increase in collagen amount and a more complex organization was visible. Despite cell migration and proliferation, in relation to the different analyzed structures, is comparable, collagen morphology and its distribution resulted different, highlighting that the effect of scaffold geometry on the collagen assembly process is more profound than on cell proliferation and distribution. In fact, in microfabricated scaffolds, collagen showed an inhomogeneous planar organization whereas in salt-leached scaffolds a fiber-like organized structure is evident. The idea to consider the ECM organization to evaluate the goodness of different scaffolds deserve further evaluations to better understand the correlation between scaffold morphology and collagen production and organization for its great potentiality.

Keywords. Bone Tissue Engineering, Extracellular matrix, Biomimetic, Confocal Laser Microscopy

(20.O5) CARTILAGE MATRIX DEVELOPMENT IS ENHANCED IN CONSTRUCTS WITH LOW AGAROSE CONTENT IN PRESENCE OF TGF-Β3

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Introduction. Agarose has been extensively used as scaffold for cartilage tissue engineering. The concentration of agarose determines its diffusive and mechanical properties, and therewith influences matrix development. TGF-β3 is also known to influence matrix development by increased matrix production and by changing the distribution of the matrix. However, it is unknown how these two factors, agarose concentration and TGF-β3, interact. This insight is of interest for engineering cartilage. Therefore, this study aims to assess the effects of agarose concentration and TGF-β3 on matrix production and distribution in tissue-engineered cartilage.

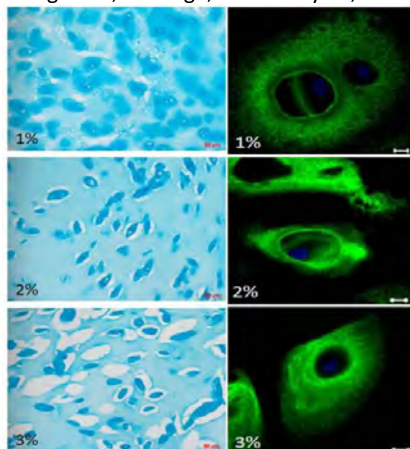
Methods. Immature bovine chondrocytes were isolated and cultured in 1%, 2% and 3% (w/v) agarose (n=6 per condition) at a density of 20x106 cells/ml in a) standard chondrogenic medium containing FBS or b) serum-free medium supplemented with 10 ng/ml TGF-β3. After 42 days, total sGAG and collagen content were measured, and Alcian Blue staining for proteoglycans and ihc for collagen type II were performed to monitor matrix distributions at the pericellular level.

Results. Constructs cultured in 1% (w/v) agarose showed a diffuse deposition of proteoglycans and collagen type II around the cells (Fig 1). In 2% and 3% agarose constructs a denser layer of matrix appeared. In FBS-supplemented

cultures collagen type II was more restricted to cell perimeter or territorial region, whereas distribution in TGF-β3-supplemented cultures was more uniform in all groups.

Conclusions. Content and distribution of matrix were enhanced in low agarose gels, probably induced by enhanced nutrient transport and increased extracellular space for matrix development. TGF-β3 improved the distribution even further. We therefore conclude that culturing in low agarose concentration and in presence of TGF-β3 is promising for engineering cartilage.

Keywords. Agarose, cartilage, chondrocytes, TGF-B3



Comments. Caption figure: Staining for proteoglycans (left; 40x; scalebar = 50 µm) and collagen type II (right; 63x; scalebar = 5µm) of cultured constructs in 1, 2 and 3% agarose in standard FBS-medium.

(20.O6) FIBRIN MEMBRANE IN BONE NEOFORMATION

Goldberg P (1)

1. ABC Hospital

This is a study performed on the parietal aspect of New Zeeland rabbits. A comparison between fibrin membranes will be analized. Materials, human fibrin, bmp2, db, autologous bone.

3 roun holes were made 2 for variables and one for control. Variables, fibrin alone, fibrin db, fibrin bmp2, fibrin autologous bone control. The hypothesis was that the fibrin membrane is an excellent carrier of bmp2 permit a greater bone formation, and the db sustains the fibrin membrane permitting the fibrous transformation. Histology, histomorphometry, and inmunochemistry were used. Euthanasia was done at 4,8,12 weeks. The results were great, a fibrous fibrin membrane was developed, an excellent bone neoformation was achieved.

Keywords. Fibrin, membrane bmp2, db

(20.P1) DECELLULARIZED HUMAN DERMIS TO TREAT MASSIVE ROTATOR CUFF TEARS: IN VITRO COMPARATIVE EVALUATION

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1. Laboratory of Preclinical and Surgical Studies, Rizzoli Orthopaedic Institute - IRCCS, Bologna – Italy; 2. Emilia Romagna Skin Bank - Bufalini Hospital, Cesena – Italy

Introduction. Extracellular Matrix (ECM) membranes were developed to heal rotator cuff tendons. However, some clinical studies failed to demonstrate an improved healing, especially with the use of animal derived tissues. The aim of this research was to evaluate a human dermis-derived membrane (HDM_derm) that was submitted to a new developed decellularization method.

Methods. Preliminarily histological, ultrastructural and biomechanical investigations on HDM_derm showed the absence of viable cells with intact ECM and vascular channels. Tensile and pull-out tests showed that HDM_derm can support tendon healing with a failure mode ×similar to other membranes as described in literature. Tenocytes (1 105 cells/ml) were seeded on 12 samples of HDM_derm; the same amount of cells were plated in the polystyrene wells as culture controls (CTR). Cell viability (WST-1) and synthetic activity were evaluated at 3 and 7 days.

Results. Tenocytes migrated and attached to the surface of the HDM_derm as early as 3 days after culture without significant differences in cell viability in comparison to CTR at both experimental times. At both experimental times A significant higher amount of collagen I was observed when cells were cultured with HDM_derm in comparison with CTR (3d: $p < 0.0001$; 7d: $p < 0.05$). In HDM_derm group, Fibronectin synthesis was significantly higher at both experimental times ($p < 0.0001$), while Proteoglycans only at 3 days ($p < 0.0001$). At 3 days TGFβ1 was significantly higher when tenocytes were plated with HDM_derm ($p < 0.005$) and at 7 days it significantly decreased ($p < 0.05$) and no differences were observed between the 2 groups.

Conclusions. Even with the limitations of in vitro static systems, data showed that the decellularization technique enabled the development of a matrix with adequate mechanical and biological properties for the surgical augmentation of massive rotator cuff tears.

Keywords. Human dermis-derived membrane, decellularization

(20.P2) CELL SURFACE GLYCOSYLATION AND GLYCOSAMINOGLYCAN COMPOSITION PROFILES IN IMMATURE AND MATURE INTERVERTEBRAL DISCS

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1. Glycoscience Group, Ireland; 2. Network of Excellence for Functional Biomaterials, Ireland; 3. AO Research Institute, Switzerland

Back pain is a predominant cause of disability, and the major cause is intervertebral disc (IVD) degeneration. Proteoglycan (PG) content of the IVD is decreased during degeneration, and PG glycosaminoglycan (GAG) composition is altered. In this study, the cell surface glycosylation and GAG composition profiles in immature and mature ovine IVD tissue were profiled in an effort to understand potential signals involved in cell-extracellular matrix crosstalk during maturation and degeneration. IVD tissue from L3-L4 and L4-L5 lumbar segments were harvested from 3 and 11-month-old animals. One portion of the tissue was fixed for histochemical profiling with a panel of lectins and neoglycoconjugates. The remaining portion was digested with proteinase K and the sulfated GAG (s-GAG) content was quantified by dimethylmethylene blue assay. The digested tissue was treated with chondroitinase and analysed by HPLC for s-GAG constituents. All cells present in the IVD had similar surface lectin and carbohydrate expression (Figure 1), but

the intensity of expression differed with cell type. HPLC analysis of the digested IVD tissue revealed that the quantity and ratio of s-GAG components of the nucleus pulposus (NP) tissue differed from annulus fibrosus (AF) tissue, and from IVD segment to segment. In support, the DMMB assay showed that NP tissue contained 3-fold more s-GAGs than AF and cartilage in 3-month sample. In the 11-month sample, no difference was observed between AF and NP, but overall content of s-GAG was higher than cartilage. The glycosylation expression level differs between AF and NP cells and s-GAG composition differs between AF and NP tissue and alters with age. As GAG sulfation is critical for maintaining water content and specific patterns of sulfation are influential in cellular signaling and differentiation events; these observations have significant implications for therapeutic regenerative approaches.

Acknowledgements. AO Foundation (S-09-7P), Science Foundation Ireland, Research Frontiers Programme (07/RFP/ENMF482).

Keywords. Glycobiology, intervertebral disc, glycosaminoglycans

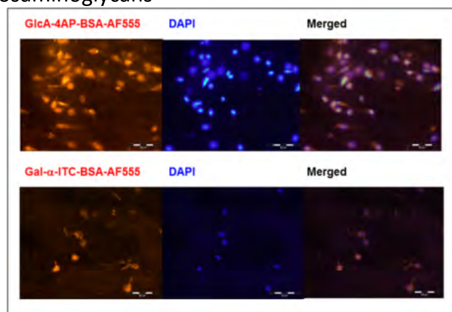

Figure 1: 40X fluorescence microscope images of IVD tissue stained with AlexaFluor555 labelled neoglycoconjugates of glucuronic acid (GlcA-4AP-BSA-AF555, red) and galactose (Gal-α-ITC-BSA-AF555, red) and counterstained with nuclear label DAPI (blue).

(20.P3) MORPHOGENESIS OF PROSTATE CANCER CELL IN 3D SYNTHETIC AND BIOMIMETIC MATRIX

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Introduction. Cell-cell and cell-matrix interactions are key modulators of cell behaviour and are crucial in tumour morphogenesis. These interactions, mediated by the surrounding matrix are well demonstrated in (three-dimensional) 3D more than (two-dimensional) 2D cultures. In a 3D environment, cells interact with their ECM in all directions and this induces specific cellular responses. These features render a more physiological condition for cells therefore a better representation of the cancer pathophysiology. In this study, we aim to characterise prostate cancer cells (CaP) cultured in PEG-based synthetic matrix in the form of hydrogels and compare their phenotypes to the conventional 2D cultures.

Methods. CaP cell line, LNCaP cells were embedded in PEG-based hydrogels as described by Lutolf et al (2007) via Factor XIII catalysed cross-linking reaction. The 3D culture was maintained for up to 28 days and cell proliferation was assayed during this period. Other analyses comparing 2D and 3D cultures include morphology examination and; protein and gene expression of typical CaP markers.

Results. LNCaP cells grow slowly to form multicellular spheroids (MS) and become irregular in shape as the culture continues (Figure 1A). Apoptosis and hypoxia are also detected in the core of the MS. When protein expressions of the MS were compared to 2D cultures, we found that in both cultures, cells produce prostate specific antigen and retain their epithelial markers, E-cadherin and Cytokeratin 8 (CK8, Figure 1B). However, the localization of CK8 and androgen receptor (AR) differs between the cultures.

Conclusions. The morphology of MS and localization of the cellular proteins indicate tissue-like development which is not observed in 2D cultures. It is apparent that the PEG-based hydrogel promotes cell morphogenesis but does not compromise the expression of functional proteins. This 3D model offers a potential useful tool for drug discovery and translational studies.

Keywords. Prostate cancer, synthetic matrix, 3D cultures

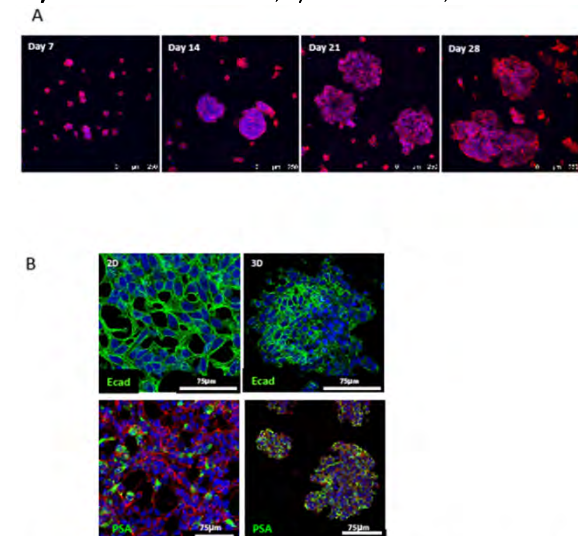

Figure 1. (A) 3D projection (150um stack) of confocal images of LNCaP cultured in 3D hydrogels from day 1 to day 28. Spheroids labeled with actin (red) and nuclear (blue). (B) 2D and 3D cultures in normal growth media. Green staining represents protein of interest, actin cytoskeleton (red) and nucleus (blue).

(20.P4) EXTRACELLULAR MATRIX AS A BIOSCAFFOLD FOR THE CONSTRUCTIVE REMODELING OF THE SKELETAL MUSCLE MICROENVIRONMENT

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Extracellular Matrix (ECM) scaffolds derived from decellularized tissues have been used in both pre-clinical and clinical studies to promote the constructive, functional remodeling of defects in a variety of soft tissues including damaged larynx, esophagus, heart, and skeletal muscle. These naturally occurring acellular scaffolds are composed of growth factors and matricryptic peptides which are released following implantation during the process of scaffold degradation. These ECM degradation products have been shown to promote the recruitment and mitogenesis of multipotent perivascular cells as well as other cell types in vitro. Multipotent perivascular cells are found ubiquitously throughout vascularized tissues and can be isolated according to their surface marker phenotype. Multipotent perivascular cells possess myogenic potential and upon their transplantation into sites of skeletal muscle injury induce enhanced regenerative capacity. The effect of ECM degradation products on endogenous multipotent

perivascular cells has not been examined. The present study used a murine skeletal muscle critical size injury model in which a unilateral resection of the tensor fascia latae and rectus femoris quadriceps muscles is conducted, followed by the implantation of an ECM scaffold. The use of this model allowed for the spatial and temporal examination of ECM mediated skeletal muscle constructive remodeling and recruitment of endogenous multipotent perivascular cells.

In uninjured skeletal muscle multipotent perivascular cells were anatomically associated along the outer periphery of blood vessels and capillaries. Following injury in untreated animals, multipotent perivascular cells remained predominantly in their normal vessel-associated anatomic location. In contrast, following injury and treatment with an ECM scaffold, multipotent perivascular cells populated the ECM scaffold outside the context of blood vessels or capillaries, suggesting that the perivascular progenitor cells participate in the ECM mediated constructive remodeling process.

Keywords. ECM, perivascular cells, skeletal muscle

(20.P5) NEUROBLASTOMA AND EMBRYONIC LUNG FIBROBLASTS REQUIRE DIFFERENT MODIFICATIONS OF AN ALGINATE-BASED THREE DIMENSIONAL CULTURE SYSTEM TO MAINTAIN CELL VIABILITY AND GROWTH

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Introduction. Three dimensional (3D) alginate-based (Alg) culture systems represent a structural platform for the improved modelling of cell-cell and cell-extracellular matrix (ECM) interactions. However, alginates are typically non-adhesive to cells and can cause cell death [1, 2]. This study was designed to elucidate the most favourable conditions to enable extended culture of two types of fibroblasts in modified alginate capsules.

Methods. IMR-32 neuroblastoma or MRC5 embryonic lung fibroblasts were encapsulated in alginate with the addition of chitosan (Cs), and with incorporated adhesion substrates: fibronectin (Fn) or hyaluronic acid (HA). Metabolic activity of encapsulated cells was measured using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at defined time points during 10-20 days of culture. Cell morphology and survival were assessed through light microscopy and fluorescent live/ dead staining.

Results. There was higher metabolic activity of IMR-32 cells than MRC5 cells (1.4-fold, day 2, $p=0.065$; 8-fold, day 9, $p<0.05$) in Alg-Cs-HA matrix. The addition of Fn and removal of Cs from matrix resulted in increased MRC5 but not IMR-32 metabolic activity (3-fold, day 6, $p<0.05$). Light micrographs revealed elongated aggregates of MRC5 cells in Alg-Fn matrix and round aggregates of IMR-32 cells in Alg-(Cs)-HA matrix. Lung fibroblasts in Alg-Cs-HA and neuroblastoma in Alg-Fn matrix underwent shrinking and eventually disintegrated. Enhanced green fluorescence of viable cell aggregates and less necrotic cells was observed in growth-supporting matrices.

Conclusions. Our study shows that the composition of artificial ECMs should be 'tailored' to the cultured cell type and is critical to cell survival and cell-cell interactions. This individualised approach enables prolonged 3D culture within a clearly defined matrix with structural consistency and retention of cells.

Acknowledgments. Thanks to my supervisors for support and AstraZeneca, EPSRC for funding.

References.

1. Alsberg E et al. ProcNatlAcadSci. 99, 12025, 2002.
2. Fischbach C et al. PNAS 106(2), 399, 2009.

Keywords. Alginate, extracellular matrix, 3D culture systems

(20.P6) A NOVEL DEVICE FOR THE AUTOMATIC DECELLULARIZATION OF BIOLOGICAL TISSUES

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Introduction. Biological decellularized matrices revealed to be a promising scaffold with non-immunogeneic behaviour for tissue engineering applications [1,2]. Anyway these substrates are obtained through a process that requires numerous manual labour-intensive steps (repeated cycles, different solutions), posing major problems in terms of safety, reliability and reproducibility. To address these issues we developed a novel apparatus for the automatic decellularization of tissues, able to autonomously manage the entire process according to a predefined protocol (solutions and timeline) in multi-compartment chambers.

Methods. Key requirements of the apparatus were: (1) automatic exchange of substances, (2) mechanical stirring, (3) thermal regulation, (4) control over process parameters. Furthermore, the device was designed to be versatile, modular and economically sustainable looking towards a clinical use. Main elements are: decellularization chambers, hydraulic system and support-movement system. To promote an efficient cell removal throughout the sample, an alternated 180° rotation has been imposed to the chambers.

Results. The device has been realized and bench-tested with positive outcomes. Appropriate electronics was implemented to manage the automated exchange of fluids and the engine movement to provide mechanical shaking. Commercial Falcon™ tubes were chosen as decellularization chambers, providing an easily available, sterile, and disposable tool to house the biologic sample. Modified caps were manufactured to connect each tube to the hydraulic circuit. Tubing and materials for non-disposable components were chosen according to biocompatibility and autoclavability. The device is currently employed in the decellularization of porcine arterial blood vessels.

Conclusions. Looking at biological acellular scaffold as a promising solution for tissue engineering we developed a novel device for the decellularization of tissues. We believe that the automation of the process is a key step to ensure a reliable, validated and efficient GMP process.

Acknowledgements. Partially supported by Regione Lombardia.

References.

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2. Gilbert TW, Sellaro TL, Badylak SF, Biomaterials 27:3675-83, 2006.

Keywords. Automation, decellularization, extracellular matrix, biological scaffold

(20.P7) CHARACTERIZATION OF THE RETICULIN FIBRES BUNDLES IN NEUROBLASTOMA BY MEANS OF INTEGRATED QUANTITATIVE IMAGE ANALYSIS.

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Introduction. Reticulin fibres (RF) quantification is a prognostic factor in several diseases involving bone marrow, mostly correlating with poor prognosis. Neuroblastoma is the most frequent solid extracranial malignancy in childhood and its prognosis depends, among other factors, on histological criteria. Our group confirmed that neuroblastic tumors with abundant stroma had a more differentiated phenotype and were associated with a more favorable outcome, compared to Schwannian stroma-poor tumors. We aim to characterize the bundles of RF in neuroblastoma by means of an automated image analysis.

Methods. We measured 24 differentiated, poorly differentiated and undifferentiated neuroblastoma samples, contained in a Tissue Microarray, and stained with the Gomori technique. We used two techniques for image intake. We divided the 1mm-cores in 6 images at 20x (DMD 108, Leica), which were then joined to obtain a single image of the whole core. We also digitized the cores by scanning the whole TMA at 40x (ScanScope XT, Aperio). The staining was measured by automated image analysis after designing the proper algorithm for both methods, able to detect and characterize the same structures.

Results. The RF bundles measured by both image intake methods showed different patterns following parameters of shape and size, related to clinical-biological data with prognostic value. Frequently, they draw lobules including the tumor cells.

Conclusions. A large amount of samples stained for RF need to be screened in neuroblastoma, this requires a fast and reliable system for image intake and data collection connected with algorithms for automated image analysis. The scanning of the whole TMA with the ScanScope XT (Aperio) is the fastest system available and provides the best image quality.

Grants. ISCIII (PI10/00015), AECC (396/2009), ISCIII (RD06/0020/0120)

Keywords. Reticulin, automated digital analysis, neuroblastoma

(20.P8) CONTROLLED RELEASE OF rhTGF-β3 FROM THE EXTRACELLULAR MATRIX MEMBRANE

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Introduction. The recombinant human transforming growth factor beta-3 (rhTGF-β3) is known as a key regulator of chondrogenesis of stem cells and cartilage formation. We have developed a novel drug delivery system that continuously releases rhTGF-β3 using an extracellular matrix (ECM) membrane. We applied it to the repair of articular cartilage, which is known to possess limited capacity for self-renewal. We hypothesized that the sustained release of rhTGF-β3 could activate stem cells, resulting in the enhancement of repair of cartilage defect. The property and efficacy of the delivery system were examined using rhTGF-β3 as a candidate drug in vitro and ex vivo model.

Materials and Methods. The ECM membrane was constructed using porcine chondrocytes ECM. The rhTGF-β3-loaded ECM membrane was manufactured by a lamination process. Five ECM membranes were combined, and 100 ng of rhTGF-β3 was loaded between the second and third layers. The rhTGF-β3-loaded ECM membrane was dried and cross-linked by UV for 1 hour. rhTGF-β3-loaded membranes were incubated with 1 ml at 37℃, 50 rpm for 28 days. The activity of released rhTGF-β3 was determined using western blot (WB) analysis and Circular Dichroism (CD). The cartilage constructs made were implanted subcutaneously into nude mice.

Results. We have developed a novel system that enables the controlled release of rhTGF-β3 from an ECM membrane. The measurement of rhTGF-β3 in vitro showed sustained releasing profile for 28 days despite the decreasing tendency in dose. We confirmed the bioactivity by the chondrogenic differentiation of MSCs, WB, and CD analysis. The rhTGF-β3-loaded ECM membrane produced better repair of cartilage defect than the unloaded one did.

Conclusions. The ECM membrane could be a useful drug delivery system for cartilage regeneration by allowing the sustained release of drug for a long period of time.

Keywords. ECM membrane , rhTGF-β3 , controlled release, cartilage regeneration

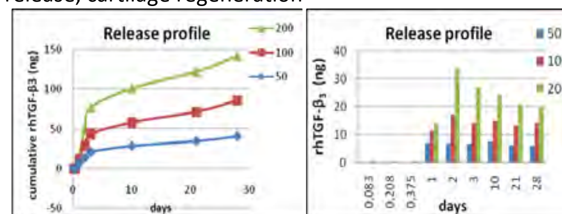

Fig1. Release profile of rhTGF-β3 from the ECM membrane. The amount of rhTGF-β3 released into PBS was quantified by ELISA until 28 days.

(20.P9) THE EFFECTS OF FIBRIN AND FIBRIN-AGAROSE IN THE CELLULARITY AND COLLAGEN FIBRILLOGENESIS OF BIOENGINEERED ORAL MUCOSA

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1. Biomedical Research Centre, School of Medicine, Universidad de Valparaiso, Chile; 2. CREAS, Valparaiso,

Chile; 3. Tissue Engineering Group, Department of Histology, University of Granada, Spain; 4. Institute of Biomedical Sciences, University of Sao Paulo, Brazil; 5. Cátedra de Histología B, Facultad de Odontología, Universidad Nacional de Córdoba, Argentina.

Several researchers have developed efficient oral mucosa constructs using different types of scaffold. However, the changes in the cellularity and collagen fibrillogenesis profile that may occur in these artificial constructs are unknown. In this study, we compared the histology and the collagen distribution in a human artificial oral mucosa developed using two different types of scaffolds: fibrin and fibrin-agarose.

For that purpose, bioengineered oral mucosa stromas were constructed from biopsy samples of human oral mucosa and the substitute generated was analyzed at different periods of time in culture. Histological analysis was carried out by light and transmission electron microscopy and the expression of collagen types I, III and VI, were assessed by immunoperoxidase technique. We found that the fibrin scaffold accelerates fibroblast growth and remodeling of the scaffold, thus enhancing collagen fibrillogenesis. In the fibrin-agarose scaffold, the morphology and organization of the fibroblasts not change along the culture period. All types of collagen analyzed were expressed in both scaffolds. However, in fibrin scaffolds, these proteins were widely distributed, and they replaced the scaffold along the follow-up period. Our results demonstrated that the substitutes generated in our laboratory had histological and molecular similarities with the native human oral mucosa stroma. In addition, we showed that the nature of the biomaterial influenced the behavior of the oral stromal fibroblasts, modulating their growth, protein synthesis and collagen fibrillogenesis.

Acknowledgments. CI 05/2006 (Universidad de Valparaiso, Chile) and CONICYT (ACT-73), Chile.

Keywords. Collagen, oral mucosa, fibrin, agarose, scaffold

21. INJECTABLE SCAFFOLDS

Chair: Wenxin Wang
Co-chair: Abhay Pandit
Keynote speaker: Peter M. Mariner
Organizer: Wenxin Wang

Synopsis: Injectable scaffolds have attracted more and more attention in tissue engineering and regenerative medicine fields because of their promising advantages over performed scaffolds, which include ease of application, confined delivery for a site-specific action and improved patient compliance and comfort. In addition, injectable scaffolds mixing with cells form a uniform distributed cell/scaffold tissue system in vivo, which provide a rich and complex environment of signals so as to promote tissue regenerate and development.

Several types of scaffold have been utilised as injectable scaffolds, which include both natural materials, such as: alginate gel, algiante tricalcium phosphate, β-tricalcium phosphate, calcium phosphate, chitosan, collagen, fibrin, fibronectin, gelatine; and synthetic or semi-synthetic materials, such as: poly(D-L-lactic acid) (PLA), poly(glycolide acid) (PGA), poly(D-L-lactide-co-glycolide acid) (PLGA), poly (propylene fumarate), poly(propylene fumarate)-diacrylate (PPF-DA), poly(caprolactone fumarate) (PCLF), poly(D-L-lactic-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG), and collagen/polymethylmethacrylate etc.

However, many challenges in the design and development of injectable scaffold-cell system still remain:

- optimisation of the kinetics of solidification process in-situ;
- development of injectable scaffold that can withstand the mechanical stresses while maintaining an environment conducive to tissue growth;
- optimisation of creation of required pore size and formation of inter-connectivity of pores within the solidified scaffold;
- protection of cell damage and viability during preparation of scaffold and delivery;
- controlled release of growth factors;
- understanding of cell-material interaction, “cell-adhesion” and biocompatibility;
- rate of biodegradation and cytotoxicity;
- phenotype tissue formation.

As a consequence, a symposium based in this area would fit well with the key theme of the conference. The symposium is aimed at bringing together all aspects of the field, from synthetic chemists interested in developing suitable materials to applied scientists, engineers and clinicians that are interested in the applications of injective scaffolds

This symposium will cover following aspects of the state of art studies and developments of injectable scaffolds:

- Design and synthesis of suitable biomaterials for injectable scaffolds including hydrogel and stimuli materials;
- Functionalization and degradation mechanism studies of polymers for injectable scaffolds;
- Application and evaluations (in vitro and in vivo) of polymers for injectable scaffolds.

(21.KP) THIOL-ENE CLICK GELS AS IN SITU FORMING, CELLULARLY-DEGRADABLE BIOMATERIALS

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1. University of Colorado and HHMI; 2. University of Colorado

Introduction. Synthetic hydrogels with engineered cell-mediated degradation sites are an important category of biomimetic materials. Here, hydrogels are synthesized from a regular, step-growth mechanism via a radically-mediated thiol-norbornene photopolymerization, allowing in situ formation in the presence of tissues and cells. This reaction combines the advantages of ideal network formation, facile incorporation of peptides without post-synthetic modifcation, and spatial and temporal control over the network evolution into a single system to make proteolytically degradable PEG-peptide hydrogels.

Material and Methods. 4-arm and 8-arm PEG macromers were end-functionalized with norbornene, and subsequently reacted with cysteine-containing peptides

(e.g., CGGRGDS, CGGDGEA, CGGIKVAV, KCGPQGIWGQCK), via a photochemically initiated reaction with ~10 mW/cm2 of 365 nm light. The macromer purity and functionality were characterized with proton NMR and MALDI.

Results. Using this thiol-ene photopolymerization, fast gelation times are achieved while maintaining high cell viability (>95% for thirty second polymerizations). The gelation process and mechanical properties were characterized using in situ rheometry. Biological results demonstrate the enzyme and cell responsive characteristics of the gels by tailoring the rate of spreading of human mesenchymal stem cells through the choice of proteolytically degradable crosslinker. Gel properties and cellular function can also be manipulated spatially within the thiol-ene hydrogels through biochemical photopatterning. Finally, in vivo performance was tested in a critical-sized rat cranial defect, demonstrating improved healing in the presence of thiol-ene gels as monitored and characterized with microCT and histology.

Conclusions. The high degree of spatial and temporal control over gelation, combined with robust material properties, make thiol-ene hydrogels an excellent platform as an in situ forming biomaterial.

Acknowledgments. Funding from NIH (DE16523) and HHMI

Keywords. hydrogels, photopolymerization, MSC

(21.O1) THE EFFECTS OF FERULIC ACID ON NUCLEUS PULPOSUS CELLS UNDER HYDROGEN PEROXIDE-INDUCED OXIDATIVE STRESS

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Disc degeneration is strongly associated with low back pain which causes increased cost of health care. It believed that the degenerative process begins in the inner nucleus pulposus (NP) of intervertebral disc with a decrease in cell number and loss of proteoglycans. Several studies have shown that overproduction of reactive oxygen species (ROS) could be associated with apoptosis of NP cells and degradation of ECM. Ferulic acid (FA), a free radical scavenger, has been reported to possess an excellent antioxidant property. In the study, we're going to elucidate the therapeutic effects of FA on hydrogen peroxide-induced oxidative stress NP cells and the feasibility of use the thermosensitive chitosan/ gelatin/ glycerol phosphate (C/G/GP) hydrogels as a controlled release system of FA for NP regeneration.

NP cells were harvested from the intervertebral discs of the adult New Zealand white rabbits and cultured in monolayer. Oxidative stress on NP cells was induced by 100μM of hydrogen peroxide (H2O2). H2O2-induced oxidative stress NP cells were further treated with 500μM of FA. The mitochondrial activity, cell proliferation, cytotoxicity, anabolic/catabolic gene expressions, extracellular matrix (ECM) related gene expressions, production of sulfated glycosaminoglycans (GAGs), alcian blue stain, caspase-3 activity and TUNEL stain of the NP cells were evaluated. The release of FA from C/G/GP hydrogels was analyzed by ultra violet-visible-near infrared spectrophotometer. The ROS production was performed by chemiluminescence assay.

The results showed that post-treatment of FA on H2O2-induced oxidative stress NP cells could restore part of the cell functions (Aggrecan and MMP-3) and promote the desired gene expression (type II collagen and BMP-7). The GAGs production could be switched back to the normal level. The addition of FA to H2O2-induced oxidative stress NP cells decreased the apoptosis. The results suggested that FA incorporated in C/G/GP hydrogels may potentially be applied for NP regeneration in the future.

Keywords. hydrogels; nucleus pulposus; ferulic acid

(21.O2) PHAGOCYTOSIS BY ACTIVATED MACROPHAGES

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1. NFB, NUIG

Introduction. Microspheres embedded within a scaffold can form a sequential-release system. However; a major issue with microspheres is phagocytosis by macrophages in vivo. Hence, the objectives of this study are to fabricate hollow collagen microspheres, identify a size that can avoid phagocytosis, embed microspheres within a scaffold and characterize the release profile, and assess the microspheres in an acute sub dermal model.

Materials and Methods. Hollow collagen microspheres were fabricated using a template-based method, embedded within a collagen hydrogel crosslinked with 4S-PEG and the release profile characterised with Cy3 labeled pDNA and collagenase was used to degrade the scaffold. THP-1 cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) and activated using lipopolysaccharide (LPS). FITC-labeled microspheres (100nm, 1 μm and 10 μm) were incubated with macrophages for 24hrs, after which cells were fixed for FACS analysis or fluorescent microscopy. Spheres within a collagen scaffold were implanted in a sub dermal model to assess the release profile of bioactive polyplexes. Spheres were loaded with polyplexes of a reporterl plasmid with either polyethylenimine (PEI) or a linear-dendritic hybrid poly(2-dimethyl amino) ethyl methacrylate or plasmid alone (n=6).

Results and Discussion. Following microscopy and FACS, 1μm spheres showed an ability to avoid phagocytosis by macrophages. The uptake of 10μm spheres by activated macrophages was visible by fluorescent microscopy, while there was a higher signal from 100nm spheres than 1μm spheres. The release profile from the scaffold showed two distinct release patterns over time. In vivo studies demonstrated the ability of the spheres to release bioactive polyplexes, showing minimal tissue response in a sub dermal model.

Conclusion. A sequential release scaffold composed of microspheres embedded within a hydrogel was fabricated, with the chosen microspheres demonstrating an ability to avoid uptake by macrophages in vitro, and characterised in vivo.

Acknowledgment. Science Foundation Ireland, Grant no. 07/SRC/B1163.

Keywords. Microspheres, Phagocytosis, Gene Therapy

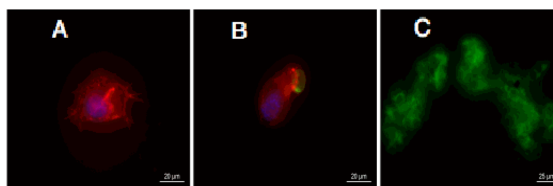

Figure 1: (A) Macrophage activated using LPS, (B) an activated Macrophage engulfing a 10µm hollow collagen micropshere, (C) hollow collagen microspheres embedded within a collagen hydrogel.

(21.O3) THERMOREVERSIBLE HYALURONAN-BASED HYDROGELS FOR NUCLEUS PULPOSUS TISSUE ENGINEERING

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1. AO Research Institute Davos

Introduction. Degeneration of the intervertebral disc is considered as a major cause of back pain. Current surgical treatments provide back pain relief but are unable to restore disc functionality. Tissue engineering has showed promise for repairing degenerated discs by providing an appropriate combination of cells and carrier. Recently, thermoreversible hydrogels have attracted interest in the spine research as they provide easy injectability (low viscosity at room temperature) together with a mild gelling mechanism (physical cross-link at 37°C). The purpose of this study was to assess the potential of a thermoreversible hyaluronan-based hydrogel (HA-pNIPAM) as nucleus pulposus cells (NPC) carrier.

Material and Methods. Cytocompatibility of HA-pNIPAM and its degradation products was evaluated with bovine NPC after 1 and 3 days. The sulphated glycosaminoglycan synthesis (DMMB assay), histology (toluidine blue staining) and gene expression profile (RT-PCR) of NPC cultured for one week in HA-pNIPAM or alginate gels was studied. Injectability and NPC survival in an ex-vivo whole organ culture model was evaluated.

Results. HA-pNIPAM and degradation products were cytocompatible to NPC (viability >80%). After one week of culture, NPC morphology and glycosaminoglycan synthesis were similar in the two hydrogels. Following NPC expansion, both hydrogels induced re-differentiation toward the NP phenotype (Fig.1); moreover, HA-pNIPAM induced a stronger hyaluronan synthase 1 up-regulation in NPC as compared to alginate gels. Finally, NPC suspension in HA-pNIPAM was injectable and NPC were >80% viable in HA-pNIPAM for one week in an ex-vivo whole organ culture model.

Conclusions. HA-pNIPAM provides an injectable carrier for NPC capable of maintaining their phenotype and promoting disc matrix generation.

Keywords. Thermoreversible hydrogel; hyaluronic acid; intervertebral disc; whole organ culture

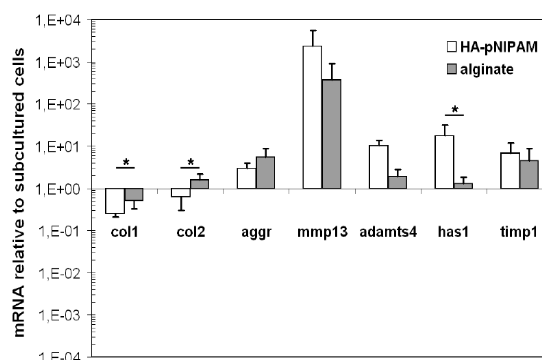


Figure 1: Gene expression profile of bovine nucleus pulposus cells after 1 week of culture in hydrogels (*notes = significant differences, p<0.05): collagen I (col1), collagen II (col2), aggrecan (aggr), matrix metalloproteinase 13 (mmp13), hyaluronan synthase 1 (has1) and matrix metalloproteinase tissue inhibitor 1 (timp1).

(21.O4) IN SITU FORMING HYALURONIC ACID HYDROGEL FOR NUCLEUS PULPOSUS REGENERATION: RHEOLOGICAL PROPERTIES AND GENE EXPRESSION EVALUATION

Chen YC (1), Su WY (2), Lin FH (1)
1. Institute of Biomedical Engineering, National Taiwan University; 2. Division of Medical Engineering Research, National Health Research Institute

Introduction. In situ forming hydrogel allows irregular surgical defects to be completely filled, lessens the risk of implant migration, and minimizes surgical defects due to the solution–gel state transformation. Additionally, the liquid solution can also be incorporated with therapeutic factors (e.g., TGF, BMP, EGF) and cells to treat or to relieve the injury/degenerated tissues.

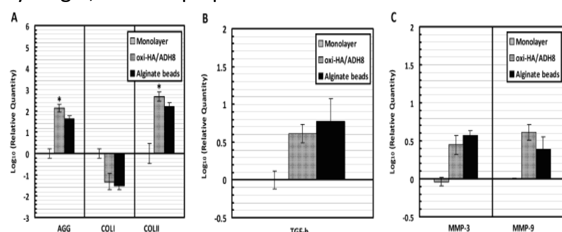
Materials and Methods. In this study, we propose a method for preparing in situ forming hydrogel composed of oxidized hyaluronic acid (oxi-HA) and adipic acid dihydrazide (ADH) and incorporated it with nucleus pulposus cells to reverse nucleus pulposus degeneration.

Results. Fourier transform infrared spectrometry and trinitrobenzene sulfonate assay were used to confirm the oxidation of hyaluronic acid. Rheometer were used to evaluate the working ability and viscoelastic properties of the hydrogel for further clinical application. The oxi-HA/ADH in situ forming hydrogel can transform from liquid form into a gel-like matrix within 3–8 min, depending on the operational temperature, and the value of the shear modulus |G*| is about 30 kPa. Furthermore, hydrogel degradation and cell assessment is also a concern for clinical application. In situ forming oxi-HA/ADH8 hydrogel can maintain its gel-like state for at least 5 weeks with a degradation percentage of 40%. Importantly, oxi-HA/ADH8 hydrogel can assist in nucleus pulposus cell synthesis of type II collagen and aggrecan mRNA gene expression (as Figure shows) according to the results of real-time PCR analysis, and shows good biocompatibility based on cell viability and cytotoxicity assays.

Conclusions. Cell-based therapy is a novel biological treatment for tissue regeneration, and the cell carrier plays an important role in cell based therapy. However, finding a suitable cell carrier is not an easy task. Based on

the results of current study, oxi-HA/ADH hydrogel could be a suitable cell carrier for nucleus pulposus cells in the treatment of nucleus pulposus degeneration.

Keywords. hyaluronic acid; biocompatibility; injectable hydrogel; nucleus pulposus



(21.O5) A NOVEL BIOSYNTHETIC HYDROGEL FOR SURGICAL APPLICATIONS

Godek ML (1), Lavigne K (1), Mast N (1), Skalla W (1), Throm A (1), Mayotte J (1), Bushnell R (1), Bennett S (1)

1. Covidien

Maintaining tissue apposition throughout wound healing sequelae without acting as a barrier to tissue remodeling requires a delicate balance between attracting and promoting cellular attachment and tissue ingrowth, and biomaterial persistence. Currently, a need exists for a material which joins tissue planes without inhibiting wound healing, a requirement that is marginally fulfilled by fibrin glues. Potential clinical indications necessitating tissue ingrowth and/or tissue plane apposition include abdominoplasties, mastectomies, and bowel sealing. Biosynthetic materials may exploit the properties of biological components to facilitate wound healing following surgical intervention, while synthetic components offer the ability to tailor characteristics such as strength and persistence. This study examined properties of novel biosynthetic hydrogels formed by crosslinking bovine serum albumin with a reactive poly(ethylene glycol), with or without the addition of charged beads, and subsequent in-vitro cellular response. The novel hydrogels exhibited similar mechanical and physical properties: gel time (99 and 113 seconds), percent swelling (22% and 20%) and material persistence (23.and 20 days) were comparable for the hydrogels (averages for hydrogels with and without charged beads, respectively). Average elastic moduli were 488 kPa ± 93 and 315 kPa ± 55 for hydrogels with and without beads respectively (n=6, standard deviations reported); hydrogels were further characterized via environmental SEM. Cellular response and cytotoxicity were assessed by culturing monocyte/macrophage and fibroblast cell lines on gel surfaces and tracking microscopically for 30+ days (n>3 for all tests). Significant to the wound healing process, macrophages and fibroblasts adhered to, proliferated on, and migrated into preformed hydrogels. Cells exhibited a preference for the bead surfaces and used them to migrate through the full thickness of the hydrogel materials; materials were non-cytotoxic over the experimental time frame. These materials may be suitable for applications which require tissue plane apposition and tissue ingrowth where a degradable material is desired.

Keywords. PEG, hydrogels, tissue adhesive, wound healing

(21.O6) THE EFFECT OF INCUBATION TIME OF PREFORMED INJECTABLE HYDROGELS ON BONE FORMATION WHEN USED AS CARRIER FOR rhBMP-2

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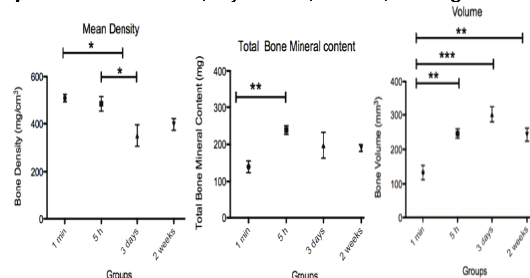
Introduction. Hydrogels has demonstrated efficacy as carriers for growth factors. Our aim was to investigate the effect of curing-time of aldehyde-modified hyaluronan (HAA) and carbazate-modified polyvinyl-alcohol (PVAC) hydrogel on bone formation.

Materials and Methods. HAA (22.25 mg/mL in PBS) and 1 g amorphous calcium phosphate (ACP)(Plasma"Biotal Ltd.) and PVAC (4.125 mg/ml) with 0.6 g ACP and 0.375 mg/ml recombinant human bone morphogenetic protein"2, (InductOs , Wyeth Europe Ltd.) were cross-linked for 2 weeks, 3 days, 5 h or 1 min before injection. Preformed gels were injected SC in five Sprague Dawley rats weighing about 250 g with one sample from each time point in each rat. The animals were sacrificed at week five. Explanted samples were radiographed and scanned by quantitative peripheral computed tomography (pQCT) (Stratec, Germany). Bone density analysis was performed of radiographs (Software scion) and pQCT data was analysed by Graphpad Prism 5.0 regarding bone mineral content, bone density and bone volume.

Results. Bone formation occurred in all samples. Radiographs revealed higher attenuation for the 5 h cross-linked hydrogel. The same result was seen in the pQCT were both 5 h and 1 min preformed hydrogels had significantly higher bone density compared to 3 days (p=0.0064, ANOVA Tukey's multiple comparison test; fig 1). 5 h yielded higher bone mineral content compared to 1 min cross-linked gel (p=0.0116 ANOVA Tukey's multiple comparison test; fig 1). The 1 min preformed gel had significantly lower volume then all the other groups (p<0.0001, ANOVA Tukey's multiple comparison test; fig 1). Figure 1. Mean bone density, total bone mineral content and bone volume (n=5;ANOVA Tukey's multiple comparison test) *p=0,05, **p=0.01, ***p<0.0001.

Conclusions. A minimum of 5 hours curing-time gives the most efficient bone formation concerning density, mineral content and volume.

Keywords. Preformed, injectable, in vivo, osteogenesis



(21.O7) INJECTABLE GELLAN GUM-BASED HYDROGELS FOR INTERVERTEBRAL DISC REGENERATION

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Introduction. Intervertebral disc (IVD) degeneration is a challenging pathology that, due to the inefficiency of the current treatments, urgently demands for the development of new regenerative approaches[1]. The best viable implant material for nucleus pulposus (NP) regeneration has yet to be identified, but it is believed that biodegradable hydrogel-based materials are promising candidates[2]. In this work, we are proposing the use of ionic- and photo-crosslinked methacrylated gellan gum (GG-MA) hydrogels as potential acellular and cellular injectable scaffolds for IVD regeneration.

Materials and Methods. Gellan gum (GG) was reacted to glycidyl methacrylate (GMA) to enable incorporation of methacrylate groups in GG structure. Ionic- and photo-crosslinked hydrogels, obtained either by immersion in phosphate buffered saline (PBS, pH 7.4) solution or by UV exposure, were physico-chemically characterized by FTIR, 1H NMR and DSC. Hydrogels swelling capacity and degradation rate were also analyzed in PBS, for the period of 30 days. Additionally, the morphology and mechanical properties of the hydrogels were assessed by SEM and DMA, respectively. Cytotoxicity of the GG-based hydrogel leachables was evaluated by carrying out a cellular viability assay (MTS test) on rat lung fibroblasts (L929 cell line) cells until 7 days.

Results. Results demonstrated that GG was successfully methacrylated and allowed to produce both ionic- and photo-crosslinked GG-MA hydrogels. The developed GG-MA hydrogels possess improved mechanical properties and lower water uptake ability and degradation rate as compared to those for GG. This work also revealed that GG-MA hydrogels are non-cytotoxic in vitro (Figure 1).

Conclusions. The results indicate that the proposed ionic- and photo-crosslinked GG-based hydrogels are promising biomaterials to be used as acellular and cellular substitutes of the NP.

Acknowledgements: Funding from EU FP7 project Disc Regeneration (Grant No. NMP3-LA-2008-213904).

References:

1. Richardson SM et al. (2007), Histol. Histopathol., 22: 1033-1041.

2. Peppas NA et al. (2006), Adv. Mater., 18: 1345-1360.

Keywords. Gellan gum, intervertebral disc, methacrylation, photo-crosslinking

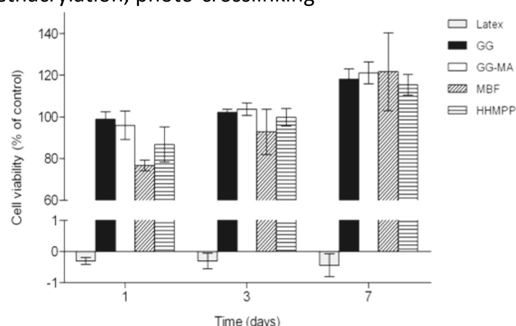

Figure 1. Cytotoxicity screening of the GG-based hydrogel leachables using L929 cells (GG: gellan gum; GG-MA: ionic-crosslinked hydrogel; MBF: photo-crosslinked hydrogel with MBF 0.1% w/v; HHMPP: photo-crosslinked hydrogel with HHMPP 0.05% w/v).

(21.O8) SELF-ASSEMBLED Β-SHEET PEPTIDE HYBRID POLY (γ-GLUTAMIC ACID) HYDROGELS

Clarke DE (1), Pashuck III TE (1), Gentilini C (1), Stevens MM (1)

1. Imperial College London

Introduction. Tissue engineering strategies frequently utilise a bio-mimetic scaffold carrier material such as a polymer- or peptide-based hydrogel (1). These materials are designed to provide mechanical support for cells and may be combined with bioactive moieties. However, disadvantages include the synthetic nature of some polymers and the fact that peptide hydrogels suffer from quick degradation rates and fail at strains exerted on them by cellular environments. Here we present a novel and alternative hybrid polymer-peptide gel system consisting of a Poly (γ-glutamic acid) (γ-PGA) polymer network physically cross-linked via grafted self-assembled β-sheet peptide sequences. This system provides a gel with predicted high failure strains, the ability to incorporate cells and biomolecules and also, being designed to gel through self-assembly, be used as an injectable system.

Material and Methods. γ-PGA was functionalised with a maleimide group, in the presence of Dicyclohexylcarbodiimide. β-sheet peptide sequences were synthesised using a manual Fmoc/tBu strategy on rink amide resin. Purity was confirmed by HPLC and Mass Spectroscopy. The peptide sequences were grafted to functionalised γ-PGA via cysteine-maleimide coupling and the degree of peptide conjugation estimated by 1H-NMR. The subsequent material was dissolved in deionised water and the solution pH increased by addition of NaOH, forming a gell.

Results/Conclusion. Naturally produced γ-PGA was successfully chemically modified and grafted with β-sheet peptide sequences. Using an induced β-sheet self-assembly mechanism, hybrid polymer-peptide β-sheet gels were formed (Figure 1). The gels can be manipulated with tweezers, suggesting that they are able to withstand significantly high strains for peptide-based hydrogels. Also, by varying the sequence of the peptide to influence its propensity to form β-sheets or changing the degree of peptide functionalisation, it will be possible to achieve a range of mechanical properties. Further characterisation is ongoing.

Reference: 1. Place, Evans, Stevens. Nature Materials 2009

Keywords. Hydrogel, β-sheet Peptide, Self-assembled, injectable, Poly (γ-Glutamic acid)

(21.O9) BIOCOMPATIBLE ALGINATE BASED HYDROGELS WITH ADJUSTABLE GELLING AND RESORBTION RATE

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1. NovaMatrix/FMC Biopolymer

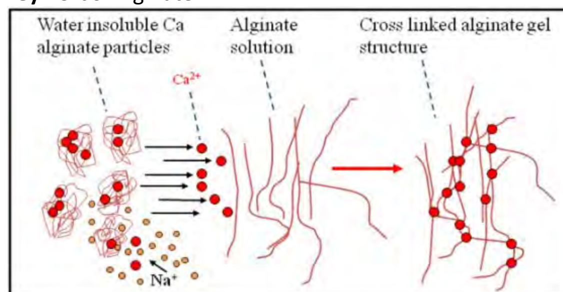
Introduction. Alginates are biocompatible polymers with the unique property to form gel structures under physiologic conditions. We have developed an alginate gelling system with the ability to be moulded in vitro or injected before gelling. This is obtained by mixing a sodium alginate solution with calcium alginate particles resulting in gel formation as a result of exchange of calcium ions between the insoluble and soluble alginate fraction. The gelling process is also accelerated by physiologic sodium levels. In order to control gelling rate and in vivo resorbtion time formulation factors like MW distribution, polymer sequence distribution (mannuronic and guluronic acid content) and degree of calcium cross-linking among others may be adjusted.

Materials and Methods. Selected calcium alginate particles and PRONOVA sodium alginate grades were mixed and gel structure build up was followed by using oscillation rheometry. In order to test gel resorbtion rate, different formulations were prepared and compared in an in vitro dissolution study. The gel system was also injected into rats in order to demonstrate biocompatibility and resorbability. Cell cultures were also entrapped within the gel to demonstrate viability.

Results. By mixing the two components gelling was initiated and the buildup could easily be followed on a rheometer. By changing the components and concentrations gelling could be carefully controlled. Avoiding sodium ions allowed long term delay before gelling occurred. Dissolution studies also demonstrated the resorbtion time could be greatly varied and this was also confirmed by animal testing.

Conclusion. Our data demonstrates that alginate formulations with a range of profiles may be successfully designed as injectable matrices for cells or other. Through formulation modifications the gel formation kinetics and resorbtion profile may be adapted for particular appications.

Keywords. Alginate



(21.O11) DOSE- AND TIME-DEPENDENT ANGIOGENESIS BY CONTROLLED DELIVERY OF MATRIX-BOUND VEGF

Sacchi V (1), Wolbank S (2), Martino M (3), Hofmann A (2), Hubbell J (3), Banfi Andrea (1)

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Introduction. Vascular Endothelial Growth Factor (VEGF) is a key factor to induce therapeutic angiogenesis. Using in vivo cell-based gene delivery we previously established that induction of normal and stable angiogenesis requires VEGF delivery at controlled and homogeneous microenvironmental levels for at least 4 weeks. Sustained release of matrix-bound factor potentially is a convenient and fully customizable approach for clinical translation of this biological concept. Therefore, here we aim to determine the requirements to induce normal and stable angiogenesis by controlled release of transglutaminase (TG)-bound VEGF from a fibrin hydrogel. Dose and duration are investigated based on these relationships:

Dose=concentration x degradation rate

Duration=volume x degradation rate

Methods/Results. As degradation rate plays a pivotal role in both processes, we first determined the relationship between different hydrogel compositions and their in vivo persistence. Fibrin gels degradation rate in vivo correlated mainly with fibrinogen concentration, rather than cross-linking enzymes. Surprisingly, we found that in vivo degradation rate was not directly, but inversely correlated with gel stiffness. In fact, keeping fibrinogen concentration constant and varying the cross-linking enzymes, the hardest gels were degraded the fastest (Fig. 1). We are testing the hypothesis that macrophages can activate their degrading activity as a function of substrate stiffness. Initial results suggest that this may explain the observed in vivo degradation behaviour.

We finally performed a pilot experiment to test if we can induce angiogenesis in vivo using the highest feasible VEGF dose (100 ug/ml) loaded onto a fibrin gel with the longest persistence. After 9 days this VEGF dose induced aberrant structures similar to small angiomas.

Conclusions. Matrix-bound VEGF can induce robust angiogenesis, even aberrant, in muscle tissue. The next steps will define the minimum VEGF dose necessary to obtain safe and stable vascular growth.

Acknowledgements. Support by the EU FP7 Project ANGIOSCAFF (CP-IP 214402).

Keywords. Fibrin hydrogel, VEGF, angiogenesis

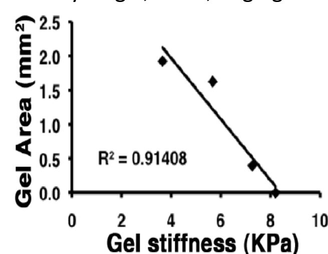

Fig. 1. Gel persistence 4 days after in vivo injection as a function of gel stiffness

(21.O12) IN-SITU CROSS-LINKED HYDROGEL FROM THERMORESPONSIVE PEG-BASED HYPERBRANCHED COPOLYMER

Dong Y (1), Zheng Y (1), Hassan W (1), Abu-Rub M (1), Pandit A (1), Wang W (1)

1. Network of Excellence for Functional Biomaterials, National University of Ireland, Galway, Ireland

Thermoresponsive gels are widely applied in tissue engineering as it can be easily injected directly into irregular cavities and hold shapes1. However, this physical thermal gel is mechanically too week for clinical applications. In this study, we reported a thermoresponsive PEG based hyperbranched copolymer which could in situ form a thermal gel at body temperature and cross-link with a nontoxic chemical cross-linker. Furthermore, by physically mixing the ECM materials into this gelation system, the cell attachment property and viability has been significantly enhanced.

A novel hyperbranched PEG based copolymers were prepared by copolymerisation of poly (ethylene glycol) methyl ether methacrylate (PEGMEMA) and 2-(2-methoxyethoxy) ethyl methacrylate (MEO2MA) with multifunctional vinyl monomer poly ethyl glycol diacrylate (PEGDA) as branching agent via in-situ deactivation enhanced ATRP. The LCST of these polymers were closed to body temperature characterized by UV-vis spectrophotometer (Fig. A). At room temperature, the polymers are soluble, while at body temperature they will form a physical gel. Moreover, the copolymer can be cross-linked with pentaerythritol tetrakis (3-

mercaptopropionate) (QT) less than 10 minutes at 37 oC (Fig. 1B). AlamarBlue® assessment with 3T3 fibroblast cells demonstrated that neither polymers nor the cross-linker are toxic even at a high concentration (Fig. 1C). Rabbit Adipose Derived Stem Cells (ADSCs) were seeded on the top-surface of the hydrogels for 48 h, and LIVE/DEAD® studies indicated that the addition of hyaluronic acid (HA) obviously enhanced the cell attachment and improved the cell growth on the hydrogels (Fig. 1D/E).

References: 1. Kavanagh CA et al., Pharmacol. Ther. 102:1, 2004

Acknowledgments: DEBRA International - funded by Debra Austria; Health Research Board (HRA/2009/121), College of Engineering and informatics Research Fellowship, NUIG, and Science Foundation Ireland Engineering Lectureship (07/EN/E015A) are acknowledged for funding.

Keywords. Injectable Scaffold;Thermoresponsive; Chemical cross-linking; PEG-based

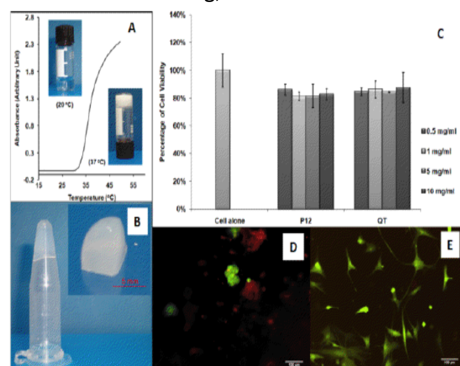

Fig. 1 A: Thermoresponsive properties of PEGMEMA-MEO$_2$MA-PEGDA. B: Chemical gelation. C: AlamarBlue® cell metabolism assay with 3T3 cells. D-E: LIVE/DEAD® viability assessment for ADSCs cell seeding on the surface of chemical cross-linked hydrogels with (E) or without (D) addition of hyaluronic acid.

(21.O13) INJECTABLE POLOXAMINE-BASED GELS FOR SUSTAINED DELIVERY OF rhBMP-2

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1. Departamento Farmacia y Tecnología Farmacéutica. Universidad de Santiago de Compostela; 2. Instituto de Ortopedia y Tejidos Musculoesqueléticos. Universidad de Santiago de Compostela

Local application of bone growth factors using minimally invasive in situ gelling solutions is an efficient and patient-friendly alternative to bone grafts. Poloxamines, i.e., X-shaped poly (ethylene oxide)-poly(propylene oxide) block copolymers with an ethylenediamine core (commercially available as Tetronic®), can provide solutions that undergo sol to gel transitions as temperature becomes closer to 37ºC. Hyaluronic acid (HA) is a natural polysaccharide and one of the main components of extracellular matrix that is found in all connective tissues of the body. Heparin (HEP) is a highly sulfated glycosaminoglycan present in the extracellular matrix and can establish for it specific interactions with some growth factors. The aim of this work was to evaluate Tetronic-HA and Tetronic-HEP gels as suitable vehicles for BMP-2. Formulations containing Tetronic 908 (20%) with HA (1.5 and 1%) and HEP (1.5%) in phosphate buffer pH 7.4 were rheologically characterized. Citocompatibility was tested against BALB/3T3 mouse fibroblasts cell line. Profiles of BSA and BMP-2 release were recorded. Cell viability in presence of the all formulations was higher than 90% at 1 and 3 days. T908-HA formulations exhibited elastic modulus (G´) at room temperature. Above this temperature, G´ and G" increased less than formulations with Tetronic alone. BSA release from T908-HA and T908-HEP showed a burst much less pronounced than T908 alone. BMP-2 release profiles resembled to some extent those of BSA showing a release profile for two weeks. In conclusion, poloxamines are pointed out as a promising material for syringeable in situ gelling systems. Combination with other polymers can serve to tune their properties in order to optimize their performance as carriers of BMP-2.

Keywords. poloxamine, in situ gelling, PEO-PPO block copolymers, BMP-2

(21.O14) CIRCULATING PROGENITOR CELL VIABILITY AND COLLAGEN HYDROGEL PROPERTIES ARE DEPENDENT ON THE INTERACTIONS BETWEEN THE CELLS AND THE BIOMATERIAL

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Introduction. Injectable hydrogels are attractive for biomedical applications to deliver therapeutic cells in a minimally invasive manner. This study investigates interactions between collagen-based hydrogels and different additives (cross-linkers, cells and microspheres).

Materials and Methods. Type-I collagen (1%) was cross-linked with a 1:1 molar ratio of EDC/NHS (6.5, 13, and 26mM). Human circulating progenitor cells (CPCs) were incorporated into hydrogels either in PBS or glycine prior to thermogelation (cell densities of 2.5×10^5, 5.0×10^5, and 1.0×10^6 per 500uL of matrix were tested). Live/dead staining was used to assess 24-hour CPC viability. Rheological properties of matrices with varying cross-linker concentrations, cell densities and with alginate microspheres were measured using a Brookfield R/S-Plus Rheometer.

Results. EDC/NHS induced cell death in a dose-dependent manner, and cell density had no effect on preventing cell death. However, the use of glycine improved CPC viability compared to addition of cells in PBS (74.4 and 44.9% respectively; $p=0.03$). Glycine viability did not differ from baseline (91.8%; $p=0.2$). Doubling the EDC/NHS concentration resulted in a 0.7-fold reduction in gelation time ($p=0.004$), while halving it resulted in a 1.8-fold increase ($p<0.0001$). Maximum viscosity increased with increasing EDC/NHS concentration. The addition of 1.0×10^6 cells reduced gelation time by 22.6% and 18.2% compared to the matrix without cells ($p=0.001$) and 2.5×10^5 cells ($p=0.01$). Maximum viscosity for matrices with cells was significantly greater than matrices without cells ($p\leq0.03$). The addition of microspheres to the matrix reduced gelation time by 10% ($p=0.03$) and increase maximum viscosity by 23% ($p=0.02$).

Conclusions. Cell-material and microsphere-material interactions should be taken into consideration for

development of delivery scaffolds. These interactions can affect the physical characteristics of the material and the function of the cellular components. A better understanding of how materials and cells (and other additives) respond to each other will help towards the goal of improving scaffolds for regenerative therapy.
Keywords. Injectable hydrogel, circulating progenitor cells, viability, microspheres, rheology

(21.P1) CHITOSAN-ALGINATE INJECTABLE SYSTEM IN PERIODONTAL AND PERI-IMPLANT BONE REGENERATION. IN VIVO STUDIES ON RAT MODEL

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1. Biomedical Engineering Laboratory, Faculty of Chemical and Process Engineering, Warsaw University of Technology; 2. Medical Devices Department, National Medicines Institute; 3. Department of Pathology, Medical University of Warsaw

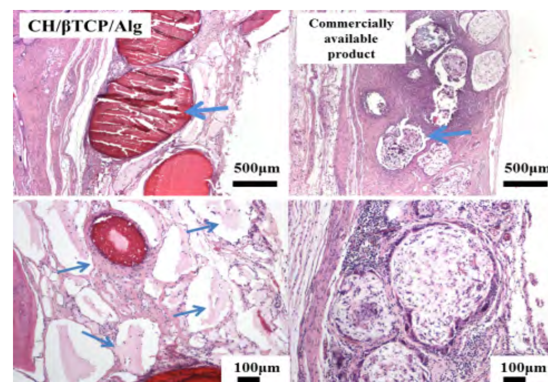
Introduction. The lack of bone in the alveolar ridge is a great challenge for the stomathognatic system rehabilitation success. Its reconstruction allows titanium implants instalation and rehabilitation of chewing and face esthtetics. Recently much attention has been paid to alloplastic osteoconductive materials which provide an alternative to autologous and xenogenic bone grafts. The non-immunogenic and resorabable materials may provide the basis for complete, predictable and reproducible bone regeneration. The aim of this study was fabrication of biodegradable, injectable biomaterial acting as a bone substitute supporting new tissue ingrowth.

Material and Methods. The injectable system was obtained by agglomeration of chitosan/βTCP microspheres and relied on alginate gel formation in presence of calcium ions released from polymer granules. The system consisted of two phases: solid – CH/ βTCP particles and liquid one acting as a microsphere carrier which tends to gelling. The material was tested according to ISO Standard 10993 - Biological Evaluation of Medical Devices. Both fabricated material and commercially available product were implanted into experimentally created critical-size (7 mm) osteochondral defects in rats skull. There were 20 rats subjected to implantation. Histological evaluation after the surgery was conducted after 4 and 12 weeks.

Results and Discussion. Studies concerning material degradability showed that biodegradation of alginate was rather rapid and after two weeks gel-phase was established as degraded, while solid one after 3 months did not show significant weight loss. The chitosan-alginate material did not reveal cytotoxic effects. Studies aimed at evaluation of irritation and delayed-type hypersensitivity, systemic toxicity and genotoxicity were negative. The histological observations of implanted materials are shown in Fig. 1.

Conclusions. It was found that the experimental injectable biomaterial allows to observe newly formed bone tissue after a month from implantation. Commercially available biomaterial caused inflammatory response and did not show osteogenic effect through the same period of time.

Keywords. chitosan, alginate, guided bone regeneration, in vivo, rat model



(21.P2) 3D OSTEOGENIC DIFFERENTIATION OF ADIPOSE-DERIVED STEM CELLS IN A THERMOSENSITIVE POLY-CAPROLACTONE SCAFFOLD

Hruschka V (1), al Ghanami RC (2), Wolbank S (1), Redl H (1), Shakesheff K (2), van Griensven M (1), Nürnberger S (1), Meinl A (1)
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In tissue engineering (TE), the natural process of regeneration is imitated by using bioresorbable scaffold matrices that support cellular attachment, migration and proliferation, along with cells capable of differentiation and upon exposure to inductive factors. One class of bioresorbable matrices are hydrogels, which are widely used in TE applications because of their three-dimensional network, their tissue-like water content, structure stability, and biocompatibility to homogeneously encapsulate cells. Based on the idea of combining a fully degradable polymer (Poly(ε-caprolactone); PCL) with a thermoresponsive polymer (polyethylene glycol methacrylate (polyPEGMA)) a scaffold was developed which liquefies at 4°C degrees and solidifies at body temperature. Adipose-derived stem cells (ASCs) can be used for TE in an autologous way and differentiate into various lineages, such as adipocytes, chondrocytes and osteocytes. In this project, we aimed to combine the novel thermoresponsive material with human ASC to generate an osteogenic TE construct, which can be expanded under 3D conditions in vitro. For this purpose, we optimized biomaterial seeding for primary ASCs and characterized their attachment, survival, distribution and persistence within the 3D material using viability assays and fluorescent imaging. The constructs were cultured in media supporting the expansion or osteogenic differentiation of ASCs for up to 4 weeks. Mineralization of 3D cultures was examined non-invasively using the fluorescent dye Xylenol Orange and confirmed by staining of histological cryosections for osteogenic markers. Human ASCs attached to the polymers and were found viable and evenly distributed in all scaffolds. Coating of the 3D material using gelatine or FBS did not improve cellular attachment, coating with poly-L-lysine even deteriorated cellular viability. In summary, the thermoresponsive scaffold presented in this study was able to support the osteogenic differentiation of mesenchymal stem cells.

Keywords. Adipose-derived stem cells (ASCs), Poly(ε-caprolactone) (PCL), Osteogenic differentiation

(21.P3) INJECTABLE HYALURONAN-BASED HYDROGELS WITH CONTROLLABLE ADHESIVE AND MECHANICAL PROPERTIES

Rojas R (1), Ossipov D (1), Hilborn J (1)
1. Uppsala University, Sweden

Introduction. Synthetic hyaluronan-based scaffolds have been designed and prepared in order to study the synergistic effects of chemistry (composition), physical properties (stiffness), and biology (adhesion) when in contact with cells or tissue. By creating a tunable library of injectable hydrogels, we can study systematically their properties use them in a wide range of applications.

Methods. Hyaluronic acid (HYA) was functionalized with a carbazate, hydrazide, aldehyde and/or thiol functional groups through chemoselective reactions and peptide-coupling techniques. Mechanical properties were tested by rheology and compression assays. Experiments to determine the cell- or tissue-material interactions were performed.

Results. Novel chemoselective reactions in the form of protected nucleophilic groups were performed to obtain HYA-carbazate or hydrazide components as well as dual-functionlized components with thiol groups. A new approach to functionalize HYA with aldehydes was used in order to prevent bioactivity loss and changes in the backbone of HYA. These components could be handled as solutions and mixed with interconnected syringes to obtain hydrogels. Rheological properties showed a high control over the mechanical properties over a range of 200 to 2 000 Pa. Different curing showed little influence in their mechanical properties. Initial testing of spinal cord sections of mice pups on these hydrogels suggests their use as a neuroprotective materials by their preservation of motor neurons compared to collagen hydrogels and cell culture membranes.

Conclusions. Controlled functionalization of HYA by using chemoselective groups was performed. Injectable hydrogels with high elasticity comparable to soft tissue were created and characterized. Crosslinking reactons were dependent on the concentration of the components. Preliminary tissue-material experiments suggest some members of this family of hydrogels as biocompatible and with potential use on neuronal tissue engineering applications.

Acknowledgements. We acknowledge the help of Nils Hailer and Nicos Schizas for the initial tissue-material testing. RR acknowledges the Swedish Institute for generous funding.

Keywords. Injectable scaffolds, hyaluronan, hydrogel, stiffness

22. INNOVATIONS IN STEM CELL-BASED CARDIAC TISSUE ENGINEERING

Chair: Nenad Bursac
Co-chair: Lauren Black
Keynote speaker: Ina Gruh
Organizer: Lauren Black

Synopsis: Stem cell based therapies for the treatment of myocardial infarction and failure have become a mainstream experimental concept in cardiac research. While ongoing clinical studies with injected stem cells show promise, implantation of a tissue-engineered cardiac patch is expected to further enhance long-term survival of delivered cells and exert a more efficient structural and functional tissue reconstruction at the infarct site. Over the past decade, neonatal rat cardiomyocytes have been the only utilized cell source for cardiac tissue engineering. While use of these cells is limited to proof-of-concept *in vitro* and *in vivo* studies, the use of stem cells for cardiac tissue engineering offers a potential for clinical translation. However, the field of stem cell-based cardiac tissue engineering is still in its infancy and awaits the development of robust and reproducible approaches for non-tumorigenic and non-arrhythmogenic functional cardiac repair. The aim of this symposium is to bring together leaders in the field of cardiac tissue engineering and cell and developmental biology with focus on the use of stem cells to create functional engineered myocardium. In particular, this symposium will center around recent innovative approaches to: 1) derive the optimal stem cell sources for cardiac tissue engineering, 2) promote *in vitro* functional cardiogenesis of stem cells by different biophysical and biochemical cues, 3) understand the structural and functional interactions between stem cell-derived and native cardiomyocytes, and 4) enhance the survival and functional integration of implanted stem cell-derived cardiac tissues *in vivo*. Overall, this symposium will review the contemporary efforts in the field of stem cell-based cardiac tissue engineering and emphasize potential strategies towards the future clinical use.

(22.KP) INDUCED PLURIPOTENT STEM CELLS FOR CARDIAC TISSUE ENGINEERING

Kensah G (1), Mauritz C (1), Schwanke K (1), Zweigerdt R (1), Schöler H (2), Martin U (1), Gruh I (1)
1. Leibniz Research Laboratories for Biotechnology and Artificial Organs LEBAO, Cluster of Excellence REBIRTH, Hannover Medical School, Hannover, Germany; 2. Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Muenster, Germany

Cardiac cells can be differentiated from stem cells, i.e. embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells. For future reconstructive therapies, contamination of stem cell-derived artificial cardiac tissue with undifferentiated cells bears the risk of tumor formation after transplantation. Therefore we implemented a cardiac-specific selection system. First, iPS cells derived from OG2 mice were used, expressing GFP under control of the Oct 3/4 promotor. Cells were transduced with a Zeocin resistance gene under control of the cardiac-specific alpha myosin heavy chain promoter. Integration was confirmed by RT-PCR. Cardiac differentiation was induced using the hanging drop method. Cardiac differentiation resulted in up to 100% beating EBs, with the onset of alpha MHC expression on day 6. Selection was performed starting on d8 - d14 of differentiation culture for 24 to 48 hours. Cells were analyzed by flow cytometry, immunofluorescence

staining and by semi-quantitative RT-PCR for pluripotency and cardiac markers. Efficiently enriched cardiomyocytes of up to 99% purity were used for the generation of bioartificial cardiac tissue which displayed spontaneous contractile activity and alignment of cardiomyocytes within the matrix. Moreover, selection of human iPS-derived cardiomyocytes was performed using a Neomycin resistance gene expressed under control of the alpha-MHC promoter. Human iPS-derived cardiomyocytes were used for the successful generation of bioartificial cardiac tissue. In conclusion, iPS cell-derived cardiomyocytes from mouse and human were efficiently enriched for cardiac tissue engineering purposes. Currently, the tissue mechanical properties (contraction frequency, forces, tissue stiffness) and factors influencing tissue formation and maturation are investigated using a multimodal bioreactor. This bioreactor combines mechanical stimulation and direct real-time contraction force measurement, electrical stimulation, perfusion of the culture chamber and the possibility of (fluorescence) microscopy during continuous cultivation. Thus, this bioreactor represents a valuable tool for the ongoing optimization of stem cell-based cardiac tissue engineering strategies for regenerative medicine.

Keywords. iPS cells, cardiac tissue engineering, bioreactos

(22.O1) QUANTITATIVE CELLULAR AND MOLECULAR SCREENING OF PLURIPOTENT STEM CELL DERIVATIVES IN AN ENGINEERED HEART TISSUE MODEL

Song H (1), Yoon C (1), Thravandiran N (1), Masse S (2), Rubart M (3), Zandstra P (1), Radisic M (1)

1. University of Toronto; 2. Toronto General Hospital; 3. Indiana University School of Medicine

Myocardium has no significant ability to regenerate and the viable tissue remaining after an injury such as myocardial infarction (MI) is often insufficient to maintain adequate cardiac output. Heart transplant is very often not an available or appropriate option. Thus there is a pressing need for alternative interventions. We are using engineered heart tissue (EHT) and quantitative molecular and electrophysiological analyses, as a test-bed to screen regenerative conditions for their potential to functionally integrate with the host tissue. EHT was prepared by seeding neonatal rat CM onto porous collagen scaffolds and subjecting the resulting constructs to electrical field stimulation (biphasic pulses, 1ms duration per phase, 1Hz) for 5 days as described. This platform was validated by injection of contractile mouse neonatal (n) CM, non-contractile cardiac FB and embryonic stem cells (ESC). As expected, FB injection interfered with electrical signal propagation, ESC formed teratoma-like structures uninfluenced in their differentiation trajectory by the in vitro cardiac microenvironment, and injected nCM improved tissue function. Interestingly ESC-derived CP (Flk+/PDGFRα+ cells), but not ESC-derived CM (generated using MHCα-driven antibiotic selection) appeared able to appropriately mature and integrate into EHT, enhancing the amplitude of tissue contraction, propagating electrical signals, supporting the viability of the host EHT and exhibiting Ca2+ transients in synchrony with the host EHT. Recently, using the EHT model system, we have began to quantitatively test the impact of in situ fibroblast-to-CM reprogramming using regulated expression of the cardiac transcription factors MEF2c, Gata4 and Tbx5. This reprograming strategy resulted in about 15 % cTnT + cells with upregulation of other cardiac genes such as Mesp1. In addition, to accelerate the screening efficiency in reprogramming, we engaged a self-organized micro-tissue platform using ESC- derived CM. These cardiac model systems are powerful tools to rapidly and qualitatively screen cellular and molecular based strategies in cardiac environment**.**

Keywords. Engineered heart tissue, Cardiac stem cell

(22.O2) TISSUE PRINTING OF CARDIAC PROGENITOR CELLS ALLOWS IN VITRO CREATION OF AN ORGANIZED CARDIOGENIC TISSUE

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1. Department of Cardiology, University Medical Center Utrecht, Utrecht, NL; 2. Deptartment of Orthopaedics, University Medical Center Utrecht, Utrecht, NL; 3. Department of Molecular Medicine, University of Rome Sapienza, Rome, IT

Introduction. Tissue engineering is emerging as a therapeutic approach to overcome the limitations of cell therapy as well as to mechanically support the ventricular wall and prevent dilation. Tissue printing technology (TP) offers the possibility to deliver, in a defined and organized manner, scaffolding materials and living cells.

The aim of our study was to evaluate the combination of tissue printing technology, cardiac-derived cardiomyocyte progenitor cells (CMPCs) and biomaterials to obtain a cardiogenic construct for in vitro or in vivo studies.

Methods/Results. Sca-1+-CMPCs were isolated and cultured as previously described. Cells were mixed in 7,5% alginate (20x106/ml) and printed with a pressure of 3-3,5 Bars. The formed biocomplex is made of 6 perpendicularly printed layers (strand distance of 2,5mm) on top of each other for a final structure of 2cmx2cm and 600µm thick. Cell-viability was not affected by the printing process. . After 1 week in culture, CMPCs retain expression of Nkx2.5, Gata-4, Mef2c, TnI, MLC2a, VE-Cadh, CD31 and VEGF-R2, as evaluated by PCR analysis and immunofluorescence, indicating CMPCs in the matrix did not influence CMPCs phenotype. Printed CMPCs can migrate out of the matrix when the bio-complesx is placed on top of a matrigel layer, and still formed tubular-like structures. The biocomplex was applied on the heart and the presence of cells was further confirmed by immunofluorescence analysis.

Conclusion. For the first time, we combined TP with human cardiomyocyte progenitor cells and developed a cardiovascular tissue for in vitro studies or as a new therapeutic tool to repair the damaged heart.

Keywords. Cardiac Tissue engineering; Cardiac Progenitor cells; Tissue Printing; Cardiac regeneration

(22.O3) GENERATION OF AN ENHANCED ANGIOGENIC CELL POPULATION FOR CELL TRANSPLANTATION IN ISCHEMIC DISEASE

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1. University of Ottawa Heart Institute

Introduction. Transplantation of circulating progenitor cells (CPCs) in cell therapy has the potential to restore function to ischemic tissue, mediated by neovascularization. Herein, we present novel findings that demonstrate how the therapeutic potential of CPCs may be enhanced by pre-conditioning in a 3D culture system.

Materials and Methods. Collagen matrix was prepared by cross-linking collagen with chondroitin sulfate-C using glutaraldehyde. CPCs were isolated from human donors, and cultured on a 3D matrix or on fibronectin as control. Flow cytometry was performed for CPC phenotype analysis. Hindlimb ischemia was induced in nude mice, followed by transplantation of CPCs (raised on fibronectin or matrix), or PBS as a control. Limb perfusion was assessed over time using laser Doppler. On day 14, serum was collected for cytokine analysis to investigate the host paracrine response triggered by treatment.

Results. Compared to culture on fibronectin, CPCs on collagen matrix were enriched in the number of CPCs expressing hematopoietic stem cell markers CD133 and CD34 (by 4.2- and 2.6-fold, respectively) and endothelial markers CD144 and CD31 (by 3.2- and 2.3-fold, respectively), $P<0.05$ for all. After PBS treatment, hindlimb perfusion was further reduced by 29% at day 4 ($P=0.05$), whereas matrix-cultured cell-injected mice showed a 51% increase that was sustained until day 14, and constituted a 42% increase vs. fibronectin ($P=0.02$). Circulating pro-angiogenic cytokines SDF-1 (2.6-fold; $P=0.03$), G-CSF (2.9-fold; $P=0.02$), VEGF (1.8-fold; $P=0.04$) and SCF (1.8-fold; $P=0.03$) were elevated, in the matrix-cultured CPC group compared to PBS.

Conclusion. The expansion of CPCs on collagen matrices is more effective for generating therapeutically relevant cell populations. Ultimately, matrix-culture may offer a novel strategy to improve CPC therapy for ischemic diseases.

Keywords. stem cell transplantation, biological scaffolds, angiogenesis, cell culture

(22.O4) THE CAPACITY OF THE EXTRACELLULAR MATRIX FOR ENHANCING CARDIAC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Many novel techniques to repair cardiac damage involve the use of the patient's own stem cells (e.g. from bone marrow, muscle or heart) but often result in limited cell implantation and inappropriate cell functionality. One innovative strategy for improving differentiation of stem cells to cardiac cells is to seed stem cells on matrices that mimic the properties of the developing or maturing heart. Recent research by Engler et al showed that merely changing substrate stiffness altered the lineage commitment of mesenchymal stem cells (MSCs). Others have shown that adhesion, proliferation and cardiac differentiation of MSCs is enhanced in the presence of Collagen V but not Collagen I, resulting from the activation of specific integrin-mediated pathways. To date no one has fully investigated the capacity of native heart-specific extracellular matrix (ECM) to aid in the differentiation of MSCs to cardiac cells. Further, there have been limited studies evaluating the cooperation (or competition) between substrate stiffness and composition within the framework of integrin-mediated pathway signaling. The goal of this work was to assess the effects of ECM composition and substrate stiffness on the differentiation of MSCs to cardiomyocytes. Native ECM was isolated from different developmental time points in the rat life cycle (neonatal to adult) via perfusion decellularization of the heart. The ECM was processed, solubilized and then adsorbed onto tissue culture polystyrene (TCPS). Preliminary results indicate that plating cells on native heart ECM enhances early cardiac differentiation compared to Collagen I coating or TCPS in the absence of traditional soluble factors to promote cardiac differentiation, such as 5-azacytidine. Ongoing experiments will determine which integrin-mediated pathways are involved in the improved differentiation. Future experiments include culturing MSCs on polyacrylamide gels with varying stiffnesses and solubilized ECM to assess the combined effects of ECM composition and stiffness on MSC differentiation to cardiac cells.

Keywords. Extracellular Matrix, Integrins, Decellularization

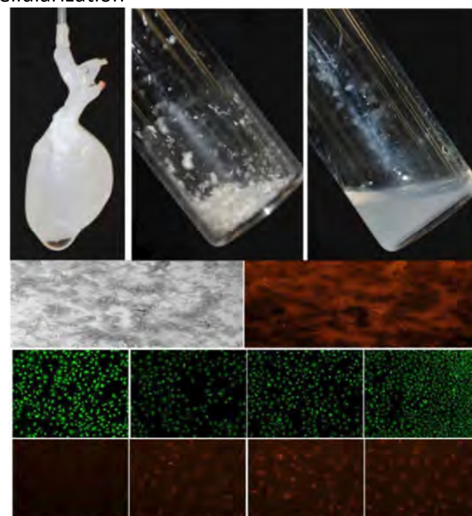

Figure 1: Top Row, left to right: A decellularized rat heart, the lyophilized powdered ECM, and the solubilized ECM after pepsin digestion. Row 2: 1 mg/ml solubilized ECM adsorbed onto a tissue culture plate in brightfield (left) and fluorescence (right). Row 3: Live dead stains for native myocardial cells on (from left to right) tissue culture plastic, adsorbed type I collagen (1 mg/ml), adsorbed solubilized heart ECM at 1 mg/ml and adsorbed solubilized heart ECM at 5 mg/ml. Bottom Row: Stain for early marker of cardiac differentiation Nkx2.5 (from left to right) tissue culture plastic, adsorbed type I collagen (1 mg/ml), adsorbed solubilized heart ECM at 1 mg/ml and adsorbed solubilized heart ECM at 5 mg/ml. Note that 1 mg/ml ECM appears to have the greatest cardiac differentiation signal

(22.O5) ELECTROMECHANICALLY FUNCTIONAL MYOCARDIUM FROM MOUSE EMBRYONIC STEM CELLS

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Surgical implantation of a stem-cell derived myocardial tissue patch is expected to yield improved cell survival and more efficient structural and functional repair of the injured myocardium. In this presentation, I will describe a novel and reproducible fibrin gel-based tissue-engineering approach to generate highly functional, 3-dimensional cardiac tissues consisting of highly aligned, differentiated and electromechanically coupled mouse embryonic stem cell-derived cardiomyocytes. Specifically, I will demonstrate that while genetically purified, α-MHC

expressing stem cell-derived cardiomyocytes require the presence of non-myocytes to form a functional 3-dimensional cardiac syncytium, purified tripotent cardiovascular progenitors expressing Nkx2.5 enhancer element represent a self-sufficient cell source capable of forming a functional cardiac tissue. After total differentiation and culture time of 21 days, localized electrical stimulation in these engineered tissues induces a rapid, uniform spread of action potentials with conduction velocities of up to 25 cm/s and resulting forces of contraction of up to 2 mN. These unprecedented levels of cardiac function are similar to those reported for neonatal mouse heart and for engineered cardiac tissues made of primary neonatal rat ventricular cells. Together, this work for the first time demonstrates successful in vitro generation of highly functional cardiac tissues derived entirely from a single, well-defined embryonic stem cell source.

(22.O6) HUMAN ENDOMYOCARDIAL BIOPSY DERIVED ADHERENT PROLIFERATING CELLS FOR CARDIAC TISSUE ENGINEERING - IN VITRO AND IN VIVO DATA

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I**ntroduction.** Cell therapy is a treatment option for cardiovascular diseases. Recently, we identified a novel cell type from endomyocardial biopsies: cardiac-derived adherent proliferation cells (CAPs). Here, we investigated their growth- and differentiation potential, surface marker-, gene- and secretion profile and immune attributes. In mice, we tested whether CAPs could improve myocarditis.

Materials and Methods. CAPs were isolated by outgrowth-method and tested for angiogenic and cardiomyogenic differentiation. Surface marker-, gene- and secretion profiles were shown using FACS, GeneChips and protein arrays. Then, CAPs were screened in a CFDA-SE proliferation assay in co-cultures with PHA-stimulated PBMC or mixed lymphocyte cultures (MLC). Pro- and anti-inflammatory cytokines and also the capacity of CAPs to induce regulatory T cell generation were measured. The therapeutic effect of CAPs was investigated in an acute mice model of Coxsackievirus-B3 induced myocarditis.

Results. CAPs could be efficiently isolated. FACS- and GeneChip analysis and comparison with reference cell types revealed a unique identity. They improved angiogenesis in HUVEC-assays, but showed no angiogenic or myogenic differentiation. In co-culture with allogeneic PBMC, CAPs showed low immunogenicity, and significantly reduced the induction of immune cell proliferation in PHA stimulated PBMC. Interaction of CAPs with PBMC resulted in elevated proportions of FOXP3(+)CD4(+)CD25(high+) cells. Furthermore, they induced no change in the normal alloantigen-driven immune responsiveness in MLC, and adding CAPs into MLC or PHA stimulated cultures resulted in highly reduced IFN-gamma and TNF-alpha levels. Co-culture with CVB3-infected HL1 cardiomyocytes led to a reduction of CVB3-induced HL1 apoptosis and viral progeny release. In the acute model of CVB3-induced myocarditis, CAPs reduced cardiac apoptosis and CVB3 viral load, and improved left ventricular function.

Conclusions. CAPs represent a unique cardiac-derived cell type promising for cardiovascular tissue engineering. They have low immunogenicity, reduce inflammatory cytokines and induce regulatory T cells. In mice, they improve acute CVB3-induced myocarditis.

Keywords. Cardiac tissue engineering, CAP cells, immunogenicity, in vivo, myocarditis

(22.O7) 3D VISUALIZATION OF UNLABELED TRANSPLANTED STEM CELL IN INFARCTED RAT HEART BY HIGH-RESOLUTION X-RAY MICROTOMOGRAPHY

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X-Ray microtomography (microCT) has been successfully used in order to provide information about bone, lung, kidney and biomaterial structure. However, some concerns persist in the scientific community for its in-vivo and ex-vivo applicability in longitudinal imaging of cell fate. Specifically, the possibility to study remote time points after cell injection seems to be limited by the lack of information on important variables such as cell proliferation and cell death. This appears to be due to the need of cell labeling using iron oxide nanoparticles that may accumulate in macrophages after death of delivered cells, and hence their signals do not precisely reflect stem cell fate (Terrovitis et al, Circulation 2008; Higuchi et al, J Nucl Med 2009). The present study shows that X-ray computed microCT in ex-vivo conditions may offer the unique possibility to detect, in the infarcted rat heart one week after injection, the 3D spatial distribution of Stem Cells, without the use of any contrast agent. Contrast between myocardium and injected stem cells relies on the presence of phase contrast and not on iron oxide nanoparticles as employed in several microCT studies performed in absorption conditions. The obtained 3D images represent a very innovative result because they definitively avoid the possibility that bright spots in 3D microCT images represent macrophages that have phagocytized iron nanoparticles, or particles that have been expelled from injected cells into the interstitium. This work provides evidence that the Authors' conclusions (Giuliani et al. , Journal of Tissue Engineering and Regenerative Medicine, in print) on cell migration away from the infarcted area are not speculative.Finally, observations made by the present investigation strongly support the contention that phase-contrast microCT represents a new 3D-imaging way to investigate the cellular events involved in cardiac regeneration and represents a promising tool for other applications in regenerative medicine.

Keywords. Phase-Contrast X-Ray MicroCT; Stem Cells; Differentiation; Synchrotron Radiation

(22.O8) CONTROLLED VEGF EXPRESSION IN A CARDIAC PATCH IMPROVES VASCULARIZATION AND CARDIAC FUNCTION FOLLOWING MYOCARDIAL INFARCTION

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Introduction. Vascularization of engineered cardiac grafts remains a critical issue for treatment of cardiac ischemia. In this study we investigated a cardiac patch generated from rat cardiomyocytes (RCM) and skeletal myoblasts (SM) expressing a specific, safe and effective VEGF level in a rodent model of cardiac ishemia. Our goal was to use controlled levels of VEGF released by retrovirally transduced SM cells to induce the formation of a stable and functional vascular network and thereby support patch survival and function.

Materials and Methods. SM were transduced with retroviral vectors expressing either mouse VEGF164 linked to a truncated version of the mouse CD8, or CD8 alone as a control. SM expressing a specific level of VEGF were then FACS-purified (Misteli, Stem Cells, 2010). Nude mice underwent myocardial infarction by ligation of the left anterior descending coronary artery. After 1 week, heart failure was confirmed by echocardiography (ECHO) and mice were implanted with patches made of polyglycerol sebacate (PGS) scaffold seeded with cells and cultured in a perfusion bioreactor. Four groups were tested: (i) cell-free scaffolds (PGS), (ii) RCM, (iii) RCM+CD8-expressing SM (CD8) and (iv) RCM+VEGF-expressing SM (VEGF). Cardiac function was assessed 4 weeks post-implantation by ECHO and end-point histological assays.

Results. High-resolution ECHO showed that only the treatment with VEGF-expressing patches significantly improved cardiac function (Fig.1A). Consistently, the RCM and SM survived only in the VEGF group, leading to a robust generation of differentiated tissue that was integrated with the underlying myocardium. Efficient normal angiogenesis was induced only by VEGF-expressing cells, both inside the patch and, interestingly, also in the underlying myocardium (Fig.1B-C).

Conclusions. Controlled VEGF expression drives effective vascularization of both the engineered patch and myocardium, leading to implanted cell survival and differentiation, and improved cardiac function.

Acknowledgments

This work was supported by the NIH-R21 grant HL089913 to GVN and AB.

Keywords. Cell and gene therapy, cardiac tissue engineering, vascularization, cardiac ischemic model

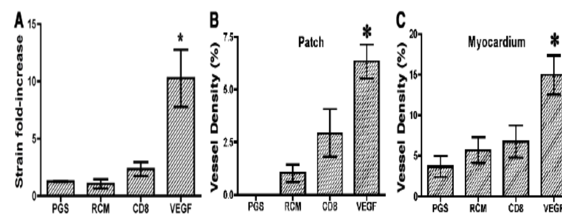

Figure 1. Cardiac function (A) and vascularization inside the patch (B) and in the underlying myocardium (C). * = p<0.01

(22.O9) EVALUATION OF A COLLAGEN MATRIX FOR GENERATION OF AN ANGIOGENIC POPULATION AND AS A VEHICLE FOR CIRCULATING PROGENITOR CELL DELIVERY IN A PORCINE MODEL OF MYOCARDIAL ISCHEMIA

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Introduction. Biomaterials with the ability to augment regenerative responses following an ischemic event are highly sought after. In this study, we examine the angiogenic potential of circulating progenitor cells (CPCs) grown on a collagen-based matrix, and test this matrix for CPC delivery in a porcine model of myocardial ischemia.

Materials and Methods. Human CPCs were isolated and cultured for 4 days on fibronectin or collagen/chondroitin sulfate blended matrices. CPCs were harvested and assessed for their adhesion and migration potential, and their ability to support tubule formation in an angiogenesis assay. Myocardial ischemia was induced in 23 pigs by ameroid constrictor placement around the left circumflex coronary artery. At week 3, animals were randomized to receive intramyocardial injection of PBS (control), CPCs, or CPCs+matrix. Animals were sacrificed at week 8. Left ventricular (LV) function (echocardiography), myocardial perfusion (rest & stress 13NH3-PET) and viability (18F-FDG-PET), and immunohistochemistry were used to assess revascularisation and heart function.

Results. Adhesion of matrix-grown cells was 4-fold greater than fibronectin-grown cells. Matrix-raised CPCs also demonstrated increased migration (by 40%) into a 3D-polymer, augmented total length of capillary-like networks (by 18%), and a 1.9-fold increase in incorporation into tubules in vitro. Randomization is ongoing for 23 animals and groups will be unblinded in April 2011. A correlation between increased myocardial blood flow at stress, increased LV function and positive smooth muscle actin staining for arterioles was observed. Improvement in LV function, myocardial perfusion, myocardial viability, and vascular density is expected to be the greatest in the CPCs+matrix group, mediated mainly through paracrine effects.

Conclusion. Our collagen matrix was successful in generating a more potent angiogenic cell population. Observing increased benefits of cell+matrix transplantation in a relevant pre-clinical model of myocardial ischemia would be a promising step towards global improvement of regenerative angiogenic therapies.

Keywords. Circulating Progenitor Cells; Matrix; Myocardial ischemia; Swine; Revascularisation

(22.O10) THE EFFECTS OF THREE DIMENSIONAL CULTURE AND STRAIN ON THE BEHAVIOR OF CARDIOMYOCYTE PROGENITOR CELLS

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Introduction. Stem cell therapy has emerged as promising treatment for myocardial infarction. A population of stem cells that resides in the human heart (cardiomyocyte progenitor cells, CMPCs) can be isolated and differentiated into beating cardiomyocytes using biochemical factors in vitro [1]. Upon injection in the heart, these cells will experience a three dimensional (3D) rather than a 2D environment and are exposed to mechanical strain. We therefore investigate how these CMPCs behave in 3D strained environment.

Materials and Methods. Human fetal CMPCs were encapsulated in a) longitudinally constrained and b) free floating hydrogels composed of collagen-Matrigel (n=3 each) (figure 1A). Resulting constructs were cultured for 9 days and analyzed for compaction and cell survival, proliferation, alignment and cardiac differentiation using immunofluorescent stainings.

Results. Based on fluorescent microscopical examination of samples, cell survival was higher in constrained constructs compared to free floating constructs. Furthermore, cell proliferation was strongly decreased in constrained constructs, and completely absent in free floating constructs. Cells cultured in constrained constructs aligned in the direction of the constraint (figure 1Ba), probably as a result of internal stress formation as demonstrated by the compaction of the constructs (22%) and the formation of intracellular stress fibers (figure 1Bb). Moreover, cells cultured in constrained constructs showed expression of a-actinin and Nkx2.5 after 9 days of culture, while this expression was lost in free floating constructs (figure 1Bc).

Conclusion. CMPCs are able to survive, proliferate and keep their cardiac expression profile in 3D constrained hydrogel-based constructs. Moreover, CMPCs are able to align in the direction of the strain, which is of great importance for proper tissue integration in vivo.

References

[1] M.J. Goumans at al., TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro, Stem Cell Research, 1: 138-149, (2007).

Keywords. Cardiomyocyte progenitor cells, hydrogel, three dimensional, static strain

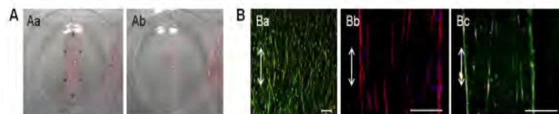

Figure 1: Panel A: Macroscopic images of CMPCs encapsulated in longitidinally constrained (Aa) and free floating hydrogels (Ab). Panel B: Immunofluorescent stainings of constrained constructs after 9 days of culture. Cell viability (Ba, green: living cells (cell tracker green) red: (remnants) of dead cells (propidium iodide)), stress fiber formation (Bb, red: phalloidin, blue: DAPI), and expression of cardiac markers (Bc, red: Nkx2.5, green: a-actinin, blue: DAPI). Ba: transversal section, Bb and Bc: longitudunal cross-sections. Bar = 100 µm, arrow denotes constraint direction.

(22.P1) LOCAL DELIVERY OF TRANSFORMING GROWTH FACTOR-β1 INDUCES IN SITU CARDIOMYOGENIC DIFFERENTIATION OF IMPLANTED BONE MARROW MONONUCLEAR CELLS

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Introduction. Bone marrow mononuclear cells (BMMNCs) can be used to treat patients with myocardial infarction, since BMMNCs can differentiate in vitro toward cardiomyogenic lineages when treated with transforming growth factor-β1 (TGF-β1). However, the in vitro cardiomyogenic differentiation culture process is costly and laborious, and the patients should wait during the culture period. In this study, we hypothesize that BMMNCs implanted in cardiomyogenically undifferentiated state to myocardial infarction site would differentiate cardiomyogenically in situ when exogenous TGF-β1 is delivered to the cell implantation site.

Materials and Methods. Heparin-conjugated poly(lactic-co-glycolic acid) nanospheres (HCPNs) suspended in fibrin gel were used as a TGF-β1 delivery system. BMMNCs were labeled with a green fluorescent dye (PKH-67) and implanted into the infarction border zone of rat myocardium using fibrin gel containing HCPNs and TGF-β1. BMMNC implantation using fibrin gel and HCPNs without TGF-β1 served as a control.

Results. TGF-β1 release from HCPNs was further sustained by suspending HCPNs in fibrin gel. TGF-β1 delivery using HCPNs suspended in fibrin gel also induced expression of the cardiomyogenic markers by rBMSCs, indicating that the released TGF-β1was bioactive. Four weeks after implantation, the expression of cardiomyogenic marker proteins by the implanted BMMNCs was dramatically greater in the TGF-β1 delivery group than in the control group. And myocardiac function was also improved in BMMNC+ TGF-β1 group.

Conclusion. In situ cardiomyogenic differentiation of BMMNCs implanted in an undifferentiated state to infracted myocardium was induced by local delivery of TGF-β1 to the cell implantation site, and improved cardiac functions.

Acknowledgements. This work was supported by the Korea Health 21 R&D Project, Ministry of Health, Welfare, and Family Affairs (grant number: A050082), the Stem Cell Research Center of the 21th Century Frontier Program (grant number: SC3220), and the National Research Foundation of Korea (grant number: 2010-0020352), Republic of Korea.

Keywords. Bone marrow mononuclear cells, Cardiomyogenic differentiation

(22.P2) CAN BONE MARROW MESENCHYMAL STEM CELLS REGENERATE THE MYOCARDIUM?

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Introduction. Bone marrow Mesenchymal Stem Cells (MSCs) are one of the current cell types being studied as a

source of factors to improve tissue repair. These cells may differentiate into many tissues types, including cardiomyocytes. The aim of this study was to create conditions of mimicking cardiomyoblasts environment.

Materials and methods. Phenotype of MSCs was confirmed by markers expression, colony forming efficiency assay, adipogenic and osteogenic assay. Conditioned media were prepared from cardiomyocyte cell line (H9C2) and by addition of 5'azacytidine to DMEM medium. Grade of MSC differentiation was assessed by morphological analysis and cell expression of characteristic markers by immunocytochemistry, immunofluorescence and flow cytometry.

Results. Conditioned media induced differentiation of MSC. After 16 days of incubation call morphology has been changed. Expression of α-smooth muscle actin, desmin, myogenin and sarcomeric actin were induced by conditioned media in comparison to the control (MSCs cultured alone). These alterations strongly indicate onset of the differentiation process to the myogenic lineage.

Conclusion. Our study shows that conditioned media which were used provide a convenient source of inductive signals to initiate differentiation of MSC towards cardiomyocytes-like phenotype. Nevertheless further studies are needed.

Keywords. Mesenchymal stem cells, conditioned media

(22.P3) THE COOPERATION OF BIOLOGICAL AND MECHANICAL SIGNALS DRIVES MURINE CARDIAC PROGENITOR CELL DIFFERENTIATION IN VITRO

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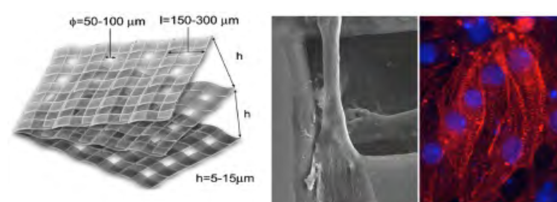
Stem cell niche has been defined as a critical microenvironment in which mechano-physical, biochemical and biological factors concur to preserve resident stem cells in their undifferentiated state. The exhaustive identification of such signals remains among the hot topics in stem cell biology. The generation of an artificial niche in vitro relies, in fact, on the definition of the most appropriate biocompatible, biodegradable scaffold to foster stem cell growth and differentiation in combination with specific biochemical factors. Indeed, stem cells can be pre-committed towards a defined phenotype matching physical and mechanical signals arising from the extracellular matrix. We show here that 3D scaffolds can enhance the cardiomyogenic potential of cardiac resident Sca-1+ progenitor cells. In particular, we demonstrate that Sca-1+ stem cell differentiation is achieved within a few days when a complex cardiogenic microenvironment is provided by coupling the biological factors arising from neonatal cardiomyocytes and 3D scaffolds having cardiac-like stiffness. Challenging the cardiac progenitors only with the appropriate tissue-specific scaffolds induced the expression of cardiomyocyte-specific proteins without the assembly of sarcomeres, while complete stem cell differentiation in co-culture with neonatal cardiomyocytes in conventional 2D conditions required a longer time. In conclusion, our study provides compelling evidence that cardiac progenitor fate can be tuned by a tight interplay between biological and physical factors and encourage in vivo studies to investigate the possibility of using 3D PLA scaffolds to fabricate pre-committed stem cell-derived cardiac patches.

Keywords. Cardiac stem cells, 3D scaffolds, cardiac regeneration



(22.P4) IMPLANTATION OF CELL-POLYMER BIOGRAFT FOR CARDIAC FUNCTION STABILISATION

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Introduction. Progress in tissue engineering for myocardial regeneration is conditioned by the creation of a suitable environment in which cells organize themselves into a functional tissue. We designed micro-fibrous scaffolds enriched with oxygen and investigated the hypothesis that epicardial implantation of bone marrow mesenchymal stem cells (MSCs) seeded on our oxygen coated Polycaprolactone (PCL) matrix would induce functional benefits.

Materials and Methods. Microfibrous PCL non-wovens were produced by electrospinning and surface-coated by an RF plasma process (CO_2/C_2H_4 gas). Bone marrow-derived MSC were characterised by FACS and 2 Mio cells were cultured on the 10x15 mm patches for 7-10 days. Cell mortality was assessed by LDH release, viability and morphology by MTT staining and SEM imaging respectively. Two weeks post LAD ligation, Lewis rats showing reduced ejection fraction (EF of 48 ± 8%) were randomized into 4 groups: MSC seeded patches glued onto the infarcted area with Tisseel fibrin glue (n=7), non-seeded patches (n=8), glue only (n=4) and sham operation (n=5). Treatments were applied via a second intercostal left thoracotomy. Echocardiography and pressure-volume loops were recorded after 28 days. Histological analyses are under investigation.

Results. CD90+, CD45- and CD31- MSC spread on the matrix into a monolayer prior to implantation. We showed that PCL patches allowed for a safe implantation without signs of rejection, encapsulation or inflammation. Patches were permanently glued onto the myocardium, no adhesion to other organs occurred. When compared to pre-treatment, MSC seeded patches induced a stabilisation of EF after 4 weeks (48±10% and 47±7% respectively). Non-seeded patches did not induce a stabilisation (EF of 46±8% and 39±4% respectively, p=0.02).

Conclusions. Our preliminary data demonstrate that epicardial implantaion of MSC-oxygen enriched PCL biografts is safe and reverses functional alterations observed in hearts after chronic myocardial infarction. Further anlyses will confirm eventual effect on myocardial regeneration.

Keywords. Myocardial infarction, heart function

(22.P5) INTRAMYOCARDIAL INJECTION OF MESENCHYMAL STEM CELLS LOADED WITHIN A SCAFFOLDING BIOMATERIAL IMPROVES CARDIAC FUNCTION AFTER ACUTE MYOCARDIAL INFARCTION

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Introduction. Efficacy of mesenchymal stem cell (MSC) myocardial transplantation after acute myocardial infarction (AMI) is limited by low MSC retention and survival after transplantation within the ischemic tissue. We hypothesized that the injection of MSCs within a biomaterial would improve efficacy of MSC transplantation. This study aimed at (1) assessing the in vitro cytocompatibility of this biomaterial, (2) evaluating the in-vivo effect on cardiac function after AMI of the intra-myocardial injection of this biomaterial loaded with MSCs.

Materials and Methods. The in vitro cytocompatibility was evaluated using cytotoxicity assays, measurements of cardiogenic marker gene expression and of contractile cell activity. Immediately after coronary artery ligation, 3.106 CFSE-labeled MSCs, alone (MSC) or in combination with the biomaterial (MSC+Bm), were injected into the myocardium of Lewis congenic rats. Injections of the biomaterial alone (Bm) or PBS were also used as controls. Cardiac function was assessed using echocardiography at different time-points over 8 weeks.

Results. Cell viability, expression of cardiogenic markers and synchronous contractile activity did not differ between cells (cardiomyocytes or MSCs) cultured alone or within biomaterial. Left ventricular Ejection Fraction (LVEF) was significantly reduced from 88.5±0.7% at baseline to 66.3±1.2% at 24h after surgery ($p<0.001$), with no statistical difference between groups. Whereas LVEF decreased from D1 (68.7±2.37) to D28 (51.9±4.14%) in the PBS group ($p<0.001$), LVEF significantly increased in the Bm (7.9%), MSC (9.6%), and MSC+Bm (9.5%) groups ($p<0.001$). Interestingly, 7 days after AMI, LVEF was higher in the MSC+Bm group (12.1%) as compared to the Bm (2.3%) or MSC (6.8%) groups ($p<0.01$).

Conclusions. These data showed that intra-myocardial injection of a scaffolding biomaterial loaded with MSCs induced an early improvement in cardiac function after AMI. This study suggests that the injection of MSCs within biomaterial is an effective strategy to enhance the effect of cellular transplantation therapy for myocardial infarction.

Keywords. Cell therapy, injectable scaffold matrix, cardiac repair

23. KOREAN-EUROPEAN SYMPOSIUM: BIOACTIVE SCAFFOLDS FOR TISSUE REGENERATION

Chair: Jin Ho Lee
Co-chair: Miguel Oliveira
Keynote speakers: Jin Ho Lee, Miguel Oliveira
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Synopsis: The importance of scaffolds must be emphasized for successful in vivo as well as in vitro tissue regeneration. Once implanted, an ideal scaffold should act not only, as a temporary substrate for cells but also, as appropriate mechanical, structural and biological environments in order to stimulate stem cells to differentiate and produce extracellular matrix. The bioactive scaffolds, which can be releasing bioactive molecules, including growth factors, cytokines and genetic material, have been focused on the current researches for target tissue regeneration. In this joint symposium between the Societies of Korean and European Tissue Engineering & Regenerative Medicine, current effort and recent advances in bioactive scaffolds for effective target tissue regeneration will be discussed. The future collaborations between the researchers from both societies will be also encouraged in this joint symposium.

(23.KP1) GROWTH FACTOR-IMMOBILIZED BIOACTIVE POROUS BEADS AS AN INJECTABLE URETHRAL BULKING AGENT

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Stress urinary incontinence, which occurs when the ordinary abdominal pressure exceeds urethral closing pressure, is associated with the lack of support of the urethra, so-called urethral hypermobility and/or intrinsic sphincter deficiency. The main idea for the treatment of stress urinary incontinence has been referred to increment of urethral resistance to intra-abdominal pressure by coaptation or narrowing of the urethral lumen. Injectable bulking agents, such as polytetrafluoroethylene particle, silicone particle, carbon particle, glutaraldehyde cross-linked collagen, and autologous fat, have been used for many years in the treatment of stress urinary incontinence. However, injection volume decrement over time caused by particle migration and resorption in the body has been considered as a main problem for the clinical applications. In this study, we prepared growth factor-immobilized porous polycaprolactone (PCL)/Pluronic F127 beads as an injectable bioactive bulking agent which can provide bulking effect and stimulate the defect tissues around urethra for the effective treatment of urinary incontinence. To this, the PCL/F127 porous beads were fabricated by combining an isolated particle-melting method (for nonporous bead formation) and the

following melt-molding particulate-leaching method (for porous bead formation). The Pluronic F127 chains exposed onto the porous PCL bead surfaces were used to bind heparin and the following growth factors (bFGF, VEGF or NGF) which may improve the sphincter muscle function around the urethra by the induction of smooth muscle, blood vessels and nerve. The morphology, growth factor release behaviors and model cell differentiation behaviors (using muscle-derived stem cells, MDSCs) of the growth factor-immobilized porous beads (in vitro), and the animal study (using an urinary incontinence rat model) to investigate the effectiveness of the porous beads as a bioactive bulking agent (in vivo) were conducted. This work was supported by the Pioneer Research Center Program through the National Research Foundation of Korea (Grant No. 2010-0002176).

Keywords. Porous beads, Growth factor, Urinary incontinence, Bulking agent

(23.KP2) DENDRIMER-BASED NANOPARTICLES FOR INTRACELLULAR DELIVERY OF DRUGS: APPLICATION IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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Among the myriad of possibilities in bone tissue engineering, the development novel strategies that can stimulate stem cell differentiation to become osteoblasts in the absence of typical osteogenic cocktails in vivo, it is still regarded as a very appealing challenge. Recently, our group has proposed a combinatorial strategy in tissue engineering principles employing carboxymethylchitosan/poly(amidoamine) dendrimer nanoparticles (CMCht/PAMAM) towards the intracellular release and regimented supply of dexamethasone (Dex) aimed at control the rat bone marrow stromal cells (RBMSCs) osteogenic differentiation, in vitro and in vivo. In this work deeper studies on the physicochemical properties and morphology of the nanoparticles were carried out. The effect of loading the nanoparticles with Dex on the viability of RBMSCs and internalization ability were also investigated. Results have revealed that Dex do not affects RBMSCs viability (ATP quantification assay), but decreases nanoparticles internalization (FACS analysis), in vitro. The osteogenic potential of Dex-loaded CMCht/PAMAM dendrimer nanoparticles and bone forming ability were also evaluated, in vitro and in vivo. 2D and 3D in vitro studies using RBMSCs cultures exposed to the Dex-loaded CMCht/PAMAM dendrimer nanoparticles were carried out. For in vivo studies, RBMSCs were incubated with Dex-loaded CMCht/PAMAM dendrimer nanoparticles during the expansion period and the cells were then seeded onto the surface of starch-polycaprolactone (SPCL) scaffolds, prior subcutaneous implantation into the back of Fischer 344 rats. This work demonstrated that cell number and ex vivo culturing strategies greatly influences the osteogenesis and de novo bone formation, in vivo. It also showed the superior performance of the dendron-like nanoparticles system for the intracellular delivery of dexamethasone as it stimulated RBMSCs to produce bone mineral within SPCL scaffolds, after 4 weeks of implantation. The use of nanoparticles system for the intracellular delivery of dexamethasone opens up a new regenerative possibility as it proved that allows controlling stem cells behaviour, in vitro and in vivo.

(23.O1) FUNCTIONALIZATION OF PLA FILMS USED IN TISSUE ENGINEERING BY INCORPORATION OF ANTIOXYDANTS: PHYSICAL PROPERTIES EFFECTS AND CELL CULTURE

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Polylactic acid (PLA) is a well-known biodegradable aliphatic polymer and has been previously used for several biomedical applications such as bone fixation devices (plates, pins, screws, etc.) and as tissue engineering scaffolds. However, for increasing the cells proliferation used in tissue engineering, four synthetic phenolic antioxidants (SPAs) including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) were added to poly (lactic acid) (PLA) film in an effort to create an active antioxidant film.

In the first time, we characterized the thermal properties by differential scanning calorimetry (DSC), mechanical properties by DMTA, surface analysis (contact angle and surface energy) by digidrop and structural properties by X-ray photoelectron spectroscopy (XPS) and fourier transform infrared (FTIR) and atomic force microscopy analysis (AFM). The addition of SPAs had not a significant effect on glass transition temperature (Tg), crystallization temperature (Tc), and melting temperature (Tm) of PLA films, but they reduced significantly the % crystallinity of PLA films. Also, the % crystallinity was calculated from FTIR. Decreasing the amount of crystallinity of PLA films containing 1% SPAs (w/w) caused the loss of some mechanical properties such as tensile modulus and Young's modulus. No significant changes were observed in the surface energy of PLA samples due to the addition of the antioxidants. Adding the SPAs didn't significantly change the hydrophilicity of PLA by introducing O-C and O-C=O groups onto the polymer surface. But their microstructures were significantly changed by addition SPAs, in term of roughness parameters (Ra and Rq). In a second time, we have investigated the biocompatibility and proliferation of human fibroblasts cell line (CRL-2703, ATCC, USA) on these films during 14 days. Biocompatibility was estimated by AlamarBlue tests at day 0, 3, 7 and 14, this results were compared with macrofluorescent images of nuclei stained by Hoechst 33258.

Keywords. Poly (lactic acid), physicochemical characterization, biocompatibility

(23.O2) DESIGNED HYBRID COMPOSITE SCAFFOLDS CONSISTED OF POLYCAPROLACTONE/TRICALCIUM PHOSPHATE AND ELECTROSPUN COLLAGEN

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One of the challenges in tissue engineering is to fabricate a reproducible three-dimensional (3D) scaffold to support cell attachment and proliferation. Solid free-form fabrication (SFF) technologies allow designing by computer both the microscopic and the macroscopic shape of scaffolds. The use of computer-based technology to easily fabricate the scaffolds for tissue engineering is advantageous because it facilitates the production of complex computer designed architectures. Unfortunately, the use of fabricated scaffolds, however, is challenging since applicable materials are limited to synthetic biopolymers, and the pore structure can be too large compared to various cells. Those provided low biophysical and biocompatible properties to the scaffold. To overcome these problems, we proposed a hybrid technology, which combines a melt-plotting system (one of SFFs) with electrospinning processes, to produce a hierarchical 3D structure consisting of micro-sized PCL/TCP strands and collagen nanofibers. To evaluate the efficiency of cell attachment, proliferation, and differentiation within the hierarchical scaffolds, we cultured osteoblasts (MG63) for regeneration of bone. The hierarchical scaffold exhibited various positive qualities. In particular, since the collagen is main component of ECM, the interactions between the cells and hierarchical scaffolds containing collagen were much more positive than those between the cells and conventional 3D PCL scaffolds. We believe that this hierarchical scaffold supplemented with the collagen nanofibers provides a good means of fabricating high-quality 3D scaffolds.

Keywords. PCL, TCP, Collagen, Scaffold, nanofiber

(23.O3) STRONTIUM SUBSTITUTED BIOACTIVE GLASSES DOWNREGULATE EXOGENOUS OSTEOPONTIN EXPRESSION

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Introduction. Bioactive glasses are biocompatible and have been used as bone graft substitute for decades. Their dissolution ions stimulate osteoblast recruitment and proliferation/differentiation in situ [1]. Strontium(Sr) stimulates osteoblast differentiation and downregulates osteoclast activity and has been used as a drug to treat osteoporosis in the form of strontium ranelate. Therefore we combined the osteoinductive properties of Sr with the osteoconductive properties of bioactive glasses and created a delivery system of strontium.

Materials&Methods. SiO_2-P_2O_5-Na_2O-CaO-SrO bioactive glass was substituted with 0, 10, 50 and 100 mol% of Ca with Sr and produced by a melt-quench route and referred to as SR0-SR100. Bioactive glass dissolution ion enriched media (BGD) is prepared as described before[2]. Osteopontin production by Saos-2 cells cultured with BGD media was measured from cell culture supernatants according to the manufacturer`s instructions (Quantikine, R&D Systems, UK). The absorbance was measured using SpectraMax® 250M5/M5e microplate spectrophotometer (Molecular Devices, USA).

Results& Discussion. In murine MCT3T-E1 osteoblastic cells, OPN is a negative regulator of proliferation and differentiation[3]. After four days in culture, cells treated with SR0 showed significantly higher exogenous osteopontin expression compared to control. At day 14 when cells start to mineralize, all groups expressed more osteopontin protein than before. However, the control group secreted significantly more OPN than all bioactive glass groups and Ca/Sr mixed groups like SR10 and SR50 expressed even less osteopontin.

Conclusion. Since Sr in bioactive glasses is known to down regulate bone resorption[4], inhibition of OPN deposition into ECM which facilitates osteoclast anchorage maybe one of the mechanisms by which Sr downregulates bone resorption.

Keywords. Bioactive glass, bone regeneration, strontium, osteopontin

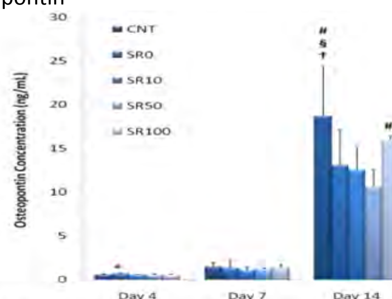

Figure 1. Osteopontin protein production by Saos-2 cells cultured with bioactive glass dissolution ion enriched culture media for 4, 7 and 14 days. * indicates $p<0.05$ compared to control. † vs. SR0, § vs. SR10 and # vs.SR50.

(23.P1) CHARACTERIZATIONS OF CHONDROITIN SULFATE-POLY(ETHYLENE OXIDE) HYDROGEL

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Introduction. Chondroitin sulfate (CS) is a anionic linear polysaccharide, consisting of alternating disaccharide repeating units of 1-3 linkage of D-glucuronic acid and N-acetylgalactosamine. It has been known to be a major component of ECM in many connective tissues. We synthesized and evaluated a CS- poly(ethylene oxide)(PEO) hydrogel for its applications in tissue engineering.

Methods. The CS-PEO hydrogel was fabricated by employing acrylated CS and thiolated PEO after modification of CS with EDC. The hydrogel was obtained by simply mixing the solutions of CS and PEO. While its chemical properties were evaluated by 1H-NMR and XPS, its physiological properties were done by rheologies, swelling and release behaviors. In vitro biocompatibility and cytotoxicity were evaluated by employing both MC3T3 cells and several biological assays.

Results. A degree of grafting of acrylates to CS was estimated to be 28% by calculating the ratio of the peak area in 1H-NMR spectra. The gelation was completed approximately within 2 min as judged by the results of rheology. While higher pHs of the medium induced higher swelling, higher concentration of the gel solution did less swelling. High cell adhesion and proliferation were observed on its surfaces and the cells inside the gel were

alive. The evaluation of cytotoxicity of the gel demonstrated similar to that of positive control Teflon. The BMP-2 and toluidine blue were release from the hydrogel for more than 5 days.

Conclusion.The CS-PEO hydrogel was fabricated in minutes via Michael type addition. The biological properties and cell cytotoxicity showed that the hydrogel have excellent biocompatibilities as biomaterials in tissue engineering. Release of BMP-2 from the gel induced better in vitro regeneration of bone tissues.

Acknowledgement. This study was supported by a grant of the Korea Healthcare Technology R&D project (A080864) from the Ministry for Health, Welfare and Family affairs, Republic of Korea.

Keywords. Chondroitin sulfate, hydrogel, poly(ethylene oxide)

(23.P2) EFFECT OF METHYLCELLULOSE ON GELATION AND DRUG RELEASE BEHAVIOUR OF SILK FIBROIN

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Hydrogel cross-linked by covalent bond or noncovalent bond is formed three-dimensional network structure and has a tendency to swell instead of dissolving in an aqueous solution. Hydrogel have been used for biomaterials such as contact lenses, wound dressing, artificial skin, drug delivery system, and biosensor. Silk is a fibrous protein polymer that are spun into fiber by silkworms and spiders. Silk fibroin is used for biomaterials, because they are impressive mechanical properties, biocompatibility and biodegradability. The formation of silk fibroin hydrogel can be adjusted by changing physical conditions, such as concentration of silk fibroin aqueous solution, temperature, pH and salts. Methylcellulose is a water-soluble polymer derived from cellulose by methylation. Methylcellulose is widely used as a binder or thickener in pharmaceutical, cosmetic, and food additives due to its unique temperature-sensitive characteristics.

In this study, we used methylcellulose to control the gelation time of silk fibroin aqueous solution. When the methylcellulose were added into the silk fibroin aqueous solution except the gelation time of silk fibroin aqueous solution was decreased. Fourier transformation infrared spectroscopy (FT-IR), scanning electronic microscope (SEM) were used to investigate the effect of methylcellulose on the hydrogelation of silk fibroin. The behavior of gelation depended on a species of added surfactant. 2- and 3-D drug release was studied using 5-aminosalicylicacid (5-ASA, mesalamine) in vitro.

Keywords. Silk fibroin, hydrogel, methylcellulose, gelation

(23.P3) ENDOTHELIALIZATION AND ANTI-PLATELET ACTIVITY OF CELL-DERIVED EXTRACELLULAR MATRIX

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In-stent restenosis and late thrombosis are representative failures of stent implantation. Previous studies have described that these are due to the hindrance of the re-endothelialization on the stent. In addition, polymers used in stent have been concerned about complications such as inflammatory response, thrombosis formation, and hypersensitivity. Unlike polymers lacking to biomimetic properties, cell-derived extracellular matrix (CDM) can be a favorable biomaterial for enhancing biocompatibility. Our hypothesis is that a native extracellular matrix (ECM) may provide a favorable microenvironment where can accelerate cellular adhesion and proliferation to endothelial cells (ECs). In this study, we have prepared the CDM from smooth muscle cells (SMCs) by decellularization method and investigated the effect on endothelialization and anti-platelet adhesion. SMCs-derived extracellular matrix (SDM) was obtained through a multi-step decellularization process from SMCs cultured on cover slip for 10 days. The SDM were characterized by immunofluorescent staining and contact angle measurement. Cell proliferations of ECs and SMCs seeded on the SDM was measured by cell counting kit (CCK-8), and the morphologies of cell and platelet were also observed by field emission scanning electron microscope (FE-SEM). The number of adhered platelet was measured by lactate dehydrogenase (LDH) kit. Based on immunofluorescent staining, SDM was successfully prepared and fibronectin was a major component in the SDM. Contact angle of SDM was higher than those of gelatin and cover slip. This difference may come from the compositional variation of each substratum. SDM coatings showed no adhesive selectivity of ECs and SMCs. No significant difference of proliferation is also observed among each surface. The result of platelet adhesion indicated that surfaces covered on SDM inhibit the adhesion and activation of platelets. Therefore, SDM can be a good approach for improving blood compatibility as stent materials.

Keywords. Stent, ECM, Endothelialization, Anti-platelet Activity

(23.P4) EVALUATION OF PHYTONCIDE AS AN ALTERNATIVE DRUG FOR DRUG-ELUTING STENT

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Drug-eluting stent (DES), metallic stents coated with drug-loaded nondegradable/degradable polymers, has become the standard treatment in percutaneous coronary intervention (PCI). Among local drug delivery based on DES, sirolimus with immunosuppressive activity and paclitaxel with potent anti-proliferative effect are the most representative drugs. This is why they can prevent in-stent restenosis which is attributed to the proliferation of smooth muscle cells. However, it has reported that there were the potential risks about inhibiting endothelialization and delaying vessel healing. Phytoncide, a volatile substance released from trees and plants, has many attractive effects such as human NK cell activity and an anti-oxidant. In particular, monoterpenoids such as α-pinene, careen, and myrceen in phytonc:ide have been known as compounds with anti-oxidative and anti-microbial activity. Hence, we investigated in vitro bioactivity and drug release behavior of phytoncide as an alternative drug of sirolimus and paclitaxel. Cell viability and anti-proliferative effects of sirolimus and phytoncide on smooth muscle cell (SMC)

and endothelial cell (EC) were measured by cell counting kit-8 (CCK-8). In vitro migration assay was performed to confirm inhibitory effect of cellular migration. For drug release in vitro, phytoncide incorporated in poly(lactic-co-glycolic acid) (PLGA) was coated on stainless steel by electrospray. Coating surface was observed by scanning electron microscope (SEM) and the drug released was characterized by UV spectrophotometry. There was no significant difference in cytotoxicity of phytoncide and sirolimus against SMC and EC, that is, both of them indicated similar IC50 values. Phytocide inhibited also the proliferation and migration of SMC. In addition, the surface of phytoncide/PLGA coated by electrospray was smooth, and various drug-release profiles were obtained according to drug concentration. Based on these results, it is expected that phytoncide would be a promising drug to replace sirolimus or paclitaxel for DES.
Keywords. Stent, Drug-eluting, Phytoncide, SMC, EC

(23.P5) FORMATION BEHAVIOR OF AG NANOPARTICLE WITH VARIOUS TEMPERATURE AND PH CONDITIONS
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Poly(vinyl alcohol) (PVA) which is widely known as a typical water soluble polymer, has been used as a biomedical material due to its a good biocompatibility. Recently, its applications has been extending to hydrogel for artificial internal organ, embolic material for care of deformed blood vessel , treatment for disease of cancer as well as wound dressing. Silver nanoparticles have an excellent antimicrobial activity and a strong effect of inhibition against inflammatory cells. Nanoparticles are intensively studied in recent years because of their potentials in catalysis, optical, mechanical and electronic devices. Nanoparticles have been prepared by various methods, such as chemical reduction, electrochemical reduction, microwave reduction, light irradiation reduction, etc. These nanoparticles properties, the catalytic and electrical activity, thermodynamic and chemical properties, are dependent on their size. The different size of nanoparticles can be synthesized by conventional physical and chemical. Generally, Specific control of size is achieved by varying the synthesis method, reducing agent, stabilizer and pH of the reaction system.
In this study, we used reported the effect of temperature and pH on the formation of silver nanopaticles. We used poly(vinyl alcohol), maltose and silver nitrate as stabilizer, reductant and silver nanoparticle precursor, respectively. The size distribution and dispersion of Ag nanoparticles formed in gelatin solution were characterized by UV-Vis spectrophotometer.
Keywords. Nanoparticles, silver, temperature

(23.P6) PREPARATION AND CELLULAR RESPONSE OF GELATIN NANOFIBERS CONTAINING SILVER NANOPARTICLES
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Gelatin is natural abundant biopolymer and widely used in food, cosmetic, pharmaceutical, and medical applications. Depending on its usage, gelatin can be fabricated in many forms, e.g., films, micro- or nanoparticles, and hydrogels. Gelatin in the form of micro- and nanofibers can also be fabricated by electrospinning technique. Electrospun nanofibers have several unique properties, such as high specific surface area, high porosity, and flexibility. These properties are suitable for biomedical applications, e.g., wound dressings, tissue engineering scaffolds, artificial vessel, and delivery carriers for DNA and drugs. The solvents for electrospinnable gelatin solution are formic acid, acetic acid, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP).
Ag (silver) metal has considerable interest because of its good conductivity, chemical stability, and catalytic and antibacterial activities. It is well known that silver ions or particles are highly toxic to microorganism and strong effect of inhibition against inflammatory cells. Therefore, silver, as an antibacterial component, have been applied for various biomedical fields by compounding it into composites or coating it on the surfaces.
In this study, gelatin nanofibers containing silver nanoparticles were prepared by electrospinning method. We used silver nitrate ($AgNO_3$), as silver compounds, and it was dissolved into gelatin solution in formic acid. In this system, formic acid was used as a solvent of gelatin, but also as reducing agent for silver ions in solution. The size distribution and dispersion of Ag nanoparticles formed in gelatin/formic acid solution were characterized by UV-Vis spectrophotometer, nanoparticle analyzer, transmission electron microscopy (TEM). The morphology of electrospun gelatin nanofibers containing silver were investigated by scanning electron microscopy (SEM). Silver nanoparticles on gelatin nanofibers were observed by TEM and confirmed by X-ray Photoelectron Spectroscopy (XPS). The effects of silver nanoparticles on the cell attachment and spreading of normal human epidermal keratinocytes (NHEK) and normal human epidermal fibroblasts (NHEF) were examined in vitro.
Keywords. Gelatin nanofiber, Silver nanoparticle, Cellular response

(23.P7) PREPARATION AND CHARACTERIZATION OF NANO/MICROFIBROUS COMPOSITE SCAFFOLDS FOR TISSUE REGENERATION
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The aim of tissue engineering is to repair and regenerate of damaged tissue using a substitute of the body tissue such as scaffold. Scaffold is to mimic the structure and biological functions of naturally occurring extracellular matrix (ECM) and plays an important role to guide cell growth and tissue development in tissue engineering. The optimal scaffold is biocompatible, degradable, structurally stable, and hydrophilic and having a highly porous with large interconnected pores1.
Gas plasma treatment is method for the chemical modification of a polymer that can generate desired functional groups such as hydroxyl and amino groups on the surface of the polymer2.
Electrospinning is a widely used simple technique to fabricate micro- to nanometer-sized fibers of various polymers. Generally, cell is difficult to infiltrate from outside to inside of the nanofibrous scaffold because it has a very small pore size in spite of its high porosity. So, we designed nano/microfibrous composite scaffold to

improve cell infiltration ability. And nano/microfibrous composite scaffolds are plasma-treated with NH3 gas to increase hydrophilicity of scaffolds.

In this study, nanofiber was fabricated by the general electrospinning and microfiber was fabricated by newly designed the melt-electrospinning. Poly(ε-caprolactone)(PCL) FDA-approached polymers, are used main component in microfiber. Silk fibroin(SF), a biocompatible natural polymer, is used nanofiber material. The nano/microfibrous scaffolds prepared from various conditions were examined by scanning electron microscopy (SEM), porosimetry, contact angle and tensile test.

References: [1] Q. Lu, K. Ganesan, D.T. Simionescu and N.R. Vyavahare, Biomaterials, 25(22), 5227 (2004). [2] K. E. Park, K. Y. Lee, S. J. Lee, W. H. Park, Macromol. Symp., 249–250, 103(2007).

Keywords. Silk fibroin, PCL, nanofiber, microfiber, composite scaffold, plasma treatment

(23.P8) IN SITU CROSS-LINKABLE AND MECHANICALLY TUNABLE GELATIN BASED HYDROGELS FOR CARTILAGE REGENERATION

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Introduction. Recently, in situ forming hydrogels are widely studied as an injectable cell delivery carrier for cartilage regeneration based on minimally invasive technique. In particular, in situ cross-linkable hydrogels formed through the enzyme-mediated cross-linking were investigated due to biocompatibility and easy to control the reaction rate in mild condition. In this study, in situ cross-linkable and mechanically tunable gelatin based hydrogel was developed as an injectable material for cartilage regeneration.

Methods. The conjugates were synthesized by a common carboiimide/active ester-mediated coupling reaction. The chemical structures were characterized by 1H NMR spectrum. Their physic-chemical properties such as gelation rate and mechanical strength were characterized with the different catalysts concentrations. In vitro cell study was investigated using primary chondrocytes for 14 days. The cell viability and chondrogenic differentiation of the encapsulated cells was determined by live/dead assay and RT-PCR. In addition, in vivo animal study was investigated using rabbit chondral defect model.

Results. The hydrogels were rapidly formed in presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). In the enzyme-triggered system, HRP catalyzes the coupling of phenol and aniline derivatives through the decomposed of H_2O_2 at the presence of aromatic proton donors. Their physico-chemical properties such as gelation rate and mechanical strength could be controlled by the variation of HRP and H_2O_2 concentrations. In the result of the in vitro/in vivo studies, the cells encapsulated in the hydrogels were well proliferated matrix and the expression level of collagen type II and aggrecan were increased dramatically.

Conclusions. In situ forming gelatin based hydrogels were developed as a cell delivery carrier for cartilage regeneration. Obtained results demonstrated that the gelatin based hydrogels have a potential as an injectable matrix for cartilage regeneration.

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Keywords. injectable hydrogels, biomimetic scaffold, cartilage regeneration

(23.P9) FABRICATION AND CHARATERIZATION OF POLY(2-HYDROXYETHYL METHACRYLATE) (PHEMA) NANOFIBERS WITH HIGH SILVER CONTENTS USING IN SITU REACTION

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Poly(2-hydroxyethyl methacrylate) (PHEMA) is interested biomaterials, because of their biocompatability and hydrophilicity, for a wide range of biomedical applications which include contact lenses, artificial bone, skin, cartilages, and drug delivery systems. Silver nanoparticles have been widely investigated due to their conductivity, catalytic activity, and excellent antimicrobial activities. It is known that these properties are depended on nanoparticles sizes, shapes and distribution. Silver nanoparticles can be used for a variety of applications such as catalyst, filter, quasi drug, and wound dressing.

In this study, PHEMA nanofibers with high silver nanoparticles contents were prepared by electrospinning. PHEMA and $AgNO_3$ were dissolved into ethanol/water mixture solution. In this system, ethanol was used as a key component in solvent of PHEMA, but also as reduction agent for silver ions. Silver ions in ethanol mixed solution were transformed to silver nanoparticles by ethanol reduction. The reduction rate of silver ions was closely related with the ethanol content in ethanol/water mixture. The formation behavior of silver nanoparticles in PHEMA solution was examined using UV-vis spectrophotometer. The surface plasmon resonance absorption of PHEMA solution containing silver nanoparticles was observed at the wavelength of 430 nm, indicating the existence of silver nanoparticles. The morphology of electrospun PHEMA nanofibers containing silver nanoparticles were investigated scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Keywords. PHEMA, Silver nanoparticles

(23.P10) DEMINERALIZED DENTIN PARTICLE AND COMPOSITE SCAFFOLD WITH POLY(LACTIC CO-GLYCOLIC ACID) FOR CRANIAL BONE REGENERATION

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We characterized functional and chemical properties of demineralized dentin particles (DDPs), and prepared composite scaffold with PLGA to investigate cranial bone regeneration. Teeth were freeze-dried and separated into dentin matrix and enamel layer. Dentin was soaked in 0.5M EDTA solution. Protein/elements content, cell responses, biocompatibility and effect of DDP/PLGA scaffold on calvarial regeneration of demineralized dentin

were measured. SEM images of EDTA decalcified DDP showed smooth surface. The net protein contents of EDTA treated DDP were increased by 1.399 times. Analysis of surface functional group showed four main absorption bands at the frequencies of wave number 562.75, 1031.68, 1640.12 and 3446.60 cm-1.The treatment with 1, 10 and 100 µg of DDP/cm2 showed nontoxic effect to DPSCs and BMSCs. Analysis of cell proliferation showed that 1 and 10 µg of DDP/cm2 treated DPSCs and BMSCs grow faster than control. ALP activity was detected more intensely in DDP treated groups. In osteogenic medium, cells formed visible minerals by day 18. With Alizarin staining, DDP treated cells occurred 5 days earlier and increased calcium deposition.SEM showed scaffold pore size ranged about 200~300 µm and porosity was 90%. DPSCs adhered highly on 1 and 3 wt% DDP-containing scaffolds, and BMSCs showed well attachment on 3 wt% DDP containing scaffold. Significant amounts of ALP, BSP, OPN, OC expression were seen on 3wt% DDP-PLGA scaffold with DPSCs. Smooth surface morphology and high protein contents of dentin can be obtained by demineralization with EDTA, and DDP/PLGA composite scaffold can be used for calvarial bone regeneration.

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Keywords. Demineralized dentin particles, PLGA, cranial bone

(23.P11) GREEN SYNTHESIS OF SILVER NANOPARTICLES BY CHITOSAN OLIGOMER

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The preparation of metal nanoparticles is a major research area in nanoscience and engineering due to their unusual chemical and physical properties, such as catalytic activity, novel electronic, optic and magnetic properties, and biotechnology. Since silver is known for its antimicrobial properties and even has shown to prevent HIV binding to host cells, it has been used for years in the medical field for antimicrobial applications. There are two general methods for the preparation of metal nanoparticles using light irradiation, or chemical reductant and organic solvents. These chemical and physical methods using chemical reducing agents are not too suitable to have application to medicine and medical area because they should have associated environmental toxicity or biological hazards. Development of adoption of sustainable processes through green chemistry using biological, biomimetic and biochemical approaches is attractive about the elimination or minimization of chemical waste. In our work, we introduce a simple and green method for the preparation of silver nanoparticles using chitosan oligomer as both the reducing and stabilizing agent in water. It's also observed how the silver nanoparticles are formed in the chitosan oligomer aqueous solution that is benign to the environment. Moreover, we expect that the use of environmentally benign and renewable materials like chitosan oligomer to prepare silver nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and biomedical applications.

Keywords. Silver nanoparticles, Green synthesis

(23.P12) EFFICIENT HEALING OF PARTIAL DEFECT OF CARTILAGE BY THE DELIVERY OF DE-DIFFERENTIATED CHONDROCYTES USING HEPARIN-BASED HDYROGEL

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Introduction. We characterized the potential of a heparin-based hydrogel in articular cartilage defect regeneration. As a realistic approach, de-differentiated chondrocytes during expansion was used as a cell source, and without adding any growth factors, cell-hydrogel construct was applied to a partial thickness defect model in rabbit cartilage, which is hard to be healed and closer to a real disease model.

Methods. Heparin-based hydrogels were prepared by a Michael-type addition reaction between thiolated heparin and diacrylated poly (ethylene glycol). Isolated chondrocytes, from knee cartilage of New Zealand white rabbits, were sufficiently expanded in monolayer culture, leading to de-differentiation, and then were cultured in the heparin-based hydrogels under a normal cell culture condition (DMEM with 10 % FBS only) without any chondrogenic factors. Re-differentiation of chondrocytes and the GAG production inside the hydrogel were analyzed *in vitro*. The proper *in-vivo* chondrogenesis was also characterized after delivering de-differentiated chondrocytes using the heparin-based hydrogel to a partial thickness defect model in rabbit knee joint cartilage.

Results. Completely de-differentiated chondrocytes were effectively re-differentiated and produced proper GAGs and ECMs inside the heparin-based hydrogels within a week without addition of any growth factors or chondrogenic components in the culture medium *in vitro*. Effective cartilage regeneration by the heparin-based hydrogel containing de-differentiated chondrocytes without exogenous growth factor was also observed within 2 months in a partial thickness defect model of rabbits whereas no proper healing of cartilage was observed in control defects.

Conclusion. Highly efficient re-differentiation of de-differentiated chondrocytes was achieved in the heparin-based hydrogel *in vitro*. Excellent cartilage regeneration was also achieved upon application of chondrocyte/hydrogel construct to a partial thickness cartilage defect in rabbits. Thus, the present heparin-based hydrogel could serve as a promising cell carrier in articular cartilage repair.

Acknowldegements. WCU program at GIST by MEST, Korea (R31-2008-000-10026-0)

Keywords. Cartilage, Heparin, Hydrogel, Partial defect

24. KOREAN-EUROPEAN SYMPOSIUM: STEM-CELL BASED TISSUE ENGINEERING

Chair: Il-Woo Lee
Co-chair: Miguel Alaminos
Keynote speakers: Gun Il Im, Ingrid Garzón
Organizers: Il-Woo Lee, Miguel Alaminos
Synopsis: Stem cells are of particular interest as valuable cell sources in tissue engineering. There have been rapid advances in variety of stem cell researches in tissue engineering to translate bench top advances into clinical therapies. In this joint symposium between the Societies of Korean and European Tissue Engineering & Regenerative Medicine, current effort and recent advances in scaffold-inclusive or scaffold-free stem cell-based tissue engineering will be discussed. The future collaborations between the researchers from both societies will be also encouraged in this joint symposium.

(24.KP1) STEM-CELL BASED TISSUE ENGINEERING

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Adult stem cells have drawn attention as an attractive cell source for tissue regeneration. Mesenchymal stem cells (MSCs) in adults are capable of self-regeneration and differentiation into several cell types. While bone marrow provides the most universal source of MSCs, other tissues such as the periosteum, muscle, synovial membrane and adipose tissue also possess MSCs. Of these, adipose tissue offers a unique source of stem cells with considerable advantages for its accessibility and abundance. The adipose stem cells (ASCs) obtained from lipoaspirates have been also proven to possess the multilineage potential. The focus of musculoskeletal tissue engineering is the regeneration of bone and cartilage.

To achieve the goal, an effective induction of differentiation poses a key challenge in the use of adult stem cells. While osteogenic differentiation of bone marrow MSCs is easily induced, ASCs have much lower osteogenic potentials than bone marrow MSCs. It can be enhanced with the used of vitamin D or BMPs. Gene transfer of osteogenic growth factors such as BMPs or transcription factors such as Runx-2 and Osterix can markedly enhance the osteogenic potential of ASCs. Recent advancement in nonviral gene transfer by electroporation has achieved a high transfection rate, and shed a light to their possible clinical application. Chondrogenic differentiation of MSCs poses greater challenge than osteogenic differentiation. TGF-□has been used to induce chondrogenesis from MSCs, but the markers of hypertrophy increase along with chondrogenic markers in this setting. Inhibitor of hypertrophy such as PTHrP can be used to induce chondrogenesis while suppressing hypertrophy from MSCs. It is even difficult to induce chondrogenesis from ASCs, requiring greater doses or different combination of growth factors. The nonviral gene transfer of SOX trio, the key transcription factors of chondrogenic differentiation, can also enhance the chondrogenesis from MSCs and ASCs.

In conclusion, great efforts have been devoted to the research to engineer bone or cartilage from adult stem cells and various strategies for tissue engineering from stem cells have been developed. Nevertheless, a lot of unsolved questions and dilemmas remain until we obtain tissues of high quality from stem cells.

(24.KP2) SELECTION OF VIABLE WHARTON'S JELLY STEM CELLS FOR CELL THERAPY

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Introduction. Wharton's jelly cells have recently been of great interest to many researchers as a possible clinical source of mesenchymal stem cells. In this context, the high plasticity and proliferation capabilities of Wharton's jelly cells make of these cells a plentiful and inexpensive source of cells potentially useful in impact fields such as tissue engineering, and biotechnology. In this work, we have used a combination of highly-sensitive techniques to determine the average cell viability levels (ACVL) and proliferation capabilities of ten consecutive cell passages of cultured HWJSC, and we have identified the genes that are associated to changes in the cell viability levels.

Methods. Wharton's jelly cells were isolated from umbilical cords and enzymatically digested by using type I collagenase. The analysis of cell proliferation and viability was measured by mitochondrial function using (WST-1) colorimetric assay on 10 consecutive cell passages of WJMSCs. The percentage of alive and dead cells in each cell passage was determined by trypan blue and LIVE/DEAD® Cell Viability Assays. Consequently, subconfluent HWJSC were subcultured using trypsin-EDTA on plated gold grids to evaluate the cell viability by electron-probe X-ray microanalysis. The stem cell profile was confirmed by flow cytometry of specific markers of stem cells and cell viability pattern was also confirmed by immunofluorescence and microarray gene expression profile.

Results. Our results showed an initial decrease of cell viability from the first to the third cell passage, with an increase until the sixth passage and a final decrease at the last cell passages. According to the ACVL, the highest cell viability levels corresponded to cell passages 6 and 5. The intracellular ionic contents of K, Na and Cl suggest that the lower cell viability levels found at passages 2, 3 and 8-10 could be associated to a process of apoptotic cell death. In this connection, the gene expression analysis revealed that a number of genes with a function in apoptotic cell death were significantly associated to ACVL.

Conclusion. In general, our data imply that the highest cell viability levels correspond to HWJSC passages 5th and 6th, and suggest that these specific cell passages should be preferentially used in cell therapy or tissue engineering protocols using this cell type.

Keywords. Wharton's jelly cells, Cell Viability, Cell Proliferation, Gene Expression Analysis

(24.O1) PRE-TREATMENT OF HUMAN MESENCHYMAL STROMAL CELLS FOR BONE TISSUE ENGINEERING

RESULTS IN SECRETION OF TROPHIC, BIOLOGICALLY ACTIVE, GROWTH FACTORS

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Introduction. Human mesenchymal stromal cells (hMSCs) are an interesting cell source for tissue engineering (TE) applications and cell therapies, both for their ability to differentiate into various cell types and for their secretome, which has immunosuppressive, anti-apoptotic and pro-angiogenic effects. For bone TE, the direct differentiation of hMSCs into osteoblasts is generally believed to account for new bone formation, but secreted growth factors could also act as chemokines for host cells and attract cells that contribute to bone formation but also to the vasculature. Here, we treated hMSCs with the small molecule db-cAMP, which enhances bone formation, to adjust growth factor secretion for bone TE, and investigated the effect of secreted factors.

Materials and Methods. Conditioned medium (CM) was prepared by culturing hMSCs with or without 1 mM db-cAMP for 3 days after which fresh medium was added for 2 more days. To test the biological activity of growth factors in cAMP-CM, various cell types were cultured in either non-CM, basic-CM or cAMP-CM, after which proliferation and gene expression were analyzed.

Results. ELISAs demonstrated an increase in bone-specific growth factors in cAMP-CM; Il-8, Il-11, BMP-2 and IGF-1. Culture of MG-63s, C2C12s and HUVECs in cAMP-CM resulted in increased proliferation as compared to non-CM and basic-CM, whereas proliferation of primary hMSCs was not affected. cAMP-CM also increased differentiation of C2C12s, as demonstrated by an increase in ALP expression. Primary hMSCs demonstrated an enhanced osteogenic gene profile in cAMP-CM after 6 hours, but gene expression was reduced to basal level after 72 hours. We found paracrine signaling via BMPs, but no mitogenic effect of IGF-1. A currently performed microarray should reveal additional mechanisms.

Conclusions. We conclude that treatment with db-cAMP results in increased secretion of specific trophic factors that are biologically active and influence both proliferation and differentiation of various cell types.

Keywords. Mesenchymal stromal cells, bone tissue engineering, trophic effect

(24.O2) THE NEOVASCULARIZATION EFFECT OF BONE MARROW STROMAL CELLS IN TEMPORAL MUSCLE AFTER ENCEPHALOMYOSYNANGIOSIS

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Objective. Patients with Moyamoya disease are in a state of chronic cerebral ischemia,and the primary goal of treatment is to improve collateral circulation through angiogenesis. During angiogenesis, the expression of vascular endothelial growth factor (VEGF) plays the most important role. In the present study, we obtained and sub-cultured bone marrow stromal cells (BMSCs) from rats and injected the labeled BMSCs directly into adjacent temporal muscle during encephalomyosynangiosis (EMS).

Materials and Methods. We divided 20 rats into a BMSCs transplantation group (n=12) and a control group (n=8). Seven days after induction of chronic cerebral ischemia, an EMS operation was performed on the right side, and labeled BMSCs (1X106/100 μl) were injected in the temporal muscle of the right side for the transplantation group, while an equivalent amount of culture solution was injected directly into the right side for the control group. Three weeks after transplantation, temporal muscle and brain tissue were collected for histological examination and western blot analysis.

Results. The capillary/muscle ratio in the temporal muscle was increased in the BMSC transplantation group compared to the control group, showing a greater increase of angiogenesis ($P<0.05$). The injected BMSCs in the temporal muscle were VEGF-positive by immunofluorescence staining. In both temporal muscle and brain tissue, the expression of VEGF by western blot analysis was not much different between the BMSC transplantation group and the control group.

Conclusions. After EMS in a chronic cerebral ischemia rat model, the injection of BMSCs resulted in accelerated angiogenesis in the temporal muscle compared to the control group. The results of this study may be applicable for enhancing revascularization in Moyamoya disease through further study.

Keywords. Bone marrow stromal cell, chronic cerebral ischemia, angiogenesis, Vascular endothelial growth factor

(24.O3) EXPANSION OF ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS MAINTAINING THEIR STEMNESS

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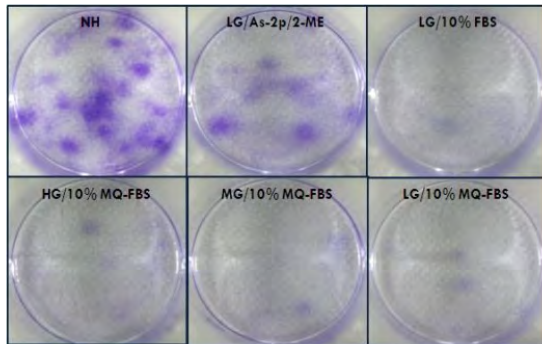
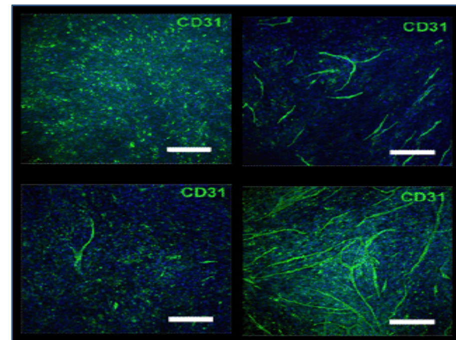
Mesenchymal stem cells (MSCs) are capable of self-renewing and have the multilineage differentiation potential that gives rise to the cells of three germ layers. It is reported that the stemness of MSCs reduces as they are expanded in vitro. Recently, some researches demonstrated that their stemness was maintained in commercial media or antioxidant-supplemented media.

In this study, we tried to find out appropriate culture media for the expansion of MSCs by performing cell culture with three experimental groups: (1) general media, (2) general media with antioxidants, (3) commercial media. The stemness was assessed by analysing cell proliferation, colony-forming activity, osteogenesis, chondrogenesis, adipogenesis, neurogenesis at 5th and 10th passage. As results, MSCs in antioxidant-added group had similar activities to commercial media, but those in general media did not. Therefore, it is strongly supposed that antioxidants play a crucial role in maintaining their stemness.

Keywords. Mesenchymal stem cell, Expansion, Stemness





(24.P1) PARACRINE EFFECTS INFLUENCED BY CELL CULTURE MEDIUM AND CONSEQUENCES FOR MICROVESSEL-LIKE STRUCTURES IN CO-CULTURES OF MESENCHYMAL STEM CELLS AND OUTGROWTH ENDOTHELIAL CELLS

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Introduction. For applications in regenerative medicine, mesenchymal stem cells (MSC) from bone marrow and outgrowth endothelial cells (OEC) from peripheral blood are considered as attractive cell types to build up complex vascularised tissue engineered constructs in which neovascularization could be promoted by release of proangio¬genic factors from MSC. Nevertheless, generation of complex tissue engineered constructs is challenging due to the diverse requirements of the cell types in terms of culture conditions. In this study the influence of different culture media on angiogenesis and osteoblastic differentiation in co-cultures of both cell types was investigated.

Materials and Methods. Co-cultures and MSC monocultures were cultivated in different cell culture media based on the osteogenic differentiation medium ODM or the endothelial cell culture medium EGM2, as indicated in table 1. After 2 weeks immunofluorescent staining, RT-PCR and FACS analysis for endothelial markers were performed to assess the effect of media on endothelial cell functionality. In addition, proangiogenic growth factors were examined by ELISA and osteogenic differentiation by Alizarin Red and RT-PCR.

Results. Co-cultures cultivated in EGM2 formed significantly more angiogenic structures compared to ODM (table 1). EBM2 and ODM-SEC resulted only in single vascular structures (table 1) in the co-culture. In addition, higher ratios of CD31- and CD146-positive cells in EGM2 were observed. A higher release of the proangiogenic factors, VEGF and PDGF, which were detected in co-cultures as well as in MSC monocultures in EGM2 might favour endothelial cell growth and function, as well as vessel stability by pericytes. Nevertheless, ODM was more suitable for the differentiation of MSC to osteoblastic lineages in the co-culture.

Conclusion. In conclusion, this study highlights the importance of medium components for cell interaction and formation of angiogenic structures in co-cultures of MSC and OEC.

Acknowledgement BMBF (German-Chinese cooperation in Regenerative Medicine)

Keywords. MSC, angiogenesis

(24.P2) COMPARISON OF OSTEOGENIC POTENTIAL OF CANINE MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW, ADIPOSE TISSUE, UMBILICAL CORD BLOOD AND WHARTON'S JELLY FOR CELL THERAPY OF BONE DEFECTS

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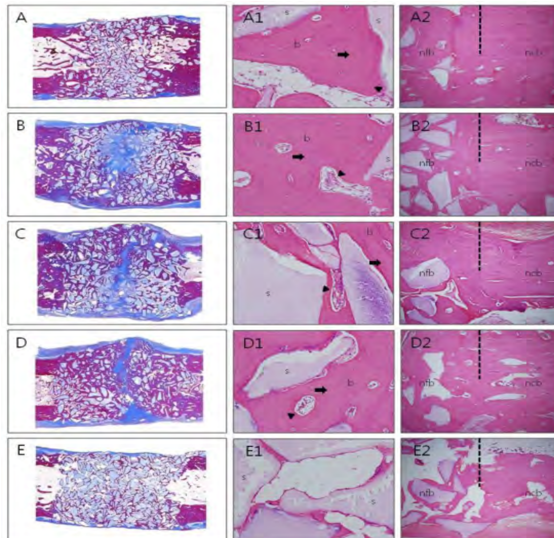
Introduction. Mesenchymal stem cells (MSCs)-based therapy has been assessed as novel tool of tissue engineering to repair bone defects. Bone marrow has been the major source to obtain MSCs for bone engineering. However, bone marrow may be unsuitable source for clinical application due to the highly invasive procedure and the decline in proliferation and differentiation potentials with increasing age. Thus, alternative sources have been intensively investigated for therapeutic applications of MSCs. The purpose of this study was to compare the osteogenic potential of canine MSCs from bone marrow (BM), adipose tissue (AT), umbilical cord blood (UCB) and Wharton's jelly (WJ) using in vitro culture and in vivo orthotopic implantation assays.

Materials and Methods. After MSCs were isolated from various origins of adult healthy dogs, proliferation potential and osteogenic potential in vitro were compared. Production of vascular endothelial growth factor (VEGF) in vitro was also compared. For the in vivo assay, MSCs from each tissue were mixed β-tricalcium phosphate and implanted into segmental bone defects of dogs. Bone matrix production was compared using radiographic and histological analyses.

Results. AT-MSCs had a higher proliferation potential and BM-MSCs produced more VEGF than other MSCs. AT-MSCs and UCB-MSCs showed a little more osteogenic ability compared with other cells in vitro differentiation. In the radiographic and histological analyses, all tested MSC exhibited similar osteogenic capacity, although new bone formation was much more detected in the implants using MSCs than the cell-free implants.

Conclusions. These results indicate that AT-MSCs, UCB-MSCs and WJ-MSCs could be alternative candidates to BM-MSC for clinical application of bone tissue engineering.

Keywords. Mesenchymal stem cell, Osteogenesis, Comparison, Dog



(24.P3) EFFECT OF FIBROBLAST GROWTH FACTOR AND PDLF CONDITIONED MEDIA ON FIBROBLAST DIFFERENTIATION FROM BONE MARROW STROMAL CELL

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Periodontal ligament fibroblst (PDLF) is essential to most periodontal tissue regeneration process. A variety of growth factors that promote the differentiation of stem cell have been identified, and among these factors, fibroblast growth factor (FGF) shows enhance the differentiation of stem cell for engineered tissue. In this study, we evaluated the potential of FGF and PDLF conditioned media to modulate the growth and differentiation activites of bone marrow stroma cell (BMSC). BMSC was obtained from mongrel dog. This study protocol was approved by the Chosun University Dental hospital Institutional Review Board. The fresh media obtained from culturing periodontal ligament fibroblasts of dog was used for PDLF conditioned media. The BMSC and PDLF were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco BRL, USA) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO2 and 95% humidity. In control group, BMSC was only cultured without FGF and PDLF conditioned media. In experimental group, FGF and PDLF conditioned media was added to BMSC and was loaded with 3ng/ml of FGF. Media was changed every 2 days for 14 days. We evaluated the effect of FGF and PDLF conditioned media on fibroblast cell differentiation from bone marrow stromal cell by using the RT-PCR. The addition of FGF and PDLF conditioned media enhanced fibroblast like cells differentiation from bone marrow stromal cell. Experimental group treated FGF and PDLF conditioned media showed high expression of the fibroblast cell-specific marker: UNCL-, S100A2-, S100A4-mRNA. The nestin mRNA showed strongly in the control group. This suggest that the FGF a;nd PDLF conditioned media can enhance the growth and differentiation of fibroblast cells from BMSC in virtro.

Keywords. BMSC, FGF, PDLF conditioned media, differentiation

(24.P4) ENHANCEMENT OF TENDON HEALING USING BONE MARROW DERIVED MESENCHYMAL STEM CELL IN ROTATOR CUFF TEAR OF A RABBIT MODEL

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Introduction. Tissue-engineering techniques have shown promise in the treatment of tendon and ligament defects. This investigation tested the hypothesis that installation of a cell-seeded, organized construct into a large tendon defect would significantly promote cellular differentiation and natural tissue regeneration.

Materials and Methods. Bone marrow were harvested from the iliac crests of two male New Zealand White rabbits. The MSCs were cultured and OPLA scaffold was encapsulated with these cells. The injury model was full-thickness, window defects (sized, 5mm x 5mm), cut in the central part of each rotator cuff tendon. The right side rotator cuff tendon defect were grafted with the autologous MSC seeded OPLA scaffold. The left side rotator cuff tendon defect was implanted with the cell-free OPLA scaffold implant and a biodegradable suture, similar to the treated side. After implantation, the samples were harvested 2, 4 and 6 weeks for analysis, which included evaluation of gross morphology, flurescent analysis, histological assessment and immunohistochemistry studies.

Results. We confirmed the existence of the living cells labeled with PKH-26 in the OPLA scaffold by staining with fluorescent staining. Numerous cells labeled with PKH-26 were integrated well into the OPLA scaffold following 2, 4 and 6 weeks after implanted. The expression of Immunohistochemical stainings for collagen I was higher in the scaffold with MSCs than in the scaffold without MSCs. The expression of Immunohistochemical stainings for collagen II, however, was not different between the scaffold with MSCs and the scaffold without MSCs.

Conclusions. We demonstrated that many MSCs in the scaffold could survive after implantation in the rabbit rotator cuff defect. Furthermore, the generation of type I collagen increased more in the scaffold with MSCs than that of scaffold alone. It was thought that MSCs promote the tendon healing by producing collagen type I when they were applied at the tendon defect.

Keywords. Mesenchymal stem cell, Rotator cuff tear, Tendon healing

(24.P5) IS SAFE SUBACROMIAL STEROID INJECTION IN THE ROTATOR CUFF DISEASE? IN VIVO EXPERIMENTAL STUDY

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Introduction. To elucidate the early effect of subacromial steroid injection upon the normal and torn rotator cuff tendon in terms of various cytokines.

Materials and Methods. Eighty rats were randomly assigned into four groups: intact tendon(IN)/intact tendon with steroid(IS), torn tendon(TN)/torn tendon with steroid(TS). Full-thickness defect was made at unilateral infraspinatus tendon insertion site in TN, TS.

Single dose of methylprednisolone (0.6 mg/kg) was injected into the subacromial space in IS, TS. The tendon was harvested at one, three days, one, two and six weeks after the operation, gene expression and immunohistochemistry study were performed for type-I/III collagen, Tumor necrosis factor(TNF)-α, and extracelluar matrix molecules including tissue inhibitors of metalloproteinase(TIMP)-1/2, fibronectin, aggrican, and matix metalloprotainase(MMP)-9.

Results. The type-III to type-I collagen ratio significantly increased at 1 weeks in steroid groups(IS, TS) compared to no steroid groups(IN, TN)($p<0.05$), then decreased to control level at 2 weeks. The gene expression of TNF-α, all extracelluar matrix molecules of steroid groups(IS, TS) significantly increased compared to no steroid group(IN, TN) at day 3($p<0.05$), following decreased to the control level at 1 week and maintained till 6 weeks.

Conclusion. Subacromial steroid injection may alter collagen composition and extracellular matrix of intact tendon and interfere with healing process in the torn tendon immediate following injection. The patient with rotator cuff disease should avoid aggressive motion and therapy immediate after subacromial steroid injection.

Summary: Subacromial steroid injection may alter collagen composition and extracellular matrix of intact tendon and interfere with healing process in the torn tendon immediate following injection.

Keywords. Rotator cuff disease, Steroid, Subacromial injection

(24.P6) EFFECT OF HUMAN AMNIOTIC FLUID STEM CELL THERAPY FOR URETHRAL SPHINCTER REGENERATION

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Human amniotic fluid stem cells (hAFSCs) can be a useful cell source for cell therapy. In this study, we investigated the potential role of hAFSCs in cell therapy for urethral sphincter regeneration. The cultured hAFSCs were injected to the pudendal-neurectomy female mouse model. Three experimental groups were established: a control group had a sham-operation without nerve transsection (Ctrl); a pudendal nerve transsection group with cell-free saline injections (Cell-) and a nerve transsection group with periurethral hAFSC injections (Cell+). The urodynamic studies including leak point pressure (LPP) and closing pressure (CP) were examined at 1, 2 and 4 weeks after treatment. And the urethra was harvested after urodynamic study. The injected hAFSCs were identified by IHC and real-time PCR. The LPP of Ctrl, Cell– and Cell+ groups at week 4 were 27.59±3.64, 15.24±2.1 and 20.24±3.25 cmH2O, respectively. The CP was 15.38±1.64, 8.35±1.1, and 14.4±3.4 cmH2O, respectively. The presence of abundant positive human nuclei, nestin, MyoD, α-SM actin demonstrated that a large number of injected AFSC can survive, proliferate, differentiate into the urethral sphincter tissue. The Ctrl group consisted of thick packed muscle layers. The cell– group showed shirinked urethral sphincter tissue. The Cell+ group showed circular smooth and striated muscle tissue regeneration at the injection site. In immunogenic response, hAFSCs showed immune tolerance and suppression through HLA-DR and CD8 analysis. In real-time PCR analysis, cell injected group showed enhanced expression of both human and mouse gene related to myogenic pathway. The injection of hAFSCs into the urethral sphincter of pudendal nerve trassected mouse promoted morphologically, immunologically and functionally competent urethral sphincter regeneration [This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A091224)].

Keywords. hAFSCs, urethral sphincter, incontinence

(24.P7) ISOLATION AND CHARACTERIZATION OF URINE-DERIVED PROGENITOR CELLS FROM THE HUMAN KIDNEY FOR UROLOGICAL TISSUE RECONSTRUCTION

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Recently, progenitor cells were isolated from human urine and investigated their potential uses for urological reconstructive procedures. However, bladder cancer patients could not be use because they might contain malignant cells. For this case, kidney can be an alternative cell source. We isolated renal urine progenitor cells (RUC), characterized and investigated their potential for urological application. RUCs were obtained by percutaneous nephrostomy, and subcultured progenitor cells were isolated. For control, sterile voided urine samples from bladder were used (BUCs). Surface phenotype of the cells was characterized by flow cytometric analysis, cell proliferation was analyzed with cell counting kit-8, and to assess pluripotency and myogencity, RT-PCR, immuno-histochemical staining, western blot were performed. For myogenic application, the optimized medium was tested.

RUC and BUC (passage 3) were positive for stem cell and mesenchymal stem cell markers, whereas negative for hematopoietic cell lineage markers.RUC showed higher exponential cell growth pattern than BUC during 7 day culture. Immunofluorescence staining revealed that RUCs showed enhancing stem cell markers (vimentin, SSEA-4 and Oct 3/4), SMC markers (α-SM actin, myosin and desmin), endothelial cell markers (CD31 and vWF) and also expressed urothelial cell markers (UPIa, CK 7 and 19). In this study we successfully isolate urine-derived progenitor cells from human kidney where free of cancer cells, determined the cells characteristics and investigated their myogenic potential for urological tissue reconstruction [This study was supported by a grant of

the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A091224)].
Keywords. renal urine progenitor cells; myogenic differentiation, urological tissue reconstruction

(24.P8) PDX1 AND CONTROLLED CULTURE CONDITION INDUCED HUMAN AMNIOTIC FLUID STEM CELLS INTO INSULIN-PRODUCING CLUSTERS

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Through genetic modification and in vitro culture system optimization, hAFSCs were differentiated into functional insulin-producing cells. hAFSCs were harvested from human amnion fluid, and isolated with stem cell marker(C-kit). The mouse Pdx1 was inserted adenovirus vector(Ad-Pdx1). The Ad-Pdx1 infection effect and in vitro culture conditions to generate insulin producing cells were analyzed. The mean infected particles per cell were 10 and calculated infectious unit was 5x105 IU/ml. When use 105 IU/ml of virus supernatant, Ad-Pdx1 infection efficiency for hAFSCs was about 50%. By day4, Ad-Pdx1 infected cells to form colonies and made islet cell-like clusters at day8. Activin A accelerated of hNgn3 expression significantly. bFGF treatment into differentiation medium stimulated the cluster formation and Ad-Pdx1/bFGF expressed endogenous PAX6 gene. The surface of Poly-L-ornithine culture environment contributed to Pax6 and Ngn3 expression at week 1, and insulin was expressed at week 3. When Ad-Pdx1 cells were cultured with Nicothinamide in medium, a series of pancreatic marker gene NGN3 was initiated at early period and mRNA level of insulin was continued. A mean±SD concentration of c-peptide on Ad-Pdx1 was 13.5±0.8pmol/300 clusters at week3. Cells embedded in alginate capsules were gradually gathered and formed clusters at 6-8days. The secreted proteins from clusters were detected at 2weeks.

The response to the transduced Pdx1 transcription factor and optimized culture systems with Activin A, bFGF, Poly-L-ornithine and Nicotinamide were induced to differentiate hAFSCs into insulin-producing beta-cells. Thus, hAFSCs offer specific potential value for cell therapy of diabetes [This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea. (A091224)].
Keywords. Pdx1, hAFSCs, beta-cells

(24.P9) NATURAL CRYOPROTECTANTS IMPROVES SURVIVAL OF CRYOPRESERVED AMNIOTIC FLUID-DERIVED STEM CELLS

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Most current cryopreservation methods used for stem cells include the potentially toxic cryoprotectant (CPA) dimethylsulfoxide (Me2SO) in the presence of animal serum proteins that prevent direct use of these cells in human therapeutic applications. To avoid any potential cryoprotectant related complications, it will be essential to develop non-toxic CPAs or reduce CPA concentration in the freezing media used. In this study, we assessed the use of disaccharides, antioxidants and caspase inhibitors for cryopreservation of AFSCs in combination with a reduced concentration of Me2SO. The thawed cells were tested for viability with MTT assays and a growth curve was created to measure population doubling time. In addition, we performed flow cytometry analysis for cell surface antigens, RT-PCR for mRNA expression of stem cell markers, and assays to determine the myogenic differentiation potential of the cells. A statistically significant ($p<0.05$) increase in post-thawed cell viability in solutions containing trehalose, catalase and zVAD-fmk with 5% Me2SO was observed. The solutions containing trehalose and catalase with 5% or 2.5% (v/v) Me2SO produced results similar to those for the control (10% (v/v) Me2SO and 30% FBS) in terms of culture growth, expression of cell surface antigens and mRNA expression of stem cell markers in AFSCs cryopreserved for a minimum of 3 weeks. Thus, AFSCs can be cryopreserved with 1/4 the standard Me2SO concentration with the addition of disaccharides, antioxidants and caspase inhibitors. The use of Me2SO at low concentrations in cell freezing solutions may support the development of clinical trials of AFSCs.

Keywords. Amniotic-derived stem cell, catalase, cryopreservation, trehalose

Table 1

Preparation of different cryoprotectant solutions

Solution	Me_2SO (% v/v)	FBS (% v/v)	Trehalose (mmol/L)	Catalase (µg/mL)	zVAD-fmk (µM)
1	10	30	0	0	0
2	5	30	0	0	0
3	5	0	60	100	0
4	5	0	60	100	30
5	2.5	30	0	0	0
6	2.5	0	60	100	0
7	2.5	0	60	100	30

(24.P10) A NEW SIGNALING PATHWAY INVOLVED IN CARDIOMYOCYTE DIFFERENTIATION FROM EMBRYONIC STEM CELL LINE P19CL6: TYROSINE MODIFICATION OF β2-TUBULIN

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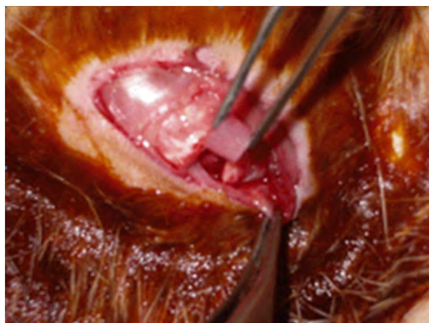
The importance of nitric oxide (NO) in cardiac development and cardiomyocyte differentiation from embryonic stem (ES) cell has been suggested; however, it remains undiscovered that what occurs at molecular level as the consequence of endogenous NO production. Employing cardiomyocyte differentiation from mouse P19CL6 ES cell line, we found out that β2-tubulin, a component of microtubule, underwent a new type of posttranslational modification, tyrosine nitrosylation. Furthermore, we revealed that the modification mediates the interaction of β2-tubulin with Op18/stathmin, a

microtubule destabilizer. Western blot with nitrotyrosine antibody exihibited a prominant immunoreactivity on 50-kDa protein, in parallel with activation of endothelial NO synthase (eNOS) and increased NO production; meanwhile, treatment with NOS inhibitor L-NAME suppressed both endogenous NO production and the immunoreactivity. Through electrospray ionization tandem mass spectrometry (ESI-MS/MS) we identified the immunoreactive 50-kDa as β2-tubulin and further revealed that β2-tubulin was tyrosine nitrosylated, including two susceptible residues, Tyr-106 and Tyr-340. More interestingly, the tyrosine nitrosylation enhanced the interaction of β2-tubulin with Op18/stathmin, a microtubule destabilizer and predisposed the modified β2-tubulins into depolymerized tubulin pool. Additionally, the immunoreactive β2-tubulins were observed enriched not only in spontaneously beating cell clusters but also in mouse embryonic heart (E11.5) containing left ventricle and aorta. In this study we for the first time discovered that as a new target molecule of NO β2-tubulin undergoes the tyrosine nitrosylation in physiological process, which was suggested in our recent report, as well as this modification could be involved in protein-protein interaction as a new NO signaling.

Keywords. eNOS, tyrosine nitrosylation, β2-tubulin, cardiomyocyte differentiation, Op18/stathmin, embryonic stem cell



25. MANUFACTURING AND CHARACTERIZATION OF SCAFFOLDS BASED ON POLYLACTIC ACID FIBRILS

Chair: M. Evstatiev
Keynote speaker: Silviya Simeonova
Synopsis: The main goal of this investigation is to offer a new technology for manufacturing of biodegradable scaffolds, based on polylactic acid fibrils free from contacts with toxic solvents. For this purpose a modified concept for microfibrillar reinforced composites (MFC) was utilized. While tissue engineering has been proven in laboratories, the use of organic solvents in scaffolds fabrication precludes its implementation in a clinical setting. In the current investigation we have obtained polymeric fibrils as 3 – dimentional (3D) scaffolds, using not toxic solvent.

(25.KP) MANUFACTURING AND CHARACTERIZATION OF SCAFFOLDS, BASED ON POLYLACTIC ACID FIBRILS

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Introduction: Tissue engineering aims to produce biological substitutes to restore or repair damaged human tissues or organs. The principle strategy behind tissue engineering involves seeding relevant cells onto porous 3D biodegradable scaffolds [1]. There are numbers of techniques developed to fabricate porous scaffolds. However, most of these methods involve the use of toxic organic solvents which are not completely removed from the scaffold even after long vacuum processing. They are harmful to the cells and reduce their ability to form new tissues. In the present work we report on a novel technique for manufacturing of scaffolds for tissue engineering free from contacts with harmful solvents. For this purpose a modified microfibrillar-reinforced composites (MFC) concept is utilized [2]. Shortly the technology includes the following steps: two polymers is melt blended and extruded, followed by cold drawing, where both blend constituents are converted into a fibrillar state. After selective dissolving of one blend component with not-toxic solvent, individual microfibrils from the second one can be isolated. The object of the present investigation were blends from biodegradable Poly(L-lactic acid) (PLA) and the soluble in not-toxic solvent Poly(ethylene-vinyl alcohol copolymer) (EVAL).

Materials and Methods: PLA (grade Natur 2002B) was supplied by Nature Works and EVOH was provided by EVAL (grade F101A) EUROPE. The drawn PLA/EVAL (40/60 wt. %) blend bristles were manufactured according to the MFC concept on an industrially relevant production line available in the Institute of Composite Materials (IVW), Kaiserslautern, Germany [3]. For selective extraction of EVAL not toxic solvent ((boiling mixture of propanol and water (50/50 by wt.)) was used. From individual PLA fibrils 3D sponges were manufactured by means of Freeze – drying. The microstructure, morphology and thermo-physical properties of the scaffolds before as well as after biomineralizations were observed by DSC, WAXS, FTIR, and SEM.

Results and Discussion: From DSC thermograms and WAXS diffractograms was determinate that after extraction of the EVAL the crystallinity of the PLA fibrils increases as compared to these in drawn samples. This is maybe due to its additional crystallization during dissolving. The absence of melting peak as well as x-Ray crystalline reflections for EVAL represent experimental proofs for completely removing of this component from the drawn blend. Additional evidence for this was drawing from FTIR spectrograms. It was observed lack of the characteristic reflexes of the EVAL.

From the SEM observation was determinate that the sponges are constructed from PLA fibrils with diameter in range from 150 nm to 1.5 μm. The pores size of the scaffolds varies between nano- and micro-scale. After bio-mineralization layers of Ca-Ph ions over the PLA fibrils were observed. This is maybe due to the nucleation and adhesion ability of the fibrils.

Conclusions: A novel technique for obtaining of biodegradable scaffolds for tissue engineering is proposed. Individual nano- and micro- PLA fibrils were obtained from oriented PLA/EVAL blends using non toxic solvents. From PLA fibrils high porous 3D scaffolds with interconnection structure were manufactured. This

material mimics the scales of the fibres that compose the matrix of the native tissues. Bio-mineralized layers of Ca-Ph ions over the PLA extracellular fibrils were successfully formed.

Acknowledgements: The authors gratefully acknowledge the financial support of the Bulgarian Ministry of Education and Science, Fund "Scientific Investigation" (Project DTK 02-70/09), as well as the Institute of Composite Materials (IVW), Kaiserslautern, Germany.

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Keywords. polymer materials, scaffolds, tissue engineering

(25.O1) ALIGNED ELECTROSPUN POLY-L-LACTIDE FIBERS FOR TENDON REGENERATION: A PRELIMINARY IN VITRO STUDY

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Introduction. Management of tendon lesions accompanied by big loss of substance represents a serious issue in orthopaedic surgery. Such lesions, which cannot be repaired with an end-to-end suture, demand for tendon augmentation procedures. Aim of this study is the synthesis of a biomimetic micro-fibrous PLLA scaffold with aligned fibres, obtained by electrospinning technique, to be used in combination with tenocytes, to produce a construct for tendon augmentation.

Materials and Methods. Aligned fibers were synthesized by electrospinning adopting a spinning disk configuration, starting from a poly-L-lactide (inherent viscosity 0.9-1.2 dL/g) 13 wt% solution in a 10:1 dichoromethane/methanol mixture.

Human tenocytes from both intact tendons (TI) and ruptured tendons (TR) were isolated and seeded on the scaffolds. Scaffolds were investigated in terms of cell viability and proliferation. Quantization and typing (type I and III) of synthesized collagen was performed after 1 and 3 weeks of in vitro culturing.

Results. Constructs showed good engraftment, with cell ingrowth and proliferation within the scaffolds, and a specific organization following the direction of PLLA microfibers. TIs and TRs showed comparable results in terms of total collagen production. TIs showed the expression of Collagen I, while Collagen III was not expressed; at the same timepoint, TRs showed a shift in collagen expression from Type III to Type I.

Conclusions. Aligned electrospun PLLA fibers demonstrated compatibility with tenocytes isolated from ruptured or intact tendons. Scaffolds promoted an aligned orientation of cells and the synthesis of Collagen I. As Collagen I is normally expressed in intact and healthy tendons, while Collagen III is expressed in ruptured or tendinopathic tendons, such results candidate this scaffold as promising for tendon augmentation strategies.

Keywords. Electrospinning; Aligned fibers; Tenocytes; Tendon regeneration

(25.O2) OPTIMIZED FABRICATION OF THREE-DIMENSIONAL RESORBABLE NONWOVENS COMPOSED OF BIODEGRADABLE POLYGLYCOLIC OR POLYLACTIDE ACID FOR THE TISSUE ENGINEERING OF HEART VALVES

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Introduction. The most prominent synthetic aliphatic polyesters are the degradable polyglycolic acid (PGA) and polylactide acid (PLA). PGA and PLA have been known for several decades; however commercial interest in them has risen in recent years due to the advancement of tissue engineered implants as substitutes for damaged organs. Different resorbable polymeric samples based on PGA and PLA were determined to examine which of them is the best-known concerning the correlation with vascular human cells, degradation parameters and negligible limitations and therefore is most suitable for the prospective fabrication of polymeric heart valve scaffolds.

Materials and Methods. For the fabrication of resorbable nonwovens polymeric granules were processed into multifilament fibers followed by a spinning process and conversion into staple fibers and later into fiber webs. The fiber webs were cross layered in 90°C direction. Myofibroblasts isolated from human umbilical cord arteries were seeded onto newly generated samples of PGA, PLA, a sandwich-structure composed of PLA/PGA, and the co-polymer PLGA and cultivated for 28 days under static culture conditions. Analysis of the cell-seeded polymeric samples included histology, immunohistochemistry, scanning and transmission electron microscopy.

Results. Scanning electron microscopy revealed a three-dimensional fibrous structure of all examined polymers. The pore size varied from 80µm to 300µm. After cultivation under static conditions all seeded polymers showed a shiny yellow surface around the samples. PGA displayed rapid shrinking after 21 days whereas all other tested samples did not shrink. Staining of the cells onto the polymers revealed expression of collagen type I and type III as well as smooth muscle actin.

Conclusion. Analyzing individual fabricated different polymeric scaffolds based on PGA and PLA, seeded with myofibroblasts from umbilical cord arteries and cultivated for several weeks, we found PLGA to be an optimal polymeric scaffold material for autologous tissue engineering of heart valves.

Keywords. tissue engineering, heart valves, resorbable scaffolds, polymers

(25.O3) IN VITRO AND IN VIVO COMPARATIVE EVALUATION OF HUMAN STEM CELLS SEEDED POLY-D,L-LACTIC ACID AND FIBROIN SCAFFOLDS

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Abstract: This study investigated the in vitro and in vivo behavior of porous scaffolds of poly-D,L-lactic acid (PDLLA) and silk fibroin (SF) prepared by salt leaching and cultured with Human Dental Pulp (DPSC) and Amniotic Fluid (AFSC) Stem Cells. Polylactic acid based polymers and copolymers have been widely employed in the production of scaffolds due to their mechanical properties, adaptability of degradation kinetics and easy processing into a variety of shapes and sizes. Silk fibroin has acquired a larger progressive interest for applications in tissue engineering due to the favourable results that have been obtained in vitro and in vivo in several proposed applications. In in vitro tests, the stem cells differentiation in osteoblastic cells has been studied. Osteogenic differentiation was evaluated by morphological, biochemical and immunocytochemical methods. Predifferentiated DPSC and AFSC seeded PDLLA and SF scaffold constructs were then implanted subcutaneously in a dorsal pocket and intraosseously in a parietal bone critical size bone of immunocompromised rats for 4 weeks and compared.

Subcutaneously, fibroin scaffold alone did not present mineralized matrix, while significant ectopic calcium deposition was found in hAFSC/fibroin constructs. On the other hand, mineralization was not evident in the PDLLA scaffolds. In ectopic implants, stem cells were present in the whole scaffold thickness in intimate contact with the scaffold walls and clearly expressing osteogenic markers. After 30 days of implantation, extended areas of mineralization were observed. Histological analysis of intraosseous graft demonstrated that the entire bone defect was completely repaired by stem cells-fibroin constructs by neo-formation of human lamellar bone. Furthermore AFSC-fibroin association showed a greater regenerative potential.

Keywords. Stem Cells, Osteogenic Differentiation, Fibroin

(25.P1) SURFACE ALKALI ETCHING OF POLY (L-LACTIDE-CO-GLYCOLIDE) BUT NOT OF POLYSTYRENE INCREASES ADHESION AND PROLIFERATION OF ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS

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Introduction. Adipose tissue-derived mesenchymal stem cells (ASCs) are multipotent adult stem cells relatively easy to obtain. These cells are able to differentiate into the mesodermal tissues and also can express, by transdifferentiation, nerve, cardiomyocyte, hepatocyte and pancreatic cell phenotypes. To use ASCs for tissue engineering a scaffold is usually need. The relationships between scaffolds and cell surfaces may be a determining factor for the results of the cell culture. We study the effects of alkali etching on different substrates on the behavior of ASCs cultures.

Materials and Methods. Adult Wistar rats were utilized following the guidelines of the European Communities Council (210/63/EU) for laboratory animal care and experimentation. ASCs were harvested from inguinal subcutaneous adipose tissue of anesthetized animals. The cells grew on Poly(L-Lactide-co-Glycolide) sheets (PLCG), and on polystyrene plates eitheretched or not with 1N NaOH, for 10 min. The culture medium was Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic.

Results. The number of adhered ASCs on alkali treated PLCG sheets was higher than on non-treated ones. Also, these cells spread more on treated than on non-treated PLCG surfaces. The cellular growth was faster on alkali treated PLCG sheets. On the contrary, we did not found any clear difference in cellular adhesion, spreading or proliferation of ASCs on treated and non-treated polystyrene plates.

Conclusions. Etching of PLCG sheets with NaOH may facilitates the cellular adhesion, increases the spread on the surfaces and improves the cell culture yields.

Supported by SAN 673/VA/23/08 grant of Junta de Castilla y León (Spain).

Keywords. Mesenchymal, stem cells, adipose, polylactic acid

(25.P2) FABRICATION OF POLYMER/STRONTIUM-SUBSTITUTED BIOACTIVE GLASS COMPOSITE MATERIAL FOR BONE TISSUE ENGINEERING SCAFFOLDS

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Introduction. In the last few years, electrospinning has gained widespread interest as a method to fabricate scaffolds for tissue engineering. The possibility of easily producing composites by including particles within the polymeric fibres made it a promising technique for the creation of materials combining biodegradability and bioactivity, two relevant features for bone tissue engineering. The aim of this study was to produce an electrospun composite material using poly-l-lactide-co-glycolide (PLGA) and particulate strontium-substituted bioactive glass, and to study its potential use as a scaffold.

Materials and Methods. Three glass compositions were produced based on work performed by O'Donnell at al. [1], where CaO was substituted with SrO in 45S5 bioglass type glasses. The glasses were melted in a Pt crucible for 3 hours at 1350°C and glass frits were ball-milled and sieved to obtain particles with sizes <45μm. The particles were incorporated in a solution of 20 wt. % PLGA 75:25 in tetrahydrofuran and then electrospun. Cytotoxicity tests of the scaffolds were performed with rat osteosarcoma (ROS) cells and the alamarBlue® assay. Scanning electron microscopy (SEM) was used to image the materials, and energy dispersive analysis (EDS) was used to characterise particles within the fibres.

Results. SEM showed that the composite fibres contained regions of increased fibre diameter, where EDS detected the aggregation of particles. Cytotoxicity tests demonstrated that ROS cells were able to grow in the presence of the material. The scaffolds displayed significant shrinkage after incubation at 37°C due to polymer contraction.

Conclusions. Electrospun PLGA/strontium-substituted bioactive glass composites were successfully fabricated and the material showed good levels of biocompatibility, making it a promising candidate to be used as a scaffold in tissue engineering applications.

Acknowledgements: The authors are grateful to the EPSRC for funding the studentship of Santocildes-Romero.

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Keywords. Electrospinning, Bioactive glass, Strontium, Scaffold, Bone tissue engineering

(25.P3) BIODEGRADABLE AND TUNABLE ELASTOMERS FOR SOFT TISSUES BIOMEDICAL APPLICATIONS

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Soft degradable elastomers having balanced amphiphilic behaviour and mid-term degradability are rarely described in the literature although, for the regeneration of soft tissues such as blood vessels, their ability to provide a structural support with similar mechanical properties to those of native tissues in dynamic environment makes them of a particular interest.[1] Among them, thermoset elastomers have the ability to degrade by combination of bulk and surface erosion which guarantee a constant shape during degradation as well as a linear drug release profile.[2] In this work we describe thermoset degradable elastomers based on photo-crosslinked poly(lactide)-poly(ethylene glycol)-poly(lactide) (PLA-PEG-PLA) triblock prepolymers. The originality of the proposed elastomers comes from the careful choice of the prepolymer amphiphilicity to provide intermediate degradation times and linear degradation profiles. Besides, we wish to illustrate the possibility to modulate the mechanical properties and degradation behaviours from the same initial tribloc depending on the presence and the nature of the cross-linker. This is illustrated with the hydrophobic and rigid 2,4,6-triallyloxy-1,3,5-triazine compared to the hydrophilic and soft pentaerythritol triallyl ether. Thermal properties, mechanical properties, degradation rates and cytocompatibility of the obtained biodegradable elastomers are presented.

Keywords. degradable elastomers, photo cross-linking, polyesters diblock, PLA, PEG, mechanical properties

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26. MASTERING SURFACE ASPECTS TO CONTROL BIOMATERIALS INTERACTIONS WITH CELLS AND TISSUES

Chair: João F. Mano
Co-chair: Rui L. Reis
Keynote speaker: Joachim P. Spatz
Organizer: João F. Mano

Synopsis: Among the characteristic of scaffolds for tissue engineering and regenerative medicine, surface aspects play an essential role in dictating the biological performance of the devices. In general, the initial acceptance or rejection of an implantable device is dictated by the crosstalk of the material surface with the bioentities present in the physiological environment; for tissue engineering, surface properties of biomaterials is also important in the cell seeding step and cell culturing prior implantation as important events will take place during such periods, including protein adsorption, cell adhesion, reorganization and proliferation and cell differentiation. Different surface properties should be considered with respect to the corresponding biological reaction, namely the chemical nature of the surface, the exposition of biochemical signals and physical/topological features. All of these points could be related with each other and also they can be displayed in the surface with some special organization (e.g. patterned organization at different length scales and geometries, gradient characteristics, or controlled randomness). Patterning methods and selective chemical modification schemes at different length scales can provide biocompatible surfaces that control cellular interactions on the micrometer and sub-micrometer scales on which cells are organized. In this symposium, the potential of chemically, biochemically and topographically micro-/nano-structured surfaces are discussed in hopes of a better understanding of cell–biomaterial interactions, including the recent use of biomimetic approaches or stimuli-responsive macromolecules. Different methodologies can be used to produce controlled topographic features that permit to establish adequate relationships with cellular behavior, that help in better understanding cellular machinery associate with surface interactions. This symposium will also address the complexity of correlating surface aspects that influence cellular behavior, which could be investigated by using high-throughput methodologies. Additionally, the focus will be on how the knowledge obtained using these surfaces can be incorporated to design implantable 3D constructs to be used in tissue engineering and regenerative medicine or in products to be used ex-vivo (e.g. in cell sheet technology or in stem cell expansion).

(26.KP) INDUCTION OF CELLULAR RESPONSES BY NANOSCOPIC ENVIRONMENTS

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Our approach to engineer cellular environments is based on self-organizing spatial positioning of single signaling molecules attached to inorganic or polymeric supports, which offers the highest spatial resolution with respect to the position of single signaling molecules. This approach allows tuning cellular material with respect to its most relevant properties, i.e., viscoelasticity, peptide composition, nanotopography and spatial nanopatterning of signaling molecule. Such materials are defined as

"nano-digital materials" since they enable the counting of individual signaling molecules, separated by a biologically inert background. Within these materials, the regulation of cellular responses is based on a biologically inert background which does not trigger any cell activation, which is then patterned with specific signaling molecules such as peptide ligands in well defined nanoscopic geometries. This approach is very powerful, since it enables the testing of cellular responses to individual, specific signaling molecules and their spatial ordering. Detailed consideration is also given to the fact that protein clusters such as those found at focal adhesion sites represent, to a large extent, hierarchically-organized cooperativity among various proteins. Moreover, "nano-digital supports" such as those described herein are clearly capable of involvement in such dynamic cellular processes as protein ordering at the cell's periphery which in turn leads to programming cell responses.

(26.O1) HIGH CELL ASPECT RATIO ALTERS STEM CELL TRACTION STRESSES AND LINEAGE

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Adult mesenchymal stem cells (MSCs) have been shown to respond to changes in their cell area and shape: cells respond to adipogenic and osteogenic media when spread cell area changes from 103 to 104 µm2, or when cultured as circles versus rectangles, stars, and polygons, respectively, by modulating myosin activity. Muscle is also a contractile MSC phenotype, and to induce this lineage using cell shape, we hypothesized that a spread but highly elongated cell morphology would be required along with muscle-mimicking substrate stiffness. Such an arrangement would generate sufficient contractile forces with the appropriate polarized orientation unique to muscle. Using microstamp printing, we patterned the adhesive protein fibronectin in shapes of varying aspect ratios (1:1, 3:1, 5:1, 10:1, and 15:1) but common area on mechanically defined thin films to regulate MSC morphology. Traction force microscopy (TFM) was used to monitor total substrate strain energy from cell-generated forces and found to depend on both substrate stiffness and cell shape. Similarly, myosin heavy chain was expressed in an elongation-dependent manner. Correlated to these contractility-based observations were shape- and stiffness-dependent lineage changes, suggesting a quantitative link between contractility and cell fate. These findings validate the use of TFM as a novel tool for assessing cell fate. Probing environments with multiple simultaneous but disparate extracellular cues, e.g. elongated pattern on osteogenic stiffness, may reveal stem cell subpopulations capable of responding only to specific cues and suggesting that any myogenic subpopulations that are stiffness-independent could be used as a regenerative therapy for fibrotic muscle diseases.

Keywords. Traction forces, mesenchymal stem cells, protein printing

(26.O2) DEFORMATION OF NUCLEI AND MICROSTRUCTURES: EVIDENCE OF DIFFERENT MECHANORESPONSIVENESS OF CANCER AND NON CANCEROUS CELLS

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Recent evidence suggests that mechanical properties of extracellular matrix, particularly rigidity, can mediate cell behavior. In this study, response of cells and their nuclei to rigidity of substrate was examined by culturing human osteosarcoma cells (Saos-2) and mouse fibroblasts (L929) on polymeric surfaces composed of microfabricated high aspect ratio pillars. Microstructured films of PLLA and blends of PLGA 50:50:PLLA (2:3) were produced by solvent casting on a PDMS template. PLLA is a highly crystalline polyester (37% crystallinity) with a high moduls of about 4.8 GPa, whereas its glycolide containing copolymers (eg. PLGA) are of much lower crystallinity and mechanical strength. Thus, it would be possible to compare mechanoresponsiveness of cancer and non cancerous cells on surfaces with different stiffness. The nucleus and cytoskeleton of the cells were examined by fluorescence microscopy. SEM was used to examine the interaction of the cells with the micropillars.

Saos-2 and L929 showed distinct differences in terms of nuclear deformation (Figure 1). Nuclei of Saos-2 showed indented borders around the pillars on both stiff and soft micropillars. In contrast, nucleus deformation in L929 cells was observed on the softer pillars only. On the stiff surfaces they gained a lobular appearence. These differences in nucleus deformation behavior of two cell types might be due to easier deformability of cancerous cells.

SEM examinations showed pillars bent to varying degrees. This was consistent with rigidity of micropillars (Figure 1). On the stiff PLLA micropillars Saos-2 and L929 cells were localized on the top of the pillars, but both types of the cells spread over softer PLLA-PLGA pillars. An interesting observation is the sagging of the cytoplasm and the nuclei of Saos-2 cells between the pillars. This may be another indication of the flexible nature of these cancer cells.

Keywords. Nucleus deformation, high aspect ratio micropillars, pillar bending,rigidity

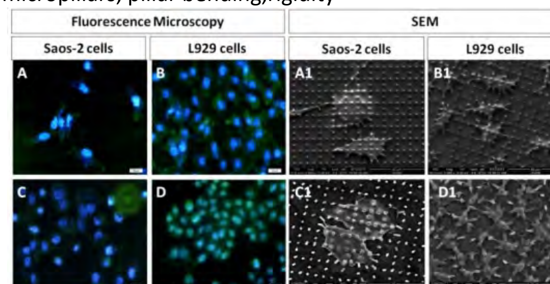

Figure 1. Fluorescence and SEM micrographs of Saos-2 and L929 cells grown on the PLLA and PLLA-PLGA 50:50 micropatterned films. Time: 2 Days. Stains: FITC-Phalloidin (green, for cytoskeleton) and DAPI (blue for nuclei). Scale bars of fluoresent micrographs, A,B,C and D: 20 µm. Scale bars of SEM micrographs, A1 and B1: 40µm, C1 and D1:30 µm.

(26.O3) CELLULAR PHENOTYPIC RESPONSES TO CULTURE ON THERMORESPONSIVE POLYMER BRUSHES

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Introduction. Biocompatibility of novel materials is determined by material surface characteristics, protein adsorption, and the cellular phenotypic response, all of which are interrelated. Recently, smart bioactive materials have emerged, capable of performing sophisticated interactions with biological tissues. Thermoresponsive polymer brushes are one category of bioactive materials that allow for the gentle detachment of cells from their culture surface. Traditionally, cells are biochemically released from tissue culture plastic (TCP), disrupting cell-cell interactions and destroying membrane-associated proteins. Thermoresponsive polymer brushes offer a unique culture system whereby cells are released by simply changing the temperature from 37oC to 25oC. In this work, we investigated changes in cellular phenotype in response to culture on thermosensitive bio-inert materials compared to TCP.
Methods. Thermoresponsive polymer brushes with a tunable lower critical solution temperature (LCST) were prepared from random copolymers of 2-(2-methoxyethoxy)ethyl methacrylate (MEO2MA) and oligo(ethylene glycol) methacrylate (OEGMA). Mouse fibroblasts (L929, ATCC) were cultured on both thermoresponsive brushes and TCP for 48 hours and then harvested for gene expression analysis using real time RT-PCR. Genes regulating cell adhesion (FN1, Dusp2, RhoA), inflammation (IL-6), and apoptosis (Bcl-2, trp53) were evaluated. Cell morphology was assessed using phase contrast microscopy at 0, 6, 18, 24, 30, and 48 hours.
Results. No obvious morphological differences were observed in cells cultured on brushes or TCP. Gene expression analysis has revealed a small upregulation of all genes on thermobrushes relative to TCP. Specifically, fold changes ranged between 1-1.6 for all genes; future studies must determine if this change is biologically significant.
Conclusions. Thermoresponsive polymer brushes are biocompatible and non-cytotoxic. Thus, they can provide an alternative method for growing confluent cellular monolayers in vitro that can be released mechanically, eliminating the use of digestive enzymes, preserving cell-cell interactions, and creating ‘cell sheets’ for potential implantation.
Keywords. cell-biomaterial interactions, smart surfaces, gene expression

(26.O4) ENDOTHELIAL PROGENITOR CELLS (TRANS)DIFFERENTIATE ON SOFT SUBSTRATES

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Introduction. Novel in situ vascular tissue engineering approaches aim at capturing and differentiating cells from the blood stream. Endothelial Progenitor Cells (EPCs) have been demonstrated to differentiate into endothelial cells (ECs) and transdifferentiate into smooth muscle cells (SMCs) under the influence of biochemical cues. The influence of mechanical cues is unknown, but relevant considering the micro-mechanical cell environment after capture. The aim of this study is to investigate the effect of substrate stiffness on EPC (trans)differentiation.
Materials and Methods. Endothelial Colony Forming cells (ECFCs) were isolated from mononuclear cells fraction of human peripheral blood and seeded on fibronectin-coated polyacrylamide gels, prepared with 3 different stiffness values: soft (3kPa), intermediate (20kPa), and stiff gels (80kPa). As control group, cells were seeded on glass. Cell behavior was assessed 48 h after seeding by measuring cell proliferation, focal adhesions (FAs), phenotypic markers (CD31 and αSMA), and collagen production.
Results. Proliferation of ECFCs was dependent on the stiffness of the substrate, being the lowest on soft gels. This behavior is correlated to differences in cell adhesion: only few FAs groups are visible on cells cultured on soft gels, compared to a greater amount on stiffer substrates.Moreover, all the cells showed a comparable expression of CD31while differences were noticed for the transdifferentiation marker αSMA: the intensity of the staining was higher on soft gels when compared to stiff gels. Finally, ECFCs always produced collagen, that passed from an intracellular to an extracellular matrix component within 3 days of culture.
Discussion and Conclusion. Preliminary results show that cell proliferation and (trans)differentiation can be manipulated with substrate stiffness. Moreover, substantial extracellular matrix is produced by the cells on all samples, but in greater amounts on stiff substrates. Further analysis with FACS should indentify the variations in marker expression within the different groups to better characterize cell phenotype.
Keywords. Endothelial progenitor cells; transdifferentiation; cell-matrix interaction

(26.O5) DIFFERENT ARRANGEMENT OF TYPE IV COLLAGEN ON MODEL –NH2 AND –COOH SURFACES ALTERS ENDOTHELIAL CELLS INTERACTION

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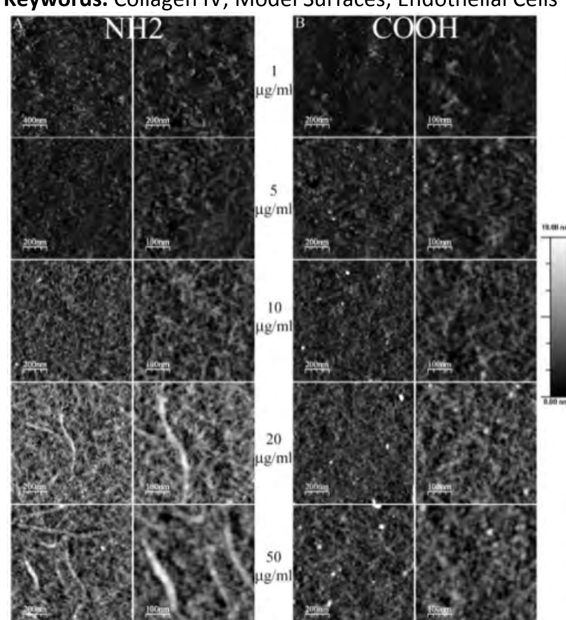
The initial cell-biomaterials interaction mimics to a certain extent the natural communication with the extracellular matrix (ECM); it starts with the adsorption of matrix proteins from the surrounding medium followed by cell adhesion, spreading and polarization. In some cases other, less soluble ECM proteins such as collagens may also associate with the biomaterial surface eliciting distinct cellular response dependent on the initial protein conformation. Recently we are interested on the behaviour of adsorbed type IV collagen (Col IV) – a unique multifunctional matrix protein that plays a crucial role in the organization of basement membrane (BM). Previously we have shown that the substratum wettability may strongly affect Col IV adsorption pattern which alters the endothelial cells interaction. Now we introduce two new model surfaces (SAMs), a positively charged -NH2 and negatively charged -COOH to learn more about the effect of substratum chemistry. The amount of fluorescently

labelled Col IV was quantified showing about twice more adsorbed protein on NH2 substrata. AFM studies revealed distinct pattern of Col IV assembly upon adsorption, resembling different aspects of a network-like structures. We further found that human umbilical vein endothelial cells (HUVEC) attach less efficiently on negatively charged –COOH surface, as judged by the altered cell spreading, focal adhesions formation and actin cytoskeleton development. Conversely, immunofluorescence for integrins revealed better Col IV recognition on positively charged NH2 substrata, moreover by both alpha-1 and alpha-2 heterodimers, apart from -COOH surface where almost no integrin clustering was observed. The expression of p-FAK was also higher on -NH2 substrata confirming better transmission of adhesive signals to the cells interior. Collectively, these results point to the prospectively of such surface functionalization for tissue engineering application.

Keywords. Collagen IV; Model Surfaces; Endothelial Cells



(26.O6) NANOSTRUCTURED FILMS EXHIBITING MULTIPLE STIMULI RESPONSIVE BEHAVIOR AND IMPROVED CELL ADHESION FOR TISSUE ENGINEERING

Costa RR (1), Custódio CA, (1), Arias FJ (2), Rodríguez-Cabello JC (2), Mano JF (1)

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Introduction. The design of smart and nanostructured surfaces has allowed to achieve significant advances in tissue engineering. Emphasis has been given to layer-by-layer (LbL) adsorption of polymers for being a simple and versatile technique. In this study, we report the use of chitosan and an elastin-like recombinamer (ELR) containing RGD to produce such nanosctructured multilayers. ELRs are smart genetically engineered polypeptides which allow conceiving ultrathin films with biologically relevant sequences.

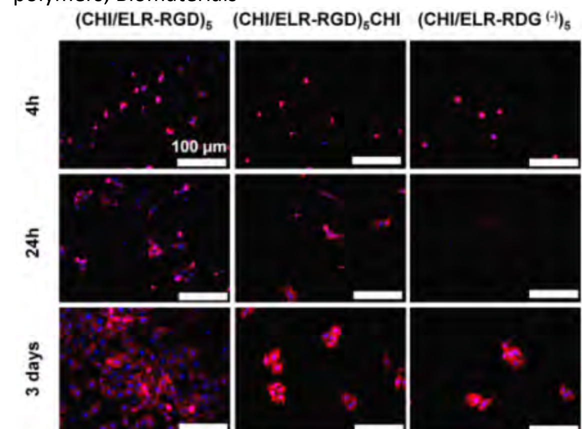
Methods. The buildup of chitosan/ELR-RGD films was monitored using a quartz-crystal microbalance. Their wettability and smart properties were studied by contact angle measurements as a function of temperature, pH and ionic strength. The topography was observed by AFM. SaOs-2 cell adhesion was also studied in vitro.

Results. Chitosan and ELR-RGD films were constructed based on electrostatic and hydrophobic interactions. Contact angle measurements revealed cyclic, acute and independent wettability changes around stimuli transition values of 50 ºC, pH 11, and ionic strength of 1.25 M. Below each transition value, the surfaces were moderately hydrophobic. Above, they acquired an extremely hydrophilic character. The transition was also evident in the topography changes, becoming rougher with increasing pH. SaOs-2 cells showed increased adhesion and activity for films ending with ELR-RGD ending films, comparing to chitosan and a nonfunctional ELR-RDG peptide.

Conclusion. This work demonstrates the potential to use natural and recombinant macromolecules to engineer multilayered and nanostructured surfaces, which can be further extrapolated to more complex devices. The potential of this work can stimulate the development of other similar biomedical products processed through LbL for pharmaceuticals delivery and structures for tissue engineering.

Acknowledgments. Fundação para a Ciência e Tecnologia (SFRH/BD/61126/2009, SFRH/BD/61390/2009) European regional development fund, MICINN (MAT 2009-14195-C03 03, MAT2010-15982, MAT2010-15310, IT2009-0089, ACI2009-0890), JCyL (VA034A09), Instituto de Salud Carlos III ("Network Center of Regenerative Medicine and Cellular Therapy of Castilla and León").

Keywords. Smart coatings; Layer-by-layer; Recombinant polymers; Biomaterials



(26.O7) BIOINSPIRED SUPERHYDROPHOBIC POLYSTYRENE SURFACES FOR SPATIAL CONTROL OF CELL ATTACHMENT/PROLIFERATION

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Introduction. In this work we propose a biomimetic and simple approach for spatial control of cells, correlating the surface wettability in the range of superhydrophobic to superhydrophilic with SaOs-2 attachment and proliferation.

Methods. Superhydrophobic polystyrene (PS) surfaces were produced by a phase separation method and the in vitro performance of SaOs-2, L929 and ATDC5 cell lines assessed in terms of cell morphology and dsDNA content, after 4 hours, 2 and 6 days in incubation. Smooth and

rough PS surfaces were chemically modified with UVO irradiation during 6, 9 and 18 minutes to control surface wettability. MTS assay and dsDNA quantification were performed on rough and smooth PS surfaces with controlled wettability with SaOs-2 cells after 4 hours, 2 and 6 days in culture. Superhydrophilic patterns were made on the superhydrophobic PS surfaces using UVO irradiation and hollowed masks. Cellular suspension of SaOs-2 cells was dropped over whole surface or only on the wettable areas and surfaces observed by microscopy after 24 hours in incubation. The surfaces were characterized by contact angle measurements, XPS analysis, optical profilometry and SEM.

Results. ATDC5 and SaOs-2 cell lines were not able to proliferate on PS superhydrophobic surfaces. After 4 hours in culture, the attachment of SaOs-2 cell line was higher on superhydrophilic PS surfaces whose serum proteins effects seemed reduced, although the proliferation was higher in surfaces with water contact angles ranging from 30 to 13°. SaOs-2 cells remained adhered after 24 hours in incubation on the patterned superhydrophilic regions in both cases (Fig. 1).

Conclusions. This work suggests that the introduction of random micro/nano roughness and further chemical modification by UVO irradiation may be an elegant and easy method to control spatially the attachment/proliferation of cells in distinct materials with possible uses in high throughput, microfluidic or even in 3D systems.

Keywords. Wettability; superhydrophobic; patterning

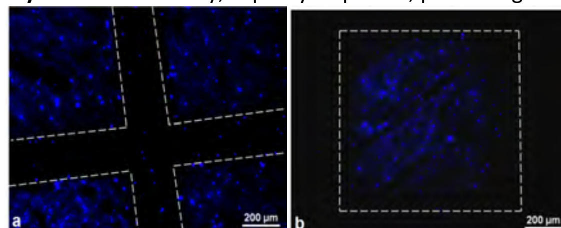

Fig.1. Fluorescent staining of SaOs-2 cell nucleus with 4', 6-diamidino-2-phenylindole (DAPI) after 2 days in culture: on samples where cell were seeded over whole surface (a), and in open-air culture where 7 µL of the cellular suspension was drop on the superhydrophilic region.

(26.O8) IMPACT OF PET MULTI-FUNCTIONALIZATION ON ENDOTHELIAL CELL BEHAVIOR UNDER SHEAR STRESS

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Over the last three decades, the envisaged strategies to develop biocompatible vascular materials have considerably evolved. Nowadays, several research group objectives are targeted towards the development of proactive materials. It is expected that these materials would promote specific interactions with the physiological environment, therefore leading either to tissue regeneration or repair. The main strategy used in our work is the co-immobilization of two different peptides onto biomaterial surface in order to improve cell functions, here at the same time cell adhesion and cell proliferation. Today, the current consensus is to distribute the bioactive ligands in a controlled fashion in the form of micro-patterns, no longer in a homogeneous or a statistically based way as previously considered the ideal approach. In this study RGDS peptides (adhesion peptides) and WQPPRARI peptides (proliferation peptides) (1) were micropatterned by photolithography process which typically consists of the transfer of a specific pattern to a photosensitive material (a photoresist) by selective exposure to a radiation source. Our work focused on characterizing connections between cells through their actin filaments, and quantifying cell attachment through the formation of focal adhesion features.

Fluorescent microscopy permits to validate homogeneous (RGDS + WQPPRARI peptides) and micro-patterned peptides grafting onto PET surfaces. Concerning biological evaluation we used primary human saphenous vein ECs (HSVECs) isolated from vein remnants provided by a cardiovascular surgery department. After 3 days of culture, a laminar flow shear stress (ss) was applied during 2h and 6h at 12 dynes/cm². We observed cells after fluorescent labeling of actin filament and vinculin on order to quantify focal adhesion. In static conditions patterning doesn't affect cell shape. However, in dynamic conditions patterning seems to improve cell orientation and focal adhesion formation in comparison with cells cultured on homogenous material (Fig.1).

Keywords. Micro-patterning, Peptides grafting, Vascular graft

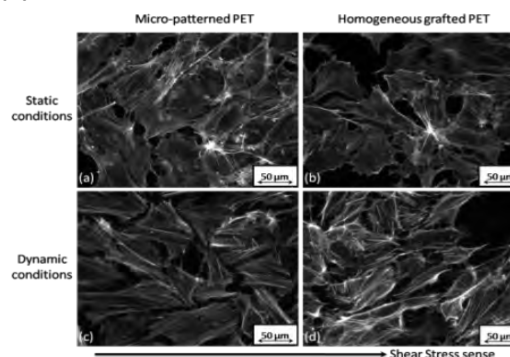

Fig.1. Fluorescent staining of actin in HSVECs cultured on: (a) micro-patterned PET before ss, (b) homogeneous PET before ss, (c) micro-patterned PET after ss and (d) homogeneous PET after ss.

(26.O9) DESIGNING ELECTROSPUN SCAFFOLDS FOR TISSUE ENGINEERING APPLICATIONS

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Introduction. There are many studies indicating that cell adhesion and proliferation is enhanced on nanofibers compared to microfibers. However, small pore sizes and limited porosities of nanofibrous materials limit cell infiltration into the materials and may also hinder transport processes and vascularization in the innermost parts of the materials. A new way of creating highly porous nanofibrous scaffolds investigated in this work was coating single microfibers with electrospun nanofibers. The nanofibers are then present to enhance cell adhesion and spreading, whereas the larger dimensions of the microfiber makes scaffolds of such fibers more porous with larger pores.

Methods. Single PLA microfibers were coated with PCL or PLA nanofibers by use of electrospinning. Possibilities of creating hierarchical structures were investigated as fiber design was studied regarding nanofiber loading, diameters and alignment on the microfibers. Scaffolds with porosities of over 95% were made and seeded with chondrocytes to study cell infiltration.

Results. The study showed that the nanofiber-coated microfibers could be designed in terms of nanofiber diameters, nanofiber alignment and nanofiber loading. This is of importance in tailoring of materials for different tissue engineering applications. It was furthermore shown that chondrocyte infiltration into scaffolds of nanofiber-coated microfibers was greatly enhanced compared to the infiltration in pure nanofiber scaffolds.

Conclusions. This work shows that coating microfibers with nanofibers is a method with great potential in the design of functional scaffolds with very high control of architecture and topography, from nano- to macroscale, i.e. from nanofiber morphology to scaffold porosity.

Keywords. Electrospinning, nanofibers, scaffold architecture

(26.O10) INNOVATIVE LAYER-BY-LAYER METHODOLOGY, BEHAVING AS AN ALL MOLDABLE PLATFORM, FOR GENERATING MACRO 3D NANO-STRUCTURED POROUS CONSTRUCTS USING FREEFORM TEMPLATES

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Introduction: An innovative multilayering concept methodology was used to develop 3D nanometric scale structures from moldable macro template obtained from the random arrangement of wax spheres showing good cell viability and proliferation rate. This work focuses on the versatility of this technique as an all moldable platform to generate 3D porous constructs having different external shapes and varied internal organizations by controlling the spatial arrangement of wax beads with different sizes.

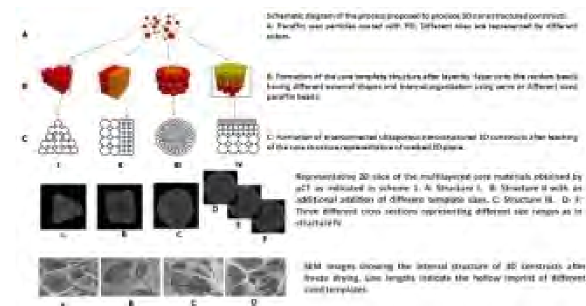
Method: 1mg/ml sodium alginate and chitosan in 0.15M NaCl were adsorbed sequentially over the wax spheres with mean diameter 420 µm coated with Poly(ethylenemine) (0.5 wt % solution in water) in a free standing packing over a perforated cylinder assembly. After 10 bilayer coatings, DCM was used for removing wax beads followed by freeze drying. Stacking of layers with different size wax beads was done to produce patterns. Micro CT, florescence and scanning electron microscopy, were used for characterization.

Results: The packing of paraffin wax spheres, critical to the formation of the 3D structure, remained stable during multilayering process even for the different internal organizations. Fine 3D constructs composed entirely of polyelectrolyte multilayers with interconnected pores were obtained after leaching and freeze drying with a potential to be used in tissue engineering.

Conclusion: Perfusion technique maintained its directional flow through the template core even though using different sizes of templates to form diverse patterns. This supports its use in forming complex 3D structures without using any binder. The layers withstood multiple changes in the system signifying excellent mechanical strength. This new approach of multilayer formation over the sacrificial paraffin wax spheres in 3D with patterns and different shape were realized. Desired results can be achieved by adding functionalities for tissue engineering

Acknowledgment: Praveen Sher thanks FCT for post-doc grants.

Keywords. Layer-by-layer, Moldable template, leaching, 3D porous constructs



(26.O11) THE USE OF PLASMA POLYMERIZATION IN THE CONTINUING DEVELOPMENT OF CELLULAR THERAPIES

Steele D (1), Low S (1), Szili E (1), Al-Bataineh S (1), Brown K (2), Mariappan I (3)

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During the late 1990's it was demonstrated that plasma polymerization; a method of coating complex geometries such as tissue cultureware with specific chemistries, could be utilized in the development of a cellular therapy for skin. This research led to an autologous clinical product, Myskin™ for burns and ulcers. Subsequently an allogeneic product, Cryoskin™ was approved on a named patient basic. Since these early years research has sought to build upon this knowledge to develop additional cellular therapies and to further refine the engineering of surfaces capable of better mediating the biomaterial-biological interface.

In describing the development of this enabling technology I will draw upon examples of the research programs from the bioengineering laboratories and cell therapy suites at the University of South Australia. These programs can broadly be categorized: In the first instance further developments of the core technology are seeking a cellular therapy for corneal epithelium utilizing a plasma polymer coated contact lens as the cell delivery vehicle. An additional development seeks to refine the biomaterial-biological interface, via incorporation of glycosaminoglycans to the plasma polymer coating, in the advance of a cellular therapy for retinal pigment epithelium. Lastly, I will outline a number of strategies designed to investigate interactions at the biomaterial-biological interface in a range of formats including microarrays and microfluidic chips, both of which are suitable for the fabrication of "lab on a chip" assays.

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Keywords. Biomaterials, Glycosaminoglycans, Plasma Polymerization

(26.O12) THROMBIN ACTIVITY IN FIBRIN SEALANTS – A CRITICAL FACTOR FOR CELL COMPATIBILITY WITH HUMAN KERATINOCYTES

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1. Baxter Innovations GmbH, Biosurgery; 2. Ludwig Boltzmann Institute for Experimental and Clinical Traumatology

Introduction. Fibrin sealant (FS) clots have been shown to promote different aspects of tissue regeneration in a similar way as their physiological counterpart, the blood clot. Since thrombin binds non-covalently to fibrin during clot formation, we evaluated the contribution of thrombin to the cell compatibility of FS. As a model cell type human primary keratinocytes (NHEK) have been employed. We analyzed cellular responses of NHEK seeded on top of fibrin clots prepared with different thrombin concentrations but identical fibrinogen component. Compatibility of NHEK with commercial FS formulations will dictate FS use as cell delivery matrix to stimulate cutaneous wound healing.

Materials and Methods. Fibrin clots were prepared with 3 thrombin concentrations (4 IU/ml, 505 IU/ml, 820 IU/ml). NHEK were seeded on top. Number of attached cells was determined on clots after different time-points by measuring lactate dehydrogenase, morphology was analyzed by phalloidin-DAPI-staining, and viability was monitored by live/death staining. Apoptosis was assessed by a caspase 3 and 7 activity assay. Expression of TrailR2 and Fas was evaluated by western blot analysis. Fibrin bound thrombin was blocked by hirudin to monitor thrombin specific effects.

Results. A dose-dependent increase in thrombin activity was observed on the surface of fibrin clots. These clots did not differ in their three-dimensional structure as shown by SEM. Clots prepared with highly concentrated thrombin (820 IU/ml) failed to support adhesion and spreading of NHEK. Apoptosis mechanisms seem to be responsible for these effects since the number of dead cells was clearly increased, and the expression of cleaved caspase 3 and 7 and of TrailR2 and Fas was strongly enhanced on fibrin clots with high thrombin activity. These effects were reversed by the addition of hirudin.

Keywords. fibrin sealants, thrombin, cell compatibility

(26.P1) ALTERED FIBRONECTIN ADSORPTION AND CELL ADHESION ON SUPERHYDROPHOBIC POLYSTYRENE

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Introduction. Wettability plays a key role in the interaction of surfaces with proteins and cells. Usually studies in this context are performed in substrates with wettabilities ranging from hydrophilic to hydrophobic. Biomimetic inspiration allows to produce surfaces with extreme water contact angles by combining micro/nano roughness in hydrophobic materials. This work investigates and compares fibronectin (FN) adsorption and MC3T3-E1 cell response on superhydrophobic polystyrene (SH-PS) and on smooth polystyrene (PS) with the same surface chemistry.

Methods. SH-PS was produced by a phase-inversion method. FN adsorption was quantified and characterized by western blot and Enzyme-Linked ImmunoSorbent Assay respectively. MC3T3-E1 cells were obtained from the RIKEN Cell Bank and cultured on the previously protein-coated substrates in serum-free conditions. Early cell adhesion was investigated by the expression of focal adhesion proteins (vinculin, paxillin, talin), cell morphology by scanning electron microscopy (SEM) and cell signaling by quantifying the phosphorylation of focal adhesion kinases (pFAK). The evolution of the cell density was followed up to 21 days.

Results. FN adsorption and cell adhesion on SH-PS were compared with the corresponding PS substrate and the control glass. Our results showed that even if superhydrophobicity hinders the contact of the material with the liquid, FN adsorption did occur on SH-PS, although in lower density and altered conformation. As a result, deficient early cell adhesion was obtained on the superhydrophobic material, where cells were characterized by an immature cytoskeleton, lack of focal adhesions and inactivation of the pathways upregulated by the pFAK (Figure 1A). Cell proliferation could be found on the SH-PS although being significantly lower than in PS (Figure 1B).

Conclusions. These results raise some doubts on the application of superhydrophobic surfaces to inhibit protein adsorption and cell adhesion in biomedical applications.

Acknowledgments. This work was supported by MAT2009-14440-C02-01.
Keywords. Superhydrophobicity, fibronectin, protein adsorption, cell adhesion

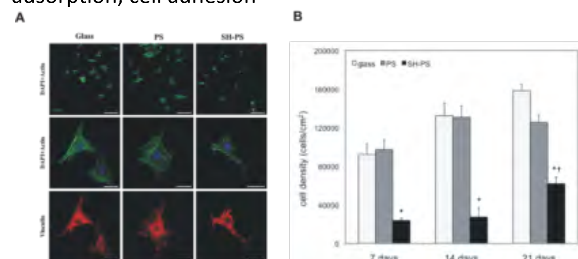

Figure 1. A) Actin cytoskeleton development and focal adhesion formation on the different material surfaces after 3 h of culture. B) Evolution of cell density on the different materials up to 21 days.

(26.P2) STEM CELL CAPTURE BY CHITOSAN MEMBRANES COATED WITH SPECIFIC ANTIBODIES

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One of the major goals in biomaterials science is to accurately control cellular interactions with implantable surfaces. [1] Covalent grafting of biomolecules is a strategy to improve the biocompatibility and bioactivity of materials. [2] In the present work we study the biological properties of chitosan, in which the surface was modified with an antibody for a specific type of cell. Using BS3 (Bis(Sulfosuccinimidyl) suberate), a crosslinker that will promote the covalent attachment of the antibody to the chitosan membranes we intend to functionalize the membranes. With this functionalized surfaces we aim to create smart surfaces that recruit a specific type of cell from a mixed cell population. Human adipose stem cells (ASCs) and SaOs-2 cells were seeded onto these surfaces to assess the biological consequences of such modifications.

It was found that after short times incubation the cells that recognize the immobilized antibody were widely attached to the surface and significantly fewer cells were detected in unmodified membranes and in membranes with antibodies that are not recognized by the cells. The results of cell adhesion studies indicated that the antibody was successfully grafted to the membranes. After 24h incubation, ASCs were adhered and well spread on membranes functionalized with CD90 and CD105, even in the absence of FBS. In opposition to unmodified membranes or functionalized with CD3 few cells adhered. The population of cells attached on the membrane was further characterized by flow cytometry. SaOs-2 adhesion for CD90 and CD105 modified membranes present also few cells indicating that only cell that recognize the antibody attach to the surface. Using PDMS stamps to immobilize antibodies we were able to produce microcontact printing features on the membranes, creating well defined regions were the cells attach. As main conclusion, were able to produce chitosan-based surfaces that recruit and immobilize with spatial control specific cell populations depending on the immobilized antibody.

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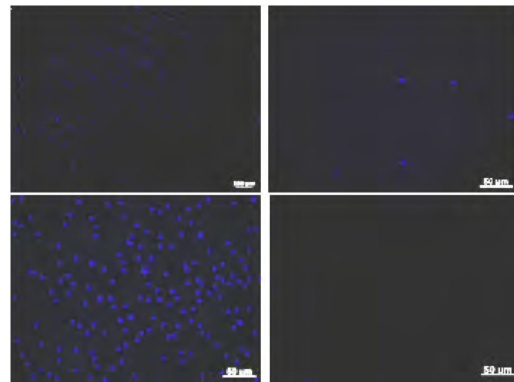
Keywords. chitosan, surface modification, cell recruitment, antibody immobilization



A) Saos-2 cells on immobilized CD90; B) ASCs on non-modified CHIT; C) ASCs on immobilized CD90; and D) ASCs on immobilized CD3

(26.P3) PLASMA-FUNCTIONALIZED BONE SUBSTITUTES FOR BETTER ADHESION AND PROLIFERATION OF HUMAN MESENCHYMAL STEM CELLS

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Introduction. The development and improvement of new materials for bone tissue engineering is essential due to the lack of autologous material and transmission of diseases by using allogenic tissue. Desirable substitutes display highly porous biodegradable materials that induce proliferation and osteogenic differentiation. In this study different chemical groups were introduced via plasma functionalization on polystyrene surfaces and bone substitutes. Human mesenchymal stem cells (hMSCs) were seeded on the modified substrates and were analyzed concerning cell adhesion and proliferation.

Experimental methods. Ammonia-, acrylic acid- and CO2-plasma was applied to functionalize polystyrene surfaces. Functionalization was proven by X-ray photoelectron spectroscopy, colorimetric methods and contact angle measurements. HMSCs were cultured for 72 h on the functionalized substrates and analyzed throughout the study using the live/dead assay (FDA/PI) and proliferation assay (WST-1). According to the results of the polystyrene surfaces, bone substitutes (ChronOS®) and polylactides-scaffolds were treated with ammonia-plasma and were evaluated respectively cell adhesion.

Results and discussion. After the application of low pressure plasma on polystyrene surfaces, a significant increase of amino and carboxy groups was measured. The metabolic activity assay WST-1 revealed a similar proliferation rate of hMSCs on the modified surfaces compared to the tissue culture dishes, which served as positive control. Especially amino functionalization showed an increased proliferation rate. Therefore, ChronOS® and polylactides- scaffolds were functionalized with ammonia plasma. Functionalized polylactides-scaffolds displayed better cell adhesion compared to non-modified ones (Fig. 1).

Conclusion. This work verifies an influence of functional groups on the adhesion and proliferation of hMSCs. Especially ammonia-plasma presents a promising modification method. Further studies concerning osteopromotive properties of plasma treated samples will be performed.

Acknowledgments. We would like to thank the Fraunhofer Gesellschaft for the financial support.

Keywords. Bone Tissue Engineering, Low-pressure plasma, Mesenchymal stem cells

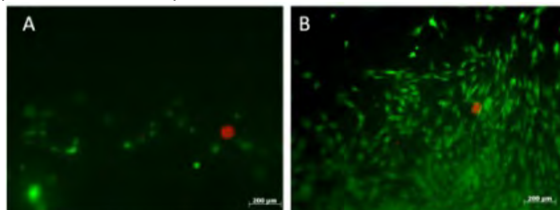

Fig. 1: FDA/PI staining of hMSCs on non-modified (A) and amino-functionalized (B) polylactides-scaffolds.

(26.P4) COMBINATORY ASSESSEMENT OF INTERACTIONS BETWEEN BIOMATERIALS, PROTEINS AND CELLS USING PATTERNED SUPERHYDROPHOBIC SUBSTRATES

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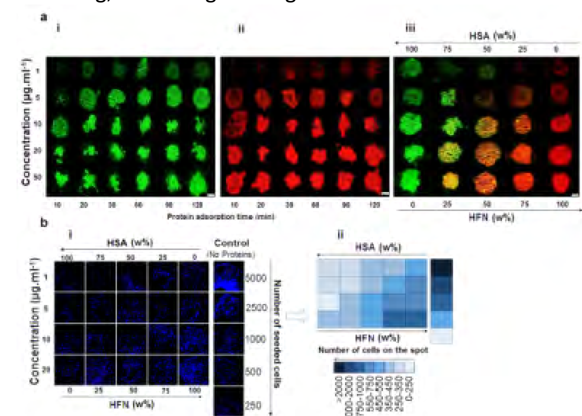
Introduction: A new single and low cost set-up for high-throughput screening analysis was developed that permits to screen the biological performance of combinations of biomaterials, cells and culture media. Patterned superhydrophobic flat substrates with wettable spots were used to produce microarray chips for such multiplexing evaluation. Different combinations of proteins was confined in the spots and milimetric volumes of different culture liquids was individually dispensed, as a result of the strong contrast of wetabilities between the initial substrate and the modified regions.

Methods: Superhydrophobic polystyrene surfaces were processed using a one-step phase-separation methodology and patterned using UV/Ozone radiation. The wettability, morphology and chemistry of the surfaces studied were assessed by water contact angle, scanning electron microscope and X-ray photoelectron spectroscopy, respectively. Different combinations and amounts of human serum albumin (HSA) and human plasma fibronectin (HFN) were absorbed onto hydrophilic regions. Cells culture studies were performed with SaOs-2 human osteoblast-like cells. The fluorescence signal were recorded by reflected /transmitted light and confocal microscope These microscopes reveals the rearrangement a number of proteins and cells in each microchip spot.

Results: As expected, the number of cells attached into each spot was higher for higher amounts of pre-adsorbed HFN or in surfaces where the relative amount of HFN in HFN/HSA was higher. Advances in this field should offer the possibility to screen individually and in the same chip different combination of biomaterials under different condition, including different cells, culture medium or solutions with different proteins or other molecules.

Conclusions: Such inexpensive and simple bench-top method, or simple adaptations from it, could be integrated in tests involving larger libraries of substances that could be tested under distinct biological conditions, constituting a new tool accessible to virtually anyone to be used in the field of tissue engineering and regenerative medicine.

Keywords. protein adsorption; superhydrophobic surfaces; cell-materials interactions; high-throughput screening; tissue engineering



27. MECHANICAL BEHAVIOUR OF CELLS, SCAFFOLDS AND ENGINEERED TISSUES

Chair: Fergal O'Brien
Co-chair: Brendan Harley
Keynote speaker: Dietmar Hutmacher
Organizers: Fergal O'Brien, Brendan Harley

Synopsis: The native ECM is instructive, providing a dynamic and spatially heterogeneous constellation of microstructural, mechanical, and compositional cues that can influence cell behavior. It has become increasingly clear that the mechanical considerations are equally important and interact synergistically with the chemical and microstructural microenvironment environment in directing cell fate. Mechanical properties of tissues, biomaterials, cells, and biomolecules have profound biological consequences in terms of implant bioactivity versus failure, transmission of mechanical stimuli, and for a wide range of processes at the tissue (e.g. stress shielding, strain transmission, deformation, failure), cell (e.g. stem cell differentiation, cell motility, cell adhesion, cell-cell interactions), and subcellular (e.g. protein-folding, cytoskeletal organization, DNA) levels. Further, mechanotransduction pathways activated in response to mechanical force are essential for the maintenance and function of tissues and can be exploited for tissue engineering applications to direct cell function. It is now clear for example that substrate stiffness can regulate stem cell differentiation. Mechanical behaviour also plays a key role in biomaterials and scaffolds for tissue engineering. Ideally, a scaffold should have mechanical properties consistent with the anatomical site into which it is to be implanted and, from a practical perspective, it must be strong enough to allow surgical handling during implantation. While this is important in all tissues, it

provides some challenges for orthopaedic applications specifically. Producing scaffolds with adequate mechanical properties is one of the great challenges in attempting to engineer bone or cartilage. For these tissues, the implanted scaffold must have sufficient mechanical integrity to function from the time of implantation to the completion of the remodeling process. However, as the field of tissue engineering has evolved, it could be argued that too much focus has been placed on trying to develop scaffolds with mechanical properties similar to the host tissue. Many scaffolds have been produced with good mechanical properties but reduced porosity and these materials, which have demonstrated potential *in vitro* have failed when implanted *in vivo* due to insufficient capacity for vascularisation. It is clear therefore that a balance between mechanical properties and a porous architecture sufficient to allow cell infiltration and vascularisation is key to the success of any scaffold.

This symposium will encompass approaches to both: 1) probe the mechanical behaviour, deformation, damage, and failure under applied forces of scaffolds as well as biological materials at the tissue, cellular and molecular levels; and 2) design of novel biomaterial systems that take advantage of mechanical considerations in order to alter their bioactivity. Submissions concerned with the practical application of biomaterials in medical devices as well as fundamental scientific investigations of a theoretical or experimental nature are welcome. These discussions will attract interest from materials scientists, engineers, biologists, clinicians, and industry representatives. This symposium will also form the basis for an upcoming Special Issue of the *Journal of the Mechanical Behavior of Biomedical Materials* (Impact Factor: 3.176) which will edited by the co-chairs.

(27.KP) SCAFFOLD DESIGN & FABRICATION - STATE OF THE ART AND FUTURE DIRECTIONS

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A paradigm shift is taking place in orthopaedic and reconstructive surgery. This transition from using medical devices and tissue grafts towards the utilisation of a tissue engineering approach combines biodegradable scaffolds with cells and/or biological molecules in order to repair and/or regenerate tissues. One of the potential benefits offered by solid free form fabrication technologies (SFF) is the ability to create scaffolds with highly reproducible architecture and compositional variation across the entire scaffold, due to its tightly controlled computer-driven fabrication. Many of these biologically activated materials can induce bone formation at ectopic and orthotopic sites, but they have not yet gained widespread use due to several continuing limitations including, poor mechanical properties, difficulties in intraoperative handling, lack of porosity suitable for cellular and vascular infiltration, and suboptimal degradation characteristics. In this chapter we define scaffold properties and attempt to provide some broad criteria and constraints for scaffold design and fabrication in combination with growth factors for bone engineering applications. Lastly, I comment on current and future developments in the field, such as the functionalisation of scaffolds designed to promote cell attachment, cell survival, vascular ingrowth, and osteoinduction.

Keywords. scaffolds, bone tissue engineering, osteoblasts, MSC

(27.O1) CYCLIC TENSILE STRAIN UPON ADULT HUMAN MESENCHYMAL STEM CELLS INFLUENCES GENE EXPRESSION LEVELS

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Introduction. It has been shown that tensile strain can alter cell behaviour. Evidence exists to confirm that human MSCs can be encouraged to differentiate in response to tensile loading forces. We have investigated the short term effects of cyclic tensile strain on gene expression for primary human MSCs in monolayer.

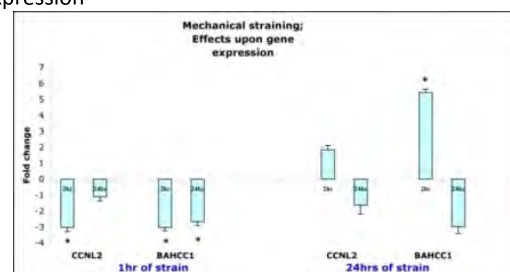
Methods. MSCs (Lonza) were cultured in monolayer on pronectin-coated 6-well plates. They were seeded at 5 x104 cells/well in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% antibiotics. Uniaxial strain, equivalent to 3% elongation, with a cycle speed of 1Hz, was applied to cells for 1 or 24 hours using a FX-4000T system (Flexcell), while controls remained static. Samples were collected at 2hours or 24hours post strain. A full human genome microarray was performed to highlight consistent differential expression of genes. Two genes of interest were then selected and real-time RT-PCR was performed (n=6) to assess reproducibility.

Results. Microarray data highlighted that 229, 269, 405 and 179 genes were differentially expressed at strain durations of 1hour (plus 1hour post strain), 1hour (plus 24hours post strain), 24hours (plus 1hour post strain) and 24hours (plus 24hours post strain) respectively. Two genes selected of interest that consistently showed differential expression were CCNL2 and BAHCC1. PCR confirmed CCNL2 was significantly downregulated after 1hour strain and 2hours latency (post strain). BAHCC1 was significantly downregulated after 1hour strain (both 2hours and 24hours latency). After 24hours strain and 2hours latency, BAHCC1 was significantly upregulated.

Conclusions. The latency period appeared to be the main factor which effected the level of gene expression in the strained samples compared to controls. We are currently investigating the role of these genes in long-term cell activity such as differentiation. We are also investigating this gene expression role in MSCs strained in a 3D hydrogel environment.

Acknowledgements. We acknowledge funding from BBSRC BB/D000548/1 and EPSRC IMPACT Pathfinder grants.

Keywords. Mesenchymal Stem Cells. Tensile Strain, Gene Expression



(27.O2) TIME-DEPENDENT COMPRESSIVE BEHAVIOUR OF HYDROGELS FOR NUCLEUS PULPOSUS TISSUE ENGINEERING

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1. University of Cambridge

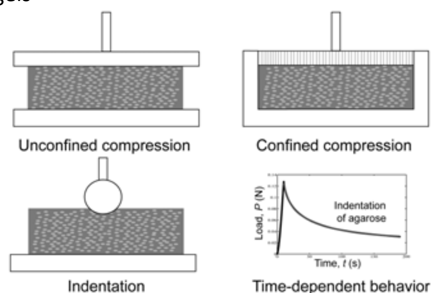
Introduction: Tissue engineering offers a paradigm shift in the treatment of back pain. Engineered intervertebral discs could replace degenerated tissue and overcome the limitations of current treatments, which substantially alter the biomechanical properties of the spine. The centre of the disc, the nucleus pulposus, is an amorphous gel with a large bound water content and can resist substantial compressive loads. Due to the similarities in their composition, hydrogels have frequently been considered as substitutes for the nucleus pulposus. However, there has been limited work characterising the mechanical behaviour of hydrogel scaffolds for nucleus pulposus tissue engineering. Most efforts have solely used unconfined compression to evaluate the equilibrium elastic modulus of the scaffold. They have not characterised poroelastic behaviour, which is thought to play a key role in nutrient transport, nor confined behaviour, which more closely replicates the in vivo state.

Methods: Here, we investigate the time-dependent compressive properties of three hydrogels (agarose, gelatin, and alginate) using confined compression, unconfined compression, and both micro- and nano-indentation in order to more fully understand how these scaffolds will behave in a semi-confined state in vivo. The time-dependent properties of these hydrogels are explored using viscoelastic and poroelastic frameworks, where applicable.

Results: Several gel formulations demonstrate comparable equilibrium mechanical behaviour to the nucleus pulposus under unconfined compression. However, under confined conditions, the same gels are an order of magnitude less stiff than the nucleus pulposus. Substantial differences in the degree of time dependent behaviour are observed. Interestingly, this is sometimes affected by the characteristic length scale of the test.

Conclusions: The methodology presented here is applicable to the testing of a variety of hydrogels. It will lead to a better understanding of how hydrogels will perform mechanically as scaffolds for nucleus pulposus tissue engineering.

Keywords. intervertebral disc, mechanical testing, hydrogels



(27.O3) CARTILAGINOUS TISSUES ENGINEERED USING INFRAPATELLAR FAT PAD DERIVED MSCs FAIL TO ACHIEVE NATIVE FUNCTIONALITY DUE TO A INABILITY TO RETAIN AND ASSEMBLE SYNTHESIZED MATRIX COMPONENTS

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Introduction: MSCs from non-cartilagenous knee joint tissues such as the infrapatellar fat pad (IFP) and synovium possess significant chondrogenic potential and provide a readily available and clinically feasible source of chondroprogenitor cells. Fibroblast growth factor-2 (FGF-2) has been shown to be a potent stimulator during ex vivo expansion of MSCs, as well as regulating subsequent differentiation potential. The focus of this work was to investigate the longer term effects of FGF-2 expansion on the functional development of cartilaginous tissues engineered using MSCs derived from the infrapatellar fat pad.

Methods: Porcine infrapatellar fat pad derived MSCs (IFP) were expanded in a standard media formulation (STD) with or without the addition of fibroblast-growth factor-2 (FGF-2, 5ng/ml). Agarose (2%) constructs (15 million cells/ml, Ø5x3 mm) were maintained in a chemically defined medium with TGF-β3 (10ng/ml) supplementation in a low oxygen (5%) environment for 42 days. Biomechanical functional properties and biochemical constituents were assessed at days 0, 21 and 42.

Results: Functional properties increased with time for both expansion conditions, with FGF-2 expanded MSCs forming the most mechanically functional tissue by day 42 (Fig1A). FGF-2 expanded MSCs had accumulated ~3 fold greater total sGAG and ~6 fold greater collagen content by day 21 indicating rapid matrix accumulation had occurred (Fig 1B&C). The release of sGAG into the media was significant over the time period for both groups, with the release from FGF-2 expanded groups being significantly higher. However, only 15-30% of the total sGAG and collagen molecules synthesised were retained or accumulated within the hydrogel itself for both STD and FGF-2 groups (Fig.1D).

Conclusions: MSCs expanded in the presence of FGF-2 rapidly accelerates chondrogenesis in 3D agarose cultures compared to control cultures. Future studies will focus on strategies to promote the assembly of matrix components into fully functional cartilaginous grafts.

Keywords. Cartilage; Functional Properties; FGF-2; Infrapatellar fat pad MSCs

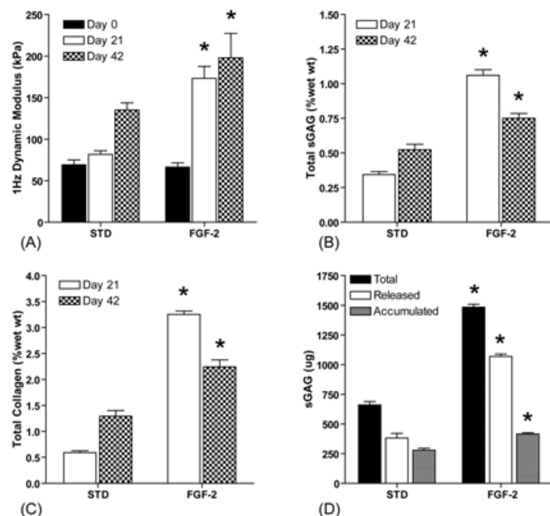

Figure 1 (A) 1 Hz dynamic modulus (B) sGAG content (C) collagen content and (D) Comparison of sGAG content generated, released into media and accumulated by the hydrogel construct at day 42. * indicates significance compared to STD group ($p<0.05$).

(27.O4) MESENCHYMAL STEM CELL FATE IS REGULATED BY THE MECHANICAL PROPERTIES OF COLLAGEN-GLYCOSAMINOGLYCAN SCAFFOLDS

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The extracellular matrix (ECM) provides biochemical and physical cues that direct cell fate. The rigidity of a substrate and the mechanical input generated by its deformation has been shown to regulate cell migration and differentiation. However many of these investigations have been carried out on two-dimensional (2-D) surfaces in the form of hydrogels. In our laboratory we have developed a homogenous three-dimensional (3-D) collagen-glycosaminoglycan (CG) scaffold which has been shown to support osteogenic differentiation. In addition it is possible to tailor the mechanical properties of CG scaffolds via different crosslinking methods**.** This study aims to investigate how substrate stiffness affects mesenchymal stem cell (MSC) infiltration and differentiation within the CG scaffold. CG scaffolds were fabricated using a freeze-drying method and then dehydrothermal (DHT) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) (3mM and 6mM) crosslinked resulting in scaffolds with the same composition but different stiffness ranging from 0.5 kPa – 1.5 kPa. The scaffolds were seeded with rat MSCs and assessed 1 day, 7 days and 14 days post-seeding. Blebbistatin (50mM), a non muscle myosin II inhibitor that blocks branching, elongation and spreading of cells on their substrate, was added to media as a negative control. Osteogenic factors were added to media as a positive control.

Results demonstrate that MSC fate is regulated by the mechanical properties of the scaffolds. Higher rates of scaffold infiltration by the cells was observed on the stiffer EDAC-crosslinked scaffolds and this coincided with higher levels of RUNX2 expression, a key transcription factor associated with osteogenic differentiation, in comparison to the more compliant DHT treated scaffolds. This suggests that the stiffer scaffolds are elucidating an osteogenic response. Ongoing research is investigating how scaffold stiffness affects the transcriptional profiles of early commitment myogenic, chondrogenic and adipogenic markers.

Acknowledgements: European Research Council & Integra Life Sciences

Keywords. Substrate Stiffness, Collagen, Mesenchymal Stem Cells, Crosslinking

(27.O5) INFLUENCE OF SCAFFOLD STIFFNESS AND MECHANICAL STIMULATION ON ECM PRODUCTION BY HUMAN PRIMARY DERMAL FIBROBLASTS

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Matrix stiffness is known to have a strong influence on cell behavior. For fibroblasts, morphology and spreading is actually driven by the substrate stiffness. In bone healing, fibroblasts infiltrate the hematoma and lay down a matrix to form the granulation tissue. However, little is known on how the cells actively modify their local mechanical surrounding e.g. by ECM production. We hypothesize that scaffold stiffness influences ECM production and thus cell-matrix interplay. External straining of the matrix further modulates ECM production.

Human primary dermal fibroblasts were seeded into macroporous collagen-I scaffolds (OptiMaix, Matricel GmbH) with different initial stiffnesses (Youngs modulus of 2.0±1.0kPa and 7.5±1.0kPa). They were transferred into an in house developed bioreactor system and mechanically loaded (3h stimulation / 5h rest) over a two week period. Changes in mechanical properties were monitored online by mechanical testing. Cell number and ECM production were assessed by histology staining using cryosections and immunoassays for procollagen type I and fibronectin.

ECM production showed dependency on the initial stiffness of the scaffold: Low initial Young modulus scaffolds (2.0±1.0kPa) resulted in a 25% higher collagen production and a four- to sixfold increase in contraction over time when compared to higher initial Young modulus (7.5±1.0kPa) (cf. Fig1a,b,c).

Additional cyclic mechanical straining resulted in additional stiffening of the soft scaffolds through enhancement of matrix contraction and ECM production. In contrast, the stiffer scaffolds showed decreasing mechanical properties over time (cf. Fig1d).

Both cell contraction and ECM production were found to be dependent upon the mechanical environment, here realized by different initial matrix stiffnesses. The cells seem to provide mechanisms that allow formation of a structure with specific mechanical properties. Apparently, our findings indicate that they recruit and organize their ECM environment - known to be the key regulator for cell behavior - according to their own needs.

Keywords. fibroblast, scaffold, stiffness, extracellular matrix

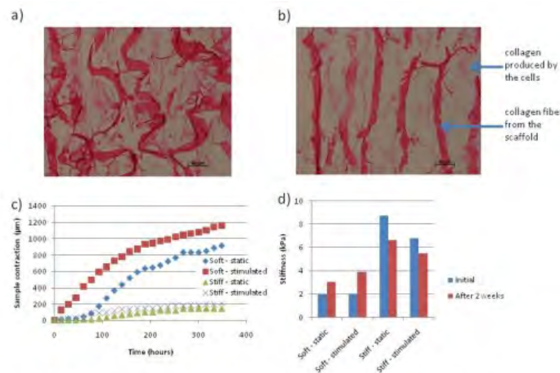

Fig. 1: Differences of contraction in soft (a) and stiff scaffolds (b) visualized by sirius red staining for collagen fibers. Monitoring of contraction (c) and changes in stiffness (d) in the bioreactor.

(27.O6) EFFECT OF SUBSTRATE RIGIDITY ON iPS DIFFERENTIATION

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Introduction: Every living cell is affected by the surrounding microenvironment, known as the extracellular matrix. Many recent studies reveal that parameters such as substrate stiffness, biochemical composition and matrix topography can influence cell response and even dictate their fate. This effect, known as mechanotransduction, affects cell proliferation, adhesion, morphology, motility and differentiation. Many studies have been performed in order to define the effect that the matrix stiffness has on the differentiation of stem cells towards specific phenotypes. Among the different stem cell populations, the recently discovered iPS (Induced Pluripotent Stem Cells), a genetically reprogrammed somatic cell line with embryonic stem cell properties, has emerged as a promising cell population for regenerative medicine and in-vitro testing for the study of biological cues. This work studies the effect of matrix stiffness on iPS differentiation.

Methods: Mouse iPS were obtained by retroviral overexpression of the transcription factors Oct3/4, Sox2 and Kfl4 in adult fibroblasts. iPS-derived embryoid bodies were plated on Fibronectin or Collagen-I coated Polyacrylamide gels (pAAg) of 0.6, 13 and 50 kPa and kept in culture during 14 days. Afterwards, iPS differentiation towards the three-germ layers has been analyzed by real-time PCR, immunofluorescense and electronic microscopy. Cell behaviour and motility has been observed by time-lapse optical microscopy in phase contrast and fluorescence modes.

Results: Substrates with tunable mechanical properties have been achieved using pAAg. Depending on the stiffness of the matrix, a distinct differentiation pattern of the iPS cells has been observed. More precisely, the greatest differentiation towards mesoderm-derived cell types has been found in the middle-rigid matrices (13 kPa), where even beating foci have been detected.

Conclusion: As a complement to the biochemical factors, adjustment of the mechanical properties and matrix composition can direct iPS differentiation in a more efficient and controlled way.

Keywords. Mechanotransduction, iPS, Stem Cell Differentiation

(27.O7) ANISOTROPIC COLLAGEN-GAG SCAFFOLD-MEMBRANE COMPOSITES WITH PDGF-BB AND IGF-1 SUPPLEMENTATION FOR TENDON TISSUE ENGINEERING

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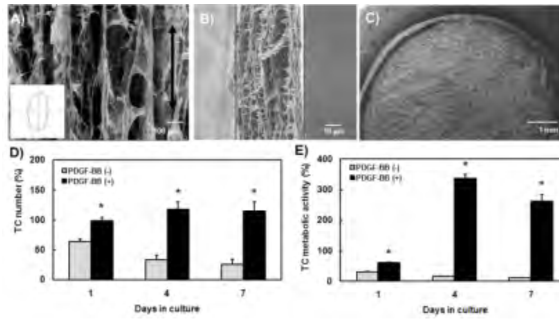
Introduction: We are developing collagen-glycosaminoglycan (CG) biomaterials for tendon tissue engineering. CG scaffolds have predominantly been used as regeneration templates for a range of soft tissues including dermis; here we describe a composite (core-shell) CG biomaterial for tendon tissue engineering. Key design elements include: an aligned microstructure to mimic native tendon; integration of agonists of tendon cell (TC) migration, proliferation, and metabolic activity to improve construct bioactivity; increased mechanical strength via integration of a CG membrane shell with the CG scaffold core to mimic mechanically efficient structures in nature.

Methods: CG composites are created from highly porous CG scaffolds and dense CG membranes from a suspension of type I collagen and chondroitin sulfate. CG membranes are fabricated via an evaporative process and integrated with CG scaffolds through lyophilization; mold thermal conductivity mismatches induce directional solidification to create aligned pores. TC metabolic activity and number are measured using fluorescent assays (alamarBlue, Hoechst 33258).

Results: SEM and stereology analyses of CG scaffolds showed longitudinally-aligned pores (Figure A); modulating freezing temperature enabled creation of aligned (pore aspect ratio: 1.6) CG scaffolds with constant relative density but a wide range of pore sizes (55-243 µm). Incorporating a CG membrane shell into the scaffold core (Figure B-C) increased scaffold tensile elastic modulus by a factor of up to 36 (0.21 to 7.64 MPa); core-shell constructs maintain adequate permeability to support TC viability after 14 days of culture. We have also found concentration dependent effects of soluble IGF-1 and PDGF-BB on TC migration into these scaffolds and subsequent proliferation and metabolic activity (Figure D-E).

Conclusions: We describe an anisotropic CG scaffold-membrane composite supplemented with PDGF-BB and IGF-1 for tendon tissue engineering. Ongoing work is optimizing membrane design and integration, exploring growth factor immobilization and spatial patterning schemes, and quantifying TC alignment, gene expression, and matrix biosynthesis.

Keywords. tendon, collagen scaffold, growth factors, composite



(27.O8) CONTRACTILITY OF STEM CELL-DERIVED CARDIOMYOCYTES IN 2D AND 3D MICROENVIRONMENT

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1. Medical College of Wisconsin; 2. University of Wisconsin Madison

Rationale: Stem cell derived cardiomyocytes (SCCMs) could become a critical source for developing a cardiotoxic compound screening system for drug discovery. Very little, however, has been reported about the contractility of SCCMs in single-cell 2D and multi-cellular 3D microenvironments. To develop high-throughput cardiotoxicity tests, basic biophysical properties of SCCMs, including their contractility, have to be characterized. Objective: To characterize the contractility of SCCMs in 2D and 3D microenvironments.

Methods and Results: Single SCCMs were cultured on polyacryamide cell culture substrata with various stiffness values (1kPa-100kPa). Traction-force microscopy was used to measure contractility of cardiomyocytes. Multicellular, miniaturized engineered heart tissues (EHTs) were fabricated with SCCMs. A proper growth regulation of non-muscle cells comprising the tissue facilitated the development of spontaneously and synchronously beating EHTs. An automated biomechanical measurement device, the Palpator™, analyzed their contractile activities to validate the cardiotoxic effects of well-known cardiac toxic compounds, including doxorubicin (1-100nM). The single SCCMs on 2D substrata generated increasing magnitudes of cardiac contractility (1-25 mN/mm2) in response to the increasing substrate stiffness. However, the SCCM derived engineered heart tissues developed significantly less cardiac contractility (~0.16 mN/mm2) compared to those by single cells.

Conclusions: SCCMs on 2D culture generated stress comparable to that generated by the human papillary muscles (~15mN/mm2), indicating a promising source of developing heart tissue equivalents that mimic physiological functions of human myocardium. There is a gap in contractility measured in 2D and 3D microenvironments. Various strategies to improve the contractility in 3D engineered heart tissues will be discussed.

Keywords. engineered heart tissue, stem cell, cardio toxicity

(27.O9) HUMAN MESENCHYMAL STEM CELLS MIGRATION ON MATRICES WITH DISTINCT ELASTICITY GRADIENT MAGNITUDES

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1. University of California, San Diego

Adult mesenchymal stem cells (MSCs) respond to extracellular niche elasticity, which varies dramatically between tissues that MSCs inhabit. Similarly as MSCs egress from bone marrow and hone to tissues, they may encounter stiffness gradients brought on either by pathological conditions, e.g. myocardial infarction ~ 8.67 ± 1.50 kPa/mm, or through normal tissue variation, e.g. skeletal muscle ~ 0.58 ± 0.88 kPa/mm. We have recently shown that MSCs can undergo directed migrate even in response to shallow, physiological (~1kPa/mm) stiffness gradients before differentiating, suggesting the importance of spatial changes in stiffness during disease. Such gradients, however, contain aphysical ranges, e.g. 1 – 15 kPa, and more refined gradients of both range and gradient strength are perhaps more appropriate to mimic tissue interfaces. To better understand how mechanical cues dictate MSC migration versus differentiation, we have engineered polyacrylamide (PA) hydrogels with tunable stiffness gradients of defined magnitude using a microfluidic device. MSCs responded to stiffness gradients with a physiological range, e.g. mimicking the myotendenous junction, but of varying strength, i.e. 1 - 30 kPa/mm. Cell morphology was also stiffness dependent with cells exhibiting increased spread areas on the stiffer regions, suggesting that the previously observed correlation between substrate mechanics, cell motility, and morphology exists over a physiological stiffness range but is independent of gradient strength. These studies imply that MSCs in vivo may contribute better to repairs in stiffer regions of tissues where they may preferentially accumulate due to tissue stiffness.

Keywords. microfluidics, photopolymerized hydrogels, mesenchymal stem cells

(27.O10) FLEXURAL STRENGTH OF UNIDIRECTIONAL AND WOVEN GLASS FIBERS REINFORCED COMPOSITES USED FOR DENTISTRY

Furtos G (1), Prejmerean C (1), Silaghi-Dumitrescu L (1), Moldovan M (1), Tamas C (1), Boboia S (1),
1. Raluca Ripan Institute of Research in Chemistry, Babes-Bolyai University, Cluj-Napoca, Romania

Introduction. Fiber-reinforced composites (FRCs) have been introduced in dentistry as metal-free alternatives and esthetic and including: prosthodontic bridges and single crowns and full coverage fixed partial dentures, periodontal splints, and orthodontic retainers, post core systems and implant prothesis. The glass fiber post is today an alternative to metallic post if esthetic qualities are required.

Methods. A series of light cured composites (Table 1) containing the same Bis-GMA/TEGDMA matrix control (1.5/1 wt.) with different glass fiber reinforcement (14.96 – 47.25 %wt.) of unidirectional or woven glass fibers were prepared. The samples 25x2x2 mm were cured for 60 sec with a halogen curing lamp Optilux 501 (Kerr) followed by additional curing by thermal treatment at 135±5°C temperature and 60 psi pressure, for 20 minutes, using a "BelleGlass" warmer. The test specimens (n = 8) were water stored at 37 °C for 24 h before the mechanical tests. Samples were tested before and after post curing by determining the flexural strength, flexural modulus and by structural investigations using SEM.

Conclusions. Reinforcing composites with glass fiber improve mechanical properties than unreinforced specimens. The flexural strength was high improved when was used unidirectional glass fiber (438.01 Mpa; 2UG) than woven glass fiber (117.78 Mpa; 3WG). Young's modulus had the same trend as flexural strength. Combination of woven glass fiber with unidirectional glass fiber (2WG+1UG) showed lower mechanical properties than unidirectional glass fiber 1UG but higher than 2WG. The effect of post polymerization and increasing of the glass fiber amount in experimental composites improve mechanical properties of FRC. SEM micrographs suggested the strongest interfacial interaction between glass fiber and matrix.

Acknowledgements. The authors thank the Romanian Ministry of Education, Research and Youth for the financial support by the IDEI 1047 Grant and PNII Contract nr. 72168/2008.

Keywords. Glass Fiber, Dental, Fiber-reinforced composites, Flexural strength

Table 1. Composition of experimental glass fiber reinforced composites

Cod	Resin (% wt)	Fiber architecture	Type of curing
Control	60% Bis-GMA 40% TEGDMA	No fibers	Light curing
Control_P			Light curing + postcuring
1 WG	60% Bis-GMA 40% TEGDMA	1 woven glass fibers bands	Light curing
1 WG_P			Light curing + postcuring
2 WG	60% Bis-GMA 40% TEGDMA	2 woven glass fibers bands	Light curing
2 WG_P			Light curing + postcuring
3 WG	60% Bis-GMA 40% TEGDMA	3 woven glass fibers bands	Light curing
3 WG_P			Light curing + postcuring
1 UG	60% Bis-GMA 40% TEGDMA	1 unidirectional glass fibers bundle	Light curing
2 UG_P			Light curing + postcuring
2 UG	60% Bis-GMA 40% TEGDMA	2 unidirectional glass fibers bundle	Light curing
2 UG_P			Light curing + postcuring
2WG+1UG	60% Bis-GMA 40% TEGDMA	2 woven glass fibers bands 1 unidirectional glass fibers bundle	Light curing
2WG+1UG_P			Light curing + postcuring
2WG+1UG_W	60% Bis-GMA 40% TEGDMA	2 woven glass fibers bands 1 unidirectional glass fibers bundle	Light curing
2WG+1UG_W_P			Light curing + postcuring
2WG+1UG_U	60% Bis-GMA 40% TEGDMA	2 woven glass fibers bands 1 unidirectional glass fibers bundle	Light curing
2WG+1UG_U_P			Light curing + postcuring

(27.O11) THE NON-ACETYLATION OF ACTIN AND TROPOMYPSIN DISRUPTS THE ACTIN CYTOSKELETON IN MAMMALIAN CELLS

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Introduction: Most of mammalian soluble proteins are N-terminally acetylated by one of the main N-acetyltransferase complexes (NAT), namely NatA, NatB and NatC. NAT complexes modify a defined subset of cellular proteins in each one. There are not many studies indicating the biological relevance of this protein modification, but there are an increasing number of publications linking N-α-terminal acetylation to cell growth, differentiation, survival and cancer. NatB is composed of a catalytic subunit, Naa20, and one accessory subunit, Naa25, which are associated to the ribosome. It has been observed in yeasts that the non-acetylation of actin and tropomyosin by NatB affects its actin cytoskeleton. It has also been described that mammalian actin and tropomyosin are N-terminal acetylated and that this modification may affect their biochemical activity; however, this has not been confirmed yet. This work studies the effects of inhibition of hNatB subunits in mammalian cells.

Methods: Mammary HeLa cells were cultured on glass and different polyacrylamide-based hydrogels of controlled mechanical properties. Inhibition of hNatB was achieved by siRNA transfection. Cell spread area, F-actin and myosin II distribution across the cell and focal adhesions were quantified by phase contrast and fluorescence microscopy, using phalloidin staining, myosin II and vinculin immunostaining, respectively. Also, cell migration and cellular traction forces were measured. Finally, pharmacological inhibitors of cell contractility, such as blebbistatin, cytochalasin B and latrunculin B, were also applied for comparisons.

Results: Results show that hNatB inhibition on HeLa cells disorganizes significantly the distribution of actin cytoskeleton stress fibers across the cell and prevents focal adhesion formation, reducing both force generation on focal complexes and cell motility.

Conclusion: The inhibition of hNatB weakens the interaction between actin and tropomyosin and may act similarly between actin and myosin, leading to a disruption of the actin cytoskeleton which alters main cellular functions.

Keywords. N-terminal acetylation, mechanobiology, actin cytoskeleton, cell mechanics

(27.O12) HIGH-THROUGHPUT MEASUREMENTS OF CELL TRACTION FORCES OF CANCER CELLS

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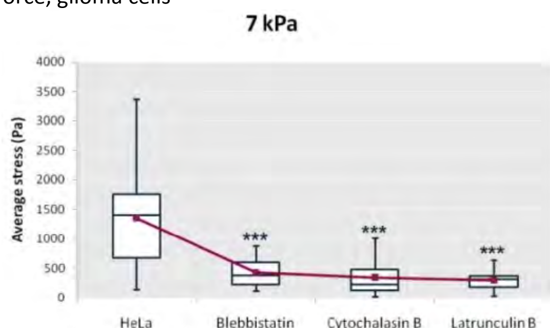
Introduction: Glioblastoma multiforme is the most common and most aggressive type of primary brain tumor in humans, associated with a median survival time of 15 months, even with aggressive therapy. Increasing evidence suggests that mechanical cues inherent to the extracellular matrix may be equally critical as its chemical identity in regulating cancer cell behavior.

Methods: We cultured different glioma cell lines (U373-MG and U87-MG) and HeLa cells on polyacrylamide hydrogels coated with different collagen or fibronectin with controlled mechanical properties. The role of the stiffness in cell traction force was studied in order to understand the key function that rigidity of the extracellular matrix plays related to invasion. Traction Force Microscopy technique was used to measure the adhesive forces that cells exert on different stiffness of

substrate simulating the extracellular matrix. Furthermore, in order to study the importance of the actin cytoskeleton for cell traction forces, pharmacological inhibition of cells' contractility was made with blebbistatin, cytochalasin B and latrunculin B.
Results: We measured systematically that cancer cells make inferior adhesive forces on compliant substrates. On the other hand, pharmacological inhibition of cells' contractility showed a great reduction of the traction forces, which was independent of the stiffness of the substrate.
Conclusion: We have proven that high-throughput traction force microscopy can be used for systematic studies of cancer cells. We observed that the mechanical properties of the extracellular matrix control cell traction forces of cancer cells. Collectively, our results provide support to assert the importance of the different properties of the extracellular matrix to regulate cell behavior and control the invasiveness of glioblastoma multiforme tumor cells.
Keywords. Extracellular matrix, mechanobiology, traction force, glioma cells



(27.O13) THE EFFECT OF LOW SHEAR STRESS ON TISSUE ENGINEERED AORTIC VALVES

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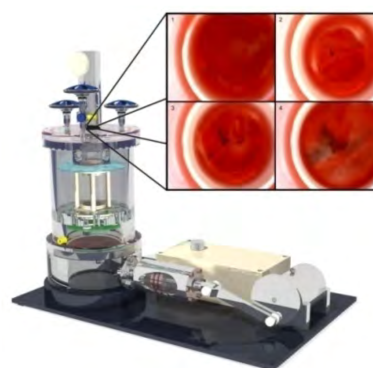
Introduction: Vascular cells are able to sense and respond to flow forces by expressions of pro-inflammatory genes. In this study we compared the cell adhesion and pro-inflammatory gene expressions (IL-1α, IL-6, IL-8, MCP-1 and VCAM) of vascular cells seeded on synthetic or natural aortic valve scaffolds in a novel self-made bioreactor. **Methods:** Polyurethane valve scaffolds (Group A, n=4) and cryopreserved/thawed aortic homograft valves (Group B, n=4) were primarily seeded with FBs (92.11 ± 11.08 x 106 cells), followed by a colonization with ECs (96.48 ± 8.05 x 106 cells) using a special rotating seeding device. Each seeding procedure was followed by an exposure to low pulsatile flow (750 – 1100 ml/min) in a newly developed Bioreactor for 5 days. Specimen for scanning electron microscopy (SEM), immunohistochemical staining (IHC) and real time PCR were taken prior to cell seeding, following each seeding procedure, and after conditioning.
Results: SEM evaluation showed a confluent cell layer in both groups. IHC staining of both groups with CD31 (ECs-antibody) and TE-7 (FBs-antibody) revealed a positive reaction of both cell layers before and after low flow conditioning. However, the results of Group A had higher colonization efficiency than Group B. At PCR, samples of both groups showed an increase of gene expressions after each seeding procedure (After endothelialisation; IL-6: 19.58 ± 8.05 % and MCP-1: 14.43 ± 4.47 %). Preconditioning after cell colonization resulted in a significant reduction of gene expressions (Step 1 in both groups: MCP-1: 11.91 ± 3.95 %; VCAM: 7.95 ± 5.37 % and Step 2 in Group B: MCP-1: 8.97 ± 3.44 %).
Conclusions: New bioreactor allows colonized cells to adapt to shear stress and to establish a strong extracellular matrix. Preconditioning of colonized vascular cells with low shear stress modulates and stabilizes the pro-inflammatory gene expression.
Keywords. Shear stress; Gene Expression; Aortic valve scaffolds



(27.O14) MIGRATION OF HUMAN MESENCHYMAL STEM CELLS IS INFLUENCED BY WNT3A AND MECHANICAL STIMULATION

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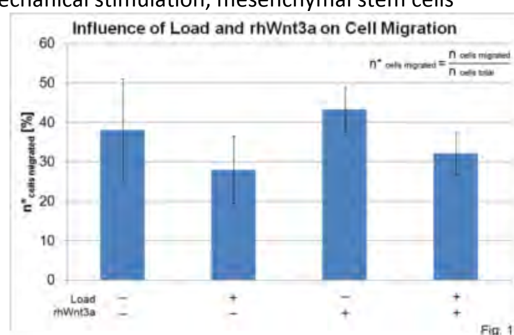
Introduction. After tissue injury, migration of pluripotent stem cells from their niches to the injury site is essential to initiate tissue repair. In various tissues Wnt signaling has been observed to play a significant role during regeneration and is assumed to improve migration. On the other hand the mechanical environment is known to influence cell behavior. Therefore we hypothesize that Wnt3a and mechanical loading have a synergistic effect on the migratory behavior of human mesenchymal stem cells (hMSCs).
Methods. Highly orientated macroporous collagen scaffolds (5mm Ø, 3mm high) were seeded with a compact layer of hMSCs on the bottom side of the scaffold. In a bioreactor system mechanical loading (10% cyclic axial compression) and rhWnt3a (c=200ng/mL) were applied individually or in combination for three days. The cell distribution inside the scaffolds was assessed from DAPI-stained cryosections and the medium was analyzed for secreted matrix-metalloprotease-2 (MMP-2).
Results. Based on our novel 3D migration assay, we found that mechanical loading alone reduced cell migration by

33%, whereas the supplement of rhWnt3a alone increased cell migration by 16%. Consequently the combination of both stimuli partially reduced the effect of mechanics, leaving a decrease of cell migration of 13% (Fig. 1). Furthermore, we found an up-regulation of MMP-2 with mechanical stimulation as reported from earlier studies using fibrin gels (Kasper et al., 2007).

Conclusions. Preliminary results showed that the migratory behavior is regulated by both the mechanical and biochemical (rhWnt3a) environments. Together with the up-regulated matrix degrading MMP-2 our results further substantiate the importance of mechanical signals for cell invasion and matrix remodeling in the early phase of tissue regeneration. A clear synergistic effect of mechanics and rhWnt3a, however, could not be identified so far. Further studies will investigate how the variation in the amount of both parameters will influence their synergistic effect.

Keywords. cell migration, Wnt/β-catenin pathway, mechanical stimulation, mesenchymal stem cells



(27.O15) CELL CONTRACTION FORCES ADAPT TO THE MECHANICAL ENVIRONMENT IN A SPECIES-DEPENDENT MANNER

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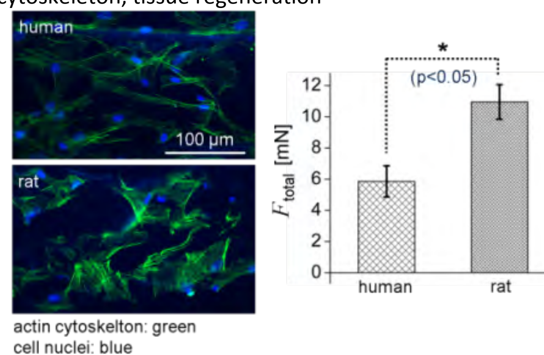
Introduction. It is known that contractile cellular forces are involved in regeneration processes like wound closure and stabilization, but also tissue vascularization. In this work we investigated the hardly known interplay between extrinsic forces, induced by a cyclic axial compression, and the intrinsic cell contractile forces.

Methods. Primary human and primary rat dermal fibroblasts were seeded into macroporous collagen scaffolds. Cell constructs were cultured for in a bioreactor over 65h and exposed to a sinusoidal mechanical stimulation (8% strain at 1Hz for 2h) every 12.5h. The total contraction force applied by the cells to the scaffold matrix was continuously measured during a three day culture time as the difference between the compression force of cell loaded scaffolds and cell free controls.

Results. A clear difference in total contraction force between the species was observed with a mean value of F[total,hum]=5.9(±2.0)mN for human and F[total,rat]=11.2(±2.0)mN for rat fibroblasts (figure 1). The mean force applied by an individual cell was calculated to be F[cell,hum]≈2.0nN and F[cell,rat]≈3.7nN respectively. With the application of mechanical stimulation, an adaptation of cell contractile forces was observed within a timeframe of 2h. When stimulation stopped, forces returned to values of unstimulated cells in a comparable time frame. Human fibroblasts showed a further 16(±7)% reduction of their comparably low contraction force while an 11(±6)% increase in response to mechanical stimulation was observed for rat fibroblasts.

Conclusions. Our investigations demonstrate a connection between the application of extrinsic forces and the adaption of intrinsic cellular contractile forces. Clear differences between the amount and the adaptation of contractile forces between rat and human cells were shown. This observation suggests that the well balanced force interplay between cell and extracellular matrix is species specific. This has to be taken into account if wound healing processes are to be compared between rat and human.

Keywords. cell contraction, fibroblasts, actin cytoskeleton, tissue regeneration



actin cytoskelton: green
cell nuclei: blue

(27.O16) CHARACTERIZATION OF ALGINIC ACID-COATED PLLA-PLGA FOAMS FOR POTENTIAL USE IN TISSUE ENGINEERING OF THE MENISCUS

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Introduction. In this study, a blend of biodegradable polymers; poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA), were used to produce a scaffold for meniscus tissue engineering. Alginic acid was introduced to serve as a substitute for proteoglycans in meniscus, both modifying surface properties and improving mechanical properties.

Methods. Two sets of PLLA-PLGA solutions (3% w/v in dioxane) were frozen at -20 oC or -80 oC. They were dip-coated with alginic acid which was then crosslinked with CaCl2. Compressive mechanical properties of the foams were determined with a mechanical tester at a displacement rate of 10 mm/min. Mercury porosimetry was performed to determine pore size distribution. Mouse L929 fibroblasts were seeded onto foams to observe biocompatibility of the foams. After a 3 week culture; the scaffolds were studied with SEM and CLSM.

Results. Upon coating with alginate the compressive modulus increased with foams prepared at both -20 oC and -80 oC. Compressive strength increased especially with the foams prepared at -80 oC (Fig. 1). Porosimetry showed that the pore sizes of the foams prepared at the

lower temperature were higher than those prepared at the higher temperature. Upon coating, the pore sizes of the foams decreased from 50-200 µm to 25-170 µm while those of the foams prepared at -80 oC decreased from 30-80 µm to 15-60 µm. CLSM and SEM analysis of the cell seeded scaffolds show that coating the foams with alginate enhanced cell attachment and ECM production.

Conclusion. Surface and mechanical properties of the alginic acid-coated PLLA-PLGA foams were significantly better than those of the uncoated ones, however, porosity decreased upon coating. The results show that the foams prepared at -20 oC have properties comparable with those of the native meniscus.

Keywords. Mechanical characterization, scaffold, meniscus, biodegradable polymers

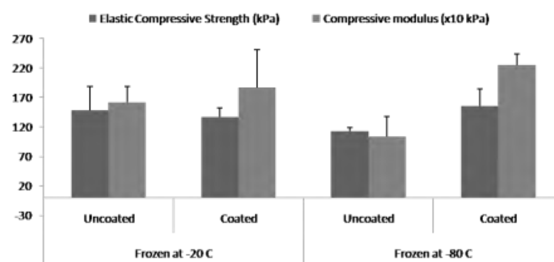

Figure 1. Influence of coating with alginic acid and praparation condition (freezing temperature) on compressive properties of the PLLA-PLGA foams.

(27.O17) FABRICATION AND MECHANICAL CHARACTERIZATION OF A NOVEL THREE-DIMENSIONAL CELL-SEEDED COLLAGEN/HYDROXYAPATITE OSTEOCHONDRAL SUBSTITUTE

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Introduction. When a cartilage injury occurs, the underlined bone changes, thickens and becomes pathologically altered. In these conditions, a successful procedure for cartilage regeneration should not involve only the cartilaginous tissue but the whole ostechondral compound. Nowadays, in the clinical practise, only cell-free composites are available. However, although the bone tissue is able to colonize an osteocompatible scaffold, the cartilage cannot. We believe that seeding cells into the chondral part of a biphasic substitute, could represent a promising solution for the ostechondral tissue repair. In this study, a novel three-dimensional collagen/hydroxyapatite ostechondral substitute was developed, cell-seeded and mechanically characterized.

Methods. A hydroxyapatite macrochanneled porous scaffold was produced by polymer sponge templating method using a reactive sub-micron powder synthesized by hydroxide precipitation sol-gel route. The ceramic scaffold was then integrated to a collagen part obtained by a freeze drying technique. Morphology and mechanical properties of scaffolds was analysed by scanning electron microscopy and compression test. Expanded swine chondrocytes were suspended within the collagen part. Samples were retrieved from culture after 1, 3, 5 weeks. All samples were processed for histological evaluation and mechanically tested to evaluate the engineered constructs stiffness.

Results. The ceramic part of the scaffold had high mechanical performance (compressive strength ~ 0.51 MPa) compared to literature data. The collagen scaffolds showed a regular structure and homogeneous porosity (~ 100 µm). The histological analysis showed cell survival and matrix production within the scaffolds' fibres. The mechanical strength of the tissue-engineered construct increased significantly after 3 weeks of culture (from 7 to 55KPa).

Conclusions. Three-dimensional collagen/hydroxyaptite scaffolds could be properly fabricated and mechanically characterized. An in vivo study, to evaluate the efficacy of the scaffolds for repairing osteochondral defects is in progress in a pig model.

Keywords. scaffold, engineered construct, compression test

(27.O18) EFFECT OF SUBSTRATE STIFFNESS AND PHYSICOCHEMICAL PROPERTIES ON HUMAN MESENCHYMAL STEM CELL ACTIVITY IN 3D SCAFFOLDS

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Introduction: Developing scaffolds with instructive properties to control stem cell behavior is an emerging strategy in tissue engineering. With the aid of mathematical models, theories were proposed to understand the influence of mechanical stress and strain on cellular behavior, while substrate elasticity was experimentally shown to direct stem cell differentiation. Yet, it is not known whether cells adhered to the surface of scaffolds perceive a different magnitude of physical stimuli compared to cells embedded in synthetic or natural extracellular matrix (ECM). We developed finite element models (FEMs) to predict the distribution of stresses and strains in 3D scaffolds displaying variable stiffness and physicochemical properties. Assuming that different stress/strain regimes result into different cell activity, we also initiated the experimental evaluation of the influence of scaffold stiffness and physicochemical properties on human mesenchymal stem cells (hMSCs).

Method: 3D scaffolds from different biomaterials (300PEOT55PBT45, 1000PEOT70PBT30, PCL, PLDLA) with equal pore architecture were fabricated by rapid prototyping. Scaffolds were imaged through microcomputed tomography and converted to FEMs to

calculate the surface and volume strain distribution and fluid shear stresses upon compression and perfusion. The influence of substrate bulk and apparent stiffness on cellular activity was investigated. Static culture experiments were performed on 3D scaffolds seeded with hMSCs exposed to basic, osteogenic, chondrogenic and adipogenic medium.

Results: Scaffolds consistently supported a lower surface strain compared to the volume strain. This implies that the mechanical stimuli perceived by cells on a 3D scaffold are of different magnitude than cells embedded in a synthetic or natural ECM. Static culture experiments showed hMSCs adhesion and distribution on 3D scaffolds varied by biomaterial. hMSCs adhered and distributed better on 300PEOT55PBT45 followed by PCL, PLDLA, and 1000PEOT70PBT30. Further experiments will aim at correlating substrate physicochemical properties and stiffness with hMSC differentiation in 3D scaffolds in a dynamic environment.

Keywords. 3D scaffolds, substrate stiffness, physicochemical properties

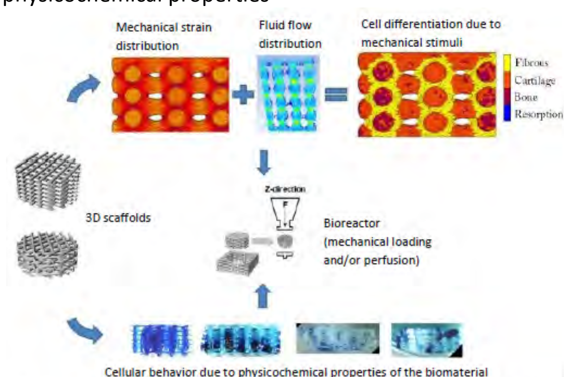

Figure 1: Top track: from uCT, Finite Element Models are developed in which the strain distribution and fluid flow can be calculated and the influence of bulk stiffness and apparent stiffness can be investigated. Taken together, these mechanical stimuli could predict cell differentiation due to the loading regime in a bioreactor. Lower track: the physicochemical properties of the scaffold have an influence on cellular behavior, which might be of interest in a dynamic loading regime as well.

(27.O19) VALIDATION OF THE EFFECT OF WALL SHEAR STRESS ON CELL ADHESION FOR A PERFUSION BIOREACTOR MATHEMATICAL MODEL

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Introduction. Mathematical modelling of tissue engineering bioreactors has been used to determine the best operational conditions. The lattice Boltzman (lB) method allows the inclusion of all the parameters into a model for a perfusion bioreactor. The effects of fluid shear stress in cell seeding and cell differentiation in such systems have been studied previously; however a math model that allows the optimization of flow rate and cell concentration, among others parameters, have not yet been developed.

Materials and Methods. The lB model included velocity and concentration evolution equations to model the behaviour of cells when seeded into a scaffold; wall shear stress and attachment were also modelled. The simulations were done in straight and circular channels. For the validation, protein-coated and uncoated tygon tubings were seeded with hMSC and cultured for 24 hours. Non-stained samples were CT imaged including sections at 0* and 180* to mimic the flow of cells through a porous scaffold.

Results. The mathematical model showed cell alignment as well as concentration gradients in the case of the curved channel; experimental work confirmed cell deposition difference between uncoated and protein-coated tubing as well as in different areas of the same tubing.

Discussion and Conclusions. The lB code produced cell attachment and wall shear stress models depending on cell concentration and flow. Although work is still ongoing to improve the resolution of some images, current CT data has produced useful information for the math model.

Acknowledgements. We want to acknowledge the Henry Moseley X-Ray Imaging Facility for the assistance on the CT scans and image analysis. This project has been supported by the BBRSC research grants BB/F013892/1 and BB/F013744/1.

Keywords. mathematical model, Lattice Boltzman, perfusion bioreactor, wall shear stress, microCT

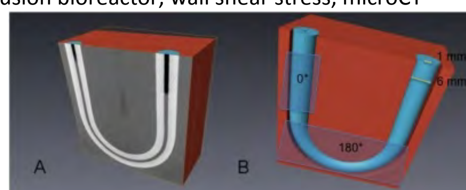

Fig. 1. (A) Reconstructed image of tubing containing cells (B) 0* and 180* sections for the model validation.

(27.O20) MECHANICAL PROPERTIES EVOLUTION OF A PLGA/PLCL KNITTED SCAFFOLD IN CULTURE CONDITIONS

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Introduction. The mechanical properties of a scaffold during its degradation are of particular importance in tissue engineering. Indeed, the scaffold must ensure the transmission of mechanical efforts until a neo-tissue is formed. In this study, we studied the evolution of mechanical properties of a scaffold designed for ligament tissue engineering under different culture conditions.

Methods. The evolution of the mechanical properties of a knitted scaffold in PLGA with an electrospun PLCL fiber membrane was studied in three culture conditions: (I) static without cells; (II) static with 250 000 rMSC seeded at D0 in normoxia conditions from D0 to D28 and (III) cyclic traction-torsion (stretching: 10%, torsion: 90°) at 0.33Hz 2 hours per day in a bioreactor from D0 to D14 at 37°C (Medium was changed twice a week). Mechanical tests of uniaxial traction were made at D0, D7, D10, D14, D21 and D28 for group I and II and until D14 for group III (n=3). Biocompatibility test (AlamarBlue) was made for group II and III (n=3) and cell colonization was investigated by staining cell nuclei (TO PRO3) under confocal microscope.

Results. The scaffold immerged into culture medium without cells kept its mechanical properties during the 21st days and then lost rapidly its mechanical quality

(Figure 1). Whereas, scaffold of group II lost mechanical quality regularly from the beginning but slower than group I scaffolds and group III scaffolds which lost more than 50% of initial ultimate strength at D14. Simultaneously, we found that stem cells were proliferated on scaffolds for the group II.

Conclusion. In conclusion, we found that cyclic stretching (10% deformation), even 2 hours per day, has a greater impact on damage polymer than cell culture which seems to degrade the polymer in the first weeks but also reinforce the structure during colonization compared to group I.

Keywords. Mechanical test, biodegradable polymer, stem cell, bioreactor

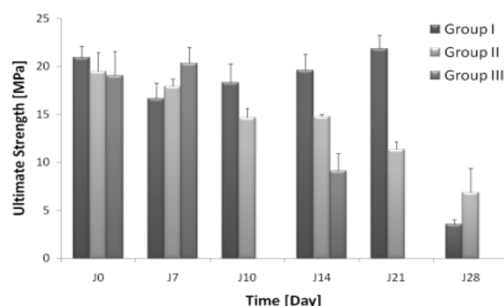

Figure 1. Ultimate strength evolution during polymer degradation

(27.O21) DEVELOPMENT OF A NOVEL COLLAGEN-BASED COMPOSITE BIOMATERIAL FOR CARTILAGE DEFECT REPAIR: COMPOSITIONAL AND STRUCTURAL OPTIMISATION

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Introduction. While articular cartilage exhibits a limited capacity for self-repair, the use of Tissue Engineering (TE) biomaterials have shown some promise in healing cartilage defects (Cheng et al., 2010). Scaffold composition and architecture can manipulate cell attachment, proliferation and differentiation (Pieper et al., 2000) by creating unique biochemical environments with distinctive ligand binding sites(Murphy et al., 2010). Collagen-Glycosaminoglycan (CG) scaffolds have been used extensively in TE due to their ability to support chondrogenesis and customisable pore architecture (O'Brien et al., 2005). In this study, we investigated the effects of varying the type of glycosaminoglycans as well as varying the mean pore size in CG scaffolds. Collagen scaffolds co-polymerised with (i) chondroitin sulphate (CG-CS) or (ii) hyaluronic acid (CG-HyA) were assessed for their chondrogenic potential in vitro.

Methods. Porous CG scaffolds were fabricated by varying the freeze-drying process(O'Brien et al., 2005) to produce mean pore sizes of 130µm and 300µm. Scaffolds were seeded with rat MSCs and cultured in chondrogenic medium for 28 days and assessed for cellular infiltration, sulphated GAG content and gene expression using RT-PCR.

Results. RT-PCR revealed sox-9 and collagen type II up-regulation in the CG-HyA group compared to CG-CS. Overall, larger pore sizes (300µm) stimulated an elevation in proteoglycan production. Interestingly CG-CS samples exhibited an inhomogeneous cellular and sulphated GAG distribution in comparison to CG-HyA of the same pore size.

Conclusions. By altering the composition from CG-CS to CG-HyA, we notice significant increases in cartilage matrix production and collagen type II gene expression (> 3 fold increase) demonstrating the potential of HyA in promoting in vitro chondrogenesis within collagen-based scaffolds. Coupled with large pores, the level of chondrogenesis is elevated even further. With these characteristics this scaffold may show potential for cartilage repair applications.

Acknowledgements. Funding was provided by Enterprise Ireland Commercialisation Fund, Technology Development Phase CFTD/2009/0104.

Keywords. Cartilage, scaffolds, collagen-glycosaminoglycans, pore size

(27.O22) PROBING MECHANICAL PROPERTIES OF DECELLULARIZED LUNG MATRIX WITH ATOMIC FORCE MICROSCOPY

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Introduction: Engineered lungs are potentially useful as an alternative in lung transplantation. It has been shown that differentiation of both adult and embryonic stem cells in culture depends on the stiffness of the substrate. Moreover, a site-specific differentiation pattern has been recently found when seeding embryonic stem cells inside decellularized lung matrices. Therefore, the mechanical interaction with the microenvironment could be critical in determining the fate of stem cells in the lung. Although the stiffness (E) of lung epithelial cells is known to be approx 1 kPa, the stiffness of alveolar extracellular matrix is poorly defined. AIM: To study the stiffness of the decellularized rat lung matrix by Atomic Force Microscopy.

Methods: Rat lungs (N=2) were harvested from exsanguinated adult Sprague-Dawley rats and treated with a detergent solution to remove cellular components. Decellularization was carried out by subsequent freezing and thawing and sodium dodecyl sulfate perfusion. After several washes with PBS, the upper lobe of the left lung was snap frozen with liquid nitrogen in tissue freezing medium (OCT). Lung section (7 micron thick) were then obtained using a cryostat and placed on top of positively charged glass slides. Several rinses with PBS were performed prior to the AFM measurements. The samples were probed with a V-shape cantilever (nominal stiff constant: 0.03 nN/nm) with a pyramidal tip. After calibration on the glass surface, the sample was indented (10 micron/s) up to 0.5-1 micron. The stiffness was computed by fitting the pyramidal Hertz model to the force-indentation curves.

Results: E of alveolar septa was 22.9 ± 22.5 kPa (m ± SD). Pleural wall (E = 46.0 ± 26.8 kPa) was stiffer than alveolar septa ($p < 0.01$).

Conclusion: These data indicate that alveolar cells mechanically interact with a matrix microenvironment one order of magnitude stiffer.

Support: SAF2008-02991 and PI081908.
Keywords. Extracellular matrix mechanics, atomic force microscopy, lung stiffness

(27.O23) EFFECTS OF DYNAMIC MECHANICAL FORCES ON HUMAN MESENCHYMAL STEM CELL THERAPY PRODUCTS

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Mechanical stimuli are important factors for cell and tissue maintenance. They may cause stem cell differentiation and more intense stimuli, vibrations, can reduce cell viability significantly [1]. When “ready to use” cell therapy products are transported, changes potentially caused by mechanical vibrations are not desirable and should be investigated, estimated and predicted. It is therefore necessary to understand, by measuring any damage, the consequences of vibrations during transport on cells packaged for immediate clinical use. This is important for cells transported in suspension, especially for cell types sensitive to freeze-thawing cycles such as stem cells. An earlier experiment used human dermal fibroblasts (hDF) [1].

The work reported here is on human mesenchymal stem cells (hMSC). Mechanically induced damage has been quantified by measuring cell viability using the trypan blue exclusion method. In addition this work has used the apoptotic cell surface marker Annexin V-FITC to characterise necrosis, apoptosis and late apoptosis. These measurements showed that cold storage alone (without vibration) causes a decrease of cell viability and that cells are susceptible to damage from some vibration regimes after only 48 hours of cold storage. Further work will investigate how cold storage and transport vibrations influence stemness, functional potential and key mechanical properties of the cells.

[1] N. I. Nikolaev, Y. Liu and D. J. Williams, 6th World Congress of Biomechanics (WCB 2010). August 1-6, 2010 Singapore IFMBE Proceedings, 2010, Volume 31, Part 4, 1137-1140.

Keywords. stem cell, transport vibrations, cell viability, cell damage

(27.O24) MECHANICAL EVALUATION OF NANO-CELLULOSE CONSTRUCTS DESIGNED FOR TISSUE-ENGINEERING OF AURICULAR CARTILAGE

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Like any cartilaginous tissue the external part of the ear, the auricle, cannot regenerate once damaged. Tissue engineering (TE) is a potential alternative to surgical reconstruction which would reduce donor site-morbidity. Adequate mechanical properties which allow for daily activities (e.g. sleeping, wearing earphones) are key. Various biodegradable ear-shaped scaffolds have been investigated, yet due to inadequate degradation rates mechanical properties deteriorated with long-term in vivo implantation. Nano-cellulose (NC) is a biocompatible but non-biodegradable biomaterial, making it a promising scaffold material for ear reconstruction. This study aims to characterize the mechanical properties of various NC constructs and compare them to human auricular cartilage to decide the best candidate for auricular TE.

Materials and Methods. Fresh human auricular cartilage (n=5) is harvested after reconstructive surgery. Three different NC scaffolds are produced by bacterial synthesis: 5%-NC (95% water, n=5), 2%-NC (n=7), microporous NC (n=6). Samples are cut to 5x5 mm2, and tested in stress-relaxation (5%-strain steps with 30 minutes relaxation) on a Zwick-Z005 machine (10 N load cell, Ø0.9 mm indenter). Material properties, including equilibrium modulus (Eeq), are computed using MATLAB.

Results. Figure 1 shows that native auricular cartilage has a higher Eeq (1184.6 ± 676.4 kPa) than 5%-NC (194.7 ± 49.0 kPa) and 2%-NC (92.5 ± 42.5 kPa). The stiffness of microporous NC was below the sensitivity of the system.

Discussion and Conclusions. NC mechanical properties are highly tunable, covering stiffnesses from < 15 kPa up to 200 kPa, where 5%-NC scaffolds have the greatest mechanical potential as a candidate for auricular TE. In future, higher concentrations will be used to further increase scaffold strength. Cell-seeding and subsequent extracellular matrix production will further improve the mechanical properties of the constructs, making them a relevant choice for auricular TE efforts.

Acknowledgements. A Swiss National Science Foundation, NRP63, and ERA-NET/EuroNanoMed project (EAREG-406340-131009/1)
Keywords. nano-cellulose, auricular cartilage, stress-relaxation

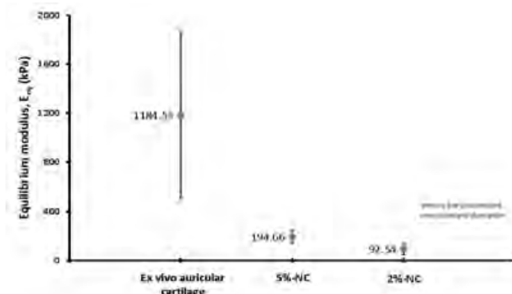

Figure 1: Equilibrium modulus of native auricular cartilage, 5%-NC and 2%-NC scaffolds.

(27.O25) GENERATION OF A SCAFFOLD WITH HUMAN BLOOD PLASMA AND SODIUM ALGINATE FOR THE GROWTH OF FIBROBLASTS

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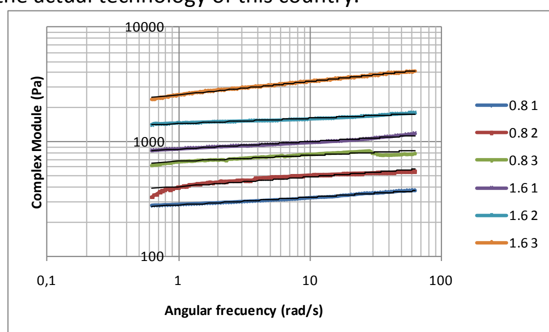
The mixture of polysaccharides and proteins has proven to be an excellent option to produce cell growth media due to the fact that polysaccharides offer support and stability to the film and proteins offer adhesion structures and cell proliferation. By determination of Potential ζ it was possible to define the interaction between alginate sodium and human blood plasma, indicating an increment at the blood plasma potential (41mV) in the presence of sodium alginate solutions (i.e. concentrations of 0.8% w/v and 1.6% w/v, with 60 mV and 64 mV, respectively) which reflects stability in suspension for the generation of films. By viscosimetry tests performed in a

Rheometer Bohlin CVOR-200 it was found that such mixtures have a pseudoplastic behavior. The mathematical modelling obtained by the Ostwald-De-Wale model shows no significative changes at the behavior of alginate solutions when plasma is added. By spraying on a solution of calcium chloride mixtures of alginate and plasma were gelled. Then it was determined the linear viscoelastic región (VLR) by strain sweep tests to find such a region below 1% strain; by Frequency sweep tests were adjusted Power Laws models for different concentrations of sodium alginate (0.8 and 1.6% w/v) and calcium chloride used (1,2,3% w / v). It was found that G 'is about 10 times greater than G'', which reflects the solid performance of the films obtained. For a concentration of 1.6% sodium alginate and calcium chloride 3% w / v which was the most resistant, strength was determined by tensile testing using a 22486.96±TA XT Plus texture analyzer and it was found a Young's modulus of 243.20 Pa± 1281.33Pa, an Ultimate tensil Stress (UTS) of 6369.38 ±243.20 Pa and a maximum strain of 0.2425 ± 0.009. Finally, it was evaluated the growth of human fibroblasts isolated from foreskin of infants and it was found a splendid growth on the surface available. The obtained support is an excellent alternative for skin losses Colombia, which can be manufactured with the actual technology of this country.



(27.P1) DEVELOPMENT OF COMPUTATIONAL TOOLS FOR THE QUANTIFICATION OF KEY PARAMETERS INVOLVED IN CELL MECHANICS OF TUMOR CELLS

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Introduction: Most diseases present a complex genetic profile with multiple changes in molecular expression. Nonetheless, physical forces and mechanical structures also contribute to the changing properties of cells and tissues and impact cellular decision making. Remarkably, cancer biologists have begun to recognize that a critical component of malignant phenotype involves changes in cell mechanical properties.

Here we have developed a set of computational tools based on image processing to systematically quantify key parameters that are related to cell mechanics. We have applied the tools to further study the mechanobiology of glioblastoma multiforme, which is only partially known, and of HeLa cells.

Methods: HeLa cells and two glioma cell lines (U373-MG and U-87-MG) were grown on polyacrylamide hydrogels with controlled mechanical properties. The following parameters were quantified: cell spread area (Naverage=70); actin, non-muscle myosin II and vinculin distribution across the cell; focal adhesions (size and number, Naverage=500); cell migration (Naverage=30) and cellular traction forces (Naverage=30). Also, studies of these parameters under the effect of pharmacological inhibitors of cell contractility with blebbistatin, cytochalasin B and latrunculin B were performed. Computational tools were developed using Image J, Matlab and NIS Elements (Nikon) software.

Results: Our quantification tools allowed us to systematically measure cell spread area, focal adhesions, cell traction forces and distribution of actin and non-muscle myosin II. Results confirmed that, on rigid substrates, tumor cell spread area is larger, cells showed more prominent stress fibers, and larger and stronger focal adhesions, and could move faster. On more compliant substrates, cell morphology is more spherical and migration is strongly inhibited.

Conclusion: We have successfully developed different computational tools for systematic and high-throughput studies of key parameters related to cell mechanics and the actin cytoskeleton, and we validated them using HeLa and glioma cell lines.

Keywords. Computational tools, mechanobiology, actin cytoskeleton, cancer cells

(27.P2) STIFFENING OF TUMOR MICROENVIRONMENT IS NECESSARY FOR THE ACTIVATION OF EMT UNDER THE EFFECT OF TGFß

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Introduction: Epithelial–mesenchymal transition (EMT) is a decisive process of primary and also metastatic tumor progression where immotile epithelial cells become mesenchymal migratory cells expressing stem cell properties. It is believed that TGFß enhances this transformation, although its mechanism of action is still unknown.

Also, tumor cells are known to remodel their surroundings, leading to stiffer microenvironments, a fact that has been proposed to favor the EMT process.

In this work, we established an in vitro system to study the role of TGFß on the EMT process under controlled mechanical properties.

Methods: AML-12 cells, mice liver hepatocyte cells, were cultured on fibronectin-coated polyacrilamide substrates of defined mechanical rigidity. Expression of the epithelial marker E-cadherin and mesenchymal marker vimentin was measured by immunofluorescence to quantify the progress of EMT. Cell spread area, cell-cell contacts, focal adhesions, actin cytoskeletal organization and cell motility were also measured.

Results: We found significant differences in cell motility, single cell and cell aggregates spread area and cell-cell contacts under different mechanical environments. As the stiffness decreases, AML-12 cells form aggregates with a smaller spread area and rounded shape, stronger

cell-cell complexes, less focal adhesions and reduced motility, confirming that the progression of EMT is related to a stiffening of the environment. Expression of E-Cadherin and vimentin verified this observation. The effect of TGFß on the response of AML-12 cells varied at different mechanical scenarios: on very stiff surfaces TGFß activated EMT, whereas AML-12 cells treated with TGFß underwent apoptosis on more compliant surfaces.
Conclusion: Our results provide solid evidence that stiffening of extracellular matrix is necessary for the activation of EMT under the effect of TGFß.
Keywords. Mechanobiology, EMT, AML-12, TGFß

(27.P3) ACTIVE COMPUTATIONAL MODELLING OF CYTOSKELETAL REMODELLING DURING CELL SPREADING

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Introduction. In the present study an active constitutive formulation for the remodelling and contractile behaviour of the actin cytoskeleton and focal adhesions is used to simulate cell spreading on a rigid substrate. We demonstrate that this active formulation accurately captures experimentally observed measurements, in contrast to traditional passive cell models.

Methodology. The formation of the actin-myosin cytoskeleton is captured by allowing stress fibres to assemble in any direction at any point in the cell. Cellular signalling drives assembly of actin-myosin stress fibres, while tension reductions in the cytoplasm lead to dissociation of the actin cytoskeleton [1]. Focal adhesion formation is predicted based on the thermodynamic equilibrium of bound and unbound integrins on the cell membrane [2]. Increased cytoskeletal tension leads to clustering of bound integrins to form focal adhesions.

Results. In the simulations initially detached cells and were allowed to actively spread for 10 minutes on a rigid substrate. As the cells spread, distinctive bands of highly aligned stress fibres are predicted to form. These bands extend from the nucleus to the cell periphery, as shown in Figure 1. Focal adhesions are predicted to cluster at the cell periphery. Cells spread on stiff substrates generate large concentrations of stress fibres and focal adhesions, in contrast to cells on compliant substrates.

Discussion. These results show the interdependence between the remodelling of the actin cytoskeleton and the formation of focal adhesions. Our finding that stiffer substrates cause more stress fibre and focal adhesion formation is supported by in-vitro observations. Our predictive model of active cytoskeletal responses to mechanical stimuli can be used to guide the design of ECMs for tissue engineering applications.

[1] Deshpande et al, 2007, PNAS 463, p787.
[2] Deshpande et al, 2008, JMPS 56, p1484.

Keywords. cell spreading, stress fibres, focal adhesions, actin, substrate stiffness, computational model

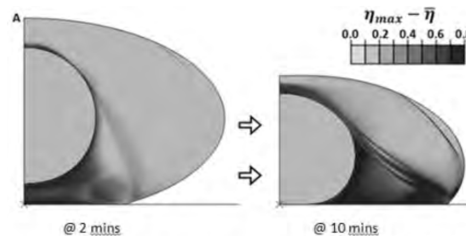

Figure 1. Stress fibre evolution during cell spreading (note: only half cell shown).

(27.P4) THE INFLUENCE OF SUBSTRATE STIFFNESS ON THE OSTEOGENIC DIFFERENTIATION OF UMBILICAL CORD STEM CELLS

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Abstract: Tissue formation and maintenance is regulated by various mechanical signals transmitted between cells, but also originated from cell-substrate interactions. Recent studies showed that cell fate in vitro could be directed by the elasticity of the substrate on which the cells are grown.
In our study osteogenic potential of mesenchymal stem cells isolated from umbilical cord (UC-MSC) was investigated in relation to the substrate rigidity

Methods: Polyacrylamide substrates of two different stiffness were used, i.e. a “soft” and a “stiff” (Young's modules of 2,66 kPa and 28,12 respectively). Both substrates and a Tissue Culture Plate (TCP) which served as a “high rigidity” control were coated with collagen I. UC-MSCs were cultured for 7 and 14 days in osteogenic medium. Cell viability (XTT assay) and cell number (PicoGreen test) were assessed, and mineralization was evaluated (quantitative Alizarin Red). Expression of Runx2, collagen I, and osteocalcin genes (selected markers of differentiation toward osteoblasts) was investigated by a real-time PCR.

Results: Cell viability and morphology was comparable on all substrates used. The genes expression was not affected by a substrate stiffness at day 7, while at day 14 expression of all three genes were the lowest on the “soft” substrate, values for collagen I were comparable on the “stiff” substrate and TCP, while for osteocalcin and Runx2, the highest values were obtained on TCP. Mineralization was observed only at day 14 - calcium deposition was positively correlated with the substrate stiffness.

Conclusion: Our results confirmed that the stiffness of the substrate may be a player in the osteogenic differentiation of progenitor cells, as shown for UC-MSCs.

Acknowledgment: to prof Zygmunt Pojda who kindly provided UC-MSCs.
This work was supported by project no. N N302157037 from the Polish funds for scientific research in 2009-2012.
Keywords. stiffness of the substrate, osteogenic differentiation, mesenchymal stem cells

(27.P5) THE USE OF A BIOREACTOR FOR PREPARATION OF TISSUE ENGINEERED CARTILAGE FOR MECHANICAL AND TRIBOLOGICAL TESTING: A METHODOLOGICAL STUDY

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Introduction: Joint diseases such as osteoarthritis present a great socio-economic cost to society. Tissue engineering (TE) techniques have the potential to provide a patient-specific treatment for the repair and regeneration of articular cartilage, restoring function and low friction properties. Research has shown that tissue engineered constructs prepared using mature cells and standard culture conditions has inferior mechanical properties compared to native cartilage, and it has been suggested that advanced bioreactor-based culture would improve biological quality and therefore tribological properties. Aim: The aim of this study was to compare conventional culture with a rotating wall vessel (RWV) bioreactor for the preparation of tissue engineered cartilage constructs for biomechanical testing.

Methods: TE cartilage constructs were cultured under standard semi-static conditions and in a RWV bioreactor. Indentation alongside start-up and dynamic friction tests were performed with PBS and synovial fluid as a lubricant whilst biological quality was assessed using histology and biochemical analysis.

Results: The extracellular matrix composition of the constructs cultured in the RWV was superior and while all the constructs exhibited a time-dependent increase in deformation and friction under test, the friction values were lower for constructs cultured in the RWV (μ20min=0.20±0.02) than those under the semi static conditions (μ20min=0.25±0.06), and also closer to those of native tissue (μ20min=0.20±0.05). Conclusion: Tissue engineered cartilage cultured using the RWV bioreactor demonstrated low friction properties in common with native tissue and superior to those shown by tissue cultured under standard conditions. This study demonstrated that the use of more advanced culture conditions can result in higher quality tissue with more consistent biological and frictional characteristics.

Acknowledgements: The authors are grateful to the White Rose DTCTERM and the EPSRC for funding, and part of the work was performed within the EXPERTISSUES Network of Excellence (EC project: NMP3-CT-2004-500283).

Keywords. Cartilage, RWV Bioreactor, Tribology, Friction

(27.P6) VALIDATED IN VITRO CYCLIC FLUID SHEAR STRESS ALTERS HUMAN TENOCYTE ECM SYNTHESIS

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Tendons exhibit mechanical properties withstanding high cyclic stresses and are also able to modify their properties in response to changing demands. A deeper understanding of tendon cell mechanosensation, key to this response, will point to new clinical therapies for the treatment of painful and disabling tendon disease and rupture. The aim of this study is to develop and validate an in vitro model for tendon mechanosensation, and to test the hypothesis that fluid flow stimuli are responsible for alterations in tendon cell ECM synthesis.

Human tenocytes (up to passage 3) were cultured in 0%FCS/DMEM conditions to inhibit usual activity. Cyclic fluid stress was applied using a see-saw rocker (Stuart SSL4) at 30, 45 and 60 rpm continuously for periods of 3 and 24 hours. Post stimulation, cell morphology and ECM synthesis (collagen and GAG) were investigated using microscopy (ImageJ) and biochemical assays (Sircol and DMB) respectively. Shear stresses at the cell layer were simulated in computational fluid dynamics (CFD) software (ACE) and validation provided using laser sheet particle image velocimetry (PIV) techniques.

Preliminary culture results show an increase in collagen synthesis at all speeds and times with shear stress stimuli. CFD predicted peak levels of shear stresses of 36 Pa and validation was provided by matching velocity profiles from PIV. The results indicate that collagen synthesis is upregulated by fluid shear stress pointing to the involvement of specific fluid flow mechanoreceptors. We will use the validated CFD shear stress distribution to inform cell morphology investigation across the culture dish, and modulate shear stress magnitude by changing medium viscosity. Understanding the mechanisms for cellular response to validated shear stresses is a key step in the development of future mechanobiological tendon therapies.

Keywords. mechanosensation, tenocytes, fluid stress

(27.P7) THE IMPLICATIONS OF THE RESPONSE OF HUMAN MESENCHYMAL STROMAL CELLS IN THREE DIMENSIONAL CULTURE TO ELECTRICAL STIMULATION FOR TISSUE RESGENERATION

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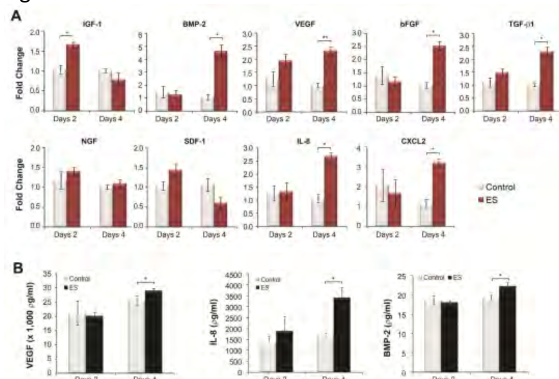
Introduction. Previously, we demonstrated that local electrical stimulation (ES) improved bone and peripheral nerve regeneration. To determine how ES induces the regeneration of different kinds of tissues, we investigated the initial ES-induced regeneration process by investigating the expression of chemokines and growth factors from human mesenchymal stromal cells (hMSCs).

Methods. In particular, we assessed the responses of hMSCs grown in three-dimensional (3D) culture on a collagen sponge, because 3D culture techniques induce cell behavior that is closer to *in vivo* cell behavior. We also compared the gene expression patterns of monolayer hMSCs with those of 3D hMSCs.

Results. Surprisingly, biphasic pulses did not affect the proliferation of hMSCs in 3D culture significantly. However, ES enhanced the gene expression and extracellular release of growth factors (BMP-2, IGF-1, VEGF), chemokines (CXCL2, interleukin (IL)-8), and chemokine receptors (CXCR4 and IL-8RB) from hMSCs grown in 3D culture. The expression of the chemokine receptor, CXCR4, was significantly different between 3D and monolayer cultures in that this receptor was expressed by cells in 3D cultures but not by cells grown in monolayer cultures.

Conclusions. These results demonstrate that ES increased the expression of a variety of growth factor and chemokine genes from 3D hMSCs, what might trigger tissue regeneration *in vivo*, independent of tissue type. Our findings also suggest that investigations using cells cultured in 3D systems may yield results that more accurately reflect *in vivo* findings than investigations using monolayer cultures.

Keywords. electrical stimulation, human mesenchymal stromal cells, three-dimensional culture, tissue regeneration



(27.P8) REGULATION OF OSTEOCYTE DIFFERENTIATION BY SUBSTRATE STIFFNESS AND SEEDING DENSITY

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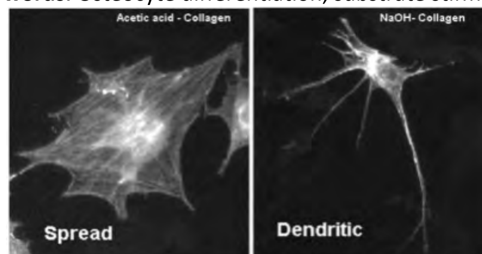
Introduction. The extra-cellular mechanical environment regulates the differentiation of many biological cells. MSC's differentiate into adipocytes, osteoblasts, neurons, or myoblasts depending on the mechanical stiffness of the underlying substrate. However, it is not yet clear how the extra-cellular mechanical environment regulates osteocyte differentiation. The objective of this study is to investigate the effect of substrate stiffness on the differentiation of pre-osteoblasts into mature osteocytes.

Methods. MC3T3 cells were plated at 10^3/cm^2, 10^4/cm^2 and 10^5/cm^2 on collagen substrates (100 ug per cm^2), neutralised with either NaOH (forming a soft gel, E < 1 kPa) or acetic acid (forming a stiff coating, E ≈ 2.4 GPa). Phalloidin-TRITC (Sigma-Aldrich) was used to examine cellular morphology at set time-points to distinguish dendritic cells, phenotypic of osteocytes. A colourimetric assay (Abcam) was used to quantify intracellular and extracellular ALP (an osteoblastic marker) from the same time-points. Immunohistochemistry and RT-PCR are used to quantitatively measure expression of DMP1 and Sost, two osteocyte specific markers.

Results. A greater percentage of dendritic cells, were observed on the softer substrate, whereas cells were spread on stiff substrate (Figure). This was particularly evident at the lowest seeding density. ALP expression peaked at day 9 for all conditions. Preliminary biochemical studies indicate that DMP1 expression is upregulated in cells cultured on softer substrates. The morphology and biochemical characteristics indicated differentiation along the osteogenic pathway from pre-osteoblast to mature osteoblast to osteocyte.

Conclusions. This study indicates that osteocyte differentiation is regulated by the ECM, specifically that of a soft collagen matrix. In vivo certain osteoblasts become embedded in newly formed osteoid, which is a soft, non-mineralised collagen matrix. We propose that this mechanical environment may play a role in triggering osteocyte differentiation. Ongoing studies using finite element methods are being implemented to delineate how substrate stiffness alters cellular mechanics and osteocyte differentiation.

Keywords. Osteocyte differentiation, substrate stiffness



(27.P9) MECHANICAL PROPERTIES OF BACTERIAL CELLULOSE IMPLANTS

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Introduction: Bacterial Cellulose (BC) is a polysaccharide composed of highly hydrated nano-fibrils and characterized by high mechanical strength, high water content and high crystallinity. Thanks to its biocompatibility, BC can be used in a wide range of biomedical applications, as artificial skin or replacement for blood vessels and meniscus. The purpose of this work has been to study the effect of the morphology of BC networks on the mechanical properties of the implants.

Methods: Several mechanical tests were performed, with a focus on tensile testing, to evaluate the mechanical properties of the BC samples: Young's modulus, tensile strength and strain were determined and analyzed. By changing the percentage of cellulose content in the BC network, it was possible to produce BC materials with mechanical properties more similar to the native soft tissue that have to be replaced. As it is the micro-structure of the network of fibrils which provides the mechanical performance of the material, a SEM analysis of the morphology of BC at a micro-level has been conducted.

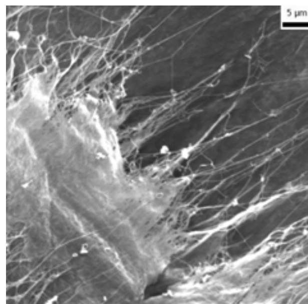
Results: Results showed that Young´s modulus, tensile strength and tensile strain vary to a great extent when changing the cellulose content of the sample. Through SEM analysis of the network of BC pellicles at a micro-level, it was possible to show the realignment of the fibers affected by tensile stress, which has a direct effect on the mechanical properties of the material.

Conclusions: The mechanical properties analyzed showed that Bacterial Cellulose is a very attractive material for new possible biomedical applications. The growth of the commercial applications of this material is strongly depending on discovery and utilization of all its unique properties, which can turn Bacterial Cellulose into an amazing biomaterial.

Acknoledgements: Magdalena Zaborowska, Aase Bodin (BBV-lab; Chalmers) Guillermo Toriz, Anders Höije, Anders

Mårtensson at (Polymer technology; Chalmers) Kristoffer Drotz (ArterionAB)

Keywords. Bacterial cellulose, Biomechanics, Biomaterials, Scaffold



(27.P10) EFFECT OF THE MECHANICAL PROPERTIES OF THE SUBSTRATE ON THE CARTILAGE PHENOTYPE IN SHEEP CULTURED CHONDROCYTES

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Background. One of the parameters that affects chondrocyte phenotype maintenance is the physical properties of the environment. Much is written about the influence of active mechanical stimulation on cartilage cells, but little is known about how does a chondrocyte respond to different stiffness in the substrate on which it is cultured. Aim: Our goal was to describe the response of the chondrocyte to the mechanical properties of the substrate.

Methods. We used collagen gels fabricated with different collagen concentration. Mechanical properties were calculated and chondrocytes cultured using gels as substrates for monolayer culture or embedded inside the collagen for 3 dimensional culture. We studied the cell proliferation rate and the gene expression of collagen types I and II for cells in passage 0, 2 and 4.

Results. Chondrocytes in gels with high collagen concentration and so, with a higher stiffness, showed a significantly increased proliferation rate. According to gene expression analysis, on the first hand type II collagen was significantly higher in low stiffness gels both in monolayer and in the 3 dimensional culture. On the other hand collagen type I was up-regulated in the stiffer gels in 3D cultures, but surprisingly, down regulated when in monolayer culture. Finally, the passage of chondrocytes in culture did modify the response of the cells to the mechanosensing.

Conclusions. The chondrocyte phenotype, according to gene expresión of cartilage markers as well as to proliferation rate can be modulated by changing the mechanical properties of the substrate and must be further studied.

Keywords. Chondrocyte, mechanobiology, hydrogels

(27.P11) THE EFFECT OF CaF2 CONTENT ON MECHANICAL PROPERTIES OF SOME BONE CEMENTS BASED ON HYDROXYAPATITE

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Introduction. The main effect of fluoride on bone is found to be related to the direct stimulating effect on the proliferation of osteoblasts and bone matrix synthesis, and to the decrease rate of bone resorption and ion exchange, when hydroxyapatite (HAP) is converted into fluoroapatite, which is more resistant to osteoclastic resorption.

Methods. HAP powders were obtained by co-precipitation method using Ca(NO3)2 and (NH4)2HPO4. After thermal treatment at 650ºC, HAP powders were investigated by FT-IR, XRD and TEM. HAP is formed primarily from particles with an average length of about 70nm and an average diameter of about 37nm. The CaF2 powder is characterized by particles with a mean diameter of about 34.6µm. Polymer matrix is Bis-GMA resin and TEGDMA in ratio 1.5/1(%wt.). Self curing system is based on N,N-dimethyl-4-toluidine and benzoyl peroxide. The test specimens (4 mm in diameter and 8 mm long) for compression testing were stored in water at 37°C for 24h before the mechanical tests, which were performed in a universal testing machine at a loading rate of 0.75 mm/min until their fracture. Samples measuring 8 mm diameter and 1 mm thickness together with aluminum step wedges were radiographed with an intraoral sensor system XIOS Plus(Sirona) at 60kV, 7mA, 0.04sec with a target-film distance at 30cm using X-Ray Soredex(Minray).

Conclusions. The highest compressive strength of 190.78 MPa was obtained for HAP based bone cement (C0, Table 1). The adding of CaF2 showed a slow decrease of compressive strength of bone cements to 170.53(C5), 136.67(C10), 165.42(C15) and 136.41(C20) Mpa. The addition of BaSO4 nanopowder improved radiopacity of these cements from 0.32 mm Al for C0 bone cement up to about 1.52 mm Al for bone cements containing CaF2.

Acknowledgements. One of the authors (G.F.) thanks the Babes-Bolyai University of Cluj-Napoca for the financial support through the POSDRU/89/1.5/S/60189 Grant.

Keywords. hydroxyapatite, bone cement, mechanical properties, radiopacity

Table 1. Composition of bone cements

Code	Composition (% wt.)	P/L (% wt.)
C0	HAP (50%), Polymer (50%)	1/1
C5	CaF_2 (5%), $BaSO_4$ (15%), HAP (30%), Polymer (50%)	1/1
C10	CaF_2 (10%), $BaSO_4$ (15%), HAP (25%), Polymer (50%)	1/1
C15	CaF_2 (15%), $BaSO_4$ (15%), HAP (20%), Polymer (50%)	1/1
C20	CaF_2 (20%), $BaSO_4$ (15%), HAP (15%), Polymer (50%)	1/1

28. MICROVASCULAR ENGINEERING

Chair: Nolan L Boyd
Co-chair: Thomas H Barker
Keynote speaker: James B Hoying
Organizers: Nolan L Boyd, Thomas H Barker
Synopsis: Providing a functional microvasculature is required for successful engineering of an organ replacement, but has yet to be achieved. It is important to recognize that the requirements to generate a microvascular system are fundamentally different to engineering a large artery replacement and should be tailored accordingly. To achieve this goal will require an interdisciplinary effort combining microvascular biology, stem cells, matrix engineering, biomechanics and micro/nanofabrication. This symposium will focus specifically on state-of-the-art approaches to generate stable, mature, perfusable microvascular circulatory systems in 3D using both in vitro and in vivo models.
The Keynote Speaker, Professor James (Jay) Hoying, is an expert in microvascular biology and tissue engineering. He will discuss the needs for and challenges to microvascular engineering as well as contemporary techniques and solutions including the manipulation of intact microvessel fragments for microvasculature generation. The organizers will primarily select submitted abstracts for presentation, but may also invite highly targeted speakers in the area (e.g. Jeffrey Hubbell depending on their availability) that address microcirculation topics such as 3D organization and assembly, microvascular maturation and stability, nano/microfluidics models, microvascular analysis methodology, in vivo and in vitro, matrix engineering for microvascular control, cell sourcing and functionality. Dr Nolan Boyd is an Assistant Professor in the Cellular Therapeutics Division of the Cardiovascular Innovation Institute at the University of Louisville. The focus of his research is investigating the mechanisms regulating endothelial recruitment and maturation of mural progenitor cells for microvascular stabilization. Dr Thomas Barker is an Assistant Professor in the Georgia Institute of Technology/Emory University Department of Biomedical Engineering. He is an expert in matrix biology and using engineered ECM protein fragments for controlling integrin-specific cell differentiation and function.

(28.KP) ENGINEERING THE MICROCIRCULATION

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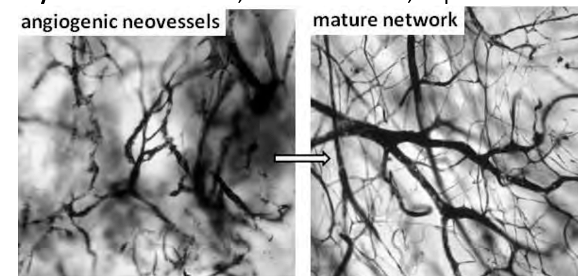
The ability to build or manipulate microvasculatures is critically important for both in vivo vascular regeneration and in vitro tissue construction. In the repair or construction of a functional microvasculature, it's important to consider that an effective microcirculation critically depends on the proper organization of stable microvessels into a perfusion-competent network. Therefore, successful neovascularizing strategies must address not only the construction of individual microvessel elements, but also the post-angiogenesis assembly of these new elements into a properly organized and adapted vascular network (see Figure). Numerous examples of tissue repair/regeneration in which perfusion is reduced even though microvessel density is elevated highlight the importance of network functionality. Moreover, any neovasculature formed as part of an implant, must make connections (or inosculations) with the host microcirculation following implantation in order for the new vascular network to persist and adapt appropriately to match implant needs. We have developed enabling technologies for manipulating microvascular networks and investigated processes and mechanisms related to the dynamics of how microvascular networks form in implanted tissues. Using a unique model of implant neovascularization, we discerned the processes by which microvascular systems grow and form in vitro (which involved the development of a custom bioreactor designed to support the culture of microvascular networks), the post-angiogenesis activities underlying the progression of pre-assembled microvessels through neovascularization once implanted, the cell-based approaches that stabilize and direct microvascular assembly in vivo, and the extent of microvessel plasticity in these assembled systems.

Keywords. microvessel, microcirculation, implant



(28.O1) REGULATION OF CATHEPSIN ACTIVITY BY MONOCYTE-ENDOTHELIAL CELL INTERACTIONS: IMPLICATIONS FOR TISSUE REMODELING

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Introduction. Inflammation and damage activate endothelium and promote monocyte adhesion, an initial step in many cardiovascular diseases (CVD). Cathepsins are cysteine proteases overexpressed in CVD that degrade elastin and collagen in the vessel wall during CVD. One disease of the microcirculation with elevated inflammation and increased circulating monocytes is sickle cell disease (SCD) characterized by vaso-occlusive crises and systemic vascular damage. This study tests the hypothesis that tumor necrosis factor alpha (TNFα), an inflammatory cytokine, promotes monocyte-endothelial cell interactions, cathepsin K activity, and related pathological remodeling of the vessel wall.

Methods. Confluent endothelial cells were conditioned with TNFα (10ng/mL) and/or Thp1 monocytes either through direct contact or indirectly through 0.2µm transwell membranes. After 24 hours, cathepsin activity was measured using multiplex cathepsin zymography that detects cathepsins K, L, S, and V in one cell extract. Cleavage of fluorescent elastin substrates were used with live cell assays.

Results. Direct monocyte contact increased cathepsin activity 10-15 fold (n=3, $p<0.05$) over indirect monocyte-endothelial cell co-cultures. Inhibition of c-Jun N-terminal kinase in TNFα stimulated endothelial cells significantly reduced cathepsin K activity by 49% and 39% in both EC only and EC-monocyte co-cultures, respectively.

Peripheral blood mononuclear cells (PBMCs) were isolated from persons with SCD, cultured with ECs, and stimulated significantly higher levels of cathepsin K, even in the absence of TNFα compared to PBMCs from non-sicklers mononuclear cells (n=4, p<.05).

Conclusions. Together, these data show that monocyte-endothelial cell interactions regulate cathepsin activity and suggest new mechanisms by which arteries and arterioles are narrowed. Continued investigation of underlying cell-cell contact signaling and contribution to proteolytic remodeling in the microcirculation will be important to properly design tissue engineered replacements with improved patency and longevity in vivo. Insights can be learned from sickle cell disease and other human diseases with exacerbated microcirculatory effects.

Keywords. proteases, cathepsins, TNF, monocytes, endothelial cells

(28.O2) IMMOBILIZATION OF RECOMBINANT FIBRONECTIN FRAGMENTS ON CHITOSAN HOLLOWED POROUS SCAFFOLDS TO PROMOTE ENDOTHELIALISATION

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Introduction. Vehicles for neural stem cells (NSCs), including porous matrices and hydrogels, are being developed to enhance the efficacy of NSC therapies in spinal cord injury. The present study aimed at developing a pre-endothelialised chitosan (CH), porous hollowed scaffold to be used for NCS transplantation. Besides contributing to angiogenesis in vivo, endothelial cells (ECs) are expected to contribute to NSC survival by mimicking the adult NSC niches. Previous work showed that colonization of CH scaffolds by ECs could be achieved by physiadsorbing fibronectin (FN) to CH. Nevertheless; FN physiadsorption was only effective in promoting cell adhesion to CH with low degrees of acetylation (DAs). In this study, rhFNIII$_{7-10}$, a recombinant fragment of hFN which includes the integrin-binding RGD site and the PHSRN domain was covalently bound to CH scaffolds to promote EC adhesion to CH with different DAs.

Methods. CH tubular porous scaffolds (DA 4 and 15%) were prepared by TIPS. rhFNIII$_{7-10}$ was produced in transformed E. coli and subsequently purified. Immobilization was performed by incubating CH scaffolds in rhFNIII$_{7-10}$ solutions (2.5 to 80 µg/mL in MES) with EDC/NHS. Functionalized scaffolds were characterized in terms of amount of immobilized rhFNIII$_{7-10}$ (^{125}I-radiolabelling), exposure of the RGD site (immunofluorescence and fluorimetry), as well as ability to promote EC adhesion and cytoskeletal organization.

Results. rhFNIII$_{7-10}$ was successfully grafted to CH scaffolds with exposure of cell-binding domains. Immobilized rhFNIII$_{7-10}$ was more effective than adsorbed hFN in promoting EC adhesion and cytoskeletal organization on DA 4%, even for concentrations as low as 2.5 µg/mL. Moreover, rhFNIII$_{7-10}$ allowed EC adhesion to DA 15% for concentrations ≥ 20 µg/mL.

Present results show that grafting of rhFNIII$_{7-10}$ can be used as a strategy to endothelialise CH scaffolds with different DAs. This is of particular interest when matrices with different degradation rates are required.

FCT (PTDC/SAU-BEB/65328/2006) supported this work.

Keywords. Engineered constructs; vascularization; fibronectin; peptide immobilization

(28.O3) ENGINEERING A FUNCTIONAL MICROVASCULATURE WITHIN A DERMO-EPIDERMAL SKIN SUBSTITUTE

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Introduction. The development of rapidly and efficiently vascularized tissue grafts is vital for tissue engineering. Especially within the first days after transplantation, rapid establishment of an intact vascular network and blood flow often decides whether or not a graft is taken. One way to accelerate vascularization of an enginered tissue is to provide it with a pre-formed vascular network. The fundamental idea behind this approach is that fast anastomosis of a pre-formed vascular network with the patient's vascular system can compensate for the delayed neovascularization, which usually results in a shortage of blood supply right after transplantation. Here we describe the generation of an engineered skin substitute, from a single human skin biopsie, displaying a network of functional and anastomosing capillaries.

Methods. Primary human endothelial cells, fibroblasts and keratinocytes were isolated from human skin biopsies and expanded in vitro. These cells were used to generate a pre-vascularized dermo-epidermal skin substitute based on fibrin hydrogels. To test the effect of pre-vascularization, the skin substitutes were transplanted on the back of immuno-incompetent rats. The quality of the engineered skin was evaluated by excising the grafts after different time-points and subsequent analysis.

Results. The microvasculature produced within an organotypic skin substitute consists of a high number of branching and continuously lumenized capillaries. After transplantation, anastomosis with the rat vasculature occured and the differentiation process of the in vitro generated microvascular structures continued by the attraction of mural cells, which are known to support stabilization and maturation of capillaries.

Conclusions. We show that a network of branching and continuously lumenized capillaries within a dermo-epidermal skin substitute can be produced in vitro, and that prevascularization of tissue substitutes (derived from fibrin hydrogels) is demanding but possible.

Keywords. Prevascularization, dermo-epidermal skin substitute, anastomosis

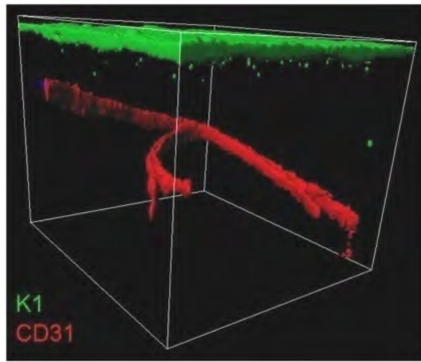

Figure. 3-dimensional illustration of engineered human epidermis (K1,green) and underlying engineered blood vessel (CD31, red)

(28.O4) MICROPATTERNING OF POLY (ETHYLENE TEREPHTHALATE) WITH PEPTIDES FOR INDUCTION OF VASCULARIZATION

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Introduction. Vascularization, the formation of new blood vessels, is important for the establishment and maintenance of complex engineered tissues. In this work, we aim to regulate and guide this process on the surface of poly (ethylene terephthalate) (PET) by the micropatterning of adhesion peptide RGDS or angiogenic peptide SVVYGLR.

Methods. The PET surfaces were micropatterned with different geometries (10, 50 and 100 um-wide stripes) using photolithography. Then RGDS or SVVYGLR peptides were covalently immobilized onto these patterns. Human umbilical vein endothelial cells (HUVECs) were cultured on these surfaces to study the alignment, elongation, as well as tube-like formation of cells from the patterned surfaces.

Results. After 24h cell culture, the HUVECs were aligned onto the stripes of peptides (either RGDS or SVVYGLR) (Fig. 1). The cell bodies, cell nuclei, and focal contacts showed a significant alignment along the direction of patterns. The cell bodies were significantly elongated when compared to cells on an unpatterned surface. As the width of the patterns becomes smaller (10 or 50 um), the alignment and elongation are more important. Confocal images revealed that HUVECs cultured on stripes with smaller widths (10 or 50 um) underwent morphogenesis and condensed into highly organized, cord-like structure along the length of the stripes. In contrast, HUVECs cultured on wider stripes of 100 um failed to undergo morphogenesis.

Conclusions. The present work proved that vascularization seems to be regulated and guided by micropatterning of bioactive peptides. The organization of cells on functionalized patterns and promotion of their morphogenesis into blood vessel-like structures are an important first step towards fabrication of complex engineered tissues. This bioactive micropatterning onto PET can be a useful experimental tool for applications in basic science and tissue engineering.

Keywords. Tissue engineering, vascularization, micropatterning, bioactive peptides

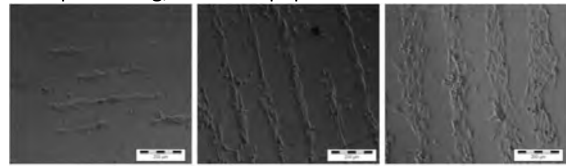

Figure. Endothelial cells aligned onto 10, 50 and 100 um-stripes of peptides.

(28.O5) ENGINEERING OF MICROVESSELS FOR THE SUPPLY OF CLINICALLY APPLICABLE TISSUE-TRANSPLANTS

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1. Fraunhofer IGB

Introduction. The lack of effectual graft vascularization still seems to represent an insurmountable hurdle for clinically applicable engineered tissue transplants. Here, we present our approach to engineer a biological vascularized scaffold (BioVaSc) that allows the generation of a wide range of human tissues.

Methods. Porcine jejunal segments were explanted by preserving their private vascular supply. Then, they were decellularized with 4% sodium desoxychylate monohydrate solution using a recirculating perfusion circuit of a bioreactor. To remove cell residues, the decellularized porcine jejunal segment was incubated in 150 ml DNase I-solution (200 U/ml) over night. To effectuate tissue sterility, the scaffold was γ-irradiated with 25 kGy. The vascular remainings within the decellularized jejunal segment were reseeded in a 2-staged procedure with human I) bone marrow derived mesenchymal stem cells (bmMSC), II) cutaneous microvascular endothelial cells (mEC), or III) peripheral blood mononuclear cells (PBMC) using the recirculating perfusion set up. To safeguard ideal culture conditions, temperature, gas exchange, pump activity and systemic pressure levels in the bioreactor were controlled computer aided and adjusted automatically when necessary. Medium samples were obtained for sterile control daily.

Results. Histological work-up confirmed the thorough decellularization of the porcine jejunal segment including its feeding artery and draining vein. Subsequent tissue incubation with DNase solution resulted in complete of cellular and DNA removal. The injected vascular cells (bmMSC, mEC or PBMC) repopulated the decellularized vascular structures and differentiated into an endothelial lining expressing the characteristic vascular surface markers CD 31, VE-cadherin and Flk-1. Pre-clinical and clinical applications confirmed vessel and microvessel function.

Conclusions. The BioVaSc represents a universal starter matrix for the generation of clinically applicable tissue transplants. It also may be applied for studying angiogenesis in vitro.

Acknowledgements. This work is funded by grants of the Federal Ministry of Education and Research (01KQ0902N, 01KQ0902O, and FKZ 0315575) and the German Research Foundation (WA 1649/3-1).

Keywords. microvessel; scaffold; transplant; vascularization

(28.O6) DELAYED ADDITION OF HUMAN MESENCHYMAL STEM CELLS TO PRE-FORMED ENDOTHELIAL CELL NETWORKS PROMOTES VASCULARISATION WITHIN A COLLAGEN-GLYCOSAMINOGLYCAN SCAFFOLD

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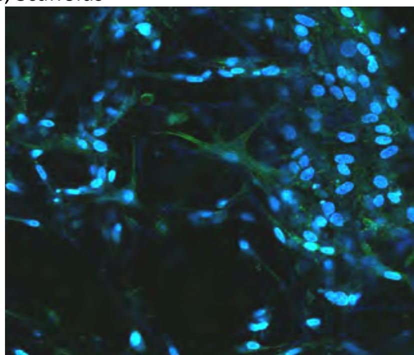
Graft failure post implantation. Current research is now focused on developing pre-vascularised constructs with potential clinical applications. This study aims to vascularise, in vitro, a highly porous collagen-glycosaminoglycan (CG) scaffold developed specifically for bone regeneration.

Human umbilical vein endothelial cells were seeded alone or in co-culture with hMSCs (4:1 ratio) on CG scaffolds. Immediate and delayed addition of MSCs to ECs was also investigated to optimise co-culture conditions. Multi-photon imaging was carried out at day 6, 10 and 14 post-seeding to assess vessel-like structure distribution throughout the 3D porous architecture of the scaffold. Haematoxylin and fast green staining was carried out on 10μM sections to observe lumen formation. Vessel-like structure formation was observed in all groups by day 6. In the co-culture group with delayed addition of MSCs multi-photon imaging demonstrated that these structures were well developed with interconnecting networks forming within the porous collagen network with structures extending ~900μM into the scaffold (Fig 1). Histological imaging revealed the formation of lumina within the nascent vessels, which were more pronounced in the co-culture group when compared to ECs alone. Vascular regression began to occur across all groups by day 14.

This study has shown for the first time that CG scaffolds are able to support vessel-like network formation using ECs with delayed addition of MSCs. Extensive network formation was observed within the scaffold at day 6 which is ideal for bone tissue regeneration as it is essential that pre-vascularization of constructs occurs efficiently to enable clinical translation. Ongoing work is focusing on in vivo evaluation of the pre-vascularised scaffolds in a rat calvarial defect model. It is envisaged that such novel vascularization strategies may overcome the current problem of graft failure post-implantation.

Acknowledgements: SFI, HRB & Integra

Keywords. Endothelial Cells, Perivascular Cells, Co-cultures, Scaffolds



(28.O7) ENGINEERING VASCULARIZED ALIGNED MUSCLE CONSTRUCTS USING MECHANICAL STRAIN

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Introduction. Skeletal muscle tissue engineering holds promise for the treatment of patients with traumatic injury. We are currently able to make small three dimensional (3D) muscle tissues with aligned muscle fibers. In order to advance towards in vivo application, which requires the growth of larger tissue constructs, vascularization is required. We hypothesize that by using co-cultures of muscle and endothelial cells merely mechanical cues are sufficient to generate a properly aligned vascularized muscle.

Methods. C2C12 myoblasts and H5V endothelial cells were cultured in a 3D hydrogel culture system with or without anchoring points. After one week the constructs were fixated and a whole mount staining was done with antibodies against CD31 and alpha-actinin. The constructs were analyzed using confocal microscopy and alignment of muscle fibers and vascular structures was investigated using Mathematica. VEGFR-2 blocking antibody was used in some experiments.

Results. Endothelial cells in 3D anchored constructs align in the direction of strain, but without the presence of muscle fibers do not form tubular structures. When muscle cells are co-cultured with endothelial cells, both the muscle fibers and endothelial cells align in the direction of strain induced by the anchoring points. When cultured without anchoring points no alignment was observed. Moreover, in the co-cultured constructs the endothelial cells form vascular structures dependent on the presence of the muscle cells. In a pilot experiment we tested whether VEGF, produced by the muscle cells, was the factor essential for the organization of endothelial cells in tubular structures, which indeed seemed to be the case.

Conclusions. Strain induced by anchoring of 3D tissue constructs induced an alignment of both muscle fibers and endothelial cells. Moreover, in the constrained co-culture tissue samples the endothelial cells organized into tubular structures, whereas this was not the case in the unconstrained situation.

Keywords. Muscle, vascularization, mechanoregulation, alignment

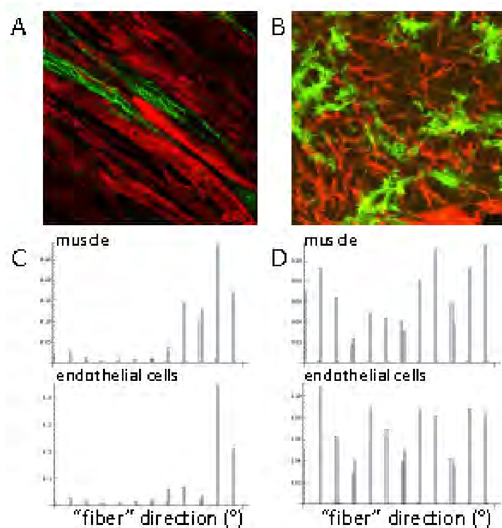

Figure 1 Confocal image of muscle constructs of C2C12 myoblasts (alpha-actinin, red) and HSV endothelial cells (CD31, green) with (A) and without anchoring points (B), with respective alignment analyses in C and D

(28.O8) CO-OPERATIVE INDUCTION OF VASCULAR AND OSTEOGENIC LINEAGES IN ENGINEERED BONE GRAFTS

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Introduction. We recently reported *in vitro* engineering of large, anatomically shaped bone grafts. Vascularized component is essential for the subsequent *in vivo* functionality of these constructs. To establish a stable vascular network within the engineered bone matrix, we co-cultured human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (MSCs) under various environmental conditions. Our main goal was to elicit synergistic interactions leading to the development of vascular capillary-like network inside the mineralized tissue matrix.

Methods. Cells were seeded into decellularized trabecular bone scaffolds and cultured for 6 weeks *in vitro* to investigate the effects of temporal variations in growth-factor availability and addition of cells on the development of vascular and bone compartments. The resulting grafts were also implanted sub-cutaneously into nude mice to determine the functionality of engineered vessels.

Results. Following *in vivo* implantation, the amounts of mineralized bone matrix were similar in all grafts, whereas the formation of vascular networks and their ability to anastomose with the host vessels were significantly different between the groups.

Conclusions. Based on the improved vessel connectivity, it was determined that the vascular development should be induced prior to osteogenesis, and that the addition of fresh MSCs during osteogenic induction improved the formation of bone tissue and the functionality of engineered vessels. We propose that this cultivation system, designed to mimic various aspects of endothelial cell – osteogenic precursor interactions, can be used as a controllable model to study heterotypic cellular interactions that couple vessel formation and osteogenesis *in vivo*.

Keywords. Microvasculature; tissue-engineered bone; mesenchymal stem cells; umbilical vein endothelial cells

(28.O10) DEVELOPMENT OF TISSUE-ENGINEERED VASCULAR GRAFTS USING NON-WOVEN PET SCAFFOLDS

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1. National Research Council of Canada

Introduction. Polyethylene terephthalate (PET) vascular grafts perform well for large-caliber vessels but fail in small-diameter applications due to thrombogenicity and mechanical mismatch. Production of tissue-engineered PET grafts to prevent thrombogenicity is limited by the low PET cell adhesion. Using a melt-blowing process, we fabricated novel non-woven PET scaffolds (ID 6.0 mm) with vas-cular-matching compliance1 and investigated a novel cell seeding method2 and flow condi-tions on cell retention, and extracellular-matrix (ECM) deposition.

Materials and Methods. Scaffolds were seeded abluminally with aor-tic smooth muscle cells (AoSMC; 2×106 cells) or AoSMC (106 cells) + fibroblasts (AoF; 106 cells), and luminally with endo-thelial (AoEC; 2×106 cells) cells using a novel device that allows micro-patterned po-sitioning of cells through a software-controlled delivery system (Fig. 1B). Scaf-folds were rotated (6-days) and then sub-jected to either a constant flow-rate (50 ml/min) or a flow-rate ramp (10-50 ml/min) in a pulsatile-pressure bioreactor (8-days). Cell retention and ECM deposition was evaluated by immunofluorescence and Western-blot (WB).

Results. Scaffolds subjected to 50 ml/min flow pre-sented a severe cell loss compared to those subjected to gradual flow rate conditioning, which were homogenously and fully sealed by AoSMC and AoEC (Fig. 1C-D), and re-sponded to pulsatile pressure (120/80 mmHg) without leakage. Western-blot analysis indicated that scaffolds seeded with AoSMC+AoF contained higher levels of elastin than those seeded with AoSMC alone (Fig. 1E).

Conclusions. The PET structures can be uniformly seeded with vascular cells using a novel automated cell seeding device. Gradual flow rate ramp-ing enhances cell retention in PET scaffolds. ECM formation improves by the presence of AoSMC+fibroblasts in the scaffold.

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2. Moreno et al.US patent application 20100233811.

Keywords. PET scaffolds, automated cell seeding, bioreactor, vascular graft

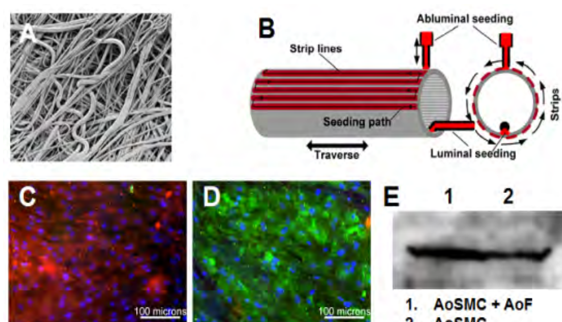

Figure. SEM of PET scaffold (A); Seeding technique (B); Fluorescently-labeled AoSMC (C) AoEC (D) in the abluminal and luminal side of scaffolds; Elastin expression by WB (E).

(28.O11) STARVED OF OXYGEN: CELLS ENGINEER 3D VASCULAR NETWORKS IN VITRO

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Engineering vascular networks within 3D tissue models is paramount for the survival of cells in large 3D constructs. This is critical for both in vitro and in vivo survival.

We have recapitulated some of the in vivo events relating to the processes of angiogenesis and vasculogenesis in in vitro 3D constructs. Our work has focused on network formation by endothelial cells in 3D collagen type I scaffolds, utilising hypoxia-induced signalling by resident cells cultured in physiological hypoxia (5% oxygen) to upregulate the angiogenic factor cascade.

Constructs with co cultures of Human Umbilical Vein Endothelial (HUVECs) cells and Human Bone Marrow Stromal Cells (HBMSCs) were cultured under normoxia (20% O2) or physiological hypoxia (5% O2), to test the effect of physiological hypoxia on angiogenic stimulus. Protein analysis showed higher levels of VEGF in co-culture constructs, cultured under physiological hypoxia (500-850pg/ml) compared to constructs cultured under normoxia (200-500pg/ml). When cultured in normoxia, the HUVECs aggregated with a cobblestone-like morphology, and cultures at physiological hypoxia aggregated within a network (figure 1).

Differences in up regulation of VEGF levels by HBMSCs at the two oxygen conditions affect the morphology of endothelial cell fusion. HUVECs formed an endothelium-like morphology (simulating that of a large vessel) at lower concentrations of VEGF, versus a capillary network at higher concentrations. We have developed a system where cells form gradients of angiogenic cascades in 3D and tested the effect of this gradient on HUVEC aggregation. We found that HUVECs show increased aggregation in areas of predicted higher angiogenic signalling concentrations.

By quantifying the localised parameters which lead to specific types of endothelial cell aggregation, we can develop strategies to predictably control this effect spatially in 3D, with the specific aim of producing extensive vascular networks in 3D in vitro.

Keywords. Oxygen, Collagen, Vascular Networks, Endothelium

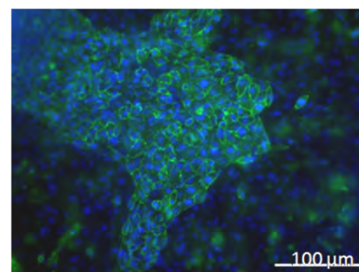

Figure. Cobblestone morphology in co-cultures of HUVECs and HBMSCs in 3D collagen constructs under normoxia (CD31 staining in green; DAPI in blue)

(28.P1) ANALYSIS OF GENE EXPRESSION AND ANGIOGENIC POTENTIAL IN COCULTURES OF PRIMARY HUMAN ENDOTHELIAL CELLS AND COMMONLY USED HUMAN OSTEOBLAST CELL LINES

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Introduction. One of the goals to accelerate the acceptance and recovery of a bone biomaterial is to generate a prevascularized construct with the patient's own cells prior to implantation in the patient. A variety of primary human endothelial cells in coculture with primary osteoblasts demonstrate varying degrees of microcapillary formation dependent on both endothelial and osteoblast cell donors and tissue from which the cells are isolated. These differences may be attributed in part to donor variability or the presence of other "contaminating" cell types. These factors make it difficult to understand and determine the mechanisms resulting in the induction of microcapillaries and to predict the success after implantation. In order to obtain knowledge of the cell-cell communication that occurs, different endothelial cell types were cocultured with a variety of human osteoblast cell lines (all cells clonally identical) and microcapillary formation and changes in gene expression were analyzed.

Materials and Methods. Primary human endothelial cells, HUVEC and HDMEC and cocultures with primary osteoblasts and osteoblast cell lines (Cal-72, MG-63, SaOS-2) were established. As well as the identification of cells in coculture, analysis of RNA and supernatants were performed as previously described. Analysis was carried out at various time points after coculture.

Results. The osteoblast cell lines differed significantly in their ability to induce microcapillary formation by endothelial cells in coculture ranging from no visible capillaries to capillaries similar in number and shape to those observed with primary osteoblasts. Large differences in factors known to involved in angiogenesis were also observed.

Conclusions. Only certain osteoblast cell lines exhibit microcapillary-inducing capabilities and gene expression profiles similar to primary osteoblasts. Thus, these selected lines may be suitable for studies to analyze cell-cell communication.

Acknowledgment. The authors would like to thank the German Federal Ministry for Education and Research for

supporting this research (Grant Nos. 0315689C and 0315771B)

Keywords. ANGIOGENIC POTENTIAL microcapillary formation coculture human osteoblast cell lines primary osteoblasts Primary human endothelial cells

(28.P2) ENDOTHELIAL PROGENITOR CELLS (CD34+) AND BONE MARROW MESENCHYMAL STEM CELLS CO-CULTURE PROMOTES 3D SCAFFOLD NEOVASCULARIZATION

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Introduction. Angiogenesis is a key factor in early stages of wound healing and is also crucial for tissue regeneration. In cases of large bone defects, to date most of the efforts have been focused on the filling of the gap with autologous bone grafts, or various bio-active materials associated or not with bone forming cells. However, the neo-vascularisation of such implants is still a limiting factor. The aim of the present study is to develop a pre-vascularised hybrid bone implant made of a polyurethane scaffold seeded with autologous cells; Endothelial Progenitor Cells (EPC) and Bone Marrow Mesenchymal Stem Cells (BMSC).

Methods. BMSCs were isolated by Ficoll-Paque density-gradient centrifugation from human bone marrow (KEK_Bern126/03). EPCs (CD133+/CD34+) were isolated from BMSC fractions using magnetic-activated cell sorting (MACS®). After cell fluorescent staining using PKH67-green® for EPC and PKH26-red® for BMSC, EPCs were seeded on 2D Matrigel® coating alone or together with BMSC. Cellular network formation was observed using confocal microscopy. In 3D co-culture set-ups, cells were seeded in different proportions in a polyurethane scaffold in presence of Platelet Rich Plasma. After 7 days of culture in different media (osteogenic, angiogenic, or mixed), cryosections were performed and stained with toluidine blue and endothelial-cell specific antibodies.

Results. On Matrigel® assay, EPCs showed the capacity to re-organize themselves in typical endothelial-like cellular networks and demonstrated improved tubular-like formation when co-cultured with BMSCs. In 3D scaffolds, we showed that the association EPC-BMSC within a polyurethane scaffold promoted the formation of Laminin, von-Willebrand-factor and PECAM positive tubular structures formation.

Conclusion: EPC-BMSC co-culture in 2D- and 3D-environment enhanced the formation of early tubular structures within the scaffolds with both cell types contributing to this cellular re-organization. In vitro pre-culture for 7 days with 50-50 cell proportion in osteogenic media containing PRGF seems to be optimal.

Keywords. Neovascularization, tubular structure, PRGF, bone marrow mesenchymal cells, endothelial progenitor cells

(28.P3) INCORPORATION OF GELATIN INTO CHEMICALLY CROSS-LINKED POLYVINYL ALCOHOL (PVA) HYDROGELS PROMOTES HUMAN ENDOTHELIAL CELL ADHESION AND PROLIFERATION

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Introduction. Cardiovascular diseases constitute the leading cause of mortality in developed countries. Although synthetic grafts are successfully used to replace large-diameter vessels, small-caliber (<6mm) substitutes are still needed for coronary and peripheral vascular procedures. We previously described a cross-linked polyvinyl alcohol (PVA) scaffold, with mechanical properties close to those of arterial blood vessels, and a successful vascular replacement in a rat model was reported. However, the absence of luminal endothelial cells might impair the long-term patency of this vascular graft. In the present study, we modified this PVA material by incorporation of gelatin to improve endothelial cell attachment.

Materials and Methods. PVA/Gelatin blend hydrogels were prepared by chemical cross-linking with sodium trimetaphosphate, using a casting process. FTIR spectroscopy and DSC were carried out to identify chemical bonds and cristallinity. Films morphology and wettability were characterized with SEM, AFM and water contact angle measurement. Mechanical data were obtained using uniaxial tensile tests. Human endothelial cells (EA.hy) viability, attachment and proliferation were investigated using the Live&Dead assay and assessed with fluorescence microscopy.

Results and Discussion. PVA/gelatin films displayed a high water content (~80%). Presence of gelatin in PVA-based hydrogels was confirmed by FTIR spectroscopy. Blend films displayed a tensile strength (1.4 MPa) and an elastic modulus (0.7 MPa) close to that of vascular tissue. SEM images revealed smooth pure PVA films, while blend films presented micrometric crater-shaped asperities. Water contact angle significantly increased with addition of gelatin (56° vs 78° with 10% w/w gelatin). Few ECs attached on pure PVA films. In contrast, ECs attached, remained viable, proliferated on PVA/Gelatin films and reached confluence on day 14.

Keywords. vascular graft / polyvinyl alcohol / hydrogel / endothelial cells

(28.P4) PROMOTING AGIOGENESIS IN 3D STRUCTURE FROM ELECTROSPUN ALBUMIN TUBES

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A vascular like conduit for oxygen and nutrient perfusion is an important design consideration for a bulky 3-D engineered tissue. Recently, several approaches have been proposed to promote vascularization of such tissues. These can be classified into two main approaches: the self assembly: where endothelial cells are included in the seeding mixture with the aim of inducing spontaneous vessel formation; and the engineered guided assembly: this involves utilizing a preformed

scaffold to promote the interaction with seeded cells in the formation of vascular conduits.

In this study, we plan to compare the effectiveness of the above mentioned approaches. An electrospun Bovine Serum Albumin (BSA) tube scaffold seeded with co-culture of Human Foreskin Fibroblasts (HFF) and Human Umbilical Vein Endothelial Cells (HUVEC) was assessed in a bioreactor system. Our aim is to define the optimized conditions for the formation of cell-seeded vessel like structures that can promote radial angiogenesis *in vitro*. Moreover, the direct influence of BSA tube scaffold on cellular organization will be comprehensively examined.

A tubular BSA scaffold of 1mm diameter was fabricated using electrospinning technique, resulting in ribbon-like micro-scale fibers arranged in a 90% porous structure, with permeability constants in the order of 10^{-13} m^2. Analyzing the scaffold's mechanical properties under wet conditions revealed similarities to soft ECM fibers, e.g. elastin, with a stiffness of 0.006 GPa and 1.1% extensibility. Moreover, BSA scaffolds allowed an immediate strong adhesion of various cell types due to the negatively charged fibers. An *in vitro* test indicated good biocompatibility for the BSA tube scaffolds. A 60% reduction of the scaffold was observed 3 weeks post-implantation. Interestingly vessel-like structures composed of endothelial cells stabilized by HFF cells surrounding was observed under the static conditions. Such a structure may lead to radial angiogenesis and vascularization of 3D tissues.

(28.P5) MESENCHYMAL CELL MEDIATED STABILIZATION OF MICROVESSEL NETWORKS

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Generation of a functional microvasculature for regenerative medicine applications is a fundamental technical hurdle that has yet to be achieved. Human endothelial cells (EC) are notoriously unstable in 3D culture. In vivo, EC require interaction with perivascular cells for vessel stabilization, yet it is unclear how these cells communicate to stabilize the microvasculature. Here we show that human embryonic stem cell derived mesenchymal cells (hES-MC) interact with human EC to form stable network-like structures in 3D collagen-fibronectin (Fn) constructs by paracrine and direct contact mechanisms.

HUVEC cultured alone in 3D collagen-Fn are unstable, even in the presence of VEGF and bFGF. With the addition of hES-MC, networks formed and persisted up to 15d. We examined paracrine mechanisms by culturing HUVEC in 3D transwell (4µm pore) culture with or without hES-MC. Only HUVEC in indirect culture formed persistent networks. We next tested if angiopoietin-1/Tie2 or HGF/c-Met played a role in paracrine stabilization. Treatment of direct and indirect cultures with Tie2-Fc or IgG-Fc control showed no effect. However, treatment of coculture with c-Met inhibitor completely blocked network formation. We next examined if HUVEC and hES-MC were in direct contact using laser confocal microscopy. HUVEC showed a range of surface contact of 2-7% while hES-MC contact ranged from 3-12%.

This data indicates hES-MC regulate the stabilization of HUVEC in 3D culture by both paracrine and direct contact mechanisms. This also shows that though VEGF and bFGF may play a role in vessel stability, they are insufficient without the addition of hES-MC. Paracrine mediated stability appears to involve HGF/c-Met signaling which is known to have pleiotropic effects such as pro-survival and tube formation. This also demonstrates HUVEC and hES-MC make direct contact which affects the morphological structure of the networks, though the mechanisms are unclear.

Keywords. microvascular, mesenchymal cell, HGF

(28.P6) HEAT STRESS-INDUCED HORMESIS ENHANCES ANGIOGENESIS IN CO-CULTURES CONSISTING OF PRIMARY OSTEOBLASTS AND OUTGROWTH ENDOTHELIAL CELLS

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Introduction. Engineering functional vasculature is a major requirement for achieving tissue regeneration. Thus, a therapeutic goal is to create systems that can rapidly and efficiently build a functional vasculature. Hormesis is a process by which a mild or an acute stressor results in the functional improvement of cells or tissues. Heat stress (HS)-induced hormesis enhances tube formation in endothelial cells [1]. Recent studies documented that outgrowth endothelial cells (OEC), a cell type isolated from human peripheral blood, in co-culture with primary osteoblasts (pOB) are able to form functional capillary-like vessels [2]. The present study focuses on the effect of repeated HS on angiogenesis in co-cultures of OEC and pOB.

Methods. Cells and co-cultures were prepared according to previously published protocols [3]. After one day of co-cultivation, cells were pre-treated at 39°C (20 min) and 2 hours at 37°C before being exposed to HS (41°C, 1 hour) (T1). These steps were repeated two times per week over 14 days. In addition, non-HS-treated cells were cultured with the conditioned culture medium collected from cells exposed to HS (T1M). These co-cultures were stained for endothelial markers, and capillary-like structures were investigated using laser scanning microscopy with comparison to controls cultured at 37 °C. The release of angiogenic factors was analysed using ELISA.

Results. Enhanced microvessel-like structures were observed in co-cultures exposed to HS, as well as in cells treated with the conditioned medium (Fig.1). T1-treated co-cultures released significantly more VEGF, but less Ang-1 compared to controls. Elevated VEGF and Ang-1 concentrations were also observed in cultures of T1M, indicating paracrine effects associated with HS-induced angiogenesis.

Conclusions. Our results suggest that repeated HS improved angiogenesis in vitro by enhancing the release of angiogenic factors in the co-cultures.

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Acknowledgements. BMBF (German-Chinese cooperation in Regenerative Medicine)
Keywords. Angiogenesis, Co-culture, Heat stress, Hormesis

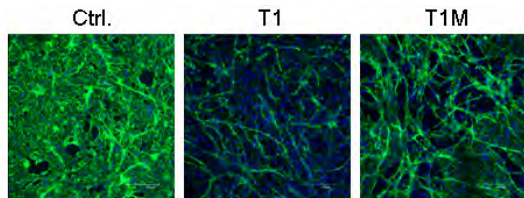

Figure 1 Immunofluorescent staining analyse the effects of HS on the formation of capillary-like structures in a co-culture system.

(28.P7) LACK OF ADRENOMEDULLIN IN MOUSE ENDOTHELIAL CELLS RESULTS IN LIVER AND KIDNEY INFLAMMATION

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Introduction. Adrenomedullin (AM) is a vasodilating peptide involved in the regulation of circulatory homeostasis and in the pathophysiology of certain cardiovascular diseases. AM is constitutively secreted by vascular endothelial and smooth muscle cells and is expressed at a high level in the lung and tumor cells. AM plays critical roles in blood vessels, including regulation of vascular stability and permeability. Besides, AM is also essential for angiogenesis. Previous studies showed that mice lacking AM develop cardiovascular abnormalities, including overdeveloped ventricular trabeculae and underdeveloped arterial walls. So that embryos lacking AM signaling die due to leaky and unstable blood vessels. Therefore, AM is indispensable for the development and/or maintenance of the vasculature during embryogenesis.

Methods. Conditional knockout of AM in the endothelial cells (AM ECKO -/-) was obtained by crossing animals whose adm gene was flanked by LoxP sequences with transgenic mice expressing Cre recombinase under the Cdh5 promoter.

Results. In the liver of AM ECKO -/- animals, large accumulations of small mononucleated cells (mostly lymphocytes) were found in the proximity of large blood vessels. In the kidneys, excessive deposits of Syrius red-positive material (collagen I) were found in the Bowman´s capsule of the glomeruli in AM ECKO -/- animals. In addition, mice lacking AM in their endothelial cells had defective angiogenesis in vivo.

Conclusions. Here we show that ablation of AM in endothelial cells caused the spontaneous development of liver immune infiltration and glomerulonephritis in mice. These findings revealed a physiological function of AM in protecting the liver and kidney from disorders such as inflammation, identifying AM as an immune suppressor in these organs. Moreover, the amount of vascularization in the matrigel implants was lower in AM ECKO -/- mice indicating a defective angiogenesis. Thus, AM may be a key player in tissue engineering protocols requiring vascularization of the scaffolds.

Keywords. Adrenomedullin, knockout, endothelial cells

29. MODULATING IN VITRO MICROENVIRONMENTS TO LET CELLS THRIVE: FROM PATHOLOGY TO PHYSIOLOGY AND THERAPY

Chair: Dimitrios I. Zeugolis
Co-chair: Michael Raghunath
Keynote speaker: Michael Raghunath
Organizers: Dimitrios I. Zeugolis, Michael Raghunath

Synopsis: With the advent of multicellular organisms, the exterior of cells changed dramatically from highly aqueous surroundings to extracellular matrix and space crowded with macromolecules. Cell-based therapies require removal of cells from their tissue context and propagation thereof in culture medium to attain therapeutically relevant numbers, whilst preserving their phenotype. However, bereft of their microenvironment, cells perform poorly under *in vitro* culturing. They lose their functionality, and with it, their therapeutic potential. Major efforts are directed towards reconstructing better *in vitro* microenvironments by modifying surface properties of cell culture dishes, or utilising semi-artificial or fully synthetic polymer structures. As the engagement of integrin and non-integrin receptors plays an important role *in vivo*, offering points of engagement via patterned nanostructures is an interesting strategy along with observations on modulation stiffness of the support on which the cells are to be propagated.

An alternative very efficient, yet unconventional, strategy is to invite cells and stem cells to create their own matrices by applying macromolecular crowding, a biophysical principle that governs the intra- and extra-cellular milieu in multicellular organisms. Interestingly, current cell culture conditions are far from being macromolecularly crowded. Cells are grown in highly dilute conditions in culture; this, in the human body, would represent a medical pathology. However, this situation can be remedied, by adding macromolecules of defined hydrodynamic radius to culture media and thus creating excluded volume effects with defined volume fraction occupancies. The development of *in vitro* culturing systems that will closely imitate the *in vivo* microenvironment could provide a valuable experimental tool to maintain cell phenotype and study mechanisms associated with tissue formation and pathophysiologies. Another important artefact of routine culture is that it is done at atmospheric oxygen levels of 18-21%. This system is so entrenched in day-to-day cell culture that it has been forgotten that these oxygen levels are remarkably disparate from oxygen levels prevailing in tissue of the adult body (e.g. physiological oxygen tension in bone is 12.5% and falls to 1% in fracture haematoma) or the

embryo. This means that cells are under oxidative stress in contemporary cell culture and that oxygen signalling pathways are overwhelmed. Not surprisingly, oxygen tension is an important component of the stem cell microenvironment. In fact, oxygen plays a significant role as a signalling molecule to regulate stem cells development into mesenchymal tissues *in vitro*, for example into cartilage, a tissue that is physiologically not perfused. Low, or actually physiological, oxygen tension benefits stem cells expansion and maintenance. Moreover, low oxygen levels induce enhanced matrix production in culture and enables *in vitro* control of tissue development.
This symposium aims to review these key modulators of the *in vitro* microenvironment (three dimensional culturing systems, macromolecular crowding and hypoxia) and how the newly developed knowledge in the field will allow us to overcome current bottlenecks in *ex vivo* tissue cell culture and thus enable wide acceptance and clinical translation of cell-based therapies.

(29.KP) BUILDING THE STEM CELL MICROENVIRONMENT IN VITRO - PLEIOTROPIC EFFECTS OF MACROMOLECULAR CROWDING

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The role of the microenvironment surrounding stem cells is increasingly acknowledged as an important biological driver of stemness and differentiation. Major efforts are directed towards the reconstruction of these microenvironments in vitro by modifying surface properties of cell culture dishes, or utilizing semi-artificial or fully synthetic polymer structures. What is often forgotten is the huge potential of cells, including stem cells, to create their own matrices and remodel them as they differentiate into certain lineages. We show here, how macromolecular crowding, a biophysical principle that governs the intra and extracellular millieu in multicellular organisms, can be reversely bioengineered in a simplistic manner using carbohydrate-based macromolecules of defined hydrodynamic radius. Macromolecular crowding creates an excluded volume effect in the culture medium. This exerts a pleiotropic effect on matrix formation via enhanced conversion of procollagen to collagen, accelerated supramolecular assembly of collagen and fibronectin, and increased extracellular crosslinking of matrices via lysyl oxidase and transglutaminase 2. This works very well in matrix producing mesenchymal and epithelial cells, but also with mesenchymal stem cells. Thus, we can effectively delegate the construction of the microenvironment entirely to the cells themselves as they either stay undifferentiated or move into different lineages upon induction. Creating fraction volume occupancies by macromolecular crowding that are close to the perfused compartment of bone marrow, we are able to drive MSCs into the osteogenic and the adipogenic lineages with a higher efficiency and speed than hitherto known and with an increased proliferation rate by up to one order of magnitude.

Keywords. stem cells, macromoleclar crowding, extracellular matrix

(29.O1) ENZYME MODULATED CONTROLLED RELEASE OF OXYGEN FROM ENCAPSULATED HYDROGEN PEROXIDE FOR ENHANCED CELL SURVIVAL UNDER HYPOXIA

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Coagulative necrosis often occurs under hypoxic conditions, and it is a major limitation in the field of tissue engineering, particularly for larger tissues and organs. In order to improve cell viability under hypoxic conditions, attempts have been made to develop controlled release oxygen systems that could provide oxygen before the implant becomes vascularized. This study involved the design of a dual layer matrix composed of encapsulated H2O2 with PLGA that was further imbedded into secondary alginate matrix having catalase immobilized to the chains through EDC/NHS chemistry. Due to the presence of catalase, decomposition of H2O2 became more active and faster within the secondary layer. Optimization studies of the formulation showed that PLGA with molecular weights of 90,000 and 110,000 g mol−1 showed high encapsulation efficiency, while the best stirring time during the encapsulation was 8 hours. Additionally, the concentration range of 1.4% to 1.8% (w/v) alginate sustained the continuous release of oxygen up to two weeks without damaging the cells. Different concentrations of H2O2 were encapsulated and viability of muscle cells was observed under low oxygen condition. It was observed that encapsulated the 4% H2O2 generated oxygen very efficiently in controlled manner and rendered the cell viability under hypoxic condition. That exhibited better results than normoxia condition. The constructs of oxygen producing matrix demonstrated the potential of this system for engineering large sized tissue in vivo.

Keywords. Oxygen, hypoxia, modulating environment, functional scaffold

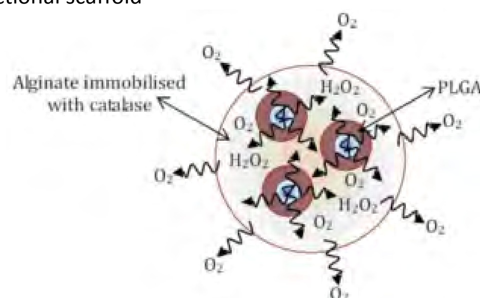

Figure. Diagram on releasing mechanism of oxygen from double layered H_2O_2 encapsulation system.

(29.O2) THE ROLE OF RETINOIC ACID RECEPTOR INHIBITOR LE135 ON OSTEOCHONDRAL DIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS

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Aim. Bone marrow derived mesenchymal stem cells (MSCs) have demonstrated the multipotential to differentiate into several cell lineages. The advantage of using MSCs for regenerative medicine is that the harvest

procedure is relatively easy. Growth factors and mechanical load have been used to modulate the fate of MSCs. However, there is the potential for small molecules to modulate stem cell differentiation, which would be more cost-effective. The present study aimed to determine the effect of retinoic acid receptor inhibitor LE135 on osteochondral differentiation of human bone marrow MSCs in pellet culture and a scaffold culture system subjected to load.

Methods. P3 human MSCs were pre-cultured for 1 day, and then cultured with or without mechanical load and/or with 1µM LE135 for 7 days. Loaded samples were exposed to cyclic axial compression performed by a ceramic hip ball 32 mm in diameter, and oscillation of the ball over the construct surface (±25°, 1 Hz). Measurements included DNA, glycosaminoglycan and mRNA expression. TGF-β1 and TGF-β3 protein were measured by ELISA.

Results. In pellet culture, exogenous TGF-β1 induced chondrogenesis of hMSCs. Addition of LE135 to TGF-β1 treated pellets diminished the chondrogenic differentiation response compared with the TGFβ group. In scaffold culture, mechanical load increased GAG synthesis, COL2, AGG, Sox9, TGFB gene expression and TGF-β1 protein synthesis. Addition of LE135 to loaded scaffolds down-regulated COL2 gene expression. Both exogenous TGF-β1 and endogenous TGF-β1 enhanced gene expression of COL10 and Sp7. LE135 did not affect COL10 or Sp7 expression.

Discussion. LE135 inhibited the chondrogenic response in both pellet and scaffold culture. Although LE135 inhibited the chondrogenic response induced by TGF-β, the osteogenic response was not affected. This suggests that the effects of LE135 are at later stages in the osteochondral differentiation pathway. Alternatively, it might be that different cells were proceeding down different lineages and were affected differently by LE135.

Keywords. Differentiation, Osteogenesis, Chondrogenesis

(29.O3) CONTROL OF ANGIOGENIC SIGNALLING BY STEM CELLS IN 3D TISSUE MODELS

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Due to the multi-potential nature of stem cells, we are seeing an increase in the use of these cells for therapeutic purposes, particularly in regenerating damaged tissues. Understanding how the stem cell microenvironment affects multiple signalling cascades will be critical if we are to control these cells to enhance their regenerative capacity. We have created dense 3D collagen scaffolds, into which we have seeded human bone marrow derived stem cells. O_2 consumption gradients were formed in these cell-seeded constructs and monitored using a real-time O_2 monitoring system. Cells within the same construct were exposed to varying O_2 tensions, and following dissection of these cell populations, the angiogenic gene signalling was measured and linked to O_2 tension, thus informing us that 20-40mmHg (or ~3-5%) was the optimal O_2 tension in which to culture these HBMSCs to enhance HBMSC-generated hypoxia-induced signalling1. By day 2 of cultures we saw a significant increase of Hypoxia- inducible factor Iα (HIF-Iα) by core cells compared to surface cells. The introduction of a channelled architecture into such 3D constructs provided a mechanism to deliver O_2 to core cells, and essentially 'switch-off' this angiogenic signalling by the seeded stem cells (figure 1). Phosphate-based dissolving glass fibres (PGFs) were introduced to stem cell-seeded constructs, which dissolved away to leave continuous channels after 24 hours1. No upregulation of HIF-Iα was detected in these cultures.

This model allows us to test varying O_2 exposure on different cells in 3D scaffolds, providing indications of stem cell signalling in low O_2 environments, i.e. when these cells are implanted in tissues in vivo. This model also provides a mechanism to regulate angiogenic signalling by creating O_2 gradients to upregulate signalling and the introduction of O_2, via channels, to switch off such signalling.

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Keywords. Stem cells, Physiological Hypoxia, Oxygen gradients, Collagen scaffolds

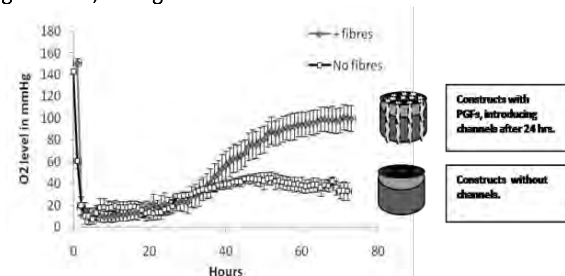

Figure. O_2 levels in the core of 3D cell-seeded collagen constructs. Levels of O_2 are increased in constructs where channels are introduced after 24 hrs.

(29.O4) PRINTING ANISOTROPIC CELL MICROENVIRONMENT FOR TISSUE ENGINEERING

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Introduction. Current cell/tissue scaffolding methods have limited spatial and temporal control over cell seeding and extracellular matrix composition. Bioprinting technology holds great potential to be used to engineer complex tissue anisotropies by producing scaffolds with controlled micro-scale spatial heterogeneity in extracellular, cellular compositions and physical properties. To test this, we printed agarose hydrogel microdroplets colored with red, green and blue (RGB) high molecular weight (35-38 kDa) fluorescent dyes and assessed the phase transitions via image processing to evaluate the anisotropy of the resulting anisotropic structure by measuring the RGB color intensities.

Methods. Microdroplet generation process was performed with multiple ejectors in sterile laminar flow hood under controlled humidity. The inter-droplet distance was determined by the size of the droplets residing on the substrate. The prepared RGB colored

hydrogels were printed in a staggered configuration. Printed staggered phases were gelled by incubation at 4 degC for 5 minutes. The diffusion and integration of the phases was assessed immediately after and 3 hours after printing by taking micographs and analyzing using ImageJ software. RGB color relative intensity values were used analytically to analyze the anisotropic gradient of the phases and phase transitions.

Results and Discussion. The printed multiphase hydrogel structure representing an anisotropic tissue unit displayed sharp RGB boundaries between the phases immediately after printing. These sharp boundaries disappeared and smooth transitions emerged within 3 hours. These results suggest that microdroplet based hydrogel printing technology can be used to create highly anisotropic structures with smooth boundaries mimicking the complex cellular and extracellular gradients in the natural tissues. Our long term goal is to develop effective bioprinting methodologies to engineer micro-scale anisotropic complex tissue structures with multiple phases, which can be incorporated into currently available biomaterials to face the challenges of incompatibility at tissue-biomaterial interfaces.

Keywords. Bioprinting, cell microenvironment, anisotropic structure, tissue engineering

(29.O5) MODULATION OF THE IN VITRO MICROENVIRONMENT OF CORNEAL KERATOCYTES USING MACROMOLECULAR CROWDING

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Introduction. As living cells provide the starting material for numerous tissue engineering applications, it is essential to maintain in culture cell phenotype and functionality and consequently their therapeutic potential. However, this success has not been translated to all cell types. Cultured corneal keratocytes, for example, lose their dendritic morphology; lose characteristic markers; and dedifferentiate to fibroblasts/myofibroblasts. Recent studies have shown that macromolecular crowding (MMC) - the addition of macromolecules to culture media - not only enhances the deposition of extracellular matrix, but also facilitates cell phenotype maintenance. Herein, we describe the influence of MMC on the culture of corneal keratocytes.

Methods. Primary corneal keratocytes were cultured under MMC (e.g. 100µg/ml dextran sulphate; and Ficoll™ (Fc) combination (i.e. 37.5mg/ml Ficoll™ 70 and 25mg/ml Ficoll™ 400). The influence of crowders on cell metabolism was monitored using AlamarBlue® assay. Deposition of extracellular matrix proteins was analysed by SDS-PAGE and immunocytochemistry for type I collagen and fibronectin.

Results. The metabolic activity (Figure-1A) of corneal keratocytes under MMC was comparable to controls up to day 4 ($p>0.05$), whilst a significant increase was evident for both MMC modes at day 6 ($p<0.001$). Densitometric analysis (Figure-1B) of SDS-PAGE gels shows that both MMC modes significantly increased collagen type I deposition ($p<0.003$) at all time points. Immunocytochemistry (Figure-1C) confirmed that MMC-enhanced the deposition of fibronectin and collagen I in a highly ordered fashion.

Conclusion. MMC significantly accelerates ordered collagen type I deposition in keratocyte culture. Keratocyte metabolic activity and/or proliferation did not increase, even up to 4 days under crowded conditions, which indicates the maintenance of keratocyte phenotype.

Acknowledgments. The authors would like to thank College of Engineering and Informatics NUI, Galway, Science Foundation Ireland (Grant-09/RFP/ENM2483) and SFI - ETS Walton award.

Keywords. Corneal Keratocyte Culture; Macromolecular Crowding; Collagen type I Deposition

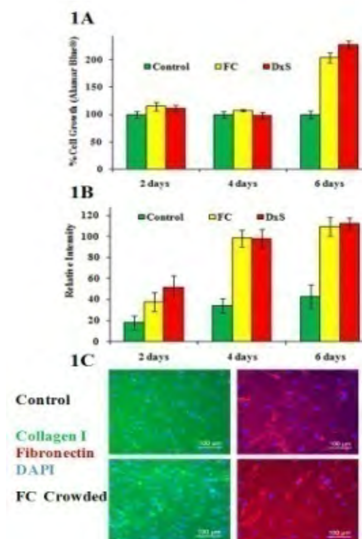

Figure 1A: AlamarBlue® assay of keratocytes under crowded (with Fc and DxS) and non-crowded condition. Figure 1B: Densitometric analysis of SDS-PAGE gels for collagen I deposition Figure 1C: Immunocytochemistry for Collagen I (green) and Fibronectin (red). Nuclei were counterstained with DAPI (blue).

(29.O6) THE OXYGEN ENVIRONMENT REGULATES BOTH THE CHONDROGENIC AND OSTEOGENIC POTENTIAL OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS.

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Introduction. Bone marrow derived mesenchymal stem cells (BM-MSCs) are capable of undergoing osteogenesis and chondrogenesis. Understanding how environmental factors regulate BM-MSC fate is critical for developing new therapies for tissue repair. The local oxygen environment is a key regulator of MSC activity and it has been previously demonstrated that chondrogenesis of BM-MSCs is enhanced by differentiaton in a low oxygen environment (Meyer et al., 2010). The objective of this study was to examine the effect of oxygen tension on both the proliferation kinetics of BM-MSCs and on their osteogenic and chondrogenic potential.

Methods. Porcine BM-MSCs were expanded in normoxic (20%pO2) or low oxygen (5%pO2) conditions. To assess chondrogenesis BM-MSCs were suspended in cylindrical

(Ø5x3mm) agarose constructs and maintained in a chondrogenic medium supplemented with 10ng/ml TGF-β3 at 20% or 5%pO2 for 42 days. To assess osteogenesis BM-MSCs were seeded in 6 well plates and treated with osteogenic medium supplemented with 20µg/ml β-Glycerophosphate at 20% or 5%pO2 for 14 days.

Results. Expansion at 5% pO2 did not influence BM-MSC colony number but did increase colony size (Fig. 1a). BM-MSC laden constructs chondrogenically differentiated at 5%pO2 accumulated significantly more sGAG compared to constructs differentiated at 20%pO2 (Fig. 1c). Indication for more robust osteogenic differentiation was observed for BM-MSCs expanded at 5%pO2, as evidenced by Alizarin Red staining (Fig. 1d).

Conclusions. Cells expanded at 5%pO2 proliferated faster, forming larger colonies, without affecting the colony forming unit efficiency. Differentiation in low oxygen tension is a more potent regulator of chondrogenesis that expansion at low oxygen tension; no significant differences were found between groups expanded at 20% or 5%pO2. Expansion at 5%pO¬2 may support a more osteogenic phenotype, as weaker Alizarin Red staining was observed for BM-MSCs expanded and differentiated at 20%pO2.

Acknowledgements. Supported by Science Foundation Ireland-08/Y15/B1336

Keywords. msc, oxygen, chondrogenesis, osteogenesis

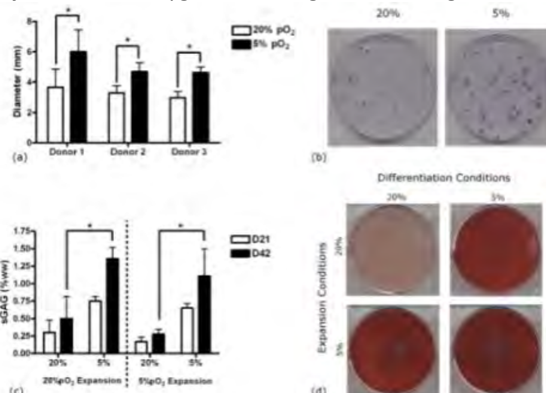

Figure 1. (a) Diameter of 10 largest colonies from Colony Forming Units-fibroblast (CFU-f) assay (3 donors, n=3 per donor). (b) Sample image of CFU-f assay at 20% and 5% oxygen. (c) sGAG accumulation of BM-MSCs differentiated at 20% and 5% oxygen at days 21 and 42. (2 donors, n=3-4 per donor). (d) Alizarin Red staining after 14 days osteogenic differentiation (1 donor, n=3). Significance * ($p<0.05$).

(29.O7) A BIOREACTOR PROTOTYPE FOR SUBJECTING CULTURED CELLS TO FAST-RATE INTERMITTENT HYPOXIA

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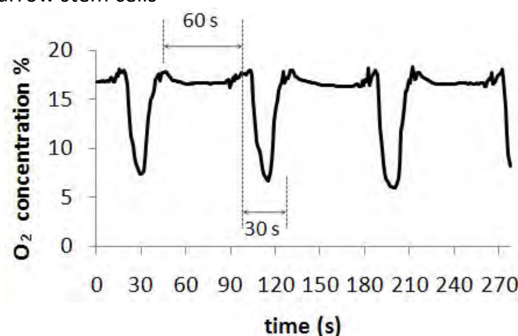
Tissue hypoxia in injured tissues is a key factor to mobilize bone marrow-derived stem cells for tissue repair. Hypoxia is particularly relevant in patients with obstructive sleep apnea (OSA) since they are subjected to high-frequency hypoxemic events (up to more than event 1 per minute). It has been recently proven that OSA results in the activation of endothelial progenitor and mesenchymal stem cells for endothelial repair. Consequently, better understanding the repairing role of stem cells in OSA requires a bioreactor capable of subjecting cultured cells to fast-rate intermittent hypoxia. Given that the available settings for the culture of cells under intermittent hypoxia conditions are limited by long equilibration times, we designed a device capable to effectively subjecting cultured cells to hypoxic/normoxic stimuli with controlled magnitude and time pattern. The bioreactor is based on exposing cells to a medium that is bubbled with the appropriate mixture of gases in two separate containers and from there it is directed to the cell culture dish with the help of a pair of two-way peristaltic pumps. Initially, the first pump transfers the normoxic medium to the culture dishes and after leaving it into contact with the cells for the desired time period, it aspirates it back to the container. The second pump then, repeats a similar process with the hypoxic medium but it leaves it to rest in the culture dish for a time period that can be independently controlled, before the whole cycle is repeated. An O2 microsensor (Unisence A/S) monitors the value of oxygen pressure at the cell culture level, as shown by the figure in an example mimicking a typical time pattern in OSA. This bioreactor can be useful to study the short- and long-term response of stem cells under controlled high-rate hypoxia/normoxia.

Keywords. intermittent hypoxia, sleep apnea, bone marrow stem cells



(29.O8) THE EFFECT OF ELECTRICAL STIMULATION ON HUMAN MESENCHYMAL STEM CELL BEHAVIOUR

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Introduction. Bioelectricity exists in all living organisms. Endogenous electric fields are known to play a crucial role in the action potentials of nerves and muscles, organogenesis, repair and regeneration. Therapeutic devices using electric stimulation, as a means to induce positive healing processes in the human body, are in existence since the 1950s. The aim of this study was to examine the effect of electrical stimulation on the behaviour of hMSCs.

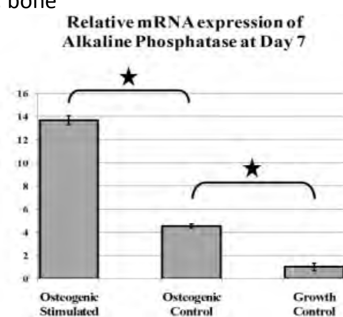
Methods. Human Mesenchymal Stem Cells were stimulated through direct coupling for 30 min or 1 h per day with regimes of voltage pulses, varying in amplitude, frequency and pulse width, in growth or osteogenic medium. Their effect upon proliferation, viability, morphology and the expression of 11 markers of differentiation was assayed after four and seven days by Alamar Blue and Live/Dead fluorescent staining, confocal microscopy and quantitative real-time RT-PCR.

Results. 10 µs pulses elevated proliferation by 68 % compared to sham-treated groups, while signals consisting of 1 µs pulses had a devastating effect upon cell numbers. 250 µs pulses non-significantly up-regulated

ACAN expression, while 1 µs ones significantly decreased CNN1 levels in growth medium. When combined with osteogenic medium, these 1 µs pulses interfered with ACAN and CNN1 levels and appeared to enhance osteogenic differentiation, as the expression of ALPL was significantly elevated after 7 days of stimulation, at the expense of GFAP expression, which was significantly lowered.

Conclusions. This study demonstrated that direct coupled electrical stimulation can enhance proliferation of hMSCs, though the effects depend heavily on the parameters. Additionally, electricity was shown to be able to influence differentiation in growth medium, though its effects were observed to be stronger when applied in conjunction with osteogenic medium. These results suggest that electrical stimulation could become a very useful tool for engineering bone and other tissue.

Keywords. electrical stimulation, hMSCs, gene expression, bone



(29.O9) HYPOXIA CHANGES THE CHEMOKINE EXPRESSION PROFILE AND MIGRATORY POTENTIAL OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS IN-VITRO

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Introduction. Bone marrow-derived stromal cells (MSC) have become a therapeutically important cell type because of their multilineage potential, immunomodulatory properties and ability to localize specifically to injury sites. When used for therapeutic purposes, MSC cultured in standard conditions must adapt from 21% oxygen to less than 1% oxygen in the ischemic tissue. To mimic the microenvironment which may occur in therapeutic situations of regenerative medicine, we aimed to analyse MSC characteristics under hypoxic conditions.

Methods. Human primary MSC from healthy donors were incubated under normoxic (20% O2) and hypoxic (0.5% O2) conditions for different time periods and several in-vitro characteristics were investigated.

Results. The MSC proliferation was inhibited under hypoxic conditions as determined by MTT assay. Culture of MSC in adipogenic induction medium for 14 days under hypoxia resulted in a reduced appearance of adipocyte-like cells containing lipid droplets and almost 50 % lower mRNA levels of fatty acid binding protein 2. The MSC immunophenotype which is characterized by expression of CD73, CD90, CD105, CD146 and CD166 was not significantly changed by hypoxia. We detected decreased SDF-1 and increased IL-8 protein levels in cell culture supernatants from hypoxic MSC. These results were confirmed at the mRNA level by quantitative real-time PCR. The migration capacity was investigated in a 24-well transwell chamber assay with 8 µm pore size. Using recombinant IL-8 as a chemoattractant in the lower chamber, we detected an almost twofold enhanced MSC migration rate after 24 hours under hypoxic conditions. In an in-vitro wound assay the MSC migration could be increased in the presence of IL-8 under hypoxic conditions.

Conclusion. In summary, hypoxia changes main characteristics of MSC which needs to be considered for clinical applications. IL-8 seems to be a chemotactic factor for MSC and enhances their migratory capacity in an autocrine manner.

Keywords. MSC; hypoxia; cytokines; migration

(29.P1) IMMUNE RESPONSE EVALUATION IN MICE WITH MELANOME AFTER ADMINISTRATION OF BONE MARROW DENTRITIC CELLS MATURED WITH MAGE AND ACTIVATED WITH GK-1 PROTEIN

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Introduction. The melanoma is considered to be the most dangerous type of skin cancer. During the last 10 years, some types of therapies were used against cancer, for instance the peptides vaccine. MAGE, one of these peptides used, is founded in melanoma and is suitable to be target of T cells during the immune responses. However, the tumor regression rate is only 20%. The immune therapy is considered to be an option in melanoma, especially when dendritic cells are used. Nonetheless, in order to increase the immune response against melanoma, it is necessary to use adjuvants. GK-1 is a component of the Taenia solium vaccine S3Pvac, and it is well known to induce stimulation of dendritic cells. The purpose of the present work is to demonstrate that bone marrow dendritic cells (BMDC) stimulated with MAGE and GK-1 are suitable to induce immune responses against melanome.

Methods. Bone marrow cells from mice were cultured with GM-SCF for 10 days; subsequently, the cells were immunostimulated with MAGE and GK-1. Flow citometry analysis showed a matured phenotype in the BMDC (class II, CD11c, CD40 and CD86). In mice, melanoma was induced by subcutaneous administration of 100000 B16 line cells. Tumors were measured during the treatment. After two weeks, lymph nodes and tumors were dissected and fixed with Samboni solution. Immunohistochemistry against TNFa, IL-4, and IFNg was made in lymph nodes, while stain with hematoxilyn and eosin was made in tumors.

Results. The immunotherapy with BMDC induced a decrease in tumor size and in their expression of TNF□ and IFNγ in lymph nodes. Besides, tumor histology showed apoptosis and necrosis areas in treated mice. In contrast, the tumors of control mice displayed extensive areas of mitotic tumor cells.

Acknowledgements: CONACYT: 50396-M, DGAPA/PAPIIT: IN213510 and IN214109-3

Keywords. Melanoma, Dendritic cells

30. NANOSTRUCTURED & BIOMIMETIC SCAFFOLDS FOR SKELETAL TISSUE ENGINEERING

Chair: Paul Hatton
Co-chair: Abhay Pandit
Keynote speaker: João F. Mano
Organizer: Paul Hatton, David Farrar, Abhay Pandit
Synopsis: Structural nanostructured materials are commonly encountered in nature where, in combination with hierarchical organisation, they are associated with impressive or unusual functional properties. The field of biomimetics seeks to develop advanced biomaterials by adopting or adapting natural materials, and/or utilising knowledge of natural materials to inform the processing of synthetic systems to improve their properties. Recent evidence suggests that nanostructured or biomimetic materials that incorporate features of the extracellular matrix of tissues are superior to simple polymer and ceramic scaffolds. There is therefore significant interest in the application of nanotechnology and biomimetics in the fabrication of tissue engineering scaffolds with tailored properties. Potential enhancements include improved mechanical properties (of the scaffold or cell-material construct), controlled bioresorption, modified cell response, and improved biocompatibility. The human skeletal system is the principal load-bearing, mechanically functional system in the body, and regenerative therapies are being investigated to alleviate pain, and restore lost function and the biomechanical continuum. This symposium will consider the current state-of-the-art with respect to nanostructured and biomimetic scaffolds, their design, fabrication, properties, cell response, and ultimately the nature of musculo-skeletal tissues (e.g. bone, cartilage and ligament) engineered using scaffolds formed from these materials.

(30.KP) BIOMIMETIC AND NANOSTRUCTURED POLYMERIC SUBSTRATES IN TISSUE ENGINEERING

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Different polymers and composites have been proposed for tissue engineering and regenerative medicine (TERM), both to be used as implantable devices or *ex-vivo*. In such applications, the materials must exhibit adequate surface and structural characteristics in order to interact adequately with cells or tissues. Biomimetic approaches offer innovative ideas in the field of TERM, in terms of biomaterials development or in the exploration of new phenomena. Examples are given, including the development of new smart systems, or the use of surfaces with extreme wettabilities (superhydrophobic) in the control cell adhesion, particle preparation or in high-throughput analysis of cell-biomaterials interactions. Nanostructured biocompatible coatings may be produced by the so-called layer-by-layer (LbL) methodology using natural-based polyelectrolytes. Bioactive glass-ceramic nanoparticles can also be combined with polymers in order to produced biomimetic nacre-like osteoconductive multilayered coatings using this technique. Advances on LbL have been achieved in our research group by transpose it into 3D systems, including in the preparation of highly porous scaffolds, in particle agglomeration or in the fabrication of liquid-core capsules for cell encapsulation. The case studies presented illustrate the possibility of developing original concepts with potential to be used in TERM coming from completely different areas, strengthening the multidisciplinary character of this field.

(30.O1) FABRICATION AND EVALUATION OF ELECTROSPUN POLY(LACTIC-CO-GLYCOLIC ACID):NANO CALCIUM CARBONATE SCAFFOLDS FOR SKELETAL TISSUE ENGINEERING

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Incorporation of nano calcium carbonate (nCC) into electrospun poly(lactic-co-glycolic acid) (PLGA) has the potential to alter the mechanical properties, degradation rates and biocompatibility via buffering the acidic polymer breakdown products. The aim of this work was to fabricate electrospun PLGA:nCC scaffolds and evaluate their biocompatibility and suitability for use in skeletal tissue engineering. PLGA (Sigma-Aldrich) was dissolved in Tetrahydrofuran and nano-particles of CC (American Elements) were incorporated into the scaffold materials at 5 wt% of the polymer. Scaffolds were characterised using scanning electron microscopy and in vitro biocompatibility was investigated. Primary bovine articular chondrocyte (BAC) isolation and expansion was carried out [1]. Cells were expanded to P2 and semi-statically seeded onto the scaffolds followed by 37 days culture. Constructs were analysed using histological, immuohistological and biochemical methods. PLGA:nCC electrospun scaffolds were shown to support the attachment and proliferation of the primary BACs, with no significant difference in cellular metabolic activity compared to the unfilled PLGA scaffold. Cells were distributed throughout the PLGA:nCC and PLGA scaffolds, although a greater cell density was observed at the periphery. Glycosaminoglycans (GAG), collagen type I and II was detected throughout both the filled and unfilled scaffolds, suggesting a cartilage-like matrix.

In conclusion, the PLGA:nCC composites supported the proliferation and differentiation of expanded BACs, although the ECM was less hyaline-like than reported for commercial non-woven scaffolds. The electrospinning process used here was a simple and elegant route for the fabrication of composite scaffolds. It was concluded that while these scaffolds may have potential for skeletal tissue engineering, they are not yet optimised for articular cartilage tissue engineering.

The authors of this work would like to acknowledge the Technology Strategy Board (TP/8/BIO/6/I/Q0034H) and EPSRC for funding.

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Keywords. Tissue Engineering, Electrospinning

(30.O2) ELECTROSPUN COLLAGEN TYPE II(-POLY-E-CAPROLACTONE CO-POLYMER) - BIOMIMETIC NANOFIBRE SCAFFOLDS FOR POTENTIAL CARTILAGE REPAIR

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Introduction. Cell-scaffold interactions determine the fate of mesenchymal stem cells (MSC) in cartilage tissue engineering approaches. Mimicking biochemical and ultrastructural aspects of articular cartilage are critical for tissue specific scaffold design. The influence of collagen II- and collagen II/PCL nanofibre scaffolds (NFS) on the chondrogenic differentiation of MSCs was investigated to generate an optimized biomaterial for future cartilage repair strategies.

Methods. Collagen II was isolated from knee cartilage of calves. NFS from various collagen II and poly-caprolactone (PCL) concentrations were electrospun and characterized by scanning electron microscopy. Optimized collagen II/PCL scaffolds were seeded with MSCs and cultured in a chemically defined, serum-free medium (CDM-) or CDM-supplemented with TGF-ß1 (CDM+) for up to 21 days. Chondrogenic marker gene expression (aggrecan, Col II etc.) and histological visualization of cartilage specific extracellular matrix molecules was investigated at defined time-points.

Results. Co-electrospinning of collagen II/PCL revealed heteropolymeric scaffolds with fiber diameters between 300–1500 nm, dependent on the collagen II/PCL ratio and concentration. Collagen II/PCL-NFS exhibited an increased ultrastructural stability in aqueous solution compared to pure collagen II NFS making chemical crosslinking redundant. In general, chondrogenic marker gene expression was evident in all scaffolds when cultured in CDM+. In CDM- chondrogenic marker gene expression was inconsistent, in particular aggrecan and Col II expression. When comparing collagen II or collagen II/PCL-NFS to pure collagen I NFS (previous data), an upregulation in early chondrogenic marker genes, i.e. SOX5 and 6, was obvious in CDM-.

Conclusion. Co-electrospinning of collagen II and PCL reveals heteropolymeric NFS with favorable ultrastructural stability, that maintain chondrogenic differentiation of MSCs under the influence of TGF-ß1. The presence of collagen II alone or as co-polymer with PCL seems to exhibit a chondro-inductive effect on MSCs. Electrospun collagen II-(PCL co-polymer)-NFS exhibit biomimetic and chondro-inductive properties that potentially can improve cell-based cartilage repair strategies.

Keywords. nanofibre, collagen type II, electrospinning, stem cell, cartilage repair

(30.O3) EVALUATION OF PCL-CERAMIC COMPOSITES SCAFFOLDS FOR BONE TISSUE ENGINEERING

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There is much written in literature about the use of PCL composites with ceramic materials such as TCP, apatite, or Bioglass for bone tissue engineering purposes (1-2). Nevertheless, to our knowledge there has been no comparison of the behaviour of scaffolds with suitable characteristics for bone tissue engineering (high porosity, interconnected pores, large pore size) made out of these materials. In this work, we developed a series of scaffolds based on PCL, PCL/hydroxyapatite and PCL/Bioglass to assess which showed more suitable for bone tissue engineering. Two series of composites scaffolds with 5%, 10% and 20% (w/w) 45S5 Bioglass® and hydroxyapatite as well as PCL controls were obtained by freeze extraction and particle leaching(3). Physical properties was measured (mechanical behaviour, morphology, composition, stability in physiological medium) and biological response was characterized using osteoblast-like cells (MC3T3-E1). Scaffolds had high interconnected porosity with pore size of ~200µm, being apt for cellular colonization. Synthesis of PCL-Bioglass® with high Bioglass content was hampered by the weakening caused by higher amounts of Bioglass®, likely due to basic hydrolysis during the preparation process. Samples with 5% mineral phase were used for biological characterization because PCL-Bioglass samples were seen to cause an increase in the pH of medium; same mineral ratio was chosen for PCL. Samples were cultured for up to 21 days; cell proliferation was measured using total DNA assay; differentiation was characterized using alkaline phosphatase levels quantification and with immunofluorescent staining of biochemical markers. Mineralization was visualized using Von Kossa staining of thin sections.

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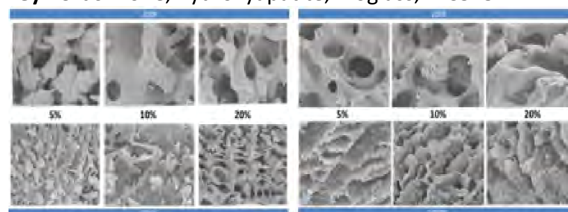
Keywords. Bone, hydroxyapatite, Bioglass, MC3T3-E1



(30.O4) CARTILAGE TISSUE ENGINEERING USING POLYCAPROLACTONE SCAFFOLDS MODIFIED WITH HYALURONIC ACID

Sousa T (1), Rodenas-Rochina J (2), Mano J (1), Gómez-Ribelles JL (2), Lebourg M (3)

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In previous works we developed polycaprolactone (PCL) scaffolds with interconnected porosity and mechanical modulus close to that of human cartilage, which supported cartilage regeneration in vivo. Nevertheless, PCL hydrophobicity and its lack of specific biological interaction do not represent mimic the physiological cartilage environment. On the other hand hyaluronic acid interacts directly with cells through specific surface receptors such as CD441, showing positive effects on chondrocyte behavior2,3.

In this work we developed two types of hyaluronic acid-based (HA) modifications of PCL scaffolds, using homogenously crosslinked HA (1 step procedure, produces gel phase within the pores) or heterogeneously crosslinked HA (2 step procedure, produces a low swelling pellicle on the surface of pore walls). Biphasic PCL/HA scaffolds were characterized physically (equilibrium water content, morphology, HA content, mechanical behavior) and their biological performance was assessed using human primary articular chondrocyte cultured up to 21 days. Material-cells constructs were characterized using qualitative observation with SEM and inmunofluorescent imaging using markers for COL I, COL II and aggrecan, and cell proliferation as well as glycosaminoglycan production was assessed respectively by Picogreen and Blyscan assays.

Observation of inmunofluorescent markers showed that HA influences scaffold colonization, promoting formation of cell clusters rather than a mere covering of the pores walls as predominant in bare PCL. More aggrecan is visualized in PCL-HA1step, and both 1step and 2 step modification seem to increase marking for collagen type II. Biochemical analysis show that HA gel phase in the pores (HA 1step) promotes the most GAG formation per cell.

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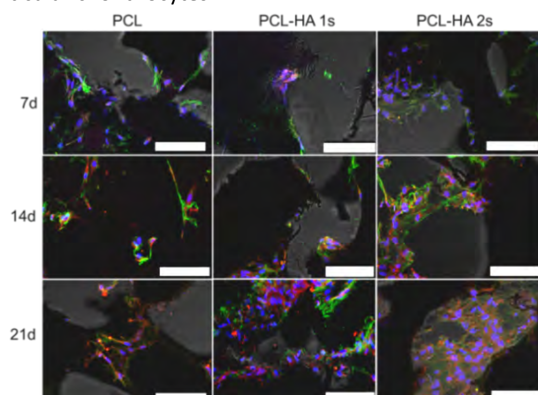
Keywords. polycaprolactone, hyaluronan, human articular chondrocytes



Immunofluorescent staining for collagen type I and II (left; COL I, green; COL II, red; nuclei, blue) Bar size 100µm

(30.O5) DEVELOPMENT OF POLY(BUTYLENE SUCCINATE) MICRO-FEATURES FOR ADIPOSE STEM CELL ALIGNMENT

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One of the major motivations for the increasing effort spent on designing microstructured materials for tissue engineering strategies is that natural tissues and the associated extracellular matrices are in fact microstructured. When an implant first contacts its host environment, a layer of proteins immediately covers the surface of the implant. The adsorptive behavior of these proteins is highly dependent on the surface properties as the micro/nano-structure and chemistry. This surface-specific adjustment can result in the presentation of different regions to cells, ultimately dictating the implant success. Thus, the aim of this work is to engineer a microstructured biomaterial with chemical properties known to elicit an enhanced biological response.

Biomimetic surfaces of the synthetic polymer poly(butylene succinate) (PBS) were engineered by hot-embossing lithography. Briefly, 2% PBS was dissolved in dichloromethane (w/v) and pressed against a heated PDMS mold. Ten micro-patterned surfaces with different groove/ridge combinations were developed and characterized by scanning electron microscopy (SEM), with features ranging from 0.50 to 4 µm. The influence of the engineered micro-features over viability, attachment and proliferation of human Adipose derived adult Stem Cells (hASC) was evaluated. hASC cultured onto the engineered surfaces demonstrated to remain viable during 7 days of culture and SEM and immunostaining showed adequate attachment and spreading of the cells cultured onto the patterned groove/ridge combinations. These microscopy results also demonstrated that hASC tend to align better on surfaces with patterns on the microscale than on the nanoscale. Moreover, DNA quantification of cultured hASC showed their ability to proliferate over the patterned surfaces. Our system combines microtechnologies and biomaterials to engineer a biomimetic surface at the micro-level. The results obtained under this study demonstrate the importance of the PBS micro-structure over the attachment, proliferation and morphology of hACS, a stem cell source with a high degree of pluripotency.

Keywords. microtechnology, poly(butylene succinate), adipose-derived stem cells

(30.O6) BONE TISSUE RESPONSE TO AN INJECTABLE NANOSTRUCTURED CALCIUM PHOSPHATE SCAFFOLD

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Introduction. Particulate calcium phosphate biomaterials are employed widely as bone graft substitutes. While generally considered successful, they are not always easy to place, may be subject to migration, and do not always support consistent tissue healing in compromised patients. New nanoscale forms of hydroxyapatite have been developed that offer direct delivery to a clinical site via injection, and it is possible that the very high surface

area:volume ratios associated with nanostructured ceramics could improve the healing response of bone tissue. Aim: The aim of this study was to investigate the response of bone tissue (healing and osteoconduction) to a new nanostructured ceramic biomaterial.

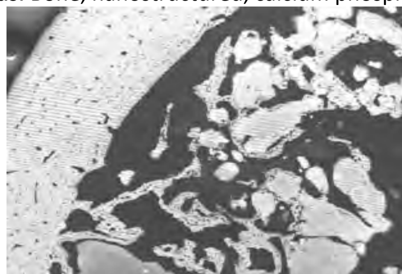
Methods. Sterile nanostructured calcium phosphates were provided in ready-to-use syringes (2 ml) by Ceramisys Ltd. (Sheffield, UK) and characterised in-house. Materials were injected into prepared defects (1 mm) in the midshaft of Wistar rat femurs and evaluated using micro-computed tomography (µCT) and histology after 2, 4 and 12 weeks implantation, and compared to untreated (sham) injuries and healthy bone.

Results. At the earlier time periods (2 and 4 weeks), bone tissue healing progressed normally in the presence of experimental material without evidence of impairment or infection. Bone tissue also grew in close apposition to the surface of nanostructured hydroxyapatite (see image below), with further evidence of significant bone growth detected using µCT, demonstrating that the material was osteoconductive. By 12 weeks, µCT showed evidence of bioresorption of the test material and associated healing and regeneration of bone.

Conclusion. Injectable, nanostructured calcium phosphates have great promise for use as osteoconductive, bioresorbable bone substitutes for use in orthopaedics, and dental or craniofacial surgery.

Acknowledgements. The authors are grateful to the Technology Strategy Board and EPSRC for funding the NANO4MED collaborative project.

Keywords. Bone, nanostructured, calcium phosphate



(30.O7) ENHANCEMENT OF OSTEOGENIC DIFFERENTIATION OF HUMAN ADIPOSE DERIVED STEM CELLS ON POLY(D,L-LACTIC ACID) SCAFFOLDS BY THE INCORPORATION OF PLATELET LYSATE-LOADED CHITOSAN-CHONDROITIN SULFATE NANOPARTICLES WITHIN THE CONSTRUCT

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Introduction. In the tissue engineering (TE) field, the production of multifunctional scaffolds, acting as templates for cell transplantation but also delivering bioactive agents in controlled manner, is an emerging strategy aimed to enhance tissue regeneration. In this work, a hybrid release system consisting in a three-dimensional (3D) scaffold based on poly(D,L-lactic acid) (PDLLA) impregnated with platelet lysate-loaded chitosan/chondroitin sulfate nanoparticles (PL/CH-CS NPs) was developed. PL is an easily obtained autologous source of growth factors that can play an enhancement role over the proliferation and differentiation ability of mesenchymal stem cells. Previous studies performed in our group revealed that the controlled release of PL from CH-CS NPs enhanced in vitro osteogenic differentiation of human adipose derived stem cells (hASCs) cultured in monolayer. Moreover, we also reported the successful incorporation of the NPs in the 3D construct and subsequent controlled release profile of a model protein.

Methods. The PDLLA scaffolds loaded with PL/CH-CS NPs were prepared by supercritical fluid foaming at 200 bar and 35 °C and after sterilization, they were seeded with hASCs and cultured in vitro up to 28 days with osteogenic and basal mediums. Cell viability and proliferation were assessed as well as histology, immunohistochemistry and gene expression (by polymerase chain reaction) characterization for osteogenic markers. PDLLA only and PDLLA loaded with empty NPs were used as controls.

Results. The controlled release profile of PL from the 3D construct allows the enhancement of initial cell proliferation and enhancement of hASCs osteogenic differentiation as shown by higher initial alkaline phosphatase values, higher mineralization and higher gene expression for osteogenic markers. Even in the absence of osteogenic medium, hASCs seeded in PL-loaded scaffolds showed signs of osteogenic differentiation.

Conclusions. The inclusion of PL/CH-CS NPs in the PDDLA scaffold enhances the functionality of the structure, suggesting the potential of the proposed system for bone tissue engineering applications.

Acknowledgments. FCT for the grants (SFRH/BD/39486/2007, SFRH/BD/64070/2009 and SFRH/BPD/34994/2007), IPS,Hospital da Prelada,EXPERTISSUES (NMP3-CT-2004-500283) and Find&Bind (NMP4-SL-2009-229292).

Keywords. adipose derived stem cells; platelet lysate; poly(D,L-lactic acid); bone tissue engineering

(30.O8) DEVELOPMENT OF NEW BIODEGRADABLE COMPOSITE SCAFFOLDS POYURETHANE-BIOACTIVE GLASS NANOPARTICLES

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The development of polymer/bioactive glass has been recognized as a strategy to improve the mechanical behavior of bioactive glass-based materials. Several studies have reported systems based on bioactive glass/ biopolymer composites. In this work we developed a composite system based on bioactive glass nanoparticles (BGNP), obtained by a modified Stober method. The BGNP presented significant increase in cell viability when compared to microparticles. The polymeric phase is extremely important to design the final properties of the composites. The highly variable synthesis chemistry of segmented PU's may be exploited to generate polymers having properties ranging from very soft elastomers to very rigid plastics. In addition to the physical properties, a great care has to be taken in the choice of the building blocks. Their degradation products have to be biocompatible, non-toxic and metabolized or eliminated by the living organism. We developed a new chemical route to obtain aqueous dispersive biodegradable polyurethane. This chemical procedure was well

succeeded in producing PU dispersions with solid content about 20%. We then associated the newly developed components (BGNP and PU aqueous dispersions) to produce membranes and scaffolds, intending to associate biocompatibility, mechanical and physical properties in a material designed for tissue engineering applications. The composites were obtained by the dispersion of BGNP in a PVA solution. Then, this mixture was added to PU with final BGNP composition of 0, 10 and 25%. Films were obtained by casting the dispersions in molds. Porous scaffolds were produced by dilution and freeze drying of the dispersion obtained. The composites obtained were characterized by FTIR, XRD, MEV and biological and mechanical tests. The films had 320% of deformation and the foams presented shape memory. The materials presented good cell viability and hydroxyapatite layer formation.

Keywords. Biodegradable polyurethane, Bioactive glass Nanoparticles, Composites, Scaffolds, Films

(30.O9) ELECTROSPUN SCAFFOLDS WITH CONTROLLED VOID SPACE

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Introduction. Electrospinning offers possibilities to create complex 3-dimensional scaffold architectures which could mimic the natural environment of cells, important to unlock the full potential of the cells in tissue engineering. However, electrospun scaffolds often lack void spaces large enough to allow for sufficient cellular ingrowth. Thus increasing and controlling the void space is highly desirable for tissue engineering scaffolds. Here we describe a method to vastly increase and control the void space while using low temperature electrospinning (LTE)1.

Method. Poly(ε-caprolactone) and poly(lactic acid) were electrospun from 10 % (w/w) chloroform solution with different pyridium formiate concentrations to vary the fibre diameter and accordingly the fibre stiffness. Mesh morphology was analysed with SEM and computer tomography and applicability of these meshes for tissue engineering was tested with C2C12 muscle progenitor cells.

Results. By using LTE an up to 20-fold increase in void space was achieved compared to conventional spinning with similar fibre diameters (see Fig.1). It was demonstrated that the void space in LTE spun scaffolds is directly related to the fibre stiffness, e.g. changing the fibre stiffness from 0.2 to 230 N/m resulted in a 2.5 times larger void space. Using these up to 99.5% porous meshes for tissue engineering resulted in a complete and homogenous cell in-growth.

Conclusions. We have demonstrated that LTE is a robust technique to produce scaffolds with porosities up to 99.5%. Adjusting fibre stiffness and using LTE offers an efficient tool to increase and control the void space, and so the pore size of electrospun scaffolds, to a range allowing a complete cell in-growth.

References.

[1] M. Simonet et al., Poly. Eng. Sci., Vol. 47, (2007)

Keywords. electrospun scaffold, increased void space, cell in-growth

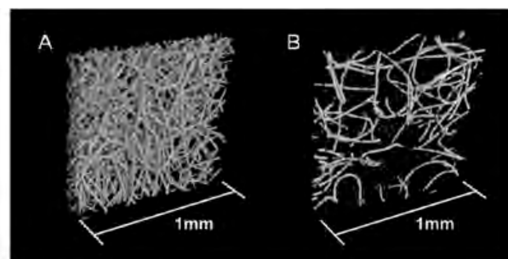

Fig. 1 200 µm slice of computer tomography scans of (A) conventional and (B) low temperature electrospun PLA mesh, both with 9 µm fibre diameter

(30.O10) BONE FORMATION IN SURFACE-MODIFIED 3D PLOTTED POLYCAPROLACTONE-SCAFFOLDS

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Introduction. 3D plotting of polycaprolactone (PCL) scaffolds has the advantage that the scaffold is fully interconnective. As PCL is not cell-interactive, four surface modifications were investigated (T. Desmet et al. Macromol Biosci. 2010, 10 (12): 1484-1494). In this study the seeding efficiency, proliferation, colonization and osteogenic differentiation was compared.

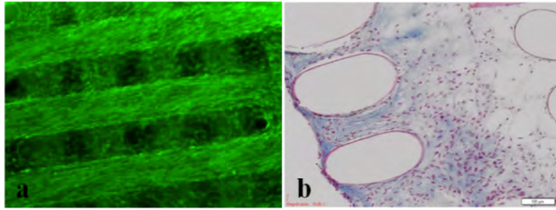
Methods. PCL scaffolds (3 mm h; 4,5 mm d) were plotted with a Bioscaffolder. PCL scaffolds were surface-modified with O2 plasma, AEMA, gelatin and gelatin-fibronectin immobilization (PCLO, PCLAEMA, PCLGelB, PCLGelBFn) and compared with commercial collagen (BDCollagen) and lactic-acid (BDOPLA) based scaffolds.

MC3T3 and human adipose-tissue derived stem cells (ATMSC) were seeded on the scaffolds and cultured in osteogenic medium for 28 days. At specific time points the cultures were evaluated by fluorescence microscopy (CaAMPI), ALP/protein content, histology (Trichrome Masson, von Kossa, Runx2) and qRT-PCR (Runx 2, collagen I, osteocalcin).

Results. Although the low seeding efficiency (40 %), the center of the PCLGelB and PCLGelBFn scaffolds are fully colonized within 14 days. In contrary, the commercial scaffolds, PCLO and PCLAEMA are only colonized at the edges (figure 1a). The proliferation of the cells is comparable on PCLO and BDOPLA respectively PCLGelBFn and BDCollagen. The osteogenic differentiation in 3D plotted scaffolds is retarded as was demonstrated with ALP/protein content, however after 14 days, the ALP levels are similar with the commercial scaffolds. mRNA expression of Runx2, collagen I and osteocalcin is upregulated in PCLGelBFn scaffolds and comparable with BD collagen scaffolds. After 28 days, the cells were differentiated with the formation of a bone-specific extracellular matrix (figure 1b).

Conclusions. The proposed surface modifications of 3D plotted PCL scaffolds are successful. The bioinspired coating outperforms the oxygen plasma treatment of the scaffolds. The 3D architecture will be fine-tuned to increase the cell-seeding efficiency and to optimize the differentiation of the ATMSC.

Keywords. 3D plotted scaffold, polycaprolactone, surface modification, colonization, osteogenic differentiation.



(30.O11) OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS SEEDED ONTO A NOVEL NANO-COMPOSITE BIOMIMETIC SCAFFOLD FOR OSTEO-CHONDRAL TISSUE REGENERATION

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Introduction. A novel biologically inspired scaffold able to promote in situ osteo-chondral (OC) tissue regeneration has been recently developed and characterized by Mg-HA nano-crystals nucleation on Type I collagen fibrils during their self-assembling in fibers. In this work, cartilaginous and sub-chondral bone layers were assessed for their ability to support chondro- and osteo-genic differentiation of human bone marrow-derived mesenchymal stem cells (hmscs).

Methods. The OC biomimetic scaffold has a composite three-layered gradient structure. The smooth cartilaginous layer consists of Equine Type I atelocollagen. The intermediate layer (tidemark-like) combines collagen (60wt.%) and Mg-HA (40wt.%), whereas the lower mineralized sub-chondral bone layer has 70wt.% of Mg-HA. To stabilize the scaffold and delay the degradation kinetic in physiological environment, BDDGE (1,4-butanediol diglycidyl ether) is added as cross-linking agent. Samples were first characterized by SEM and TEM analyses, then in vitro cultured. The OC scaffold was seeded with hmscs, and viability, osteogenic and chondrogenic differentiation were evaluated at day 0, 14, 28 and 52 by Alamar blue and specific histological staining.

Results. OC scaffold revealed a highly porous micro-architecture, with oriented columnar-like organic fibers on top layer. Nucleation of very low crystalline Mg-HA occurred according to a process typical of natural bone osteogenesis, with nanocrystals inside collagen fibrils having their c axes preferentially oriented parallel to the direction of organic fibers.

The scaffolds were completely colonized by highly viable cells, which penetrated until inner portions of the structure. Osteogenic and chondrogenic differentiations were observed starting from day 28.

Conclusions. Chemical-physical analyses highlighted the unique architecture of the three-layered gradient scaffold. In vitro results confirmed that this novel bio-inspired device represent an effective surgical treatment option to guarantee rapid recovery of articular functionality, thanks to its highly biomimetic properties.

Acknowledgments. This work has received funding from the EU- FP7/2007-2013 under grant agreement n° 214685 (MAGISTER).

Keywords. Osteo-chondral regeneration, biomimetic scaffold, mesenchymal stem cells

(30.O12) TWO-LAYER MEMBRANE OF CALCIUM PHOSPHATE/COLLAGEN/PLGA NANOFIBRES: IN VITRO BIOMINERALISATION AND OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

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In the context of guided bone regeneration it would be advantageous to have an implant that presents compositional and structural similarities to osseous tissue. In this work the elaboration of an anisotropic bilayer is described. Amorphous calcium phosphate (a-CaP) nanoparticles, produced by flame spray synthesis, were combined via electrospinning with collagen (Col) Type I and poly(lactide-co-glycolide) (PLGA). The fibres' transformation during crosslinking and biomineralisation was investigated. To obtain a bifunctional membrane dyed a-CaP/Col/PLGA fibres were electrospun on top of pure PLGA. A cell culture study with human mesenchymal stem cells was conducted to analyse differentiation of the cells and exclude any cytotoxic effects of the scaffolds by alamarBlue, alkaline phosphatase activity and confocal laser scanning microscopy. Ca and collagen contents were followed by Alizarin red S and Sirius red staining.

The fibres' morphology depended on the chemical composition. Fibres that contained PLGA were stable enough to undergo crosslinking and biomineralisation experiments. The surface appearance of fibres that contained a-CaP dramatically changed after biomineralisation. The double membrane presented PLGA fibres on its white side and a-CaP/Col/PLGA fibres on the blue side (Fig. 1a-c). In vitro proliferation of the cells seeded on the membrane was successful and neither side showed cytotoxicity. Differentiation into the osteogenic lineage was better than in 2D control. Further an augmented content of Ca and collagen was confirmed.

a-CaP nanoparticles were combined with collagen in a weight ratio similar to the chemical composition of bone tissue and the fibres were strengthened with PLGA. The hydroxyapatite formation indicates the osteoconductivity of the a-CaP-scaffolds. The anisotropic double membrane is easy to handle and can be shaped to be used as a bone wound dressing material. (Fig. 1d-f)

N. Hild et al., Nanoscale, in press.

Keywords. anisotropic bilayer, electrospinning, amorphous calcium phosphates, nanocomposite

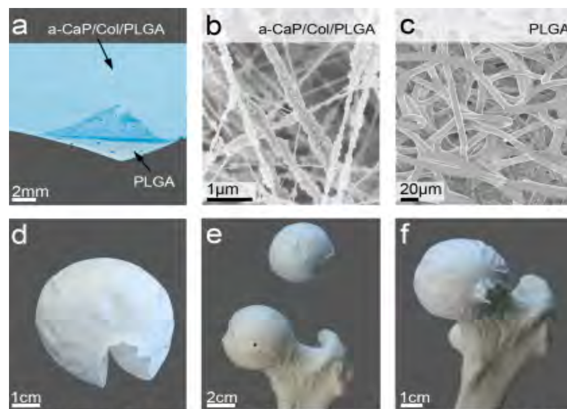

Fig. 1 (a) Bilayer with dyed a-CaP/Col/PLGA (SEM in (b)) on one side and PLGA (SEM in (c)) on the other side. The bilayer can be electrospun into a spherical shape (d) that can be positioned on a femur head model (e and f).

(30.O13) CELLULAR BIODEGRADABLE POLYMER NANOCOMPOSITE SCAFFOLDS

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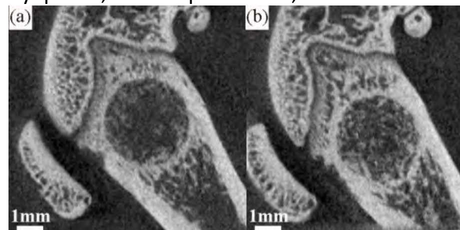
Introduction. New solvent-free processing routes for poly-L-lactide (PLLA)/nano-hydroxyapatite (nHA)-based resorbable bone replacement materials are under development in our institute. Here we describe a study of the feasibility of producing porous bone replacement scaffolds from PLLA/nHA by supercritical CO2 foaming. The specific goal has been to investigate the effects of foaming parameters and nHA content on the foam geometry, crystallinity and mechanical behavior in order to produce optimized structures for clinical trials. The most promising foams were then implanted in rat femoral condyles for in vivo testing over periods of up to 8 weeks.

Results and Discussion. PLLA/4.2 vol% nHA foams produced with a saturation pressure and temperature of 200 bar and 165 °C respectively, and a depressurization rate of 2.5 bar/s showed the most promising architectures according to established criteria for bone replacement scaffolds, with a porosity of 85 %, interconnected cells with diameters in the 200-400 µm range and a homogeneous structure. The highest compression moduli (70 MPa) and compressive strengths (2.14 MPa) were also obtained with these foams. These values compare with 50-500 MPa and 7-10 MPa for the compression modulus and compressive strength of trabecular bone. Ongoing in vivo studies have shown correspondingly promising results. After 2 weeks implantation, growth of natural rat bone into the scaffold was clearly promoted (Figure 1(a)), and after 5 weeks, the bone had colonized the entire cross-section of the scaffold (Figure 1(b)), the bone volume to total volume increasing from 6.2 to 41.06 % between 2 and 5 weeks.

Conclusion. With optimized processing parameters, foam morphologies suitable for bone replacement scaffolds could be produced by supercritical CO2 processing of PLLA/nHA nanocomposites. Extensive bone growth into these scaffolds has already been observed in vivo, and these observations will be completed with micro-CT and histological investigations after longer implantation times.

Keywords. nanocomposite, poly(L-lactic acid), hydroxyapatite, bone replacement, in vivo



(30.O14) MICRO- AND NANOSTRUCTURED HYDROXYAPATITE/COLLAGEN MICROCARRIERS FOR BONE TISSUE ENGINEERING APPLICATIONS

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Introduction. Recent studies have shown the potential of microcarriers for tissue engineering purposes, as vectors for the delivery of cells or drug release vehicles. For bone substitution, however, the use of osteoconductive or osteoinductive materials is highly desirable. This work describes a novel route for the synthesis of hydroxyapatite/collagen microcarriers with tailored micro/nanostructures, and the effect of both micro/nanostructuring and the presence of collagen on the cellular response.

Methods. Hydroxyapatite and hydroxyapatite/collagen microcarriers were obtained through the hardening in emulsion of a calcium phosphate cement in oil, stirred by a rotating paddle. The powder phase consisted of α-tricalcium phosphate particles with two different sizes, coarse and fine. Type I collagen was introduced by dissolving it in the liquid phase of the cement. An in vitro study was performed using osteoblastic-like Saos-2 cells cultured for up to 14 days, where the overall cell morphology, proliferation and differentiation (in terms of alkaline phosphatase activity) were assessed.

Results. The microstructure of the inorganic microcarriers consisted of entangled hydroxyapatite crystals, micrometric in size in the coarse and nanometric in the fine cement. When collagen was introduced in the system, the hydroxyapatite crystals on the surface were still visible, retaining the same micro- or nanostructural features. Saos-2 cells were able to attach on all types of microcarriers and showed an enhanced proliferation and differentiation in the presence of collagen. In addition the cells responded to the topography, presenting higher proliferation on the micrometric sized crystal hydroxyapatite/collagen microcarriers, and more pronounced differentiation on the nanosized crystal hydroxyapatite/collagen microcarriers.

Conclusions. A novel and simple method for the preparation of hydroxyapatite/collagen microcarriers with tailored microstructures was developed. The addition of collagen resulted in an improved interaction

with osteoblastic-like Saos-2 cells. A synergistic effect between the presence of collagen and the nanosized hydroxyapatite crystals was found.

Keywords. Hydroxyapatite, Collagen, Calcium phosphate cement, microcarrier

(30.P2) INTEGRATED STRATEGY USING IONIC LIQUIDS FOR THE DESIGN AND PROCESSABILITY OF NATURAL POLYMERIC ARCHITECTURES FOR REGENERATIVE MEDICINE

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Introduction. Natural polymers are adequate renewable resources for the design and processability of well-defined architectures for several applications, including tissue engineering. Combinations of polysaccharides and proteins may mimic the naturally occurring environment of certain tissues, providing an optimum substrate for tissue growth and regeneration. The main goal of this work was to apply the green chemistry principles, represented by the use of ionic liquids (ILs), and biorenewable sources, which combined with well-known or less conventional technologies can provide new ways to obtain controlled polymeric architectures (e.g.macro/nano porous structures, hydrogels) suitable for regenerative medicine.

Methods. To design the materials structures, chitosan-silk/IL and chitin/IL solutions were processed as hydrogels/sponges and macro/nano structures using gelation/freeze-drying and supercritical fluid technology, respectively. The preparation conditions were adjusted according to each technique/method. The ability of the developed materials to support adhesion and proliferation of human osteoblastic-like cells (SaOs-2) and dermal fibroblasts (hDFs) was assessed up to 21 days of cell culture.

Results. The chitin porous structures (CPS) showed interesting features such as very low density (0.039 g/L to 0.063 g/L), porosity between 84 and 90% and a heterogeneous porous formation with pores from micro- to nanoscale and cristallinity about 21%. Chitosan/silk-based hydrogels (CSF) showed a soft and rubbery consistency, microporous surface and viscoelastic behavior. The in vitro biological performance of both structures revealed their positive influence in adhesion, viability and proliferation of cell SaOs-2 (porous structures) and hDFs (hydrogels) for the studied culturing time.

Conclusions. The findings suggest that the IL platform provides a versatile approach to obtain different polymeric architectures with interesting properties. Both CSF and CPS structures demonstrated their potential to be used as wound dressings or as supports for skin tissue engineering.

Acknowledgements. Silva SS, JM Oliveira, AR Duarte thank the Portuguese Foundation for Science and Technology (FCT) for their Post doc fellowships.

Keywords. ionic liquids, natural polymers, polymeric architectures, tissue engineering

(30.P3) CHARACTERIZATION OF NOVEL ALGINATE NANOCOMPOSITES WITH SILVER NANOPARTICLES FOR BIOMEDICAL APPLICATIONS

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Biomaterials present an indispensable component in tissue engineering as well as in many other applications in regenerative medicine, in which they can be used alone or in conjunction with cells. These materials, potentially implanted in human body, need to be sterile before the implantation, as well as to remain sterile inside the body. Silver nanoparticles (AgNPs) express antimicrobial activity but due to tendency for agglomeration, need to be stabilized and hydrogels can provide suitable matrixes. These hydrogel nanocomposites could be potentially used as antimicrobial agents, wound dressings and soft tissue implants. In this work, we have produced alginate solutions with AgNPs by a novel electrochemical method followed by production of hydrogel nanocomposites in forms of discs and microbeads. Presence of AgNPs was confirmed by transmission electron microscopy (TEM) and UV-Vis spectroscopy, which showed surface plasmon absorption band maxima at ~400nm. Biomechanical properties of packed beds of nanocomposite microbeads were studied in a biomimetic bioreactor under dynamic compression at 10% strain in two regimes: at a loading rate of 337.5μm/s and at sequential increments of 50μm displacement every 30min. Packed beds of alginate microbeads with and without AgNPs exhibited similar compression and equilibrium unconfined compression moduli, showing negligible effects of AgNPs. On the other hand, alginate nanocomposites demonstrated release of silver ions and AgNPs inducing antibacterial activity against Staphilococcus aureus. Microbeads, in particular, were shown to be effective due to high surface to volume ratio so that the concentration of AgNPs had to be optimized in order to avoid cytotoxic effects on monolayers of bovine calf chondrocytes. These results were confirmed in short-term bioreactor studies of alginate microbeads with immobilized bovine calf chondrocytes mixed with nanocomposite microbeads. This work has shown potentials of novel alginate nanocomposites for biomedical applications, possibly reducing the need for antibiotics.

Keywords. silver nanoparticles, alginate hydrogel, microbead, cytotoxicity

(30.P4) POLY(L-LACTIC ACID)/HYDROXYAPATITE/SILICA COMPOSITE SCAFFOLDS FOR BONE REGENERATION

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Introduction. New reinforced poly(L-lactic acid) (PLLA) scaffolds with hydroxyapatite (HAp) and mesoporous silica ($SiO2$) nanoparticles for bone tissue engineering were fabricated and characterized with respect to

morphology, reinforcement potential, mechanical performance and bioactivity.

Methods. A series of poly(L-lactide)/hydroxyapatite/SiO2 composite scaffolds were prepared by freeze extraction and particle leaching and reinforced through mixing with HAp nanopowder, < 100 nm, (Sigma Aldrich) and mesoporous silica nanoparticles (UVM-7), particles size of 20-30 nm and pores around 3 nm [1]. Nanoparticles were dispersed in dioxane by sonication before and after the addition of PLLA (PURASORB PL 18, Purac Biomaterials). Scaffolds were prepared with (95/0/5), (95/5/0), (90/10/0), (90/9.5/0.5) %(w/w) of PLLA/HAp/SiO2 respectively. Porosity, crystallinity, and the elastic compressive modulus were analyzed. Bioactivity was assessed by soaking them into a simulated body fluid solution (SBF) for different days.

Results. Porosity (88%) and the degree of crystallinity (53%) of all the scaffolds are essentially constant, regardless of the quantity and nature of the nanoparticles. Although PLLA/HAp composites showed improved compressive modulus compared to PLLA, this toughening was not as pronounced as expected, due to the agglomeration of the HAp particles. When combining silica and HAp, this modulus increased from 4.7 MPa (pure PLLA) to 6.9 MPa (PLLA/HAp/SiO2, 90/9.5/0.5) due to the larger interfacial area between the nanometer-scale mesoporous SiO2 particles and the PLLA. PLLA/HAp/SiO2 composites in addition show stronger bioactivity. Nuclei grown on the pores surfaces after SBF soaking are mainly of calcium and phosphorous, with Ca/P ratio close to stoichiometric hydroxyapatite 1.67.

Conclusions. Freeze extraction and sonication allows for a good dispersion of the nanoparticles in the PLLA matrix. All composites are bioactive and show improved mechanical properties especially that with 90/9.5/0.5 content of PLLA/HAp/SiO2, which is the best candidate for bone tissue engineering among the designed scaffolds.

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Keywords. Poly(L-lactic acid)/hydroxyapatite/silica composites, bioactivity, bone tissue engineering

(30.P5) HYBRID SCAFFOLDS OF POLY (VINYL ALCOHOL)/BIOACTIVE GLASS NANOSTRUCTURED BY CHEMICAL CROSSLINKING

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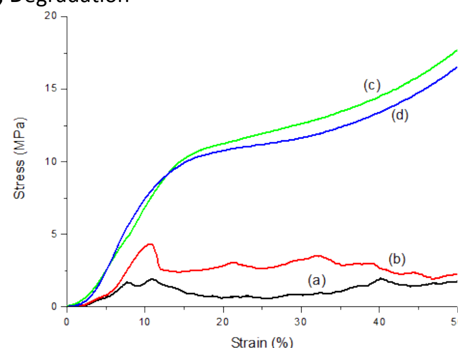
Porous composites of biodegradable polymers with bioactive glasses are of particular interest in tissue engineering, where the ceramic phase can improve or fulfill the osteogenic features. Poly(vinyl alcohol)/Bioactive glass hybrid foams (PVA/BaG) were previously developed by our group, using the sol-gel route. It was observed that hybrids with high polymer content show fast dissolution in aqueous media. The degradation rate may be tailored by the polymer molecular weight or the use of a cross linking agent. In this work it was investigated the effect of different concentrations of glutaraldehyde on the chemical and physical structure of PVA/BaG hybrids, and on their consequent mechanical and degradation behavior. The effect of cross linking on the structure of the hybrids was analyzed by X-ray diffraction, Fourier Transform Infrared Spectroscopy, Scanning Electron Microscopy and X-ray Micro-computed Tomography analysis. Mechanical properties of hybrids were evaluated by compression tests and the degradation behavior was evaluated by the weight loss upon soaking into deionized water at 37oC for 21 days. Cytotoxicity assay was also performed by the MTT method with VERO cell culture. The results indicated an increase in the yield strength and deformation and a gradual decrease in the weight loss of the obtained hybrids with the increase in the concentration of glutaraldehyde. The hybrids cross linked with 5wt% GA presented a homogeneous pore size distribution in the range 10-450m and cell viability measured by the MTT test adequate for biomaterials application.

Keywords. Biocomposite, Hybrids, Cross linking, Bioactive glass, Degradation



Stress-strain compression curves obtained for PVA/BaG hybrids cross linked with (a) 0%; (b) 1% and (c) 5wt.(%) of glutaraldehyde, and (d) cross linked with 5% GA and treated with NH_4OH.

(30.P6) DIFFERNENTIATION OF HUMAN MESENCHYMAL STEM CELLS IN MICROPOROUS NANO-CELLULOSE SCAFFOLDS

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Introduction. Microporous nano-cellulose is a potential biomaterial for scaffolds used in tissue engineering applications. The aim of this work is to study the differentiation of human mesenchymal stem cells (hMSCs) towards an osteoblastic phenotype in microporous nano-cellulose scaffolds. The aim was also to

further enhance the mechanical and osteoinductive properties of the nano-cellulose scaffolds by adsorption of anionic polymers onto the nano-cellulose providing nucleation sites for hydroxyapatite (HAP) crystals.

Materials and Method. The porous and crystalline morphologies of the scaffolds were analyzed using SEM. The deposited mineral was analyzed with SEM/EDX, X-ray crystallography, ESCA and FTIR. The mechanical properties were determined using tensile measurements. To investigate the osteoinductive properties of the mineralized scaffolds they were seeded with hepatic derived hMSCs. Cultures were continuously analyzed with MTS assay and ALP assay to determine relative cell number and degree of differentiation respectively. Immunohistology was used to study the expression of the osteoblast specific proteins Osteoadherin and Osteocalcin.

Results. Results from SEM and SEM/EDX confirmed HAP crystals deposited onto the nano-cellulose, resulting in an environment resembling native bone tissue's mineralized extra cellular matrix. Results from the in vitro cell study shows proliferation and differentiation of the hMSCs in the porous mineralized scaffolds. Osteoadherin and Osteocalcin expression by the cells in the scaffolds were confirmed by immunohistology.

Conclusion. The study shows that human mesenchymal stem cells can proliferate and differentiate in the mineralized modified microporous nano-cellulose scaffolds, which makes this novel material a promising biomaterial for bone repair applications.

Acknowledgments. Supported by the 7th framework programme the EU – EuroNanoMed - programme EAREG.

Keywords. Nano-cellulose, Human Mesenchymal Stem Cells, Hydroxyapatite

(30.P7) MULTI-SPIRAL POLY(CAPROLACTONE)/ β-TCP NANOFIBROUS SCAFFOLD FOR BONE TISSUE ENGINEERING: IN-VITRO AND IN-VIVO STUDIES

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Introduction. The purpose of this study was to evaluate the suitability of nanofibrous poly(ε-caprolactone)/β-tricalcium phosphate (PCL/β-TCP) multi-spiral structure as a scaffold for the in-vitro proliferation and osteogenic differentiation, as well as ectopic bone formation of bone marrow mesenchymal stem cells (BM-MSCs) in the rat subcutaneous tissue.

Methods. β-TCP was synthesized and characterized. Then, the electrospinning parameters for nanofibrous PCL/β-TCP membrane production were optimized. Electrospun composite membranes were curled into spirals and were assembled into a cylindrical scaffold using about 70 spirals. The in vitro degradation and SEM analyses were performed. Rat BM-MSCs isolated from rat femurs were seeded and cultured both on planar membranes and on multi-spiral scaffolds. The adhesion and proliferation status of the cells were determined using the MTT method. In-vitro osteogenic differentiation of the BM-MSCs was evaluated using histology and IHC. For in-vivo experiments, cells cultured and partially differentiated on multi-spiral scaffolds were transplanted into the epigastric groin fascia of Wistar rats. Constructs were explanted and evaluated using histology and IHC at time points.

Results. Synthesized β-TCP and the electrospun PCL/β-TCP membranes were extensively characterized. BM-MSCs attached, proliferated and synthesized extracellular matrix on the easily-recoverable nanofibrous membranes. The composite scaffold supported the in-vitro osteogenic differentiation of the cells, confirmed by positive-staining to osteogenic markers, such as Von Kossa and anti-Osteopontin. Animal studies indicated the cellular constructs did not cause significant inflammatory reaction suggesting their biocompatibility. The porous composite scaffold was infiltrated with tissue, deposited a calcium-rich matrix during in vivo osteogenesis and induced neovascularization at the subcutaneous site.

Conclusions. Findings support the notion that, the multi-spiral PCL/β-TCP nanofibrous composite scaffold could be a potential three-dimensional substrate for bone tissue engineering using bone marrow mesenchymal stem cells.

Acknowledgments. The Scientific and Technological Research Council of Turkey (TUBITAK 108M501).

Keywords. Bone tissue engineering, bone marrow-mesenchymal stem cells, multi-spiral scaffold, poly(ε-caprolactone), β-TCP, composite biomaterials

(30.P8) CO-CULTURE OF HUMAN BONE MARROW STROMAL CELLS (HBMSC) AND HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS (HDMEC) ON NANO-HYDROXYAPATITE (HA) SURFACES

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Introduction. Nanostructured materials recently developed for bone implant, such as ceramics, polymers and metals have a more dynamic response when compared to counterpart materials with larger particle size. Recent studies have shown that HA nanocrystalline have better functional properties, such as surface reactivity and ultra-fine structure, which are important to create suitable local conditions for bone formation when implanted in an osseous environment. Bone formation depends on several complex processes, including a tight communication between osteoblasts and endothelial cells. Taking this into account, the main aim of the present work was to study the interactions between HBMSC and HDMEC when co-cultured on nanoHA surfaces.

Methods. Structures with a nano-hydroxyapatite surface were prepared from nano-hydroxyapatite (nanoHA) powders (Fluidinova S.A.; nanoXIM_HAp202), applying the sintering temperature of 1050ºC. HBMSC and HDMEC isolated and co-cultured were maintained in endothelial medium. The cultures were examined regarding the metabolic activity (MTT assay), cell viability (Calcein-AM; CLSM), morphology (CLSM), endothelial (CD31, VE-Cadherin, vWF) and osteoblastic (Collagen type1, ALP, BMP-2, OPG, M-CSF) related gene expression (RT-PCR) and ALP activity.

Results. Under the applied conditions HDMEC and HBMSC in single culture and co-culture were able to maintain their viability, morphology, metabolic activity and individual functionality during the culture time. An earlier expression of endothelial and osteogenic markers expression was enhanced in co-culture conditions.

Conclusions. Nano-structured HA materials may be considered a promising class of implants for bone regeneration, since they provided an adequate environment for osteoblast and endothelial cells adhesion and migration, improving cells response.

Acknowledgments. M.S. Laranjeira would like to thank FCT — Fundação para a Ciência e Tecnologia (Portugal) for their support in this work through PHD grant (SFRH/BD/29056/2006) and Fluidinova S.A. (Maia). The support of ADI through the project NanoforBone (NORTE-01-0202-FEDER-005372) is also acknowledged.

Keywords. Human bone marrow stromal cells, Human dermal microvascular endothelial cells, co-culture, bone regeneration, nanotechnology, biomaterial

(30.P9) INTERACTION OF OSTEOBLAST AND ENDOTHELIAL CELLS IN THE PRESENCE OF NANO-HYDROXYAPATITE PARTICLES

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Introduction. Nano-Hydroxyapatite (nanoHA) has a variety of proposed applications in bone repair/regeneration strategies. Most of the published work regarding the biological profile of nanoHA relies on films, composite scaffolds, dense or porous bulk or cements manufactured with HA nanoparticles. A clear deficit exists on studies addressing the biological assessment of individual nanosized HA particles. Considering the close association of angiogenesis and osteogenesis in bone formation events, this study aims to address the biological profile of nanoHA particles on isolated and co-cultured osteoblast and endothelial cells.

Methods. NanoHA was prepared by a hydrothermal synthesis and characterized by XRD analysis, FTIR and TEM. MG63 osteoblast-like cells (103 cell/cm2) and human dermal microvascular endothelial cells (HDMEC, 104 cell/cm2) were cultured isolated or co-cultured in endothelium medium. At day 1, nanoHA was added (0.1-100 µg/ml) and cultures were maintained for 14 days. Culture medium was changed at day 7. Cultures were characterized at days 2, 7 and 14 for cell viability/proliferation (MTT assay), pattern of cell growth (CLSM) and gene expression of osteoblastic and endothelial markers (RT-PCR; Collagen type1, ALP, BMP-2, OPG, M-CSF, CD31, VE-Cadherin and vWF).

Results. Monocultures of MG63 cells and HDMEC presented high viability and the expected pattern of cell growth and gene expression. Co-cultures exhibited the formation of cell clusters of endothelial cells surrounded by osteoblast cells and inductiun and/or earlier expression of osteoblastic and endothelial markers. The presence of nanoHA caused a dose-dependent increase in the viability/proliferation of osteoblast cells, but decreased values were found in HDMEC. Co-cultures showed a slight increase in the cell viability/proliferation and mixed effects in the gene expression profile of osteoblastic and endothelial markers.

Conclusion. Results suggest that NanoHA modulates the behaviour of osteoblastic and endothelial cells and also affects the interaction between these two cell types.

Keywords. nano-Hydroxyapatite particles, osteoblast cells, endothelial cells.

(30.P10) PREPARATION OF COLLAGEN-HYDROXYAPATITE BIOCOMPOSITE SCAFFOLDS BY CRYOGELATION METHOD FOR TISSUE ENGINEERING APPLICATIONS

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1. INEB/FEUP; 2. INEB/FEUP

Introduction. The available treatments for repairing injured bone tissues could suffer many problems and are often unsatisfactory. Recent efforts of bone repair focus on development of porous three dimensional scaffolds for cellular adhesion and proliferation. In this work, the possibility of applying cryogelation as an alternative technique for preparation of 3D scaffolds based on collagen/ nanosized hydroxyapatite composite (coll/nanoHA) for bone tissue engineering applications was explored. This approach used ice crystals as templates to produce porous structure without the involvement of organic solvents or any additives during the materials' production.

Methods. Collagen/nanohydroxyapatite aggregated scaffolds (70:30; 50:50; 30:70 mass percentage) were prepared, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as crosslinker agents. The materials were freeze-dried and sterilized with gamma radiation. The samples were analyzed by Scanning Electron Microscopy (SEM), infrared spectroscopy (FT-IR), Hg permeability and cellular viability with osteoblast-like (MG63) by indirect contact test.

Results. SEM analysis revealed that all the scaffolds had high porosity and that the nanohydroxyapatite aggregates were randomly dispersed throughout the materials' pores. FTIR analysis showed the presence of all major peaks related to collagen and hydroxyapatite and also indicated possible interaction between nanoHA aggregates and collagen molecules. The porosity analysis revealed an enhancement on the total area as the percentage of nanoHA increased. On the biocompatibility analyses, all the different compositions of Coll/nanoHA scaffolds showed cellular viability over 80% after 3 days, except the composition 50:50 (m/m%) (Fig. 1).

Conclusion. The obtained scaffolds were highly porous and showed increased surface area as the nanohydroxyapatite concentration enhanced. The materials showed interaction between the organic and inorganic components through the carbonyl groups and behaved as biocompatible with respect to osteoblasts culture.

Keywords. Cryogel, collagen, nanohydroxyapatite, bone regeneration

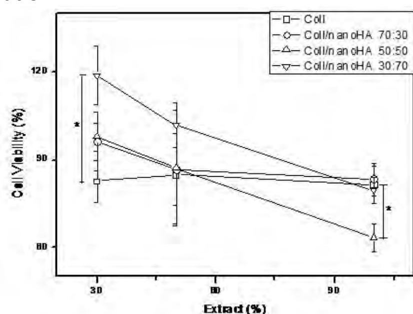

Figure 1: Cellular viability of MG63 with extracts of different Coll/nanoHA scaffolds. *One Way Anova, $p<0.05$ compared to pure collagen.

31. NANOTECHNOLOGY AND REGENERATIVE MEDICINE

Chair: Alexander M. Seifalain
Co-chair: Hossein Ghanbari
Keynote speaker: Alexander M. Seifalian
Organizers: Hossein Ghanbari, Alexander M. Seifalain
Synopsis: Recent innovatory advances in nanotechnology have resulted in the emergence of novel techniques and advanced new nanomaterials with improved properties, capable of being used in several biomedical applications. Merging this advanced technology with other emerging innovatory fields such as stem cell technology and regenerative medicine can be a real breakthrough in current medical practice. Development of advanced diagnostic and therapeutic tools based on these novel technologies hold great promises in overcoming unsolved problems of traditional medicine. Application of nanoparticles or other nanostructures in nanomedicine including cell labelling, advanced imaging tools, and gene and drug delivery systems based on nano-carriers has opened new horizons to the field of regenerative medicine. Creating smart nanotopographic surfaces for tissue engineering by the use of nanotechnology is an interesting field which can be used to control and direct stem cell proliferation and differentiation. The advantage of smart scaffolds with specific nanotopography over other scaffold materials is that their structure is precisely controllable at microscopic and nanoscopic scale level in order to control cell behaviour, proliferation and differentiation. These scaffolds cab be modified using different techniques to enhance their biocompatibility, to tune their biodegradation rate, and to improve their mechanical properties to sustain stresses at a specific site of application. Using nanotechnology, the physicochemical properties of the scaffold materials for various applications in tissue engineering field can be drastically improved. It is known that the nanotopography and other physical characteristics of the scaffolds such as porosity and pore structure, volume and size are responsible to regulate cell function. Highly porous scaffolds offer significant surface area for cell attachments. The key factor responsible for successful cell adhesion, proliferation and differentiation is pore interconnectivity. Good pore interconnectivity provides sustainable environment for a uniform cell distribution within the scaffold and plays an essential role in regulating the diffusion of cell nutrients and waste disposal. These features can be controlled and improved at nano-scale level by the use of advanced nanotechnological techniques. Recently, a novel family of nanocomposite polymers with improved biomechanical and surface properties has been developed for tissue engineering application. This includes a family of biocompatible nanocomposite polymers with tuneable biodegradation rate and porosity for several *in vitro* tissue engineering approaches, and a group of compeletely non-biodegradable scaffolds with smart surfaces for *in vivo* tissue engineering approaches. These nanocomposite polymers offer potential applications in the fields of tissue engineering liver, small intestine, cartilage, nerve conduits, and heart valve.
This symposium aims to bring together all leading experts who are working to apply nanotechnology-offered materials and techniques in the field of tissue engineering and regenerative medicine. This can potentially expand our knowledge and ability to intervene at nano-scale level in order to control cell's behaviour, proliferation and differentiation for tissue engineering and stem cell therapy purposes.

(31.O1) NASAL SEPTUM HEALING PROCESS IS IMPAIRED BY DOUBLE WALL CARBON NANOTUBES IN SHEEP

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1. Inserm U577; 2. CNRS ; 3. CHU

Introduction. Previously, we showed that Biphasic Calcium Phosphate (BCP) implant was able to repair nasal septum in vivo and that Double Wall Carbon Nanotubes (DWNTs) had a deleterious effect on nasal epithelial cells in vitro. Here, we explored whether DWNTs could alter the healing process when nasal septum is being repaired by BCP implant.
Methods. Ten sheep had nasal septum skeleton repaired by two macroporous BCP implant (75% hydroxyapatite and 25% betaTCP) in distinct places separated by 1 cm of normal septum. The posterior implant had previously soaked for 24 hours in a DWNTs solution (25 µg/mL). Both implants were exposed to nasal content on one side through a mucoperichondrial perforation measuring half of their surface. After 45 days, the mean rate of soft tissues covering the perforated side was assessed by imaging software (NIS-Elements AR 3.00). The healing process was assessed by optical microscopy (H.E.S., Movat pentachrome, Masson Trichrome), histomorphometry, immunohistochemistry (cytokeratins 5-6-7-903, lectins UEA-I, MUC5AC) and transmission electronic microscopy. $P<0.05$ was considered statistically significant (Mann-Whitney test).
Results. Two implants without DWNTs were partially excluded vs seven with DWNTs. The mean rates of soft tissues covering the perforated side were 48.9± 24.7% and 82.2±10.4% depending or not on DWNTs presence ($p<10-2$), respectively. Most of the macropores were occupied by connective tissue consisting of fibroblast-like cells surrounded by collagen fibrils and a vascular

network arising from the perichondrium. Perichondrium thickness was higher with both implants compared with normal septum (p<10-5), and with DWNTs presence (p<10-4). Sub-mucosal inflammatory infiltration was evidenced in the presence of DWNTs. The respiratory epithelium remained mainly well differentiated in both cases. The profiles of immunostaining were correlated with the degrees of differentiation by optical and electronic microscopy.

Conclusions. DWNTs seemed to impair the biocompatibility of the implant and its capacity to close septal perforation.

Keywords. Ceramic, carbon nanotube, nanoparticle, nasal septum, healing process

(31.O2) NANOTOPOGRAPHICAL MAINTENANCE OF MULTIPOTENT MESENCHYMAL STEM CELLS

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1. University of Glasgow; 2. University of Southampton

Introduction. The discovery of stem cells has lead to rapid advances in regenerative medicine, a field which aims to utilise the properties of stem cells as a means to repair or replace damaged tissue. Currently however, a method for providing large quantities of high quality autologous stem cells for downstream use in regenerative medicine has yet to be established. In vivo, the stem cell niche provides appropriate cues which help to regulate stem cell fate. By mimicking these cues in vitro; stiffness, topography, and chemistry have all been shown to affect many biological processes including adhesion, proliferation and differentiation. In this study, a novel nanopit topography was investigated relative to a planar control for its affects on maintenance and growth of Mesenchymal stem cells (MSCs) in vitro.

Methods. We have examined using immunofluorescence, microarray analysis and real-time qPCR the effect of discrete nanotopographies on maintenance of human MSCs multipotency. Furthermore, we applied an innovative metabolomic approach to determine the metabolic state of MSCs under both control and test nanotopographies. Critically, no chemical supplements were used in these studies alleviating issues of non-FDA approved and presence of animal-derived products not safe for use in humans.

Results. We have identified a novel nanotopography, which in the absence of chemical cues, provides a substrate conducive to maintenance of MSCs. Furthermore, the stem cells were shown to continue to undergo proliferation over a period of two months allowing for critical growth of the stem cell population in vitro.

Conclusion. We demonstrate the potential of topographically patterned substrates for regenerative medicine providing chemical free, inexpensive and easily reproducible platforms for the control of MSCs and the wider stem cell population.

Acknowledgements. We thank Kate Murawski for providing the mesenchymal stem cells. RJ McMurray is funded by a University of Glasgow Lord Kelvin/Adam Smith Scholarship.

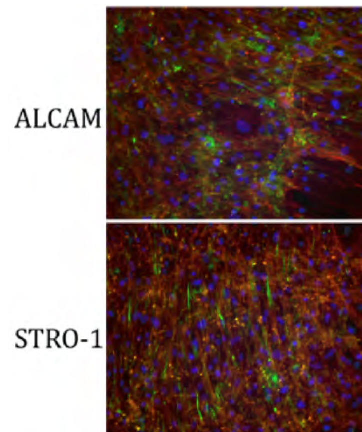

Fig 1. MSCs cultured on the novel nanotopography show continued expression of Stro-1 and ALCAM, stem cell markers, at 8 weeks.

(31.O3) NANOCERIA PROTECTION AGAINST OXIDATIVE DAMAGE IN CARDIAC PROGENITOR CELLS

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1. National Institute for Materials Science (NIMS); 2. University of Rome Tor Vergata

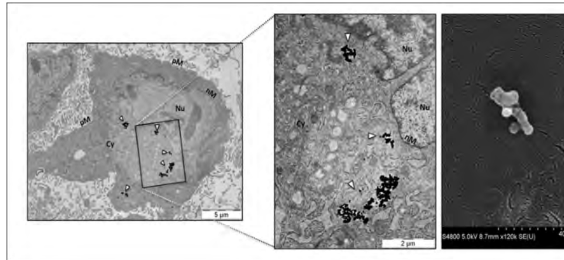
Over the last few years, nanotechnology has significantly spread over the field of regenerative medicine. The extensive usage of nano components, particularly in combination with polymeric scaffolds for TE, has opened to new generations of nano-biomaterials for medical applications. Consequently, studying how cells interact with the composite scaffold and if embedded nano-components are internalized and exert any direct effect on cell behavior, is crucial for obtaining biomaterials with predictable and promising properties. It has been reported that ceria nanoparticles (CeO2) reduce the cytotoxic effects of intracellular oxidative stress conditions via changes of the oxidation state Ce4+ è Ce3+. Recently, Cerium oxide nanoparticles embedded into PLGA scaffolds have been demonstrated to favor and direct CPCs adhesion and proliferation, as compared with PLGA alone, as a result of the modulation in scaffold nano-roughness and surface functionality.

Here we studied Linneg/Sca-1pos Cardiac Progenitor Cells (CPCs) response to CeO2, in terms of nanoparticle cyto-compatibility, internalization route, and effect on cell morphology and differentiation. In particular, the possibility that CeO2 could confer protection to cells from oxidative stress resulting from Reactive Oxygen Species (ROS) production was investigated. For this purpose, CPCs were cultivated in the presence of various concentrations of CeO2 having mean particle size of 5-8 nm. After 24 hours incubation, the CeO2containing medium was replaced by fresh medium and H2O2 was administered to induce oxidative stress, followed by analysis up to 7DIV.

The study showed that 24 hour exposure to 10, 25, and 50 □g ml-1 of CeO2 did not affect CPC survival and function. On the contrary, all concentrations were effective in protecting CPCs from H2O2-induced damage in the long run, which could be ascribed to the efficient antioxidant mechanism elicited by ceria nanoparticles. In

conclusion, these results undisclose enormous possibilities for the use of nanoceria in cardiac TE therapies.

Keywords. ceria, cardiac progenitor cells, antioxidative stress, ROS



(31.O4) COMPARATIVE STUDY ON THE METHODS FOR DETERMINATION OF BONE YOUNG'S MODULUS USING AFM NANOINDENTATION

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Progression of nanoscale material testing techniques has produced new perspectives for studying the effect of musculoskeletal diseases on the weight-bearing tissues. Growing understanding of small scale mechanical behavior enhances prevention and treatment of osteoporosis and osteoarthrosis, as well as engineering of artificial prosthesis.

Nanoscale indentation testing may be conducted with an atomic force microscope (AFM). Several different calibration and data analysis methods have been introduced for the AFM-nanoindentation. ISO-standard 14577 gives additional boundary values for the inspection. The main goal of this paper is to review the most used calibration methods, especially for analyzing the cantilever spring constant and tip contact area. A particular interest on the testing of bone microstructure is taken into account.

Also, this paper describes the innitial method of analyzing bones structures using nanoindentation with the Atomic Force Microscope, developed by the research team and relates the issues and problems encountered in the validation process.

Keywords. Atomic force microscope, nanoindentation, Young's modulus, trabecular bone, cortical bone, spring constant

(31.O6) PERSPECTIVE OF ELECTROSPINNING TECHNOLOGY FOR NANOMEDICINE

Kim GM (1)

1. CEIT & TECNUN (University of Navarra)

Burgeoning interest in the medical applications of nanotechnology has generated a new field so-called NanoMedicine. NanoMedicine may have extraordinary, far-reaching and highly specific medical intervention at the molecular scale for the diagnosis, treatment and prevention of disease and traumatic injury, and ultimately for the improvement and preservation of human health. Research in the NanoMedicine includes the development of diagnostics for rapid and precise monitoring, targeted and localized drug delivery, scaffolds design for tissue engineering (TE) and regenerative medicine and gene / cell delivery systems.

This work demonstrates the biocompatible and/or biodegradable 3-dimensional (3D) fibrous polymeric scaffolds for NanoMedicine, which are manufactured by means of an electrospinning (ES). ES is a well recognized nanotechnology to produce synthetic nanofibrous structures. The resulting structures have morphology and diameter for fibers in a range comparable with those found in the extracellular matrix of human tissues. Therefore, nanofibrous scaffolds are intended to provide the appropriate environments for cell attachment, migration, proliferation and differentiation when compared with traditional scaffolds. In addition, the process versatility and the high specific surface area of nanofibers facilitate their use as immobilization and release systems for biological active agents, e. g. , drugs, DNA, RNA as well as enzymes. Based on the 3D multifunctional fibrous scaffolds we will present state-of the art strategy for reconstruction of neo-tissues and -organs, e. g. , skin, nose, ear, blood vessels, nerve guide systems, bladder etc. In addition, the implantable scaffold-guided targeting and localized delivery systems for bioactive agents will also be demonstrated for the future nanomedical therapy.

The NanoMedicine is still in its infancy, however, in the near future its promise should be fulfilled to dramatically improve the quality of human life and even to extend it.

Keywords. Nanomedicine, electrospinning, nanofiber, scaffold

(31.O7) A GENE-ACTIVATED MATRIX TO ENHANCE ANGIOGENESIS DURING DERMAL REGENERATION

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The use of scaffolds in skin tissue engineering is accompanied with low regeneration rates and high risk of infection. In this study, we activated an FDA-approved collagen scaffold for dermal regeneration by incorporation of copolymer-protected gene vectors (COPROGs) to induce a temporary release of VEGF. In vitro results show that the presence of COPROGs did not affect the distribution, attachment, proliferation and viability of cells in the scaffold. A transient release of VEGF was observed for up to 3 weeks. Moreover a high amount of VEGF was found in the cells and associated with the scaffold. In a full skin defect model in nude mice, VEGF levels were significantly increased compared to controls in VEGF gene-activated scaffolds 14 d after implantation, but not in skin from the wound edge. Results showed an increased amount of non-adherent cells, especially erythrocytes, and von Willebrandt factor (vWF) and a yellow red appearance of gene activated scaffolds in relation to controls. This suggests the presence of leaky vessels. In this work we show that the bioactivation of collagen scaffolds with COPROGs presents a new technology that allows a local release of therapeutic proteins thus enhancing the regenerative potential in vivo.

Biomaterials. 2011 Mar;32(7):1996-2003. Epub 2010 Dec 14.

Keywords. non-viral gene vector, gene therapy, tissue engineering, angiogenesis

(31.O8) DEVELOPMENT OF CHITOSAN-BASED DELIVERY SYSTEM TO RECRUIT MESENCHYMAL STEM CELLS

Goncalves R (1), Molinos M (1), Pereira AC (1), Antunes J (1), Barbosa M (1)

1. INEB

Introduction. Human mesenchymal stem cells (MSC) have been used in regenerative therapies due to its ability to differentiate into multiple lineages, as well as their immune-suppressive potential. MSC recruitment to specific injury sites may be modulated using biomaterials-based systems.

Methods. By layer-by-layer technique, Chitosan (Ch) and Poly-glutamic acid (PGA) were assembled over Ch ultra-thin films into 2D structures, by electrostatic interaction at pH 5. The chemokine, stromal-derived factor-1 (SDF-1) was combined within these polyelectrolyte complexes (PECs) and delivered up to 3 days. This system was characterized using Imaging Ellipsometry, protein radiolabeling and ELISA. In vitro, delivered SDF-1 was able to recruit MSC.

Results. Soluble SDF-1 can be successfully combined into Ch/PGA PECs without affecting PECs assembly, with the maximum concentration of 1. 7-2. 3 ng/cm2. The PECs thickness ranges from 120 to 150 nm with and without SDF-1. Using these complexes, SDF-1 is delivered during 72h, with a burst in the first 6h (50-60% of the chemokine is released); nevertheless, after 72h, 17 to 27% of SDF-1 was retained into PECs. The SDF-1 delivered by these systems increase specific MSC migration 8 to 12 times.

Conclusion. Ch/PGA PECs can constitute chemokine reservoirs and efficiently recruit MSC. This strategy can be applied in vivo to potentiate MSC migration to an injury site.

Acknowledgments. This work was supported by DISC Regeneration project (FP7, Project no. CP -IP 213904)

Keywords. polyelectrolyte complexes, cell recruitment, mesenchymal stem cells

(31.O9) EFFECT OF PDMS NANOPATTERNED SUBSTRATES ON EMBRYONIC STEM CELLS DIFFERENTIATION INTO NEURONAL LINEAGE

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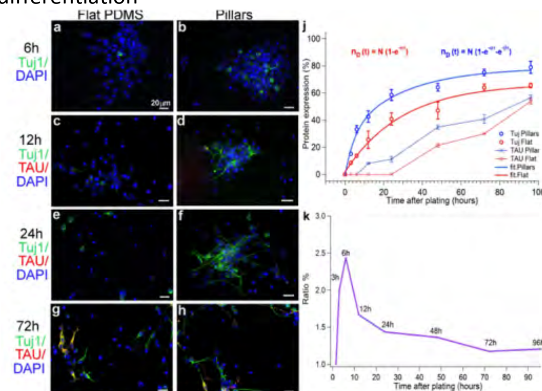
1. IOM-CNR laboratorio TASC; 2. SISSA

Embryonic stem (ES) cell differentiation in specific cell lineage is a major issue in cell biology particularly in regenerative medicine. Differentiation is usually achieved by using biochemical factors which concentration and mechanism are not completely understood and with sides effects difficult to overcome. Using a substrate which mimics brain extracellular matrix it could be possible to induce ES-cells differentiation into neurons without adding any biochemical factors. Therefore, we produced patterns in polydimethylsiloxane (PDMS) consisting of groove and pillar arrays of sub-micrometric lateral resolution as substrates for cell cultures. Neuronal precursors from ES cells were obtained using a Stromal Cell-Derived Inducing Activity protocol and we analyzed the effect of different nanostructures on differentiation into neuronal lineage. Neuronal precursors adhered on PDMS more effectively than on glass coverslips. After 48 hours of culture on PDMS pillars with a 500nm period, neuronal differentiation increased and almost doubled with respect to flat PDMS substrates. Neuronal yield was enhanced by increasing pillars height from 35 to 400 nm. With pillars, 500nm period and 360nm height, the neuronal yield reached almost the 80% 96 hours after plating (fig1). However the largest differentiation enhancement of pillars over flat PDMS was observed during the first 6 hours of culture (fig 1k). These shown results that PDMS nanopillars accelerate ES cells differentiation into neurons.

Keywords. nanotopography, nanotechnology, regenerative medicine, embryonic stem cells, neuronal differentiation



(31.O10) NANOMAGNETIC GENE TRANSFECTION: THE EFFICIENCY OF OSCILLATING MAGNET ARRAYS AND NANOPARTICLES FOR GENE DELIVERY

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Introduction. Nanomagnetic gene transfection has been effectively used with both viral and non-viral vector systems. In these systems plasmid DNA or siRNA is attached to magnetic nanoparticles and incubated with cells while an oscillating magnet below the surface of the cell culture pulls the particles into contact with the cell membrane stimulating endocytosis. In this study human osteoblastic/ osteosarcoma cell line, MG63, has been used to demonstrate the efficiency of the nanomagnetic gene transfection using oscillating magnet arrays, based on its extensive use for in vitro research on bone cancers, tissue engineering and regenerative medicine.

Methods. MG63 cells were transfected with nTMag and PolyMag nanoparticles coated with pEGFP green fluorescent protein reporter construct using oscillating magnet arrays (magnefect-nano system) for 30 min. The cationic lipid transfection agent Lipofectamine 2000TM was used for comparison.

Results. At 48hr post-transfection, fluorescent microscopy and Fluorescence Activated Cell Sorting (FACS) analysis showed that the magnefect-nano oscillating system enhances overall in vitro transfection levels in MG63 cells in comparison with the Lipofectamine 2000TM ($p<0.001$) (Figure 1). Transfection efficiency was up to 49% and cell viability was largely unaffected.

Conclusions. The oscillating magnetic field of the magnefect-nano system outperforms the best currently available cationic lipid-based agent. Furthermore, it retains the advantages of magnetic transfection such as short transfection times, lower reagent concentrations and little or no cytotoxicity. Magnetic nanoparticle-based gene transfection shows promising results for non-viral gene delivery for both in vitro and in vivo applications.

Acknowledgements. A Fouriki thanks Keele University, nanoTherics Ltd. and EPSRC for funding support.

Keywords. nanomagnetic gene transfection, nanoparticles, gene therapy

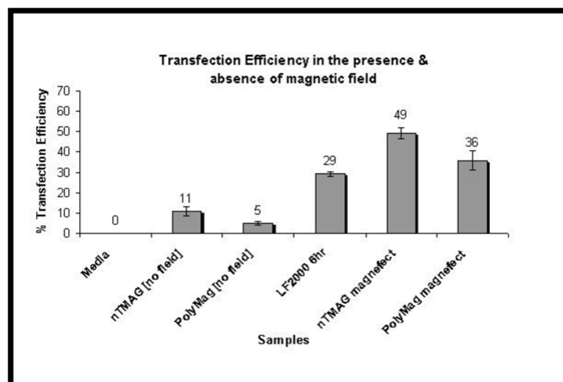

Figure 1: FACS results showing transfection efficiency of the tested groups. N= 3 per group.

(31.O11) MICROFLUIDIC PLATFORM FOR SIMULTANEOUS GENERATION OF FOUR INDEPENDENT GRADIENTS: TOWARDS HIGH THROUGHPUT SCREENING IN TISSUE ENGINEERING

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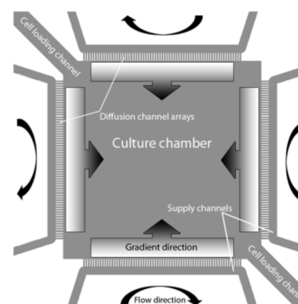
Current strategies in bone tissue engineering are based on combinations of a biomaterial carrier with osteogenic cells and/or growth factors. The use of growth factors is associated with drawbacks of limited availability, biological instability and high costs. To overcome these drawbacks and accommodate the increasing need for repair of damaged and degraded tissues, alternative approaches are required. One interesting class of simple compounds are bioinorganics, such as strontium and zinc. Some of these compounds are present as trace elements in the human body and have shown to be involved in various biological processes. Although bioinorganics play an important role, their application in bone regeneration has not yet been systematically studied. We hypothesize that mixtures of elements exist that, at low concentrations, can be applied in bone regenerative strategies. Since there are over seventy known trace elements, the systematic investigation of thousands of different combinations needs an approach different from classical cell culture. Therefore, we propose a new microfluidic cell-culture platform that allows for high-throughput live imaging and analysis.

The platform consists of a microfluidic chip (schematic image) that is built of a square chamber, in which cells are cultured, with four surrounding independent concentration gradient generators and two cell-loading channels. Supply channels, on the sides of the chamber, are connected to the chamber through arrays of smaller channels that have a low µm2 cross-section. This allows for the diffusion-based delivery of chemicals introduced into the supply channels. As a result, four perpendicularly overlapping concentration gradients form across the chamber. Experiments using fluorescent dies showed that stable gradients were formed after roughly 10 minutes. To investigate cell viability, the platform was used for time-lapse imaging of the proliferation of C2C12 myoblasts over three days. Functional experiments on proliferation and differentiation of cells under influence of different compounds are ongoing.



(31.O12) MECHANOTRANSDUCTION AND SKELETAL STEM CELL DIFFERENTIATION IN RESPONSE TO BIOACTIVE NANOTOPOGRAPHY

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Abstract. A detailed understanding of the effects of nanotopography on cell adhesion and morphology and the consequences of those changes in the nucleus and gene expression will be presented and may offer new approaches to modulating and controlling, stem cell differentiation. This information will benefit materials scientists working on next-generation tissue engineering scaffolds.

Stro-1-selected skeletal stem cells were used to study mechanotransductive events. Nanopits with a controlled degree of disorder, were compared to planar and ordered nanopit controls known to change stem cell fate (Dalby et al. , Nature Materials, 2007). To examine mechanotransductive events, cell, nucleus and adhesion morphology were quantified and phenotypic changes investigated. Nucleus organisation was investigated by lamin nucleoskeletal staining and chromosome territory analysis. Orbitrap Mass Spectroscopy was used to study nanotopography derived changes in metabolism and this was coupled to microarray to study biochemical events. Significant changes in cell adhesion, nucleus and lamin morphologies in response to the different nanotopographical substrates were observed. These changes relate to changes in packing of chromosome territories within the interphase nucleus leading to changes in transcription factor activity and phenotypical signalling. We found that these changes in nucleus oranisation and differentiation can be reversed by reducing adhesion size (siRNA vinculin / competitive

integrin binding) or depleting intermediate filaments (hence reducing tension). Furthermore, microarray data shows interplay between canonical, biochemical signalling and cell phenotype and this is compared to the cell matabolome.

Different nanotopographies result in different morphological changes in the cyto- and nucleoskeleton as well as the chromosomes themselves. We propose that both biochemical) and mechanical signalling is important in tuning stem cell fate driving targeted phenotype development. These studies provide us with a better understanding of cell-surface interaction and new insights of how to control cell differentiation providing a basis for rational biomaterials design.

Acknowledgements. BBSRC grant BBG0088681.

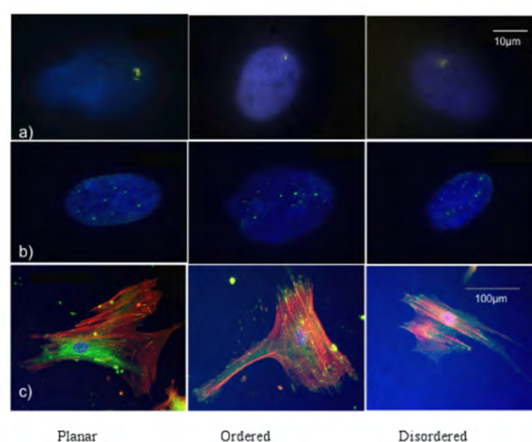

Fig. 1: Effect of different nanotopography patterns on Chromosome 1 territory (FISH) (a) Lamin A staining shows differences in the organisation of the interphase nucleus (b) cell attachment /morphology using Actin(red filaments)/Vinculin (green adhesions) /DAPI (nucleus) staining(c).

(31.O13) IN-SITU TISSUE ENGINEERING HEART VALVE: THE ROLE OF NANOTECHNOLOGY AND REGENERATIVE CAPACITY OF CIRCULATING PROGENITOR STEM CELLS

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Introduction. In cardiovascular system, endothelial progenitor cells (EPCs) are cells with known regenerative capacity which potentially can be used in developing partial or complete tissue engineering devices such as heart valves. An advanced nanocomposite material with specific nanotopography and surface characteristics has been developed for tissue engineering applications, based on polyhedral oligomeric silsesquioxane nanoparticle and poly(carbonate-urea)urethane (POSS-PCU). In this study we assessed its potential application in in-situ tissue engineering using the heart valve prototypes made with this nanocomposite and human EPC culture in a pulsatile bioreactor system.

Materials and methods. Heart valve prototypes were fabricated using 18% POSS-PCU polymer and dip-coating technique. The leaflet's surface was further biofunctionalised using GRGDG peptide motives. Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque (GE Healthcare, Sweden) centrifugation and then EPCs were purified using MACS isolation technique after labelling with CD34 microbeads (Meltenyi Biotec, Germany). The valves were mounted in the bioreactor and were incubated with EPCs for 24 hours, in a pulsatile circulatory bioreactor system. The valve leaflets were studied under light, confocal and scanning electron microscopy for cell attachment and fluorescence-activated cell sorting (FACS) technique was used to analyze cell surface markers.

Results. Characterization of POSS-PCU revealed specific surface nanotopography, morphology and chemistry, achieved mainly by self assembly of the POSS nanoparticles on the surface. Surface studies also proved the presence of functional peptides on the surface. EPCs were attached to the valve leaflets made from POSS-PCU nanocomposite after 24 hour of dynamic culture. The cells revealed endothelial progenitor cell surface markers, being positive for CD34, CD133 and VEGFR2 surface markers.

Conclusions. Under pulsatile flow conditions, human peripheral blood EPCs were attached onto the heart valve leaflets made from POSS-PCU nanocomposite, with biofunctionalised surfaces. These results support the potential use of this material in *in-situ* tissue engineering heart valves.

(31.O14) HIGHLY MAGNETIC AND FLEXIBLE ACTUATOR FOR ARTIFICIAL MUSCLE APPLICATIONS

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The combination of force and flexibility enables virtually all body movements in living organisms. Presently used technical machines in contrast, are based on rigid, linear or circular geometries. As a possible alternative, magnetic elastomers can be realized through dispersion of micro- or nanoparticles in polymer matrices and have attracted significant interest as soft actuators in artificial organs, implants, and devices for controlled drug delivery. At present, magnetic particle loss and limited actuator strength have restricted the use of such materials to niche applications.

This contribution reports on the direct incorporation of metal nanoparticles into the backbone of a hydrogel and application as an ultra-flexible, yet strong magnetic actuator [1]. This magnetic hydrogel could be used in tissue engineering for designing artificial muscles. Flame spray synthesized carbon coated cobalt nanoparticles [2] were directly incorporated into the polymerization of 2-hydroxyethyl methacrylate (HEMA). To overcome the problem of inhomogeneously dispersed nanoparticles in the polymer, the carbon shell of the particles was covalently functionalized [3] with an organic component similar to the monomer. Later favored the in-situ polymerization of the magnetic particles with the monomer and resulted in the formation of a highly

magnetic, mechanical stable and homogenously dispersed polymer. Since pure metals have a far higher magnetic behavior than oxides, the resulting increased force/volume ratio afforded significantly stronger magnetic actuators with high mechanical stability, elasticity, and shape memory effect. Depending on the final shape, different polymer blocks (60 wt% nanoparticles) could be produced offering an alternative approach to flexible and magnetic actuators towards artificial muscles.

References:

1) R. Fuhrer, et al., Small, 2009, 5, 383-8
2) E.K. Athanassiou, et al., Nanotechnolgy, 2006, 17, 1668-73
3) R.N. Grass, et al., Angew. Chem. Int. Ed., 2007, 46, 4909-12

Keywords. actuators, hydrogel, nanocomposites, magnetic materials

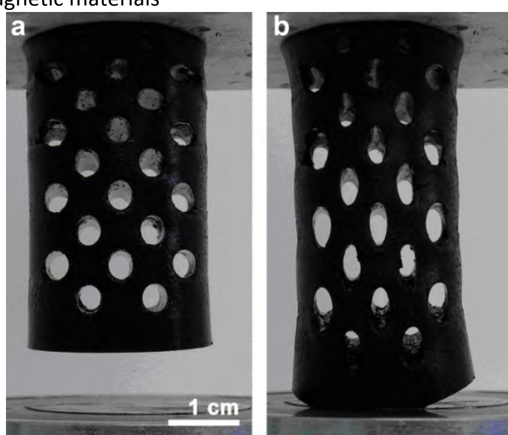

Figure 1: Photo of as prepared soft magnetic hydrogel (a). By applying an external magnetic field the polymer can contract or elongate making it appropriate in application such as for magnetic actuator or even artificial muscles (b).

(31.P1) PLASTICITY CERAMIC COATING FOR THE BIOMEDICAL

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Introduction. The general problem of applying of ceramic coating on metal is low adhesion of layers. Promising approach to increase of implant reliability is creation of composition materials of HA-polymer type. Composition calcium – phosphate chitosan coating (Ca- P/Ch) applied with method of electrochemical precipitation is developed.

Methods. Powders and coating have been analyzed by means of X-ray phase analysis, sorbtomer Tristar 3020 and SEM Philips 515.

Results. According to results of electron microscopy the obtained samples are powder-like mixtures with inclusion of granule with 10 – 100 nm grains. Obtained biological hydroxyapatite manifests high degree of similarity with human bone and can be used as material for filling of bone defects as well as for obtaining of nanostructured Ca/P coatings. CaP-Ch coating is formed from isolated pores with spherical form of various diameter with sizes from 1 up to 20 µm. Pores have closed character and developed spatial structure. Micropores are characterized with developed inner surface with through nanopores with sizes from 20 up to 150 nm, reaching metallic substrate. Chitosan and HA are uniformly distributed in forming composite material (Fig. 2). Data of X-ray -phase analysis show that coating is mixture of amorphous phase and nanodimension monoclinic structure of hydroxyapatite. Defining of elasticity and strength of coating applied of a metallic plane at its bending by the GOST 6806-73 method was carried out on a «Izgib» device. Investigations showed that composite coating are not delaminated at bending of substrate on the angle more than 900.

Conclusions. Thus, result of this work is development of a plasticity nanocomposite ceramic coating that essentially extends field of its application.

Keywords. ceramic, hydroxyapatite, chitosan

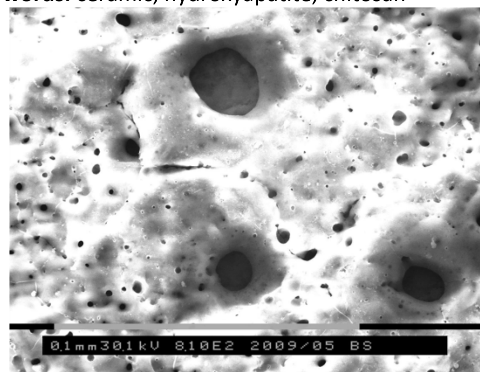

Figure 2 – Microphotographs of Ca-P/Ch coating

(31.P2) UP-REGULATION OF GLYCOSAMINOGLYCANS IN THE INTERVERTEBRAL DISC USING FUNCTIONALISED HYALURONAN PARTICLES

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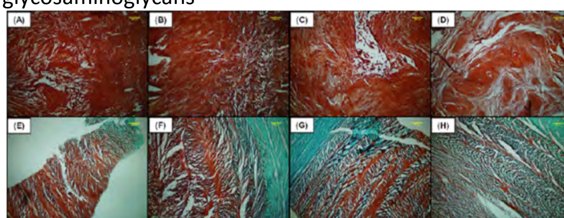
The degeneration of the intervertebral disc (IVD) is marked by loss of water due to a decrease of glycosaminoglycans (GAGs) content in the tissue. Glucuronyltransferase I (GT-I) and xylosyltransferase I (XT-I), two key enzymes involved in the synthesis of GAGs, have been shown to be down-regulated in degenerated cartilage1. However, the role of these enzymes in GAG synthesis has not been reported in ageing intervertebral discs. The hypothesis tested herein is that hyaluronan (HA) nanoparticle mediated delivery of GT-I and XT-I plasmids would increase the GAG production by IVD cells. The specific objectives of this study were (1) to identify an age-associated down-regulation of GT-I and XT-I in bovine IVD tissue and (2) to reverse this down-regulation by pGT-I and pXT-I delivery. IVD from six month, 1, 2 and 8 year-old bovine tails were processed for Safranin O/Fast green staining, immunostaining of XT-I and GT-I, and biochemical and HPLC analyses. IVD cells and adipose derived stem cells (50,000cells/cm²) were treated 24h after seeding with pXT-I, pGT-I and pLuc (control) pDNA HA nanoparticle4 complexes. At days 2 and 4, cell viability and proliferation, GAG quantification, gene expression, and enzyme activity analyses were evaluated. A significant decrease of sulfated GAGs associated with a

general disorganization of the tissue was observed in bovine IVD correlated with aging (6 month-old IVD 6092±393µgGAG/µgDNA - 8 year-old IVD 1033±530µgGAG/µgDNA) (Figure 1). After 2 and 4 days of transfection with HA particle complexes, over 80% of viability and luciferase activity were noted across the treatment groups. The delivery of GT-I resulted in a 440±178 fold up-regulation of this gene. Down-regulation of GAGs associated with a change in XT-I and GT-I expression was observed within ageing IVD. The up-regulation of GAGs by delivery of XT-I and GT-I showed promising results for reversing this phenomenon.

Reference. 1 Venkatesan, N. , et al. , FASEB J, 2009. 23(3):p. 813-22

Acknowledgments. Science Foundation Ireland, Research Frontiers Programme (07/RFP/ENMF482), 07/RFP/ENMF482 STTF 09, AO Foundation (S-09-7P)

Keywords. intervertebral disc, gene therapy, glycosaminoglycans



(31.P3) SYNTHESIS OF BIOACTIVE TITANIUM MATERIAL FOR THE LOCAL DELIVERY OF GENTAMICIN SULPHATE AND IN VITRO ACTIVITY EVALUATION

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Introduction. Controlled release technology overcomes the drawbacks of traditional drug dosage form, and offer more effective methods to optimize antibiotic dosage deliver to specific sites or prolong delivery duration. By systemic therapy, antibiotics may not be delivered efficiently and adequately without side effects. In this project are developed biomaterials allowing a local release, over an adjustable period of time, of an antibiotic (Gentamicin sulphate, GS) fixed covalently on the surface of the latter thanks to chemical anchoring of nanoparticles functionalized by the antibiotic. A cleavage of the nanoparticle-antibiotic bond, activated by a pH decrease, induces a controlled release of the bioactive molecule.

Methods. α-norbornenyl poly(ethylene oxide) macromonomer was synthesized by anionic ring-opening polymerization of ethylene oxide and was ω-functionalized with carboxylic acid or GS linked by an imine bond. Nanoparticles were obtained by Ring-Opening Metathesis CoPolymerization (ROMP) of norbornene with macromonomers in dispersed medium and were grafted onto titanium discs functionalized with amine groups using aminosilane.

Minimum Inhibitory Concentration (MIC) measurements were carried out after GS release at acidic pH.

Results. Nanoparticles were synthesized with total conversions allowing a quantitative determination of the GS concentration. The grafting was controlled by Scanning Electronic Microscopy allowing estimating the GS surface density to 0.08 µg/cm².

The GS-delivery efficiency was proved by bacterial inhibition tests using staphylococcus epidermidis. MIC of GS was compared with MIC of buffer solutions in which GS was released from GS-macromonomers, functionalized nanoparticles and grafted discs. A delivery only at acidic pH was observed.

Conclusion. The synthesis of bioactive biomaterials and their characterization were presented. We equally demonstrated the in vitro activity of such materials. In vivo experiments on rabbits are now in progress.

Acknowledgments. The French “Agence Nationale de la Recherche” (ANR) and the GIS “Advanced Materials in Aquitaine” (AMA) are thanked for financial support.

Keywords. drug delivery system; nanoparticles; biomaterial; pH-controlled release

Schematic of the bioactive material and the GS release in acidic pH condition

(31.P4) EVALUATION OF IN VITRO BIOACTIVITY OF BIOACTIVE GLASS MICRO AND NANO SIZED PARTICLES

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1. Federal University of Minas Gerais

Bioactive glasses (BG) bond to bone by forming a hydroxyapatite (HA) layer in vivo. In solution, the surface of BG undergoes a time-dependent modification. The formation of a HA layer in vitro on a material surface is believed to indicate its bioactive potential in vivo. Parameters such as surface charge, composition, structure, and morphology will be important in the formation of the Ca-P layer as well as in the interaction between the material surface and the surrounding medium, proteins and cells. The BG surface zeta potential variations in an electrolyte solution correspond to, and may directly influence, Ca-P layer formation. In this study, the bioactivity of BG micro and nano sized particles was investigated and correlated with the time-dependent variations in zeta potential of BG immersed in SBF solution. Fourier Transformed Infrared Spectroscopy, X-Ray Diffraction and Scanning Electron Microscopy analysis were used to confirm the formation of HA layer. The cell viability by MTT assay and alkaline phosphatase was used to evaluate the behavior of particles in direct contact with osteoblast cells. The zeta potential variations were larger and occurred faster for nanoparticles (NP), suggesting that the kinetic of HA formation in particles is influenced by the particle size. The NP presented significant increase in cell viability when compared to microparticles (MP). These results support the hypothesis that BGNP are more bioactive than BGMP.

Keywords. Bioactive glass, Nanoparticles, Hydroxyapatite layer, Bioactivity

(31.P5) SYNTHESIS AND CHARACTERIZATION OF BIOACTIVE GLASS NANOPARTICLES OBTAINED BY MODIFIED STOBER METHOD

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1. Federal University of Minas Gerais

Since developed by Hench as a promising bone repairing material with high bioactivity and biocompatibility, bioactive glass has attracted extensive studies. Various investigations were directed to obtain sol–gel derived bioactive glasses as bulk, powder or porous scaffolds. Nanotechnology gives a new strategy to develop bioactive glasses. Nanoscaled biomaterials exhibited an enhanced biocompatibility and bioactivity. Stober developed a method for the synthesis of spherical and monodisperse silica nanoparticles from aqueous alcohol solutions of silicon alkoxides in the presence of ammonia as a catalyst. In this work a modified Stober Method was used in order to get bioactive glass nanoparticles (BGNP) with dispersion capability and bioactivity. The size, composition, shape and dispersion capability of the obtained particles were studied by Fourier Transformed Infrared Spectroscopy, X-Ray Diffraction, Scanning Electron Microscopy, Nitrogen Adsorption Analysis, and Dynamic Light Scattering. The BGNP obtained have spherical shape and medium size of 20 nm, as prepared, and 90 nm, after heat treatment. They present good dispersion ability in polar solvents. A significant increase in cell viability was observed when osteoblast cells were cultured in the presence of nanoparticles when compared to microparticles, indicating that BGNP could offer superior performance for developing bone tissue engineering scaffolds.

Keywords. Bioactive glass, Nanoparticles, Stober Method, Size measurement

(31.P6) SILK NANOFIBERS AS BIOMATERIAL FOR CORNEAL REGENERATIVE MEDICINE

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Introduction. Silk is a natural protein fiber produced by many insects and arachnoid species. Silk fiber, as typified in a fiber derived from silk worm, Bombyx mori, has been used as a suture in medical field for a long time. These days, research has being actively conducted into silk as biomaterial, and not only fibroin but also sericin and other silk proteins, e. g. hornet and spider silk, have been drawing great attention. In this study, several types of silk protein were processed into a nanofiber and a feasibility study of silk nanofibers as biomaterial for corneal regeneration was carried out.

Materials and methods. Silk fibroin and sericin derived from *Bombyx mori* and hornet silk were provided from National Institute of Agrobiological Sciences in Japan. To prepare the solutions for electrospinning, fibroin and hornet silk were dissolved in HFIP (hexafluoro-2-propanol) and sericin was dissolved in DMSO (dimethyl sulfoxide) at several concentrations. The electrospinning experiment was performed using a simple handmade device.

Results. Fibroin was readily processed into nanofibers as reported by many researchers. In our study, a nanofiber with a diameter of 121 nm was deposited on a flat aluminum foil when a HFIP solution with a concentration of 2wt% was involved in. Standard deviation of diameters of the fibers was 14 nm and it means that relatively uniform fibers were produced at the condition. However, a solution with a concentration of 1. 7wt% or less produced only beaded fibers and particles. Results of electrospinning of sericin and hornet silk and also cell experiments on the nanofibers will be reported.

Acknowledgements. Generous financial support from AFFC, Grants-in-Aid for Scientific Research (B) of MEXT, Challenging Exploratory Research of JSPS, Management Expenses Grants of NIMS, and CREST of JST are gratefully acknowledged.

Keywords. Silk, nanofiber, cornea

(31.P7) FIBRONECTIN FRAGMENTS-FUNCTIONALIZATION INCREASES BINDING AND UPTAKE OF NANOMETER-SIZED MICELLES

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Introduction. An ideal carrier for drug or gene delivery should be nanometer sized and should show, as viruses, a high efficiency to cross cell membranes. Viruses first bind to cell surface proteins, carbohydrates and lipids, leading to activation of several signalling pathways while the cells respond by internalizing the virus using an endocytic mechanisms of the cell. In this context, integrins are involved in the binding of different virus types, among them reo- and adeno-viruses. Fibronectin is an extracellular matrix glycoprotein that binds to different integrins, promoting integrin endocytosis and recycling. Taking advantage from this biological process we sought

to develop novel micelles, easily internalizable by cells and with a high capacity to cross cell membranes.

Material and Methods. Fluorophore-labeled poly(ethylene glycol-bl-propylene sulfide) block copolymers with thiol-reactive pyridyl disulfide groups were functionalized with recombinant fibronectin fragments to form funcitonalized micelles with a diameter of 50-80 nm. The binding and uptake by different cell types were investigated by using flow-cytometry and confocal microscopy.

Results. When NIH3T3 or human mesenchymal stem cells were treated with either functionalized or unfunctionalized micelles a higher internalization of fibronectin fragments-functionalized micelles was observed, thus confirming the potential of integrin targeting to increase micelle uptake. Moreover, the involvement of endocytotic pathways was demonstrated by the inhibition of uptake at 4°C and by confocal microscopy and using a blocking antibody. Finally, absence of toxicity was confirmed by CyQuant assay.

Conclusion. In this work we explored the potential of integrin-targeting by using fibronectin fragments to increase the uptake of nanometer-sized micelles. The use of facile chemistry to conjugate recombinant fibronectin fragments to poly(ethylene glycol-bl-propylene sulfide) block copolymers and the strong binding of fibronectin fragments to integrins makes this approach an interesting tool in regenerative medicine and drug delivery.

(31.P8) PREPARATION OF CYCLOSPORIN A-LOADED NANOFIBERS AND THEIR USE FOR SUPPRESSION OF T CELL FUNCTION IN VITRO AND IN VIVO

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Introduction. Nanofibers have turned out as convenient and perspective carriers of diverse drugs for their local administration. One of the possible use of nanofibers is their application for a local suppression of harmfull inflammatory reactions.

Methods. Nanofibers with a defined content of immunosuppresive drug cyclosporin A (Cys A) were prepared from a polymer poly(L-lactid) (PLA) by an original needleless electrospun technology. The kinetic of Cys A release was determined in aqueous solutions (culture medium) or in vivo after application on skin allografts. The pharmacological activity of Cys A released from nanofibers was assessed by its effect on T cell proliferation and IL-2 production. The ability of Cys A-loaded nanofibers to inhibit a local inflammatory reaction was determined in a model of skin grafting or after mechanical ocular damage.

Results. Incorporation of Cys A into polymer PLA used for nanofiber fabrication had not effect of diameter size, density, porosity and architecture of nanofibers. Analysis of drug release behaviour showed that a considerable amount of Cys A was released within the first 12 h after soaking of nanofibers into medium, but a significant amount of the drug was retained and was released for more than 4 days. Adding of Cys A-loaded nanofibers into cultures of concanavalin A-activated spleen cells significantly inhibited T cell proliferation and IL-2 production. On the contrary, production of nitric oxide by activated macrophages or growth of non-T cells was not inhibited in the presence of Cys A-loaded nanofibers. Covering of inflammatory sides with Cys A-loaded nanofibers significantly inhibited a local inflammatory reaction.

Conclusion. Nanofibers prepared from biocompatible and biodegradable polymer PLA and loaded with Cys A can be used for suppression of T cell functions in vitro and for a suppression of harmfull inflammatory reactions in vivo.

Keywords. Cys A-loaded nanofibers, imunosuppression, inflammatory reactions

(31.P9) CONTROLLABLE ARCHITECTURES OF NANOFIBROUS SCAFFOLDS PREPARED BY ADVANCED ELECTROSPINNING PROCEDURE

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Introduction. World laboratories deal with a fabrication of three-dimensional scaffolds designed for potential applications in the regenerative medicine. Inner structure of such materials is crucial for viability, proliferation and migration of seeded cells and its integrated behavior in a body. Well-designed ordered structure can improve efficacy and applicability of new healing methods, in general. Novel electrospinning procedure was developed to obtain controllable architectures of nanofibrous scaffolds.

Methods. Electrospinning method was used to fabricate nanofibers which were deposited to uniaxial direction onto a patterned collector. These ordered nanofibers were then collected by a sliding concave tool. Such mechanism was constructed as a novel advanced collector which can eventually be used for an automatic industrial scaffolds production. Moreover, nanofibers were collected perpendicularly layer by layer leading to ordered three-dimensional structured scaffold. Two types of spinning solutions were prepared for process optimization and scaffold characterization.

Results. Uniaxially aligned nanofibers made of PVA and HA/PEO blend with diameter (250 ± 50) nm and (90 ± 30) nm, respectively, were collected from the patterned collector. This method improves the orientation order parameter up to extremely high values (S > 97 %) by additional straightening of nanofibers via collection tool movement. Fabricated samples have 3D block shape (14 x 14) mm2 with thickness of few millimeters and controllable inner structure composed of perpendicularly ordered nanofibers.

Conclusion. Thick nanofibrous layers having almost perfectly aligned nanofibers in all the structure are obtained on the collecting tool surface. The scaffolds made by this novel method are easily manipulated, ready to use directly after deposition, and very promising for future applications. The structured scaffolds made of lower solubility materials will be further fabricated for biological characterizations.

Acknowledgement. This research was conducted under financial support provided by the Ministry of Industry and Trade of the Czech Republic.

Keywords. Electrospinning, Aligned Nanofibers, Scaffold, Biomedicine Applications

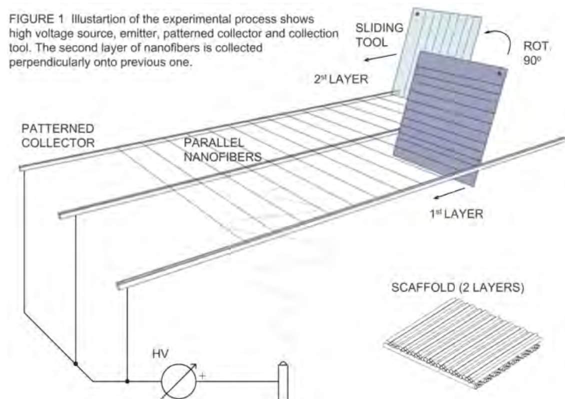

FIGURE 1 Illustartion of the experimental process shows high voltage source, emitter, patterned collector and collection tool. The second layer of nanofibers is collected perpendicularly onto previous one.

(31.P10) CNTS/Fe_3O_4 NANOCOMPOSITES AS BINDING-SUBSTRATES FOR LUMINESCENT IMMUNOASSAY

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Introduction. Luminescent immunoassays are normalize and more sensitive than chromogenic enzyme-linked immunosorbent assay. Carbon nanotube (CNT) shows strong electrocatalytic activity and chemical stability. These unique properties make CNT a potential candidate for biosensors and electrochemical sensors. In this study, the magnetic composites of CNTs and magnetite (Fe3O4) were synthesized and applied to luminescent immunoassays.

Methods. CNTs were blended with the mixture of sulfuric acid for oxidation. The oxidized CNTs were added into FeCl3/FeCl2 (molar ratio=1:1) aqueous solution to prepare the magnetic composites of CNTs/Fe3O4. To add EBNA1 solution on the CNTs/Fe3O4 composites to immobilize EBNA1. Luminescent immunoassays were applied for the measurement of anti Epstein-Barr virus (EBNA1) antibody. The crystallinity of CNTs/Fe3O4 composites was identified by X-ray diffraction (XRD), and the structure was observed using the transmission electron microscope (TEM).

Results. Figures 1(a) and 1(b) represent the TEM images of CNTs/Fe3O4 nanocomposites. From TEM images, the average diameter of CNTs/Fe3O4 was about 20 nm. Figure 2 shows the XRD pattern of the mixtures and reveals the characteristic peaks of CNT/Fe3O4 (2θ=30. 2, 35. 6, 43. 3, 57. 3, 62. 8) which corresponds to the (220), (311), (400), (511), and (440) planes of Fe3O4, respectively. Figure 3 represent the 1uminescent immunoassay results of anti-EBNA1 antibody measurement. The results show 1uminescent immunoassay of CNTs/Fe3O4 nanocomposites could detect up to 1eu. 1EU of the commercial kit was negative control.

Conclusion. These results indicate that the Fe3O4 nanoparticles were magnetite with high purity from XRD data. And the Fe3O4 nanoparticles possessed of spinel structure and intercalated on the surface of CNTs. And the luminescent immunoassay of CNTs/Fe3O4 could detect negative control of commercial ELISA kit. The luminescent immunoassay of CNTs/Fe3O4 could be validated with the commercial ELISA. Luminescent immunoassay of CNTs/Fe3O4 nanocomposites showed more sensitive than commercial chromogenic enzyme-linked immunosorbent assay.

Keywords. carbon nanotube, luminescent immunoassays

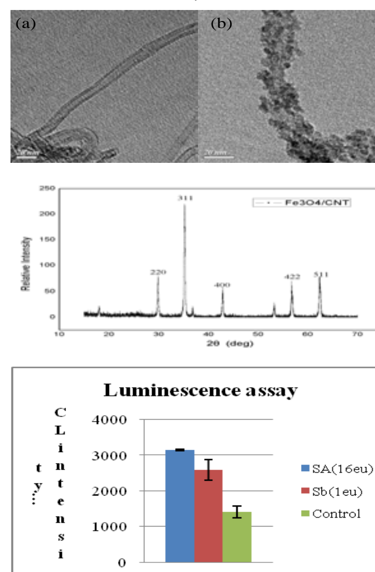

Fig. 1: TEM images of the (a) CNT and (b) CNTs/Fe_3O_4 composites. Figure2. XRD patterns of CNTs/Fe_3O_4 composites. Fig. 3: Luminescent immunoassay of CNTs/Fe_3O_4 composites.

(31.P11) ELECTRICAL STIMULATION OF HUMAN MESENCHYMAL STEM CELLS CONTAINING CARBON NANOTUBES AND IN THE PRESENCE OF GROWTH FACTORS

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Over the past 10 years, carbon nanotubes (CNT) have made an exciting entrance to the field of biomaterials. Indeed, previous research has shown that cells can take up CNT [1-3], which is leading to many exciting opportunities for drug delivery and cell tracking. Moreover, the fact that CNT are electroactive opens up the possibility of electrically stimulating cells. The authors have previously shown that electrically stimulated human mesenchymal stem cells (MSC) containing CNT can differentiate towards a cardiomyocyte lineage. In parallel, other researchers have shown that embryonic cells cultured in the presence of growth factors differentiate towards a cardiomyocyte lineage [4]. In this study, we wanted to take a further step, by electrically stimulating MSC containing CNT in the presence of growth factors. Initially, MSC at the beginning of P2 were exposed to 0. 032mg/ml COOH-functionalised SWNT for 24 hours as previously described [3]. Confluent cultures were trypsinised, counted and 3,500 cells/cm2 were seeded onto 4-well plates. After 24 hours CNT containing cells were exposed to 50ng/ml BMP-2/20ng/ml bFGF/20ng/ml HGF cocktail in the MSC growth media. After 7 days in culture all cells were fed with MSC growth media and electrically stimulated (0. 15V/cm, 2 ms duration) for a further 7 days. After the combination treatment of growth factors and electrical stimulation, 74% of the MSC containing CNT aligned perpendicular to the direction of the current, adopted a rod-like morphology and appeared broader than control MSC containing CNT. In terms of

altered gene and protein expression, a 35-fold increase in GATA-4 mRNA levels was observed, while a 4-fold increase in GATA-4 protein and a 6-fold increase in cardiac troponin T protein (Figure 1) was observed compared to controls. Although the data is promising, further work is still required to determine whether it is possible to differentiate an MSC in to a functional beating cardiomyocyte.

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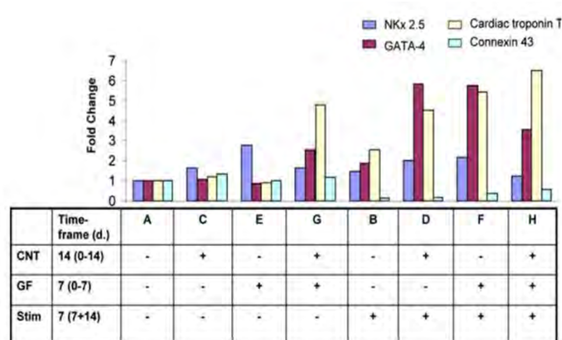
Acknowledgments. The authors would like to thank Science Foundation Ireland Research Frontiers Programme (RFP/2005/ENG004) for providing financial support to this project and also CSET 03/CE2/B312 for additional funding.

Keywords. Carbon Nanotubes, Growth Factors, Electrical Stimulation, Stem Cells, Cardiomyogenesis



	Time-frame (d.)	A	C	E	G	B	D	F	H
CNT	14 (0-14)	-	+	-	+	-	+	-	+
GF	7 (0-7)	-	-	+	+	-	-	+	+
Stim	7 (7+14)	-	-	-	-	+	+	+	+

Figure 1. Densitometric analysis of protein expression of cardiac markers in MSC treated with different conditions (A-H). Cardiac marker expression was normalized to CuZn SOD levels and presented as fold increase over control MSC cultures

32. NEURAL TISSUE REGENERATION

Chair: Ana Paula Pêgo
Co-chair: Abhay Pandit
Keynote speaker: Schlosshauer B
Organizers: Ana Paula Pêgo, Abhay Pandit

Synopsis: The nervous system may be subject of damage by a variety of insults. Depending on its extent, nerve damage can have serious and unrecoverable consequences to the patient with total loss of functionality in the more severe cases. Much research in the field of neuroscience aims at the understanding of the fundamental cellular and molecular mechanisms that underlie each clinical condition. Among the main challenges currently facing neuroscientists is the development of effective therapies based on the advances achieved in basic research.

The application of nerve conduits as grafts to bridge small peripheral nerve defects has already reached the clinic. However, solutions are awaited to promote the regeneration of both the peripheral and central nervous system resulting from a broad range of neuropathologic conditions. Cell-seeded nerve guides to bridge large peripheral nerve gaps, in situ gelling materials that promote the survival and controlled differentiation of transplanted cells or nanoparticle-based systems for targeted delivery of therapeutic molecules are examples of biomaterial-based approaches expected to have a major impact in the field in the coming years. This symposium will focus on the recent contributions of biomaterials to the field of neuroscience and how they may constitute key tools for the development of cutting edge therapies for neural tissue regeneration.

(32.KP) MULTIPLE BIOMATERIAL IMPLANTS AND NEURO TISSUE ENGINEERING

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Abstract. In the peripheral nervous system lesioned axons can regenerate if the continuity of the nerve tissue is preserved. Currently, nerve gaps are bridged by autologous nerve transplants with considerable side effects. To improve the therapeutic performance of resorbable nerve tube implants as alternative to autologous nerve transplants, we employ three tissue engineering strategies based on the combination of microstructured biomaterials, signalling molecules and regulatory glia cells. The three strategies are: 1) The instantaneous induction of Schwann cell bands as guiding cues inside the nerve tube. 2) The desensitization of regrowing axons with regard to inhibitory proteins of fibrotic and glial scars. 3) The induction of neovascularization in the implant biomaterial to foster rapid nutritional support. Implant tubes (1 mm diameter) were made from resorbable synthetic or extracellular matrix derived polymers. Schwann cell bands were formed on hundreds of longitudinally microstructured polymer filaments (0.02 mm diameter) which were inserted into the tube lumen. Desensitization of axons will be achieved by siRNA nanoparticles attached to the implant biomaterial. The siRNA shall silence a central messenger pathway onto which various repulsive signals converged. Otherwise the activation of this pathway leads to the collapse of actin filaments and consequently, of axonal growth cones. Neovascularization was achieved in a novel biomaterial sponge which might surround the internal implant tube. The indicated approaches were evaluated by gel retardation assays, RT-PCR, western blotting, single cell- and explant culture systems, time lapse video recording in vitro, a chorioallantois membrane transplantation system in chick eggs to monitor angiogenesis, rat sciatic nerve implantation, histology, immuno fluorescence- and confocal laser scanning microscopy and other techniques. Our results suggest that the novel tissue engineering approaches have the potential to substantially improve the therapeutic performance of peripheral nerve implants.

Partly supported by the german ministry BMBF (0313728) and the European programme EuroNanoMedicine.

Keywords. peripheral nerves, biomaterial, nerve guide tubes, regeneration , Schwann cells, siRNA

(32.O1) SCAFFOLD-DRIVEN REGENERATIVE THERAPY FOR THE SPINAL CORD INJURY - BIOMIMETING NEUROGENESIS IN THE CNS

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Abstract. The strategy of combining the transplantation of neural stem cells (NSCs) with biodegradable polymeric matrices has several advantages over transplantation of simple cell suspensions in the treatment of spinal cord lesions. A scaffold can serve as a vehicle for the cells while sequestering neurite growth promoting factors, providing a favorable environment for NSC proliferation and differentiation.

In this context we have developed a chitosan porous hollowed scaffold (DA=4%) to be used for NSC transplantation. Firstly, the scaffolds were seeded with endothelial cells (ECs) and cultured (supplemented EBM-2) for 5 days. Subsequently, the NS-5 NSC line was expanded under serum-free and adherent monoculture, suspended in a fibrin gel (Fb) in the form of neurospheres and were transferred to the inner part of endothelized scaffolds. Besides contributing to angiogenesis, ECs are expected to contribute to NSC survival and neuronal differentiation, by mimicking the NSC niches where NSCs are closely opposed to vascular ECs. 8-12 weeks old nude rats were subjected to a laminectomy (T7-9 vertebral level) by complete transection of a 4 mm region encompassing T8 and scaffolds implanted. Scaffolds with Fb in the presence or absence of EC, and scaffolds with NSCs alone were used as control. Animals were allowed to recover for 12 weeks and their functional performance assessed once a week (BBB score).

The scaffold alone did not contribute to functional recovery but in the presence of NSCs approximately 70% of the animals reached a score > 6 (extensive movement of two joints and slight movement of the third) versus only 20% with scaffold alone. The ECs also lead to similar functional recovery but their effect was not cumulative to the one of NSCs. The correlation of the functional data with axonal regeneration and cell integration parameters is underway.

FCT (PTDC/SAU-BEB/65328/2006) supported this work.

Keywords. spinal cord injury, neural stem cells, in vivo model, scaffolds

(32.O2) AN IN VIVO PLATFORM FOR CELL MIGRATION AND TOPOGRAPHICAL GUIDANCE OF AXONS

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Introduction. This study investigates the use of extruded collagen fibres (EGFs) as an intraluminal filler, to improve existing nerve guidance conduits (NGCs). It is hypothesized that EGFs increase the surface area available for cell adhesion, enhancing cell migration, and providing topographical guidance cues for regenerating axons. This study involves the characterisation of the EGFs in vitro and in vivo as a platform for cell migration and topographical guidance of axons.

Methods. The EGFs were fabricated in a multi step process. EGF structure was analysed using standard surface characterisation techniques. Neuronal interaction and cell migration, on the EGFs, was assessed using rat PC12 cells and 3T3 fibroblast cells respectively. Parameters assessed can be seen in (fig. 1). Following in vitro characterization, 18 EGFs were enclosed within a collagen NGC, and implanted in a rat sciatic nerve model for 16 weeks (fig. 1). 16 weeks post implantation, simultaneous retrograde tracing and nerve morphometry analyses were carried out.

Results. In vitro assessment of the neural interaction, of the EGFs, showed a significant increase in neurite length and higher alignment versus control collagen fibres (n=3, p<0.05) (fig. 1) and 3T3 cells successfully migrating across the fibres (fig. 1). 16 weeks post implantation, successful nerve regeneration was seen across a 10 mm nerve gap (fig. 1). Preliminary retrograde tracing results show a significant number of labelled neurons in the EGFs versus the non micro-grooved fibres; suggesting an increase in the number of regenerated axons in the EGF group

Conclusion. EGFs show the ability to increase aligned nerve growth, act as a platform for cell migration and topographical guidance of axons and to successfully bridging a 10 mm nerve gap.

Acknowledgements. Science Foundation Ireland, Grant No. 07/SRC/B1163 and Enterprise Ireland - Proof of Concept Grant (PC/2008/399)ç

Keywords. conduit, microtopography, peripheral nerve, contact guidance, in vivo

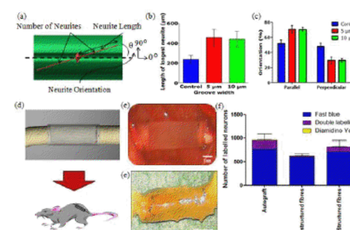

Figure 1 (a) Parameters for the assessment of the neural interaction of EGFs; (b+c) EGFs showing significant increase in length of longest neurites and more parallel neuronal outgrowth versus control non-MG collagen fibres (n=3, p<0.05)(d) Schematic of EGFs in a NGC implanted within a rat sciatic nerve model (e) Day 0 implanted NGC with EGFs (f) NGC 16 weeks post implantation showing a tissue cable bridging a 10mm nerve gap; (g) Preliminary retrograde tracing results showing an a significant increase in the number of labelled neurons in EGF group versus control non EGF group, suggesting an increase number of regenerated axons

(32.O3) ALIGNED POLYCAPROLACTONE FIBRES FOR PERIPHERAL NERVE REPAIR

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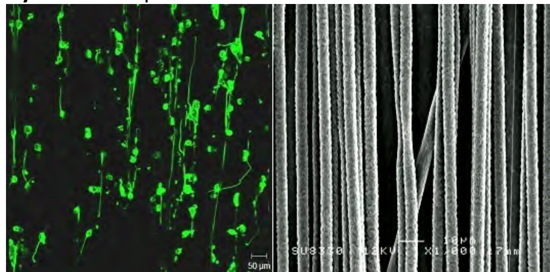
Abstract. The ability of the peripheral nervous system to regenerate following injury is limited. Autografting is the gold standard treatment but there are a number of drawbacks to this procedure including donor site morbidity and lack of donor material. Nerve Guidance Conduits (NGC) can be surgically implanted to bridge the gap left by injury to provide mechanical strength and physical guidance cues but these devices do not encourage re-innervation beyond a few millimeters. NGCs

can incorporate a number of features to improve the basic design. The use of an aligned fibrous scaffold in an NGC is a potential method to guide neurite outgrowth and bridge nerve gaps. Both aligned nanofibres and microfibres have been shown previously to facilitate nerve regeneration. However, further study is needed to understand the relationship between fiber diameter and nerve regrowth. The aim of this work was to investigate the effect of fiber diameter on neurite outgrowth. We fabricated three different sizes of aligned polycaprolactone fibres by electrospinning by varying the electrospinning parameters (polymer concentration, solvent, flow rate, applied voltage, needle-to-collector distance, and needle size) and compared maximum neurite length, number of neurites per neuron and the percentage of neurite bearing neurones on fibres with diameters of ~ 8 µm, ~ 5 µm, and ~ 1 µm when NG108 neuronal cells were grown in vitro. Neurite guidance was evident on all fibres diameters. However, the longest neurites were observed on fibres with the largest diameters. There was no significant difference in the number of sprouting neurites, but the number was lower in comparison on flat surfaces (TCPS, PCL film and glass). In summary, the study shows that fibre size influences neurite outgrowth and that micro-size fibres support better directed neurite elongation verses nano-size fibres.

Keywords. Peripheral nerve



(32.O4) BIOMIMETIC PEPTIDE SCAFFOLDS FOR NEURAL REGENERATION

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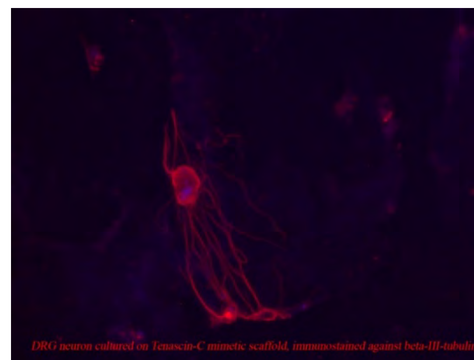
Abstract. Neural tissue is among the tissues with poor regenerative capacity mainly due to the loss of proliferative capacity of neurons and inhibitory environment of the central nervous system. For this reason, any damage leading to neuronal loss is barely tolerable. Even the remaining neurons at distal parts are not able to establish new interactions to tolerate lost cells because of the inhibitory extracellular matrix elements that prevent neurite extension. Besides, the damaged part of the system is populated by glial cells and glial scar is formed resulting in the loss of neural functions. Hence, any attempt to regenerate neural tissue should consider to overcome the inhibitory environment and prevent glial scar in order to be succesfull. Taking this into consideration, we have designed several bioactive materials mimicking the neurite extension promoting elements of the extracellular matrix (ECM). Laminin, Tenascin C and heparan sulphate proteoglycans are among the components of neural ECM playing role in promotion of neural differentiation in development and neurite extension. We have synthesized peptide amphiphile molecules that consists of a hydrophobic alkyl tail, beta sheet forming sequence and biofunctional sequence derived from the bioactive regions of above mentioned neurite promoting proteins. These peptide amphiphiles self assemble into nanofibers by electrostatic interactions in solution and form nanofibrous networks resembling ECM. Such nanofibrous networks encapsulate water and form gels at macroscale and can be used as biomimetic scaffolds. Besides presenting neurite promoting signals, these scaffolds have similar mechanical properties (stiffness) with neural ECM which is an essential element that should be considered when designing scaffolds for neural cells. We found that these scaffolds support DRG neuron viability and neurite extension, neurite extension of PC12 cells and neural differentiation of mesenchymal stem cells.

Keywords. biomimetic scaffolds, neural regeneration, neurite extension, peptide amphiphiles



DRG neuron cultured on Tenascin-C mimetic scaffold, immunostained against beta-III-tubulin

(32.O5) 3D MICRO-STRUCTURED COLLAGEN CONDUITS FOR NERVE REPAIR

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Introduction. Peripheral nerve injury leads to sensation and movement loss that is devastating to the patient. In cases of complete nerve transection (neurotmesis), it is often necessary to use an autologous nerve graft to bridge the injury site. Various attempts have been made to develop artificial conduits that can replace autologous grafts, but such conduits are usually only effective at bridging short gaps. Here, we have developed and tested a new form of nerve repair conduit, based on 3D micro-structured collagen.

Methods. Two forms of conduit were fabricated using different thicknesses of plastic compressed collagen sheet. Low density (LD) guidance-surface conduits were made from spiralling a single standard thickness (100µm) collagen sheet. High density (HD) conduits were made from spiralling three thin collagen sheets. The HD conduits have 3x as many layers (and guidance interface) as the LD conduits. For assessment in vivo, a segment of the rat sciatic nerve was removed and a HD or LD conduit

(0.5 cm in length) was implanted. Separate control experiments involved either direct repair of a sciatic nerve injury by coaptation, or insertion of an autologous graft. At 2 weeks, the implanted conduit and attached proximal and distal nerve portions were removed, fixed in formaldehyde and processed for immunocytochemistry.

Results. Transverse and longitudinal sections revealed immunoreactive axons within the conduits, with axons confined to the interfaces between the spiralled collagen sheets. In the same regions an extensive cellular infiltration was present, which included numerous Schwann cells.

Conclusions. The results indicate that interfaces between spiral layers of plastic compressed collagen can act as guidance and support structures for Schwann cell migration and axon regeneration. Ongoing studies are analysing the differences between the HD and LD conduits, and the topographical cues that maximise axon growth.

Keywords. collagen, conduit, nerve repair

(32.O6) ALIGNED FIBRIN AND SCHWANN LIKE CELLS: A READY-TO-USE NERVE CONDUIT

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Introduction. The gold standard in peripheral nerve regeneration is the autologous nerve transplant. Due to limiting factors like length and diameter of the graft and the disadvantage of a second incision site, new approaches are sought. The ideal nerve conduit provides a nanoscaled guiding structure and activated Schwann cells forming bands of Bungner for enhanced axonal outgrowth.In this study we describe electrospinning of aligned fibrin nanofibers in an attempt to create a biomimicking tissue-like material seeded with Schwann like cells (SCLs) in vitro for use as scaffold in vivo to treat peripheral nerve injuries.

Material and Methods. Lyophilized human fibrinogen and thrombin (Tisseel©) blended with PGLA were used to demonstrate fibrin electrospinning for aligned nanofibers. Quality and alignment of electrospun fibers were analyzed by scanning electron microscopy (SEM). Schwann like cells were differentiated from adipose derived stem cells and evaluated with Flow Cytometry concerning their differentiation and activation status (P75, GFAP, P0, S100, MAG). Electrospun matrices were seeded with SCLs and rolled to be placed in a silicone tube without damage to the cells. Formation of Bungner-like structures of SCLs on the aligned matrices was evaluated with AlexaFluor Phalloidin/Propidiumiodide staining.

Results and Discussion. Electrospun fibrin fibers were highly aligned and homogenous. SCLs showed expression of P75, GFAP and S100 in presence, as well as increased expression of MAG and P0 in absence of forskolin, indicating a change of activated status into beginning myelination. SCLs adhered well to electrospun fibrin and formed Bungner-like structures. Pilot animals with 8 mm dissected sciatic nerve showed proper axonal regeneration and no signs of inflammation or degradation of the scaffold after 6 weeks.

Concluding, aligned electrospun fibrin seeded with SCLs is promising for peripheral nerve regeneration.

The project was partly supported by the FHplus project “NewTissue” (818412) and the European FP7 program Angioscaff.

Keywords. Peripheral Nerve Regeneration; Fibrin; Schwann like Cells

(32.O7) EVALUATION OF A FIBRIN GEL FOR TRANSPLANTATION OF EMBRYONIC STEM-DERIVED NEUROPROGENITORS INTO THE INJURED SPINAL CORD

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Introduction. Neural stem cell (NSC) transplantation is among the most promising therapeutic strategies for the treatment of spinal cord injuries (SCIs). Yet, NSCs simply injected in the spinal cord usually results in poor cell survival and only few cells differentiate into neurons, partially due to the SCI inhibitory microenvironment. As a result, biodegradable porous matrices and hydrogels are currently being developed to increase the efficacy of NSC transplantation. Here we assessed the ability of a fibrin gel (Fb) to be used as a vehicle for transplantation of ES-derived NSCs into the injured spinal cord.

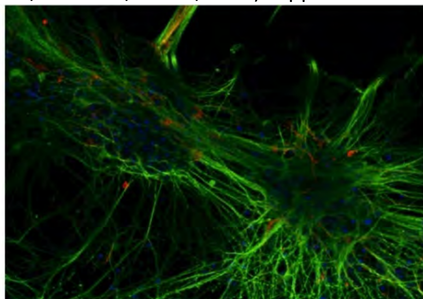
Methods. Neural progenitors generated in serum-free medium and adherent monoculture from mouse ES cells (ESC line 46C) and the NS-5 cell line (NSCs) were used. Neurospheres (Diameter approx. 300 µm) were suspended in Fb (6 mg/mL fibrinogen), and cultured for 14 days in RHB-A:Neurobasal/B27 to induce neuronal differentiation. Cell morphology was followed by phase contrast microscopy, while cell viability and phenotype were assessed using Calcein AM/PI staining and IF against Nestin (NSC), bIII tubulin (early neuronal), GFAP (astrocytes), and O4 (oligodendrocytes), respectively. Subsequently, the efficacy of this cell-matrix system to promote functional recovery following transplantation was evaluated using a rat model (complete transection) of SCI.

Results. Results showed abundant cell sprouting from the neurospheres throughout the incubation period. At day 14 of culture, few PI+ cells were found, mostly in the centre of the neurospheres. CLSM imaging at this time point revealed the presence of Nestin+ cells, as well as a network of bIII tubulin+ cells radially migrating from the center of the neuropheres. GFAP+ cells could also be observed, but in significant lower number than bIII tubulin+ cells. Moreover, transplantation of NSCs (NS-5)

within an Fb gel using a porous chitosan tubular conduit led to improved functional recovery (BBB score).

Keywords. Spinal cord injury; Neural stem cells; Hydrogel; Engineered constructs

FCT (PTDC/SAU-BEB/65328/2006) supported this work.



ES-derived NSCs seeded in fibrin after 14 days of cell culture; CLSM image of samples immunostained for bIII tubulin (green) and GFAP (red). Nuclei were counterstained with DAPI. Original magnification: 40 ×.

(32.O8) TUNING POLY(TRIMETHYLENE CARBONATE-CO-E-CAPROLACTONE) FIBERS FOR NERVE REGENERATION

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1. INEB; 2. Institute of Composite and Biomedical Materials

Introduction. Poly (trimethylene carbonate-co-ε-caprolactone) (P(TMC-CL)) scaffolds have been proposed as conduits for nerve regeneration. Electrospun fibrous scaffolds can guide/modulate cell response, depending on fiber morphology. The incorporation of anti-inflammatory drugs in these structures is expected to enhance the regeneration process after a lesion. The present work explores the formation of P(TMC-CL) fibers by electrospinning and the possibility of adjusting fiber diameter and incorporate an anti-inflammatory drug.

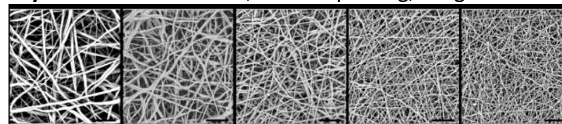
Materials and Methods. P(TMC-CL) (11 mol% TMC; Mn= 8.2x104, Mw/Mn= 1.61) solutions were prepared using dichloromethane (DCM) and N,N-dimethylformamide (DMF) at volume ratios of (1:0), (6:1), (3:1), (3:2), and (1:1). To prepare electrospun fibers, P(TMC-CL) solutions (10% (w/v)) were extruded from a needle (0.8mm) at a feed rate of 1 ml.h-1. Electric field was set to 1kV.cm-1. Ibuprofen (IBU, 5% (w/w)) was added to polymer solutions 4 hrs before electrospinning. IBU release (PBS, 37°C, 120rpm) was quantified by UV/Vis spectroscopy at 230nm. Fiber morphology was analyzed (SEM) and fiber diameter calculated from image analysis.

Results and Discussion. P(TMC-CL) fibers with an average diameter below 1 µm were obtained from all the P(TMC-CL) solutions tested, independently on the applied solvent:co-solvent ratios. A shift towards smaller fiber diameter was observed when increasing the amount of DMF in the solution. Ibuprofen was incorporated in P(TMC-CL) fibers with a loading efficiency above 80%. For solutions with a volume ratio of DMF above (3:1) fiber formation is impaired. Ibuprofen release from the loaded fibers was found to occur in the first 24hrs after incubation in PBS. A first order release was detected when fibers were prepared from DCM solutions.

Conclusions. P(TMC-CL) fibers with adjustable diameter were successfully prepared. An anti-inflammatory drug was loaded in the fibers without compromising fiber morphology. The bioactivity of the released drug is being explored.

Acknowledgements. Portuguese Foundation for Science and Technology (SFRH/BD/46015/2008).

Keywords. Inflammation, electrospinning, drug release



(32.O9) INVESTIGATION ON THE PROPERTIES OF POLY-ε-CAPROLACTONE SOLVENT CASTED FILMS AFTER SURFACE MODIFICATION AS A POTENTIAL MATERIAL FOR NERVE REGENERATION

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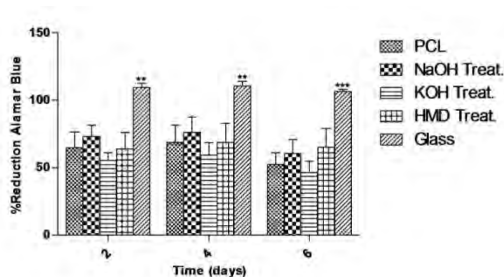
Introduction. Poly-ε-caprolactone (PCL) is a biocompatible and biodegradable polymer suitable for tissue engineering. Solvent casted films are characterised by a particular micropitted surface. Previous works demonstrated that neural cells are able to adhere and proliferate on these scaffolds. However, surface modification is necessary in order to improve the material biocompatibility. Particularly, hydrolysis and the presence of functional groups have been found to influence the cell-material interaction.

Methods. A solution of PCL/dichloromethane 3% wt/v was casted on glass coverslips and the solvent was left to evaporate. Films were treated with three different solutions: sodium hydroxide (NaOH) 10M, potassium hydroxide (KOH) 10M and hexamethylendiamine (HMD) in isopropanol (IPA) 10% wt/v. Mechanical tensile tests were carried out (n=5) to characterise the degradation of the material and a NG108-15 cells proliferation assay using Alamar Blue was carried out for 6 days (n=3) to investigate the films biocompatibility.

Results. A remarkable decrease ($p<0.05$) in the Young's modulus was noted in case of HMD treatment (69.60±13.83MPa). In case of hydroxide treatments, the Young's modulus was not significantly affected (respectively 102.30±3.78MPa and 108.90±7.13MPa) compared to untreated films (105.50±5.91). This can be explained as hydrolysis is surface-orientated degradation reaction, while mobile amines can lead to a bulk degradation of the material. Finally, it was found that after 4 days the NaOH treatment allowed the higher proliferation, while HMD treatment showed a comparable result to untreated PCL films. After 6 days, cells proliferation decreased in case of hydrolysis treatments, while the HMD treated films showed the higher percentage of reduction.

Conclusion. It could be hypothesised then that even if the cells growth is slower on aminolysed samples, the adhesion force is higher compared to the other films.

Keywords. biocompatibility, surface modification, cells response



(32.P1) SCAFFOLD MEDIATED NON-VIRAL GENE KNOCKDOWN OF GLIAL SCAR GLYCOSAMINOGLYCAN SYNTHESIS

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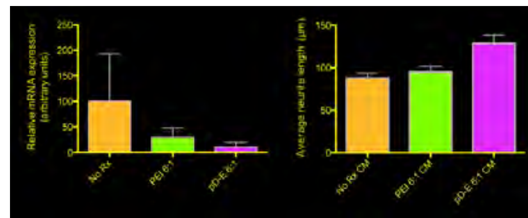
Introduction. Glial scar mediated inhibition of axonal regrowth following spinal cord injury is attributed to increased glycosaminoglycan (GAG) chain production by reactive astrocytes, driven by the enzyme Xylosyltransferase-I (XT-I). The bacterial enzyme chondroitinase ABC, although partially effective in alleviating scar-mediated inhibition by removing GAG-side chains from core proteins, has a short half-life. The overall objective of this study is to provide sustained knockdown of XT-I using small interfering RNA (siRNA) and a non-viral delivery vehicle comprised of hyperbranched DMAEMA/ethylene glycol dimethacrylate (EGDMA) copolymer (pD-E). The specific objectives are to: (1) demonstrate efficient siRNA delivery into reactive astrocytes using pD-E, and subsequent knockdown of XT-I, (2) compare this effect to other commercially available delivery vehicles, and to (3) test whether this knockdown reverses the neurite inhibitory properties of reactive astrocytes.

Methods. XT-I siRNA was used naked or complexed using different weight ratios of Poly(ethylene imine) (PEI) or pD-E. Neu7 astrocytes, which overexpress GAGs, were incubated with polyplexes 24 hours after seeding. At 48 hours, the astrocytes from different siRNA treatment groups were assessed for GAG quantity, XT-I activity and mRNA expression, and their conditioned media (CM) were used in neurite outgrowth assays of dorsal root ganglia.

Results. There was a significant reduction in mRNA expression of XT-I from cells treated with pD-E siRNA polyplexes (at 6:1 weight ratio), whereas no significant change in expression was observed with either naked siRNA or PEI siRNA polyplexes. This change in gene expression correlates with neurite outgrowth assays, where CM from pD-E treated cells resulted in neurite outgrowth significantly higher than CM from untreated cells, whereas no increase in neurite length was observed with the other treatment groups.

Conclusion. Polymer mediated delivery of XT-I siRNA to reactive astrocytes provides a mechanism by which the glial scar inhibitory pathway can be reversed to promote axonal regrowth.

Keywords. siRNA; Xylosyltransferase; CSPG



(32.P2) NEURAL POTENTIAL OF HUMAN CHRONIC MYELOID LEUKEMIA HEMATOPOIETIC CELLS IN MICE BRAIN

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Introduction. Adult hematopoietic stem cells (HSC) are able to survive in the CNS of animal models and to “transdifferentiate” into neural cells upon exposure to signals generated by CNS tissue injury (Bonilla et al, 2002). This fact has been difficult to prove when studying human HSC in animal models due to the lack of a good marker to follow the fate of the transplanted cells. Chronic myeloid leukemia (CML) is a stem cell disorder characterized by immune-scape and resistance to apoptosis due to an acquired chromosomal translocation, the t(9;22). This translocation generate a specific BCR-ABL chimeric gene that may be identified by Fluorescence in situ hybridization (FISH), and could facilitate tracking the fate of CML human cells in xenogeneic transplant experiments.

Methods. We isolated CD34+ positive cells from two untreated chronic phase CML patients by Macs immunomagnetic selection. These cells were infused in the white matter of the parietal hemisphere of six adult Swiss mice, six neonates and six NOD.Cg-Prkdc scid Il2rgtm1Wjl/SzJ immunosuppressed mice. Mice were sacrificed at 7, 15 and 30 days. LSI t(9;22) BCR/ABL Dual Fusion Dual Color Translocation Probe (Vysis) was used to perform the FISH. Neural differentiation was checked by colocalization of the FISH signal with antibetaIII-tubulin and antiGFAP by immunofluorescence.

Results. The mice received 1 to 2x105 CD34 selected cells. No cells with the bcr-abl translocation could be observed at 7 day post-transplant in either the Swiss or the NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice. In the 6 neonatal transplanted mice scarce bcr-abl positive cells could be observed at day seven, but not thereafter. We did not observe colocalization of the bcr-abl signal with betaIII-tubulin or antiGFAP.

Conclusions. Human CML hematopoietic stem cells are not able to survive in the CNS of either normal mice or naturally or artificially immunosuppressed mice. CML cells are not a good candidate to study the HSC neural transdifferentiation potential.

Acknowledgements. This work was supported by FIS PI071130, and RETICS RD/0010/2012 grants from the ISCIII, Spain

Keywords. Hematopoietic stem cells, CNS, Chronic Myeloid Leukemia

(32.P3) MODULATION OF PROLIFERATION/DIFFERENTIATION FATES OF NERVE

CELLS ON EXTRACELLULAR MATRIX COMPONENTS OF THE NERVOUS SYSTEM

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Introduction. Nerve tissue damage impairs the correct neurotransmission and its regeneration requires signalling and scaffold molecules to facilitate proliferation and differentiation of the principal cells involved in this function, neurons and oligodendrocytes. Here we studied the influence of ECM matrix components (proteins and GAGs) on proliferation and differentiation fates of neurons and oligodendrocytes as an approach to determine an optimal material for nerve tissue regeneration.

Methods. The established cell lines included in the study were SH-SY5Y neurons and HOG oligodendrocytes. We used a 2D culturing method system including different proteins and GAGs integrated in the ECM. Cell proliferation and viability in the non-differentiated condition was analysed by spectrophotometry. After inducing cell differentiation, the mature phenotype of cells was assessed by systematic morphologic observation, by quantification of molecular markers detected with immunocytochemistry and by genetic identification of mRNA coding for proteins of mature nerve cell types.

Results. Both cell lines can proliferate spontaneously, but when cells are cultured with specific differentiation media they can reach a certain mature phenotype and suppress their proliferating potential. Among those matrix components explored, laminin potentiates the differentiation of SH-SY5Y neurons and seems to provide with consistent axon growth as shown by NF200 expression. On the other hand, HOG cells mostly proliferate as undifferentiated oligodendrocytic precursors on most ECM proteins and GAGs without evident morphological changes. Those HOG cells that show potentially myelinating membrane expansions increase their expression of a myelin protein, MBP and its expression is more pronounced when culturing on laminin-containing substrates.

Conclusions. Laminin-containing substrates present relevant properties for nerve tissue regeneration. This ECM scaffold protein enables consistent axon growth in neurons, promotes oligodendrocyte proliferation and allows their differentiation. Therefore, laminin seems to be a valid candidate for biomaterials designed for nerve tissue regeneration.

Acknowledgements. This study has been supported by grants from CONNECT and ETORGAI, Basque Government. Submitted by 212.8.106.224

Keywords. Neuronal cells, oxidative stress

(32.P4) RECOVERY THRESHOLD OF DIFFERENTIATED NEURONS UNDER OXIDATIVE ENVIROMENT

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Introduction. Oxidative stress is implicated in the neuronal damage associated with a variety of neurodegenerative disorders and spinal cord injury. Here, we analyze the effect of H2O2 generated during inflammatory process in the nervous tissue as an approach to define the basal conditions to evaluate procedures for tissue repair.

Methods. we used the human neuroblastoma cell line SH-SY5Y differentiated using retinoic acid (ATRA) into neuronal-like cells, which acquire morphological, neurochemical (NF200 and MAP2 expression) and electrophysiological properties characteristic of neurons. Once differentiated, neuronal cells were exposed to oxidative stress with different concentrations of H2O2 (range: 0.1 - 1.0 mM) during 1 h. In this study, we determined the reactive oxygen species (ROS) levels, the glutathione (GSH) levels and cell viability.

Results. Exposition of neurons to H2O2 reduce cell viability in a dose-dependent manner, which was in correlation with the dose-dependent increase of ROS levels (figure 1). However, the cell viability could be recovered in cells exposed up to 0.25 mM. In fact, we observe an increase in GSH levels in cells exposed to the H2O2 intermediate concentrations (0.1 and 0.25 mM) when compared to control values.

Conclusions. The oxidative stress significantly reduced the viability of neurons, but at the similar intermediate concentrations as occurs in inflammatory conditions, the neuronal viability could be recovered using an appropriate neuroprotective procedure.

Acknowledgements. This study has been supported by grants from the UPV/EHU-GIU10/16, Basque Government (Etortek), Gangoiti Barrera Foundation and Histocell S.L.

Keywords. Neuronal cells, oxidative stress

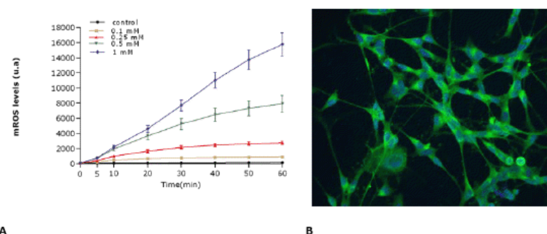

Figure 1. (A) Significant increase of ROS levels in the differentiated SH-SY5Y cells exposed to H_2O_2 (0.1-1.0 mM). (B) NF200 expression of SH-SY5Y differentiated using ATRA.

(32.P5) EFFECT OF SURFACE MORPHOLOGY ON ORIENTATING THE NG108-15 CELLS APPLIED IN GROOVED NERVE CONDUIT

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1. The University of Manchester

Introduction. Synthetic nerve guide conduits are extensively acknowledged as an alternative treatment for stimulating nerve regeneration. The aim of the study was to evaluate the influence of microgrooves, with three different designs, on the orientation of nerve cells and to assess the mechanical strength of films.

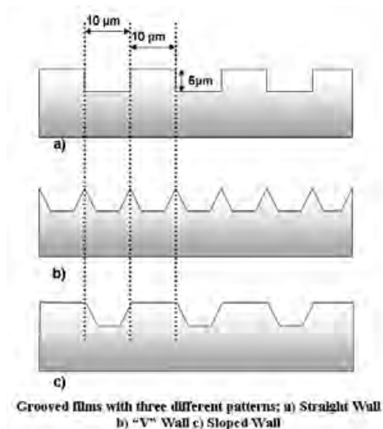
Methods. Polycaprolactone and poly lactic acid (80/20 %) in dichloromethane (3% w/v) was cast onto the patterned silicon substrates. The substrates were fabricated with the groove dimension of 10-10-5µm, (space-width-depth), by microlithography and a) Wet etching to produce both "V" and slopped wall, b) Reactive ion etching to create an upright wall. A non-grooved film was used as a control. Films were imaged by scanning electron microscopy and tensile testing performed in the direction

of the parallel to the length of the groove. Cell culture on the films was carried out with the 10,000 cells/sample.

Results. Statistical analysis demonstrated that the maximum stress, Young's Modulus and strain of the grooved films are not significantly different when compared to the non-grooved films. Assessment of cell orientation in relation to the grooves was observed after 2, 24 and 48 hrs from initial seeding. Cells on grooved film highly aligned in the grooved direction, specifically on "V" and slopped wall.

Conclusion. This study has demonstrated that the presence of grooves affects the orientation of nerve cells and causes cell alignment. Cells' are preferentially aligned when in contact with "V" wall and slopped wall grooves; which cells attachment occurred between the grooves ,however cells mostly attached on the groove spaces and grow in random direction, on the upright wall. This is a highly promising result as this guidance conferred from the grooves to the cells will assist with the directional re-growth of damaged nerves.

Keywords. Nerve regeneration, Surface morphology, groove film



Grooved films with three different patterns: a) Straight Wall b) "V" Wall c) Sloped Wall

(32.P6) SCHWANN CELLS PROLIFERATION IS INCREASED WHEN CO-CULTURED WITH ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS

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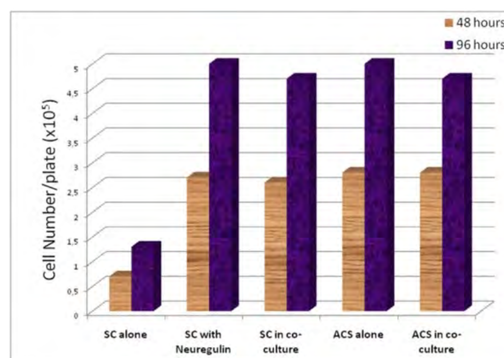
Introduction. Schwann cells (SCs) grafts have been experimentally used for the regeneration of injured nerves. In order to improve SC growing in cultures we evaluate the effect of adipose tissue-derived mesenchymal stem cells (ASCs) co-cultured with SCs and compare these results with neuregulin effect.

Methods. SCs from rat predegenerated sciatic nerves were cultured either alone, adding neuregulin or co-cultured with ASCs obtained from inguinal fat. Morphological, immunohistochemical and quantitative studies were performed at 48 and 96 hours. Cultures were maintained in DMEM plus 1% antibiotic and 10% FBS, the medium being changed every two days until subconfluence, when cells were subcultured.

Results. SCs in culture appeared bipolar with small somata and small and elongated nuclei. They tended to contact on their ends forming chains or clustering around other cell types. They were S100 and vimentin positive. ASCs in culture showed fibroblast-like shape with large rounded nuclei and distinct nucleoli. They were CD 105 and vimentin positive, became larger along time and contacted with the neighboring cells. SCs cultured with neuregulin proliferate about 4 times faster than alone. A similar proliferation increase was observed in SCs co-cultured with ASCs. In this co-culture, a few triangular SCs appeared and ASCs also proliferate faster, but their CD 105 positivity turned weaker and became vimentin negative at 4 days, what could mean the initiation of ASC differentiation. Fibroblasts appeared in a small proportion in all the different cultures. Cell counting is shown in the figure and ANOVA revealed significant differences between 2 and 4 days within every kind of culture, whereas across the different cultures, significant differences were found between simple SC cultures, grown without neuregulin, and the rest of the cultures.

Conclusion. SCs co-cultured with ASCs increase their proliferation rate about 4 folds in 4 days, in a similar manner as when neuregulin is added, suggesting possible secretion by ASCs of growth factors for SCs. Supported by a Junta de Castilla y León grant # SAN 673/VA/23/08

Keywords. Nerve Regeneration, Scwann, ASC



(32.P7) USE OF POLY(DL-LACTIDE-E-CAPROLACTONE) PLC MEMBRANES AND MSC CELLS FOR PROMOTING NERVE REGENERATION IN AN AXONOTMESIS RAT MODEL

A Gärtner (1,2), T Pereira (1,2), I Amorim (2), C Lopes (2), PAS Armada da Silva (3), AL Luís (1,2), S Geuna (4), AC Maurício (1,2)

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Many studies have been dedicated to the development of scaffolds for improving post-traumatic nerve regeneration. The goal of this study was to improve the nerve regeneration, associating a Poly(DL-lactide-ε-caprolactone) PLC, Vivosorb® membrane with Mesenchymal Stem Cells (MSCs) isolated from Wharton's Jelly of Umbilical Cord , in peripheral nerve reconstruction after crush injury. PLC, Vivosorb® membranes were previously tested *in vitro*, to assess their ability in supporting MSCs survival, expansion, and differentiation. These membranes showed good biocompatibility and proved to be a suitable support for this cellular system

delivery in peripheral nerve axonotmesis. MSCs from Wharton's jelly umbilical cord were cultured and differentiated in neural-like cells with MSC neurogenic medium. The culture medium was replaced every 24h during 3 days, to obtain neural-like cells. Immunocytochemistry was done for anti-NeuN, anti-glial fibrillary acidic protein and anti-growth associated protein-43.
For the *in vivo* testing, Sasco Sprague adult rats were divided in 5 groups of 6 animals each: Group 1 sciatic crush injury without any other intervention (Group 1 - Crush), Group 2, the crushed sciatic nerve was infiltrated with a suspension of 1250 MSCs (total volume of 0.5 ml) (Group 2 – CrushCell), Group 3, the crushed sciatic nerve was encircled by a PLC, Vivosorb® membrane covered with a monolayer of non differentiated MSCs (Group 3 – CrushCellNonDiffPLC) and Group 4, the axonotmesis lesion of 3 mm was enwrapped with a PLC, Vivosorb® membrane covered with a monolayer of differentiated MSCs (Group 4 – CrushCellDiffPLC). A group of 6 animals was used as control without any sciatic nerve injury (Group 5 – Control). Motor and sensory functional recovery was evaluated throughout a healing period of 12 weeks using sciatic functional index (SFI), static sciatic index (SSI), extensor postural thrust (EPT), and withdrawal reflex latency (WRL). Stereological analysis was carried out on regenerated nerve fibers.
Keywords. MSCs, differentiation, neural-like cells

(32.P8) HYALURONIC ACID BASED SCAFFOLDS FOR NEURAL TISSUE ENGINEERING

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Introduction. Regeneration of cortical regions of the brain demand materials with a three-dimensional porous structure capable of being invaded by neural cells and being vascularized in order to maintain the viability of the cells within the scaffold. It is thus of great interest to study the interaction of the cells involved in the structure of the blood-brain-barrier inside such scaffolds.
Materials and methods. We prepared three-dimensional porous matrices of crosslinked hyaluronic acid (HA) having two different well-defined geometries of inner pores. In the first scaffold , a basically random isotropical distribution of spherical interconnected pores and in the second scaffold , an architecture of parallelly alligned, mutually orthogonal interconnected cylindrical pores was generated, respectively This structure was achieved by providing the HA matrix with a acrylate elastomer skeleton. Co-cultures of two different cell lines involved in the central nervous system were studied within these scaffolds: a human endothelial cell line from the brain microvasculature (hCMEC) and an astrocytic human cell line (U373), using monocultures as controls. Viability, inflammatory effects, specific protein expression and cytokines secreted during co-culture were anylized in a time span of 21 days.
Results and conclusions. The hyaluronic acid based scaffolds were efficiently colonized by the cells seeded on them. Endothelial cells cultured in the materials exhibited a normal phenotype, showing no expression of the inflammatory marker E-selectin. The distribution of the cells throughout the scaffold was identified with the vWF and GFAP markers for inmunofluorescence of hCMEC and U373 respectively, while secretion of VEGF, ang-1 and ang-2 showed some effects attributable to the interaction of both kinds of cells. No significant differences between the results obtained on both types of porous structures could be thus far ascertained by these experiments.
Keywords. Hyaluronic acid, scaffold, brain, tissue engineering

(32.P9) MARKERS OF NEURAL CREST CELLS IN THE ADULT ENTERIC NERVOUS SYSTEM

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1. Universidad de Zaragoza

Introduction. An increasing body of evidence has accumulated in recent years supporting the existence of multipotent neural progenitor cells in the adult mammalian gut. The repair of the intestinal enteric plexuses should occur, in the adult, from poorly differentiated or undifferentiated cells capable of becoming neurons under the influence of certain stimuli. Presumably these cells are similar to neural crest cells, which, during embryonic development will be differentiated, to the stage of mature neurons. The aim of our study is to make a escreening of intestinal nerve plexus, using a battery of neural crest markers, in order to locate the niche of neural stem cells.
Material and methods. Four to 3 month old rat Wistar (Jackson Laboratoires) were used in accordance with institutional guidelines. (CEICA Acta nº CP 06/05/2009). To carry out immunocytochemical techniques we used: 1) the method in Vision. 2) Technical fluorescence double labeling. The antibodies used were: C-Ret, sox 10, nestin and doublecortin, combined with proliferation markers (BrdU and Ki 67).
Results. All neural crest markers studied showed positivity around or inside the enteric ganglia. Some of the nestin positive cells mark the periphery of the enteric ganglia and in Inside of these colocalize with GFAP. Sox 10, p75 and doublecortin mark some cells within the intestinal ganglia. C-Ret colocalizes with PGP9.5 (pan-neuronal marker), but it does not with GRAP (glial marker) or with c-Kit (specific marker of ICCs). The few cell divisions observed are located on the periphery of the enteric ganglia.
Conclusions. The neural progenitors of the enteric nervous system of rat intestine express markers of neural crest cells.
Acknowledgements. This research received financial support from Aragon Institute of Health Sciences (I+CS) (PIPAMER 001/11) and the European Social Fund (ESF), DGA (B83)
Keywords. Neural stem cells, intestinal adult stem cells, neural crest progenitors.

(32.P10) ULTRASOUND-STIMULATED PERIPHERAL NERVE REGENERATION WITHIN HYDROPHILIZED ASYMMETRICALLY POROUS NERVE GUIDE CONDUIT

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The sufficient functional recovery of damaged peripheral nerves has been considered as one of the clinical challenges. Artificial nerve guide conduits (NGC) to bridge the gap between severed nerve stumps are widely accepted as a useful alternative that creates a favorable environment for nerve regeneration. Recently, we developed a novel method to fabricate a NGC with asymmetrical pore structure and hydrophilicity using poly(lactic-co-glycolic acid) (PLGA) and Pluronic F127 by a modified immersion precipitation method. From the animal study using a rat model (sciatic nerve defect of rat), we recognized that the unique PLGA/F127 tube provided good environments for nerve regeneration. In this study, we applied low intensity pulsed ultrasound as a simple and non-invasive stimulus at the PLGA/F127 NGC-implanted site transcutaneously in rats to investigate the feasibility of ultrasound for the enhanced nerve regeneration through the tube. The nerve regeneration behaviors within the ultrasound-stimulated PLGA/F127 NGCs were compared with the NGCs without the ultrasound treatment as well as normal nerve by histological and immunohistochemical observations. It was observed that the PLGA/F127 tube-implanted group applied with the ultrasound had more rapid nerve regeneration behavior (~ 0.71 mm/day) than the tube-implanted group without the ultrasound treatment (~ 0.48 mm/day). The ultrasound-treated tube group also showed greater neural tissue area as well as larger axon diameter and thicker myelin sheath than the tube group without the ultrasound treatment, indicating better nerve regeneration. The better nerve regeneration behavior in the our NGC/ultrasound system may be caused by the synergistic effect of the asymmetrically porous PLGA/F127 tube with unique properties (selective permeability, hydrophilicity and structural stability which can provide good environment for nerve regeneration), and physical stimulus (stimulation of the Schwann cells and activation of the neurotrophic factors). [This work was supported by the National Research Foundation of Korea (Grant No. 2010-0000496)].

Keywords. Peripheral nerve regeneration, nerve guide conduit, ultrasound, asymmetrically porous tube

(32.P11) GENOMIC IMPRINTING STATUS IN HESCS DURING NEURONAL DIFFERENTIATION

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Abstract. Gene expression and DNA methylation at imprinted loci has been used as an indicator of epigenetic status in the cell differentiation. It has been reported that epigenetic status of imprinted genes are predominantly stable in undifferentiated hESCs with some variations between hESC lines. However, much less is known about the expression and the epigenetic status of imprinted genes during differentiation of hESCs into a specified cell type.

To begin to answer these questions regarding expression and regulation of imprinted genes during directed differentiation of hESCs, we analyzed temporal expression profile and the epigenetic status of imprinted genes in hESCs undergoing neuronal differentiation. Temporal expression of 30 imprinted genes was examined by quantitative real-time PCR method and upregulation was found in most genes in hESCs during differentiation. In addition, allele-specific expression of 12 imprinted genes was examined and mostly unaltered monoallelic or biallelic expression was observed during differentiation. We also studied methylation patterns at the four DMRs responsible for regulating imprinted genes within the domain and at two CpG islands upstream of one imprinted genes. We found that monoallelic imprinted gene expression was consistent with maintenance of normal differential methylation during differentiation. These data provide the first demonstration that the epigenetic status of imprinted genes is generally stable in hESC during differentiation into neuronal lineage, also offering an in vitro model to investigate temporal expression and epigenetic status of imprinted genes during differentiation.

Keywords. differentiation, imprinting, embryonic stem cells, dopaminergic neuron

(32.P12) SIKVAV-MODIFIED POLY(2-HYDROXYETHYL METHACRYLATE) HYDROGEL SCAFFOLDS WITH ORIENTED CHANNELS FOR SPINAL CORD INJURY TREATMENT

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Spinal cord injury (SCI) often results in a loss of motor and sensory function as a consequence of the inability of axons to regenerate across the lesion. To create scaffolds that promote cell-surface interactions and that can serve as a permissive bridge for the re-establishment of damaged connections after spinal cord injury, poly(2-hydroxyethyl methacrylate-co-2-aminoethyl methacrylate) [P(HEMA-AEMA)] hydrogels with parallelly oriented channels and modified with the laminin-derived cell adhesion ligand SIKVAV have been developed.

Radical copolymerization of 2-hydroxyethyl methacrylate (HEMA) with low amounts (1 wt.%) of (2-aminoethyl methacrylate) (AEMA) and ethylene dimethacrylate crosslinking agent in the presence of ammonium oxalate crystals was used to produce a reactive superporous polymer hydrogel with oriented channels. The hydrogel, denoted as P(HEMA-AEMA), was activated with gama-thiobutyrolactone and 2,2´-dithiopyridine to immobilize the Ac-CGGASIKVAV-OH peptide. The viability and morphology of rat mesenchymal stem cells seeded on SIKVAV-modified P(HEMA-AEMA) hydrogels were analyzed to evaluate cell-surface interactions in vitro. In

an in vivo study of acute rat SCI, a hemisection was performed at the Th8 level, and a hydrogel was implanted to bridge the hemisection cavity. Histological evaluation was done 8 weeks after implantation.
SIKVAV-modified P(HEMA-AEMA) hydrogels were shown to support cell adhesion and proliferation in vitro. Histological evaluation revealed the good incorporation of the implanted hydrogel into the surrounding tissue, the progressive infiltration of connective tissue and the ingrowth of neurofilaments, Schwann cells and blood vessels into the hydrogel channels. Moreover, the parallel orientation of the hydrogel channels promoted axonal sprouting across the lesion.
The results show that SIKVAV-modified P(HEMA-AEMA) hydrogels with oriented channels have bioadhesive properties and are able to bridge a spinal cord lesion after implantation into a hemisection cavity.
Comments. Suported by: GAČR: P304/11/0731, P304/11/P633, 108/10/1560; KAN200520804, 1M0538 and IAA500390902.
Keywords. hydrogel, spinal cord repair, laminin, scaffold

(32.P13) LOCALIZATION OF CHOLINE ACETYLTRANSFERASE OF THE PERIPHERAL TYPE IN THE PRIMARY SENSORY NEURONS OF THE GUINEA PIG TRIGEMINAL GANGLION; COMPARISON WITH CGRP AND SP-CONTAINING CELLS
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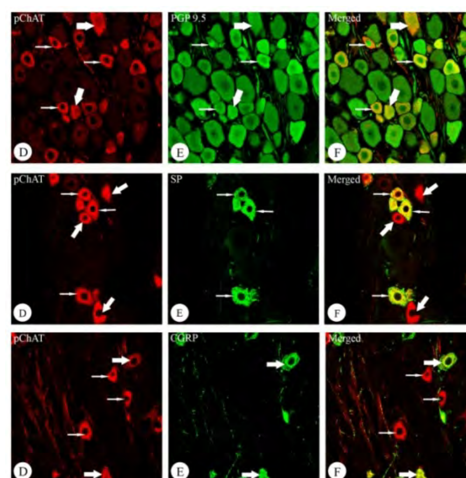
It has been demonstrated that a splice variant form of choline acetyltransferase (pChAT) is expressed in peripheral organs, including sensory ones, preferentially than the common type (cChAT). In order to identify the possible functional significance of pChAT in sensory functions, we characterized immunohistochemically pChAT-immunoreactive (IR) trigeminal ganglionic neurons in the guinea pig. We observed that the pChAT-immunoreactivity occurred in an almost uniform pattern and in a considerable number of all trigeminal neurons throughout the trigeminal ganglion (TG). The vast majority of pChAT-IR neurons were of small to medium-sized cell bodies, although large-sized neurons also observed. Most pChAT reactivity was mainly in the cytoplasm with few number of pChAT-IR neurons had nuclear staining. Double immunofluorescent study showed that a great proportion of substance P (SP)- and calcitonin gene-related peptide (CGRP)-immunoreactive trigeminal cells showed pChAT-immunoreactivity, although those with SP was outnumbered those with CGRP. The intracellular expression of pChAT (which differs from that of cChAT) probably reflecting a difference in physiological roles between pChAT and cChAT in ACh production in distinct intracellular compartments. The present data suggest also that pChAT plays roles not only in nociception, but also in other sensory functions such as mechanoreception mediating tactile sensation.
Keywords. Trigeminal ganglion; Substance P; Calcitonin gene-related peptide; Protein gene product 9.5; Guinea pig; Colocalization



(32.P14) EVALUATION OF THE SURFACE TOPOLOGY AND CELL BEHAVIOUR ON SOLVENT-CAST POLY (E-CAPROLACTONE) FILMS FOR PERIPHERAL NERVE GUIDANCE
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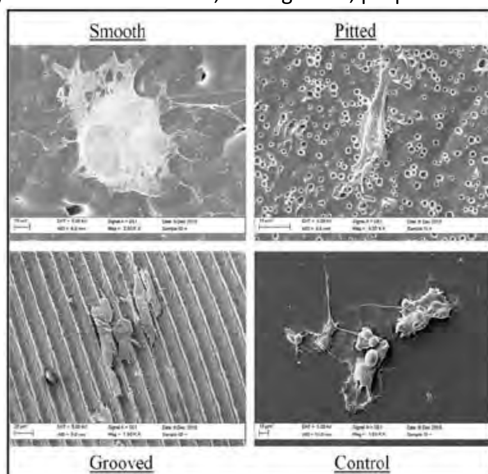
Introduction. Synthetic nerve guidance conduits are a promising alternative to autologous nerve graft treatment in peripheral nerve injury. PCL is already used for existing biomedical applications, but is a novel material for nerve guidance. This study will assess how altering the surface topology of ultrathin (42-93 µm) PCL films affects their mechanical properties as well as cell behaviour, particularly cellular alignment and orientation.
Methods. Smooth, pitted and grooved (width and spacing 10 µm, depth 5 µm) PCL films were cast from a PCL/dichloromethane solution (3% wt/v) onto silicon wafers. Scanning electron and atomic force microscopy were used to confirm the fabrication of film patterns. Tensile testing quantified the mechanical properties of the films. Motor neuron-like NG108-15 cells were cultured on the films for 3, 7, 10 and 14 days, before imaging with fluorescence and scanning electron microscopy.
Results. The air surface of the pitted films had a porosity of 53.2% and average pit diameter of 2.12 µm. Smooth films lacked pits, while the grooved films were successfully made through the solvent-casting process. Smooth films (11.9±1.8MPa) were stronger than pitted (5.9±1.5MPa) and grooved (4.4±1.3MPa, $p<0.01$). Grooved (50.2±16.6MPa) and pitted (61.2±22.4MPa) were more flexible than smooth (106.4±25.5MPa, $p<0.01$). Yet, grooved films still possessed greater tensile strength than fresh rat sciatic nerve (2.7±1 MPa). NG108-15 cells proliferated on each PCL pattern, confirming the biocompatibility of the material. Interestingly, scanning electron and fluorescence microscopy suggested cellular alignment only occurred on the grooved films, orientating in the same direction as the microgrooves.
Conclusion. The combination of strength and flexibility of grooved and pitted films make them attractive for surgical applications. Evidence of cellular alignment and organisation on the grooved films suggests their use in nerve conduits could improve nerve regeneration as axonal alignment is necessary for optimal function in peripheral nerves.

Keywords. nerve conduit, microgroove, properties



33. PHYSICAL METHODS AND TECHNIQUES FOR THE EVALUATION AND QUALITY CONTROL OF BIOMATERIALS AND ARTIFICIAL TISSUES

Chair: María del Mar Pérez Gómez
Co-chairs: Juan de Dios García López-Durán, Miguel Alaminos
Keynote speaker: Scott Prahl
Organizers: María del Mar Pérez Gómez, Juan de Dios García López-Durán, Ana-Maria Ionescu, Juan de la Cruz Cardona Pérez, Razvan Ionut Ghinea, Ana Yebra Rodríguez

Synopsis: Over the years we have seen the rapid development in the field of tissue engineering and regenerative medicine which continues to expand further in scope and depth. While much have been achieved through new insights and refined methods, the characterization of the basic physical properties are essential for the therapy and diagnostic techniques and also for the design process of artificial tissues. Typically, the most common physical properties studied are the optical and mechanical ones.

The optical properties are obtained using solutions of the radiative transport equation that express the optical properties in terms of readily measurable quantities. These solutions are either exact or approximate and correspond to the direct or indirect methods. Direct methods place stringent constraints on the sample to match the assumptions made for exact solution. Indirect methods relax the sample constraints but require approximations that are often invalid for tissue samples. The theory used in indirect methods usually falls into one of three categories: Beer's law, Kubelka-Munk, or the diffusion approximation. Beer's law neglects scattering and is inappropriate for thick scattering materials. The Kubelka-Munk method and variants are still used, but are limited in their accuracy. Methods based on the diffusion approximation or another similar approximation tend to be more accurate. These methods remain popular because they are easy to use, place relatively minor constraints on the type of sample, and are amenable to analytic manipulation. Other methods such as the IAD and the Monte Carlo methods have been developed to provide more accurate optical properties and can be made arbitrarily precise at the cost of increased computation time. The mechanical properties of tissue depend on many factors, including the type of tissue and the presence of disease or pathology.

Confocal microscopy technique has gained popularity in the scientific and industrial communities with typical applications in life sciences and material science, in therapy and diagnostic. It has been used to determine different properties of biomaterials and also to reconstruct three-dimensional structures from the obtained images. Basically, in the field of tissue engineering and regenerative medicine, the physical properties (optical and biomechanical) of the artificial tissue and biomaterials must resemble the properties of the tissue to be replaced. As it is generally true that a method developed for one problem can be applied to a very different set of problems, it is important that the general idea may be shared among wider audience in the field. Our aim is to boost interaction among different branches of physical properties of biological materials so that researchers may gain access to new developments in other fields. For this purpose we arrange this symposium to provide a forum for the discussion and dissemination of all aspects of design, implementation and application of any methods and techniques developed for the evaluation and quality control of artificial biological tissues and biomaterials. We hope that this symposium will bring fruitful results owing to your cooperation.

(33.KP) USING LIGHT TO QUANTITATIVELY MONITOR COMPOSITES DURING PHOTOCURING

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Introduction. Photocured polymers are widely used in dental applications and as tissue engineering constructs. The physical and mechanical properties of the composite change during curing; the appearance of the composite also changes. This talk summarizes insights gained into the photopolymerization process by quantitatively monitoring appearance.

Methods. The composite were 50:50 weight ratio of BIS-GMA:TEGDMA mixed with various sizes of strontium glass as filler and different concentrations (0-0.175 wt%) of camphorquinone (CQ). The curing irradiance was 100-400mW/cm2. Total reflection and total transmission spectra of filled and unfilled 1mm thick composite samples were measured before and after curing. Total transmittance was measured every 50ms from 400-900nm during curing. The refractive index of the unfilled resins was also measured during curing using an Abbé refractometer. Before and after reflectance and transmittance values were converted to scattering and absorption properties using the inverse adding-doubling technique. Transmission data during curing was analyzed by using red light to monitor changes in scattering and blue light to assess changes in absorption.

Results. The absorption spectrum of uncured composite measured matched the absorption spectrum of CQ in

unfilled resin. Uncured composite with and without CQ had the same scattering coefficient spectrum. The absorption and scattering coefficient at 469nm of composites with five different CQ concentrations decayed exponentially as a function of radiant exposure. The refractive index increased with radiant exposure.

Conclusion. The optical properties of a composite affect the light distribution within the composite during curing. Changes in absorption are caused by changes in CQ absorption. Mie scattering calculation indicate that scattering changes are directly attributable to index of refraction changes of the resin during curing.

Comments. Keynote speaker presentation Dr. Prahl SA

Keywords. Optical properties

(33.O1) THREE-DIMENSIONAL ANALYSIS OF HEART VALVE SCAFFOLDS SEEDED WITH VASCULAR CELLS

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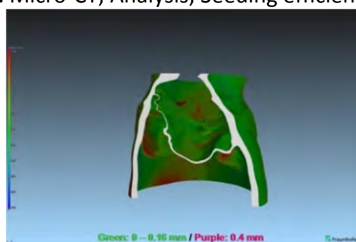
Introduction. Micro computerized tomography (µ-CT) is a new method that can provide a non-destructive, high-resolution, three dimensional analysis of objects. The aim of this study was to evaluate for the first time the feasibility of using µ-CT scanner as a standard 4D-evaluation method for tissue engineering applications.

Methods. Polyurethane aortic valve scaffolds (PAVSs) were produced at the iTV-Denkendorf by the spraying technique. Human vascular fibroblasts (FBs) and endothelial cells (ECs) were isolated from saphenous vein segments and expanded in culture. PAVSs were primarily seeded with human FBs and secondarily seeded with FBs, followed by a colonization with ECs. PAVSs were scanned before and after cell colonization with a µ-CT scanner. Colonization efficiency was additionally controlled by scanning electron microscopy (SEM) and immunohistochemical staining (IHC).

Results. SEM results showed a confluent cell layer on the PAVSs. IHC staining with CD31 (EC-Antibody) and TE-7 (FB-Antibody) revealed a positive reaction of both cell layers after a rest period of 9 days. Micro-CT Data allowed a quantitative visualization of the colonized cell layer.

Conclusions. Visualization and quantitative analysis of tissue engineered prostheses are possible with µ-CT scanner.

Keywords. Micro-CT; Analysis; Seeding efficiency



(33.O2) USING MACHINE LEARNING TECHNIQUES AND GENOMIC/PROTEOMIC INFORMATION FOR PROTEIN-PROTEIN CLASSIFICATION

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In modern biomedicine, one major objective is the extraction of functional models capable of predicting the function of genes from completely sequenced genomes. Although genomics has already provided a huge amount of molecular interaction data that have been used to construct maps for specific cellular networks, information regarding the biological roles of the products of these genes still remains as a major challenge. The contemporary proteome research is striving hard to elucidate the structure, interactions and functions of the numerous proteins that make up cells and organisms. Most proteins provide a specific function only by interacting with other proteins. It is a generally accepted fact that most essential biological processes involve protein-protein interactions (PPIs). Therefore the exhaustive identification of these PPIs, in the form of PPI maps, is crucial to systematically defining their cellular role. Such maps provide a valuable framework that can lead to a better understanding of the mechanisms underlying protein function and cellular processes and to the design of new and effective therapeutic approaches.

In this work, we propose a new approach to PPI dataset processing based on the extraction of genomic and proteomic information from well-known databases and the application of data mining techniques which will provide very accurate models with high levels of sensitivity and specificity in the classification of PPIs. One of the most analyzed organisms, Yeast will be studied. We processed a very high-confidence dataset by inserting up to 26 specific features obtained from well-known databases; half of them were calculated using two new similarity measures. Then, by applying feature selection, we obtained a final set composed of the most relevant features for predicting PPIs. The prediction capability of a Support Vector Machine (SVM) model has been validated by an analysis of the predictions in experimental, computational and literature-collected datasets.

Keywords. Protein to protein interaction. Intelligent systems, variable selection

(33.O3) NANOSTRUCTURED FIBRIN-AGAROSE CORNEAL CONSTRUCT: RHEOLOGICAL PROPERTIES FOR POTENTIAL CLINICAL APPLICATION

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Abstract. Since the cornea behaves as a viscoelastic structure that provides protection to the intraocular contents, any artificial substitute for a human cornea designed to replace part or the full thickness of damaged or diseased corneas must be resistant enough to undergo mechanical stress. The purpose of this study was the evaluation of the rheological behavior of the different artificial models of the human cornea as an essential part of the quality-control process of these bioengineered tissues.

Artificial substitutes of the human corneal stroma were developed using fibrin and fibrin-agarose with different agarose concentrations (0.025%, 0.05%, 0.1%, 0.2%, 0.3%) with human keratocytes immersed within. Samples of the different stromal substitutes were studied weekly until 8 weeks of development in culture. After each week in culture, samples were subjected to a novel nanostructuring technique and measurements of the elastic (G’) and viscous (G”) moduli were performed for using a controlled shear-stress rheometer (Bohlin CS-10, UK). The highest values of G’ and G” were found for the corneal subtitutes with higher agarose concentration (0.1%, 0.2% and 0.3%) (Figure 1).

Strikingly, the fibrin substitute with 0.1% agarose, in the first weeks of culture, showed G’ values equals to a half or one third of the values of the substitutes with higher concentrations of agarose, whereas from the fourth week on, these values are comparable.

The statistical analysis determined a high correlation between the agarose concentration and the elastic and viscous moduli with statistical significance ($p<0.05$) and also, an inverse correlation between the time in culture and the viscous modulus. In conclusion, these preliminary results suggest that the nanostructured artificial corneal stroma substitutes show good viscoelastic stability during time of development in culture.

Acknowledgements. Grants MAT2009-09795, FIS PI08/614.

Keywords. nanostructured cornea; elastic modulus; viscous modulus.

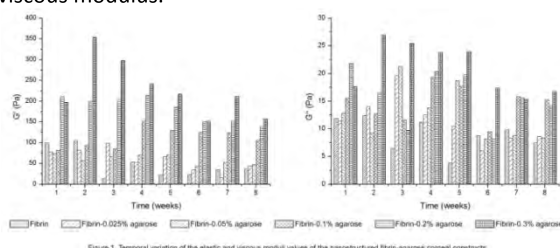

Figure 1. Temporal variation of the elastic and viscous moduli values of the nanostructured fibrin-agarose corneal constructs.

(33.O4) NON-INVASIVE QUALITY CONTROL FOR ISLET TRANSPLANTATION USING RAMAN SPECTROSCOPY

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Abstract. Type 1 diabetes patients with poorly controllable glucose levels, can be treated by intrahepatic transplantation of donor islets of Langerhans. During isolation, islets are exposed to mechanical stress and their cell-matrix relationship is disrupted, which may induce apoptosis. Before islets are transplanted into the patient, their quality needs to be assessed. Current quality control requires fixation and labeling and does not allow time-lapse studies on the same tissue. In this study we explore the feasibility of using Raman spectroscopy to perform functional studies on pancreatic islets and to monitor their quality over time.

We first used Raman spectroscopy to measure purified insulin and glucagon, the two main hormones produced by pancreatic islets. Raman bands at 520 and 640 cm-1 can be assigned to cysteine and tyrosine, amino acids that are present in insulin. Tryptophan, one of the building blocks of glucagon, causes specific bands at 759 and 1552 cm-1 (fig.1). These bands can be used as markers for the identification of beta and alpha cells in islet preparations. We subsequently measured human islets and compared their spectral characteristics to those of insulin and glucagon. Tryptophan-specific Raman bands were observed in the islets spectrum, suggestive for the presence of glucagon-producing alpha cells. Bands suggestive for the presence of insulin were not observed in the average islet spectrum, possibly because insulin is a weaker Raman scatterer (fig 1). High resolution local measurements on individual islet cells are currently performed to identify the presence of insulin-vesicles inside these cells.

Currently, we are extending these studies by investigating the effects of different substrates and extracellular matrix components on islet function using Raman spectroscopy. Our data provides the first steps towards a non-invasive and label-free method to study the quality of pancreatic islets, before transplantation in patients with type 1 diabetes.

Keywords. Raman spectroscopy, Quality control, Islet transplantation, Diabetes

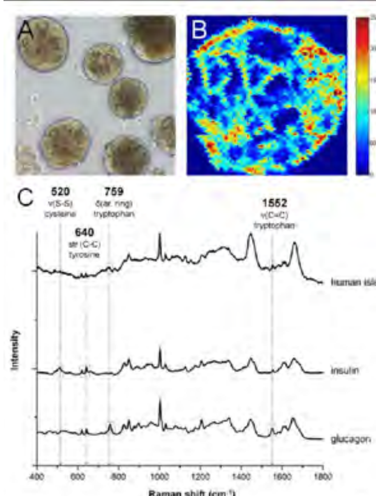

Figure 1 A) White light microscopy of human islets. B) Raman image showing the distribution of the 759 cm-1 tryptophan band, suggestive for the presence of glucagon, inside an islet. C) Average Raman spectrum of human islets compared to purified insulin and glucagon.

(33.O5) TAILORABLE, HIGHLY-ALIGNED POLY-DL-Γ-GLUTAMIC ACID FOR LIGAMENT TISSUE ENGINEERING

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Introduction. A tissue-engineered ligament requires appropriate mechanical properties, resistance to bulk degradation, and biocompatibility. Poly-DL-γ-Glutamic Acid (γ-PGA) is a water-soluble, non-toxic polypeptide produced by many Bacillus species, and undergoes enzymatic degradation rather than hydrolysis. We have modified γ-PGA by esterification[1] and utilized tensile deformation to produce highly-aligned molecular structures with ultra-high tensile strength[2]. This has enabled us to tailor γ-PGA’s mechanical properties over a broad range, targeting that of native ligaments.

Methods. To reduce the high water solubility of γ-PGA, we esterified[1] the side carboxylic groups using alkyl or aryl bromide to yield γ-PGA-Ethyl, γ-PGA-Propyl, and γ-PGA-Benzyl. Modified γ-PGA films were cast from a 5 % wt/wt solution in 1,1,1,3,3,3-hexafluoroisopropanol. Dumbbell shaped samples (l=4.5mm, w=1.2mm, t≈100µm) were cut and oriented using tensile deformation[2] at controlled temperatures above their Tg to draw ratios (λ=ε+1) of λ=2,4,6,8,10. Following orientation, samples were retested at RT. Cytotoxicity of scaffolds was assessed with ISO10993:5 cytotoxicity testing. Human foreskin fibroblasts (HFF, passage 2) were also cultured on oriented scaffolds for 7 days, and then visualized with LIVE/DEAD assay.

Results/Conclusions. Esterification of γ-PGA polymers decreases hydrophilicity, while tensile deformation allows for tailoring of mechanical properties over an exceptional range as shown in Figure. Both Young's modulus and tensile strength increased after drawing while strain was maintained at or above 7% for all polymers. Therefore, we can tailor these polymers over a wide range for the desired ligament applications. These enzymatically degradable materials were found to be non-cytotoxic and support cell growth. Taken together, our results hold promise for use in ligament tissue engineering, where mechanically appropriate, biodegradable scaffolds are required for joint stability.

References.

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Keywords. Tissue Engineering, Scaffold, Biomaterials, Mechanical Properties

	γ-PGA-Acid	γ-PGA-Ethyl		γ-PGA-Propyl		γ-PGA-Benzyl	
	λ=1	λ=1	λ=max	λ=1	λ=max	λ=1	λ=max
E, GPa	4.93	0.85	**3.85**	1.37	**2.54**	1.24	**5.36**
σ, MPa	94	15	**196**	29	**109**	28	**149**
ε, %	2	542	**16**	361	**36**	106	**7**

(33.O6) MICROFOCUS X-RAY COMPUTED TOMOGRAPHY BASED CHARACTERIZATION OF CALCIUM PHOSPHATE – STEM CELLS CONSTRUCTS COMBINED WITH EMPIRICAL MODELLING AS A TOOL FOR THE PREDICTION OF BONE FORMATION

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Introduction. For the evaluation of the bone forming capacity of stem cell – biomaterial combinations, both in vitro biomaterial screening/selection and in vivo assessment are required. For combinations of human periosteal derived cells (hPDCs) with clinical calcium phosphate (CaP) scaffolds, this study combined microfocus X-ray computed tomography (micro-CT) for initial material and explant characterisation with an empirical model to correlate material properties and in vivo bone formation.

Materials, methods and results. Five clinical scaffold types composed of CaP grains in an open collagen network (NuOss™, CopiOs™, Bio-Oss®, Collagraft™ and Vitoss®) were scanned using micro-CT. Image analysis showed significant differences in the structural properties, i.e. volume fraction and specific surface area of the grains, and the average particle size. After hPDC seeding and 8 weeks of ectopic implantation, the scaffolds were explanted and bone formation was quantified by micro-CT and histomorphometry, showing good a correlation (R = 0.994). Additionally, the bone interconnectivity was assessed using micro-CT, together with a spatial localisation of the bone formed within the scaffold structure (Fig. 1), showing a clear correspondence between the spatial CaP particle distribution and the amount of newly formed bone. A partial least square regression model was established, allowing the prediction of the bone forming capacity of the different CaP scaffolds using the material characteristics obtained from micro-CT, X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR).

Conclusions. After validation, micro-CT image analysis enables an in-depth 3D and quantitative characterization of the material properties of CaP scaffolds and their in vivo bone formation capacity in combination with hPDCs. The results from the micro-CT image analysis, together with XRD and FTIR analysis, have been used to develop a predictive model linking specific scaffold properties to the scaffold's bone forming capacity, which can provide a rationale for initial scaffold selection based on the materials characteristics.

Keywords. micro-CT image anlysis; emprical modelling; bone forming capacity; calcium phosphate – stem cell constructs

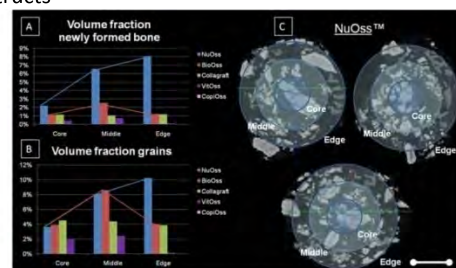

Fig. 1: For the different scaffold types (A) the micro-CT-based volume fraction of the newly formed bone distributed over the core, middle and edge of the scaffold and (B) the micro-CT-based volume fraction of the CaP particles distributed over the core, middle and edge of the scaffold, showing the same distribution as the volume fraction of newly formed bone. (C) Typical cross-sectional micro-CT images of a NuOss™ explant, indicating how the core, middle and edge region are defined (scale bar = 1 mm).

(33.O7) SELECTED ION FLOW TUBE MASS SPECTROMETRY AS A TOOL FOR MONITORING THE VOLATILE COMPOUNDS IN THE HEADSPACE ABOVE MESENCHYMAL STEM CELL CULTURES

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Selected ion flow tube mass spectrometry (SIFT-MS) is a real-time technique capable of quantifying and monitoring trace gases in a given volume at parts per billion (ppb) levels [1]. The goal of this project is to characterise biogenic volatile organic compounds (BVOCs)

produced by stem cell cultures and thereby develop SIFT-MS as a non-invasive tool for monitoring their production. Briefly, in the present experiments, a sample vapour is introduced into the helium carrier gas in the flow tube of the SIFT-MS instrument, within which the trace compounds in the sample react with selected precursor ions that have been injected into the carrier gas. The product ions of the reactions, which characterise the trace compounds, are detected and counted by a downstream mass spectrometer system.

Our headspace vapour analyses have shown that acetaldehyde, a known carcinogen which is present in DMEM medium, is removed when bone marrow-derived mesenchymal stem cells (MSCs) are incubated in the medium. These results mirror previous data obtained from SIFT-MS analyses of telomerase-producing lung fibroblast cells, but contrast with the finding that CALU-1 lung cancer cells produce acetaldehyde in amounts proportional to cell number [2]. In addition to these experiments, we have commenced preliminary investigations into BVOCs produced by bacteria, namely acetoin and diacetyl, with a view to developing a SIFT-MS-based early-warning system for the detection of microbial contaminations in stem cell culture.

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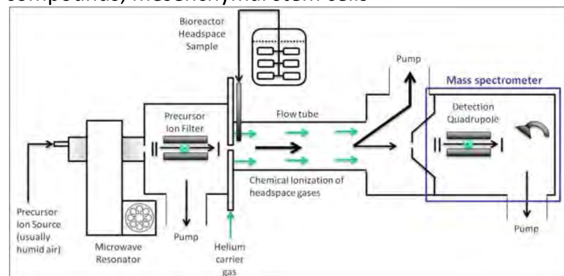
Keywords. Mass Spectrometry; biogenic volatile compounds; mesenchymal stem cells



(33.O8) LONG-TERM STORAGE OF ENGINEERED TISSUES VISUALIZATION OF CPA DIFFUSION IN COLLAGEN SCAFFOLDS

Bernemann I (1), Spindler R (1), Manuchehrabadi N (1), Choi J (2), Wolkers W (1), Bischof J (2), Glasmacher B (1)

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Introduction. Effective long-term storage of native and engineered tissues poses a specific challenge for biomedical applications. While significant progress has been made in cryopreservation of cells in suspension, cryopreservation of 3D-tissues is still a major problem. The diffusion of cryoprotective agents (CPA) into tissue is one of the major hurdles for succesful cryopreservation. In 3-dimensional native and cell seeded engineered scaffolds CPAs like dimethyl sulfoxide (DMSO) should be homogeneously distributed to protect them from freezing damages. A local excess of CPA in the construct will damage the cells due to the general toxic effects of CPAs, whereas insufficient CPA concentrations will lead to cryopreservation damage. This study was performed to measure and visualize the effective diffusion of DMSO within engineered collagen scaffolds using computer tomography (CT).

Materials and Methods. Collagen scaffolds with an average pore size of 100µm (dimension: 30x30x10 mm3) and a porosity of 98% were self-manufactured [2]. The scaffolds were stored in phophate buffered saline (PBS). Scaffolds were transferred directly in 10% (v/v) DMSO in PBS. Computer tomographic images were acquired immediately every 1.5 minutes over a period of 3 hours. Grey scale values that were determined from the images were converted in Hounsfield units (HU). The conversion from HU to DMSO concentration indicates CPA dispersion within the tissue. The DMSO loading process of the scaffold could thus be measured and visualized in real time.

Conclusion. The study showed that incubation times of more than 3h are required to achieve homogenous CPA distribution in collagen scaffolds of this size.

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Acknowledgments. This work is supported by funding from the Deutsche Forschungsgemeinschaft (DFG) for the Cluster of Excellence REBIRTH (from Regenerative Biology to Reconstructive Therapy).

Keywords. Long-term storage, CPA diffusion, Freezing of Tissue Engineered Constructs, Computer Tomography

(33.O9) CONTRAST TRANSFER FUNCTION FOR EVALUATING THE OPTICAL QUALITY OF BIOENGINEERED HUMAN CORNEAL STROMA

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Optical characterization of biomaterials used for the construction of corneal substitutes by tissue engineering is essential to ensure a proper functionality of these specifics tissues due to since they are intended to replace the principal refractive component of eye responsible for image-forming. In this sense, the present work aims to describe a new developed non-invasive method based on the Contrast Transfer Function (CTF) for evaluating the optical quality of these tissues. Bioengineered substitutes of the human corneal stroma were developed using fibrin and fibrin with agarose (0.025%, 0.05%, 0.1%, 0.2%, 0.3%) with human keratocytes immersed within and evaluated every two weeks until 8 weeks. Twelve bar patterns CTF frequencies (0.05, 0.06, 0.08, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 0.65, 1.00 and 2.00 cycles/mm) were generated by a laptop's LCD monitor and the images of these bar patterns through the samples were captured using a high-resolution B/W PixelFly CCD camera (1360x1024 pixels). CTF was calculated by image analysis, and the cut-off spatial frequency and the area under CTF (CTFA) were determined.

The results showed that the optical quality increased with increasing agarose concentration (CTFA ANOVA $p=0.022$ and cut-off frequency ANOVA $p=0.003$) and time of development in culture of the corneal substitutes (CTFA

p=0.013 and cut-off frequency p=0.025). This proposed non-invasive method and experimental set-up allow evaluating the optical quality without modifying the conditions of culture. Furthermore, the use of a laptop's LCD monitor to present the bar patterns is a versatile technique, enabling quick and easy modification of the spatial frequency of the bar patterns. In summary, the method proposed in this study could be used to evaluate the optical quality of any translucent biomaterial.

Acknowledgements. Grants MAT2009-09795, FIS PI08/614.

Keywords. Contrast Transfer Function, Human Corneal Stroma Substitute, Fibrin, Agarose

(33.O10) FLUORESCENT MICROPARTICLE INCORPORATION IN CHONDROGENIC CELL AGGREGATES TO MONITOR LOCAL OXYGEN TENSION

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Introduction. High cell density aggregate cultures provide a three-dimensional environment to achieve chondrogenic differentiation in vitro [1], but at the same time can introduce large gradients in nutrient and oxygen concentration. These gradients, which are determined by the interplay between diffusion and consumption, may however result in insufficient nutrient and oxygen supply within the aggregate and may in turn lead to a decrease in cell viability.

To study this problem, we have developed a method to incorporate oxygen sensitive particles (OSPs) within cell aggregates to provide spatial information on oxygen tension and hence define optimal oxygen conditions.

Materials and Methods. Fluorescent OSPs were produced as described by Acosta et al. [2]. For efficient and robust OSP integration in a cell aggregate a noncovalent streptavidin biotin interaction based method was used [3].

OSP distribution and the effect of the incorporated particles on aggregate size, morphology, cell number and viability were assessed for the prechondrogenic mouse clonal cell line, ATDC5. Investigated particle concentrations were 0, 0.1%, 0.2%, 1% and 10% (particle number / cell number).

Results. No statistically significant difference could be detected with reference to cell aggregate size, morphology or cell number, when up to 1% of OSPs was incorporated within aggregates. LIVE/DEAD viability images showed no difference in cellular viability of OSP integrated aggregates as compared to control conditions without OSPs (fig. 1).

Discussion and Conclusions. A method for consistent OSP incorporation into cell aggregate cultures was validated within the investigated concentration range and can now be further explored as a continuous local oxygen monitoring sensor to follow oxygen tension dynamics during cell condensation and to optimize aggregate culture conditions.

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Comments. Dennis Lambrechts, Greet Kerckhofs, Hans Van Oosterwyck and Jan Schrooten are also members of the institution: Prometheus division of skeletal tissue engineering Leuven, as indicated with (3)

Keywords. Aggregation, Avidin-Biotin, Oxygen, Optical sensor

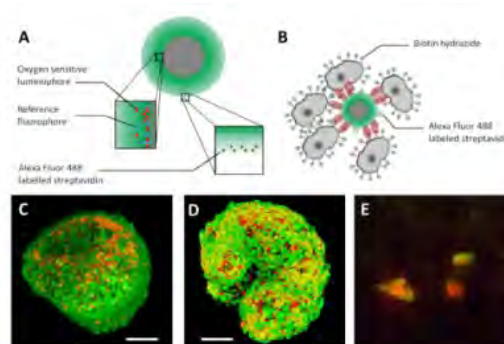

Fig 1: (A) OSP design and (B) integration scheme. (C) Confocal LIVE/DEAD viability image of an ATDC5 aggregate with 0.2% of OSPs and (D) control without particles. (E) Confocal image of OSPs integrated in a cell aggregate (green: Alexa Fluor 488 used for particle detection, red: oxygen sensitive luminophore, blue: reference fluorophore).

(33.O11) EFFECT OF PLASMA TREATMENT ON POLYLACTIC ACID (PLA) PROPERTIES USED IN TISSUE ENGINEERING

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Abstract. Polylactic acid (PLA) is a well-known biodegradable aliphatic polymer and has been previously used for several biomedical applications such as bone fixation devices (plates, pins, screws, etc.) and as tissue engineering scaffolds. Although, PLA is known to be biocompatible and widely used in the field of medicine, its low wettability and surface energy affect cell attachment and proliferation and remain an important issue.

However, to be as competitive, its properties must be improved and in particular its surface properties. In the present work, a microwave plasma assisted surface treatment has been applied to improve the wettability of the PLA. From an experimental design, we defined the most significant parameters of the process. The effect of this treatment on the mechanical, thermal, morphological, surface composition and barrier properties of the PLA was carried out using different Ar-N2-O2 gas mixtures. Three compositions of the N2-O2 mixture diluted in argon were used: 100% O2, 50:50% N2:O2, and 94:6% N2:O2. We found that this treatment does not improve the barrier and mechanical properties, but did not alter them too. Indeed, the plasma processing

only modifies the top surface without affecting the bulk properties. The study of surface properties using Atomic Force Microscopy (AFM) and X-ray Photoelectron Spectroscopy (XPS) in conjunction with the measurement of water contact angle enabled us to understand the phenomena responsible for improving the wettability. Also, from contact angle, XPS and AFM results, it is shown that the discharge atmosphere can have a significant effect on the PLA surface modifications. We concluded that the improvement of wettability is mainly due to an etching effect that modifies the surface properties of the polymer. Functionalisation could have taken place though it has been confirmed by XPS analysis. Therefore, this functionalisation contributes also to the improvement of the wettability and explains the aging effect.

Keywords. Plasma, poly (lactic acid), physicochemical characterization, experimental design

(33.P1) A METHOD TO SCREEN AND EVALUATE TISSUE ADHESIVES FOR JOINT TISSUE ENGINEERING

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Introduction. In tissue engineering, tissue adhesives like fibrin glue are used for cell-, transplant- and tissue fixation. Besides clinical approval and biocompatibility, the adhesive strength is important, and its analysis is crucial for tissue adhesive selection and development. However, most standardized test methods do not consider the structure of defect tissues. Therefore, our aim was to develop a method to screen and evaluate tissue adhesives for joint tissue engineering.

Methods. Tissue holders were designed, manufactured and used to prepare defined and viable bone-, cartilage-, bone-cartilage- and cartilage-defect cylinders from porcine femoral condyles, and to fix test tissues to the jigs of a universal testing machine (Fig.1). Different operation modes were applied to analyze the adhesive strength of tissue adhesives that stick tissue cylinders together or fix clinical applied fibrin/PLGA-cell-transplants or tissues in cartilage-defect cylinders (Fig.1). We analyzed the capability of the method to evaluate adhesive properties of fibrin glue, and we performed a screening of self-manufactured collagens to show the potential of the method to identify new tissue adhesives.

Results. Evaluation of the test cylinders revealed that they are viable and re-useable. Fibrin glue had a high adhesive strength in test modes filling cartilage-defect cylinders with cartilage tissue (30.0kPa) or fibrin/PLGA-cell-transplants (33.0kPa). Adhesion to bone (7.0kPa) was remarkably lower than adhesion to cartilage (2.6kPa). Out of seven self-manufactured collagen formulations, a candidate with superior adhesion to bone and cartilage, but a shortcoming in fixing fibrin/PLGA-cell-transplant was identified.

Conclusions. The method considers the tissue engineering requirements and is suitable to screen and identify tissue adhesives for joint repair applications. Moreover, the technique is translatable to other species or modes of measurements like torsion analysis.

Acknowledgements. This study was supported by grants from the Investitionsbank Berlin and the European Regional Development Fund (grant no: 10140057).

Keywords. tissue adhesives, adhesive strength measurement, joint cartilage repair

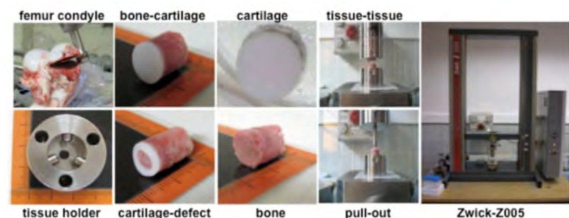

Figure 1: Porcine femur condyle, tissue holder, test cylinders, operation modes and testing machine.

(33.P2) MINIMAL-INVASIVE CARIES REMOVAL USING POLYMER BURS: A DIGITAL IMAGE EVALUATION

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Introduction. In minimal-invasive dentistry, infected-irreversibly denatured dentine should be removed selectively in order to preserve caries-affected dentin, which is a demineralized collagen matrix. It constitutes suitable collagen scaffold to be remineralized, as it possesses remineralization/healing potential. This is hardly achievable clinically with the currently available caries-excavation techniques, being the potentially remineralizable tissue often sacrificed.

Objective. To assess the efficiency and tissue selectivity for carious dentin removal using new designed polymers burs vs. conventional carbide burs.

Methods. Extracted carious human molars were longitudinally sectioned in the point of maximum lesion depth. Following a split tooth design each half of the tooth was assigned to carbide or polymer burs. Visual-tactile clinical parameters were employed for caries excavation. Digital images were taken before and after caries removal. Lines delimiting caries affected, caries infected, sound dentin and caries removal were segmented in images. A digital image processing software was employed to calculate the effectiveness and selectivity of carious infected dentin removal.

Results. Carbide burs (mean ratio:-0.44) removed the largest amount of caries-affected dentin leading to overexcavation effect, and did not leave carious dentin. Polymer burs (mean ratio: 0.09) produced the least amount of overexcavation and the major coincidence lines, in some cases underexcavation was attained.

Conclusions. The ideal caries-excavation tool was the polymer bur that selectively removes the irreversibly destroyed tissue, but left the potentially remineralizable tissue, even when it revealed a tendency towards underexcavation.

Acknowledgements. CICYT/FEDER MAT2008-02347/MAT, JA-P08-CTS-3944, P07-CTS-2568, MAT2009-09795, SS White Burs Inc.

Keywords. caries removal, caries affected dentin, polymer burs, bioremineralization

(33.P3) OPTICAL CHARACTERIZATION IN DIFFERENT BOVINE DENTIN ZONES

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Introduction and Objective. The success in restorative dentistry is based on functional and esthetic results. To achieve esthetics, the knowledge of the concept of optical characterization is essential. The purpose of this study was to evaluate optical properties of different bovine dentin zones to establish suitability criteria for aesthetic restorative materials.

Methods. Twenty bovine teeth were used. Five discs of 0.5mm of thickness were prepared for each dentin zone analyzed. The dentin zones evaluated were: cervical deep (CDD), medial deep (MDD), incisal deep (IDD) and medial superficial dentine (MSD). Each specimen was polished with silicon carbide papers from 220 to 4000 grits and finally with alumina slurry of 1, 0.3 and 0.05µm. Difuse reflectance was measured against white and black backgrounds, using a spectroradiometer with D65 daylight simulator source (geometry difusse/0º). The scattering and absorption coefficients (S and K) and transmittance were calculated using Kubelka-Munk's equations. Kruskal-Wallis, Mann-Whitney tests, and VAF coefficient were applied to statistically analyze the data. A qualitative evaluation of the different dentin zones was made using Scanning Electronic Microscope (SEM) images.

Results and Conclusions. Generally, the statistical analysis (VAF>92%) showed a similar spectral behavior of the scattering and absorption coefficients and transmittance for the different zones analyzed. The comparison among zones indicated that the spectral values of the transmittance presented statistically significant differences $(p<0.05)$ for each dentin zone analyzed. It was found that CCD and MDD had higher values of transmittance than IDD and MSD. SEM images showed different distribution and orientation of dentin tubules in the zones evaluated which could explain different translucency values. This finding should be considered to select appropriated dental materials to replace this dental structure in its different areas.

Keywords. scattering, absorption, translucency, bovine dentin

(33.P4) ON COLOR THRESHOLD CALCULATIONS IN ORAL TISSUES AND DENTAL MATERIALS

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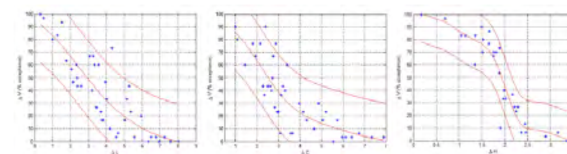
Introduction. Biological tissues and consequent restorative materials designed to replace them are complex systems subjected to external stimuli. A deeper understanding of their behavior requires extensive experimental studies. In this sense, mathematical models and computational methodologies can be used to simulate the expected behavior of these complex processes. Traditionally, in some specific cases, simple mathematical models are supposed to identify phenomena behavior, and are thus used to estimate a number of properties. In recent years, computational intelligence has opened the door to solve more complex problems under no previous supposition. In this sense, Fuzzy Logic Approximation has proved to be a powerful tool for some specific applications in the biomaterials and artificial tissue fields. The present study aims to apply a novel Takagi-Sugeno-Kang (TSK) Fuzzy System to determine Lightness, Chroma and Hue acceptability limits in dental materials.

Methods. A total of 58 dental ceramic disks were fabricated and the color of each sample was measured. All disks were combined to create a total of 1653 pairs. The pairs were judged in ascending series by a panel of 30 observers. For the 50:50% limits calculations a TSK Fuzzy Approximation with Gaussian membership functions and constant consequents was used to perform the approximation of the percentage of answers against the instrumentally measured color difference.

Results. The values of percent ''unacceptable'' by the observers against the corresponding instrumentally measured color difference and 95% confidence (Figure). For Lightness, the determined 50:50% acceptability limit was 2.92 (1.22-4.96, r2=0,76), for Chroma it was 2.52 (1.31-4.19, r2=0,71) and for Hue it was 1.90 (1.63-2.15, r2=0,88).

Conclusions. The TSK Fuzzy Approximation proved to be a useful tool that satisfactory fulfilled the color threshold calculation procedure for oral tissues and dental materials, suggesting its use for this type of research and for other potential applications in adjacent fields.

Keywords. oral tissues, dental materials, fuzzy logic, color threshold



(33.P5) DETERMINING BONE CHEMICAL AND MINERAL COMPOSITION FROM FTIR AND ATR-FTIR SPECTROSCOPIES

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Bone is a composite biomaterial mainly composed of packed collagen fibres and nano-sized hydroxylapatite crystals. Bone mineral characteristics such as crystallinity, carbonate and phosphate mineral content may vary as a function of age, sex, diet, bone location or pathological state of organisms. Quantitative measurements of bone mineral composition can provide insights into the bone mineral formation in health and disease. Fourier Transform Infrared (FTIR) and Attenuated Total Reflectance Infrared Fourier Transform (ATR-FTIR) Spectroscopies are well suited techniques to evaluate the variations in the chemical composition and mineralogy in bone. The advantageous application of these techniques to characterize bone properties and state of development as well as health state and disease is here described and

applied to specific cases of study. We have applied this methodology to study changes in bone mineral composition caused by different processes including aging and environmental pollution. For instance, results obtained from deconvoluted IR spectra provide representative information about the evolution of bone mineral composition as a function of age. The clearest change observed is an increase in bone mineral crystallinity with aging, indicating that crystals increase in size and/or perfection as bone maturation proceeds. This change is also correlated with an increase in the degree of mineralization on bone tissue, determined as the intensity ratio between phosphate and amide I bands. Bone samples were also analyzed by X-ray diffraction to confirm the mineralogy of the bone and its crystallinity. The present communication aims to give a more complete comprehension in the study of bone mineral using FTIR and ATR-FTIR to investigate and determinate changes or variations on its composition. Comparative results of spectra using both spectroscopies are discussed throughout this study.

Keywords. biomaterial, bone, mineral composition, FTIR, ATR-FTIR

(33.P6) INTELLIGENT SYSTEMS FOR CLASSIFICATION OF MYOCARDIAL STEM CELL DIFFERENTIATION BY THE ANALYSIS OF DIFFERENT GROWTH FACTORS

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Stem cells are a special type of undifferentiated cells that are capable of dividing indefinitely without losing its properties and get specialized cells through the process known as cell differentiation. In human and higher mammals, stem cells sustain tissues composed of cells that have a limited lifespan and lose the ability of self-renewal such as blood and epidermis cells, by providing new cells differentiated.

Many articles are focused on experimental studies about stem cell differentiation, in all of them the role of the growth factors is specially emphasized. Moreover, there are different mathematical models applied in this field of biomedicine that offer a description of the stem cell differentiation problem and simulate the effects of the growth factors on this process. The main limitation of these models is the absence of a detailed study and description of the variables or factors considered and the influence of them on the differentiation of the stem cells. This generates a great diversity and variability in the experimental and clinical results. Therefore, it is necessary the development of predictive models that facilitate the design of experiments and contribute to the understanding of the stem cells' behaviour. In this work, we propose to use intelligent systems to characterize the procedure of myocardial stem cell differentiation analyzing different growth factors. This task is performed in two phases: a) Making a hybrid, heterogeneous and hierarchical feature selection method to select the relevant variables in myocardial stem cell differentiation and performing and statistical analysis (in the problem of stem cell differentiation, feature selection is a key upstream. This process allows us to find the most important factors in the system); b) Development of intelligent systems for classification of myocardial stem cell differentiation (a priori two different techniques will be analyzed: decision tress and Support Vector Machines -SVM).

Keywords. Intelligent systems for classification; myocardial stem cell differentiation

(33.P7) NON-DESTRUCTIVE MONITORING OF THE EFFECT OF CULTURE CONDITIONS ON CORNEAL STROMAL CELL DIFFERENTIATION IN HYDROGELS

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Introduction. Collagen hydrogels have been extensively used as scaffolds for corneal tissue engineering. However, corneal stromal cells differentiate into fibroblasts in the hydrogel in vitro culture, rather than keratocytes. The aim of this study is to optimize culture conditions via chemical cues (media supplements) in order to control keratocyte phenotype, which improves the current state of corneal stromal tissue engineering models. Novel, non-destructive monitoring protocols were established to reveal the stromal cells' response under the different culture conditions in terms of the rate of contraction and mechanical properties.

Materials & Methods. A human corneal stromal model was constructed by seeding 5x105 stromal cells in 0.5 mL collagen gel. A non-destructive spherical indentation technique was used to examine the mechanical properties of the individual collagen hydrogel specimens under keratocyte or fibroblast media respectively every 3 days up to 28 days. The amount of gel contraction caused by the cells was measured by optical coherence tomography in parallel. The quantitative PCR with respect to the expression of keratocytic markers was conducted to cross-validate the observed physical properties.

Results & Discussion. It was confirmed that the culture conditions can induce the corneal fibroblasts to partially trans-differentiate into a keratocyte phenotype. Stromal cells cultured in hydrogels under keratocyte media with insulin and without serum exhibited constant elastic modulus and gel dimension, indicating that contraction was suppressed and that the quiescent characteristic of keratocytes was restored, which was cross-validated by the expression of keratocan and ALDH3; whilst stromal cell-gel cultured with serum demonstrated continuously increased modulus and reduction of thickness, typical of fibroblast phenotype.

Conclusions. The alteration of supplements in culture media can facilitate the differentiation of corneal stromal cells from fibroblasts towards a keratocytic lineage. This can potentially enhance the field of corneal tissue engineering using collagen hydrogel models. The non-destructive monitoring protocols provide convenient tools for observing biological phenomenon for prolonged culture periods in the same specimen.

Acknowledgements. Mr Ian Wimpenny, KMF Precision Sheet Metal, Newcastle-Under-Lyme, UK.

Keywords. Cornea, keratocyte, mechanical deformation

(33.P8) COMPARING THREE METHODS FOR GLOSS MEASUREMENTS OF DENTAL-RESIN COMPOSITES

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Abstract. Gloss can be defined as an uneven geometrical light distribution reflected by the surface of a specimen, with an increased flux in the specular direction. The importance of surface gloss among the appearance properties of aesthetic dental materials has been described in the literature. Experimental devices, called "glossmeters", that measure this specular gloss, have been standardized and are widely used in industry. Nevertheless, many researchers reported the limitations of this type of measurements and proposed several alternatives.
The aim of this study is to compare gloss measurements of six commercial dental-resin composites of different shades (CT and A3.5B Filtek^{TM} Supreme XT (3M ESPE) and A1, A2, A3.5 and T Tetric© EvoCeram (Ivoclar Vivadent)) using three different methods.
After photopolymerization, gloss was measured with a commercial glossmeter (Multi-Gloss 268, Minolta) and also calculated from data obtained with a spectrorradiometer (SpectraScan PR-704, Photo Research Inc. Chatsworth, USA) as:
a) the ratio between the reflected flux, in a given diagram centred on the specular direction at the surface of the sample and the reflected flux, in the same conditions, at the surface of a standard sample.
b) the proportion of specular reflection to diffuse reflection.
Results show that, although the results differ depending on the measurement method used, there is a correlation between them. This suggests the need to standardize the gloss measurement method, since gloss values based on different methods should not be compared directly.
Acknowledgements. Grant MAT 2009-09795
Keywords. Gloss measurements, dental-resin composites

(33.P9) OPTICAL POLARIZATION MEASUREMENTS OF DENTAL TISSUES AND NANOCOMPOSITES

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Abstract. Dental research has been transitioning gradually from classical restorative dentistry to regenerative dentistry. Regeneration of a functional tooth is a promising strategy for replacing an irreversibly diseased tooth and thus knowledge of its physical characteristics is essential in the design process. Polarization measurements for biological tissues must be performed since many of them are intrinsically birefringent. Furthermore, since birefringence can be related to other physical properties, these measurements could provide extensive information concerning the tissues analyzed. This study aims to measure the polarized light scattered off dental tissues and dental-resin composites in order to study their optical polarization behavior.
A He-Ne laser, two linear polarizers and a detection system based on a photodiode were used. The laser beam was passed through a linear polarizer placed in front of the sample, beyond which were placed the second polarizer (analyzer) and the photodiode connected to the multimeter. First, the maximum laser-light intensity was attained without the sample. This indicated that the transmission axes of the polarizers were parallel. Then, the sample was placed between the polarizers and the polarization shift of the scattered laser light was determined by rotating the analyzer until maximum light intensity was reached. Human enamel and dentine, and two dental resin composites (nanocomposite and hybrid) were studied under repeatability conditions (20 measurements) and the associated dispersion was calculated. The measurements were repeated for three different locations on the sample. Preliminary results (Table 1) show that for the dental resins and the human enamel, the polarization shifts are not likely to be significant, contrary to what happens for the human dentine. These values could be used in the design and development process of a functional tooth.
Acknowledgements. supported by MAT2009-09795 and FIS2007-66671-C02-02 projects from Ministerio de Ciencia e Innovación of Spain.
Keywords. lasers, polarization, nanocomposites, dental tissues, optical and physical properties

OPTICAL POLARIZATION MEASUREMENTS OF DENTAL TISSUES AND NANOCOMPOSITES

Table 1 Average values and uncertainties associated of the polarization shift in dental tissues and resin composites

Dental Material	Average Value (°)	Uncertainty (°)
Human Dentine	(7)	2
Human Enamel	2	2
Nanocomposite	2	3
Hybrid	2	3

(33.P10) INFLUENCE ON TRANSPARENCY OF A NANOSTRUCTURING TECHNIQUE FOR THE GENERATION OF ARTIFICIAL CORNEAS

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Abstract. Corneal transparency has been the subject of numerous studies over the years since understanding the function of the native tissue is very important in the design process for engineering a corneal tissue replacement.
This study aims the transparency evaluation of different artificial models of the human cornea generated by tissue engineering using a nanostructuring technique as an essential part of the quality-control process of these bioengineered tissues. This new technique allowed a partially dehydration of the fibrin-agarose biomaterial and induced complex interfibrillar changes at the nanometrical scale (nanostructuring) that could modify the properties of this biomaterial.
First, 12 types of bioengineered corneal stroma substitutes were generated in laboratory: human fibrin stromas with increasing concentration of agarose (0%, 0.025%, 0.05%, 0.1%, 0.2% and 0.3%) non-nanostructured (N-NCC) and nanostructured constructs (NCC). For nanostructuring, samples were transferred to a specific

chamber in which 4-6 layers of Whatman 3MM absorbent papers were put above and below the sample. To prevent the stromal construct to stick to the paper, a nylon porous membrane was put between the sample and the paper layers. Then, a flat crystal surface was set on top of the system and a total of 1,000Pa of pressure was applied. The transparency was calculated from the spectral reflectance measurements of the artificial corneal constructs using a spectroradiometer. The transparency temporal variation had a similar spectral behavior, with lower values for shorter wavelengths, which gradually increased to medium wavelengths and longer. The transparency values of the N-NCC ranged from 80 to 97%, while the values of the NCC ranged from 90 to 97%.
In conclusion, nanostructuring did not modify the spectral behavior nor the high transparency levels of both N-NCC and NCC (good-quality corneas are at least 60% transparent), supporting their use clinically.
Acknowledgements. Grants MAT2009-09795, FIS PI08/614.
Keywords. nanostructured cornea; transparency

34. PLACENTAL TISSUES - A NEW AVENUE IN REGENERATIVE MEDICINE

Chair: Ornella Parolini
Co-chair: Sebastián San Martín
Keynote speaker: Ornella Parolini
Organizer: Ornella Parolini
Synopsis: The clinical success of a regenerative cell-mediated therapeutic approach is strictly dependent upon the source of cells and the achievement of immune tolerance. Placental tissues combine two appealing features for cell-mediated therapy, namely the potentiality of cell differentiation, due to the early embryological origin of these tissue such as the amniotic membrane and the immunological intrinsic characteristics of the placenta, where during pregnancy the mother's immune system tolerates a genetically and immunologically foreign foetus.
Therefore, the study of placenta-derived cells (PDCs) constitutes a novel field of research which is attracting an ever-increasing level of interest for its potential to provide cells which are not only available in large supply, easily procured and free of ethical barriers for their use, but which also display high plasticity and interesting immunomodulatory features. In vitro experimental research has demonstrated that PDCs do not induce an allogeneic or xenogeneic immune response, inhibit lymphocyte proliferation induced by alloantigens or mitogenic stimulus and modulate dendritic cell differentiation. Promising in vivo preclinical data has shown that placenta-derived cells can engraft in different organs after xenotransplantation into neonatal swine and rats, suggesting an active tolerance process. In animal models of Parkinson's disease and cerebral ischemia, PDCs have been shown to offer neuroprotection and functional recovery. Similarly, PDCs produced significant locomotor improvement in bonnet monkeys and rats with spinal cord injury, a condition in which the inflammation-mediated "secondary injury" plays a greater role than the primary crush. Furthermore, PDCs have been demonstrated to exert anti-fibrotic effect in pre-clinical animal models of different diseases. They indeed reduced lung fibrosis in mice challenged with bleomycin, irrespective of transplantation route (intra-tracheal vs intra-peritoneal) and transplantation type (allogeneic vs xenogeneic).
Finally it was demonstrated that amniotic membrane from human placenta at term, applied as a patch onto ischemic rat hearts preserves cardiac dimensions and function, while when applied to the liver surface is able to reduce the severity and progression of liver fibrosis in a rat model of bile duct ligation. Together, these considerations have made cells derived from the placenta prime candidates for application in regenerative medicine, where they could help to regenerate damaged or diseased tissues while also minimizing the risk of graft versus host disease. Although further work needs to be undertaken to fully characterize the potential of these cells for clinical application, the data obtained to date are extremely encouraging. This symposium will provide an overview on the most recent findings in the field of placenta-derived cell research: from basic research, to their pre-clinical applications, to implementation of regulations on cell products. The topics presented will be as follows:
1. Updated overview regarding identification and characterization of placenta-derived stem cells
2. *In vitro* studies of immunomodulatory properties of mesenchymal placental cells
3. Mesenchymal placental cells as immunomodulators *in vivo*
4. Applications of placenta-derived cells in tissue(s) regeneration
5. Placenta-derived cells and inflammatory disease
6. From research to GMP-level tissue banking and placenta-derived cell preparation

(34.KP) PLACENTAL TISSUES – OPENING NEW DOORS IN REGENERATIVE MEDICINE

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Introduction. For many years, scientists have been fascinated by the placenta due to its nutritive and protective roles during fetal life and its role in fetomaternal tolerance. Nowadays, however, the placenta is also proving to be an appealing material for regenerative medicine. In keeping with the theme of this congress, the placenta indeed represents a source of "cells and tissues" that are promising candidates "as material for advanced therapies". Different stem/progenitor cells have been isolated from various placental tissues, and these have been shown to display multilineage differentiation potential as well as interesting immunomodulatory features. The amniotic membrane (AM) is a tissue with well-known surgical applications which are based on its anti-scarring, anti-inflammatory and wound healing properties, with the additional advantage that allogeneic transplantation of AM does not induce immune rejection. All of these

considerations have prompted scientists to investigate placental cell-based therapies in pre-clinical animal models, with encouraging results obtained to date. In particular, our group is addressing this topic mainly for treatment of pathologies related to inflammatory and fibrotic mechanisms.

Methods. Isolation of fetal membrane-derived cells and preparation of AM fragments. Transplantation of these materials into animal models. Histological evaluation of fibrosis. Measurement of cardiac functions.

Results. Transplantation of either allogeneic or xenogeneic fetal membrane-derived cells reduced lung fibrosis in bleomycin-challenged mice. Successful outcomes were also obtained when AM fragments were applied as patches onto rat hearts with cardiac ischemia and onto the liver surface of rats with fibrosis induced by bile duct ligation. In all of these pre-clinical settings, we found that donor cells in host tissues were rare or absent, suggesting that placental cells might exert reparative effects through yet unknown paracrine factors.

Conclusions. Our results reinforce the hypothesis that placenta constitutes a valuable source for regenerative medicine approaches.

Keywords. human placenta, amnion, transplantation, regenerative medicine

(34.O1) CARTILAGE REGENERATION USING MESENCHYMAL STROMAL CELLS FROM THE FETAL MEMBRANES OF HUMAN TERM PLACENTA

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Introduction. Placenta derived mesenchymal stromal cells (plMSCs) have attracted increasing attention in regenerative medicine. In this study, we report the characterization of amnion (hAMSCs) and chorion (hCMSCs) derived MSCs from the fetal membranes of human term placentas, and the specific environmental conditions that are necessary to tailor the cartilage forming ability of these cell sources.

Methods. hAMSCs and hCMSCs were ex vivo expanded up to passage 4. Bone marrow MSCs (hBMSCs) were used as controls. Morphofunctional characterization of these cell sources included assessment of cell growth kinetics, pluripotent marker expression, cell-cell and cell-matrix receptor profile and mesodermal differentiation potential. The effect of oxygen tension and growth factor supplementation on chondrogenesis was assessed using a micromass culture system.

Results. Cell proliferation was significantly higher in hCMSCs compared to hAMSCs and hBMSCs. Cell-matrix and cell-cell receptor profiling revealed marked differences in the expression of members of the integrin and cell-cell adhesion receptor families. Both hBMSCs and plMSCs, displayed trilineage mesodermal differentiation (osteo/adipo/chondro) potential. Adipogenic conversion was significantly lower in hAMSCs and hCMSCs compared to hBMSCs. Under hypoxia, hCMSCs exhibited enhanced proliferation while maintaining mesenchymal and cell-matrix marker profile. When induced to undergo chondrogenesis under TGF beta3 and GDF5 stimulation, differences in the expression of hyaline cartilage and fibrocartilage markers were observed, suggesting that GDF5 and TGF beta3 mediate differential chondrogenic maturation of these progenitors.

Conclusion. hCMSCs represent an attractive cell source for cartilage repair. They have strong cartilage formation ability with very little adipogenic potential. This information sets the basis to design specific protocols for precisely inducing chondrogenic differentiation with a defined phenotype.

Keywords. fetal membranes, mesenchymal stromal cells, cartilage regeneration

(34.O2) HUMAN AMNIOTIC FLUID STEM CELLS (hAFSCs) ARE ABLE TO RECRUIT HOST'S PROGENITOR CELLS AFTER IN VIVO IMPLANTATION AND SECRET ANGIOGENIC FACTORS

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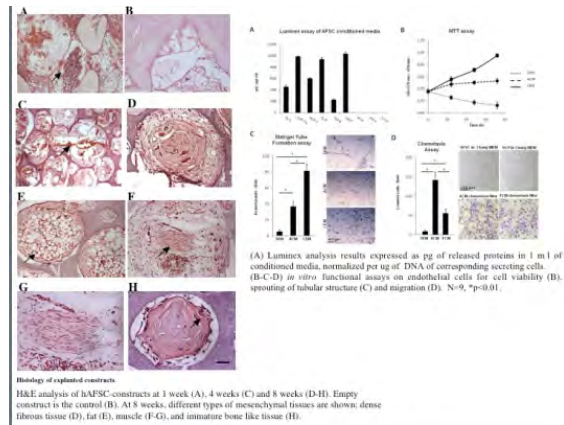
Introduction. The amniotic fluid is a source of multipotent stem cells with a therapeutic potential for human diseases. We report in vitro and in vivo studies of human amniotic fluid stem cells (hAFSC), a very appealing reserve of stem cells.

Methods. Isolation and characterization of hAFCS from amniotic fluid. Cellular ectopic implantation in nude mice of hAFCS. In vivo analysis of cell recruited. Lumiex analysis of hAFSC secretoma. In vivo assay for angiogenic activity.

Results. Cultured at low cell density, (hAFSCs) were still able to generate (CFU-F) after 60 doublings, thus confirming their staminal nature. After extensive in vitro expansion hAFSCs maintained a stable karyotype. The expression of genes, such as SSEA-4, SOX2 and OCT3/4 was confirmed at early and later culture stage. Also, hAFSCs showed bright expression of mesenchymal markers and immunoregulatory properties. hAFSCs, seeded onto scaffolds and subcutaneously implanted in nude mice, played a role in mounting a response resulting in the recruitment of host's progenitor cells forming tissues of mesodermal origin. Implanted hAFSCs migrated from the scaffold to the skin overlying implant site but not to other organs. Moreover, we demonstrated that the hAFSCs secretoma is responsible for the vascularising properties. We identified in hAFSC conditioned media (ACM) pro-angiogenic soluble factors, such as MCP-1, IL-8, SDF-1, VEGF. Our *in vitro* results suggest that ACM are cytoprotective, pro-differentiative and chemoattractive for endothelial cells. We also tested ACM on a model of hind-limb ischemic mouse, concluding that ACM contain mediators that promote the neo-arteriogenesis.

Conclusion. Given their in vivo: (*i)* recruitment of host progenitor cells, (*ii*) homing towards injured sites, (*iii*) multipotentiality in tissue repair (*iiii*) strong angiogenic response in murine recipients, in terms of host guided-regeneration of new vessels, hAFSCs are a very appealing source of cells that may be useful for clinical application in regenerative medicine.

Keywords. stem cells, amnion, tissue regeneration, cell isolation



(A) Luminex analysis results expressed as pg of released proteins in 1 m l of conditioned media, normalized per ug of DNA of corresponding secreting cells. (B-C-D) *in vitro* functional assays on endothelial cells for cell viability (B), sprouting of tubular structure (C) and migration (D). N=9, *p<0.01.

Histology of explanted constructs.

H&E analysis of hAFSC-constructs at 1 week (A), 4 weeks (C) and 8 weeks (D-H). Empty construct is the control (B). At 8 weeks, different types of mesenchymal tissues are shown: dense fibrous tissue (D), fat (E), muscle (F-G), and immature bone like tissue (H).

(34.O3) AMNIOTIC MEMBRANE OPPOSES TGFß SIGNALLING AND INDUCES C-JUN EXPRESSION IN HACAT CELLS. MOLECULAR LESSONS FROM THE RE-EPITHELIALISATION INDUCED BY AM IN DEEP LARGE-SURFACE WOUNDS

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Amniotic Membrane (AM) is a tissue of particular interest as a biological dressing due to their structure, biological properties and immunologic characteristics. We have used AM at the HUVA as a wound dressing in several patients with deep and extensive wounds inducing a robust eptithelialisation. In all cases, a well-structured skin was formed after AM treatment of the wound. TGFß has an important role in wound healing; it is initially released at the wound bed by platelets. TGFß induces migration of monocytes and fibroblasts to the fibrin clot and further release of TGFß by monocytes. Additionally, TGFß induces a powerful cell cycle arrest in keratinocytes. All together this prevents the reepithelialization of the wound before dermis is fully repaired. In big massive wounds, the high concentration of TGFß induced by inflammation may be the cause that prevents reepithelialization, even when wound cavity has been filled by granulation tissue. The application of AM may antagonise these TGFbeta effects. To test this hypothesis we used an epithelial model: HaCaT cells. We studied the effect of AM on the TGFß induced genetic response. HaCaT cells treated or not with AM were stimulated with TGFß. AM treatment triggered the activation of several signalling pathways, including MAP kinase pathway. Additionally, HaCaT cells treated with AM for 24 hours showed a dramatic change in their genetic responses to TGFß. AM modifies the genetic program that HaCaT cells exhibit in response to TGFß. These effects were compared to results obtained using primary h. keratinocytes and Mv1Lu cells. In Mv1Lu cells, AM induced a potent migration response that was prevented by SP600125, an inhibitor of SAP/JNK kinase that prevents c-Jun phosphorylation.

Keywords. Amniotic membrane, TGFß, Wound Healing.

(34.O4) ENDOTHELIAL DIFFERENTIATION POTENTIAL OF AMNION-DERIVED MESENCHYMAL STROMAL CELLS

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Amnion-derived mesenchymal stromal cells (AMSC) currently play an important role in stem cell research, as they are immunoprivileged cells of fetal origin, easily accessible and available in large supply. We tested their endothelial differentiation potential using functional studies and analysis of gene and protein expression. For isolation of AMSC, the amnion of human term placentas was treated with collagenase/DNase. AMSC were cultured in DMEM and 15% FCS (non-induced AMSC) or endothelial cell medium ECM-2 (induced AMSC/iAMSC). They were characterized by the ability to take up DiI-AcLDL and to form networks in an angiogenesis (Matrigel) assay. Gene expression was analysed using Affymetrix 1.0 ST arrays (n=3). For detection of angiogenic proteins, protein from non-induced and iAMSC was analysed with an angiogenesis array kit (RayBiotech, n=5).

Angiogenic stimulation with ECM-2 enhanced the proliferation potential and viability of AMSC. They changed their fibroblast-like morphology towards an endothelial, cobblestone-like phenotype. They did not express the mature endothelial cell (EC) markers vWF and VE-Cadherin, however, they took up DiI-AcLDL, a characteristic of EC, and formed networks similar to EC in the Matrigel assay. Gene expression analysis of non-induced vs. iAMSC revealed a differential expression of 200 genes ($p<0.005$, FC>2). Interestingly, iAMSC downregulated typical angiogenic genes such as tenascin C (FC -27.9), Tie-2 (-16.8), VEGF-A (-5.7), CD146 (-3.8) and FGF-2 (-2.2, $p=0.02$), while they upregulated genes with anti-angiogenic functions such as the FGF signaling antagonist Sprouty1 (10.6) and angioarrestin (8.2). Analysis of protein expression confirmed downregulation of FGF-2 and Tie-2 (to 26.9±8.1% and 13.3±0.5%, respectively), and upregulation of the anti-angiogenic protein endostatin (226.3±3.9%) by iAMSC.

In conclusion we could show that angiogenic induction of AMSC resulted in phenotypic and functional changes similar to endothelial cells. However, AMSC seem to resist a complete differentiation into mature endothelial cells by upregulation of anti-angiogenic genes and proteins.

Keywords. placenta, mesenchymal stem cells, endothelial differentiation

(34.O5) IMMUNE PORPERTIES AND REGENERATIVE POTENTIAL OF HUMAN MESENCHYMAL STEM CELLS DERIVED FROM UMBILICAL CORD TISSUE

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Introduction. Mesenchymal stem cells (MSC) are adherent fibroblast-like cells with multipotent properties. In addition to bone marrow and fat tissue, they can easily be isolated from the Wharton´s jelly region of umbilical cords (UCMSC). This study investigated cardiovascular and endothelial differentiation of UCMSC and seeding

feasibility on synthetic scaffolds as well as their ability to grow under GMP-compliant culture conditions. In addition, it was investigated whether UCMSC influence the phenotype of human cardiac stem cells (CSC).

Methods. UCMSC cultured in xeno- and serum-free media and tested for their immunosuppressive properties in CSFE assays with allogeneic mixed lymphocyte reactions (MLR). Endothelial and cardiomyocyte differentiation was driven by incubating the cells in vascular growth factors and oxytocin, respectively. Cells were also seeded on Titanium-coated expanded polytetrafluorethylene (Ti-ePTFE) scaffolds and analyzed for viability, proliferation, and morphology. CSC were derived from fresh heart explant cultures after cardiac transplantation.

Results. The cardiomyocyte phenotype of differentiated UCMSC was verified morphologically and with an optimized set of cardio markers. Endothelial-like UCMSC expressed von Willebrandt factor and established network structures in Matrigel™. UCMSC effectively suppressed MLR via expression of prostaglandin E2. Furthermore, UCMSC can be seeded on Ti-ePTFE scaffolds and maintain their viability and morphology. In addition, conditioned medium of UCMSC promote maturation of CSC into cardiomyocytes. Finally, GMP-compliant growth media qualify for long-term cultures and expansion of UCMSC.

Conclusion. The umbilical cord tissue is an easily accessible source for mesenchymal stem cells. Due to their potential to give rise to cardiovascular cells and due to their immunosuppressive properties UCMSC constitute an attractive tool in regenerative and transplant medicine. UCMSC might be used as undifferentiated cells to trigger endogenous repair by CSC or as cardio-differentiated cells on biocompatible scaffolds in tissue engineering.

Acknowledgements. This work is funded by Else Kröner-Fresenius-Stiftung, Bayerische Forschungsstiftung, Federal Ministry of Education and Research.

Keywords. Umbilical cord tissue, mesenchymal stem cells, transplant medicine, regenerative medicine

(34.O6) TISSUE ENGINEERING A FETAL MEMBRANE

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Introduction. Pre-term birth is the leading cause of perinatal and neonatal mortalities, 40% of which are attributed to the pre-term premature rupture of the fetal mebrane (pPROM). The aim of this study was to construct an artificial fetal membrane (FM) by combination of human amniotic epithelial stem cells (hAESCs) and a compressed collagen scaffold containing encapsulated human amniotic stromal fibroblasts (hASFCs). Such a tissue engineered FM may have the potential to plug structural defects in the amniotic sac after antenatal interventions, or to prevent pPROM.

Methods. The hAESCs and hASFCs were isolated from amniotic membrane (AM). Magnetic cell sorting was used to enrich the hAESCs by positive ABCG2 selection. Substrates (compressed collagen gel containing hASFCs and denuded AM) were characterised by electron microscopy and rheology to quantify their topographical and mechanical features. We investigated the use of a laminin / fibronectin (1:1)-coated compressed collagen gel as a novel scaffold to support the growth of hAESCs. Differences in cell-based biocompatibility were quantified by measuring growth and differentiation of hAESCs by hematoxylin and eosin staining and immunohistochemistry.

Results. Collagen fibers within the compressed gel were dense, homogeneous, and similar in structure to those within denuded AM. No significant difference in rheology between collagen and AM was also observed. hAESCs were successfully expanded upon the compressed collagen and hASFCs were shown to maintain viability within the collagen gel. The resulting artificial membrane shared a high degree of similarity in cell morphology and protein expression profiles to normal fetal membrane.

Conclusion. This study provides the first line of evidence that a laminin / fibronectin (1:1)-coated compressed collagen gel containing hASFCs can adequately support hAESCs expansion and differentiation to a degree that is comparable to the normal fetal membrane with similar mechanical properties.

Acknowledgements. Supported by the BBSRC (BB/F019742/1).

Keywords. tissue engineering; scaffold, compression collagen, amniotic membrane, stem cell

(34.O7) NON-CLASSICAL TYPE I HLAS AND B7 COSTIMULATORS REVISITED: ANALYSIS OF EXPRESSION AND IMMUNOMODULATORY ROLE IN UNDIFFERENTIATED AND DIFFERENTIATED MSC ISOLATED FROM HUMAN UMBILICAL CORD WHARTON'S JELLY

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Introduction. Wharton's jelly (WJ), the main constituent of umbilical cord, emerged as a reliable and uncontroversial source of mesenchymal stem cells (MSC). WJ-MSC show unique ability in crossing lineage borders, therefore being capable to trans-differentiate towards mature cytotypes derived from the three germ layers. As other fetal-associated cells, WJ-MSC express several immunomodulatory molecules, essential during the initial phases of human development and for the processes linked to the tolerance of the mother to the semi-allogeneic embryo. Very few data are present in literature on the maintenance of the immune privilege of the naïve cells after performing differentiation. Our previous work highlighted the expression of non-classical HLA molecules as HLA-G in WJ-MSC, together with a favorable combination of B7-1 and B7-2 costimulators.

Methods. The aim of this work was extending the knowledge on the expression of immunomodulatory molecules by naïve and differentiated WJ-MSC. WJ-MSC underwent differentiation to osteoblasts, adipocytes and hepatocyte-like cells. Differentiated cells were analyzed, by RT-PCR, ICC and histological stains for the acquisition of the desired phenotypical features. We investigated

also the differential expression of immune-related molecules in undifferentiated and differentiated cells. Moreover, we exploited the in vitro ability of such cells to suppress allogeneic lymphocyte proliferation in MLR experiments.

Results. WJ-MSC expressed diverse immunomodulatory molecules which span from non-classical type I HLAs (i.e. HLA-E, -F, -G) , to further members of the B7 family, and of the CEA superfamily, for all of which in vivo immunomodulating functions are known. For some of these factors, we demonstrated for the first time their expression in WJ-MSC. In addition, we demonstrated for the first time that the expression of these molecules is maintained after performing osteogenic, adipogenic or hepatogenic differentiation.

Conclusions. Present data should disclose new promising features for the in vivo application of WJ-MSC in allogeneic cell therapy settings.

Keywords. mesenchymal stem cells, umbilical cord Wharton's jelly, immune modulation, hepatocyte differentiation

(34.O8) ROLE OF POLYURETHANE FOAMS IN SUPPORTING PROLIFERATION AND OSTEOBLAST DIFFERENTIATION OF PLACENTA-DERIVED MESENCHYMAL STEM CELLS

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Introduction. Human placenta may represent a valid source of stem cells, as it is available without invasive procedures and because of the phenotypic plasticity of many of its cell types. This work was aimed at evaluating the proliferation and osteogenic differentiation of human amnion mesenchymal cells (AMCs) and chorion mesenchymal cells (CMCs) cultured onto polyurethane (PU) foams, coated or not with a-tricalcium phosphate (aTCP).

Methods. A PU foam (PUf) was synthesized with gas foaming technique, by reacting a polyether-polyol mixture with MDI prepolymer (Bayer, Germany), using water as expanding agent. The PUf was characterized for porosity (≈ 90%) and average pore size (268 micrometer) by micro-CT (Skyscan 1172, Aartselaar, Belgium), and density (0.127±0.003 g/cm3). PUf discs were coated by immersion in a a-TCP suspension under magnetic stirring. AMCs and CMCs were isolated from two human term placentas, seeded (2.5×105 and 5×105 cells/well) onto PUf samples, aTCP-coated or not, and cultured in the presence of osteoinductive (NH Osteogenic Differentiation medium, Miltenyi Biotech) or control medium (EMEM, Lonza) up to 20 days. Cells morphology was investigated by SEM, scaffold colonization and cells differentiation were evaluated by hematoxilin-eosin, Alizarin Red and Von Kossa staining.

Results. The 3D model generated by micro-CT demonstrated a homogeneous foam morphology with regular pore distribution (Figure 1a). SEM observation showed good cells colonization both onto the PUf matrix and the aTCP-coated PUf (Figure 1b), with cells well adherent to the scaffold pores. The osteogenic differentiation medium appeared to promote cell differentiation to the osteoblastic phenotype, as highlighted by Alizarin Red staining performed on PUf matrix (Figure 1c).

Conclusions. The proposed PU foams, in particular when coated with aTCP, are able to support hAMCs and hCMCs osteogenic differentiation, thus appearing as good candidates as scaffolds for bone regeneration.

Aknowledgments. Italian Institute of Technology (IIT), Project "NanoBiotechnology - Research Line 1".

Keywords. Placenta-derived Mesenchymal Stem Cells, Polyurethane scaffolds, Bone Tissue Engineering

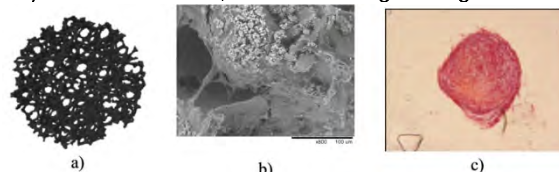

Figure 1: PUf 3D model by micro-CT analysis (a), representative SEM image of AMCs cultured onto αTCP-coated PUf using Osteogenic Medium (seeding density of 5x105 cells/well and scale bar = 100 μm) (b), and histological image of Alizarin red of hCMCs (seeding density of 2.5x105 cells/well) cultured onto PUf matrix with Osteogenic Medium (c).

(34.O9) MASSIVE POST-TRAUMATIC WOUNDS INDUCED EPITHELIALISATION BY AMNIOTIC MEMBRANE

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Abstract. Large-surface or deep wounds often become senescent in the inflammatory or proliferation stages and cannot progress to re-epithelialisation. This failure makes intervention necessary to provide the final sealing epithelial layer. The best current treatment is autologous skin graft, although there are other choices such as allogenic or autologous skin substitutes and synthetic dressings.

Amniotic Membrane (AM) is a tissue of interest as a biological dressing due to its biological properties and immunologic characteristics. It has low immunogenicity and re-epithelialisation effects, anti-inflammatory, anti-fibrotic, anti-microbial, and non-tumourigenic properties. These properties are related to its capacity to synthesize and release cytokines and growth factors.

We report the use of AM as a wound dressing in two patients with large and deep traumatic wounds. Negative Pressure Wound Therapy (NPWT) followed by AM application was capable of restoring skin integrity avoiding the need for skin graft reconstruction. AM induced the formation of a well-structured epidermis. To understand this effect, we designed some assays on human keratinocyte-derived HaCaT cells. AM treatment of HaCaT induced ERK1/2 and SAP/JNK kinases phosphorylation; and c-jun expression, a gene critical for keratinocytes migration; however, it did not affect cell cycle distribution. These data suggest that AM

substantially modifies the behaviour of keratinocytes in chronic wounds allowing effective re-epithelialisation.

Keywords. Wound healing, Massive wounds, Amnitotic Membrane

(34.P1) IPLASS: FOSTERING TRANSLATION OF PLACENTAL CELL RESEARCH INTO CLINICAL THERAPIES

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Introduction. Recent reports indicate that cells isolated from human term placenta display stem cell and immunomodulatory functions. Further advances have now prompted researches to explore the mechanism(s) behind these effects and the potential for these cells in preclinical and clinical applications. The International Placenta Stem Cell Society (IPLASS) was officially founded in June 2010, with the aim of creating a network of researchers, skills and ideas to promote research on all aspects concerning knowledge, experimentation and clinical use of placenta-derived stem cells, to thereby accelerate their progression toward clinical application.

Methods. The Society accomplishes these aims through cooperative research activities, interaction between members, promotion of staff mobility through organization of biennial scientific meetings, endorsement of scientific events, promotion and support of young researchers' education, and maintenance of a dedicated web site to promote effective connection among members.

Results. Results from IPLASS initiatives are now being manifested. We see cross-exchange of knowledge, competencies and data between basic immunologists with emphasis on the placenta's role in fetomaternal tolerance as a key to understand its immunomodulatory capabilities. Researchers interested in cell therapy are applying placental cells to a number of disease models in animals. Companies with interest in banking and/or clinical trials using placental cells are being identified for cooperation. So far, placental cells have proven beneficial in diseases involving inflammatory and fibrotic degeneration.

Conclusion. With continued support from its members, IPLASS will grow as a scientific association to foster the increase and dissemination of knowledge concerning placental cells, with the goal of elucidating the mechanisms underlying their therapeutic effects. However, this will require increased membership from researchers and support from companies for sustaining basic and academic science. Constructive input so far received from members augurs well for the second IPLASS meeting, envisioned to take place in 2012 in Vienna, Austria.

Keywords. placenta stem cells, amnion, cell therapy, regenerative medicine

(34.P2) EXPRESSION OF SLAM FAMILY MEMBERS IN DECIDUAL STROMAL CELLS

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Abstract. The SLAM family is a group of receptors that works as adhesion molecules in the immune synapse. These molecules are expressed broadly in blood cells and modulate immune functions of immuno-competent cells. Decidual stromal cells (DSC) are the mayor component at the decidua in close contact with the leucocytes that bath the maternal foetal interface and are related with modulation of the immunocompetent cells functions at this interface. Here we analyzed the expression of SLAMf members on DSC fresh cells lines from normal placenta. As assumed, these cells showed discrete expression of hSLAMF1, hSLAMF5 (hCD84), hSLAMF6 (hLY108), hSLAMF8 (hBLAME) y hSLAMF9 (SF2001). Our next step is to study whether the expression SLAMf is related with modulation of leucocytes functions or with DSC cells differentiation.

Keywords. decidual stromal cells, SLAM

FAMILIA SLAM	DSC
SLAM (SLAMF1) CD150	+
CD48 (SLAMF2)	-
LY9 (SLAMF3) CD229	-
CD244 (SLAMF4/2B4)	-
CD84 (SLAMF5)	+
LY108 (SLAMF6) CD352	+
CRACC (SLAMF7)	-
BLAME (SLAMF8) CD353	+
SF2001 (SLAMF9)	+

Expression of SLAMf members in DSC cell lines from normal decidual

(34.P3) BIOLOGICAL PROPERTIES OF MESENCHYMAL DERIVED HORSE AMNION CELLS AND IN VITRO LABELING EFFICIENCY WITH MAGNETIC RESONANCE CONTRAST AGENTS

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Extra-fetal derived cells may represent attractive alternative candidates for regenerative medicine uses, with the potential to circumvent some limitations of adult stem cells. For the first time, biological properties of mesenchymal presumptive stem cells from horse amnion (AMCs) are described and, to investigate amnion-derived cells as valuable candidates for cell therapy strategies in pre-clinical experiments, AMCs were labeled with magnetic resonance contrast agents (superparamagnetic iron oxide particles - SPIO - and manganese chloride) and studied in vitro for 8 days to determine cell toxicity by proliferation capacity and apoptotic test.

Five horse amnions were processed and isolated AMCs displayed typical fibroblast-like morphology. The mean

doubling time (DT) was 1.17 days and the mean frequency of CFU-F was of 1:283 seeded cells.

Immunocytochemical studies showed that AMCs were positive for expression of specific embryonic markers (TRA-1-60, SSEA-3, SSEA-4 and Oct-4). Meanwhile, RT-PCR performed at passage (P) 1 and P5 showed expression of CD29, CD105, CD44, CD166 with negativity for CD34 at P1, although this marker began to be expressed by P5. The cells also expressed MHC-I at both P1 and P5, but lacked MHC-II expression until P5. AMCs demonstrated high plasticity, differentiating in vitro toward the osteogenic, adipogenic, chondrogenic and neurogenic lineages. SPIO labeling procedure revealed as the more efficient and non toxic tool because SPIO-labeled cells showed no significant changes in the DT assay and in the rate of apoptotic cells compared with that of control unlabeled cells at 8 days after labeling (21.43% vs 18.44% respectively).

Our preliminary data show that equine amnion holds evident promises as a source of presumptive stem cells which may have widespread clinical applications if employed at early passages. Magnetic resonance imaging may emerge as an ideal non-invasive imaging technology to monitor the fate of labeled cells in vivo during stem cell therapy.

Keywords. Amnion derived cells, Equine, Magnetic resonance, labeling agents

(34.P4) COMPARISON OF CELLULAR BEHAVIOR IN FRESH AND CRYOPRESERVED HUMAN AMNIOTIC MEMBRANE BEFORE AND AFTER XENOTRANSPLANTATION IN RAT LIVER

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Introduction. Human amniotic membrane (hAM), the innermost layer of the fetal membranes, has been applied extensively in medicine and in animal disease models due to its anti-inflammatory, anti-fibrotic and pro-regenerative effects. Most currently available data derives from use of cryopreserved hAM. However, comparative studies of cell viability, morphology and immunoreactivity after transplantation of cryopreserved versus fresh hAM are lacking.

Methods. Fresh and cryopreserved hAM fragments were applied onto the rat liver surface. Histological liver assessment was performed 1, 3 and 7 days thereafter. Morphology and immunoreactivity of hAM was assessed before and after transplantation using hematoxylin-eosin and antibodies against tissue antigens including Vimentin, HLA-ABC and HLA-DR. Cell viability was determined using an antibody against intact human mitochondria.

Results. Cell viability evaluation in the epithelial and stromal regions of fresh and frozen membranes before transplantation showed reduced viability in cryopreserved membranes, mainly in the epithelial layer. Both total cell count and sub-total cell count in the epithelial and stromal layers showed lower cell numbers in cryopreserved hAM compared with fresh membranes. However, only the differential count of epithelial cells presented a statistically significant difference. Both membrane preparations showed positivity for HLA-DR and HLA-ABC, although with fainter signals in cryopreserved membranes. After 1 and 3 days post-surgery, both membrane preparations showed areas of contact to liver without evident signs of intraparenchymal inflammation. At day 7, membranes were still adherent to the liver capsule in association with interposed palisades of tightly packed fibroblast-like cells and the integrity of both xenotransplanted hAM was reduced forming a compact connective tissue band with cells no longer being detectable.

Conclusion. When comparing fresh and cryopreserved hAM, no substantial differences in morphology were observed after transplantation. Thus, both preparations of hAM might be equally suitable in clinical approaches.

Keywords. Amniotic membrane, placenta, histology

(34.P5) AUTOCRINE TGFSS PRODUCTION INDUCES EPITHELIAL TO MESENCHYMAL TRANSITION IN HUMAN AMNIOTIC EPITELIAL CELLS

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Amniotic membrane (AM) is the most internal layer of foetal membranes. It consists of a thin epithelium, a basement membrane and a stroma of non-vascular connective tissue. The epithelium makes a simple, continuous, uninterrupted line of columnar, cuboid cells also known as hAECs (human Amniotic Epithelial Cells), that are in contact with the amniotic fluid. hAECs are pluripotent and express stem cells markers. Previously, we have shown that AM induces re-epithelialization in massive wounds. In order to know more about hAECs, we isolated and cultivated them using DMEM supplemented with FBS. Observation of hAECs, either directly at the AM or in early culture passages, revealed a typical epithelial morphology. Either cells showed the presence of typical epithelial markers (E-Cadherin, ß-Catenin and Cytokeratin), and typical embryonic stem cells surface markers such as TRA 1-60 and TRA 1-81. With increasing number of passages however, hAECs underwent morphological changes adquiring a mesenchymal shape with evident formation of stress fibers. Moreover, epithelial and embryonic stem cells markers were lost and typical mesenchymal markers, such as Vimentina or αSMA, appeared. Finally, many genes associated with epithelium-to-mesenchymal transition (EMT), such as Snail, MMP9, PAI1 increased their expression with time. On the other hand, expression of KLF4, a positive transcription factor for E-cadherin promoter, decreased with time. When freshly extracted hAECs were culture in the presence of the TGFß receptor inhibitor SB431542 cells did not undergo EMT. Furthermore, conditioned medium from hAECs was able to elicit TGFß related signalling events in HaCaT cells, (human keratinocytes). All together, these results suggest that cultured hAECs undergo EMT mediated by autocrine production of TGFß. More results will be discussed at the poster.

Keywords. TGFß, EMT, Human Amniotic Epithelial Cells, Gene expression

(34.P6) CHONDROGENIC DIFFERENTIATION OF STEM CELLS WITHIN INTACT HUMAN AMNIOTIC MEMBRANE

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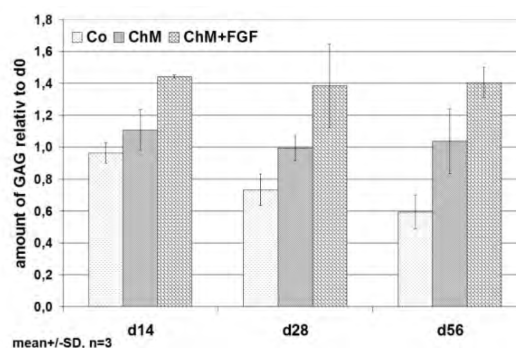
Tissue engineering strategies usually require cell isolation and combination with a suitable carrier substrate. Alternatively, the cell sheet technology enables transplantation of expanded cells without the use of carrier materials. In contrast, our approach is to differentiate stem cells within intact amniotic membrane (AM), which constitutes a pre-formed sheet of stem cells, without prior cell isolation. We have previously demonstrated osteogenic differentiation of stem cells within intact human AM in vitro. Beside bone, regeneration of cartilage is an important scope in orthopedic trauma. For this reason, also the chondrogenic differentiation potential of human AM was investigated.

In vitro chondrogenic differentiation of human AM biopsies was induced by culture in Mesenchymal Stem Cell Chondrocyte Differentiation Medium (ChM, Lonza) supplemented with transforming growth factor beta 3 (Lonza), and optionally supplemented with fibroblast growth factor 2 (FGF-2, R&D Systems; ChM+FGF) for 8 weeks. To determine chondrogenesis, cartilage-specific collagen II and glycosaminoglycans (GAG) were demonstrated by alcian blue staining of paraffin embedded histological sections. Furthermore, the amount of GAG was quantified using BlyscanTM Sulfated Glycosaminoglycan Assay (Biocolor). Regardless of the medium applied, Alcian blue stainings of all AM-samples revealed accumulation of GAGs in the membranes. Nevertheless, stainings of samples cultured in control medium (Co) were less intense, whereas ChM+FGF appeared to show the most intense staining. Quantitative evaluation showed that in Co the amount of GAG was decreasing during culture (Fig.). When culturing in ChM, the GAG-amount was increased compared to Co at any timepoint and remained stable throughout the culture period. Noticeable, increased GAG production was obtained when adding FGF-2 to the medium. Chondrogenic differentiation of intact human amniotic membrane could be induced by chondrogenic medium (ChM) and further increased with supplemented FGF-2. Thus, these results are another promising step towards using intact human amnion with its residing stem cells for tissue engineering.

Keywords. amniotic membrane, differentiation, chondrogenesis



(34.P7) DECIDUALIZATION CHANGES THE PHENOTYPE OF HUMAN DECIDUAL STROMAL CELLS

Leno-Durán E (1), Prados A (1), Muñoz-Fernández R (2), Martínez-González PJ (1), García-Morales D (1), Ruiz-Ruiz MC (1), García-Olivares E (1)

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Decidual stromal cells (DSC) are the main cellular component of the decidua, the maternal tissue which is in close contact with the fetal trophoblast. During normal pregnancy, DSC differentiate (decidualize) by the effects of the progesterone, changing their morphology to a rounder shape and secreting prolactin. We have previously demonstrated that human DSC exhibit immune functions that may be involved in the maternal-fetal immunological cross-talk. Furthermore, we have also shown that DSC are closely related to the mesenchymal stem cells and can differentiate into adipocytes, chondrocytes and osteoblasts. The physiological differentiation of DSC (decidualization) is, however, induced by the progesterone. Decidualized cells become rounder and secrete prolactin. In the present work, we have studied by flow cytometry the effects of decidualization on the antigen phenotype and functions of DSC. We have isolated and maintained human DSC lines in culture. Some lines were cultured with progesterone and cAMP for 14 days. Decidualization was confirmed by the detection of prolactin in the culture medium. We have observed that decidualization decreased the expression of CD54, CD29 and BAFF by DSC, increased the expression of HLA-G, but it did not change the expression of CD73, CD10, CD21, CD106 and CD14 on these cells. Decidualized DSC secreted more IL-15 and IL-10, but less IL-6 than non-decidualized cells. Both cultured medium obtained from decidualized and non-decidualized cells decreased the spontaneous apoptosis of decidual lymphocytes. Nevertheless, decidualization induced apoptosis in DSC, although these cells were resistant to most of apoptosis-inducer substances. The differences between decidualized and non-decidualized DSC may help to explain the different therapeutic effect of these cells in inflammatory situations.

Keywords. Decidualization, decidual strmal cell, phenotype

(34.P8) MESENCHIMAL STEM CELL DERIVATION FROM UMBILICAL CORD TISSUE OBTAINED FROM AN ALTRUISTIC CORD BLOOD DONATION PROGRAM

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1. F. Banc de Sang i Teixits de les Illes Balears; 2. Terapia celular e ingenieria tisular (TERCIT). IUNICS. Palma
Mesenchimal stem cells (MSC) promise a great potential for regenerative medicine due to their unique properties of self-renewal, high plasticity and modulation of immune response. These properties have made MSC as attractive in regenerative medicine, immunotherapy and gene therapy. Bone marrow was the original source of MSC, and has been used to generate most of the MSC used in clinical trials. However the invasive procedure that is needed to use for its recovery can limit their accessibility. Moreover, the increasing demand in tissue regeneration has put in evidence the necessity of a source of MSC as an “off-the-shelf” product for quick and effective treatment. One of the first candidates was the umbilical cord blood (UCB). However, several studies showed that the yield of MSC recovery from UCB was far too low to be considered as a reliable source for experimental and clinical use.
As a potential source of MSC we tested umbilical cord as well as placental tissue and compared with UCB. All the samples were obtained from donations included in our altruistic program of UCB procurement. Our results showed that is possible to obtain MSC from UC (UC-hMSC) with a 100% success rate by using a combination of mechanical fragmentation and enzymatic digestion. The MSC thus obtained show a phenotype very similar to the observed in the MSC from bone marrow origin: CD45-CD31-CD34-HLA-DR-CD105+CD90+CD73+. In addition, we have demonstrated that UC-hMSC, like the MSC from bone marrow, are able to differentiate into osteocytes, adipocytes and chondrocytes and also exert a suppressive effect on the proliferative capacity of peripheral lymphocytes stimulated with PHA. We conclude that it is possible to implement a structured program of MSC derivation by using the same logistic that it is used to obtain UCB.
Keywords. MSC, Umbilical cord, regenerative medicine

(34.P9) ESTABLISHMENT OF PROTOCOLS FOR ISOLATION AND CHARACTERIZATION OF CELLS FROM HUMAN AMNION: A NEW SOURCE OF CELLS FOR REGENERATIVE MEDICINE

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The amnion is a thin, avascular membrane derived from epiblast fromembryo. It is composed of an epithelial layer and an outer layer of connective tissue. Cells from this tissue are a good choice for regenerative medicine because of both their phenotypic plasticity and immunomodulatory capability. Is it readily available and easily procured without invasive procedures, and its use does not elicit ethical debate. However, because the complexity of this tissue needs to be defined, the region of origin and methods of isolation of cells derived from the human amnion. The aim of this work is to develop new protocols for human amnion isolation and phenotipically characterize the derived cell population.
Human amniotic epithelial cells (hAECs) are isolated from term placentas which would normally be discarded after delivery. It is very important to gently massage the amniotic membrane to remove blood clots after peeling it from the underlying chorion layer of the placenta body. The membrane is equilibrated in pre digestion buffer prior to be digested with Tripsin-EDTA solution. Digestion is performed twice for 40 min. each. After digestion is completed, membrane leftovers are withdrawn and the supernatant is centrifuged to obtain a cell pellet which is finally resuspended in fresh medium and plated to be grown under normal eukaryotic cell conditions. For isolation of cells the amniotic membrane is stripped from the underlying chorion and digested with trypsin. The cells are specifically released by brief trypsin digests of 40 minutes each. Isolated cells readily attach to plastic. Culture is commonly established in a simple medium, where the cells proliferate robustly and display typical cuboidal and elongated morphology and are viable after freezing. Cells do not proliferate well at low densities. Set up of new protocols for isolation of amniotic cells may contribute to the field of amnion derived cells considering their potential in tissue regeneration
Acknowledgment. CI 05/2006 (Universidad de Valparaiso, Chile) and CONICYT (ACT-73), Chile.
Keywords. amnion, stem cells, placenta

(34.P10) THERAPEUTIC EFFECTS OF DECIDUALIZED HUMAN DECIDUAL STROMAL CELLS IN MURINE MODEL OF COLITIS

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Some works have demonstrated that human mesenchymal stem cells (MSC) have therapeutic effects in different inflammatory and autoimmune disease. Decidual stromal cells (DSC), the main cellular component of the decidua, the maternal tissue which is in close contact with the fetal trophoblast, are closely related to MSC. During normal pregnancy, DSC differentiate (decidualize) by the effects of the progesterone. Decidualized DSC change their morphology to a rounder shape and secrete prolactin. In this work, we have isolated and maintained human DSC lines in culture. Some lines were cultured with progesterone and cAMP for 14 days. Decidualization was confirmed by the detection of prolactin in the culture medium. To induce colitis, trinitrobenzene sulfonic acid (TNBS) was administered intrarectally in BALB/c mice. Mice with colitis were treated intraperitoneally with decidualized or non-decidualized DSC after onset of disease and clinical scores were evaluated. Decidualized DSC decreased mortality, whereas non-decidualized cells increased mortality of mice with colitis. This different therapeutic effect may reflex the different roles of decidualized and

non-decidualized DSC in the immunological regulation of pregnancy.

(34.P11) HUMAN DECIDUAL STROMAL CELLS SECRETE CXCL13, EXPRESS BAFF AND RESCUE B LYMPHOCYTES FROM APOPTOSIS: DISTINCTIVE CHARACTERISTICS OF FOLLICULAR DENDRITIC CELLS

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1. Universidad de Granada; 2. Inst. de Parasitología y Biomedicina "López-Neyra"

We have previously demonstrated that decidual stromal cells (DSC) show phenotypical and functional coincidences with follicular dendritic cells (FDC): DSC express FDC-associated antigens, and both types of cells express α-SM actin, are contractile cells and are related to the mesenchymal stem cells (MSC). Furthermore, in immune-mediated pathological situations, DSC and FDC are detected in ectopic locations. In this work, we isolated human DSC and FDC lines and showed by flow cytometry that these two types of cells exhibited a common antigen phenotype. They both expressed BAFF, an FDC characteristic molecule. We also observed by ELISA that both types of cells secreted CXCL-13, a characteristic cytokine secreted by FDC. Moreover, we demonstrated by flow cytometry that DSC, like FDC, inhibited spontaneous apoptosis of B lymphocytes, a typical functional feature of FDC. Both types of cell lines increased the expression of CD54 when they were cultured with TNFα, a cytokine involved in the FDC maturation. Finally, we showed that FDC, like DSC, were differentiated in culture by the effect of progesterone and cAMP, factors which are involved in the differentiation (decidualization) of DSC. In these decidualization culture conditions, both types of cells changed their morphology from a fibroblatic to a rounder shape and secreted prolactin (PRL), although this latter activity was more intense in DSC. Our results show that although DSC and FDC are cells of different mesenchymal lineage, they share functional and phenotypical characteristics. We discuss these shared properties in the context of the DSC and FDC physiology and of immune responses in pregnancy.

Keywords. FDC MSC DSC

35. POLYMERIC VECTORS FOR GENE THERAPY

Chair: Wenxin Wang
Co-chair: Abhay Pandit
Keynote speaker: Caitriona O'Driscoll
Organizer: Wenxin Wang

Synopsis: The success of gene therapy is largely dependent on the development of the gene delivery vector. Over 30 years, gene delivery, especially via the nonviral route (*e.g.* transfection), has become a powerful and popular research tool for elucidating gene structure, regulation, and function. The ability to safely and efficiently transfer foreign DNA into cells is a fundamental goal in biotechnology. For this purpose, polymers are prepared by a combination of numerous functionalities and defined molecular structure. However, current synthetic DNA delivery systems are versatile and safe, but substantially less efficient than viruses. Indeed, most current systems address only one of the obstacles to DNA delivery by enhancing DNA uptake. In fact, the effectiveness of gene expression is also dependent on several additional factors, including the release of intracellular DNA, stability of DNA in the cytoplasm, unpackaging of the DNA–vector complex, and the targeting of DNA to the nucleus.

The key issue of this symposium will be a critical discussion concerning the cutting-edge technology on polymer's design and synthesis, functionalization and structural improvements for DNA or gene delivery in vitro and in vivo. Furthermore, this symposium will also discuss the establishment of efficient and safe gene delivery in vitro/vivo by a number of new techniques and concepts in targeted or controlled delivery of genes.

(35.KP) THE FUTURE OF NON-VIRAL GENE THERAPEUTICS – THE DELIVERY DILEMMA

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Increasing knowledge regarding the genetic basis of disease and the recent discovery, in 2001, that small interfering RNA (siRNA) silenced specific genes in mammalian cells ignited a revolutionary interest in siRNA as a new generation of therapeutics for the treatment of a wide spectrum of disease conditions. Specific siRNAs to target particular genes can be synthesised and significant progress has been made in the chemistry to improve stability and decrease the potential for off target side effects. The major challenge limiting the success of RNAi-based drugs is the design of a robust, effective system for in vivo targeted delivery. The physicochemical properties of siRNA and the complex physiological environment pose significant barriers to delivery. Due to safety concerns relating to viral vectors, emphasis has been concentrated on the design and development of non-viral vectors based on smart materials to help achieve safe and effective delivery.

The application of modified cyclodextrins (CD) as non-viral vectors provide the opportunity to overcome the barriers to systemic delivery of siRNA. CDs are cyclic oligosaccharides composed of glucose units; they are relatively simple and inexpensive, and are well accepted non-toxic excipients in more conventional drug delivery systems. Cyclodextrins are relatively large oligomers and can act as molecular scaffolds onto which a range of functional groups including polyethylene glycol (PEG) chains and cell specific targeting ligands can be grafted. Modified CDs synthesised by our group can form vesicles or artificial liposomes. Vesicle-forming CDs are a major advance on previous classes of CDs and are capable of encapsulating even large polar drugs within their aqueous vesicle interior. In addition, polycationic examples of these CDs, through electrostatic interactions, condense RNA into nanoparticles for efficient cellular transfer. The presentation will review progress to date with the CD vectors relative to alternative technologies.

(35.O1) MODULATING INFLAMMATION: TRANSFECTION OF MACROPHAGES BY COLLAGEN HOLLOW SPHERES LOADED WITH POLYPLEXES

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Introduction. Macrophages are key cells of inflammation as they secrete proteases and inflammatory cytokines. Conventional therapies to modulate inflammation based on the direct injection of biomolecules have been unsuccessful due to their short half-life in vivo. Therefore, gene therapy offers new opportunities to treat chronic inflammation. Unfortunately, human macrophages are difficult to transfect by non-viral reagents because of their reduced viability after transfection. The objective of this study was to use collagen hollow spheres as a reservoir of polyplexes to transfect human macrophages while maintain their viability.

Materials and methods. Polyplexes were formed by pDNA (G-Luc plasmid) and a pDMAEMA PEG based polymer. Different ratio polymer/pDNA (5:1, 8:1, 10:1) and two different sizes of spheres (diameter 1.24 and 4.5 µm) were tested. First, structure of collagen hollow spheres was analyzed by scanning electronic microscopy and their loading capacity was evaluated by Pico GreenTM Assay. Then, the ability to transfect human activated macrophages was assessed over four days by detection of the Luciferase activity. Last, cell metabolic activity was evaluated by AlamarBlue™ assay.

Results and discussion. Collagen hollow spheres were loaded by polyplexes up to an amount of 80µg of pDNA/mg of microspheres. Macrophages transfection detected by luciferase activity revealed an expression of this enzyme with the ratio 10:1 (polymer/pDNA) regardless of sphere size. In addition, collagen hollow spheres preserved cell viability because transfected macrophages exhibited a high viability (more than 80%) whereas polyplexes added directly in the culture medium were toxic.

Conclusions. These results show that collagen hollow spheres can be considered as a potent reservoir for controlled gene delivery. Contrary to existing polymers, these reservoir systems allow for transfection of macrophages without toxicity. Hence, these systems appear promising for the delivery of an inhibitory inflammatory therapeutic gene of interest.

Acknowledgments. Health Research Board: Grant HRB RP/2008/188.

Keywords. Macrophages, Gene Therapy, Transfection, Collagen Hollow Spheres

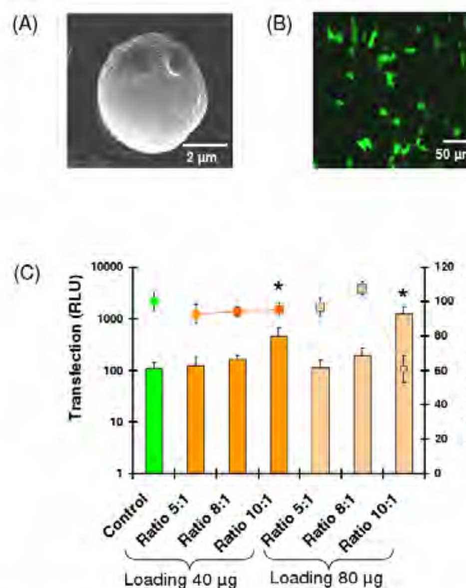

Figure 1: Transfection of macrophages by 4.5 µm collagen hollow spheres loaded with polyplexes. **(A)** Collagen hollow sphere structure. **(B)** Fluorescence microscopy showing transfection (GFP detection). **(C)** Quantification of transfection and viability. Squares represent viability; histograms represent transfection capabilities. (*: $P<0.05$, Student test).

(35.O2) DEVELOPMENT OF NEW POLYMERIC MICELLES FOR GENE DELIVERY AND DEMONSTRATION IN B16F10 MURINE MELANOMA

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Introduction. The common method to deliver DNA is the use of viral vectors, but numerous problems exist. Based on this, we prepared new polymeric micelles as non-viral vectors for DNA/RNA, not toxic and highly efficient in complexing, stabilizing and delivering the nucleic acids.

Methods. PEG-b-PPS (AB) diblock-copolymer was formed by reaction of PEG-PPS living thiolate with 2,2'-dithiodipyridine. PEG-b-PPS-b-PEI (ABC) triblock-copolymer was formed from PEG-PPS thiolate by conjugation with a with a linear poly(2-ethyl-2-oxazoline) pyridyl disulfide block, then deprotected by acid hydrolysis. When suspended in water, the ABC block copolymer self-aggregates to form micelles of diameter between 100 and 250 nm. If mixed micelles of AB and ABC block copolymers are suspended in water, the size is dramatically decreased (30 nm). Both platforms were high efficiently conjugated with GFP plasmid or with siRNA against nucleolin (one of the most important player in tumor proliferation) and the complexes have been transfected into B16F10 cells. The GFP expression has been evaluated by Flow Cytometry. Nucleolin knockdown was determined by rtPCR. In vivo gene delivery was tested by intratumoral injections of micelles-pOVA in melanoma bearing mice: pOVA expression was determined by rtPCR, respect to naked plasmid. The effect of pOVA delivery on tumor growth was evaluated in tumor bearing mice previously immunized against ovalbumin. Not immunized mice containing melanoma

tumor were injected instead with micelles-siRNA . In both of the cases, injections were repeated every day and the tumor volume was measured.

Results and discussion. Micelles efficiently transfect GFP in B16F10 and pOVA in murine melanoma. Tumors treated with micelles-pOVA in pre-immunized mice show infiltration of CD8+T cells e consequently strong tumor growth reduction. Up to 80% of nucleolin knockdown was obtained in vitro by micelles-siRNA transfection and a strong inhibition of tumors growth was observed in vivo.

Keywords. non-viral vectors, polymer micelles, transfection, B16-F10 melanoma

(35.O3) A MICRO RNA APPROACH FOR THE REPROGRAMMING OF HUMAN EPIDERMAL KERATINOCYTES

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Introduction. Somatic cells can be induced to revert to a pluripotent state however to date most reprogramming strategies have employed viral vectors for the delivery of foreign genetic material. Reprogramming cells in the absence of viral vectors whilst reducing the amount of genetic material used will offer significant advances for regenerative medicine. Here we report the reprogramming of human epidermal keratinocytes (HEK) using a microRNA (miRNA) inhibitor, hsa-mir-145.

Methods. HEK were nucleofected using the Amaxa nucleofection system and maintained either in Epilife media or serum-free hESC media post-nucleofection.

Results. An efficient method of delivering miRNA inhibitors to HEK was established whereby 90.79% ± 0.38 (n=6) were successfully nucleofected with 20µM of miRNA inhibitor control.

The specific miRNA inhibitor, mir-145 was identified due its relationship with the major pluripotency markers Oct4, Sox2 and Klf4. In an attempt to exploit this relationship HEK were nucleofected with a hsa-mir-145 inhibitor.

It could be deduced that inhibition of mir-145 elevated the levels of all four of the definitive reprogramming factors Oct4, Sox2, Klf4 and C-Myc in all cases. In addition subsequent culture in hESC media resulted in a noticeable change in cell morphology with cells clustering together to form colonies. Furthermore the expression of Sox2, Nanog, Klf4 and C-Myc was further elevated when incubated with hESC media post-nucleofection.

Conclusions. This investigation has demonstrated, for the first time that a mir-145 inhibitor can actively induce the expression of normally silenced pluripotency genes in HEK. Thus confirming cell fate can be reversed without the use of potentially harmful DNA or viruses. The extent to which these cells have been reprogrammed has yet to be fully characterised however this study has highlighted the potential of a miRNA approach to cellular reprogramming.

Acknowledgements. Funding from the EPSRC for this study is gratefully acknowledged.

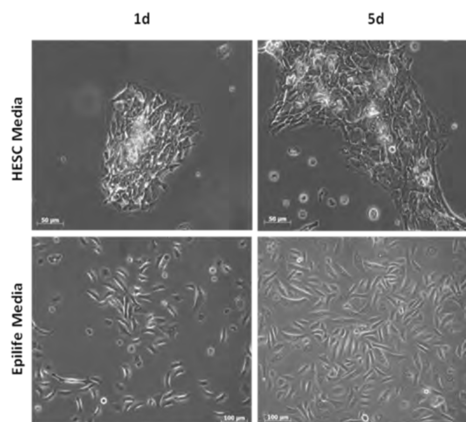

Figure 1: Light microscopic observation of HEK post-nucleofection with mir-145 inhibitor. Scale bar represents 100µM.

(35.O4) NEW POLYMER SHOWS HIGH TRANSFECTION CAPABILITY – INTRODUCING SINGLE CYCLIZED CHAINS

Dong Y (1), Newland B (1), Yao J (1), Zheng Y (1), Wang W (1), Pandit P (1)

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Introduction. Typical hyperbranched polymer preparation via multivinyl monomers (MVMs) all involve copolymerization reactions with very low proportions of branching agent (MVMs) which result in largely classic branched structures with accompanying cyclizations1. By comparison, the "one pot" transfection agents we have synthesized consist predominantly of single chains cyclised upon themselves into a knotted structure. Far higher transfection than the linear counterpart was at once seen, then, with the addition of PEG, lower toxicity than the commercially available transfection agents Poly(ethylene imine) (PEI) and the PAMAM dendrimer SuperFect®.

Methods. The monomers dimethylamino ethyl methacrylate (DMAEMA), ethylene glycol dimethacrylate (EGDMA) and poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) we used to synthesize the polymers termed PD-E 8%PEG and PD-E 14%PEG by in situ deactivation enhanced atom transfer radical polymerization (DE-ATRP2). After standard polymer and polyplex characterization a range of cell types (3T3 fibroblasts, human embryonic kidney (HEK293) cells, HeLa, adiposed derived stem cells (ADSC), nucleus pulposus cells (NP) and differentiated PC12s) were subjected to transfection and cytotoxicity analysis using both G luciferase and GFP (for FACS studies) plasmid DNA.

Results. A range of w/w ratios were analyzed for highest transfection with the optimum being plotted against the best comparative polymer dPAMAM (SuperFect®) (other polymers analyzed include PEI, PDMAEMA and poly(L-lysine) (data not shown)). PD-E8%PEG exhibited higher transfection than dPAMAM over all cell lines with the exception of ADSC with statistical difference shown in 3T3, NP and PC12 cells. Further to that higher cytotoxicity was only observed in HeLa cells.

Conclusion. These new "one pot" single cyclised chain structured polymers show favorable transfection properties - higher transfection ability and lower

cytotoxicity than the conventional polymer structures we tested.

References.

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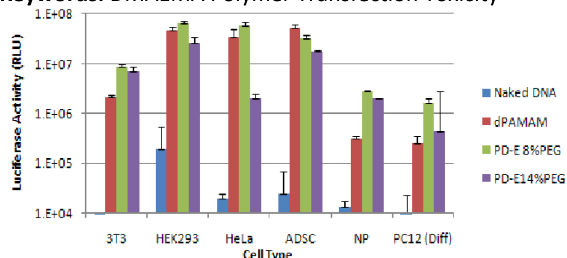
2. Wang W. et al., Macromolecules 40: 7184, 2007

Acknowledgments. Science Foundation of Ireland, Strategic Research Cluster (SRC), Grant number 07/SRC/B1163 and DEBRA.

Keywords. DMAEMA Polymer Transfection Toxicity



(35.O5) TRANSFECTION OF HELA CELLS WITH CATIONIZED GELATIN/CAP NANOPARTICLES

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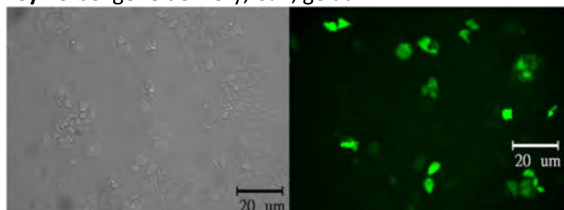
Gelatin is a nature polymer and it can be surface modified easily for many medical applications and it has been wildly used in food and drug industry. The surface modification with positive molecule (cholaminchloride hydrochloride) can increase the interaction between the cells and particles by electrostatistic force. In addition, Calcium phosphate co-precipitation with DNA was one of the most widely used trasfection methods. When CaP go into cells by endocytosis, it will be broken down and help the DNA escaped from the lysoome. The purpose of the study was using gelatin and calcium phosphate to prepared biodegradable and low cytotoxicity nanoparticles as a gene delivery carrier. In this study, we used DLS to analyse to particles size. Transmission electron microscope studied the inner structure and Atomic force microscope observed the surface morphology of the nanoparticles. The transfection test was used HeLa cell and the report gene was used pEGFP-C1. 5x104 cell/ well were seeded in 24 well plates.

The result showed that the nanoparticles had higher biocompatibility than the commercial product, LipofecamineTM2000 and the particle size distribution was around 100 ~300 nm. Because the zeta potential rose to +15 mV by cholaminchloride hydrochloride, the particles had more ability to bind the negative charge DNA and approach the cell membrane. Calcium phosphate can help the nanoparticles escaped from the lysosome, so it successful increased the transfection rate to 40%.

Keywords. gene delivery, CaP, gelatin



(35.O6) BMP-2 PLASMID DNA INCREASES BONE FORMATION IN MSC-SEEDED ALGINATE CONSTRUCTS

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1. Department of Orthopaedics, University Medical Center Utrecht, The Netherlands; 2. Faculty of Veterinary Sciences, Utrecht University, The Netherlands

Introduction. In the field of bone regeneration, BMP-2 is considered one of the most important growth factors. It has strong osteogenic activity and is widely used by clinicians in e.g. spinal fusions. The short half-life of BMP-2 protein however directs supraphysiological doses, leading to severe side effects. This study investigated the possibility of bone formation as a result of safe, low-cost, prolonged presence of BMP-2 using plasmid DNA based gene therapy instead of using BMP-2 protein. Therefore an injectable alginate hydrogel is combined with BMP-2 cDNA and BCP particles.

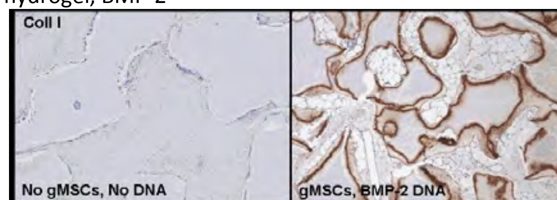
Methods. Alginate/BCP based constructs were implanted intramuscularly in 4 goats to investigate the effect of BMP-2 cDNA delivery on bone formation. The groups consisted of 2 goat MSC-seeded groups with or without His-tagged BMP-2 plasmid DNA and 2 non-seeded constructs with or without plasmid DNA. After 16 weeks samples were embedded in paraffin and MMA for histomorphometry.

Results. His-BMP-2 positive cells were found in all samples containing BMP-2 plasmid, indicating that both seeded and resident cells have taken up the cDNA and produce BMP-2 protein. Immunohistochemistry for collagen I(fig 1) and histomorphometry for bone volume showed that cell-seeded constructs contained much more bone compared to unseeded constructs which contained hardly any bone. In seeded constructs the addition of BMP-2 plasmid DNA clearly resulted in elevated osteogenic differentiation and bone formation. In unseeded constructs BMP-2 had mildly stimulatory effects on bone formation. Fluorochrome incorporation revealed that the onset of all bone formation was around or after week 9.

Conclusions. Transfection of seeded or resident cells leads to stable expression of BMP-2 during 16 weeks. The addition of BMP-2 cDNA to cell seeded constructs has a positive effect on osteogenic differentiation and bone formation, as seen by collagen I production and bone histomorphometry in vivo.

Acknowledgements. TeRM Smartmix, Prof Tabata and Huipin Yuan.

Keywords. Bone tissue engineering, gene delivery, hydrogel, BMP-2



36. RECENT DEVELOPMENTS IN SCAFFOLDING TECHNOLOGIES AND CELL BASED THERAPIES IN SPINAL CORD INJURY REGENERATION

Chair: António J. Salgado
Co-chair: Eva Sykova
Keynote speaker: Eva Sykova
Organizer: António J. Salgado
Synopsis: Spinal Cord Injury (SCI) results in a devastating condition with enormous social and personal costs, leading to life-long disability and a broad range of secondary complications. It is commonly characterized by a primary injury that leads to a cascade of cellular and biochemical reactions which cause further damage. The latter is known as "secondary injury" being characterized by microvascular alterations, edema, ischemia, necrosis, free radicals formation, lipid peroxidation, excitatory neurotransmitters accumulation, inflammatory response and other molecular changes contributing to further neural damage. Current approaches used in clinical practice are mainly based on the use of pharmacological agents, like methylprednisolone. Other approaches have also have been proposed in order to develop valid strategies for SCI repair such as biomolecular and biomaterial based therapies. However, due to the complexity of SCI repair it is unlikely that just one of the above referred strategies will be adequate to tackle the problem. In recent years tissue engineering and cell based approaches have been suggested, through a number of possible routes, as promising alternatives to therapies currently available in the clinics. In fact it is probable that only following integrative and combinatory concepts, such as those presented by Tissue Engineering (TE) that it will be possible to develop a successful approach. Therefore the present symposium aims at discussing the most recent developments in this field, namely those focusing on:

1) New processing techniques for the development of scaffolds/hydrogels for SCI applications
2) Innovative tissue engineering strategies for SCI regeneration
3) Nanotechnology based approaches in SCI
4) Novel sources and applications methods of adult/embryonic stem cells for SCI based therapies

(36.KP) STEM CELLS, BIOMATERIALS AND OTHER CURRENT STRATEGIES FOR THE TREATMENT OF SPINAL CORD INJURY: A PRECLINICAL AND CLINICAL STUDY

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Introduction. Embryonic and adult stem cells have been investigated for their therapeutic potential in brain and spinal cord injury (SCI). Mesenchymal stem cells (MSC) from bone marrow and human fetal spinal precursors (SPC) have the capacity to migrate towards lesions and induce better regeneration.

Materials and Methods. We used a balloon-induced compression lesion in adult rats, followed by the transplantation of MSC or SPC labelled in culture with iron-oxide nanoparticles; in vivo MRI was used to track their migration and fate. After acute (7 days post-injury) or chronic (5 weeks post-injury) transplantation, animals were tested using the BBB (motor) and plantar (sensory) tests once a week for up to 6 months. Animals with chronic injury were implanted with a "spinobridge", either hydrogels or nanofibers, seeded in vitro with cells.

Results. The implantation of both cell types resulted in significantly smaller lesions and higher BBB scores, while MRI tracking proved that the cells migrated into the lesion and survived there for several months. SPC implantation resulted in greater improvement, and a number of implanted cells differentiated into motoneurones and astrocytes. Improvement of motor and sensory scores in chronic SCI was only achieved after the implantation of biomaterials seeded with MSC or SPC. Compared to control rats or rats with a bridge only, this strategy led to improved scores starting 4-5 months after implantation. The results of our clinical trial show that implantation is safe and has a beneficial effect if administered within 4 weeks after injury (1).

Conclusions. The treatment of chronic SCI requires a combination of strategies, e.g. stem cells, bridging lesion cavities with biomaterials, enzymes such as chondroitinase, growth factors or antibodies against Nogo.

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This work was supported by the grants AV0Z50390703, 1M0538, LC554, IAA500390902, P108/10/1560, 203/09/1242.

Keywords. Spinal cord injury, hydrogel scaffolds, nanotechnologies

(36.O1) URINARY BLADDER MATRIX WITH LASER ACTIVATED CHITOSAN BASED BIO-ADHESIVE FOR SUTURELESS NERVE REPAIR

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1. Bio/Polymer Research Group, School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, Australia; 2. Centre for Vascular Research, The University of New South Wales, Sydney, Australia; 3. McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, USA

Biocompatibility of biomaterial-based scaffolds is pivotal for successful tissue repair and reconstruction in tissue engineering applications, particularly in spinal cord injuries. In vivo implantation of a potential biomaterial may trigger irreversible host immune responses leading to loss of function and impaired healing. Suitable biomaterials that can serve as nerve conduits for transplanting olfactory ensheathing cells (OECs) into damaged spinal cord to induce nerve repair and regeneration are required. Consequently, this study incorporated extracellular matrix derived from porcine urinary bladder (UBM), which has been shown to facilitate the recruitment of marrow-derived stem cells, with SurgiLux®, a chitosan-based laser activated adhesive to potentially fabricate a therapeutic scaffold for sutureless nerve repair. Furthermore, this study examined the influence of incorporated UBM-SurgiLux®

biomaterials on OECs at the cell-material interface by adopting a cell cycle, apoptosis and proteomics approach.

Cellular response at the material interface revealed a normal maintenance of regular morphology and enhanced growth of OECs compared to chitosan films alone. Cell cycle analysis revealed a significant difference in the DNA content of cell populations cultivated in the presence and absence of UBM-SurgiLux® films. Additionally, detection of early stage of apoptosis using Annexin V assay revealed significant deviations from standard culturing conditions as chitosan induced cell population to undergo early apoptotic activation. A comparative protein profile expression was performed which revealed secreted proteins that may be responsible for the enhanced cellular growth exhibited with UBM-SurgiLux® films. Furthermore, the phenotypic response of macrophages on UBM-SurgiLux® biomaterials was evaluated using Flow cytometry analysis and real-time PCR.

These results provide new insights into the nature of OECs and their response to their microenvironment which is of paramount significance for the success of UBM-SurgiLux® biomaterials that promote regeneration of neural tissue.

Keywords. Biomaterials, Olfactory ensheathing cells, chitosan, extracellular matrix, spinal cord injury

(36.O2) CLINICAL GRADE PREPARATION OF HUMAN AUTOLOGOUS SCHWANN CELL FOR TREATMENT OF SPINAL CORD INJURY

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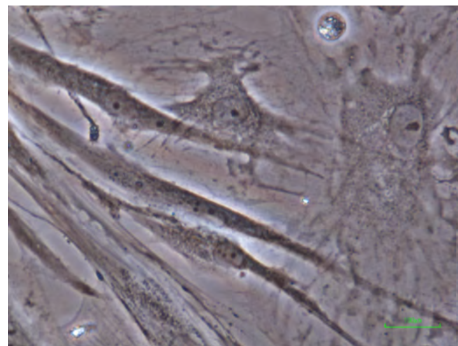
Introduction. Clinical grade cultivation of human schwann cell by the utilization of human autologous serum instead of fetal bovine serum (FBS), and also avoiding any growth factors, can increase safety level of this procedure in cases of clinical cell transplantation. The aim of this study was demonstration of the feasibility of clinical grade schwann cell cultivation.

Materials and Methods. In this experimental study after obtaining consent from close relatives we harvested 10 sural nerve from brain death donors and then cultured in 10 seperated culture media plus autologous serum., we also prepared autologous serum from donor's whole blood. Then cultured cells were evaluated by S100 antibody staining for both morphology and purity.

Results. Cell purity range was from 97% to 99% (mean = 98.11 ± 0.782%). Cell count was 14055.56 ± 2480.479 per micro liter . There was not significant correlation between cell purity and either the culture period or the age of donors (P> 0.05). The spearman correlation coefficient for the cell purity with the period or the age of donors was 0.21 and 0.09, respectively.

Conclusions. We demonstrated the feasibility of clinical grade Schwann cell cultivation by using of human autologous serum instead of fetal bovine serum and also without the using of growth factors. We also recommended all cell preparation facilities to adhere to GMP and other similar quality disciplines specially in preparation of clinically-used cell products.

Keywords. Autologous serum, cultivation, Schwann cell, spinal cord



(36.O3) DENDRIMER-BASED NANOPARTICLE DELIVERY SYSTEM FOR THE SUSTAINED AND INTRACELLULAR DELIVERY OF METHYLPREDNISOLONE TO CNS CELLS: POTENTIAL APPLICATION IN SPINAL CORD INJURY TREATMENT

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Spinal Cord Injury (SCI) is a traumatic condition where the post-trauma regeneration is difficult to accomplish due to the extremely harsh environment that follows the injury. One possible approach that could successfully act on the neuroprotection and/or regeneration of the lesioned area would be the use of cell-specific intracellular drug delivery systems. Methylprednisolone (MP) is an anti-inflammatory drug currently employed in the clinical practice to treat SCI. However, it presents low efficacy even when used in high doses, causing several adverse side effects in patients. Thus, we are proposing the use of a dendrimer-based nanoparticle system composed of a polyamidoamine (PAMAM) core and grafted with the natural polymer carboxymethylchitosan (CMCht). Being so, CMCht/PAMAM dendrimer nanoparticles (NPs) were synthesized and MP was incorporated in the NPs. MP-loaded NPs were labeled with fluorescein isothiocianate to evaluate internalization and intracellular trafficking. Characterization results indicated that MP-loaded NPs possess diameters around 109 nm and negative zeta potential values at the physiological pH. When incubated with glial cells (200µg/ml) the MP-loaded NPs were easily internalized by all CNS cell types reaching 100% internalization 24 hours after NPs addition. MP release profile was assessed by HPLC. Results revealed an initial burst within the first 24 hours followed by a sustained release for periods up to 14 days. Finally, the anti-inflammatory profile of these NPs was assessed in pure microglial cell cultures using 1 mg/mL and 1.5 mg/mL NPs addition. The MP released from the NPs induced a significant decrease on cell viability (around 50% when compared to the control). These results indicate that these NPs might be used to modulate the action of inflammatory cells in SCI sites. Additionally, as they are also internalized by astrocytes and oligodendrocytes one

may hypothesize whether the behavior of these cells may also be modulated by MP-loaded NPs.
Keywords. Nanoparticles, intracellular drug delivery, neuroprotection, spinal cord injury

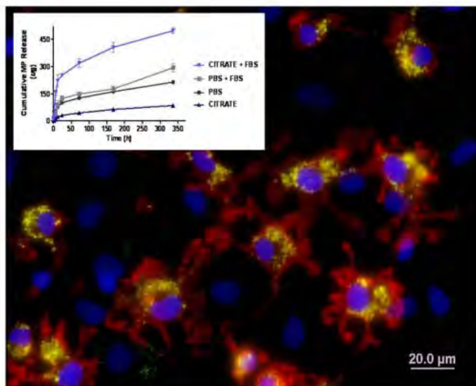

Figure 1 – Fluorescence microscopy image of microglial cells internalizing methylpredisolone loaded CMCht/PAMAM dendrimer nanoparticles in a glial cell culture (immunocytochemistry was performed using CD11b antibody). Also shown above the MP release profile for 14 days in two buffer solutions (PBS pH 7.4 and citrate buffer pH 5.0).

(36.O4) DEVELOPMENT AND CHARACTERIZATION OF A PHB-HV 3D SCAFFOLD FOR A TISSUE ENGINEERING AND CELL-THERAPY COMBINATORIAL APPROACH FOR SPINAL CORD INJURY REGENERATION

Samy SM (1,2), Silva NA (1,2), Correlo VM (1,2), Silva RM (1,2), Fraga JS (3) , Pinto LA (3), Pinto LG (3), Castro A (3), Gimble JM (4), Sousa N (3), Salgado AJ (3), Reis RL (1,2)

1. 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal; 2. Institute for Biotechnology and Bioengineering, PT Government Associated Lab, Braga, Portugal; 3. Life and Health Sciences Research Institute(ICVS), School of Health Sciences, University of Minho, Braga, Portugal; 4. Stem Cell Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA

Spinal cord injury (SCI) leads to devastating neurological deficits and disabilities that result in physical and lifestyle constraints that totally reconfigure the realities of daily life. Given the complexity of SCI, its treatment will require a combinatorial approach such as that presented by tissue engineering. The objective of the present work was to develop a PHB-HV-based 3D scaffold aimed for SCI regeneration. The PHB-HV 3D structures were processed through an emulsion freezing/freeze-drying technique. Following the processing phase, the scaffold morphology/porosity and mechanical properties were assessed. Subsequently, direct contact assays were performed using CNS-derived cells, OECs and MSCs (HUCPVCs, BM-MSCs and ADASCs). The cells were seeded on the 3D structures at different densities and allowed to grow for periods up to fourteen days with weekly assessments of phenotype, viability and proliferation. The morphological/porosity analysis showed that PHB-HV 3D scaffolds disclosed a fully interconnected network of pores, with a porosity of 88.1±0.3% and an average pore size of 163.5±0.1µm. Mechanical analysis revealed that the scaffolds are anisotropic structures, exhibiting on average a longitudinal and transversal compressive modulus of 1.3MPa and 3.8MPa, respectively. Finally direct contact assays revealed that the developed 3D structure could support the in vitro culture of CNS-derived cells (neurons, astrocytes and oligodendrocytes), OECs and MSCs from different sources.

The present work showed that freeze-drying was an adequate processing technique for the production of PHB-HV-based 3D scaffolds. Furthermore the developed scaffolds morphology/porosity, and mechanical properties analysis revealed a random morphology and a fully interconnected network of pores. Finally, it was possible to observe that scaffolds here in presented supported to the growth of different cell populations relevant for SCI regeneration. Future work will be focused on the in vivo proof of concept using the PHB-HV 3D scaffolds as the template for SCI regeneration.

Keywords. 3D scaffolds, Tissue engineering, Cell-based Therapy, Spinal Cord Injury regeneration

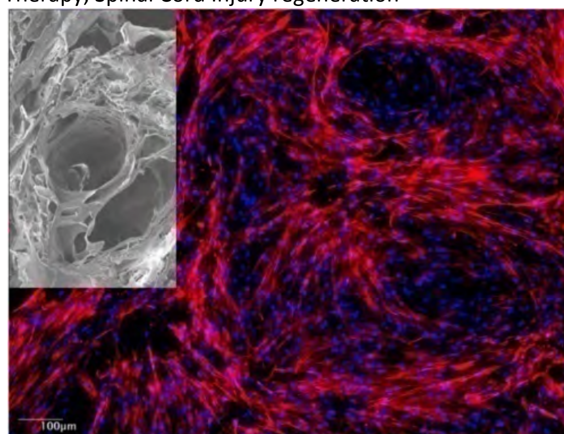

Figure 1: Adipose-derived adult stem cells (ADASCs) seeded on PHB-HV 3D scaffold. Insert: PHB-HV 3D scaffold SEM image, 100x magnification (top left).

(36.O5) IMPROVING TISSUE ENGINEERED SKELETAL MUSCLE THROUGH INCORPORATION OF A NEURAL INPUT

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There are a number of methodologies for tissue engineering the contractile component of skeletal muscle (SkM) however a truly biomimetic *in vitro* model requires a functional neuromuscular input. Here we use two established models of SkM tissue engineering to act as a base for addition of a neural input. Primary muscle-derived cells (MDCs) were seeded within either a collagen (1) or a fibrin (2) construct. The collagen construct is believed to represent a more “adult” SkM phenotype whilst the fibrin construct is believed to represent a more “developmental” phenotype. Primary motoneurons (MNs) were then plated on top of the SkM constructs at a density of 50,000 cells per fibrin construct or 1 million

cells per collagen construct. These co-cultures were maintained for up to 15 days before analysis by confocal microscopy and Q-PCR.

Microscopy revealed that both the collagen and fibrin models demonstrate a physiological architecture more representative of the *in vivo* tissue compared with conventional 2D cell culture controls. Survival of neurons co-cultured with MDCs in both 3D models has been confirmed by confocal microscopy of immunostained constructs. Exploratory neurites developed in close association with the cultured myotubes. The formation of putative synaptic contacts was attested to by the expression of both synaptic vesicle protein 2 and alpha-Bungarotoxin. Q-PCR gene expression analysis suggests improved muscle fibre maturation/ hypertrophy in co-culture as indicated by increased expression of major contractile proteins compared with MDC only cultures. mRNA for the acetylcholine receptor epsilon-subunit, found at the neuromuscular junction, was likewise significantly increased.

This work describes the establishment and optimum culture conditions for 3D myotube-motoneuron co-cultures in two well-characterised, 3D, *in vitro* systems. Motoneurons not only survive within these constructs, but also interact with the developing muscle fibres, leading to the formation of putative synaptic structures and improved SkM construct maturation.

References:

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(36.P1) THE THERAPEUTIC POTENTIAL OF A NOVEL SELF-ASSEMBLING PEPTIDE IN SPINAL CORD INJURY

Liu Y (1), Ye H (1), Bayon Y (2), Fehlings MG (3)

1. Genetics and Development, Toronto Western Research Institute, Toronto, ON, Canada; 2. Covidien – Sofradim Production, Trevoux, France; 3. Genetics and Development, Toronto Western Research Institute; Division of Neurosurgery, University of Toronto, Toronto, ON, Canada

Introduction. Spinal cord injury (SCI) results in irreparable neurological insults partially due to the limited axonal regeneration. Scar tissue and gaps formed in the nervous tissue during phagocytosis of dying cells are the major barriers that prevent axonal regeneration. Given the poor regenerative capability in the injured spinal cord, it is necessary to supply an appropriate tissue-engineered scaffold that can serve as a bridge for endogenous cell migration and axonal elongation. Self-assembling peptides (SAPs) can form a network of nanofibers that has similar scale to the native extracellular matrix, and therefore can provide an "in vivo" environment for tissue regeneration. In the present study, we investigate the therapeutic effects of one of the novel SAP, K2(QL)6K2 (QL6), in SCI.

Materials and Methods. In adult rats, the QL6 or saline was injected locally 24 hours after a 35g clip compression SCI. GFAP, Iba1 antibodies and TUNEL immunostaining were used to evaluate astrogliosis, inflammation and apoptosis, respectively. Tract-tracing technique with DBA was used to determine whether QL6 injection would support axonal regeneration. Compound action potentials were recorded from the dorsal column white matter to assess improvement of the axonal conduction after QL6 treatment.

Results. The QL6 was successfully injected into the cord. It aggregated in the epicenter and diffused rostro-caudally into the penumbra zone. QL6 administration significantly reduced post-traumatic apoptosis seven days post-SCI, although it had minimal effects on reducing inflammation and astrogliosis. Electrophysiological recordings confirmed that the axonal conduction was increased after QL6 injection, including increased magnitude in the compound action potentials and their faster propagation across the injured sites.

Conclusions. This work represents the first detailed examination of the therapeutic effects of QL6 in SCI. Several aspects of the SCI-induced pathology have been alleviated, resulting in the functional recovery in the axonal conduction. Further investigation will incorporate long-term neurobehavioral and histological endpoints.

Keywords. Spinal cord injury; Self-assembling peptides; Axonal regeneration

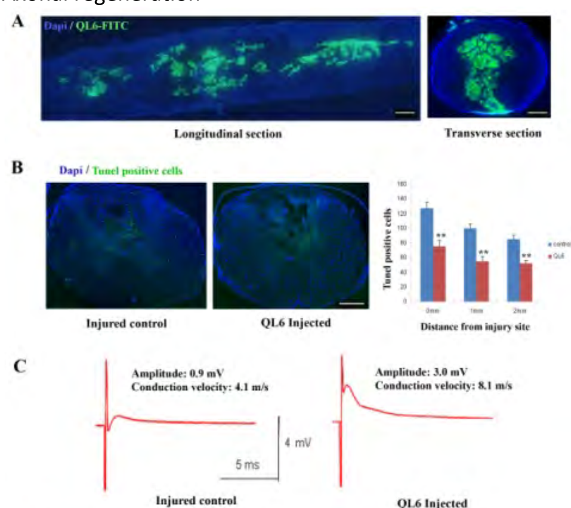

Figure: Effects of QL6 on apoptosis and functional recovery after SCI. A. The QL6 was successfully injected into the cord. It aggregated in the epicenter and diffused rostro-caudally into the penumbra zone. Transverse section is taken from the epicenter. B. QL6 administration significantly reduced post-traumatic apoptosis seven days post SCI. Images are representative sections taken 2mm rostral to the epicenter. Scale bar 350 μm for A and B. ** P < 0.001. C. Both the amplitude and the conduction velocity of the component action potentials increased after QL6 injection.

37. RELATING IN VIVO BIOCOMPATIBILITY WITH IN VIVO OUTCOME

Chair: Jöns Hilborn

Co-chair: Gilson Khang

Keynote speaker: Gilson Khang

Organizers: Jöns Hilborn, Gilson Khang

Synopsis: *In vitro* evaluation of scaffolds and scaffold materials is today performed in 2D culture dishes to evaluate critical factors such as cytotoxicity, gene-toxicity,

cell adhesion, migration, differentiation and effect of added soluble factors. This gives predictive tools for designing scaffold materials to comply with just this i.e. monolayers of cells on static 2D substrates. The major complication when transferring materials to scaffolds *in vivo* is scarring and fibrosis as has been observed frequently in almost all organs but has rarely been studied systematically.

Thus, although contractions and fibrosis has been observed in diverse organs, with very few exceptions, quantitative data on organs other than skin are practically absent from the literature. Lack of quantitative reports on the topic has contributed to widespread misunderstanding of the relative importance of this critical healing process in regeneration of adult organs that has not been accounted for when designing scaffolds. Our understanding of the underlying mechanisms eliciting this response in soft tissue is poor. Presently, there is no real/viable alternative to predetermine the local host response to a new material or device. Therefore development and testing of materials is based on "blind trial and error" attempts *in vivo* in animals and for humans for each and every implant material. Therefore this session will be devoted to the relation of materials design with *in vivo* responses such as immunological aspects, mechanical stimulation and the relating inflammatory that results in functional tissue and not scar.

(37.KP) BIOCOMPATIBILTY OF PLGA-BASED SCAFFOLDS FOR TEMPS IN TERMS OF SAFETY

Khang G (1)

1. Chonbuk National University

Implanted biomaterials and drug delivery vehicles have been reported to induce sequential events of immunologic reactions in response to injury caused by implantation procedures and result in acute inflammation marked by a dense infiltration of inflammation-mediating cells at the material-tissue interface. Poly(lactide -co-glycolide)(PLGA) is a member of a group of poly(α-hydroxy acid) that is among the few synthetic polymers approved for human clinical use by FDA. Consequently, it has been extensively used and tested for scaffold materials as a bioerodible material due to good biocompatibility, relatively good mechanical property, lower toxicity and controllable biodegradability. PLGA degrades by nonspecific hydrolytic scission of their ester bonds into their original monomer, lactic acid and glycolic acid. During these processes, there is very minimal systemic toxicity, however, in some cases, their acidic degradation products can decrease the pH in the surrounding tissue that result in local inflammatory reaction and potentially poor tissue development. Currently, biomaterials are endowed with biocompatibility through three different methods which are: coating with hydrophilic molecules, modifying surface characteristics using physiochemical methods and impregnating bioactive substances. In our laboratory, the natural/synthetic nano-hybrid scaffolds have been investigated such as small intestine submucosa (SIS), demineralized bone particles (DBP), DBP gel, fibrin, keratin, hyaluronic acid, collagen gel, silk and a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer (PMEH) with PLGA to reduce cellular inflammatory response. In this lecture, we introduced synthetic/natural nanohybrid as DBP/PLGA and SIS/PLGA scaffold in terms of scaffold design for the reduction of host response and the augmentation of tissue formation. This information will be supporting the basic strategy for the scaffold design with better improved biocompatibility.

Keywords. PLGA, scaffold, safety, in vivo/in vitiro

(37.O1) EVALUATION OF THE IN VIVO INFLAMMATORY DYNAMICS OF BOTH IMPLANTABLE SYNTHETIC AND TISSUE-BASED BIOLOGIC MESHES FOR USE AS ABDOMINAL WALL REPAIR BIOMATERIALS IN A SUBCUTANEOUS IMPLANTATION MODEL

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1. University of Liverpool; 2. Covidien - Sofradim Productions

Introduction. Meshes for tissue repair are manufactured from a broad spectrum of materials, synthetic or biological. Modifications in chemical or biological composition result in changes in foreign body reaction. In this study inflammatory dynamics for both synthetic and biological meshes were investigated to determine responses in changing key features: chemistry and knitting pattern (synthetics), and tissue origin and cross linking chemistries (biologics).

Materials and Methods. Materials ($1cm^2$) were implanted subcutaneously (SC) into the backs of 6 week old male wistar rats (4 materials per animal, n=6/material/time point), for 2, 5, 7, 14 and 28 days. Tissue processing was carried out after resin infiltration observing histopathology (H&E, Van Gieson, Von Kossa) and quantitative immunohistochemistry. Collagens I and III were identified to qualitate neotissue matrix. Materials included synthetics derived from PP, PET, PGA and crosslinked or non-crosslinked biologics from porcine dermis or small intestinal submucosa (SIS).

Results. All meshes stimulated a foreign body reaction allowing precise deduction of their inflammatory characteristics. Histopathology demonstrated differences in the magnitude and kinetics of cellular infiltration, neo-vascularisation and interface thickness. Immunohistochemistry allowed quantification of the immune cell profile of materials throughout their implantation. The predominant cell infiltrates for all materials were of a myeloid rather than lymphoid origin demonstrated by staining using macrophage markers CD68 (Fig.1), CD163 and T-lymphocyte antigen CD5. Tissue repair index was evaluated to compare the quality of the neo-tissue from the ratio of collagen I:III (Fig.1). A greater acute inflammation reaction was observed with synthetic vs. biological meshes with the exception of the SIS mesh.

Conclusion. By using inflammation and wound healing indicators, this study showed that synthetic meshes elicited the greatest cell response comprised predominantly of myeloid cells; the largest numbers of macrophages being recruited by the multifilament PET mesh. The SIS mesh also stimulated a similarly strong inflammatory reaction.

Keywords. In vivo, inflammatory response, biocompatibility, hernia

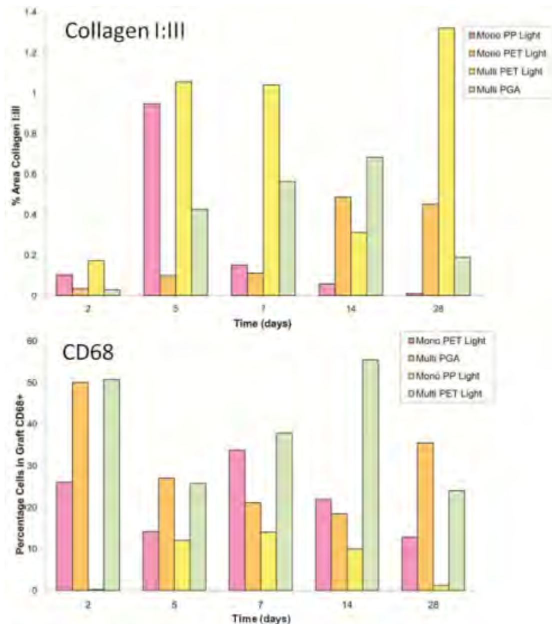


Fig.1 Quantitative Immunohistochemical Analysis of CD68 (mature tissue macrophages) and wound maturation index Collagen I:III. All cell counts are normalized to total cell number in the explanted graft

(37.O2) EFFECT OF PLGA SCAFFOLD PENETRATED DEMINERALIZED BONE SOLUTION FOR CHONDROGENESIS: IN VIVO TEST

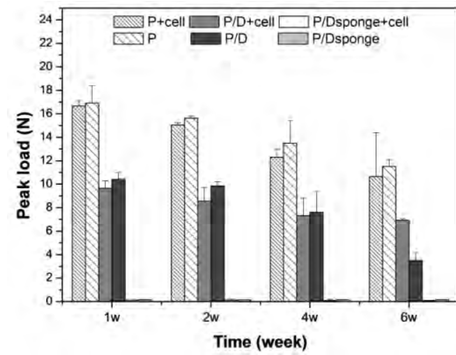
Sim CR (1), Seo HS (1), Lee YM (1), Song JE (1), Lee D (1), Khang G (2)

1. Dept of BIN Fusion Tech; 2. Dept of Polymer Nano Sci Tech

Articular cartilage repair remains a challenge to surgeons and basic scientists. The field of tissue engineering allows the simultaneous use of material scaffolds, cells and signaling molecules to attempt to modulate the regenerative tissue. Adult articular cartilage tissue has poor capability of self-repair. For the effective articular cartilage repair and regeneration, numerous studies have been focused on tissue engineering approaches motivated by the clinical need. In this study, we developed synthetic/natural hybrid scaffolds with PLGA and demineralized bone solution (DB solution) for the application of articular cartilage regeneration. Composite scaffolds of PLGA penetrated DB solution (PLGA-pen-DBP) were manufactured by a solvent casting/salt leaching method soaked in DB solution. The scaffold mechanical strength, histology and immunohistochemistry were performed to elucidate in vitro and in vivo cartilage development and the deposition of cartilage-specific extracellular matrices. we observed better to keep the characteristic of cartilage cell in the PLGA-pen-DBP scaffolds than that PLGA scaffolds. Histology and immunohistochemistry staining showed that attached cells at PLGA-pen-DBP scaffolds had been more increased than PLGA scaffolds for 6 weeks. This study suggests that PLGA-pen-DBP scaffold may serve as a potential cell delivery vehicle and a structural basis for in vitro tissue engineered articular cartilage.

This research was supported by WCU(R31-20029) and Musculoskeletal Bioorgan Center (0405-BO01-0204-0006).

Keywords. Chondrocyte, PLGA, Demineralized bone particle (DBP).



(37.P1) REDUCTION OF INFLAMMATORY REACTION OF USING PURIFIED ALGINATE MICROCAPSULES

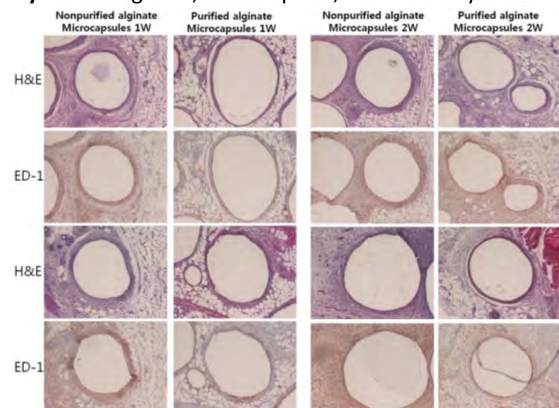
Hwang JH (1), Kim OY (1), Kim AR (1), Yoon KH (2), Lee D (1), Khang G (3)

1. Department of BIN Fusion Tech; 2. Department of Internal Medicine; 3. Department of Polymer·Nano Sci Tech

Alginate, a polysaccharide extracted from brown seaweed, remains the most widely used biomaterial for immobilizing cells to be transplanted, because of the good viability of encapsulated cell and the relatively ease processing for cell encapsulation. However, the main drawback is the immune rejection in vivo. To overcome this problem, we have developed using modified Korbutt's method for alginate purification. After alginate microcapsules manufactured, NIH/3T3 fibroblast and RAW 264.7 macrophage cells were seeded in purified and non-purified alginate microcapsules, and then cell viability were analyzed by MTT assay. RT-PCR was performed to assess mRNA expression for inflammation cytokines such as TNF-α. Purified and non-purified alginate microcapsules were implanted into a wister rat, and the implanted alginate microcapsules were extracted after 1, and 2 weeks. Tissues surrounding implants were harvested and evaluated by histology through H&E staining and immunohistochemistry through ED-1 staining (Figure 1). In this result, purified alginate microcapsules was removed contamination element by process of purify and reduced inflamantion. Therefore purified alginate anticipated that highly potent for numerous applications in biomaterial.

This research was supported by WCU (R31-20029) and MHWFA(A040004).

Keywords. Alginate, microcapsule, inflammatory reaction



(37.P2) APPLICATION OF 3-DIMENSION CULTURE IN COMPOSITE INTERVERTEBRAL DISCS USING DBP/PLGA SCAFFOLD AND DBP SPONGE

Yoo H (1), Song Y (2), Yoo SC (1), Song JE (1), Lee D (1), Khang G (2)

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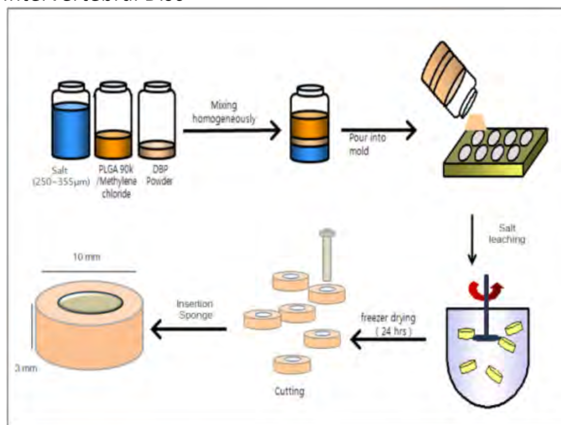
Tissue engineering strategies have the great potential to improve intervertebral disc repair. Determining a suitable biomaterial scaffold for disc regeneration is difficult due to the complex structure of the tissue. We developed the synthetic/natural hybrid scaffolds with poly (lactide-co-glycolide) (PLGA) and DBP (demineralized bone particle) for give similar environment of intervertebral disc in tissue engineering. Composite scaffolds of DBP/PLGA scaffolds and DBP sponges were manufactured by using solvent casting/salt leaching method and freeze-drying method in Figure 1. We evaluated the 3-dimensional scaffolds on proliferation and phenotype maintenance of disc cells. The composite scaffolds were characterized by SEM and MTT. RT-PCR and histology stains were assessed to figure out the disc cells. In assay results, we confirmed cell viability was well attached and disc cells were strongly expressed their specific mRNA in composite scaffolds. These results indicate the composite scaffolds are useful for disc regeneration and application of tissue engineering.

This research was supported by WCU (R31-20029) and MBC (0405-BO01-0204-0006).

Keywords. DBP, PLGA, DBP/PLGA composite scaffold, Intervertebral Disc



(37.P3) PLGA/DBP MATRIX COMPOSITE SCAFFOLD FOR CARTILAGE TISSUE ENGINEERING

Kim HN (1), Kim HE (1), Lee SK (1), Kim OY (1), Ahn WY (1), Lee D (1), Khang G (2)

1. Dept of BIN Fusion Tech; 2. Dept of Polymer·Nano Sci Tech

Abstract. Polymer scaffold have been employed to support cell growth and extracelluar matrix (ECM) secreation. Natural/synthetic biomaterial composite 3-D scaffolds were prepared a solvent casting/salt leaching method and designed by varying the DBP (demineralize bone particle) contents from 10 to 80 wt %. Articular chondrocytes of Newzealand White rabbits were cultured in vitro in these scaffolds. Cell viability (MTT), glycosaminoglycan (GAG) assay, RT-PCR, hematoxylineosin (H&E) and safranin-O stanining were evaluated to investigate the effect of the DBP on the cell viability and extracellular matix. As DBP contents of PLGA/DBP scaffold become higher, the rate of cell growth (Figure 1) and ECM secretion increase. Also the phenotype of the cells is maintained better in PLGA/DBP scaffolds containing DBP 20, 40 and 80 wt %. H&E and Safranin-O staining of tissue-engineered implants indicated progressive tissue formation with time. The characteristic alignment of cells were not observed at early times in implants but some oriented structures were apparent at 4 and 6 weeks. To further chacraterize in vivo inflammatory response surrounding the implants, histological examination was performed on 1 weeks after implantation. remakrable inflammation was observed in tissue surrounding the PLGA scaffold, although this inflammatory reaction was progressively diminished with an increase in DBP content in PLGA scaffold. The fibrotic thickness was significantly lower in PLGA/DBP scaffolds. These results demonstrated Chondrocytes prefer PLGA/DBP scaffolds with the DBP 40 and 80 wt % for better proliferation and ECM production. It is believed that DBP would significantly affect the cell metabolism.

This research was supported by WCU (R31-20029) and MBC (0405-BO010204-0006).

Keywords. DBP, PLGA, Chondrocyte

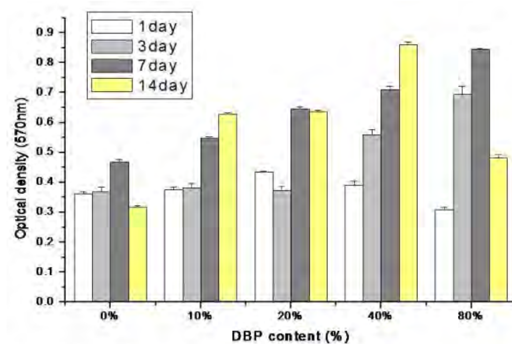


Fig 1. Cell viability of chondrocytes in DBP/PLGA scaffold analyzed by MTT assay after 1, 2 and 3 weeks post-seedi *in vitro* (n=2)

38. RELEVANT MODELS FOR PRE-CLINICAL EVALUATION ON THE PATH TO CLINICAL TRANSLATION

Chair: Julian Dye

Co-chair: Tomas Egana

Keynote speaker: Eustace Johnson

Organizers: Tomas Egana, Julian Dye

Synopsis: For the past decade, tissue engineering techniques have been developed and several models have been commercialised. However, the clinical impact of many commercial products has been disappointing. A critical issue for tissue regeneration is the interaction of the host with intended scaffold, cells or constructs. This symposium aims to address the intersecting issues of:

1. Potential of mammals to regenerate tissue, and more specifically, the potential of the host response to tissue engineered interventions, to result in regeneration or restoration of function.

2. Potential of progenitor and stem cells to integrate and organise. Although regeneration of certain tissues will

very likely need stem cell differentiation and integration (e.g. within the nervous system), stem cells fall into a spectrum of 'potentiation' strategies spanning protein or gene therapies. These might stimulate host proliferative and regeneration responses by appropriate scaffold, or scaffold in combination with specific cytokines and growth factors, providing a normalised and spatially structured healing environment. The design of therapies needs to be cognisant of embryological development and differentiation of the target tissue and organ.
3. The need for suitable models to develop these themes. Cumulative pre-clinical and clinical experience shows the pressing need for relevant and effective pre-clinical models to evaluate the current and new generations of therapeutic approaches.
Full thickness skin reconstruction is an illustrative 'test case' for how these ideas form the symposium theme. Clinically, dermal repair is characterised by degrees of scarring or fibrosis and it is unclear whether regeneration is possible to any extent. Although scarring is markedly influenced by various secondary factors such as mechanical environment (joint or relatively immobile area, body region, skin mobility) and infection, it is also appreciated that healing by scar-less regeneration may be attainable.
Research issues:
Can therapeutic interventions orchestrate and optimise the endogenous wound healing process to a useful extent? How much do therapeutic wound healing interventions depend ultimately on in situ differentiaton of mobilised stem/progenitor cells? Can mobilised stem/progenitor ever result in skin adnexia reconstruction, or forming stem cells niche structures? Can stem or progenitor cells within a tissue-construct and engrafted then integrate and establish organised self renewable anatomical structure?
How do we measure these outcomes? How do we determine whether stem cell niches have been created? Can we quantitatively measure how closely reconstructed tissue anatomy resembles original structure, or is a qualitative anatomical assessment needed?
Experimental exploration of these issues needs appropriate understanding, models and histological examination. Engrafted tissue territory, either acellular scaffold, or potentiated (e.g. scaffold with growth factors, genetic transfection vectors, or cellularised) is likely to stimulate some type of chemokinetic, angiogenic and inflammatory signalling, leading to a host-graft interaction. Accelerated angiogenesis may modulate inflammatory responses, and therapeutic potentiation mechanisms could regulate the regenerative outcome through contributing to chemokine/cytokine/growth factor networks.
The symposium invites contributions to explore this topic through instructive examples, possibly regeneration of target tissues like skin, bone, adipose...
Keynote speaker - Dr Eustace Johnson, has a distinguished experience in clinical interface research, in the spinal injury unit, Oswestry, UK. He has worked on experimental and preclinical approaches for clinically relevant solutions to significant injuries such as spinal disc prolapsed, spinal cord lesion and pressure ulceration. He has described the regenerative potential of intravertebral disc, and is currently exploring the regenerative potential of MSC for various of these problems. Second speaker - Dr Johan Van Neck, who has long experience in pre-clinical models and in realising the potential of RGTA (heparan-mimetic) to modify the extracellular milieu, stimulating a non-healing chronic wound to develop a reparative (possibly regenerative) healing response. Contributions from the organisers' groups and others will extend the theme.

(38.KP) THE REPARATIVE ACTIVITY OF MESENCHYMAL STEM CELLS: WHICH IS THE RIGHT CELL FOR THE RIGHT JOB?

Johnson WE (1)
1. Life & Health Sciences, Aston University

Bone marrow-derived mesenchymal stem cells (MSC) have enormous potential as an autologous cell therapy in regenerative medicine. However, MSC are a somewhat poorly characterized and heterogeneous cell population, with evident intra- and inter-patient variability in terms of their growth, differentiation potential and paracrine activity. As clinical treatments involving MSC transplants or MSC-based tissue engineered products are already underway, there is an urgent need to examine and test how MSC phenotype relates to wound healing activity.
With a view to treating patients with spinal cord injury (SCI), we have developed *in vitro* methods to examine the regenerative potency of human MSC. Using these assays we have demonstrated (i) MSC can be isolated and culture expanded from patients with chronic SCI; (ii) human MSC express and secrete well known and novel growth and neurotrophic factors; (iii) in the context of the extracellular milieu present in the damaged spinal cord, MSC stimulate nerve growth but that this stimulatory activity is mediated by direct cell-cell interaction rather than by paracrine activity alone. These findings support the use of direct MSC transplants for neural repair after SCI. Paralysed patients suffer additionally from the consequences of SCI through the development of pressure ulcers. Taking a proteomic approach and using established scratch wound assays, we found that human MSC secreted a variety of cytokines known to influence keratinocyte and dermal fibroblast migration, along with angiogenesis. Perhaps surprisingly, the stimulatory effects of the MSC secretome on these various cell types were associated more with the secretion of extracellular matrix components.
There was donor-to-donor variability in the capacity of the MSC populations to exert these different wound healing effects and, in the context of autologous cell therapies in orthopaedics, similar variability has been seen in the capacity of MSC to differentiate to form cells of mesenchymal lineages, e.g. osteoblasts or chondrocytes. Therefore, we recently initiated the use of high throughput screening techniques to identify which MSC populations are most likely to result in desired outcomes following transplantation in a number of different clinical contexts.
These studies, along with other research in the field, will form a framework to consider problems associated with providing patients with an optimal and well defined autologous cell therapy or tissue engineered product.

(38.O1) EVALUATION OF TISSUE-ENGINEERED AORTIC CONDUIT TRANSPLANT MATURATION IN A GROWING SMALL RODENT MODEL

Assmann A (1), Akhyari P (1), Delfs C (1), Flögel U (2), Jacoby C (2), Lichtenberg A (1)

1. Clinic for Cardiovascular Surgery, Heinrich Heine University, Duesseldorf, Germany; 2. Institute of Heart and Circulatory Physiology, Heinrich Heine University, Duesseldorf, Germany

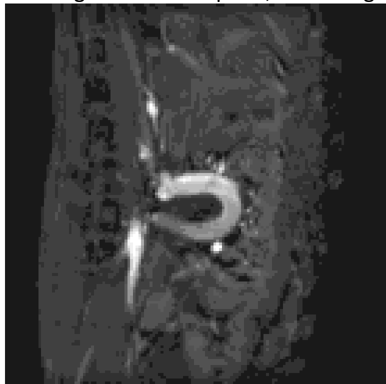
Introduction. In order to advance the search for the ideal heart valve substitute, optimization of tissue-engineered prostheses is a promising ambition. Recently reported early degeneration of decellularized grafts, particularly in children, is supposed to occur in terms of immune response and thrombogenesis. Therefore we aimed for creating a model, with which functional as well as histological and molecular aspects of tissue-engineered aortic conduit transplant maturation can be examined.

Materials and methods. After inhalative anesthetization small Wistar rats (70–80 g) were treated with full-dose heparin. The abdominal aorta was exposed, clamped, and a U-shaped decellularized aortic conduit was infrarenally connected by end-to-side anastomoses. Finally the native aortic part in between was ligated, the abdomen was closed, and the animals were recovered. Rat model miniaturization enabled control of operative success by high resolution rodent MRI.

Results. After passing a learning curve of 10 animals (70% lethality due to paraplegia, bleeding and lower limb ischemia) operative mortality could be reduced to 20%. Animals, which survived the procedure, showed unimpaired conduit perfusion in MRI examinations on day 1 to 5 after surgery (Figure 1).

Conclusions. Our miniaturized rat model is a worthwhile tool for in vivo assessment of tissue-engineered aortic conduit transplant maturation, considering functional as well as histological and molecular aspects. Since transplant fate is observed in a growing organism, adaptive development potential of decellularized grafts in context of changing geometries can be examined.

Keywords. aortic conduit transplantation, growing rat model, tissue-engineered transplant, microsurgery



(38.O2) ROLE OF MACROPHAGE PHENOTYPE IN THE REMODELING OF ECM SCAFFOLDS FOR TISSUE RECONSTRUCTION

Badylak SF (1,2), Brown BN (1,2), Kukla KA (2,3), Turner NJ (1,2)

1. University of Pittsburgh, Pittsburgh, Pennsylvania, USA; 2. McGowan Institute for Regenerative Medicine, Pittsburgh, Pennsylvania, USA; 3. Carnegie Mellon University, Pittsburgh, Pennsylvania, USA

Background. Activated macrophages are described as having an M1 or M2 phenotype based upon their effector molecule production, cell surface markers, gene expression and function. Macrophage phenotype has been shown to be predictive of tissue remodeling outcome. An M1 macrophage phenotype dominance is associated with scar tissue formation and/or encapsulation of implanted materials. An M2 phenotype dominance is associated with formation of functional, site appropriate host tissue. The goals of the present study were: to evaluate tissue remodeling, matrix metalloproteinase (MMP) and tissue cytokine expression, and macrophage polarization following soft tissue implantation of two biologic scaffold materials in the same animal; and to determine whether macrophage polarization is localized to the site of implantation.

Materials and Methods. A partial thickness abdominal wall defect model in the rat was repaired with a biologic scaffold composed of urinary bladder matrix (UBM), UBM cross linked with carbodiimide (CDI-UBM) or autologous tissue. The test articles were harvested at days 1, 3, 7, 14, and 28 days and evaluated by quantitative morphometrics, MMP expression, and gene expression patterns consistent with M1 (INOS, CXCL-10, IL-12) and M2 (arginase, CD-36, IL-10), and cell surface marker expression at the implantation site.

Results. Each test article was associated with a distinct remodeling response and macrophage phenotype population. The macrophage response to each test article did not affect the response to other test articles implanted at alternative sites in the same animal. UBM was associated with a predominantly M2 macrophage phenotype; whereas CDI-UBM was associated with a predominantly M1 phenotype and foreign body giant cell reaction. The autologous tissue was associated with a mixed M1/M2 macrophage phenotype population. Each test article was associated with a distinct gene expression profile, cell surface markers for macrophage phenotype, and M1/M2 gene expression pattern.

Discussion and Conclusions. Macrophage phenotype is associated with different methods of biologic scaffold remodeling, which in turn affects downstream remodeling outcomes. The macrophage phenotype response is a localized phenomenon in situ and does not appear to have systematic effects.. An improved understanding of causative factors of macrophage phenotype differentiation may lead to the design of scaffolds for regenerative medicine that promote restoration of functional, site appropriate tissue as opposed to inflammation and scar tissue formation

Acknowledgments. Funding for this study was provided by the NIH (R01 AR054940 and F31 EB007914).

Disclosures: None of the authors have any conflict of interest to disclose.

Keywords. ECM, macrophage, bioscaffold, tissue remodeling

(38.O3) INFLUENCE OF IN VIVO MICROENVIRONMENT ON COLLAGEN HYDROGEL INDUCING MESENCHYMAL STEM CELL CHONDROGENESIS

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Mesenchymal stem cells(MSCs) are multipotent progenitor cells that possess the ability to differentiate multi cell lineages in vitro but directing and controlling their differentiation in vivo remains a challenge. The influence of in vivo niche on MSCs chondrogenesis needs experimental evidence. In this study, we explored the potential chondrogenesis of BMSCs encapsulated collagen hydrogel and the ability of regenerating neo-cartilage in subcutaneous site and articular cavity of New Zealand White rabbits. The CD45-/CD90+ MSCs derived from new born rabbit were enculpsed encapsulated in collagen hydrogel and then delivered to diffusion chamber or dialysis-tubing bag before in vivo implantation. At 1, 3, 5 months , reconstruct samples were retrieved from in vivo and assessed by distribution and proliferation of the seed cells, histological and immunohistochemical analysis and quantitative PCR of chondrogenic marker gene. Without growth factors administered and in vivo host cells contact, the multipotential MSCs encapsulated in collagen hydrogel differentiated into cartilage specific cells. However the degree of chondrogenesis is site depended. We observed chondrocyte-like cells within cartilage lacuna producing an extracellular matrix (ECM)-enriched cartilaginous tissue exhibiting all of histological markers in articular cavity, while sporadic cells in the deep center of construct under chondrogenesis in the ectopic site . The qPCR of sox9, collagen type Ⅱ and aggrecan clearly indicate that the articular cavity have a hyaline cartilage inducing microenvironment, which promoted the marker genes expression level close to that of nature cartilage. These results provide experimental evidence that in vivo MSC chondrogenesis and neocartilage formation affected by microenvironment cues. The "dialysis-tubing bag model" established in this study is a practical model for reveal the differentiation mechanism of stem cell committed by a tissue-specific microenvironment.

Keywords. Microenvironment, Collagen hydrogel, Mesenchymal stem cell, Chondrogenesis, Cartilage

(38.O4) RAT WOUND MODELS TO TEST THE EFFICACY OF A HEPARAN SULFATE MIMETIC IN PROMOTING WOUND REGENERATION

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Introduction. Poorly healing skin wounds represent a significant clinical problem and financial burden to health care systems. Numerous strategies are invented to improve wound regeneration. However, obtaining evidence regarding their efficacy is complicated. Patient studies frequently suffer from poor design, patient enrolment and/or compliance. Animal models often lack key clinical characteristics, however, allow a detailed study of the wound regeneration process. We describe the benefits and limitations of several rat animal models to study wound repair and focus on the effects of a heparan sulfate glycosaminoglycan mimetic, OTR4120, in improving wound regeneration.

Materials and methods. Full thickness skin wounds and ischemia-reperfusion wound models are described in detail. Wound regeneration is studied in normal and diabetic animals. Experimental diabetes is induced by a single intra peritoneal injection of streptozotocin.

Results. Benefits and drawbacks for using these skin wound models are presented. In diabetic rats, wound regeneration clearly is impaired. However, the administration of OTR4120 significantly stimulates wound regeneration. Remarkably, this stimulatory effect of OTR4120 administration also is observed on wound healing in normal animals. OTR4120 administration results in a reduced inflammatory response, reduced matrix metalloproteinase expression, increased angiogenic capacity and an increased collagen synthesis. Furthermore, the increased ratio of collagen type III to I in wounds of diabetic animals is reversed to normal in OTR4120-administered ulcers. Also short and long-term restoration of wound biomechanical strength is significantly enhanced following OTR4120 administration.

Conclusion. Both surgical and ischemia rodent wound models do contribute to a better understanding of complex wound healing, however, have clear limitations regarding their clinical translation. Heparan sulfate mimetic OTR4120 administration is beneficial in improving skin wound regeneration in both normal and diabetic animals. In addition, limited case series confirm the positive effect of OTR4120 administration on wound regeneration in chronic ulcer patients.

Acknowledgments: This research was supported by the NutsOhra Foundation.

Keywords. regeneration, heparan sulfate, wound, animal model

(38.O5) REGULATION OF NITRIC OXIDE LEVELS BY THE NITRIC OXIDE DONOR LA419 AS A POSSIBLE STRATEGY FOR STROKE'S TREATMENT

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Introduction. Nitric oxide (NO) synthesized by endothelial cells has a wide range of functions that are vital for maintaining a healthy vascular system. During the initial phase of ischemia, NO generated by endothelial NO synthase (eNOS) provides protective and vasodilatador effects. This makes that current trends in the treatment of acute stroke are directed towards pharmacological modulation of NO release. In this field, NO donors are considered as promising therapeutic agents. However, the most prominent problems of NO donors are drug tolerance and NO resistance that limits their clinical application. Recently, LA419, a new organic nitrate containing a thiol group, has been designed to treat clinical conditions associated with NO deficits. The thiol group in the molecule LA419 was expected to protect the formed NO from being degraded by free radicals and, thus, to avoid nitrate tolerance and NO resistance. LA419 acts through a mechanism that involves the eNOS signaling pathway. LA419 can restore the function of eNOS, which in turn increases NO generation and cGMP levels. Moreover, experiments have shown that LA419 has anti-ischemic, antithrombotic, and antiatherosclerotic

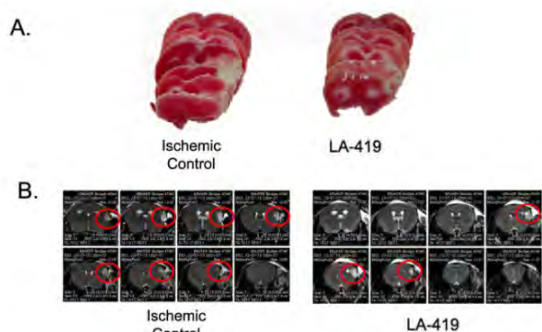
effects at doses that do not influence blood pressure. The present study was performed to investigate the in vivo effect of LA419 in the infarct size in a focal ischemia model in mouse.

Materials and methods. LA419 (30mg/Kg) was administered ip 15 min after permanent occlusion of the middle cerebral artery (pMCAO). Another group of animals was pretreated during 21 days before pMCAO, using Alzet mini-osmotic pumps (30mg/kg, daily). The infarct was delineated 48 hours after using vital stain triphenyl tetrazolium chloride (TTC) and magnetic resonance imaging (MRI).

Results. Treated animals with LA419 showed significant smaller infarct sized.

Conclusions. These findings point to the therapeutic potential of LA419 in preventing the process of ischemic stroke.

Finnancial support: LACER, S.A., MCINN (SAF 2010-15173)
Keywords. Stroke, nitric oxide, LA419



Treatment with LA419 decreases the infarct volume after pMCAO in mice.
Mice were subjected to 48h of pMCAO and infarct volumes were quantified from TTC-stained (A) and MRI (B) serial coronal sections. Representative sections are shown for each group. Untreated mice reveal a larger area of ischemic tissue in the infarcted neocortex when compared to the LA419 treated.
Data are mean ± SEM, n=6-9; (*, $P<0.05$).

(38.O6) TISSUE ENGINEERING OF NASAL SEPTAL CARTILAGE: DEVELOPMENT OF A NOVEL ORTHOTOPIC ANIMAL MODEL

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Introduction. Cartilage defects of the nasal septum affect shape and stability and thus respiratory function of the nose. Currently the gold standard to reconstruct such defects is to use autologous material such as auricular or rib cartilage. To avoid donor site morbidity tissue engineering seems a promising option for this type of clinical problem. Therefore the aim of this study was to develop an orthotopic surgical model of nasal septum repair.

Materials and methods. In male Lewis rats (n=90) septoplasties were performed by creating a periosteum-bone flap on the nasal dorsum. Then nasal cartilage was accessed without damaging the adjacent mucosa. Marine collagen scaffolds were seeded with rat chondrocytes in vitro for 5 days. Engineered samples and unseeded scaffolds were implanted after creating a surgical defect within the septal cartilage. In the control group this defect was not filled with any scaffold. After 1, 4 and 12 weeks samples were retrieved and evaluated macroscopically as well as microscopically. Inflammatory reaction as well as production of novel matrix products was analysed histologically and immunohistochemically.

Results. Transplantation of engineered cartilage samples significantly reduced the number of macroscopically visible septal perforations. Also implantion of unseeded marine collagen scaffolds reduced the probability of a septal perforation following our surgical procedure while almost all untreated animals developed a septal perforation. Histological evaluation demonstrated mild to moderate inflammatory reactions to the transplants, furthermore newly developed cartilaginous matrix was visible in the treated animals.

Conclusion. We developed a robust and reproducible animal model of nasal septum repair. This is essential for the detailed evaluation of engineered cartilage for this specific application. As inflammatory reactions were mild and septal perforations were avoided in a significant number of animals marine collagen scaffolds seem to be promising scaffolds for nasal cartilage repair.

Supported by the Network of Excellence EXPERTISSUES.
Keywords. Nasal cartilage repair, animal model, collagen

(38.O7) A NOVEL CELL-LINE MODEL SYSTEM FOR MOUSE CARDIAC PROGENITOR (CPC) CELLS

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The view of the adult heart as a terminally-differentiated organ has been challenged with the identification of putative cardiac stem/progenitor cells (CPCs). Despite the unknown ontogenic origin of CPCs, commitment to cardiac lineages has been tracked back to a myocardium scarce cell fraction(s) displaying the stem-cell associated markers Sca-1 and/or c-Kit/MDR1. The rationale for this work is to address whether an immortalized line of Sca-1+CPCs is a representative in vitro model of the native counterparts. The characterization of the Sca-1+CPC-line will be presented. Briefly, this cell-line consistently expressed early cardiac transcription factors and stem-cell associated molecules while no transcripts characteristic of mature cardiomyocytes were found. Upon subcutaneous transplantation into Nude mice, cells were contained within lumps that developed at the injection site up to 14 weeks post-injection. Immunohistochemical analysis of the lumps indicated a recruitment of host cells, likely induced by the grafted-cells. Sca-1+CPCs were not detected in immunocompetent mice on the same experimental setting. The CPC-line response to the native cardiac environment was also evaluated following intramyocardial injection into sham-operated and myocardial-infarcted (MI) syngeneic mice. Cardiac function was assessed by transthoracic echocardiography and MI size quantified on Masson's trichrome stained-sections at 14 days posttransplantation. MI-transplanted hearts revealed higher shortening and ejection fractions and a reduced extension of left ventricle remodeling while compared to vehicle-injected MI animals. Moreover, transplanted cells were identified in the

infarcted hearts up to 21 days post-injection, despite the profound modifications on the cardiac architecture, and were also detected in hearts of non-infarcted transplanted animals. Examination on how the transplanted cells integrate/interact with the recipient cardiac cells is underway. Validation of the herein cell-line as CPC-representative would entail the first described model for CPCs and thus a tool for dissecting cardiac cell-fate, a source for high-throughput molecular analysis and a potential platform for pharmacological screening.

Keywords. Cell-line, cardiac progenitor cells, intramyocardial injection, myocardial infarction

(38.O8) USE OF A LARGE FEMORAL BONE DEFECT IN A RAT MODEL FOR ASSESSMENT OF BONE FORMING CAPACITY OF VARIOUS BIOACTIVE SCAFFOLDS

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Introduction. Biomaterials tailored for bone formation are continuously being developed. Our aim was to develop a large cortical bone defect in a rat model to mimic a clinical situation that allows assessment of bone formation of various carriers for growth factors such as bone morphogenetic protein-2 in a hyaluronan-derived hydrogel.

Materials and methods. Longitudinal defects measuring 6 x 2 mm were drilled anterolateral in the shaft of both femurs in 24 male Sprague Dawley rats. Aldehyde-modified hyaluronan was dissolved to 26.7 mg/ml in PBS. Hydrazide-modified polyvinyl-alcohol was dissolved to 3.3 mg/ml in formulation buffer with 0.3 mg/ml (rh)BMP-2 (InductOS; Wyeth, US). 0.25g/ml hydroxyapatite (TAH, Industries, US) was added to each polymer and the components were cross-linked into a hydrogel. Left leg was left empty (control) while the defect in the right leg was filled with hydrogel. Rats were sacrificed at 10, 20, 30 and 40 days. Legs were scanned by quantitative peripheral computed tomography (pQCT) (Stratec, Germany) and data was analysed regarding bone mineral content, bone density, bone area and perimeter.

Results. Bone regeneration was seen in all groups also in the empty defects, although treated defects had significantly higher bone mineral content (BMC) compared to empty controls at all time points (p=0.0001), BMC increased significantly over time (p=0.0001) and bone area and perimeter were significantly larger in treated groups (p=0.0001) (Two-way ANOVA Bonferronis posttest; fig 1), but was not affected by time (p=0.1320). There was a trend of increasing density over time (p=0.0542, ANOVA).

Conclusions. This bilateral animal model with a large cortical defect is well functioning for fast screening of biomaterials tailored for bone. Hydrogels as carriers for BMP-2 accelerate bone healing at a primary stage before 10 days.

Keywords. In vivo, bone defect, osteogenesis

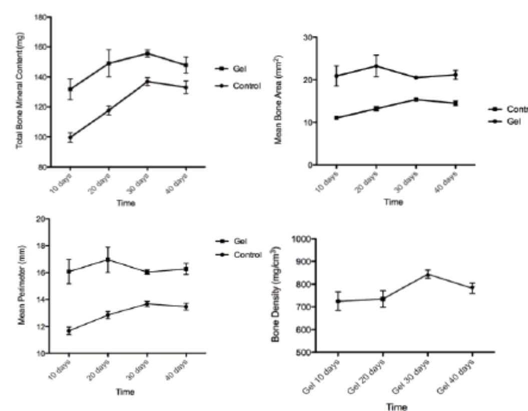

Figure 1. Total bone mineral content, bone area, perimeter of bone and mean bone density.

(38.O9) COMBINATION OF MRI AND 99MTC-NTP 15-5 SPECT FOR THE ASSESSMENT OF INTERVERTEBRAL DISC DEGENERATION AND TISSUE ENGINEERING

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The development of regenerative cell-based biotherapies for degenerated intervertebral disc (IVD) has recently been contemplated and animal models and methods of evaluation are required. Histology is classically proposed but destructive property of this method does not allow a longitudinal follow-up study. We were interested in using a new approach based on a scintigraphic imaging (SPECT: Single photon emission computed tomography) in vivo using a radiotracer (99mTc-NTP-15-5) that exhibits a high affinity for the proteoglycans.NZW rabbits (1,6,12 and 30-month-old; n=3) were used. Early aging of lumbar IVD was determined by MRI and scintigraphic analysis. MRI images were analyzed using T2 weighted signal intensity (T2wsi) and scored according to the Pfirrmann's grading. The distribution of 99mTc-NTP-15-5 in IVD was evaluated by in vivo scintigraphic imaging (g-imager Biospace) on 3 rabbits of each age (ratio IVD on muscle fixation was measured).

MRI assessments of rabbit IVDs reveal a grading decrease with increasing age in IVD signal intensity (T2wsi) associated with an increase in Pfirrmann's grade. These data indicate the existence of an aging /degeneration process of rabbit IVD. As a function of escalating ages, a differential uptake of 99mTC-NTP 15-5 in IVD was observed. Uptake was maximal at 1 month of age, and was followed by a dramatic decrease as early as 6 months. After six-month, the intensity of decrease was less pronounced. These in vivo results highlight the interest of combining 99mTc-NTP-15-5 scintigraphic imaging of proteoglycans with MRI. Whereas MRI T2wsi reflects the hydration level, 99mTc-NTP-15-5 scintigraphy allowed the functional assessment of IVD at the proteoglycan level. Such multi-imaging approach could be useful for the longitudinal study of engineered IVD following the intradiscal transplantation of mesenchymal stem cells using an injectable biomaterial and should be

also paid further attention particularly to detect the early IVD degeneration.

Keywords. Intervertebral disc, radiotracer, proteoglycan, SPECT, tissue engineering

(38.O10) STUDYING MECHANISMS INVOLVED IN ARTICULAR CARTILAGE REPAIR USING AN OSTEOCHONDRAL CULTURE MODEL

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Introduction. Untreated cartilage defects may progress into early osteoarthritis due to the low intrinsic repair capacity of cartilage. These defects can be treated with surgical procedures and/or tissue engineering strategies. Often this results in repair tissue consisting mainly of fibrocartilage. To study involved mechanisms, we have developed and validated an osteochondral culture model.

Materials and methods. Defects were created in osteochondral biopsies from bovine MCP joints. Viability, matrix activity and osteoclast activity were assessed using lactate dehydrogenase (LDH) secretion, alkaline phosphatase (ALP) activity and tartrate resistant acid phosphatase (TRAP) staining respectively. qPCR was performed after 7 and 28 days of culture on biopsy cartilage and cartilage-only explants. Defects were filled with different cell-scaffold constructs which were harvested for immunostaining and qPCR after 28 days of culture.

Results. We achieved to control defect depth, based on histology. High initial levels of LDH and ALP decreased towards a relatively stable level. The expression of cartilage related genes decreased over time, which was more pronounced in explants than in biopsy cartilage. In the different cell-scaffold constructs we detected clear differences in defect filling as well as in integration with adjacent tissues.

Conclusions. We developed and validated a representative model to study mechanisms involved in cartilage repair. Based on LDH secretion we hypothesize that initially necrosis occurred due to the drilling procedure. Decreased ALP activity can be addressed to decreasing amounts of debris in the medium or to lack of mechanical loading. Gene expression in biopsy cartilage remained more stable than in cartilage-only explants. We conclude that we have developed a useful model to study different cartilage repair methods and the mechanisms of action.

This study was financially supported by the Dutch Arthritis Foundation and grant NMP3-SL-2010-245993, FP7: GAMBA.

Keywords. Osteochondral model, cartilage repair mechanisms, in vitro model

(38.P1) HISTOLOGICAL EVALUATION OF THE EFFECTIVENESS OF 5-ASA LOADED N-SUCCINYL-CHITOSAN SYSTEMS IN TNBS-INDUCED COLITIS MODEL OF RAT

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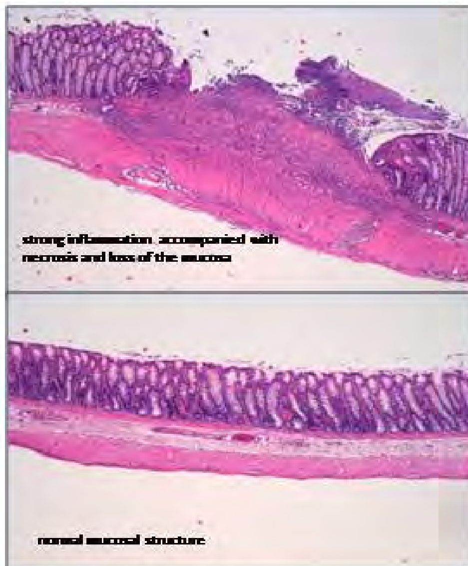
Introduction. 5-aminosalicylic acid (5-ASA) is a drug used to treat inflammatory bowel disease (IBD). Orally administered it is absorbed in the upper gastrointestinal tract, but therapeutic concentrations are not reached in the distal tract. To achieve a colon specific delivery it is necessary to develop a controlled release formulation. N-Succinyl-chitosan (SucCH) is a chitosan derivative that exhibit pH-dependent swelling behavior. Swelling of SucCH in the stomach is minimal and thus the drug release is minimal. Due to increase in pH, the swelling degree increases as the hydrogels pass down the intestinal tract, and drug release occurs.The aim of this work was to prepare two 5-ASA loaded SucCH systems: microparticles (MP) and freeze-dried system (FD). Their effectiveness for the treatment of IBD was examined using TNBS model.

Materials and Methods. To induce the model of chronic inflammation in the rat colon, the method described by Morris et al. was followed with some modifications. The development of inflammation was evaluated in respect to the clinical activity score, colon/body weight ratio, myeloperoxidase activity and histological evaluation.

Results. To select an optimal schedule to induce the inflammation by TNBS, the development of the inflammation at different days after TNBS administration was examined and compared to the control group that received 50% (v/v) ethanol. Therefore the effectiveness of 5-ASA for the treatment of the inflammation was evaluated by administering 5-ASA formulations, rats were divided into 4 groups (SucCH suspension, 5-ASA suspension, FD and MP).Histological findings indicated that untreated TNBS group showed presence of strong inflammation accompanied with necrosis and loss of the mucosa. Similar results were also obtained with SucCH and 5-ASA. Indeed histological findings for FD and MP showed decreasing of inflammation followed by regeneration and normal mucosal structure.

Conclusion. Histological evaluation confirms the usefulness of the two systems in the treatment of IBD.

Keywords. 5-aminosalicylic acid, N-Succinyl-chitosan, TNBS-induced colitis, histological evaluation



(38.P2) ALCOHOLIC LIVER DISEASE: DEVELOPMENT OF NEW EXPERIMENTAL MODELS AND STUDY BIOLOGICAL AND MORPHOSTRUCTURAL OF NEUROLOGICAL AFFECTATION

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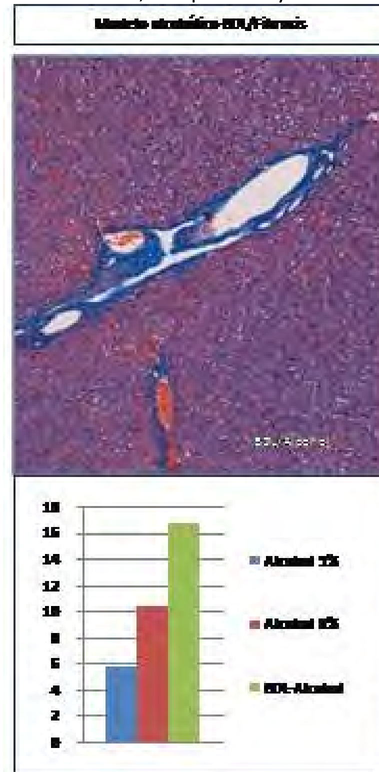
Introduction. Hepatic encephalopathy (HE) is a neuropsychiatric syndrome present in patients with liver disease. Although alcohol consumption is responsible for a high percentage of hepatic cirrhosis, there is not an adequate animal model of cirrhosis and alcohol-induced HE. Our aim is to develop an animal model of fibrosis, cirrhosis and hepatic encephalopathy induced by alcohol that allow to reproduce the neurological alterations showed in patients with liver disease and to study the molecular mechanisms responsible, as well as to assess potential therapeutic treatments to reverse them.

Materials and methods. We have developed an animal model of cirrhosis by treating rats with several concentrations of ethanol (5, 8 and 10%) and to bile duct ligation (BDL) Rats were sacrificed at 5, 8 and 11 weeks after surgery and ethanol treatment, and biochemical parameters were determined in blood. Histological analysis of livers were performed using Hematoxylin/Eosin, Masson Trichrome and Gomori Reticulin staining for morphometry study (quantifying the degree of hepatic fibrosis/cirrhosis). Ethanol levels in blood were measured in order to assess the presence of ethanol in treated rats.

Results. The weight of ethanol-treated rats did not differ from control rats in all ethanol concentrations used. In BDL rats and BDL rats treated with ethanol there was an increase in blood ammonia and in serum inflammation parameters (IL-6). The morphometry analysis indicated that concentrations of 5 and 8% of ethanol together with BDL induced periportal liver fibrosis. We are now analysing if a concentration of 10% ethanol would be enough to induce liver cirrhosis.

Conclusion. The animal model developed is a good model of fibrosis, cirrhosis and hepatic encephalopathy induced by alcohol that reproduce the alterations in hyperammonemia, inflammation and neurological alterations showed in cirrhotic patients.

Keywords. Hepatic Encephalopathy, bile duct ligation, cirrhosis, Inflammation, morphometry



(38.P3) ECHO-GUIDED PERCUTANEOUS INTRAMYOCARDIAL INJECTIONS IN NORMAL RABBITS AND RABBITS WITH EXPERIMENTAL CARDIOMYOPATHY

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In most cardiological studies involving small animals, cell/gene therapy is delivered using a left thoracotomy, with intramyocardial injection (IMI) under direct visualization. However, this delivery route is invasive, carries peri-surgical mortality, is impractical for repeated injections, and does not prevent inadvertent injection into the cavity. We tested the hypothesis that in rabbits echo-guided IMI is feasible, reproducible and with low grade of mortality and undesirable effects. We used echocardiography (7.5 MHz probe, supine position, parasternal long and short-axis views) with simultaneous ECG to guide percutaneous injections (4-5 sites, PBS 0.9 ml) accurately into myocardial wall of 14 anesthetized rabbits (9 normal rabbits and 5 rabbits with doxorubicin induced cardiomiopathy, 2 mg/kg/week, 8 weeks). We optimized this system by adding a dye (Chinese ink, 0.1

ml) which enabled us to confirm the IMI and to monitor accidental intra-chamber injection, as well as post-mortem confirmation of the injection points in 4 normal rabbits that were euthanized immediately after (dye group). 24hECG-Holter and serial echocardiography (pre, during and 2-weeks after IMI) were used to monitor the effects of IMI in the rest of rabbits. Using this protocol we could guide successfully the IMI in all rabbits. Ventricular premature complexes were frequently induced during IMI but sinusal rhythm was soon recuperated and stable during the 24h period. The only undesirable effect attributable to IMI was the presence of a mass in the left ventricular outlet inducing mild aortic stenosis in a rabbit. In the rest of rabbits, qualitative and quantitative echocardiographic exams before and 2-weeks after IMI were not significantly differents. Post-mortem studies in dye group showed a good correlation with echocardiographic images. Any rabbit died during the study.

In summary, we demonstrated that echoguided IMI is feasible, repeatable and does not induce significant electrical or echocardiographic alterations in normal or cardiomiopathic rabbits.

This study was supported by the Fundación Séneca-Agencia de Ciencia y Tecnología de la Región de Murcia (Spain), Proyect number: 11935/PI/09

Keywords. Echocardiography; doxorubicin; experimental heart failure; rabbit

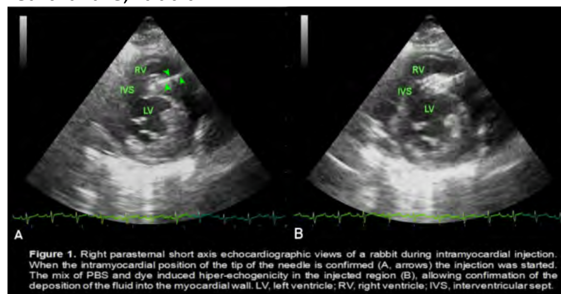

Figure 1. Right parasternal short axis echocardiographic views of a rabbit during intramyocardial injection. When the intramyocardial position of the tip of the needle is confirmed (A, arrows) the injection was started. The mix of PBS and dye induced hiper-echogenicity in the injected region (B), allowing confirmation of the deposition of the fluid into the myocardial wall. LV, left ventricle; RV, right ventricle; IVS, interventricular sept.

(38.P4) HUMAN RETINA DEGENERATION DURING ORGANOTYPIC CULTURE

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Introduction. To characterize the survival pattern and early degeneration of neural and glial cells, in an organotypic culture of adult human retina.

Materials and methods. Two postmortem human globes from donors aged 57 and 59 years old were dissected, and 16 retinal explants were obtained. Twelve were explanted on Transwell® culture dishes and cultured for 3, 6 and 9 days. Culture medium was composed of Neurobasal A supplemented with B-27, FBS and L-glutamine. Furthermore, 4 post-detachment explants were used as freshly retina controls. Vertical sections stained with toluidine blue, and singly or doubly immunostained, with specific markers of neuronal and glial cells, were evaluated.

Results. Retinal morphology was properly preserved in post-detachment controls. On the contrary, retinal degeneration was remarkable during culture. Pyknosis of photoreceptor nuclei, ganglion cells vacuolization, reduction of plexiform layers thickness, photoreceptor outer segments (OS) disruption, and marked reduction in the number of nuclei at both nuclear layers were progressively apparent during culture. Within 3 days, remarkable changes included cone OS swelling with impairment of their pedicles; loss of axon and dendrites of horizontal and rod bipolar cells, respectively. At 9 days, horizontal cells presented pyknotic nuclei and their terminal tips were gone. Similar degenerative processes in the OPL for rod bipolar cells and a loss of axon terminal lateral varicosities in IPL were found. Glial fibrillary acidic protein staining did not reveal an increased gliosis of Müller cells. However, some Müller were calbindin inmunoreactive after 6 days of culture.

Conclusions. Morphological changes not previously described in human cultured retinas, emphasize the important relationship of the retina with the pigment epithelium. These results may suggest the importance of time in retinal detachment treatment.

Acknowledgments. MICINN (BFU2009-07793/BFI), (RETICS RD07/0062/0012 and RD07/0062/0013) to NC. IF was supported by Junta de Castilla y León, Spain.

Keywords. Retinal detachment, retinal degeneration, synaptic connectivity, photoreceptors

(38.P5) COMPARATIVE STUDY OF INFARCT SIZE AND THE LYMPHOCYTE POPULATION IN A PORCINE MODEL OF MYOCARDIAL INFARCTION TREATED WITH ANTI-TNF-ALPHA

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Introduction. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine with pleiotropic biological effects. It is released in response to acute myocardial infarction from macrophages, monocytes and cardiomyocytes within minutes. The precise impact of TNF- α signaling on myocardial ischemic injury remains controversial but the inhibition of TNF- α signaling via quenching antibodies has been reported to protect against myocardial ischemia/reperfusion injury. In this study we aim carry out a preliminary evaluation of the effect of “ethanercept” an anti-TNF- α soluble receptor, to reduce infarct size and microvascular obstruction in a porcine model of myocardial infarction.

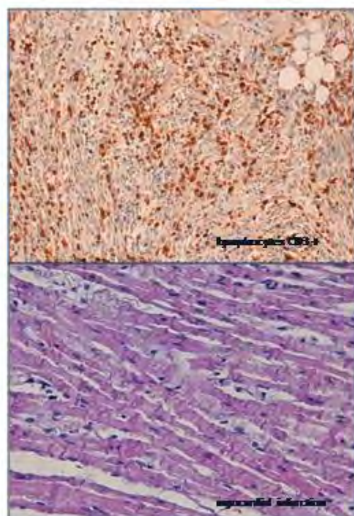
Materials and methods. We have studied infarct size, histological structure and the number of T lymphocytes in 16 pigs that had previously been induced myocardial infarction by means of mid left anterior descending artery occlusion using percutaneously introduced angioplasty balloon. Six pigs were given anti-inflammatory treatment

with TNF- α and 10 pigs served as controls. We have also studied microvascular obstruction detected by intracoronary injection of thioflavin S (4% solution) to define the region of microvascular obstruction.

Results. The mean infart size and mean microvascular obstruction marked by Tetrazolium (TTC) and Thioflavin S on postmortem examination and measured by morphometry in 6 pigs treated with TNF- α was the 2±2% ($p=0.02$ vs. controls) and 7±13% ($p=0.2$) respectively. The mean infart size and microvascular obstruction in 9 pigs treated by TNF- α was the 4,32 and 1,32 respectively. We also studied the amount of CD3 positive lymphocytes T measured by morphometry. In the control group we found that 17020 lymphocytes T CD3+, whereas in the group treated by TNF- α was 5275. We found that in all cases but one, the number of lymphocyte was much lower than that found in the control group.

Discussion. In a porcine model of myocardial infarction we found that the infarct size, microvascular obstruction and the number of lymphocytes T was higher in the control group than in the group treated with TNF- α. Further studies are needed to confirm this promising preliminary data.

Keywords. Myocardial infarction, microvascular obstruction, infarct size, lymphocytes T, TNF-α



(38.P6) CHARACTERIZATION OF OCTODON DEGUS RETINA, A DIURNAL RODENT

Fernández-Sánchez L (1), Esquiva G (1), Lax P (1), Cuenca N (1)

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Introduction. Octodon degus is a rodent with a cone-dominant retina also displaying diurnal crepuscular activity. Our purpose was to describe neuronal phenotypes and visual functions in this diurnal rodent, and compare them with the retinas of other nocturnal mammals.

Materials and methods. Octodon degus retinas were singly or doubly immunostained with antibodies against calcium-binding proteins and classical neurotransmitters and then visualized by confocal microscopy. Visual function was studied by means of electroretinogram.

Results. The outer nuclear layer thickness ranged from 2 rows of photoreceptors at the ora serrata to 4-5 rows in the central retina. The inner nuclear layer contained 3-4 rows of neurons. Recoverin, ChAT, TH and connexin36 showed cellular immunoreactivity patterns similar to other mammalian retinas. However, horizontal cells were labeled with antibodies to parvalbumin, as in the primate retina, but also to was found in rod bipolar cells and aαcalbindin, as in other rodents. PKC subtype of amacrine cells, as expected, but also in a cone bipolar cell subtype and the outer segments of blue cones. Finally, a substance P-immunoreactive plexus was found at the stratum S5 of the IPL, as a difference with other mammals. ERG a- and b-wave thresholds were higher in dark-adapted Octodon degus as compared with other rodents. The double-flash protocol showed percentages of cone contribution to the mixed dark-adapted a- and b-waves over fivefold higher in Octodon degus than those found in rats and mice. Finally, Octodon had a significantly higher flicker fusion than that observed in rats and mice.

Conclusions. The Octodon degus retina exhibits a number of differences compared with nocturnal rodents regarding visual function, morphological features and cellular characteristics, but also several similitudes to diurnal animals. Therefore, it constitutes an interesting experimental subject for retinal research.

Supported by grants from BFU2009-07793/BFI, RETICS RD07/0062/0012, FUNDALUCE, ONCE, Fundación Médica Mutua Madrileña.

Keywords. Animal models, retina, electroretinogram, retinal markers

(38.P7) PHOTOSENSITIVE RETINAL GANGLION CELLS DEGENERATE IN P23H RATS, AN ANIMAL MODEL OF RETINITIS PIGMENTOSA

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Introduction. Within the mammalian retina exist nonrod, noncone photoreceptors, that express the photopigment melanopsin. These photosensitive cells consist of a subset of retinal ganglion cells, which mediate nonvisual photoreceptive task such as the regulation of the circadian rhythms and the pupillary reflex. The aim of this study was to investigate morphological changes in these cells in the model of retinitis pigmentosa, P23H rat.

Materials and methods. For this study we used homozygous P23H line 3 rats aged between 4 and 18 months. Sprague-Dawley rats (SD) were used as controls. Whole retinas were stained by immunohistochemical technique with avidin-biotin complex (ABC) and immunofluorescence. Melanopsin cells from each retina were drawn using a camera lucida. Quantification and morphometric analysis were carried out using the software NIH Image J.

Results. In P23H rats, the number of melanopsin retinal ganglion cells (mRGCs) progressively decreased with age. At 18 months of age, the number of mRGCs was around 70% fewer than observed at 4 months of age ($p<0.001$, Student t test). Dendritic arborization and soma size were also smaller with aging in mRGCs. At 18 months of age, dendritic area was around 90% shorter and soma diameter around 10% smaller than observed at 4 months

of age (p<0.001 in both cases, Student t test). In SD rats, although the soma size of mRGCs slightly fell at 18 month of age (4.5% smaller; p<0.001, Student t test), mRGCs showed no differences with aging in neither, the relative number of cells, nor the dendritic arborization.

Conclusions. Melanopsin cells hardly change with aging in SD rats. However, in the P23H model of retinitis pigmentosa there is a drastic degeneration of these cells with the progression of the disease.

Supported by: MICINN (BFU2009-07793/BFI), MSyC (RETICS RD07/0062/0012, RETICEF RD06/0013/0019), FUNDALUCE, ONCE, Fundación Médica Mutua Madrileña.

Keywords. Melanopsin, ganglion cells, P23H, retinitis pigmentosa

39. REPAIR, REPLACE AND REGENERATION IN THE EYE

Chair: Carl Sheridan
Keynote speaker: Che J Connon
Organizer: Carl Sheridan

Synopsis: The eye has been the focus of significant advances in translational research both at the cellular and tissue engineering level. The symposium will reflect these advances with talks covering different diseases of the eye. Numerous corneal complications have been treated by corneal transplantation which is one of the most successful transplant procedures to date and is now also where successful adult stem cell transplantation procedures are being performed.

The replacement of cataract lens with an artificial lens is a procedure which has recovered vision in millions of people worldwide but still remains one of the leading causes of blindness in the world. As such, the development of different artificial lenses remains at the forefront of Ophthalmology research.

New cell based therapies are emerging using both embryonic and adult stem cells to repair or replace specific cells within the neural retina and its support tissue. These techniques are encompassing the latest cell biology and biomaterial advances and are at the forefront of translational research in the hope of treating numerous retinal diseases such as Age-related Macular Degeneration.

(39.KP) WHAT MAKES A GOOD SUBSTRATE FOR CORNEAL TISSUE ENGINEERING?

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Introduction. The limitations of amniotic membrane (AM), the most commonly used substrate for ocular surface reconstruction, are becoming increasingly apparent (poor transparency, structural and chemical variation, poor scalability). Therefore understanding what it is that makes a good substrate for ex vivo expansion of corneal epithelial cells needs urgent attention. We present recent developments in both our understanding of what it is that makes AM successful as well as creating new substrata with well defined mechanical properties.

Materials and methods. Substrata (AM and compressed collagen gels) were characterised by electron microscopy, rheology and x-ray diffraction to quantify their mechanical, topographical and chemical features. Differences in cell-based biocompatibility were quantified by measuring growth and differentiation of limbal epithelial cells by immunohistochemistry and Western blotting. Substrate stiffness was enhanced by UV cross linking in the presence of riboflavin.

Results. AM collected from near to the placenta presented a different topography to AM taken from far from the placenta and was significantly better at supporting stratified and undifferentiated limbal epithelial cells. Compressed collagen gels were successfully used to model the AM and decouple the substrates mechanical and topographical features. Increasing compression time increased the substrates stiffness whilst scanning electron microscopy showed no difference in the gels topography. Substrates with increased stiffness promoted differentiation. Riboflavin and UV treatment increased substrate stiffness in a dose dependent manner.

Conclusions. These results have important implications for both basic and clinical research. From a tissue engineering perspective, our work demonstrates that compressed collagen gels can be used to model stiffness of biological substrates (such as AM) and decouple the mechanical from topographical effects on cell behaviour. We also conclude that the mechanical properties of collagen gels and AM are improved by increasing substrate stiffness which can influence limbal epithelial stem cell differentiation.

Supported by the BBSRC (BB/F019742/1)

Keywords. Substrate, stiffness, differentiation, cornea

(39.O1) THE INJECTABLE OXIDATED HYALURONIC ACID-BASED HYDROGEL AS A VITREOUS SUBSTITUTE

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Introduction. Vitrectomy is a common procedure for treating ocular-related diseases. The surgery involves removing the vitreous humor from the center of the eye, and vitreous substitutes are needed to replace the vitreous humor after vitrectomy.

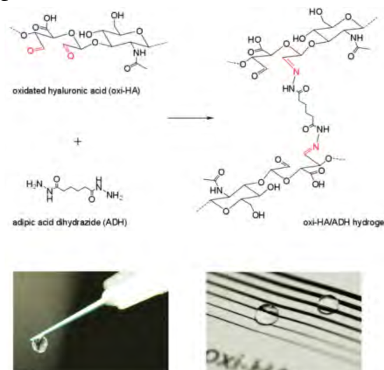
Materials and methods. In the present study, we developed a colorless, transparent and injectable hydrogel with appropriate refractive index as a vitreous substitute. The hydrogel is formed by oxidated hyaluronic acid (oxi-HA) cross-linked with adipic acid dihydrazide (ADH). Hyaluronic acid (HA) was oxidized by sodium periodate to create aldehyde functional groups, which could be cross-linked by ADH.

Results. The refractive index of this hydrogel ranged between 1.3420 and 1.3442, which is quite similar to human vitreous humor (1.3345). The degradation tests demonstrated that the hydrogel could maintain the gel matrix over 35 days, depending on the ADH concentration. In addition, the cytotoxicity was evaluated on retina pigmented epithelium (RPE) cells cultivated

following the ISO standard (tests for in vitro cytotoxicity), and the hydrogel was found to be non-toxic. In a preliminary animal study, the oxi-HA/ADH hydrogel was injected into the vitreous cavity of rabbit eyes. The evaluations of slit-lamp observation, intraocular pressure, cornea thickness, electroretinography (ERG) and histological examination showed no significant abnormal biological reactions for 4 weeks.

Conclusions. This study suggests that the injectable oxi-HA/ADH hydrogel should be a potential vitreous substitute.

Keywords. Vitreous; Hyaluronan; Injectable; Vitreous substitute



(39.O2) FIBROIN-BASED MATERIALS SUPPORT CO-CULTIVATION OF LIMBAL EPITHELIAL AND STROMAL CELLS

Bray LJ (1), George KA (2), Hutmacher DW (1), Chirila TV (2), Harkin DG (1)

1. Queensland University of Technology; 2. Queensland Eye Institute

Introduction. The silk protein fibroin (*Bombyx mori*) provides a potential substrate for use in ocular tissue reconstruction. We have previously demonstrated that transparent membranes produced from fibroin support cultivation of human limbal epithelial (HLE) cells (Tissue Eng A. 14(2008)1203-11). We extend this body of work to studies of human limbal stromal cell (HLS) growth on fibroin in the presence and absence of serum. Also, we investigate the ability to produce a bi-layered composite scaffold of fibroin with an upper HLE layer and lower HLS layer.

Materials and methods. Primary cultures of HLE and HLS cells were established in DMEM/F12 medium with 10% fetal bovine serum (FBS). Cultures were subsequently passaged onto transparent fibroin membranes or within 3D scaffolds prepared from partially-solubilised fibroin. Primary cultures of HLE and HLS cells were also established separately in serum supplemented media and cultured together upon bi-layered silk fibroin or single-layered amniotic membrane (gold standard). Tissue constructs were paraffin-embedded and analysed via immunohistochemistry.

Results. HLE and HLS cultures grown in 10% FBS were able to adhere to and proliferate on silk fibroin 3-D scaffolds and transparent films respectively. HLE silk constructs expressed ΔNp63+ and CK3/12+ comparably to amniotic membrane. HLE and HLS cells were able to be co-cultivated on composite fibroin scaffolds and amniotic membrane.

Conclusions. HLE and HLS cultures can be grown on fibroin-based materials and can be co-cultivated in a bi-layered scaffold of silk fibroin. These results encourage progression to studies of efficacy in a pre-clinical animal model.

Keywords. Silk fibroin; cornea; limbus; transplantation

(39.O3) OPTIMISATION OF PRIMARY CELL CULTURE CONDITIONS FOR RETINAL AND IRIS PIGMENT EPITHELIAL CELL TRANSPLANTATION ON ARTIFICIAL SUBSTRATES

Kearns V (1), Vasilev K (1), Nian S (1), Sheridan C (1), Williams R (1)

1. University of Liverpool

Introduction. A potential treatment for age-related macular degeneration (AMD) could be subretinal transplantation of a functioning retinal pigment epithelium (RPE). Iris pigment epithelial cells (IPE) have similar properties to RPE cells. They are relatively easy to obtain surgically and may be an alternative cell source for RPE replacement. We have demonstrated that surface-modified ePTFE can support a functional monolayer of an RPE cell line. The aim of this work was to investigate the ability of ePTFE membranes modified by plasma polymerisation to support the growth of a differentiated monolayer of primary human RPE cells (hRPE), primary rat and porcine IPE (rtIPE and pIPE).

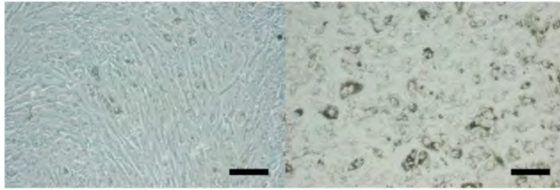
Materials and methods. ePTFE membranes were coated with n-heptylamine (HA). For experiments with rtIPE, substrates were coated with fibronectin. hRPE and pIPE were isolated from cadaver eyes and grown for several passages. rtIPE were isolated from rat eyes and seeded directly onto substrates. Cells were seeded onto HA-ePTFE and tissue culture plastic (TCPS) substrates in a high-serum medium. Medium was replaced at 48h with a low-serum, retinoic acid-containing medium. Cells were observed by light microscopy. hRPE were stained for F-actin and markers of tight and adherens junction formation.

Results. Actin belts, typical of differentiated epithelial cells, formed around hRPE cells on HA-ePTFE by 14d and on TCPS by 28d. Tight and adherens junctions were uniformly distributed over all substrates by 28d. rtIPE and pIPE cells attached and spread on HA-ePTFE substrates. They were heavily pigmented and exhibited epithelial morphology (Fig. 2). Melanin was distributed around cell nuclei.

Conclusions. Surface modification of ePTFE by plasma polymerisation can promote the attachment of IPE and formation of a differentiated layer of RPE cells. This strategy may contribute towards development of a transplantation treatment for AMD.

Acknowledgements: A private local charity and the Foundation for the Prevention of Blindness for financial support.

Keywords. Retinal pigment epithelium, iris pigment epithelium, transplantation, vision


Primary pig IPE grown for 20 days on HA-ePTFE (left) and TCPS (right) substrates. Scale bars represent 100 μm.

(39.O4) SPECTRAL DOMAIN OPTICAL COHERENCE TOMOGRAPHY (SD-OCT) COMPLEMENTS HISTOLOGIC EVALUATION OF SUBRETINAL CELL CARRIERS FOR RETINAL PIGMENT EPITHELIAL TRANSPLANTATION

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Introduction. Cell-based therapy of the RPE could be improved through co-transplantation of cultured cells on a carrier substrate (prosthetic Bruch's membrane). Using a rabbit model we studied subretinal implantation of 2 biostable artificial substrates suitable for RPE culture, which differed in surface topography, thickness, rigidity and porosity.

Materials and methods. Acellular Bruch's membrane prostheses were either an etched pore polyester membrane (PET/ Corning, Inc.), or a surface-modified expanded polytetrafluoroethylene membrane (ePTFE/ Millipore, Inc.). Standardized implants were inserted into the subretinal space with a custom-made metallic shooter instrument (Geuder) following a core-vitrectomy in a consecutive series of 25 female, 2-2.5kg Chinchilla-Bastard rabbits. They were followed up on days 3, 7 and 14 with SD-OCT and fundus photography (Spectralis®/ Heidelberg Engineering, Inc.). The animals were perfusion fixed 2 weeks post-surgery. Samples were embedded in Epon 812 and cut into semithin sections.

Results. Both prosthetic materials could be implanted in the subretinal space. Following surgery, the neural retina overlying the implants was attached by 1 week in SD-OCT, but showed material-dependent alterations (atrophy) of outer retinal reflexion bands. Retinal and outer nuclear layer (ONL) thinning on SD-OCT plateau-ed by 7 days. All implants showed a hyperreflective band on SD-OCT above the implant by 14 days. The IR mode showed a transitional zone around the implant and RF imaging revealed only minimal vitreal condensations around the retinotomy site at 2 weeks. The area around the implant showed subretinal scarring and ONL atrophy, but continuous inner retinal layers in both implant types. Initial comparison of SD-OCT and histology showed encouraging results.

Conclusions. The data suggests that PET and ePTFE substrates can be implanted safely into the subretinal space and are tolerated well over at least 2 weeks. Future studies aim for co-transplantation of RPE cultured on these substrates.

Keywords. Rabbit, retinal pigment epithelium, transplantation, spectral domain optical coherence tomography

(39.O5) DECELLULARIZED CORNEAS BY USING SDS OR NACL: FROM PIG TO HUMAN

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Introduction. Decellularized corneal xenografts emerged as a promising option for the generation of artificial corneas by tissue engineering. Numerous decellularization protocols have been proposed to remove the cells from animal corneas; however, there is no agreement about the best method to achieve this purpose. Moreover, a well-optimized protocol is needed to conserve the organization of the corneal stroma, which must promote and support the recellularization process with human cells into the acellular corneal stroma. In the present study, we have evaluated two different decellularization protocols based on SDS or NaCl with the purpose of conserving composition and structure of the decellularized corneas, recellularizing the optimum acellular cornea with human keratocytes for using in regenerative medicine.

Materials and methods. Fresh porcine corneas were obtained from 40 adult pigs. Pig corneas were decellurarized using 1.5 M NaCl or sodium dodecyl sulfate (SDS) for 12 hours. Once decellularized, the corneas were washed in PBS and dehydrated. Decellularized corneas were evaluated using histological assays. Lastly, NaCl-decellularized corneas were recellularized with human keratocytes and corneal epithelial cells, and were histologically analyzed.

Results. All decellularization protocols correctly removed the cells from the stroma. Nevertheless, some cellular debris remained after the treatment into the xenografts. The integrity of the collagen lamellae was better conserved when NaCl was applied in comparison with SDS. After recellularization, human corneal cells spread and grew into the decellularized corneas.

Conclusions. Our results showed that the most accurate decellularization method for the porcine cornea is 1.5 M NaCl for 12 hours, obtaining a proper scaffold to grow human corneal cells. This method allowed us to generate partial human artificial corneas based on porcine xenografts, which could have potential usefulness for clinical and experimental uses.

Acknowledgements. Supported by SAS PI-462-2010 from Junta de Andalucia, Spain and FIS PI08/614 from Instituto de Salud Carlos III, Spain.

Keywords. Corneal tissue engineering, decellularization, acellular cornea

(39.P1) CROSSLINKING TREATMENT INDUCES STROMAL FIBER ALTERATION IN RABBIT CORNEAS

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Introduction. Corneal pathologies, such as progressive keratoconus, are treated by new techniques as ultraviolet (UV) radiation. However, it may induce adverse effects and morphological alterations. Therefore, our purpose was to analyze morphological changes in corneas exposed to crosslinking (UV radiation plus riboflavin administration after corneal epithelium removal).

Materials and methods. New Zealand adult rabbits were randomly assigned to groups A and B (5 animals/group). Under anesthetic, corneal surface was scraped, irradiated for 30 min (370 nm wavelenght UV radiation), and then B12 vitamin was applied (group A, crosslinking). In group B, animals received the same treatment except radiation exposure. Contralateral eyes received no treatment and were used as controls (group C). Six weeks later, animals were sacrified and corneas were extracted and processed for histological study with standard procedures.

Results. In crosslinking group A, corneal stroma showed areas with a highly altered pattern of collagen arrangement, along with the appearance of scattered orcein-stained fibers between collagen fibers. Ultrastructural study showed the presence of electrodense bands with highly packed thin fibers located between collagen layers, which resembled immature elastin fibers. Besides, Descemet's membrane showed a dense amorphous fibrillogranular material with a wavy appearance close to endothelial cells, which presented cytoplasmic vacuolization. Ultrastructural study in group B showed cytoplasmic vacuolization of keratocytes, with extensive rough endoplasmic reticulum dilatation, that might seem like dehiscences between collagen layers. Stromal dense bands were also observed in these animals, although they seemed less abundant, whereas no orcein-stained bands were detected.

Conclusions. Corneal crosslinking induced the appearance of electrodense bands in the stroma along with areas with orcein-stained fibers. Descement's membrane and posterior endothelium were also altered.

Acknowledgements: Proyecto Copernicus-Santander a FJR, y fondos de la Fundación Oftalmológica del Mediterráneo.

Keywords. UV radiation, keratoconus, stromal fibers

(39.P2) CELL THERAPY APPLIED ON CANINE EYE INJURY: USED MESENCHYMAL STEM CELLS

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Introduction. Mesenchymal Stem Cells (MSC) are being used in the treatment of bone and myocardium regeneration in dogs. Prior to clinical application of these cells in other common canine pathologies, as corneal ulceration, we are optimizing the technique in a rabbit model. We are also carrying out the characterization of canine cells. Besides, we are developing an alternative method to the surgical intervention through the use of these cells in an accurate scaffold.

Materials and methods. Cell doubling time was calculated for ten passages and at different densities of seeding in MSC derived from bone marrow (BM) and adipose tissue (AT). At third passage, canine MSCs from both sources were cultured in specific inductive media. To determine osteogenic differentiation, ALP activity and mRNA expression were quantified. To assess adipogenic differentiation mRNA expression was carried out. To evaluate the chondrogenesis sGAG was analysed. Alizarin red and oil red O staining were performed. For the scaffold construction, the cells were encapsulated in autologous plasma gelificated with $CaCl_2$. The viability of these cells was studied. Ulcer was made and cells were labelling with BrdU to prove the migration of encapsulated cells to the ulcer.

Results. Cell doubling time was higher in MSCs derived from BM than AT. BM and AT derived cells showed different behaviour in all differentiations. BM derived cells had higher osteogenic potential than AT. In contrast, AT derived cells had higher adipogenic and chondrogenic potential than BM. The gelificated plasma made possible to obtain elevated viability of the encapsulated cells in the in vitro study. Besides, MSCs were able to migrate to the ulcer.

Conclusions. MSCs from BM displayed faster growth and osteogenic potential than AT. MSCs derived from AT had higher adipogenic and chondrogenic potential. MSCs showed migration capacity from the encapsulated plasma to the eye injured at different times.

Keywords. Canine, Ulceration, Mesenchymal Stem Cell

(39.P3) MELATONIN SLOWS RETINAL DEGENERATION AND CIRCADIAN DYSFUNCTION IN THE RETINITIS PIGMENTOSA P23H RAT MODEL

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1. University of Alicante

Introduction. Melatonin has been shown to prevent retinal degeneration in ocular diseases, including retinitis pigmentosa (RP). Ocular pathologies and blindness are associated with circadian disorders. Here we evaluate the effects of exogenous melatonin on both inner retina and circadian patterns in the P23H rat model of autosomal dominant retinitis pigmentosa.

Materials and methods. P23H rats (line 3) were treated with melatonin (2 mg/kg BW/day) or vehicle (ethanol) throughout the drinking water during all the experiment (20 days to 18 months old). The body temperature (BT) was continuously monitored and their retinas hitologically examined.

Results. Exogenous melatonin produced a noticeable improvement in the BT circadian patterns recorded in P23H rats. The rhythm fragmentation (intradaily variability) observed in these animals decreased with melatonin-treatment. On the other hand, melatonin preserved retinal structure. Despite photoreceptors were already disappeared at 18 months old, retinal layers in melatonin-treated rats were more organized, well defined, and structured than in control animals. Moreover, the number of melanopsin-positive retinal ganglionar cells (ipRGC) was preserved by melatonin. Thus, in 18 months old animals treated with melatonin the number of ipRGC was around 2,5 fold higher than in

vehicle-treated animals (15,5 ± 6,4 and 6,1 ± 1,3 cell mm-2, respectively; $p < 0,05$, Student t test).

Conclusions. Melatonin administration slows degeneration in inner retina and preserves CGR sensitive to melanopsin and circadian patterns in P23H rats. That neuroprotective effect of melatonin suggests its potential use in the clinical treatment of RP.

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Keywords. Melatonin, P23H, retinitis pigmentosa, chronodisruption

(39.P4) COMPARISON OF GROWTH AND MONOLAYER FORMATION OF PRIMARY BOVINE RPE AND IPE ON POLYURETHANE (PU) MEMBRANES

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1. University of LIverpool, UK; 2. Biomer Technology Ltd, UK

Introduction. A potential surgical treatment for age-related macular degeneration (AMD) involves the implantation of functioning retinal pigment epithelium (RPE). This requires identification of the appropriate cells and scaffold. Iris pigment epithelium (IPE) has been shown to have several of the same functions as the RPE. The aims of this study were to manufacture and characterise porous polyurethane (PU) substrates with primary bovine RPE and IPE cells.

Materials and methods. PU™5% icing sugar was added to a 5% solution (weight/weight) of b9 (Biomer Technology Ltd.) in dimethylacetamide (DMAC). A thin layer was solution cast in a 70°C oven for 24h. Freshly harvested bovine RPE (bRPE) and IPE (bIPE) cells were seeded on the PU membranes and tissue-culture polystyrene (TCPS). Cells were fixed at confluence and stained for F-actin, zonula occludens-1 (ZO-1) and cytokeratin. Nuclei were counterstained with DAPI. bRPE were challenged with bovine photoreceptor outer segments (POS) to assess the monolayer function in terms of phagocytosis.

Results. PU membranes were <m thick and the diameter of the pores wereμ5 m. The PU membrane supported the growth of bRPE and bIPEμapproximately 1 cells. Cytokeratin staining demonstrated epithelial phenotype of both cell types . Nuclei were dispersed evenly throughout the layer and there was evidence of ZO-1 junctional proteins at the cell borders. The functional capacity of the bRPE cells was demonstrated by phagocytosis of POS.

Conclusions. Porous PU membranes can be produced. IPE cells were shown to attach and form a monolayer of epithelial cells on the PU membranes in a similar way to RPE cells. Primary RPE cells grown in the PU membranes maintained their functional behaviour in terms of phagocytosis. These results are encouraging and justify further study of the functional behaviour of the IPE cells on the PU.

Acknowledgements: EPSRC, Biomer Technology Ltd. and Health Technologies KTN for financial support.

Keywords. Age-related macular degeneration, AMD, retinal transplantation

(39.P5) DEVELOPMENT OF DECELLULIZED CORNEA FOR CORNEAL TRANSPLANTATION

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The cornea can be damaged by diseases and injuries such as keratoconus, bullous keratopathy and scarring that eventually cause visual impairment and blindness. The only effective treatment for irreversible corneal damage is corneal transplantation. However, an absolute shortage of donor corneas has been a major problem in many countries. Globally, there are over 10 million people suffering from corneal blindness, while approximately only 120,000 corneal transplants are undertaken annually. In the present study, we developed the decellularized cornea by high hydrostatics pressurization (HHP) as novel artificial cornea for corneal transplantation. Porcine corneas were hydrostatically pressed at 980 MPa at 10 or 30̊C for 10 min to obtain an opaque cornea. After HHP treatment, theses cornea were washed by washing medium. Decellularized samples were subjected to histological study (H-E staining). The transparency, thickness and mechanical strength of them were investigated. Futhemore, decellularized corneal were implanted into a rabbit corneal stroma. There was no change in the thickness of the cornea immediately after the pressurization, but the cornea has swollen during the washing process. By H-E staining, the complete removal of epithelial and stromal cells was confirmed in all of the pressurized corneas. As the animal study, when the implantation of the acellular porcine cornea to rabbit cornea was carried out, the immune reaction was not occurred and the turbid cornea became clear. These results indicate that the decellularized cornea by HHP method would be useful as corneal scaffold for tissue regeneration.

Keywords. Artificial cornea, Decellularized cornea

40. CANCER AND TISSUE ENGINEERING

(40.P1) MCF-7 CELLS AS A 3D MODEL IN THE STUDY OF HUMAN BREAST CANCER

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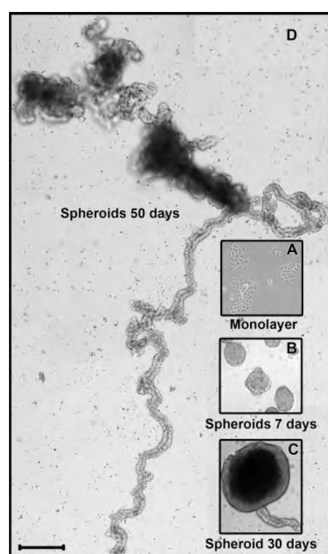
Moving from cell monolayers to three-dimensional (3D) cultures is motivated by the need to work with cellular models that mimic the functions of living tissues. Its growing use in breast cell lineages enables a greater comprehension of processes that require insertion in a

tridimensional context. Formation of the luminal space fits into this aspect since the cell to cell interaction and its spatial relation with the extracellular matrix can only be emulated in vitro when in a 3D environment. The lumen formation in breast adenocarcinoma lineages (MCF-7) was previously described by our group which indicates the relevance of autophagy and apoptosis in its formation. The MCF-7 cells were cultivated in a 3D environment forming spheroids, which were harvested after 7, 30 and 50 days of culture (Figure). Expression of the LC3 protein (an indicator of autophagosome) and p-AKT (cell survival) were analyzed using western blotting, and these proteins were also located using immunofluorescence and visualized by laser scanning confocal microscopy. The e-cadherin RNAm expression was also analyzed by real time PCR. The elevated expression of p-AKT after 7 days of culture indicates a greater probability of cell survival and proliferation. Orthogonal projections, obtained from confocal optical sections, showed a strong p-AKT immunolabelling of cortical cell layer. The LC3 protein presented the most intense expression after 30 days of culture with a reduction in expression after 50 days. Under confocal microscopy, immunolabelling of the LC3 protein was concentrated in the central region of the spheroids, a region that precedes lumen formation. In this work, we showed that the 3D environment changes the expression of the analyzed proteins, which probably play a role during adaptation of MCF-7 cells in this environment. With the gradual selection and differentiation, the spheroids are reorganized allowing the formation of lumen.

Keywords. 3D Cell Culture, Confocal Microscopy, 3D reconstruction, Breast Cancer Model



(40.P2) DEVELOPMENT OF A HUMAN THREE-DIMENSIONAL TUMOR TEST SYSTEM

Heymer A (1), Wagner K (1), Walles H (1)

1. Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Oncology Project Group

Introduction. New drugs have to be tested for their quality and efficiency before any clinical application. In this study preliminary tests for the establishment of a human epithelial tumor test system have been accomplished.

Methods. A biological scaffold of collagen I/III (BioVaSc) as well as a collagen type I hydrogel were seeded with different concentrations of carcinoma cell line CAL27 and cultivated up to three weeks. Seeding efficiency and cell viability were assessed histologically. In order to prepare the co-culture conditions of CAL27 with human microvascular endothelial cells (hmvECs) the proliferation and specific marker expression of both cell types were analyzed in different cell-type-specific media mixtures. At the end, a 3D co-culture model on the BioVaSc was established.

Results. CAL27 firstly adhered well on the BioVaSc, and with seeding 100,000 cells/cm² after 7 days of culture a cell monolayer was observed, with some cells migrating into the matrix. However, after 14 days only very few cells could be detected. This behavior was noticed even stronger on collagen hydrogels.

As an optimal medium for the co-culture-system endothelial medium was assessed. Endothelial cells proliferated in almost all culture media mixtures comparable, whereas CAL27 showed a reduced proliferation in all mixtures except for endothelial medium. In all media mixtures neither the tumor marker CEA nor the endothelial markers vWF and CD31 could be detected in CAL27. In contrast, these cells could be characterized with cytokeratin-pan as epithelial cells. Endothelial cells expressed cytokeratins, CD31 and vWF. The co-culture of both cell types on the BioVaSc for 7 days revealed a monolayer of endothelial cells on the apical and CAL27 cells on the basolateral side of the matrix.

Conclusions. For the establishment of a long-term stable test system the co-culture conditions, either with other cell types or under dynamic conditions, have to be further optimized.

Keywords. tumor test system, in vitro 3D model, co-culture

41. STEM CELL AND TISSUE ENGINEERING THERAPIES TO ACCOMPLISH REGENERATIVE DENTISTRY

Chair: Franklin Garcia-Godoy
Co-chair: Peter E. Murray
Keynote speaker: Peter E. Murray

Synopsis: Artificial teeth are in high demand because so many people are missing their natural teeth. Dentists are able to make artificial teeth which look real. The problems with artificial teeth is that they do not have the same chewing function as real teeth, they make talking more difficult, they can change the taste of food, and they lack the sensation of natural teeth. Because of these problems, almost everyone with artificial teeth would prefer to have replacement natural teeth. Dental researchers are using stem cells and tissue engineering therapies to accomplish regenerative dental treatment,

which can regenerate teeth, gum, skin, bone, and other tissues.
The objective of this symposium is to discuss the benefits and limitations of the stem cell and tissue engineering approaches that can be to be used to regenerate teeth. The learning objectives of the symposium are:
• Describe how regenerative dentistry can solve dental problems.
• Understand how stem cells and tissue engineering can regenerate teeth and other tissues.
• Discuss the advantages and limitations of using stem cell and tissue engineering procedures.
The long-term impact of developing regenerative dental treatments is that they will significantly benefit billions of people around the world by giving dentists the ability to regenerate teeth and other missing tissues.

(41.KP) PRECLINICAL TRIALS OF REGENERATIVE DENTAL PROCEDURES

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Introduction. The regeneration of dental tissues could benefit millions of dental patients each year. In a preclinical trial, we compared the effectiveness of four types of dental procedures to regenerate dental tissues within the root canals of teeth.
Materials and Methods. Following IACUC approval, the root canals of 54 maxillary and mandibular teeth in three non-human primates (M. fasicularis) were cleaned and shaped to a size 40.04 using protaper and profile files (Dentsply Tulsa, Oklahoma City, OK). The types of regenerative procedures used were: Group 1. Stimulate a blood clot. Group 2. Stimulate a blood clot and implant a rigid Collagen scaffold (BD Biosciences, Franklin Lakes, NY). Group 3. Stimulate a blood clot and inject a Pepgen P-15 scaffold (Dentsply, York , PA). Group 4. Gutta percha was used as a non-regenerative negative control procedure. An MTA liner was placed above the blood clot and the tooth access cavity was restored with glass ionomer (Fuji II, GC, Tokyo, Japan). After 5, 30 or 60 days, the teeth were processed for light microscope histology at x200 magnification according to ISO criteria. Data was analyzed by ANOVA statistical tests (P values) at a significance of 95%.
Results. No regeneration was observed after obturation with gutta percha. Some cells were observed in the root canals of teeth following the blood clot revascularization procedure. Greater numbers of cells were observed in the root canals of teeth where a rigid collagen scaffold or an injectable P15 scaffold had been implanted ($P<0.05$) (Fig. 1). No adverse events were observed.
Conclusions. The use of injectable and rigid scaffolds was more successful than using the blood clot revascularization procedure to accomplish regenerative dental therapy. Our ongoing work examines the preclinical effectiveness of scaffolds and growth factors to deliver reliable and safe regenerative procedures to dental patients.
Keywords. Teeth, dental, tissue regeneration, preclinical trial

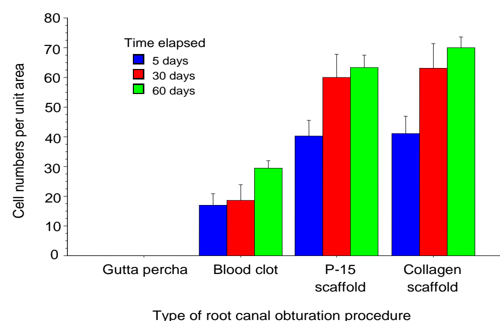

Fig. 1. Bar chart of the numbers of cells per microscope field within the root canals of teeth following regenerative dental procedures.

(41.O1) BIODEGRADABLE DOUBLE LAYER SCAFFOLD FOR PERIODONTAL ENGINEERING

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Introduction. Periodontium is often affected by periodontal disease, which is currently treated by tooth extraction, gingival flaps, root planning and conditioning, application of growth/differentiation factors or filler materials and guided tissue regeneration (GTR). Recently, Tissue Engineering as emerged as an alternative and advantageous approach for periodontal regeneration.
This work describes the development and characterization of a new scaffold composed by a starch+poly(e-caprolactone) (SPCL) membrane, which aims at acting as GTR barrier, and a SPCL fibre mesh functionalized with calcium and silicon with osteoinductive properties. The final aim is to combine this scaffold with adipose tissue derived stem cells (ASCs) to obtain a tissue engineered construct for periodontal regeneration.
Materials and Methods. The SPCL membrane was obtained by solvent casting and the fibre mesh (WSFM) by wet-spinning, which were combined by compression into a double-layer scaffold. The developed materials were characterized by scanning electron microscopy (SEM), tensile tests and Fourrier Transmission Infra-red (FTIR) analysis. Degradation behavior under the effect of relevant enzymes, alpha-amylase and lipase, was also evaluated. Finally, it was studied the proliferation and differentiation of canine ASCs seeded onto scaffold by dsDNA quantification and by SEM.
Results. SEM revealed an inwardly adherence between layers without decreasing roughness and fibres interconnection.
FTIR analysis confirmed presence of Si-O-Si and Si-OH bonds in functionalized WSFM. Scaffold exhibits high mechanical properties. Degradation tests showed that weigh loss was gradual in all conditions (enhanced by enzymes), being expected the material remain functional during the required two months of in vivo regeneration. Preliminary results from cell culturing experiments showed increased cell proliferation, according to DNA

increasing and SEM observations, suggesting that this material provides a good support for ASCs.

Conclusions. This work is a contribution to reach an effective technique for periodontal therapy, using an innovative combination of cells and bioactive matrix.

Acknowledgements. João Filipe Requicha acknowledges the Portuguese Foundation for Science and Technology for his PhD scholarship (Grant No. SFRH/BD/44143/2008).

Keywords. Periodontium, Guided tissue regeneration, Tissue engineering

(41.O3) POTENTIAL OF PLASMA RICH IN GROWTH FACTORS (PRGF-ENDORET) FOR IN SITU REGENERATIVE MEDICINE

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Plasma Rich in Growth Factors (PRGF-Endoret) involves the use of patient's own biologically active proteins, growth factors and biomaterial scaffolds for therapeutic purposes. This technology provides a new approach for the stimulation and acceleration of tissue healing and bone regeneration. The versatility and biocompatibility of using patient-derived fibrin scaffold as an autologous, biocompatible and biodegradable drug delivery system opens the door to a personalized medicine that is currently being used in numerous medical and scientific fields including dentistry, oral implantology, orthopaedics, ulcer treatment, sports medicine and tissue engineering among others. In this conference, we will review the state of the art and new directions in the use of this endogenous technology in the repair and regeneration of injured tissues by means of a controlled and local protein and growth delivery. The next generations of engineering strategies together with some of the most interesting therapeutic applications will be discussed together with the future challenges in the field.

(41.P1) OSTEOBLAST-LIKE CELL ATTACHMENT AND PROLIFERATION ON HYDROXYAPATITE AND CALCIUM PHOSPHATE COATED ZIRCONIA

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Introduction. Zirconia implants are currently under investigation in clinical experiments and are available on the market because of the demand for more esthetic **Results.** The purpose of this study is to compare the proliferation and osteogenic potential of hydroxyapatite aerosol deposition coated zirconium oxide with smooth zirconium oxide surfaces and IBAD CaP coated zirconium oxide surface.

Materials and Methods. MG63 osteoblast-like cells were cultured on zirconium oxide discs with a (n=20) (1) smooth surface, (2) hydroxyapatite (HA) coated surface, and (3) calcium phosphate (CaP) coated surface. The surface roughness and contact angles of each group of specimens were identified. Dissolution test of Ca and P was done to test the delamination of the coatings. The morphology of cells cultured was examined using scanning electron microscopy (SEM). X-ray photoelectron spectroscopy (XPS) was employed for the analysis of surface chemistry. Specimens were also evaluated by means methyl tetrazol sulfate (MTT) assay to compare the proliferation ability, and alkaline phosphatase (ALP) assay to compare the differentiation ability.

Results. Attached cells with more defined lamellopodia and flattened morphology were observed on the HA coated specimens. All the zirconia surfaces exhibited similar O and C peaks, as determined by XPS. HA coated and CaP coated surfaces exhibited Ca and P peaks whereas the smooth surface exhibited an Al peak. The contact angles of the surfaces, cell proliferation activity, and alkaline phosphatase activity showed no significant differences.

Discussion and Conclusions. To the present, zirconia implants have been developed and are under use for commercially. On the other hand, surface properties of zirconia implants have not been investigated as intensely as titanium. This study may determine the influence of HA coated and CaP coated zirconium oxide on cell viability and proliferation and also improve the osseointegration stability of zirconia implants of the same surface treatment.

Keywords. zirconia, hydroxyapatite coating, CaP coating, dental implant, osseointegration

(41.P2) ELECTROSPUN POLY-L-LACTIC ACID ALIGNED SCAFFOLDS FOR PERIODONTAL LIGAMENT REGENERATION

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Introduction. Periodontal disease is a chronic inflammatory process characterized by destruction and loss of connective and mineralised tissues. However, current therapeutic techniques, including guided tissue regeneration, do not predictably result in full regeneration of the lost periodontal structures. A tissue engineering approach may have the potential to fully regenerate the periodontal tissues. Much interest has been directed towards the construction of aligned biomaterial scaffolds. Aligned fibre scaffolds reflect the general topographic arrangement of collagen fibres of the native extracellular matrix of periodontal ligament which may influence cell behaviour.

The aim of this study was to fabricate aligned scaffolds and investigate the response of periodontal ligament fibroblasts (PDLF) to these biomaterials.

Materials and Methods. An 8% (w/v) poly-L-lactic acid (PLLA) polymer solution was prepared and electrospun to produced aligned scaffolds. Random PLLA fibre scaffolds were fabricated as a control. Periodontal ligament fibroblasts (PDLFs) were seeded onto the PLLA scaffolds and cultured for varying periods of time. The resultant constructs were analysed by scanning electron microscopy (SEM), confocal microscopy and immunohistochemistry.

Results. Scaning electron microscopy confirmed alignment of the fibers and PDLFs were observed to attach to the scaffold fibres. Phalloidin staining of the PDLF actin filaments, confocal microscopy photographs, showed that cells were oriented and elongated along the long axis of the fibres. Also, a higher level of cellular activity was observed by PDLFs cultured on aligned fibres.

In addition, a greater staining density for collagen I was seen in the aligned fibres constructs. These results indicate that fibre alignment may exert an effect on cells phenotype of periodontal ligament fibroblasts.

Conclusions.Electrospun PLLA aligned scaffold may be a suitable candidate for periodontal tissue engineering.

Acknowledgment. The authors are grateful to the Government of Saudi Arabia and King Saud University, Dental College for sponsoring D. Alotaibi.

Keywords. Periodontal fibroblast, PLLA, electrospining

(41.P3) MASTIC GUM/ POLY(CAPROLACTONE) AND MISWAK/ POLY(CAPROLACTONE) ELECTROSPUN NANOFIBROUS HYBRID MEMBRANES FOR PERIODONTAL LIGAMENT ENGINEERING

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Introduction. This research aims to investigate the effects of incorporating two natural oral disinfectant/anti-plaque agent, namely mastic gum (mg) and miswak (mw) into electrospun nanofibrous poly(caprolactone) (PCL) on the viability and proliferation of human periodontal ligament fibroblastic progenitors (hPDLFs) in-vitro.

Materials and Methods. Mastic gum harvested as a spice from the cultivated mastic trees (Pistacia lentiscus var. chia) was purchased from the local market (origin: Chios, Greece) and used as is. Miswak obtained from the twig of Salvadora persica tree was purchased from the local market (origin: AETM, S.Arabia) and its extract was used. All other chemicals, polymers and cell culture reagents were supplied from Sigma Chemical Company (USA). Either mastic gum or miswak extract containing PCL nanofibrous membranes were fabricated after optimizing the electrospinning parameters. Produced membranes were characterized by SEM and FTIR. Human PDLFs established and characterized at our laboratory were used in cell culture experiments under standard and osteogenic culture conditions. hPDLFs were immunophenotypically characterized. Cell viability and proliferation on the nanofibers was assessed using the MTT mitochondrial dehydrogenase activity assay.

Results. Electrospinning parametres such as the solvent system, applied voltage, needle size, needle-plate distance, concentration of PCL, mg and mw, etc. were optimized for mg/PCL and mw/PCL separately. SEM micrographs confirmed that the electrospun fibers had uniform morphology and were couple hundred nanometers in-size. FTIR analysis indicated the differences between the two hybrid membrane systems. MTT findings demonstrated a dramatic hPDLF cell viability increase of two-folds for mg/PCL, and a three-folds for mw/PCL nanofibrous membranes. Histology and immunophenotype characterization indicated that hPDLFs on both mg/PCL and mw/PCL membranes could be differentiated into the osteogenic phenotype.

Conclusions. The results indicate that incorporation of mastic gum and miswak into electrospun nanofibrous PCL membranes positively influences hPDLF cell proliferation and shows potential as a novel substrate for use in periodontal applications.

Keywords. Mastic gum, miswak, poly(caprolactone), electrospinning, nanofiber membranes, periodontal ligament engineering

(41.P4) THE APPLICATION OF TISSUE ENGINEERING ON DENTIN REGENERATION

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The human dentition is indispensable for nutrition, communication and physiology. Caries, pulpitis, apical periodontitis and trauma may lead to loss of tooth, causing the problem of pronunciation, mastication, and appearance difficulties. A biological tooth substitute that could replace lost teeth would provide a vital alternative to currently available clinical treatment. Stem cells are multipotent cells which are capable of self-renewing and differentiating into multi- cell lineages. In this study, tissue engineering approach will be addressed to evaluate this possibility to reconstruct tooth. To achieve this goal, we separately dissociated Wistar rat Tooth Bud Cells and Mesenchymal Stem Cells (MSC). Then two kinds of cells were seeded either in monoculture (Tooth Bud Cells only) or co-culture (MSC + Tooth Bud Cells) condition to evaluate cell viability and gene expression in vitro. Co-culture Cells and Tooth Bud Cells were then respectively seeded onto biodegradable bilayered scaffold and implanted in subcutaneous layers in SCID mice. After implantation for 12 weeks, the results were studied by X ray, histology and immunohistochemistry.

From the results, the cell phenotype of rat msenchymal stem cells was identified by flow cytometry. Cells viability and gene expression were elevated in co-culture group. At 12 weeks, the mineralization in Co-culture Cells group and Tooth Bud Cells group was visualized in X ray, Von Kossa and Alizarin Red S. At 4, 8, 12 weeks, the dentin matrix protein-1 (DMP-1) were deposited and stained in Co-culture Cells group and Tooth Bud Cells group by immunohistochemistry, representing dentin-like structure formation. In addition, Co-culture Cells group expressed stronger than Tooth Bud Cells group. It seems like that rat Mesenchymal Stem Cells and Tooth Bud Cells could reorganize themselves on biodegradable bilayered scaffold into arrangements that favor formation of mineralization / dentin-like tissue.

Keywords. Tissue Engineering ; Mesenchymal Stem Cells ; Tooth Bud Cells ; Bilayered Scaffold

(41.P5) DETERMINING A CLINICALLY RELEVANT BONE ENGINEERING METHOD. AN "ALL IN ONE" STUDY IN NUDE MICE

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Introduction. In craniofacial traumatized or contaminated bone tissues, but also in ectopic area, osteoprogenitors are essential for providing osteoinductive properties to the Biphasic Calcium Phosphate (BCP). Numerous bone tissue engineering (BTE) strategies have been described but they didn't completely address the clinical issues with

respect to efficacy and cost. In an attempt to determine the most clinically relevant strategy, we sought to compare some of the main successful BTE procedures in an "all in one" study in nude mice ectopic subcutaneous site.

Materials and Methods. Thirty mice were implanted with BCP combined or not with rat bone marrow mesenchymal stem cells (MSC). GROUP A: BCP + Autologous bone graft (ABG), GROUP B: BCP alone, GROUP C: BCP + unfractionated total bone marrow (TBM), GROUP D & E: BCP + 90 000 MSC/cm² osteogenically committed (D) or not (E) by coculture on BCP, GROUP F & G: BCP + 45 000 MSC/cm² osteogenically committed (F) or not (G) by coculture on BCP, GROUP H & I: BCP + 90 000 MSC/cm² osteogenically committed (H) or not (I) extemporaneously loaded on BCP, GROUP J: Highly confluent committed MSC alone. The bone formation was assessed by scanning electronic microscopy after 8 weeks.

Results. Only 4 groups demonstrated an ectopic bone formation. The highest rate (6/6) of bone formation was seen in group A and group C. A lower rate (3/6) was seen in group D and group H No bone formation was detected in the remaining groups.

Conclusion. This study clearly demonstrated the positive effect of associating TBM to a BCP for ectopic bone formation. Given its usability and efficacy, a total bone marrow-based strategy could be considered as a relevant alternative to ABG but also to current BTE procedures that use committed or not MSC.

Acknowledgments. "Région Pays de la Loire" (BIOREGOS II), Biomatlante.

Keywords. stem cell ; tissue engineering ; calcium phosphate ceramics

(41.P6) MECHANICAL AND BIOLOGIC BEHAVIOR OF BETA-TRICALCIUM PHOSPHATE IN THE SURGERY OF MAXILLARY SINUS

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Introduction. Mechanical behavior can play a key role in biomaterials for maintenance of graft tissues. The aim of this study was to report the outcome of sinus lift surgery with use of biomaterial for bone regeneration in implant dentistry.

Materials and Methods. 25 patients with edentulous maxilla atrophy were treated with 115 Galimplant® implants. In all cases, beta-tricalcium phosphate KeraOs® were used for sinus floor elevation. Implants were inserted 6 months after sinus lift. During this implant surgery, graft biopsies were obtained for trephine. Implants were loaded after a healing free-loading period of 6 months. Radiographic control were realized after a year of follow-up.

Results. Radiography and histologic assessments demonstrate a sufficient mechanical integrity in the anatomical site and partial remodelling of biomaterial without inflammation and/or fibrosis. Clinical results indicate a success rate of implants of 98.3% and 100% of prosthetics status, after a mean functioning period of 28.4 months.

Conclusions. This study indicate that beta-tricalcium phosphate can be used with success as biomaterial with a important mechanical and biological properties in the treatment with dental implants by sinus floor elevation.

Keywords. sinus floor elevation, biomaterials, beta-tricalcium phosphate, implant dentistry

(41.P7) THE THERAPEUTIC LYMPHANGIOGENESIS USING STEM CELL AND VEGF-C HYDROGEL

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Introduction. Lymphedema is defined as a manifestation of lymphatic system insufficiency. Although lymphedema is mainly managed by comprehensive non-operative therapy, it needs lifelong care. This study was designed to examine whether therapeutic efficacy could be improved by human adipose-derived stem cells (h-ADSCs) and VEGF-C incorporated gelatin hydrogel (VEGF-C Hydrogel) in a lymphedema animal model.

Materials and Methods. We prepared biodegradable hydrogel from gelatin which can provide continuous release of VEGF-C. H-ADSC viability on VEGF-C hydrogel was examined by MTT assay. Hindlimb lymphedema animal model was made by lymphatic dissection after dye injection. Experimental group was divided into 5 groups; Normal(N), Lymphedema (L), ADSC after lymphedema (A), VEGF-C hydrogel after lymphedema (V) and ADSC+VEGF-C hydrogel after lymphedema (AV). H-ADSC was applied at lymphatic dissection area and cover with VEGF-C hydrogel. We investigated foot edema thickness and lymphatic vessel density at various time points.

Results. We have confirmed that the VEGF-C was continuously released up to 28 days by ELISA. From in vitro cell culture, ADSC viability into VEGF-C Hydrogel was displayed by MTT. From in vivo study, the h-ADSCs with VEGF-C hydrogel group showed significantly decreased dermal edema thickness when compared with h-ADSC only and VEGF-C hydrogel only group. Immunohistochemical analysis also revealed that h-ADSCs/VEGF-C hydrogel group have a significantly observed many lymphatic vessel regeneration compared with other groups. H-ADSC was co-stained with lymphatic endothelial marker LYVE-1.

Conclusions. H-ADSCs and VEGF-C hydrogel combined therapy played an important role for therapeutic lymphangiogenesis in lymphedema animal model

Keywords. Lymphangiogenesis, Stem cell, VEGF-C hydrogel

(41.P8) CHARACTERIZATION OF MESENCHYMAL STEM CELLS (MSCS) OBTAINED FROM HUMAN CHIN BONE

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Introduction. MSCs reside in bone marrow and have the capacity of auto-renewal and multiple lineages differentiation. They are most frequently obtained from iliac crest requiring a puncture which is a painful procedure of high postoperative morbidity, and disability. Preliminary results of our research group suggested that,

cells of BM with similar morphology to MSCs could be obtained from chin fragments that do not exceed 3 mm long. This new site can provide a better surgical accessibility, minor morbidity and provides an adequate amount of cells for regeneration of different tissues. However, it is essential to characterize them and to determine their differentiation potential. Objective: Characterize adherent cells obtained from human chin bone fragments and analyze whether they could relate to the MSCs described in the literature for implementation in bone jaw defects.

Methodology. Prior to surgery, patients were informed about all procedures and approved by written consent. Seven patients that required chin bone graft accepted to donate a 3 mm size sample. The fragment was disintegrated and was cultured in a plate of 12 mm in supplemented DMEM medium under defined in vitro conditions. Cells were expanded for the CFU-F (colony-forming unit-fibroblast) and flow cytometry assays.

Results. Cells were morphologically related with MSCs. Percentage of CFU-F was similar in all samples (27%±3) and rewards were consistent with those described in the literature. Flow cytometry showed MSCs phenotype in 6 samples. (Table 1)

Conclusions. Chin bone is a good option to obtain MSCs. Small fragments can easily been isolated and expanded to achieve enough cells for an adequate treatment on bone regeneration defects.

Acknowledgment. This work was supported by Estrategia de sostenibilidad 2009-2011 and Convocatoria interna Facultad de Medicina, Universidad de Antioquia.

Keywords. chin bone, stem cell

Sample	Age	Gender	ethnic group	Size of fragment	Type of bone	Day with 60-70% of confluence (First subculture)	Cells obtained in dishes 12mm	Flow cytometry			
								CD73 /CD105	CD90 /CD73	CD45 /CD34	CD34/HLA-DR
P001	25	M	African race	3 mm	Bone marrow	33 days	6000	45,90%	69,20%	95%	95%
P002	32	F	Mestizo			20 days	5000	98.4%	98.4%	3.14%	3.04%
P003	23	M	Mestizo			25 days	5.000	98.6%	98.7%	0.89%	0.60%
P004	22	M	Mestizo			15 days	4.000	93.4%	98.8%	0.25%	0.14%
P005	27	M	Mestizo			19 days	6000	89.2%	98.9%	0.19%	0.10%
P006	19	F	Mestizo			16 days	5.000	99,40%	98,90%	0,14%	0,30%
P007	27	M	Mestizp			7 days	7.500	94,80%	94,50%	1,96%	1,56%

(41.P9) EFFECT OF SURFACE TREATMENT ON TITANIUM FOR IMPLANT DENTISTRY APPLICATIONS

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Introduction. An increasing number of titanium materials are being used in implant dentistry. The aim of this study was to study the effect of different treatments on the surface of a commercially titanium.

Materials and Methods. The material studied was a titanium/aluminium/vanadium alloy grade 5. Discs of machined titanium alloy (5-mm diameter×1-mm height) were obtained. The surface treatments used were acid etching (ES), sandblasting (SS) and dual treatment of sandblasting + acid etching (SES). The machined surface (MS) was used as reference. Topography were characterized with a scannig electron microscope.

Results. Microscopic images showed parallel marks (MS); microholes (ES); irregular surface with peak and valleys with presence of some blasting particles (SS), while that in SES treatment were evidenced a irregular pattern with microholes in low magnification, and nanopeaks and nanoporous with higher magnification.

Conclusions. This study indicates that treatment of surface implants increased the topographic changes and is an important procedure to improve biologic applications in implant dentistry.

Keywords. Surface treatment, titanium, dental implants, nanosurface

(41.P10) COMPARATIVE MULTILINEAGE DIFFERENTIATION ANALYSIS OF OSTEOPROGENITOR CELLS AND BONE MARROW MESENCHYMAL STEM CELLS

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Introduction. Tissue engineering is a promising approach for regenerative procedures in oral and maxillofacial surgery. This study investigated the suitability of oral skeletal tissue as an applicable source of progenitor cells and an alternative to the iliac crest bone marrow. The aim was to compare multilineage differentiation potential of osteoprogenitor cells and bone marrow mesenchymal stem cells (BM-MSCs).

Materials and Methods. Osteoprogenitor cells were isolated from explant cultures of intra-orally harvested bone chips during routine oral surgery. BM-MSCs were obtained from iliac crest bone marrow aspirates and used as positive control for multilineage differentiation analysis. Cells were immunocytochemically characterized by the expression of characteristic surface antigens including CD73, CD90, CD105 and the lack of CD14, CD34, CD45. Differentiation capacities into the osteogenic, adipogenic and chondrogenic lineages were investigated using cytochemical tests (alkaline phosphatase activity, Oil Red O and Alcian blue staining) and RT-PCR analysis.

Results. Osteoprogenitor cells showed characteristics of BM-MSCs like plastic adherence and expression of defined surface antigens. Their differentiation capacity into the osteogenic, adipogenic and chondrogenic lineages was comparable to the one of BM-MSCs.

Conclusions. These findings suggest that osteoprogenitor cells have a similar differentiation potential to BM-MSCs' in vitro. Oral skeletal tissue may be considered as a suitable source of cells for tissue engineering therapies in regenerative dentistry.

Keywords. regenerative dentistry, alveolar bone, mesenchymal stem cells, differentiation potential

(41.P11) OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION OF PERIODONTAL LIGAMENT STEM CELLS

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Introduction. Periodontal Ligament stem cells are multipotent stem cells with the capacity to differentiate into osteoblasts and adipocytes. The objective was to evaluate the potential of PDLSCs to differentiate into osteoblasts and adipocytes using two commercial induction media.

Methods and materials. Periodontal ligament (PL) was extracted from teeth of healthy adult subjects aged 21–45 years. PL was gently removed and immersed in a

digestive solution for 1 h at 37Cº. After digestion, cells were placed in three flasks with different mediums. Medium 1: Medium Osteodiff (Miltenyi®); Medium 2: Medium Adipodiff (Miltenyi®); Medium 3: Eagle's alpha minimal essential medium, 15% fetal bovine serum, Lenexa, KS, USA), and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 lg/ml. amphotericin B; Sigma Chemical Co., St. Louis, MO, USA). Flasks were incubated at 37ºC in a 5% CO2 and the medium was changed twice a week. At day 30, the matrix mineralisation was determined with the Alizarin Red S dye. The adipogenic differentiation was determined with Oil Red Staining.

Results. After 35 days, PDLSCs cultivated with Medium 1 formed and developed mineralization nodules (clusters), as revealed by Alizarin red staining. This staining was stronger than those obtained with controls without induction media. Furthermore, we observed accumulation of intracellular neutral lipids positively stained with Oil Red when PDLSCs was cultivated with Medium 2.

Conclusion. This study demonstrates the ability of periodontal ligament stem cells to differentiate into osteoblasts and adipocytes with commercial induction media such as Osteodiff (Miltenyi®) or Adipodiff (Miltenyi®).

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Keywords. periodontal ligament stem cells, dental stem cells, cell therapy

(41.P12) AN *IN VIVO* STUDY OF OSSEOINTEGRATION OF DENTAL IMPLANTS WITH NANOSURFACE

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Introduction. Microrough titanium surfaces can to promote enhanced periimplant bone apposition.The aim of this study was to evaluate the bone response to nanosurface implants in a rabbit femur model.

Materials and Methods. 12 implants, 6 implants with nanosurface and 6 implants with sandblasted surface, were bilaterally placed in the distal area of the femur of 6 white New Zealand rabbits, remaining for 90 days in vivo. After sacrifice, the implants in bone were processed to slides and were histologically and histomorphometrically (bone-to-implant contact) evaluated.

Results. Histologic results indicate a good bone response to implants without inflammation and/or fibrosis that permit to stablished an adequate interaction with cellular behavior based in a topographically micro-nano structured surface. The histomorhometry demonstrate a higher degree of bone-to-implant contact in nanosurface (53,7%) compares with sandblasted surface (50,9%).

Conclusions. This study indicates that implants with nanosurface were biocompatible and can be used with success for rapid osseointegration and consequently biomechanical fixation.

Keywords. nanotechnology, dental implants, osseointegration, nanosurface

42. THE EXTRACELLULAR MATRIX IN TISSUE ENGINEERING: PASSIVE OR ACTIVE PLAYER?

Chair: Robert P Mecham
Co-chair: Julia Buján
Keynote speaker: Robert P. Mecham
Organizer: Spanish Society of Histology and Tissue Engineering (SEHIT)

Synopsis: The extracellular matrix (ECM) defines the mechanical properties of all tissues. Like constructing a building, interstitial cells during development deposit a changing combination of ECM molecules to deal with the changing forces that the tissue experiences as the organism matures. In addition to playing a key structural role, the ECM provides instructional signals that induce, define, and stabilize cell phenotypes. This includes providing an instructive niche that is critically important for stem cells. This reciprocal instructive interaction between the cell and its ECM is critical in directing the developmental transitions that occur in embryogenesis, postnatal development, and response to injury. How cells interpret these regulatory signals is critically important for tissue engineering.

Constructing a complex, mechanically appropriate ECM requires instructions for ECM assembly, knowledge of the available building materials, and information about the stresses that the final material will have to endure. One way this could occur is through a process where all of the required information is genetically hardwired into the cells participating in the construction project, with no deviation from the blueprint design. Alternatively, the project could be fashioned over time through changing instructive signals from the microenvironment that tell the cells what mix of ECM proteins need to be added at that particular instance. Current studies with genetically engineered mice support the latter possibility and show that there is more flexibility in the building plan than previously thought. Thus, to fully understand tissue function, we need to understand the interplay between mechanical signals, cell behavior, and ECM composition during remodeling and development. The artery wall, for example, is designed for optimal performance given the mechanical demands of the closed, high pressure, pulsatile, cardiovascular system. When these demands are changed, either through genetic alterations or adult-onset acquired disease, the vessel wall adjusts its ECM to meet the new demands and to normalize the stresses experienced by the smooth muscle cells. By characterizing the complex relationship between mechanical forces, ECM production and cellular differentiation, we will be able to design strategies to encourage advantageous remodelling in diseases and to recreate this process in tissue engineering. This session will address the relationships between tissue development, mechanics as imparted by the mix of ECM molecules deposited in the extracellular space, and the role of ECM in stem cell biology.

(42.KP) VASCULAR EXTRACELLULAR MATRIX AND VESSEL WALL DEVELOPMENT

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An important factor in the transition from an open to a closed circulatory system was a change in vessel wall structure and composition that enabled the large arteries to store and release energy during the cardiac cycle. The component of the arterial wall in vertebrates that accounts for these properties is the extracellular matrix network organized by medial smooth muscle. In most animals with a closed circulatory system, pulse pressure and overall blood pressure increase as the cardiovascular system matures during fetal development and early postnatal life. As blood pressure increases during this period, the smooth muscle cells (SMC) change the types and amounts of extracellular matrix (ECM) they produce in order to strengthen the vascular wall and maintain the appropriate mechanical properties. By changing the amount of elastin in the vessel wall of mice through elastin gene deletion or elastin transgene expression, we show that cardiac and vascular development are physiologically coupled, and we provide evidence for a universal elastic modulus that controls the parameters of ECM deposition in the vessel wall. The major changes associated with elastin insufficiency are an increased number of smooth muscle cell layers in the vessel wall and a substantial increase in blood pressure. Interestingly, these animals live a normal life span with no evidence of cardiac hypertrophy. The unique cardiovascular remodeling seen in elastin insufficient mice indicates that the developing vascular cell can adapt its building process to accommodate environmental changes and produce an altered overall wall structure that operates at different physiologic setpoints.

(42.O1) ENGINEERING THE GROWTH FACTOR MICROENVIRONMENT WITH FIBRONECTIN DOMAINS TO PROMOTE TISSUE HEALING

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Growth factors (GFs) are key molecules for tissue morphogenesis and healing. However, while they are really promising molecules for a use in regenerative medicine applications, they often fail to prove cost-effective or even clinically efficacious during clinical trials. One of the reasons for this poor translation may lie in the rapid breakdown and clearance of GFs from tissue sites in vivo, leading to the development of strategies controlling their release. We recently found that the 12th to 14th type III repeats of fibronectin (FN III12-14) promiscuously bind GFs from the platelet-derived GF, fibroblast GF, transforming GF-β and neurotrophin families. However, the reasons for such promiscuous binding capacity were still unclear, while evidences from the literature suggested that the close proximity of the major integrin-binding domain, allows joint integrin/GF-receptor signaling triggered by a complex FN/GFs. Accordingly, we found that FN fragments containing both the integrin- and GF-binding domains could drastically enhance GF activities in vitro. In addition, testing which integrins were involved within these synergistic effects, we found that α5β1 integrin was mainly involved. By the use of FN fragments and fibrin, we could engineer a specific microenvironment allowing sequestration of multiple wild-type GFs, while triggering synergistic signaling between GF-receptors and integrins. In a delayed wound healing model in mouse and in a calvarial bone defect model in rat, GFs delivered with the FN fragment microenvironment were drastically improved in their ability to induce tissue healing, even though a single low dose of GFs was used. Specifically, we established integrin/GF-receptor synergistic activities as a key parameter for GF translation into regenerative medicine treatments and demonstrate a method to exploit this phenomenon. This work highlights the absolutely critical role of the ECM microenvironment in modulating signaling of GFs and in driving these molecules forward toward more widespread clinical use.

Keywords. Growth Factors, Intergins, Microenvironment, Signaling.

(42.O2) ENHANCED IN VITRO PRODUCTION OF ENDOTHELIAL CELL MATRIX BY MACROMOLECULAR CROWDING

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Aim. Production of extracellular matrix (ECM) in amounts relevant to tissue engineering is currently difficult to achieve. Fastidious cells, such as pancreatic islets, need ECM to survive outside their in vivo environments. Previous literature has shown that islet ECM appears to be mostly produced by endothelial cells. However, in vitro endothelial ECM production is limited in quantity. Macromolecular crowding is able to enhance the deposition of ECM by mesenchymal stem cells and fibroblasts, but not yet reported in endothelial cells. This study examined the impact of crowding on the in vitro deposition of endothelial ECM with the objective of producing sufficient concentrations for pancreatic tissue engineering purposes.

Methods. Immunofluorescence (IF) was used to characterise matrix deposited by human umbilical vein endothelial cells (HUVECs). Deposition was enhanced by crowding with a Ficoll 70/400 cocktail. Detergents sodium deoxycholate (DOC) and Nonidet P-40 (NP40) were compared for their ability to decellularise HUVECs from the ECM.

Results. ECM components identified in the HUVEC cell layer included collagen IV, fibronectin, nidogen, perlecan and heparan sulfate. Non-crowded conditions resulted in ECM that remained largely intracellular, whereas crowded cells mostly secreted their matrix into the extracellular space. DOC was more efficient than NP40 in removing cellular debris and genetic material from the ECM. Collagen IV, fibronectin and perlecan remained adherent to the culture plates after DOC decellularisation.

Conclusions. Ficoll crowding successfully enhanced the uniform deposition of endothelial ECM onto the culture plate and DOC decellularisation did not markedly impact

on the ECM composition. Future studies will include culturing of islets on the endothelial ECM as well as the incorporation of deposited endothelial ECM into a 3D scaffold system.

Acknowledgements. This research was supported under Australian Research Council's Discovery Projects funding scheme (project number DP0986447) and the National University of Singapore Faculty Research Committee Grant (Engineering in Medicine).

Keywords. Endothelial cells; Macromolecular crowding; Extracellular matrix; Islets of Langerhans

(42.O3) ENGINEERING DECELLULARIZED POLYMERIC-BASED HYBRID CONSTRUCTS FOR BONE TISSUE ENGINEERING APPLICATIONS

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Introduction. Synthetic polymers represent promising scaffold materials in bone tissue engineering owing to tailored-made physical properties, but their use is limited due to poor bioactivity. To overcome this limitation, we investigated a novel perfusion bioreactor-based strategy that relies on the combination of a polyesterurethane (PEU, DEGRAPOL®) scaffold with a decellularized, cell-laid mineralized extracellular matrix (mECM) providing bioactive properties. The osteoinductive potential of the resulted hybrid substrates was then investigated in vitro on human mesenchymal stromal cells (hMSCs).

Materials and Methods. hMSCs were cultured on 3D porous scaffolds in a perfusion bioreactor for 4 weeks using osteogenic medium to induce mECM deposition. The amount of DNA, collagen and calcium in the hybrid substrates was determined before and after the decellularization process, consisting of three freeze/thaw cycles followed by a washing step in the bioreactor. Expanded hMSC were then reseeded and cultured up to 16 days to assess their osteoblastic differentiation by means of gene expression and calcium deposition. Plain PEU and ceramic scaffolds were used as controls.

Results. The decellularization process efficiently washed away cellular components throughout the internal regions of the hybrid constructs (94% DNA reduction) without affecting the amounts of collagen and calcium embedded in ECM. The resulting decellularized constructs supported the osteoblastic differentiation of freshly seeded hMSC by upregulating, similarly to ceramic scaffolds, the expression of typical osteoblastic gene markers (Bone sialoprotein 6-fold; Osteocalcin 4-fold; Osteopontin 4-fold) and increasing calcium deposition as compared to plain PEU.

Conclusions. Our results show that the developed bioreactor culture and devitalization protocols can provide an effective, standardized process for engineering acellular hybrid mECM+polymer constructs with enhanced osteoinductivity in vitro on hMSC. Ongoing experiments will evaluate the bone forming capacity of these hybrid constructs in an ectopic model in mice.

Acknowledgment. The authors thank ab medica SpA for providing the DEGRAPOL® biomaterial.

Keywords. Extracellular matrix; Human mesenchymal stem cells; Osteogenic differentiation; Bioreactor

(42.O4) COLLAGEN I SCAFFOLD SEEDED WITH VALVE INTERSTITIAL CELLS, A PROMISING APPROACH FOR HEART VALVE TISSUE ENGINEERING

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Introduction. Type I collagen in the form of gel, foam or nanofibres was evaluated for heart valve tissue engineering. The purpose of our study was to evaluate the adhesion, growth and differentiation of pig valve interstitial cells (VIC) cultured under static and dynamic conditions on laboratory produced porous collagen I scaffold.

Materials and Methods. Disc-shaped porous collagen scaffolds were prepared from 1% bovine collagen (Devro plc) suspension using a freeze-drying method and subsequently chemically cross-linked [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide]. Scaffolds were seeded on both sides with VICs at a density of 563x103 cells/cm2 and cultured under static conditions for up to 1 week. Subsequently, scaffolds were placed for 4 weeks into a dynamic culture system with pulse flow of culture medium and conditioned by gradually increasing the flow of medium from 100 to 130 ml/min. Control samples were cultured only under static conditions. After 4 weeks, VICs in the samples were stained by hematoxylin-eosin, resorcin-fuchsin, immunocytochemically for alpha-actin and vimentin, and evaluated by light and confocal microscopy.

Results. VICs covered the scaffold surface nearly confluently, penetrated into deeper layers to an average depth of 100 µm and were strongly positive for vimentin and smooth muscle alpha-actin which proved the presence of both activated myofibroblast-like and quiescent phenotypes. VICs also produced new extracellular matrix. All these factors, including VIC phenotypic development, seemed to be influenced by dynamic loading.

Conclusions. Laboratory produced 1% collagen I scaffold that mimics the natural architecture of ECM in vivo may be a promising approach in the tissue engineering of heart valves. Collagen scaffolds support VIC proliferation, differentiation and ECM production and do not appear to have cytotoxic effects on the seeded cells.

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Keywords. heart valve, tissue engineering, collagen, valve interstitial cells, bioreactors

(42.O5) AORTIC HEART VALVE PREPARED FROM HUMAN PERICARDIUM UNDER STATIC AND DYNAMIC CONDITIONS

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Introduction. Allogenous or xenogenous heart valve prostheses evoke an immune reaction in the patient`s body, gradually undergoing degeneration, eventually necessitating replacement of the prosthesis. The development of a functional autologous biological heart valve replacement is therefore a plausible solution to this problem and a current challenge for tissue engineering.

Materials and Methods. Three individual samples of human pericardium were fixed around a plastic holder and sewn into the shape of an aortic heart valve. Each valve was placed into a chamber and perfused with cell culture medium using a peristaltic pump. The medium flow rate was increased weekly from 300 to 400, 500 and 600 ml/min. Pericardium without conditioning was used as a control. After 4 weeks the samples underwent histological and biomechanical assessment.

Results. In the conditioned biological heart valve an increased number of alpha-actin positive cells was found in the central part of the valve cusps and also in the vessel wall. In addition, mesenchymal cells positively stained for a proliferation marker Ki-67 were present. In the control samples we observed only rare alpha-actin positive cells in the superficial layer of the valve cusps and vessel wall, but no proliferating cells were found. For biomechanical evaluation we measured the secant modulus using a uni-axial tensile test (MTS machine). The secant modulus was slightly lower in the conditioned samples compared to control when different orientations of loading were used.

Conclusions. Conditioning of the autologous aortic heart valve made the pericardium slightly more pliable and supported the proliferation and differentiation of mesenchymal cells into a phenotype similar to that found in natural aortic heart valves.

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Keywords. autologous heart valve, pericardium, biomechanics

(42.O6) USING NATURAL EXTRACELLULAR MATRIX PLATFORMS TOWARDS ENGINEERING OF THICK CARDIAC-LIKE TISSUE CONSTRUCTS

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Introduction. We have previously reported the successful isolation of thin porcine cardiac ECM (pcECM) manifesting bio-mechanical properties relevant for myocardial tissue engineering. As cardiac tissue could reach a thickness of 12-15 mm, such thin constructs offers limited regeneration capacity. Generation of thicker myocardial-like tissue constructs is limited due to diffusion limitations (~100 µm), and the lack of proper vascular network. Thus the development of support systems enabling the cultivation of thicker constructs is required. Present work focuses on the optimization of the decellularization procedure for thicker tissue constructs and the development of a novel supportive bioreactor system.

Methods. Our previousely reported decellularization procedure was optimized to obtain thick pcECM (10-15 mm) by increasing trypsin activity and incorporating sonication and/or perfusion through built-in vasculature. Isolated thick pcECM was evaluated by Masson trichrome and oil-red staining of histological cross-sections, SEM and two-photon microscopy. Immunogenicity was evaluated using TNFα secretion by mouse bone marrow derived macrophages (BMM). Vascular network preservation was evaluated using corrosion casting and feeding of fluorescently labeled dextran.

Results. Increased trypsin activity together with sonication and/or perfusion enabled a better decellularization procedure. No cellular remains were observed with Masson trichrome staining. Oil-red showed remaining of adipocytes. SEM and multiphoton microscopy showed preserved structural characteristics, supportive of cellular growth. Realtime RT-PCR analysis of the TNFα/GAPDH expression ratio in BMM, revealed low stimulation of pcECM, compared to native cardiac tissue exposed cells. Vascular network functionality was preserved to the first three-four branches from the main coronary vessels.

Conclusions. We have successfully isolated thick non-immunogenic pcECM preserving its ultrastructural properties and its inherent vascular network. A novel bioreactor system is currently being developed that would enable the control of pulsate flow, electrical and mechanical pre-conditioning.

Acknowledgements. This research is funded with the most generous support of the Israel Science foundation (grant no. 1563/10).

Keywords. Extracellular matirx (ECM), myocardial tissue engineering, Bioreactors

(42.O7) DECREASED MECHANICAL PROPERTIES OF HEART VALVE TISSUE CONSTRUCTS CULTURED IN PLATELET LYSATE AS COMPARED TO FETAL BOVINE SERUM

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Introduction. Numerous studies emphasize the production of endogenous extracellular matrix (ECM), or the use of ECM analogs as scaffold, to provide optimal cellular environments and mechanical properties in engineered tissue. Here we concentrate on matrix

production and tissue strength while changing culture conditions towards autologous alternatives for heart valve tissue engineering.

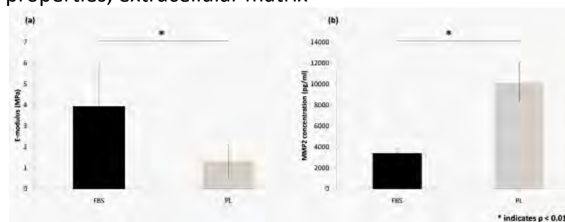
Materials and Methods. A 3D tissue model, consisting of human myofibroblasts seeded into a biodegradable scaffold was used to study mechanical properties and tissue composition. For this purpose we harvested myofibroblasts of seven patients. These cells were used to engineer per patient 10 heart valve tissue constructs for the culture in medium supplemented with either platelet lysate (PL) or fetal bovine serum (FBS). After 4 weeks of culture, these tissue constructs were analyzed for mechanical properties and matrix composition, quantitatively and qualitatively.

Results. Under both medium conditions (medium supplemented with PL or FBS) the cellular phenotype remains unchanged, and similar amounts of collagen, GAGs, and collagen crosslinks were found. Nevertheless, mechanical testing showed that the ultimate tensile strength and elastic modulus in PL constructs was on average approximately three times lower as compared to FBS (Fig. a). Additional tests indicated that this difference might be explained by a different collagen fiber architecture possibly due to increased production of matrix-degrading proteases by cells cultured in PL (Fig. b).

Conclusion: Our results indicate that despite comparable tissue composition mechanical properties are drastically reduced in PL. These results could not be predicted from earlier 2D studies. The tissue strength is presumably related to matrix organization. Hence, PL is not preferred for engineering strong and organized heart valve tissue constructs.

Acknowledgements. This research is supported by the Dutch Technology Foundation (STW), Applied Science Division of NWO, and the Technology Program of the Dutch Ministry of Economic Affairs.

Keywords. heart valve, tissue engineering, mechanical properties, extracellular matrix



(42.O8) TROPOELASTIN: CELL INTERACTIONS IN TISSUE ENGINEERING

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Tropoelastin is the soluble precursor to elastin, the main elastic protein found in mammals. Elastin facilitates the reversible deformation of elastic tissues and can withstand decades of repetitive forces (1). The tropoelastin protein monomer has a defined three-dimensional shape that exquisitely balances multiple roles, including organized assembly and cell interactions. Ideal elastic behavior clearly places tropoelastin in the same category as other natural elastomers such as rubbers and resilin. Single molecules of tropoelastin behave as perfect elastic molecules, since they can be stretched to several times the resting length and overstretching is a fully reversible process (2). Recoil restores the shape of tropoelastin.

We recently described how tropoelastin's extensional elasticity can serve to expand undifferentiated cells, including progenitors and mouse hemopoietic stem cells. Mechanotransduction is facilitated through the actin-myosin cytoskeleton. An elastic circuitry extends continuously from the extracellular tropoelastin to deep inside the cell, involving elastic communication from the tropoelastin extracellular environment though intracellular myosin II to the nucleus (3). We find that there are distinct regions of the molecule that are responsible for elasticity and cell binding. The cell-binding C-terminus is connected to the elastic region that dominates tropoelastin (3,4). We have identified a specific non-canonical, integrin-binding motif at the tip of the human protein that dominates interactions with a variety of human cells (4,5,6,7). We are using, adapting and modifying these specific interactions to generate functional elastic scaffolds that promote cell attachment, spreading and proliferation.

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(42.P1) CHANGES IN EXTRACELLULAR MATRIX CONSTITUENTS IN VENOUS INSUFFICIENCY

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Introduction. Elastic fiber formation is believed to be a complex process. Our previous work suggests that the

development of varicosity involves a restructuring of the elastic component of the vein wall. Elastin expression diminishes with age. The aim of this work is to evaluate the changes of the elements involved in elastic fiber assembling.

Materials and Methods. Healthy (n=12) and varicose saphenous veins (n=20) were subjected to qRT-PCR and immunohistochemical analysis, using specific primers and antibodies of fibulins-4 and 5 (Fib-4, Fib-5) and fibrillins-1 and 2 (FBN-1, FBN-2). Study groups were established according to the age.

Results. The mRNA expression of the elastic components did not changed in the different groups. Fib-5 protein level showed the highest expression, and presented similar pattern of expression that Fib-4 one. In varicose veins, it was localized around the elastic fibers, in vascular smooth muscle cells (VSMCs) and accumulated in the extracellular matrix, and overcoat in VSMCs in control veins. FBN-1 had the weakest expression. Both fibulins showed the highest expression in the group less than 50 years in healthy veins, whereas it was increased in elderly patients in pathogenic veins. In the other hand, FBN-1 had similar expression in all the groups, with less expression in younger healthy patients. We did not find expression of FBN-2 in our experiment conditions.

Conclusions. Our findings suggest that the restructuring of the elastic component in the development of the varicose condition involves alteration at translational but not transcriptional levels.

Keywords. Elastic fiber, varicose veins, vascular matrix

(42.P2) DIFFERENTIATION OF HUMAN BONE MARROW STROMAL CELLS ON CELL-DERIVED MATRIX

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Extracellular matrix (ECM) is a complex network of proteins and proteoglycans that can provide cells with a physical support and micro-environment. ECM interacts with surrounding cells and serves as signaling cues for cell migration, proliferation, and differentiation. In this study, cell derived matrices (CDMs) obtained from fibroblasts and preosteoblasts were named FDM and PDM, respectively and their intrinsic potentials were evaluated in terms of osteogenesis and chondrogenesis of human bone marrow derived stromal cells (hBMSC). Our hypothesis is that naturally driven CDM may be very useful in simulating biomimetic micro-environment for stem cells and thus play a specific role in regulating stem cell differentiation. From the early results of gene expression (osteopontin, type I collagen, Cbfa1, type II collagen, aggrecan), it seemed that cellular response was significantly different as the type of substrates (control, gelatin, FDM, PDM) varied. After 3 weeks of culture in the defined medium, the hBMSCs were more osteogenic on the PDM, whereas chondrogenic differentiation was more preferential on the FDM as compared to other substrates. Histological analysis also supported these results. This work suggests that naturally assembled 2D matrix can offer a physical cue in directing stem cell differentiation.

Keywords. Extracellular matrix, decellularization, bone marrow stromal cells, chondrogenesis, osteogenesis

(42.P3) GROWTH FACTOR-IMMOBILIZED EXTRACELLULAR MATRIX FOR THE CULTURE OF FUNCTIONAL CELLS

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Introduction. Functional culture substratum is very important to establish a practical culture method for tissue engineering. In particular, reduction of required amount of growth factors and induction of tissue-like structure are expected [1]. In this study, we studied about growth factor-immobilized natural extracellular matrix.

Materials and Methods. We synthesized a heparin-immobilized gelatin (h-gelatin) which enables the immobilization of various types of native growth factors. Furthermore, extracellular matrix from decellularized rat liver (D-ECM) was prepared by using 4% Triton X-100. Human umbilical vascular endothelial cells (HUVEC) and primary rat hepatocytes was used for the estimation of them for the application of tissue engineering.

Results and Discussion. Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) could be immobilized to not only h-gelatin but also D-ECM. Mitochondrial activity HUVEC on VEGF-immobilized h-gelatin was higher than that on gelatin. Furthermore, gel formation of h-gelatin by tissue transglutaminase could be observed. VEGF and HGF immobilization to D-ECM was higher than that to h-gelatin.

Conclusion. Growth factor-immobilized extracellular matrix has a potential for the application to tissue engineering field.

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Keywords. Growth factor, Extracellular matrix, HUVEC, Hepatocytes

43. THE SPANISH CELL THERAPY NETWORK ACTIVITIES: FROM BENCH TO BEDSIDE

Chair: Javier García-Sancho
Co-chair: José Mª Moraleda
Keynote speaker: Salvador Martínez
Organizer: Ana Sánchez García

Synopsis: The Spanish Cell Therapy Network (TERCEL) that combines the most outstanding groups on basic and clinical research that, under the financial support from the National Institute of Health, provides the scientific knowledge and the medical skills to make possible the translation of cellular therapy from the bench to the bedside.

In this symposium we will present a key note conference by Dr. Salvador Martinez that will introduce the subject and also will present his own work on neural regeneration using mononuclear bone marrow cells in a Phase I-II clinical trial for ALS patients that is at the present

recruiting patients at the Arrixaca University Hospital in Murcia, Spain.
Communications by other TERCEL members will provide the background of regenerative medicine applied to Cardiovascular, Neural, Osteo-articular, Autoimmune, and other diseases and also the GMP facilities and regulatory constraints to make possible the clinical translation.

(43.KP) BONE MARROW STEM CELLS: THERAPEUTIC MICRO-DEVICES FOR NEURODEGENERATIVE DISEASES

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In the last two decades up to 48 potentially therapeutic agents have been tested searching for therapeutic activity in ALS patients. Only Riluzol has demonstrated some activity when tested in clinical trials. Then, it seems necessary to explore different therapeutic approaches. We have been pioneers to demonstrate that cellular therapy using bone marrow mononuclear stem cells (BMCs) produce a neurotrophic effect in animal models of motoneuron degeneration and postulating this as a therapeutic option. In this study the grafted bone marrow cells migrated into the anterior horn of the spinal cord, forming cellular nests around the motoneurons soma and produced GDNF, an active growth factor that protect these neurons from death (Cabanes et al., 2003). A clinical trial based on these preclinical results was warranted. We proceeded to design a safety study as the first step to explore this neurotrophic therapy.Therefore we were especially interested to demonstrate similar cell-to-cell neurotrophic mechanisms in grafted human spinal cord, searching for motoneurons survival and location of bone marrow cells in “cellular nests” around motoneurons. Anticipating in our mechanistic hypothesis the cellular processes that would modify motoneurons microambient and favors their survival. This stimulated us to perform accurate pathology explorations. We will present data supporting the reproducibility of the cellular mechanisms operating in the mouse model in human ALS patients, suggesting the possibility of utilize bone marrow stem cells as therapeutic biological micro-devices to protect neurodegeneration by molecular and cellular neurotrophic mechanisms.

Work by the Instituto Carlos III (TERCEL RD06/10/0023 and FIS EC07/90762) the Fundación Diógenes and the Club Rotary-Illice.
Keywords. stem cells, Regenerative Medicine, neurodegenerative diseases, Terapia Celular

(43.O1) HUMAN MESENCHYMAL STROMAL CELLS FOR BONE FORMATION AND SPINE FUSION

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Mesenchymal stromal cells (MSC) can be isolated from the marrow cavity, display multipotency and have been shown to have the ability to form new bone when transplanted. When cultured with scaffolds they are capable of participating in bone formation. Bone autografts are commonly used in the repair of non-unions, tumors, osteonecrosis and in spinal fusion. However they are associated with morbidities related to the harvesting procedure and also show unpredictable repairing potential so new treatment approaches are needed. In order to induce and study bone formation in vivo we have developed an animal model using cultured human (h-) MSC and several biomaterials used as scaffolds and then implanted subcutaneously into immunocompromised mice.
To analyze whether cells could reside during several weeks into the scaffolds, they were transduced with a F-luc vector and bioluminescence analysis was performed weekly during 6 weeks. Then mice were sacrificed and histological analysis performed. Hydroxiapatite-TCP was the scaffold with the highest bioluminescence signal during the study period. In addition, h-MSC-derived cells were detected at the end of the 6 weeks and were positive for alkaline phosphatase showing osteoblastic differentiation. Also blood vessels could be seen within the implant.
Based on these features a clinical trial was designed in order to analyze safety and feasibility of autologous MSC expanded in vitro in GMP facility and with the hydroxiapatite-TCP carrier for patients undergoing spinal fusion. Cell dose is 0,5x106 to 1,5x106 CSM/kg of body weight, and preliminary data on both cell expansion and clinical outcome of enrolled patients will be presented.

Keywords. stem cells, regenerative medicine, terapia cellular

(43.O2) TREATMENT OF REPERFUSED ISCHEMIA WITH ADIPOSE-DERIVED STEM CELLS IN A PRECLINICAL MODEL OF MYOCARDIAL INFARCTION

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Introduction. To determine the long-term effect of the transplantation of adipose-derived stromal cells (ASC) y a preclinical model of ischemia/reperfusion (I/R). Adipose cells have demonstrated their usefulness in models of the disease, although never in a clinically-scalable one.

Materials and Methods. I/R was induced in 20 Goettingen minipigs pigs by 120 minutes coronary artery occlusion followed by reperfusion, and 9 days later, were allocated to receive trans-endocardial injection of 213.6±41.78 millions of GFP-expressing ASC or culture media as control. Heart function determined by echocardiography was performed together with histological studies 3 months after transplantation.

Results. Transplantation of ASC induced a statistically significant long-lasting (3 months) improvement in cardiac function and geometry, greater than the effect provoked by reperfusion alone. Functional improvement was associated with an increased revascularization degree (both capillaries and arteries/arterioles) and a positive heart remodeling with lower scar fibrosis and cardiac hypertrophy in animals treated with ASC. Although transplanted cells were not able to remain within the injured hearts, they induced a positive change

in the MMP/TIMP imbalance, strongly suggesting a paracrine mediated action of ASC.

Conclusion. Our results shown that ASC increase perfusion and reverse adverse remodeling in a clinically relevant model of I/R. ASC thus constitute an attractive candidate for the treatment of myocardial infarction.

Keywords. stem cells, Regenerative Medicine, Cardiac ischemia/reperfusion, Angiogenesis, Heart remodeling

(43.O3) ADIPOSE DERIVED STEM CELLS (ASC) FOR TREATMENT OF CROHN'S FISTULA

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Complex perianal fistulas are extremely difficult to manage due to the limitations of currently available treatments. The use of ASC therapy could represent an alternative treatment. We performed an initial phase I clinical trial of nine implants with autologous ASCs in five patients with Crohn's disease. Eight weeks after treatment, signs of repair were apparent and 75% of treated fistulas had closed and were considered healed. After these promising results a phase II clinical trial was designed to evaluate the safety and efficacy of this novel stem cell therapy. Forty-nine patients with complex perianal fistulas of cryptoglandular origin (n = 35) or associated with Crohn's disease (n = 14) were recruited and randomized for treatment with autologous ASCs (dose 20 – 60 million cells) in combination with fibrin glue or fibrin glue alone. The proportion of patients who achieved fistula healing was significantly higher with autologous ASCs than with fibrin glue (17 (71%) versus 4 (16%) respectively, risk ratio = 4.43 (95% confidence interval 1.74, 11.27); $p < 0.001$). A phase III multicenter clinical trial to evaluate the safety and efficacy of autologous ASCs to treat complex perianal fistulas not associated with Crohn´s disease is currently being completed. However, although the data of this phase III are not yet published the trial was complicated by the unexpected finding that the clinical outcomes of patients getting fibrin glue alone were better than anticipated, making it harder to show the effectiveness of autologous ASCs as demonstrated in phase II studies. In the near future we will know whether or not the treatment is effective. A further three clinical trials in phase I/II are ongoing to evaluate ASCs for the treatment of fistulas associated with Crohn`s disease: One with autologous ASCs, one with allogeneic ASCs and one with allogeneic ASCs to treat specifically recto-vaginal fistulas. Although the efficacy of ASCs in the treatment of fistulas has yet to be clarified; the results obtained in the treatment of nonhealing wounds with MSC therapies in experimental models continue to arouse great expectations in the clinical practice.

Keywords. stem cells, Regenerative Medicine, Terapia Celular

(43.O4) INDEPENDENT MEDICAL TRIALS ON REGENERATIVE MEDICINE SUPPORTED BY A BIOMEDICAL INSTITUTION

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In the last few years the ISCIII has provided funds to the Spanish Network for Regenerative Medicine to support the construction and validation of the platform of GMP facilities that provided the required scientific and regulatory expertise to translate of stem cell based therapeutics to the clinic. This measure has been very important after the 2003 European community law that considered expanded cells as drugs and to be produced under pharmacological regulations.

On those grounds, the unit in Valladolid pioneered as the first GMP facility for expanded bone marrow derived mesenchimal stem cells produced in Valladolid (MSV) for cardiac regeneration on the NOGA trial.This start was follow by the use of MSV on different osteo-articular pathologies on inter-vertebral disc incompetence and grade II gonartrosis. Consolidated protocols like engineered skin ad limbar stem cells expanded over amniotic membrane, that were successfully used by several clinic groups in Spain, were also validated in our GMP unit as pharmaceutical products an are used now days on several clinical trials.Preliminary results of those trials will be presented in the symposium.

Keywords. Regenerative Medicine, stem cells, terapia cellular

(43.P1) DEVELOPMENT OF PROTOCOLS REQUIRED FOR APPROVAL OF A LABORATORY OF CELL THERAPY

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Introduction. In the field of the biotechnology, there is included the use of the cellular therapy as a new biomedical science capable of offering new treatments to diseases or human dysfunctions that up to the moment do not have treatments or the existing ones are not effective. The cellular therapy is defined as the use of alive cells, mature cells, progenitors or mother cells in order to restore or improve the function of damaged organs as consequence of traumatic injuries, degenerative chronic diseases or tumour diseases. This study shows the validation protocols requiring the production of cell therapy products.

Material and Methods. In the translation of basic research to the clinic, attended by several factors: production rules, facilities, personnel and procedures. The owner of a marketing authorization for advanced therapy medicinal products should ensure a system. Its components can be examined during the process of procurement, manufacturing, packaging, storage, transport and delivery.

Results. 1. Production Processes: It is required that the part of those processes are properly validate. 2. Validation of aseptic processing. Demonstrate the

cleanliness of the environment, staff and material. 3. Analytical Methods: The analytical methods are beyond the scope of these requirements. 4. Cleaning Procedures: To check to ensure the absence of cross-contamination. 5. Environmental Control Systems: Means those environmental control systems to ensure that the environmental conditions of temperature, pressure differential and control of microbiological and particulate loads are adequate. 6. Machinery and Equipment: This section includes all the equipment and machinery that affect or operate directly in production processes or quality control.

Conclusion. Validation processes required for the manufacture of cellular medicine have been conducted in the unit cell production Cabimer (Centro Andaluz de Biologia Molecular y Medicina Regeneraativa), from obtaining results within the range of GMP.

Keywords. Cell, validation, good manufactures production, cell medicine

(43.P2) DEDIFFERENTIATED SMOOTH MUSCLE CELLS NEXT TO INTESTINAL NERVOUS TRUNCKS

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Introduction. The dedifferentiation of smooth muscle cells is a natural repair process that includes the elimination of contractile apparatus and nucleus activation to prepare the cell toward proliferation or redifferentiation. The dedifferentiation process causes the cell regress to an embryonic state. Smooth muscle cells (SMCs) and Interstitial Cells of Cajal (ICCs) in the small intestine emerge during embryonic period from Kit + mesenchymal precursor cells. Previous studies suggested that Kit + cells adjacent to myenteric neurons might decide to become IC-MY (Interstitial Cells-Myenteric plexus) because enteric neurons express stem cell factor, the ligand for Kit (Torihashi S. et al 1996, 1997). This process could be reversible in the presence of certain microenvironmental factors.

Materials and Methods. Human biopsies and different mammalian vertebrate specimens were analyzed by electron microscopy.

Results. In our results we have observed the dedifferentiation process characterized by an increase in the number of organelles next to the nucleus, a clear disorganization of the cytoskeleton, the appearance of vesicles that fuse together until the vesicle membrane breaks, and cytoplasmic fragments are detached from the SMC. This dedifferentiation process is often found next to nerve trunks.

Conclusions. We suggest dedifferentiation of smooth muscle cells as an homeostatic repair process characterized by the regression to an embryonic state mediated by the influence of signalling pathways from enteric neurons.

Acknowledgements. This research received financial support from Aragon Institute of Health Sciences (I+CS) (PIPAMER 001/11) and the European Social Fund (ESF), DGA (B83).

Keywords. Dedifferentiation; intestinal smooth muscle cell; Interstitial Cells of Cajal

(43.P3) DIABETIC ULCER HEALING IS STIMULATED BY OTR4120

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Introduction. Heparan sulfate (HS) regulates the bioavailability of HS-bound polypeptides and maintains the balance between tissue integrity and tissue disruption allowing the cellular tissue components to unfold their natural mechanism to achieve tissue homeostasis. In chronic wounds, HS is disrupted in highly proteolytic environment. OTR4120 is a heparan sulfate (HS) mimetic. It replaces the degraded HS and takes its role in restoring tissue homeostasis. Previously, we showed that the OTR4120 improved pressure ulcer healing in non-diabetic rats. This study describes the profound effects of OTR4120 on diabetic wound healing.

Materials and Methods. Experimental diabetes was induced by an intra-peritoneal injection of streptozotocin (STZ). Six weeks after STZ-diabetes induction, rats were subjected to pressure ulcer formation by external clamping a pair of magnet disks on the dorsal skin for a single ischemic period of 16 h. Immediately after magnet removal, rats received an intramuscular injection of OTR4120 weekly for up to one month.

Results. Compared to the untreated non-diabetic rats, ulcer healing was impaired in untreated diabetic rats. However, ulcers in OTR4120-treated diabetic rats healed significantly more rapid than wounds in untreated diabetic rats. OTR4120 treatment reduced inflammation, reduced matrix metalloproteinase expression and increased collagens synthesis. Furthermore, the increased ratio of collagen type III to I in diabetic ulcers was reversed to normal in OTR4120-treated ulcers. Also short and long-term restoration of ulcer biomechanical strength was significantly enhanced following OTR4120 administration.

Conclusion. OTR4120 treatment is beneficial to improve diabetic ulcer healing.

Acknowledgments. The authors thank Prof. D.W. van Bekkum (LUMC, Leiden, the Netherlands) for his assistance in the development of the pressure ulcer model, and Prof. D. Barritault (OTR3, SAS, Paris, France) for providing OTR4120. This research was supported by a grant from the Nuts Ohra Foundation (the Netherlands), Grant No. SNO-T-0-0501-159.

Keywords. diabetes, pressure ulcers, matrix, Heparan sulfate

44. THE USE OF MAGNETIC NANOPARTICLES FOR TAGGING, TRACKING AND ACTIVATION IN REGENERATIVE MEDICINE

Chair: Alicia El Haj
Co-chair: Gerjo Van Osch
Keynote speaker: E. Sykova
Organizer: Alicia El Haj

Synopsis: Magnetic based technologies have begun to be used in many aspects of regenerative medicine in a variety of applications. Cell labelling with magnetic particles can allow cells to be targeted, tracked and controlled in their behaviour and phenotype. Ultimately these strategies can lead to injectable solutions for regenerative medicine therapies. The challenges lie in the different clinical applications and the complexities of controlling magnetic field strengths and the profiles combined with labelling of cells with varying magnetic particles of differing sizes and magnetic core strengths. In this workshop, the applications where magnetic technologies are used will be presented and the differing strategies explored. The future for these technologies in reaching clinical use will be discussed.

(44.KP) NANOPARTICLES IN STEM CELL RESEARCH AND THERAPY

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Nanotechnology offers promising perspectives in biomedical research and clinical practice. To cover some of the latest nanotechnology trends in regenerative medicine, this review will focus on the use of nanomaterials for tissue engineering and cell therapy. Cell therapy is a modern approach in regenerative medicine for the treatment of various diseases or injuries. To follow the migration and fate of transplanted cells, superparamagnetic iron oxide (SPIO) nanoparticles have been developed for cell labeling and non-invasive MRI monitoring of cells in the living organism, with successful application in, e.g, cell therapy of CNS injury, tumors or infarcted myocardium, cartilage and liver transplantation and monitoring the survival or rejection of pancreatic islets in both laboratory animals and human patients. SPIO nanoparticles typically consist of a crystalline iron oxide core and a dextran or monolayer polymer shell. Coating with a protective layer helps to prevent the aggregation of the nanoparticles, induces their efficient internalization into the cell and minimizes any deleterious effects on cellular function. Mesenchymal stem cells (MSCs), olfactory ensheathing glia (OEGs) and neural progenitor cells (PNCs) have the capacity to migrate towards lesions and induce regeneration. Cells labeled with iron-oxide nanoparticles were transplanted into rats with a cortical lesion, a middle cerebral artery occlusion model of stroke, or an acute or chronic balloon-induced spinal cord compression lesion. In vivo MRI, used to track their migration and fate, proved that MSCs, OEGs as well as PNCs migrated into the lesion after either intravenous or intraspinal administration. All implanted animals had significantly smaller lesions and better scores on BBB (motor) and plantar (sensory) tests. Noninvasive MRI monitoring can thus help to optimize transplantation.

(44.O1) WORKING TOGETHER: THE COMBINED APPLICATION OF A MAGNETIC FIELD AND PENETRATIN FOR THE DELIVERY OF MAGNETIC NANOPARTICLES TO CELLS IN 3D

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Introduction. Novel therapeutic molecules, such as synthetic oligonucleotides for gene therapy, have the potential to cure many diseases including cystic fibrosis and cancer. The main problems associated with the direct injection of these molecules into a patient's body include enzymatic degradation, poor cellular uptake and lack of specificity. Magnetic nanoparticles (mNPs) present as a promising drug delivery vehicle to solve these problems because they protect against enzymatic degradation, and due to the attachment of functional biomolecules, can facilitate specific targeting and uptake into diseased cells. Furthermore, an externally applied magnetic field (MF) allows imaging and targeting of mNP within the body (ref 1). This project has developed an optimal mNP species for in vivo drug delivery that facilitates both targeting (via an externally applied MF) and cell uptake (via the attachment of the cell penetrating peptide, penetratin). This NP design was tested in 3D cell-seeded collagen gels, thereby providing a more realistic prediction of clinical performance than has been previously gained through traditional 2D studies.

Materials and Methods. The effect of a MF and/or penetratin attachment on the uptake of 100 and 200nm fluorescent mNPs into a fibroblast-seeded 3D collagen gel was quantified by inductively coupled plasma mass spectrometry (ICP-MS). The most suitable mNP species was further investigated by fluorescence microscopy, histology, confocal microscopy and TEM.

Results. Results show that gel mNP-uptake occurred on average twice as fast in the presence of a MF, and up to three times faster with penetratin attachment (Fig 1). In addition, a MF increased the distance of mNP travel through the gel, while penetratin increased mNP cell localisation.

Conclusion. This work is one of the first to demonstrate that a MF and CPPs can be effectively translated for use in 3D systems, and if applied together, will make excellent partners to achieve therapeutic drug delivery in vivo.

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Keywords. magnetic nanoparticles, 3D cell culture, drug delivery

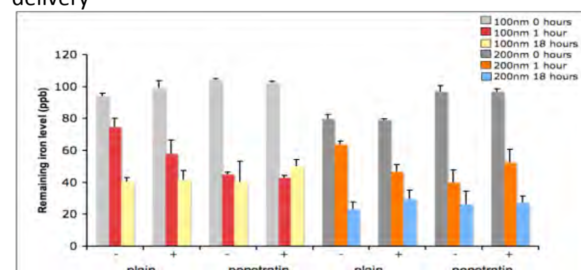

Figure 1. mNP uptake into 3D cell-seeded gels. 100nm mNPs are represented in the left hand side by light grey (0 hours), red (1 hour) and yellow (18 hours) bars. 200nm

mNPs are represented in the right hand side by dark grey (0 hours), orange (1 hour) and blue (18 hours) bars. On the x-axis, samples treated without or with a magnetic field are indicated by - or + respectively, and staggered x-axis labels "plain or penetratin" indicate samples without or with the attachment of penetratin respectively.

(44.O2) MAGNETIC NANOPARTICLE TECHNOLOGY FOR USE IN CONTROLLING DIFFERENTIATION OF EMBRYONIC STEM CELLS

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Mechanical force is known to be a key regulator controlling the proliferation and differentiation of stem cells. The transduction of mechanical signals often occurs through mechanosensitive membrane receptors that transduce physical forces into biochemical signals that then activate second messengers and subsequent downstream signalling events. The Wnt signalling pathway is known to play an important part in the differentiation of many cell types and the development of many tissues including bone. Wnt signals are transduced through a number of cell surface receptors and co-receptors, one of which is the Frizzled receptor.

In this preliminary study we demonstrate the use of magnetic nanoparticles to activate the frizzled receptor in order to promote the osteogenic differentiation of human embryonic stem cells (hES). Cultures of the hES cell line SHEF-1 were labelled with 250nm magnetic nanoparticles coated with Anti-Frizzled (Fz) antibodies. Cultures were stimulated for 9 hours over 20 days in an oscillating (vertical) magnetic force bioreactor (MICA Biosystems) to create a compressive force on the cell membranes. Alizarin red staining and semi-quantification showed significantly more extracellular calcium deposition in stimulated cultures in osteogenic media with nanoparticles compared to un-stimulated cells in osteogenic media. Interestingly no effect was observed in cells cultured in spontaneous differentiation media. This study provides the first evidence of mechanotransduction through the Frizzled receptor. Furthermore it appears that a primary biochemical osteogenic cue is required before osteogenic differentiation can be accelerated by mechanical stimulation through the frizzled receptor. This preliminary study has demonstrated that using this technique, it is possible to enhance the osteogenic differentiation of stem cell cultures.

Keywords. Nanoparticles, mechanotransduction, wnt signalling

(44.O3) IN VIVO TRACKING OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLE-LABELED CD133+ STEM CELL TROPISM TO DYSTROPHIC MUSCLE TISSUES USING MICRO-CT IMAGING

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Introduction. Cell therapy is an emerging approach of regenerative medicine with significant efforts in clinical areas. Stem cells cannot be easily observed directly when injected systemically, and their behaviors need to be visualized indirectly. Micro-CT is a non invasive technique that exploits the attenuation of X-rays and offers the possibility to obtain a 3D visualization of the in vivo distribution of systemically injected stem cells. We focused on muscular dystrophy, in order to discover the mechanisms involved in muscle homing of stem cells.

Materials and Methods. FeO-nanoparticles labeled blood derived human CD133+ cells were injected into the femoral artery of dystrophic animal models and detected in vivo in muscle tissues of injected limb at different times by using micro-CT with high spatial resolution. Real-Time PCR analysis was performed to obtain a quantification of cells migrated from blood stream inside muscle tissues and organs.

Results. Immediately after the injection cells were concentrated in the injected quadriceps, while after 2 hours they reached the ischio-crural muscles in the posterior part; at 24 hours injected stem cells were also present in gastrocnemius; cells number increased 24 hours after the injection, indicating a progressive distribution and migration of cells. Within 2 hours after the injection, QPCR analysis confirmed micro-CT data, showing a large amount of cells in QA, ischio-crural and GAS. Intra-arterially injected cells were also found in filter organs.

Conclusions. Intra-arterially injected cells continue to migrate within the muscles of the injected limbs with a specific spatiotemporal distribution. We tried to explain these data in several different ways. Firstly, intra-arterially injected stem cells clustered within capillaries of muscles nearly the site of injection and migrate subsequently in other muscles after spontaneous clusters dissolution. Secondly, injected CD133 cells represent an heterogenous population of stem cells with different capacity of muscle tissue homing.

(44.O4) HIGHLY EFFICIENT MAGNETIC STEM CELL LABELING WITH NEW SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES FOR IN VIVO TRACKING BY MRI

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Introduction. Tracking of transplanted cells is essential to monitor safety and efficiency of cell-based therapies. Magnetic resonance imaging (MRI) offers very sensitive,

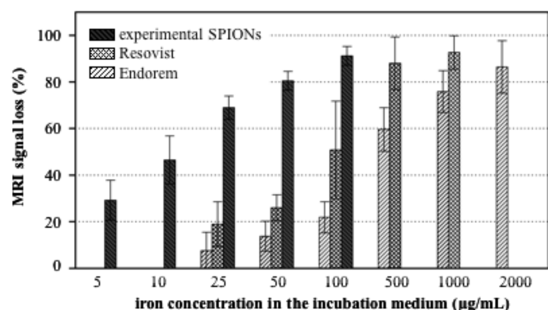
repetitive and non-invasive in vivo detection of magnetically labeled cells. However, magnetic stem cell labeling with clinically approved superparamagnetic iron oxide nanoparticles (SPIONs) is still problematic because of low labeling efficiencies and the need of transfection agents. The objective of this study is to investigate magnetic labeling of human mesenchymal stem cells (MSC) with new experimental SPIONs with regard to labeling efficiency, effects on stem cell properties and in vivo tracking by MRI.

Materials and Methods. Human MSC were labeled dose-dependently with commercially available SPIONs Endorem and Resovist and with new experimental anionic SPIONs. Labeled cells were investigated by ICP-OES, Prussian Blue staining, TEM and 7T-MRI. Proliferation, multilineage differentiation and chemotaxis assays and flow cytometry were conducted to determine detrimental effects of magnetic stem cell labeling. Labeled cells were tracked in vivo by 7T-MRI following intramuscular injection in rats.

Results. Very efficient labeling of human MSC without transfection agents was only achieved with the experimental SPIONs. Intracellular iron content per cell and MRI signal loss was increased significantly for cells labeled with these SPIONs compared to Endorem and Resovist (see Fig.). Intracellular SPIONs were visualized with Prussian Blue staining and TEM. Labeling of human MSC with SPIONs did not affect cell proliferation, differentiation into the adipogenic and osteogenic lineage and exposition of typical cell surface antigens (CD44+, CD73+, CD105+, CD166+, CD14-, CD34-, CD45-). However, chondrogenic differentiation and cell migration were significantly impaired with increasing SPION incorporation. The SPION-labeled MSC were detectable in vivo by 7T-MRI for several weeks.

Conclusions. Though careful titration of SPION incorporation and MRI detection is essential, the new SPIONs are a promising tool for efficient mesenchymal stem cell labeling and in vivo tracking.

Keywords. magnetic stem cell labeling, in vivo cell tracking, superparamagnetic iron oxide nanoparticles, tissue engineering



(44.O5) INVESTIGATING VARIOUS MRI CONTRAST AGENTS AT DIFFERENT CONCENTRATIONS FOR THE PURPOSE OF TAGGING AND IMAGING OF MESENCHYMAL STEM CELLS AND CHONDROCYTES FOR ACI

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Introduction. Autologous Chondrocyte Implantation (ACI) is a realistic alternative to current osteoarthritic therapies, a disease that affects some six million people in the UK. ACI involves the isolation of chondrocytes from the patients' knee, expanded and then re-implanted into the defected area. However, further optimisation of this technique is required. Having a practical means of visualising and monitoring the implanted cells in vivo can provide us with the ability to quantify and track cells thus aiding the enhancement of the therapy. In this report, we describe our techniques to optimise the use of magnetic nanoparticles for MRI imaging in ACI.

Materials and Methods. Methodology includes mesenchymal stem cells (MSC) and chondrocytes being cultured and passively loaded with MRI contrast agents ranging in size and dosage. The magnetic nano-particles (MNP) used were superparamagnetic nano particles (SPIONS) ranging in size between 50-1000 nm in diameter. Contrast-agent-loaded-cell populations were then implanted into cadaveric porcine knees and visualised by MRI (Magnetic Resonance Imaging).

Results. Both MSC and chondrocytes were found to uptake MNP at variable efficiencies depending on particle size and dosage in culture. A relationship was established between the MNP size and loading for the optimal in vivo visualisation using MRI.

Conclusion. Our results demonstrate the potential for magnetic nanoparticles to be used in clinical trials for ACI.

Keywords. ACI, magnetic nanoparticles, MRI

(44.P1) CHARACTERIZATION OF MAGNETIC IRON NANOPARTICLES COATED WITH POLY(ETHYLENEGLYCOL) (PEG) AS AN ANTIENEOPLASIC DRUG CARRIER

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Abstract. During the last years, there has been increasing research interest in therapeutic nanoparticles due to a large number of beneficial properties and potential applications of nano-meter sized materials [1,2]. This field of investigation allows improving properties of magnetic nanoparticles, especially in their drug loading and delivery capabilities. In the present work, we describe how superparamagnetic iron nanoparticles can be used as nuclei for the engineering of composite particles aim with an external poly(ethyleneglycol) (PEG) coating, and are capable of loading a number of pharmaceutical drugs. Iron nanoparticles were synthesized according to Huiping Shao's thermal decomposition method [3]. After the iron nanoparticles synthesis, we made the characterization of their size, magnetic properties, cristallinity, hydrophobicity, surface charge and composition. Finally, the designed nanostructures were used to incorporate an antineoplasic drug, 5-fluorouracil, on the surface of the nanoparticles, after they were coated with two different molecular weight of PEG (2000 and 35000). In both cases absorbance spectra and electrokinetics demonstrated that the coating was produced. Absorbance in UV-VIS range was used to assess the drug adsorption on the nanoparticles surfaces. Preliminary in vitro tests in peripheral blood cells show that nanoparticles can be introduced into them. Results are presented for both types of PEG loading for a specific drug concentration.

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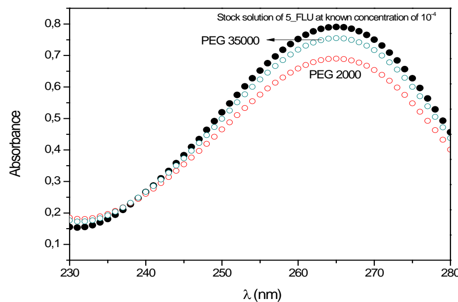
Acknowledgments. Financial support from Junta de Andalucia, Spain (Project PE-2008-FQM-3993)
Keywords. magnetic nanoparticles, antineoplasic, drug carrier, poly(ethyleneglycol)



(44.P2) MECHANORECEPTOR ACTIVATION VIA MAGNETIC PARTICLE REGULATES DIFFERENTIATION OF HUMAN BONE MARROW STROMAL CELLS

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Introduction. Previous studies in our group have shown that remote activation of magnetic particle tagged specific mechanosensitive receptors can induce adult human mesenchymal stem cells to differentiate down the osteogenic lineage (1). However, the inter-relationship between chemical and mechanical signalling to promote differentiation is not clear (2,3). In this report, we test the hypothesis that human bone marrow derived MSCs require different stimuli at different stages of the osteogenic differentiation pathway to promote maximal response. Our aim is to define and characterise the protocols which are required for controlling stem cell behaviour in vitro and in vivo for tissue engineering.

Materials and Methods. hMSCs were treated with (Group T1) or without (Group T2) 2 days static osteogenic induction using dexamethasone, ascorbic acid and β-glycerophosphate, followed by magnetic conditioning using the Magnetic Force Bioreactor (MICA Biosystems) 1 hour per day targeting the mechnoresponsive receptor - integrin B1. After 7 days culture, cells were harvested and the relative mRNA expression of RUNX2, Collagen I, ALK and BGLAP were measured by qRT-PCR.

Results. The levels of RUNX2, Collagen type I and BGLAP expression were upregulated in the group (T1) with 2 days static osteogenic chemical induction compared to the group without static chemical preconditioning (T2). In contrast, the levels of ALK gene expression were lower in T1 with preconditioning compared to T2, the group without previous static osteogenic induction.

Conclusions. These results demonstrate that cells at various stages of differentiation have different sensitivities to mechanical conditioning. In the case of the markers for early differentiation, expression is enhanced with the preconditioning using well defined chemical induction followed by mechanical stimulation. Our results demonstrate the role of magnetic mechano-conditioning in defining stem cell differentiation pathways and optimising the temporal induction towards terminal osteogenic utilising combinations of chemical and mechanical conditoning.

Reference: 1. Kanczler JM, Sura HS, Magnay J, Green D, Oreffo RD, Dobson, JP, El Haj AJ. 2010. Controlled differentiation of human bone marrow stromal cells using magnetic nanoparticle technology. Tissue Engineering Part A. 16(10), 3241-50. 2. Thomas GP, El Haj AJ. 1996. Bone marrow stromal cells are load responsive in vitro. Calcified Tissue International 58, 101-108. 3. Mouw JK, ConnellyJT, Wilson CG, Michael KE, Levenston ME. 2007. Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells. Stem Cells. 25;655-663.
Keywords. mechanical conditioning, human BMSC, magnetic nanoparticles.

45. TISSUE ENGINEERING IN UROLOGY

Chair: Ricardo Fernández-Valadés
Co-chair: Ingrid Garzón
Organizers: Ricardo Fernández-Valadés, Ingrid Garzón
Synopsis: Several tissues and organs of the urinary system can be generated by tissue engineering, and some promising results have been obtained both in vitro and in vivo.
All works focused on the development and analysis of bioengineered urinary system tissues and the usefulness of stem cells and cell therapy in urology are welcome in this symposium, including, among others:
- Urinary system cells culture.
- Biomaterials in urology.
- Generation of artificial urethra, bladder, ureter, kidney.
- Metabolic and functional tests.
- In vitro and in vivo analyses.
- Clinical trials in urology.

(45.O1) MATURATION AND DIFFERENTIATION OF BLADDER SMOOTH MUSCLE CELLS AND MYOBLASTS CULTURED ON NOVEL PLLA-COLLAGEN SCAFFOLDS

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For tissue engineering applications that require a smooth muscle layer, e.g. bladder or abdominal wall skeletal muscle, scaffold designs are often inadequate for their intended application. In this study, biodegradable collagen-PLLA composite textiles coated with novel collagen formulations were analysed in vitro to assess the ability of smooth muscle cells (SMCs) and myoblasts to mature with natural phenotypic expression. Experimental meshes were 1-2mm thick, 15mm diameter disc-shaped 3-D composite sponges with interconnected pores comprising PLLA textile combined with different formulations of cross-linked porcine collagen type I (Fig. a). SMCs were extracted from fresh porcine bladder and seeded on scaffolds for 1, 7 and 21 days and cell

proliferation, viability and their expression of -SMA protein was determined. To assess the functional capacity if the scaffold to support skeletal muscle cell differentiation, myoblasts were differentiated in 2% horse serum for 1 and 7 days and then analysed by real time RT-PCR for MyoD, myogenin and myogenic regulatory factor 4 (MRF4). Expression of the myogenic differentiation protein of the myosin heavy chain (MHC) was then determined using immunohistochemistry.
The PLLA-collagen scaffolds supported SMC growth, scaffold C with a low GTA was shown to have the greatest cell proliferation after 21 days (Fig. b). The maintenance of the SMC phenotype for cell direct contact with scaffolds was confirmed at day 7 and 21 (-SMA). These results indicated that the scaffold is not only able to support cell growth but also maintain cell phenotype. The myogenic differentiation study showed that scaffold C also induced a significantly higher expression of the myogenic markers myogenin and MRF4 as well as the protein of MHS indicating that they supported myogenic differentiation (Fig. c). A PLLA textile meshes that has been coated with oxidized collagen are suitable for smooth and skeletal muscle tissue engineering.
Keywords. PLLA; Muscle tissue engineering; collagen

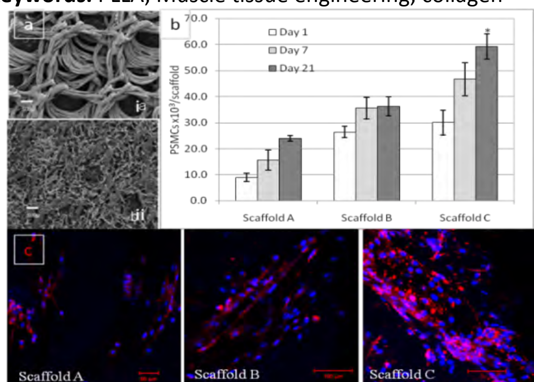

Fig. a. SEM of PLLA knitted mesh with coated collagen (i) mesh with the collagen sponge (ii); b. PSMCs proliferated on scaffolds analysed by CyQuant cell proliferation; c. immunohistochemical expression of MHC protein.

(45.O2) MINCED BLADDER MUCOSA FOR GENERATION OF A TUBULAR CONDUIT TO THE BLADDER

Reinfeldt G (1), Fossum M (1)
1. Karolinska Institutet

Introduction. In Reconstructive Urology, an abdominal stoma is sometimes needed for bladder emptying. Intestinal tissue is often used for these purposes. Drawbacks such as mucus- and stone formation, infections, disturbances in salt- and water balance and shortage of intestinal tissue can be limiting factors. We have previously shown that autologous, minced bladder mucosa can be transplanted for creating epithelialized tubular tunnels in an in vivo pig model. The aim of present study was to create conduits for bladder emptying.
Materials and Methods. Bladder mucosa was harvested by removing 1/5 of the bladder by open surgery in four female Yorkshire pigs. The mucosa was minced to small pieces of approximately 0,125 mm3 and attached to 10 cm long 22 Fr silicone-latex tubes with fibrin glue for a 1:3 expansion. In the same intervention, tubes were placed with one end into the bladder and the other end beneath the lower abdominal skin. After 4 weeks, tubes were removed and CT-scans were performed before harvest of conduit for microscopic evaluation. Tubes transplanted without minced mucosa served as controls.
Results. 3D-reconstructions of CT-scans identified bladders with normal configuration with conduits from the bladder to the skin. An 8 Fr catheter was introduced through the conduit to empty the bladder. Histological studies and immunoassay showed that transplanted epithelium had expanded (from 33% to 80% circumference) and reorganized to a continuous transitional urothelium facing the lumen. No epithelium was found in controls.
Conclusion. The study supports a mincing technique for in vivo expansion of autologous bladder mucosa around a three-dimensional mold. The technique could be used for tissue regeneration of tubular conduits for reconstruction of the urethra or for cutaneous stomas for bladder catheterization. In a human setting the method would be easy to perform, does not require laparotomy nor in vitro cell culturing for tissue expansion.
Keywords. guided tissue engineering, tubular conduit, in vivo, urothelium, animal model

(45.O3) ESTABLISHMENT OF A HUMAN IN VITRO MODEL OF THE RENAL PROXIMAL TUBULE

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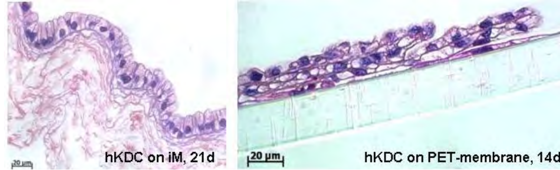
Introduction. The kidney is the key organ in drug excretion. The most decisive segment is the proximal tubule (PT) where cells transport high amounts of a wide variety of substances and can also alter them enzymatically. Transport and metabolism influence drug bioavailability and can lead to nephrotoxicity but no validated in vitro model of the PT exists.
In cooperation between the Fraunhofer IGB and ATRM an in vitro model of the PT was established which is based on human kidney-derived progenitor cells cultured on acellular natural matrices derived from porcine small intestinal submucosa.
Materials and Methods. Human kidney-derived cells (hKDC) were cultured on intestinal matrices (iM) for 21 days and compared to hKDC on PET membrane inserts, the standard model for transport studies. Tissue samples were analysed histologically, immunohistologically, by lectin staining and scanning electron microscopy. Albumin uptake was also investigated. The co-culture with human microvascular endothelial cells was integrated to further promote hKDC differentiation and simulate the natural barrier of peritubular capillaries.
Results. hKDC seeded on iM form a monolayer of cubic to high-prismatic cells, the characteristic morphology of PT cells. In contrast, hKDC cultured on inserts show a flat morphology, multilayer formation and agglomerates peeling off leading to a discontinuous cell layer. This is unsuitable for transport studies and was not observed on

the iM culture. Renal proximal markers (N-Cadherin, Aquaporin-1) could be detected throughout the hKDC culture on iM whereas markers of distal tubule cells (E-Cadherin, Aquaporin-2) were only very rare. Lectin staining confirmed this distribution. Basal membrane and microvilli formation proved strong polarization of hKDC on the iM. Functionality was shown by albumin uptake. Furthermore, the co-culture with endothelial cells was successfully established.

Conclusions. hKDC on iM could be an excellent model of the renal PT. Further experiments will focus on transport capacities and sensitivity to nephrotoxic substances.

Keywords. renal proximal tubule, in vitro model, decellularized matrix, kidney



(45.O4) TWO-LAYERED TISSUE-ENGINEERED URETHRA USING ORAL EPITHELIAL AND MUSCLE-DERIVED CELLS

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Introduction. As a congenital anomaly, hypospadia is one of the widespread disease in children. To cure the disease, urethral reconstruction has been challenged using many methods and approaches. We tried to make a novel tissue-engineered urethra autologeouslly reconstructed by oral mucosal epithelial-cell and muscle-derived cell (MDC) layer.

Materials and Methods. A small piece of oral tissue was harvested from the male beagle dog by punch biopsy and divided to mucosal and muscle part. Continuously, the divided parts were isolated to the mucosal epithelial cells and MDCs. Oral epithelial cells were cultured to the thin epithelial-cell sheets as a urethral mucosal layer with positive expression of Pancytokeratin. MDCs were seeded to the collagen gel that held an orientation with textile construction, which was migrated to the MDC textile sheet as the urethral muscle layer with positive expression of Desmin. After cultured for two weeks, the individual layers were attached to reconstruct the two-layered tissue-engineered (TE) urethra. Urethroplasty was performed with autologous tissue-engineered graft in the urethral removal for dogs as the experimental group and without graft as the control group.

Results. After seven days implantation, the viable cells in TE urethras were demonstrated by labeling with red and green colored PKH in the removed grafts. The graft survival rate in the experimental group significantly prolonged without complication of fistula compared with the control group. Furthermore, urethral fistulas arose in all dogs in the control group within seven days after operation. In addition, urethrography at seven days after implantation revealed no stricture in all dogs in the experimental group.

Conclusions. Two-layered TE urethras were established with minimally invasive approach performed by oral punch biopsy. This technique could accelerate to regenerate for the replacement model for urethra.

Acknowledgement. The textile sheets of collagen with orientation were developed and kindly provided by Atree, Inc., Nara, Japan.

Keywords. Two-layered tissue-engineered urethras, epithelial cell sheet, textile sheet of collagen with orientation

(45.O5) ELECTROSPUN SCAFFOLDS IN POLY(E-CAPROLACTONE) AND POLY(HYDROXYALCANOATE) AS SUPPORT FOR BLADDER AUGMENTATION CYSTOPLASTY: PRELIMINARY IN VIVO

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Introduction. Invasive bladder cancers and neurogenic bladders often need urinary diversions or augmentation cystoplasty, with the intestine as the best option. Unfortunately, its use is affected by many surgical and medical complications. We aimed at evaluating the feasibility of in vivo augmentation cystoplasty with fibrous scaffolds made of poly(ε-caprolactone) (PCL) blended with poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV).

Materials and Methods. Polymeric solution of PCL and PHBV (50:50) was electrospun at constant feed rate of 0.5 ml/h by applying a voltage of 15 kV (needle-to-collector distance 10 cm). Female Wistar rats underwent a longitudinal bladder incision and the scaffold was anastomosed to the native bladder to allow an augmentation equal to 50% of the original volume. 3 groups of 10 rats each one underwent surgery. One group was euthanized at 15 and one at 30 days, respectively. The third group underwent sham operation. The bladder was taken out, fixed and included. H&E, and IHC for smooth muscle actin, desmin, pan-Cytokeratin (AE1/AE3) and CD35 were performed.

Results. Electrospun scaffold was comprised of polymeric fibers free of beads, the average diameter and the 2D void size were 2.9±0.5 µm and 8±3 µm, respectively. 15 days after surgery both native urothelium and smooth muscle grew harmonically from the anastomotic lines on the external surface of the prosthesis: the hyperplastic urothelium was the internal layer and the smooth muscle was external. 30 days after surgery the implants were covered by the urothelium for the 90% of their surface with the smooth muscle tending to form myofibrils and to grow into the scaffold.

Conclusions. These are promising results to develop tissue-engineered urinary diversions made of PCL and PHBV for the management of bladder cancers and neurogenic bladder refractory to all conservative treatment. Further mid- and long-term in vivo experiments, mechanical and physiological tests are ongoing in our department.

Keywords. Bladder augmentation; neurogenic bladder

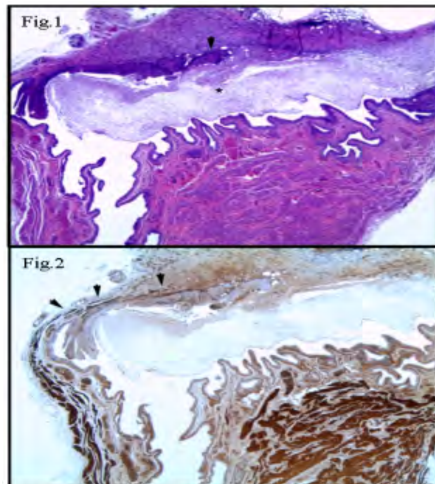

Fig. 1 — Rat bladder 30 days from surgery. A moderately hyperplastic urothelium covers the 50% of the support (arrow head). The partially re-adsorbed scaffold is dislocated in the lumen (asterisk). A foreign body reaction is evident in the bladder wall (H&E, 25x).
Fig. 2 — Smooth muscle fibres grew under the hyperplastic urothelium (arrow head) (anti-desmin, 25x).

(45.P1) DYNAMIC PERFUSION FOR INTACT-BLADDER DECELLULARIZATION: ANALYSIS OF THE ACELLULAR BLADDER MATRIX DISTENTION PROPERTIES

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Introduction. The development of a dynamic perfusion system for full-thickness intact-bladder decellularization is presented, enabling the organ mechanical distention to facilitate cell removal from the extracellular matrix (ECM). The volume-pressure relationship (cystometry) was experimentally measured previous and after the decellularization to investigate any alteration in the functional and biomechanical properties of the ECM.

Materials and Methods. Dynamic Decellularization Protocol: in the perfusion system, cell removal from whole bladder harvested from male rabbits was obtained using a combination of physical and chemical treatments. Bladders were consecutively deep immersed and intraluminally infused with three different detergents under gentle shaking [1]. Two pinch valves are automatically and alternately opened/closed replicating the natural cyclic filling/voiding dynamics (Fig. 1A). The switch of the valves is managed as a function of the volume infused by a peristaltic pump. During the voiding, the pump recirculates the fluid avoiding any manual operation during the whole process. Measurement of Compliance: a custom-made system (Fig.1B) was developed for cystometry measurement, equipped with a pressure transducer for the intravescical pressure recordings. Bladder is filled at a constant flow rate through a peristaltic pump up to a maximum internal volume, determined experimentally to not overstretch the bladder wall and compromise the integrity and the biomechanical properties of the ECM. The intravescical pressure was acquired during bladder filling via I/O hardware and a dedicated software interface.

Results. The efficacy of the developed dynamic protocol is under investigation in terms of cell removal and biomechanical properties of the acellular ECM (Fig.1C,D). This study represents a first step to the attainment of a naturally-derived scaffold retaining in vivo-like biochemical composition, tissue ultrastructure, and biomechanical behavior for bladder regeneration.

References. [1] Gilbert TW et al. Biomaterials; 27(2006):3675–3683

Acknowldgements. This work is supported by the Italian Ministry of Education, University and Research grant PRIN-2008YZNAHR.

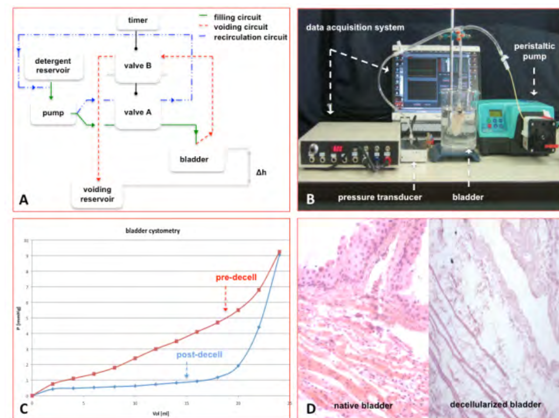

Fig.1: (A) schematics of the hydraulic circuit of the automatic dynamic perfusion system for the whole intact-bladder decellularization; (B) picture of the assembled device for cystometry measurement via pressure transducer; (C) bladder cistometry curves showing alteration of the relationship between intravescical volume and pressure during filling of the isolated bladder before (red) and after (blue) the decellularization process: up to a 20 ml volume value, while in the native bladder a linear pressure drop is accompanied to a volume increase, in the decellularized tissue a slight pressure drop is associated with a volume increase; over the 20 ml value, an increase in the pressure curve slope was observed for the acellular matrix, indicating decreased distension property with respect to the native bladder; (D) histological specimens of native bladder (left) and decellularized bladder tissue (right): in the native bladder, hematoxylin and eosin and Masson's trichrome staining indicate the intact epithelium, smooth muscle bundles, cellular nuclei, and collagen content in the bladder wall; in the decellularized tissue the complete elimination of the cellular nuclei is evident with the epithelium and the porous ECM of the muscle bundles, composed mainly of collagen fibers, preserved although partially degraded

(45.P2) INFLUENCE OF MESENCHYMAL STEM CELLS ON ENHANCED CYTOKINES EXPRESSION IN THE TISSUE ENGINEERED BLADDER WALL

Pokrywczynska M (1), Jundzill A (1), Bodnar M (2), Adamowicz J (1), Marszałek A (2), Drewa T (1), Olkowska J (1)
1. Department of Tissue Engineering, Chair of Medical Biology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun; 2. Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun

Introduction. The idea of constructing the tissue engineered bladder wall using stem cells obtained from non urinary tract components is obligatory in case of bladder cancer. Mesenchymal stem cells (MSCs) possess anti-inflammatory properties and participate in tissue repair. We used MSCs to support bladder wall regeneration. The aim of this study was to evaluate the role of cytokines expression in the rat urinary bladder wall remodeling.

Materials and Methods. Femoral bones and urinary bladders were harvested from 10 male rats. MSCs cultures from the bone marrow were established and acellular matrices from the lamina propria of the bladder submucosa (BAM, Bladder Acellular Matrix) were prepared. MSCs were seeded on BAM in density of 1x106 cells/cm2 and cultured for 5 days. Transplantation procedures were performed on 20 female rats divided into four equal groups. In the 1st and 2nd group of animals the bladders were reconstructed using MSCs seeded bladder acellular matrix (BAM) and unseeded BAM respectively. In the 3rd group of animals MSCs were injected into the bladder wall. Animals of the 4th group did not undergo any transplantation procedures. All the animals were sacrificed after 3 months of observation. Bladder samples were embedded for histological and immunohistochemical evaluation (IL-2, IL-4, IL-10, IFN-γ, TNF-α, TGF-β).

Results. Reconstructed bladders in the 1st group revealed proper function. In the 2nd group implant shrinkage and disfiguration of the bladder was observed. Angiogenesis, innervations and muscles regeneration were significantly higher in bladders grafted with MSCs seeded BAM. Different pattern of the cytokines expression in response to applied type of intervention was observed.

Conclusions. Regeneration of the bladder tissue by MSCs depends on MSC-stimulated cytokines which enhance the regeneration mechanism. These findings may improve understanding of the role of MSCs in bladder wall regeneration process.

(45.P3) HISTOLOGICAL STUDY OF ORAL MUCOSA GRAFTS USED FOR URETHRAL RECONSTRUCTION

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1. Division of Pediatric Surgery, University Hospital Virgen de las Nieves, Granada, Spain; 2. Tissue Engineering Group, Department of Histology, University of Granada, Spain

Introduction. The urethra is an organ that may be affected by multiple congenital or acquired diseases. In most of these cases, it is necessary to replace part of the urethra in order to restore its normal function using different tissue grafts (skin, oral mucosa, bladder, etc.). However, the structural and molecular processes that occur in these tissues after implantation are not clearly understood. The objective of this work is to determine the histological changes that may occur in the oral mucosa grafts after implantation in the urethra.

Methods. 26 male Wistar rats were included in this study. First, a 0.5-cm longitudinal incision was performed on the urethra of each animal, and an autologous oral mucosa graft was implanted in the urethra at this level. Then, animals were euthanatized after 10 to 120 days and grafted tissues were analyzed using light and electron microscopy and immunohistochemistry for epithelial differentiation markers (pancytoquertin, CK1, CK4, CK13, filaggrin).

Results. Our results showed that the oral mucosa grafts exposed to urinary environment, tended to change from an oral-like stratified epithelium, to a urethral-like pseudostratified epithelium. The implanted graft progressively lose the oral mucosa markers (CK1 and filaggrin very positive) and tended to behave like the native urethral mucosa (CK1 and filaggrin negative expression). The adaptation of the graft to the recipient area is high and the integration is complete after the following-up period.

Conclusions. These results suggest that the oral mucosa grafts tended to integrate in the recipient urethra by a process of transdifferentiation, and implies that these tissues could have clinical usefulness.

Supported by grant FIS PI07-619 by Instituto de Salud Carlos III, Ministry of Science and Innovation, Spain.
Keywords: Oral mucosa grafts, Urethra reconstruction, Hypospadias repair

(45.P4) MICROSCOPIC ANALYSIS OF A BIOENGINEERED MODEL OF THE HUMAN BLADDER WALL BASED ON FIBRIN-AGAROSE

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Introduction. In the urinary tract exist several diseases that often require tissue replacement. Throughout history urologists have used a wide variety of materials to replace it but always dependent on the availability of normal tissue for grafting. In this work, we have generated a novel model of bioengineered bladder wall using stromal and epithelial cells isolated from the human bladder and fibrin-agarose biomaterials.

Materials and Methods. Urothelial cells, whose cultures were generated by enzymatic treatment of normal human bladder biopsies, were seeded on top of the fibrin-agarose stromal substitutes. This substitute of the bladder stroma was constructed using fibrin-agarose scaffolds with fibroblasts immersed within. We analyzed this artificial structure using transmission electron microscopy.

Results. In urothelial cells of the artificial mucosa, we observed microtubule structures that reflected the existence of a well-developed cytoskeleton. At the level of the cell membrane, immature junctions were also recognized. Fibroblastic cells inside the fibrin-based stromal gel, with high fiber and dense material, showed an abundant vacuolar and reticular system and the

presence of nucleolus inside the nucleus as a signal of their activity status.

Conclusions. The final result after microscopic evaluation model, using optical microscopy and transmission electron microscopy, revealed that the use of urothelial cells and fibroblasts obtained from biopsies, and the use of a fibrin-agarose stromal substitutes, allowed the efficient development of an artificial mucosa consisting of a well differentiated epithelium and stroma with a structure similar to the natural urinary mucosa.

Supported by FIS PI070331 by the Spanish Instituto de Salud Carlos III.

46. TISSUE ENGINEERING OF SKIN: FROM BASIC RESEARCH TO NOVEL THERAPIES

Chair: Ernst Reichmann
Co-chair: Esther Middelkoop
Keynote speaker: Ernst Reichmann
Organizer: Tissue Biology Research Unit

Synopsis: *Overview.* Large full-thickness skin defects resulting from burns, soft tissue trauma, congenital giant nevi, and disease leading to skin necrosis, represent a major unsolved clinical problem. Despite the fact that engineered skin substitutes and their application on human patients have become a reality, scientists and surgeons are still struggling to develop an optimal therapeutic approach that would replace today's gold standards. This symposium will focus on the state of the art of tissue engineering of human skin. It will consider the current knowledge, and discuss the problems and gaps, and how these may be overcome to hasten the translation from basic science to clinical application.

Purpose. Questions that will be considered:

1. What are the clinical needs that may direct new approaches in skin tissue engineering and regenerative medicine?
2. What are the main challenges for translating basic science to novel clinical applications?
3. What are the limits of the current gold standards?
4. Advantages and disadvantages of “off-the-shelf products”?

Who should attend? Everybody who is interested in the development of novel skin substitutes, such as plastic surgeons, burn surgeons, dermatologists, and of course all scientists that are interested to translate basic research into clinical application..

(46.KP) TISSUE ENGINEERING OF SKIN: FROM BASIC RESEARCH TO NOVEL THERAPIES

Reichmann E (1)

1. TBRU, University Childrens Hospital, Zürich, Switzerland

Large full-thickness skin defects resulting from burns, soft tissue trauma, congenital giant nevi, tumor resection, and disease leading to skin necrosis, represent a common and significant clinical problem that is far from being solved. The main challenges encountered are the following two:

First, there is donor site shortage for autologous skin transplantation when the defect exceeds 50–60% of the total body surface area. The typical clinical example is a massive deep burn.

Second, most conventional skin grafting techniques to provide autologous coverage are based on transplanting split-thickness skin (the today's gold standard). Split-thickness skin contains all of the epidermis but only part of the dermis, and that frequently leads to scarring. Rarely, scarring is mild and irrelevant. Often, particularly in children, there is hypertrophic scarring or keloid formation that is frequently disabling and disfiguring.

There are still two major challenges concerning the development of novel skin substitutes:

1) On its way to an optimized and long lasting structure and function, a dermo-epidermal substitute has to be efficiently and appropriately vascularized. Attempts to reach this goal have entered a period of significant progress; however, a final breakthrough is still missing.

2) Much is still unknown about the mechanisms by which tissues form and heal, yet insights from developmental biology and other biological disciplines are already guiding the development of ‘‘instructive matrices” that work with nature's own mechanisms of organogenesis and repair. Biologically active matrices containing cells that constantly produce a physiological set of biologically active factors, in their appropriate concentrations and locations, in combination with secure, automated and highly reproducible techniques, to produce a new generation of complex skin substitutes both, in a desired number and in a constant quality, are now the guidelines of modern ‘‘skingineering’’.

(46.O1) DEVELOPMENT OF A VASCULARIZED SKIN SUBSTITUTES

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Introduction. Due to the lack of an analogue for the vascular system, current skin substitutes cannot be used to test the capacity of a given substance to penetrate through the skin into the bloodstream. However the integration of such a vascular system into a skin substitute would amplify the possible applications in research fields such as toxicity testing, by providing a model for the critical barrier between the skin and the vascular system. The objective of this study was to integrate a full thickness skin substitute (accredited according to DIN ISO-EN-10993-5) into a biological vascularised scaffold (BioVaSc), based on an acellularized part of a porcine jejunum. This BioVaSc could already be used for the formation of renal and liver tissue and was successfully implanted into a patient as a trachea patch.

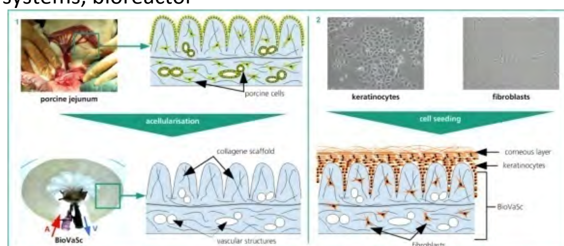
Methods. Primary human keratinozytes and human fibroblasts were seeded on the BioVaSc and cultured under submersed conditions for seven days. To initiate the differentiation of the keratinocytes the construct was subsequently cultured at an air-liquid interface for another 12 days. The formation of skin tissue on the vascularized scaffold was determined using hemalaun/ eosin (HE) and immunohistological staining.

Results. Histological HE and immunohistological staining revealed a stratified epidermal layer of keratinozytes with a corneous layer on one top of the BioVaSc and equally distributed fibroblasts inside of the scaffold.

Conclusion. In this work we could show that the BioVaSc inoculated with human keratinozytes and fibroblasts was able to facilitate the formation of a functional skin substitute. In future experiments we will combine the vascularized skin substitute with a new developed bioreactor that enables the supply of the vascularized skin substitute through the vascular system and the culture at an air-liquid interface.

Acknowledgements. The author kindly thanks the Dr Mildred Scheel trust and the Fraunhofer society for founding this project.

Keywords. skin substitute, vascularization, in-vitro test systems, bioreactor



(46.O2) IN VIVO VASCULARISATION AND COLONIZATION OF DERMAL SCAFFOLD BASED ON PLA50-PEO-PLA50 TRIBLOCK COPOLYMER

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The main consideration when developing a skin substitute strategy for promoting skin repair and regeneration is to check whether the polymer scaffold is compatible with neovascularization and consequently with skin wound healing1. The aim of this study was to design a degradable dermal scaffold that would support the development of microvessels and to visualize and characterize the microvascular network in vivo formed across the scaffold. Scaffolds were obtained according to a salt-leaching method using porogen soluble salt. The scaffolds were fabricated by using copolymer films (homemade PLA50-PEO-PLA502) obtained by the solvent evaporation and covered directly into a brass mold with a properly amount of NaCl particles sieved between 150 and 500 µm.

Porous scaffolds were implanted in mice inguinal fold for a period of 3 months. Tissue integration and scaffold degradation were evaluated by using histological staining. The formation of a vascular network was observed after injection of an isolectin drip. Neovessels were characterized by using immuno-histologic staining. In order to determine if these neovessels were functional, vascular permeability (FITC dextran) and tissue composition (antibodies directed against CD31, α-SM actin and collagen IV) were evaluated. Data showed the formation of well-differentiated vascular network. This vascular network was organized according the scaffold organization. Arteries and veins were functional and presented no pathologic signs.

As a conclusion, this work highlighted that a PLA50 -PEO-PLA50 scaffold could be a support for in vivo vascularization and tissue colonization. It confirms that this scaffold is a good candidate for skin tissue engineering and especially for help to wound healing.

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Keywords. Skin engineering, porous scaffold, angiogenesis, degradable polymer

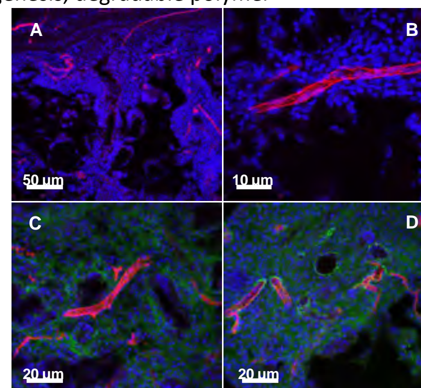

Figure: In vivo immunostaining of vessels formed within the polymer scaffold: One month polymer scaffold stained for isolectin B4 (red) and Hoescht (blue) (A and B); One month polymer scaffold stained for isolectine B4 (red) α-SM actin (green) and Hoescht (blue) (C and D).

(46.O3) SKIN REPAIR USING A BIORESORBABLE DERMAL SUBSTITUTE: TREATMENT OF ACUTE SKIN WOUNDS

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Progresses in tissue engineering and biomaterials allow us to design a new dermal substitute that would temporarily replace the tissue loss in skin wounds, and that would stimulate its own colonization from surrounding healthy tissue as soon as its hydrolytically degrades. Our approach consists in using TRIBLODERM, a porous bioresorbable polymer (poly(α-hydroxy-acid)s derived from lactic acid (PLA50-PEO-PLA50) to develop dermal substitutes for skin wounds healing. In this study, the biocompatibility, safety, and potential of TRIBLODERM matrix as dermal scaffolds were determined in a series of in vitro and in vivo systems. The therapeutic potential of TRIBLODERM device has been evaluated in porcine model of acute skin wounds. The comparison with MATRIDERM (a commercially collagen-based dermal matrix used in clinical trials) has demonstrated comparable clinical outcomes and histological results as well as the studies of neovascularization, reepithelialisation, and inflammation. And we showed a decrease in wound contraction compared to MATRIDERM. These results suggest that the TRIBLODERM matrix could form the basis of an elegant wound healing treatment strategy. Our project appears

truly new and hopeful because the contraction is a major problem in the wound healing process and may have important consequences on articulation function. Our dermal substitute offers two perspectives: first, TRIBLODERM matrix showed usefulness for wound healing and in particular in contraction limitation. The second perspective is about the interest of this TRIBLODERM device for many biomedical applications thanks to its biocompatibility and mechanical properties.

Keywords. bioresorbable polymer, wounds healing, cytocompatibility, neovascularisation, porcine model, contraction

(46.O4) NOVEL BIODEGRADABLE POROUS SCAFFOLD FOR SKIN WOUND HEALING

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Introduction. Burn, abrasion or injury wounds, substantial loss of dermal tissues, heal with wound contractures and the formation of scar tissues. To enhance the growth of skin cell is a world-wide issue and costly procedure for each age range.

Methods. A novel porous scaffold with collagen, hyaluronic acid (HA) and gelatin was fabricated for skin wound repairing.

Results. The water absorption capacity of air-dried sponge-like collagen/HA/gelatin scaffold was over 20 g water/g dried scaffold. The in vitro degradation rates of this scaffold by lysozyme, hyaluronidase, or collagenase I showed good biodegradation abilities. The average pore diameter of the dried scaffold estimated by SEM image was 132.5 ± 8.4 µm. After FBs seeded 14 days, the SEM image illustrated surface fractures inside the scaffold, indicating the material biodegradable properties. We investigated the therapeutic effects of scaffold on an in vivo rat model of excision skin wound on the back. The quantitative image analysis of the area of the excision wound was performed and indicated that the scaffold promoted the wound healing rate. From histological observations, treatments with scaffold ameliorated wound healings, including increasing neutrophils infiltrates and higher density of new generate epidermis and thickening of the epidermis.

Conclusions. We made the sponge-like scaffold from suitable biomaterials, collagen, HA and gelatin, which are biodegradable and biocompatible in the human body and adjusted the mechanical strength by cross-linking with EDC. Our co-culture model represented an alternative system for test¬ing pharmacological products in the place of laboratory animals, including mouse, rat or rabbits.

Keywords. porous sponge-like scaffold, biodegradable, biocompatible and in vivo

(46.O5) AUTOMATED PRODUCTION OF AN ENGINEERED SKIN EQUIVALENT

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Background. Skin equivalents play an increasingly vital role in testing substances for toxicity and other detrimental effects on human health. In many industries such as the development of cosmetics or even implants, passing of these tests is compulsory before product launch. Recently, great progress has been made in understanding the fundamental biological laws underlying Tissue Engineering. Yet, automation technologies essential for a reproducible, upscaled and thus economic generation of TE products are still in their fledgling stages. Aim of the project was the development of a production system which could produce 5.000 fully functional skin equivalents per month.

Results. The developed production system consists of three modules and each of two areas, one with clean room B and one with A conditions. Clean room B conditions are for the lock, where media is filled into feeder tanks and disposables can be channeled into clean room A, where cell are isolated from the biopsy (module B) proliferated (module C) or the 3D tissue growth is accomplished (module D). Before filling or channeling, a sterilization of all components via dry fog technology is possible.

Conclusions. The unique challenge in automation of biological processes requires the application of online process control to guarantee accurate production chain. One main focus was the early detection of contamination in the production facility. A further challenge results in the monitoring of the material flow between the production processes. Natural fluctuations in the biological growth processes of the skin cells depend on multiple factors, e.g. donor age, cell density and cell vitality. This results in diverse amounts of cells and vitalities in the production process. The automated production process is individually adapted to optimize the performance of the production facility. To guarantee premium products an online quality control at the end of the production chain is mandatory.

Keywords. in vitro skin model, upscaled production, automatable and industrially feasible manufacturing process

(46.O6) PLATELET LYSATE INDUCES IN VITRO WOUND HEALING OF HUMAN KERATINOCYTES ASSOCIATED WITH A STRONG PRO-INFLAMMATORY RESPONSE

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Introduction. Platelet rich derived preparations were shown to enhance the healing of chronic, diabetic ulcers. Wound healing is a complex series of events that are triggered by the onset of an inflammatory cascade. In the current work, we investigated effects of platelet lysate on wound healing in an in vitro model in association with the pro-inflammatory response.

Materials and Methods. Wound scratch assays were performed using confluent NCTC 2544 keratinocytes and incubated with serum-free MEM , MEM+ FBS10% , Platelet lysate (PL) 5% + MEM, PL5% + MEM+FBS10%, PL5% + MEM, and PL5% + MEM+FBS10% and evaluated at 6 and 24hrs. Cytoskeletal changes reflecting cell migration were also shown. Modified wound scratch assays were performed by pre-stimulating cells with 250 U/ml of IL-1. IL-8 expression from scratched cells was evaluated using western Immunoblotting(WB). To evaluate the pro-inflammatory effects of PL, cells were stimulated with serum-free media, IL-1 , PL, and IL-1 +PL and analyzed for the expression of the antimicrobial peptide NGAL and IL-8 using WB and Q-RT-PCR. Under the previous conditions, activation of p38 MAP kinase and NF-kB pathways was also evaluated in addition to iron binding activity assays for NGAL.

Results and Conclusion. PL5%, used as the only medium supplement, significantly enhanced wound closure after 6 and 24 hrs. Cells treated with PL presented a distinct actin cytoskeletal reorganization. Upon addition of a pro-inflammatory stimulus, PL maintained its enhancement effect. Enhanced wound closure rates were found to be associated with an increased expression of IL-8 especially after 6 hrs. In the absence of scratches, cells simulated with PL5% showed an enhanced expression of IL-8 and NGAL that was intensified in the presence of an inflammatory stimulus as well as an activation of the NF-kB and p38 MAPK pathways. Our data show that PL5% can enhance wound closure in association with a strong pro-inflammatory response.

Keywords. platelet lysate, pro-inflammatory, wound healing, keratinocyte migration, IL-8, NGAL

(46.O7) EARLY EFFECTS OF IONIZING RADIATION IN TISSUE ENGINEERED MUCOSA

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Tissue Engineered Mucosa (TEM) was originally designed to reconstruct large oral defects remaining in the oral cavity after oncological resection. Most patients that undergo oncological resection will receive radiotherapy as part of their treatment. Little is known about the pathobiology of the early effects of ionizing radiation (IR) on oral mucosa. This study focused on the biological response of TEM in time after exposure to a single dose of IR. We evaluated the epithelial proliferation and differentiation pattern post-irradiation, the expression of basement membrane components, cell-cell adhesion and the attachment of the epithelium to the underlying connective tissue. TEM was created by seeding human fibroblasts and keratinocytes onto an a-cellular dermal scaffold. After two weeks of culturing the TEM at the Air/Liquid interface, TEM was irradiated with a single dose of 16.5Gy, 0Gy was used as control. TEM was harvested 1, 6, 24, 48 and 72h post-irradiation.

DNA damage as a result of exposure to IR was determined using 53BP1, a DNA DSB marker. 1h and 6h post-irradiation the number of 53BP1 positive cells was significantly increased when TEM was irradiated with 16.5Gy. The epithelial layer proved to become significantly thinner after exposure to 16.5Gy. The proliferation index (PI) was oserved to significantly decrease over time when exposed to 16.5Gy.

The strong resemblance of TEM to native mucosa indicates that TEM can be used to study early effects of IR on oral mucosa. We were able to detect IR-induced DNA damage. In addition, we found thinning of the epithelial layer and a significant decrease of the proliferating capacity post-irradiation. This study might provide new insights in the mechanism underlying early irradiation damage in TEM. Future research will include the use of radioprotective agents to improve the quality of TEM after IR.

Keywords. oral mucosa, ionizing radiation

(46.O8) DEVELOPMENT OF A TISSUE-ENGINEERED HUMAN SKIN MODEL FOR DETECTING IRRITANT-INDUCED INFLAMMATION

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In order to establish appropriate risk to human health, cosmetic industry had relied on animal tests for screening of cosmetic products and their chemical ingredients. However, 7th amendment to the European Council Directive 76/768/EEC has prohibited the use of animals for toxicological testing since March 2009, resulting in an increasing need for alternative models. Several in vitro 3D culture models have been developed as alternatives, but they are largely confined to epidermis and do not provide information beyond basic toxicity assessment. The aim of this research is therefore to develop a tissue-engineered human skin model which contains both epithelial and mesenchymal components and reflects the paracrine inflammatory interactions observed in real skin, as an improved alternative. The model comprises normal human keratinocytes (NHK) and dermal fibroblasts (HDF). The effects of sodium dodecyl sulphate (SDS) and potassium diformate (Formi®), as model irritants, were studied on the release of IL-1a, IL-6 and IL-8 by ELISA. Activation of NF-kB in HDF was also investigated by immunofluorescence staining and confocal microscopy. MTT-ESTA assay was conducted in parallel to ensure that the cytotoxic concentrations for each compounds were known, and that the inflammatory effects studied were below these concentrations. Results indicated that subtoxic concentrations of SDS and Formi® induced the release of IL-1a by keratinocytes. Subsequently, fibroblasts responded by activation of NF-kB and the release of IL-6 and IL-8 in large quantities. Reporter plasmids were then constructed containing a secreted version of luciferase (from Metridia longa), driven by IL-6 or IL-8 promoters transfected into fibroblasts. SDS/Formi conditioned medium from keratinocytes was shown to activate luciferase secretion enabling the non-invasive inflammatory detection of irritants using an in vitro model.

Keywords. 3D models; skin; irritation; inflammation

(46.O9) THE ENGINEERING OF PREVASCULARIZED MATRICES

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Introduction. Initial take, development and function of transplanted engineered tissue substitutes are crucially dependent on rapid blood perfusion. Thus, the development of fast and efficiently vascularized tissue grafts is essential for tissue engineering and regenerative medicine.

Methods and Results. In the last years, we have developed a 3D-cell culture system using human dermal microvascular endothelial cells and biodegradable fibrin hydrogels, and we are now able to, reproducibly, create highly organotypic capillary networks in engineered tissue substitutes. Our results have shown that the in vitro process of capillary formation follows the principles of both vasculo- and angio-genesis and that capillary lumenization requires the deposition of a basement membrane, intensive pinocytosis, the generation of intracellular vacuoles and their successive fusion. Pre-clinical transplantation studies have shown that the vascular structures are stabilized by mural cells of the recipient animal and perfused by host blood. This suggests that the human capillaries not only survive, but also mature and anastomose acquiring full functionality, in vivo. Recent accurate immunohystological and a computerized quantitative analysis reveal that both blood and lymphatic vessels are formed in the 3D hydrogels and that the in vitro formed capillaries have a physiological lumen size. In vivo maturation, anastomosis and functionality of the pre-formed human lymphatic vessels are, currently, under investigation. Importantly, our system perfectly suits tissue engineering of skin, for which the vital need of rapid vascularization is encountered with the use of large transplants. Indeed, our recent data shows that human keratinocytes proliferate and stratify onto the pre-vascularized hydrogels both in vitro and in vivo.

Conclusions. Our findings suggest that the in vitro engineering of pre-vascularized skin grafts is within reach and that future clinical application, for skin substitutes can be envisaged.

Keywords. Pre-vascularization, Tissue engineering, Tissue engineering of skin

(46.O10) MODIFIED PLASTIC COMPRESSION OF COLLAGEN GELS: A NEW METHOD TO OPTIMISE THE MATRIX FOR LARGE SCALE TISSUE ENGINEERED SKIN TRANSPLANTS

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Skin tissue engineering for the generation of large transplantable dermo-epidermal skin replacements is dependent on a three-dimensional matrix that supports the biological function of skin cells and provides mechanical properties/stability to allow for surgery. Until now collagen type I hydrogels promised the best biological functionality but their mechanical weakness and tendency to contract and degrade did not allow for the generation of large transplants or long term stable in vitro skin models. We show that, by a modified plastic compression method, collagen hydrogels can acquire mechanical and biological stability while conserving the ideal biological functions. Tissue engineered skin substitutes based on compressed collagen gels can be handled easily in large sizes for transplantation and give rise to a near normal homeostatic skin. Additionally, improved stability reduces in vitro degradation and therefore compression improves the quality of long term in vitro skin cultures.

Keywords. collagen gel, plastic compression, clincal, transplantation, skin, keratinocytes, fibroblasts, matrix

(46.O11) A MATHEMATICAL MODEL OF COLLAGEN LATTICE CONTRACTION

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Since Bell and coworkers introduced the fibroblast populated collagen lattice (FPCL) contraction model in 1979, many modifications have been made in the manufacturing and analyzing FPCL contraction. An objective of FPCL contraction system was to model the closure of an open wound by wound contraction. The FPCL system follows fibroblasts and collagen interactions, which mimic those in wound granulation tissue. A mathematical model, which simulates the forces responsible for FPCL contraction, is proposed to simulate the forces responsible for wound contraction. Ideally the model will predict tissue structure from basic cell extracellular interactions.

Keywords. fibroblasts, collagen, lattices

(46.O12) CHARACTERIZATION OF ANGIOGENIC TRANSFORMATIONS AND THE ROLE OF PROTEASES IN REVASCULARIZATION OF SKIN GRAFTS

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Objective. Despite advances in tissue engineering of human skin, the exact revascularisation processes during taking still remain unclear. Therefore it was the aim of this study to investigate the transformations during engraftment and to identify the proteolytic co-players during the revascularisation process. **Methods:** The modified dorsal skinfold chamber with autologous skin grafting was performed in C57BL/6J mice (n=5). Crossover transplantation was carried out in a B6/GFP model to originate the vascular structures in vivo. Further, the expression of proteases, angiogenic factors and vascular markers within wound bed and skin graft was visualized by immunohistochemistry.

Results. Reperfusion according to the original vascular pattern of the skin graft was observed at day 3. Angiogenesis in the wound bed was triggered by HIF-1a from day 1 to 3. This was accompanied by a temporary angiogenic response of the graft vessels between day 4 and 7. The B6/GFP model confirmed the origination of this angiogenic reaction from autochthonous graft vasculature. Additionally GFP-positive vessels were detected growing in from wound bed and orientating along the existing vascular structures of the graft. Next to this, MT1-MMP was detected at sprout tips of in-growing

vessels. Further proteolytic activity was accredited to MMP2 located at the wound bed/graft connection site. MMP9 was found to be accompanying regressing vessels in the graft.

Conclusions. These in vivo data indicate the connection of angiogenic bed vessels to the graft vasculature resulting in reperfusion of the graft. Additionally, we observed a temporary angiogenic response within the capillaries of the graft after reperfusion. Furthermore we identified MT-MMP1 as sprout-tip located protease indicating its role as sprout growth "facilitator" and potentially in "lysing" the existing graft capillaries in order to connect to them. Angiogenesis was further associated with increased levels of MMP2 and vascular regression triggered by MMP9.

Keywords. dorsal skinfold chamber, revascularization, angiogenic transformations, proteases

(46.O13) RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR (NEPIDERMIN) TREATMENT IMPROVES WOUND HEALING IN A DIABETIC RAT MODEL

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Introduction. Diabetes mellitus is associated to a deficient cicatrization of cutaneous ulcers, due to functional alterations in epidermic, conjunctive and immunologic tissues. Local administration of epidermic growth factor (EGF) could improve this alteration. The effects of local treatment with Nepidermin (rh-EGF) in a diabetic excisional-wound rat model were investigated.

Methods. Animals (n=64) were divided in 4 groups: healthy rats (HR), streptozotocin-induced diabetic rats treated with 0,5 (DR-0,5), 2,0 (DR-2,0) µg/mL Nepidermin or with placebo (DR-Plcb). A cutaneous excisional defect (Ø=1,5cm) was made, and peri-/intralesional injections of each treatment were administered 3 days/week for 15 days. Animals were sacrificed at days 3, 7, 14 and 21. Cicatrization tissue samples were processed for histological and morphometrical studies, and blood serum was obtained at sacrifice time for IL-12 and TNF-α quantification by ELISA. mRNA levels of these cytokines and collagen I and III were measured by qRT-PCR.

Results. HR achieved total cicatrization at day 21, while DR-Plcb did not reach continuity solution of the defect. DR-0,5 and DR-2,0 groups significantly accelerated reepithelialization, dermic growth and inhibited contraction. TNF-α studies showed a decrease of this cytokine over time in HR. Untreated diabetic rats levels of TNF-α followed an irregular pattern. Levels of diabetic rats treated with Nepidermin were closer to HR group. DR-Plcb group had higher levels of IL-12 than HR, while Nepidermin treatment seems to induce a disregulation in the activation process of this cytokine. Collagen I/III-mRNA ratio remained close to 1, with a tendency to collagen I predominance over time.

Conclusions. Local administration of rh-EGF accelerates and improves excisional wound cicatrization in diabetic rats, and seems to induce a disregulation of immunologic response, being the group of 0,5 µg/mL treated rats the closest to the observed response in control group.

This work was supported by an Art. 83 grant, Praxis Pharmaceutical S.A.

Keywords. rh-EGF, wound healing, diabetic ulcer, diabetic rat model

(46.O14) HYPERGLYCEMIA LEADS TO DIFFERENTIAL GENE EXPRESSION IN WOUNDED KERATINOCYTES

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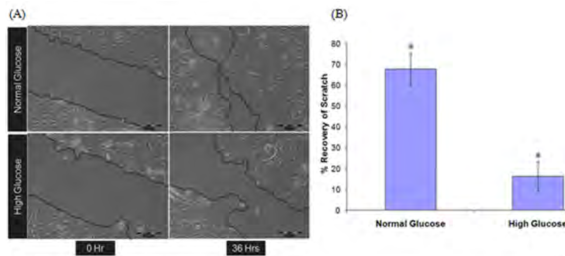
Introduction. Diabetic patients suffer from compromised wound healing characterized by chronic inflammation, reduced angiogenesis and reduced reepithelialization. The diabetic wound healing pathology can be understood at the 'molecular level' with differential regulation of gene expression in epithelial cells involved in wound healing. This study investigates altered gene expression in wounded keratinocytes cultured in high versus normal glucose conditions. Altered gene expression data will be used to dissect the link between specific gene expression, wound healing and ambient glucose concentrations. Restoring gene expression to normal levels may normalize diabetic wound healing.

Experimental methods. *In Vitro* Scratch Wound Model and Microarray: Confluent mono-layers of primary human skin keratinocytes grown in normal (6.5 mM) and high (25 mM) glucose conditions were subjected to a scratch test. RNA was extracted from the wounded cells and used for a microarray. Differences in gene expression between normal and high glucose cells were compared while using data from non-scratched cells as a control to focus on genes involved in wound healing. RT-PCR was used for validation.

Results and Discussion. Wounded keratinocytes cultured in normal glucose conditions were found to heal the scratch wound significantly better than wounded keratinocytes cultured in high glucose conditions (Figure 1. A and B). Microarray data analysis revealed that a number of genes and molecular signaling pathways involved in wound healing were altered in high glucose wounded conditions. Figure 1 (C and D) details the examples of the up- and down- regulated genes which were validated by real time PCR. Focusing on genes with altered expression during wound healing and not just when exposed to high glucose concentrations increases the chances of focusing on a therapeutically relevant gene.

Acknowledgments. This work was funded by Science Foundation Ireland - Research Frontiers Program, under Grant Number (06/RFP/ENM005) and Health Research Board, under grant number (HRB RP/2008/188).



(C) Genes upregulated in hyperglycemic wounded keratinocytes

Gene Name	Gene Symbol	Fold Change in Microarray	Fold Change in qPCR	Biological Processes
1557486_at	IGSF4D	14.58	1.80	Extracellular recognition and intercellular adhesion
206025_at	TNFAIP6	7.87	10.69	cell adhesion and migration
221577_x_at	GDF15	6.73	15.00	tissue differentiation and maintenance
202831_at	GPX2	6.65	10.85	inhibits cellular migration
201601_at	IFITM1	5.82	2.41	negative regulator of cell proliferation
213348_at	CDKN1C	5.03	21.26	regulator of cell growth

(D) Genes downregulated in hyperglycemic wounded keratinocytes

Gene Name	Gene Symbol	Fold Change in Microarray	Fold Change in qPCR	Biological Processes
239637_at	homologous to RAB18	40.32	8.310501	ER-Golgi trafficking
1563881_at	homologous to MAGUK	33.94	5.939043	scaffold proteins
239605_x_at	TGFBR1	31.89	1.922384	positive regulation of cell proliferation
242186_x_at	LPHN3	22.11	1.943466	cell adhesion
230401_at	NUPL2	12.34	1.22078	protein export from nucleus
1554287_at	TRIM4	10.85	3.809324	ubiquitin-protein ligase activity, zinc ion binding
201109_s_at	THBS1	2.345	2.655244	cell-to-cell and cell-to-matrix interactions

Figure 1. *In vitro* scratch assay and examples of differential regulation of wound healing of gene expression during wound healing in hyperglycemic conditions. (A) Monolayer of keratinocytes, both in normoglycemic and hyperglycemic growth conditions, were scratched and allowed heal for 36 hrs. (B)Percentage recovery of the scratch was quantified. * indicates statistical significance by one-way ANOVA ($P < 0.05$, $n = 3$). It is evident that keratinocytes migrate more efficiently to heal the scratch wound in normal glucose than high glucose. (C) Genes upregulated in hyperglycemic wounded keratinocytes (D) Genes downregulated in hyperglycemic wounded keratinocytes

(46.P1) BIOCIDAL ACTIVIVITY OF IODINE RELEASED FROM DIRECTLY UV PHOTOCROSSLINKED POLYVINILPIRROLIDONE ELECTROSPUN MATS

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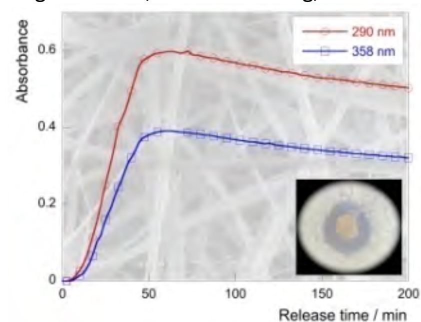
Introduction. Electrospun nanofibrous mats are particularly adequate for skin wound dressings. Their high porosity and surface to volume ratio ensure gas permeability, contribute to a correct fluid management and enable a sustainable release of biocidal agents. The typically small pore dimension is, by itself, a first barrier to infectious agents. In this work, we studied the release of iodine (a wide spectrum biocidal) from directly ultraviolet-crosslinked nanofibers of polyvinylpyrrolidone (PVP, a water soluble hydrophilic polymer). Their antimicrobial activity was evaluated.

Experimental Methods. Solutions containing 18% PVP and 5% of a PVP complex with elemental iodine (PVP-I), in water-ethanol (1:1) were electrospun using an applied field of 1.3kV/cm. The resulting non-woven mats were irradiated under UV for different periods of time in order to photocrosslink the polymer. The release of iodine, from the mats into water, was analysed by UV-visible spectrophotometry. The biocidal activity was evaluated by the Bauer-Kirby disk diffusion test in confluent Staphylococcus aureus, Staphylococcus epidermis, Pseudomonas aeruginosa and Candida albicans cultures.

Results and Discussion. Under UV irradiation, the light yellow fibre mats turn lighter with exposure time. The mats become insoluble in water after a minimum irradiation time of 60min. The absorbance peaks in the UV spectrum at 291 and 358nm (due to the I3- species) increase with release time, reach a maximum and then slowly decrease. The mat crosslinked during 4h yields the lowest release rate with the maximum being reached after 50min. This sample produced inhibition zones visible in all confluent cultures after 48h of incubation on agar. No inhibition zone was visible around a PVP nanofibrous control, without PVP-I.

Conclusion. PVP nanofibers incorporating iodine were photocrosslinked without any photoinitiator. Their biocidal activity makes them good candidates for wound dressings and as an epidermal equivalent in skin substitutes.

Keywords. Nanofibers, iodine-release, UV-crosslinking, microbiologic cultures, wound-dressing, skin substitute



(46.P2) IN VITRO SHOCK WAVE TREATMENT INCREASES MITOCHONDRIAL MEMBRANE POTENTIAL IN U937 CELLS

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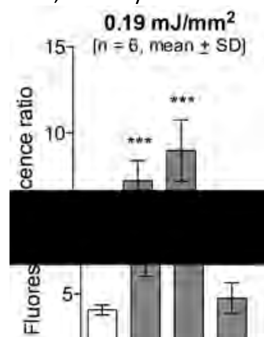
Shock wave treatment is used for a variety of indications, such as chronic soft tissue wounds, tendinopathies and bone healing disorders. Although treatment shows highly beneficial effects, underlying mechanisms remain largely unknown. In order to study the effect of shock waves (SW) on single cell types, organelles or receptors in vitro, reproducible experimental set-ups are needed. Shock wave energy dependent uptake and release of molecules is enabled by transient cell membrane permeability. This could reproducibly be shown with the recently introduced in vitro shock wave trial (IVSWT) experimental set-up. Effective in vitro treatment parameters were identified. These are used in this study to assess changes in normalized mitochonondrial membrane potential (MMP), indicating mitochondrial energetic state.

106/mL human leukemic monocyte lymphoma cells (U937) were transferred to a 15 mL polypropylen tube, fixed in the IVSWT set-up and subjected to 300 pulses, at a frequency of 3 Hz, and an energy of 0.19 or 0.07 mJ/mm2. To assess normalized MMP after shock wave treatment, cells were stained with two fluorescent mitochondrial stains – MitoTracker Green FM and

CMXRosamine (MMP insensitive and sensitive, respectively; Molecular Probes, Invitrogen). Fluorescence intensity ratio of CMXRosamine to MitoTracker Green FM – displaying normalized MMP – was analysed by flow cytometry.

Treatment with higher energies resulted in significantly higher mitochondrial membrane potential compared to untreated controls (Fig.1,C), whereas potential measured directly after SW (T1) was even increased after 2h post treatment (T2) and reaching baseline level 1 day post treatment (T3). Treatment with energies not causing transient permeability of the cell membrane did not change mitochondrial membrane potential significantly. The energy dependent increase of mitochondrial membrane potential following shock wave treatment might be one of the important factors leading to the beneficial effects of extracorporeal shock wave treatment.

Keywords. Shock wave treatment, mitochondrial membrane potential, monocytes



(46.P3) KERATINOCYTES DELIVERED ON MICROCARRIERS SIGNIFICANTLY REDUCE WOUND CONTRACTION IN VIVO IN THE PORCINE MODEL OF WOUND REPAIR

Martin Y (1), Eldardiri M (1), Sharpe JR (1)

1. Blond McIndoe Research Foundation

Introduction. Large full thickness injuries, caused by burns or penetrating traumatic injuries, can lead to the formation of contractures. These cause pain to the patient and can significant reduce mobility. Wound contraction can be reduced by treatment with skin grafts or dermal substitutes, particularly when in combination with delivery of autologous keratinocytes (1). Autologous keratinocytes can be delivered to the wound bed either as a sprayed cell suspension or on biodegradable gelatin Cultisphere™-G microcarriers (Percell Biolytica). In an in vivo study, we compared the effect on wound contraction following treatment with widely meshed split thickness skin graft (STSG) in combination with either autologous keratinocytes sprayed in single-cell suspension (SAK) or delivered on microcarriers (MCAK).

Materials and Methods. Eighteen 4 x 4 cm square full thickness wounds were created in three large white pigs and the wounds treated with STSG (expanded 6-fold) with our without SAK or MCAK. Contraction was measured at 21 days using Visitrak™ (Smith&Nephew).

Results. Histological analysis showed a comparable quality of epithelial repair in all treatment groups by H&E staining and immuno-staining for makers of basal keratinocytes (cytokeratin 14) and the basement membrane (collagen VII and laminin) (Fig 1). Contraction was significantly reduced when wounds were treated with MCAK (Fig 1). Interestingly, SAK-treated wounds contracted 22% ± 8.6% less and MCAK-treated wounds 34% ± 10.1% less, when compared to contraction observed in wounds treated with STSG alone.

Conclusions. The reduction in wound contraction observed in this in vivo study shows great potential for clinical benefit in the treatment of full thickness wounds in patients.

References. (1) Reid, MJ, et al. Wound Repair Regen 15, 889, 2007

Acknowledgments. This work was supported by Sparks and The Charles Wolfson Trust.

Keywords. skin, in vivo, wound contraction

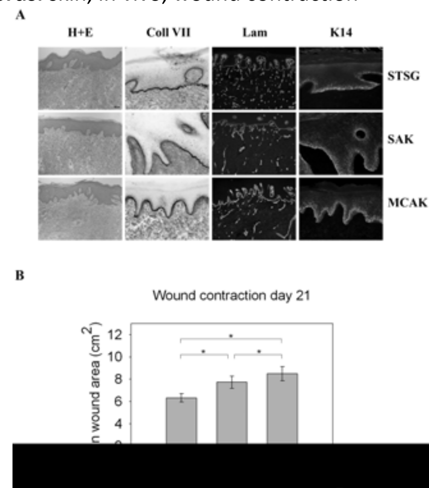


Fig 1. Immuno-staining for H&E, collagen VII (Coll VII), laminin (Lam) and cytokeratin 14 (K14) showed comparable quality of epithelial repair (A). Contraction of wounds was significantly reduced after 21 days of treatment with MCAK (B).

(46.P4) EXPLORING THE USE OF BIOMATERIALS TO OBTAIN A DERMIS SURROGATE TO BE EMPLOYED BY THE FOOTWEAR INDUSTRY

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Introduction. Progress made in the development of materials to replace human skin served us as the starting point to carry out experiments focused on obtaining a dermis surrogate that can be considered as a future substitute, after tanning, of the skin employed by footwear industry. This material could provide an alternative source of raw material that can overcome the difficulties of obtaining skin directly from animals.

Materials and Methods. Dermal fibroblasts were isolated from pig skin biopsies. Fibrin gels containing embedded live fibroblast were obtained by polymerisation of fibrinogen from pig plasma cryoprecipitate. Scaffolds were produced by plasma lyophilisation after glutaraldehyde crosslinking, according to Meana et al. Samples were visualized with optical microscopy and SEM. For histological analysis, samples were paraformaldehyde fixed and included in paraffin. Sections were stained with hematoxilin/eosin. Picrosirus and Trichrome staining were used to visualize type I collagen.

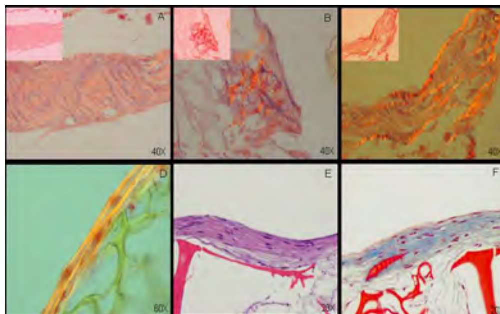
Results. Collagen I deposition increased when ascorbic acid and TGF-B1 were added. However, gradual fibrin degradation produced dermal structures with low

consistency and thickness. Cell growth and collagen production caused matrix contraction, reducing its size. Use of semi-rigid matrices from crosslinked plasma avoided contraction and scaffold degradation. Fibroblast tended to grow mainly over the outer scaffold surface, coating the inner cavities without filling them, and producing low extracellular matrix amount.

Conclusions. Biomaterials employed in biomedicine like fibrin or crosslinked plasma scaffolds are a good start point to be used as 3D structure to sustain growing of a dermal equivalent that can be used to substitute natural skin in a future with an industrial purpose. To achieve this purpose, we need to optimize and adapt the material characteristics (porositiy, stiffnes) to our objective, and improve the process in order to increase the final amount of collagen I and in vitro dermis characteristics.

Keywords. dermis equivalent, collagen type I, tanning.

Work supported by Spanish MICINN (grant CTQ2007-65569).



(46.P5) PSORIATIC SKIN SUBSTITUTES: EFFECTS OF RETINOIC ACID ON KERATINOCYTE PROLIFERATION AND DIFFERENTIATION

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Introduction. Psoriasis is well-known to be a retinoid-sensitive disease. In vivo, retinoids can modulate epidermal proliferation with an anti-proliferative effect in hyperproliferative systems and cause hyperproliferation in normoproliferative systems. The aim of this study was to compare the development of psoriatic skin substitutes cultured with retinoic acid to those cultured in a retinoic acid-free medium to observe the effects of this growth factor on in vitro keratinocyte proliferation and differentiation.

Methods. Psoriatic and healthy skin substitutes were produced using the self-assembly method. Biopsies were taken 21 days after being raised to the air-liquid interface and they were examined by histology and immunohistochemistry.

Results. Macroscopically, psoriatic skin substitutes cultured with retinoic acid showed a thinner and less extensive epidermis suggesting a diminution of cell proliferation. These results were confirmed by the measurement of the epidermal thickness and by the count of ki67 and p63 positive cells. Moreover, the expression of all tested cell differentiation markers was restored in psoriatic keratinocyte substitutes cultured in presence of retinoic acid. No significant change in epidermal thickness or in the expression of late differentiation markers were observed in healthy keratinocyte substitutes cultured with or without retinoic acid, however, some changes were reported for proliferation and early differentiation markers.

Conclusion: Results suggest that retinoic acid can modulate epidermal proliferation and differentiation of keratinocytes in a new in vitro psoriatic skin model.

Acknowledgements. This study was supported by the Canadian Institutes of Health Research. RP was recipient of a research fellowship from the “Fonds de la Recherche en Santé du Québec”, Québec, QC, Canada. JJ held a scholarship from “Fonds d’enseignement et de recherche” of the Faculté de Pharmacie, Université Laval, Québec, QC, Canada.

Keywords. Skin, Tissue engineering, psoriasis, skin substitutes

(46.P6) TISSUE ENGINEERING OF BASED-FIBRIN MOUSE SKIN WITH LANGERHANS CELLS DERIVED FROM BONE MARROW

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1. Medicine School UNAM, UAM-Iztapalapa

Introduction. The migration process of Langerhans cells is regulated by different citokines, nonetheless the exact mechanisms that induce the migration and activation of Langerhans cells are not elucidated due the presence of another kind of dendritic cells. In this sense, with the purpose of clarify some of the mechanism carried out in this process, our laboratory developed a model of a mouse skin construct that include Langerhans cells derived from bone marrow without other dendritic cells in the skin.

Methods. In order to obtain Langerhans cells, bone marrow cells were collected from male Balb/c mice and cultured with TNFa (50 U/mL) and TGFb (1ng/mL) during 7 days. The derived cells were incubated with antibodies to evidence the expression, by cytofluorometry, of Langerin, class II molecules of the major histocompatibility complex, CD11c and E-cadherin. On the other hand, from mice skin were obtained keratinocytes and fibroblast. Then, both cells were culture in a fibrin scaffold. Forty-eight hours after keratinocytes were placed, 40,000 Langerhans cells derived from bone marrow were added.

Results. The cytofluorometry showed ±30% of cells that were Langerin+/E-cadherine+. These cells were well located in a basal and suprabasal position in the epidermis of the skin constructs, showed long and slender dendrities between keratinocytes and the expression of Langerin, class II molecules and E-cadherin persisted.

Conclusions. The model created by us is appropriated to study the biology of Langerhans cells in an environment free of other dendritic cells o immune cells.

Acknowledgements. CONACYT: 50396-M, DGAPA/PAPIIT: IN213510 and IN214109-3; Posgrado en Biología Experimental UAM-I

Keywords. Langerhans cell, fibrin gel, skin model

(46.P7) TAYLORING BIOABSORBABLE SCAFFOLDS SUITABLE FOR SKIN REGENERATION

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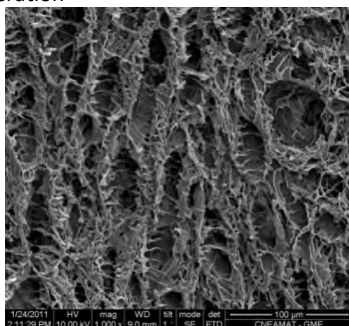
Advances in medicine and tissue engineering promote the development of new materials, particularly bioabsorbable polymers that could be used as scaffolds for skin regeneration. On considering substrate materials, it is imperative to choose one that exhibits good biocompatibility, not eliciting an unresolved inflammatory response nor demonstrate extreme immunogenicity or cytotoxicity. Polyhydroxyalkanoates (PHAs) are bioplastics produced by microorganisms that proved to be bioabsorbable; particularly, the biocompatibility of polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-valerate (PHBV) has been studied by a number of different research groups for a variety of medical applications, which include controlled release, surgical sutures, wound dressings, lubricating powders, orthopedic uses, etc. Other important features of the scaffolds are the size and interconnection of the pores since it must allow cells to migrate, grow and keep its normal functionality in the same way as the extracellular matrix does.

Thus, the aim of this contribution is to present advances in the preparation of PHBV scaffolds for skin regeneration made by thermal induced phase separation (TIPS). Pore size and its interconnection can be tailored by controlling the thermodynamic and kinetic parameters of the phase separation. Scanning electron microscopy and Hg intrusion porosimetry allows determining pore size and shape as well as the interconnectivity of the pores.

Finally, the biodegradation kinetics of the scaffolds was tested in vitro in order to evaluate the influence of the concentration of lipases and the morphology of the scaffods.

Keywords. phase separation, biopolymer, PHBV, scaffold, skin regeneration



Alligned pores in a PHBV scaffold made by TIPS.

(46.P8) SWEAT GLANDS – A NEW SOURCE OF STEM CELLS

Nagel S (1), Petschnik AE (1), Salem H (2), Paus R (3), Kruse C (1), Danner S (1)

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Introduction. In mammalian skin, stem cells reside in the hair follicle bulge area, epidermis, dermis, subcutaneous adipose tissues and are proposed to be present in glandular skin appendages such as sweat glands and sebaceous glands. Since it has already been shown that other glandular tissues like pancreas and salivary glands are sources of mulitpotent stem cells, we characterized human sweat gland-derived cells particularly with regard to stem cell characteristics and multilineage differentiation potential.

Methods. Isolation of human sweat glands was based on mechanical and enzymatic digestion of axillary skin. Cultivation was performed on collagen-coated cell culture dishes and the resulting cell population was investigated at protein- and mRNA level. Spontaneous differentiation and induced differentiation by means of 3D cultivation in organoid bodies was analyzed. Their neuronal differentiation capability was tested with a novel xenogenic co-culture system using brain biopsies. Moreover, clonal cell lines were established and subjected to the same analysis procedures.

Results. In sweat gland-derived cells the expression of various stem cell markers could be revealed. It was also possible to show spontaneous differentiation into cells of the three embryonic germ layers and enhance the differentiation in the neuronal and epithelial lineage applying defined differentiation protocols.

Conclusions. We identified the human sweat gland as a source of stem cells with multilineage differentiation potential, high proliferation activity and remarkable self-renewal in vitro. Human sweat gland-derived stem cells (hSGSC) are an attractive novel type of adult glandular stem cells that has the advantage of being abundant and highly accessible. Consequently, hSGSCs are an interesting candidate for cell-based therapies in burn or chronic wound healing.

Keywords. sweat glands, stem cells, skin

(46.P9) A NEW HOPE FOR BURN VICTIMS: AUTOLOGOUS SWEAT GLAND-DERIVED STEM CELLS

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Background. It could be shown that glandular stem cells from pancreas and parotid gland can accelerate wound healing in a full skin defect model. Recently isolated and characterized human sweat gland-derived stem cells (hSGSC) are an even more attractive novel type of adult glandular stem cells that have the advantage of being abundant and highly accessible. Therefore, hSGSCs are an interesting candidate for burn wounds healing models.

Methods. 20% partial-thickness scald burn was made on the back of nude mice. 2 hours after burn injury, hSGSCs were applied in two different ways. On the one hand the cells were injected directly into the burn margins at different sites. On the other hand the burned skin was replaced by a hSGSCs-seeded scaffold. Control groups, were treated with PBS and the scaffold itself respectively. Analysis of wound healing, angiogenesis and reepithelialization was done after one week for the

injection group and after two weeks for the scaffold group.

Observations. Analyzing the burn wound healing revealed an enhanced wound closure in the hSGSCs treated groups, together with a highly significant angiogenesis percentage compared to the control groups.

Conclusions. In this study we could show for the first time that multipotent hSGSC accelerated wound healing and vascular regeneration in a burn model. This data suggests that these cells possess a promising potential to improve wound healing in burn subjects.

Keywords. sweat gland, adult human stem cells, skin, wound healing

(46.P10) PRO-INFLAMMATORY STIMULATION OF ADULT HUMAN STEM CELLS MAY ENHANCE WOUND REPAIR IN SCAFFOLD BASED THERAPIES

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1. Fraunhofer Research Institution for Marine Biotechnology, Lübeck, Germany; 2. Department of Plastic and Hand Surgery, Technical University of Munich, Klinikum rechts der Isar, Munich, Germany

Wound repair is a complex and highly regulated succession of events,which are activated and controlled by different molecules, including cytokines, chemokines and growth factors. The repair process can lead to scar formation or at worst to chronic wounds. In regenerative medicine, the ideal goal is to generate a scenario, by which the structural and functional properties of the injured skin becomes restored ad integrum.

Previously, we and others demonstrated, that adult stem cells are able to promote wound healing through a paracrine effect. Therefore, not only the differentiating cells themselves, but also their secreted factors contribute to wound closure. In order to additionally amplify this paracrine effect, we developed a scaffold seeded with pre-stimulated human skin-derived stem cells. The bacterial lipopolysaccharid surface molecule (LPS), a main trigger of inflammatory processes in vivo, was chosen as the trigger. The effect of the stimulation was studied after 4h, 24h and 48h respectively. Via RT-qPCR a time-dependent increase of TNFa, IL1 and IL6 was revealed in the stimulated cells. Further, the supernatants of the stimulated and the non-stimulated cells were compared with respect to the secretion of various factors. It could be shown, that the stimulated cells did not only express and secrete pro-inflammatory cytokines like TNFa, IL1, IL6 or IL8 among others, but were also able to produce angiogenetic factors like Angiopoietin 2, VEGF and Serpin E1. We detected that the inflammatory response subsided and the amount of angiogenetic factors increased over time. Prospectively,the effect of pre-stimulated human stem cells on wound repair will be investigated in vivo on full thickness wounds in a nude mice model.

Taken together, this is the first work describing the use of a pro-inflammatory, cell-seeded scaffolds as a possible means to enhance wound repair.

Keywords. adult human stem cells, skin, inflammation, wound healing

(46.P11) CULTURED KERATINOCYTES ON URINARY BLADDER MATRIX SCAFFOLDS (UBM) INCREASE ANGIOGENESIS AND HELP RAPID HEALING OF WOUNDS

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Introduction. Urinary Bladder Matrix (UBM) is an extracellular matrix (ECM) scaffold. It is now used in wound care management of partial and full-thickness wounds where conventional methods for wound care usually fail to give satisfactory results. In this study we are comparing the healing of full-thickness excisional wounds in New Zealand Rabbits using either UBM scaffolds alone or in combination with cultured Keratinocytes. The wounds were compared grossly and histologically.

Materials and Methods. It's a comparative controlled study including 40 full-thickness wounds in two groups. Group (A) wounds: treated with UBM scaffolds, Group (B) wounds: treated with UBM scaffolds with cultured Keratinocytes. The wounds were examined grossly after 1, 2, and 3 weeks, and were examined histologically at the end of the 3rd week using ordinary H&E staining techniques.

Results. All the wounds healed completely by the end of the 3rd week. Early wound contraction was significantly less in group B. More angiogenic response was evident in all specimens of group B.

Conclusion. Our study shows that adding cultured keratinocytes to the rough surface of the UBM scaffold may be beneficial in reducing early wound contraction and improving wound vascularity in treatment of full-thickness wounds.

Keywords. Urinary Bladder matrix scaffolds, Wound care, cultured keratinocytes

47. TRANSLATIONAL BONE ENGINEERING

Chair: Karl-Heinz Schuckert
Co-chair: Martijn van Griensven
Keynote speaker: Peter ten Dijke
Organizers: Karl-Heinz Schuckert, Martijn van Griensven

Synopsis: Osteogenesis is a specially orchestrated complex of growth factors, biomaterials and cells. This symposium will deal with bone engineering in all aspects from the basis up to patients care. The key note speaker will address "Upscaling in bone engineering: from rodent to large animal to human". Several different animal models will be discussed. It is started with a drill hole model for fast screening in the orthopedic area. A calvarial dome model is shown for the oral-maxillofacial area. More clinically relevant non-union models are discussed in rats and sheep with different types of therapies such as growth factors, biomaterials, cells and combinations thereof. Dr. Karl-Heinz Schuckert will present clinical cases using customized biomaterials and growth factors in the field of oral-maxillofacial surgery.

He will point out pitfalls and decision criteria. Furthermore, Prof. Peter ten Dijke will elucidate the molecular biological mechanisms involved in signal transduction of bone morphogenetic proteins. He will discuss interactions of transcription pathways and how they are dysregulated in bone diseases that have either hyperostosis or osteopenia. These insights will be important to optimize bone engineering strategies. MicroRNA's are important upcoming molecules that have been shown to be involved in osteogenesis as well. Data will be presented on this new and highly actual topic. The rest of the symposium will be filled with presentations from submitted abstracts that fit to the topics presented here to have an up-to-date overview of current research themes in bone tissue engineering.

(47.KP) BMP-INDUCED OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

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1. Leiden University Medical Center

Bone morphogenetic proteins (BMPs) are used clinically to induce new bone formation in spinal fusions and long bone non-union fractures. However, large amounts of BMPs are needed to achieve these effects. BMPs were found to increase the expression of antagonists, which potentially limit their therapeutic efficacy. Yet, the relative susceptibility of osteoinductive BMPs to different antagonists is not well characterized. We found that BMP-6 is more resistant to noggin inhibition, and more potent in promoting osteoblast differentiation in vitro and inducing bone regeneration in vivo, when compared to its closely related BMP-7 paralog. Noggin was found to play a critical role as a negative feedback regulator of BMP-7 but not BMP-6-induced biological responses. Using BMP-6/7 chimeras, we identified lysine 60 as a key residue conferring noggin resistance within the BMP-6 protein. A remarkable correlation was found between the presence of a lysine at this position and noggin-resistance among a panel of osteoinductive BMPs. Introduction of a lysine residue at the corresponding positions of BMP-2 and BMP-7 allowed for molecular engineering of recombinant BMPs with increased resistance to noggin antagonism.
Fibrodysplasia ossificans progressiva (FOP) is a rare disabling disease characterized by heterotopic ossification for which there is currently no treatment available. FOP has recently been linked to a R206H mutation in the BMP type I receptor ALK2. Expression of the mutant ALK2-R206H receptor (FOP-ALK2) results in increased phosphorylation of the downstream Smad1 effector proteins and elevated basal BMP-dependent transcriptional reporter activity, indicating FOP-ALK2 is constitutively active. Mesenchymal cells expressing the FOP-ALK2 receptor are more sensitive to undergo BMP-induced osteoblast differentiation and mineralization. In vivo bone formation was assessed by loading human mesenchymal stem cells (hMSCs) expressing the ALK2-R206H receptor onto calcium phosphate scaffolds and implantation in nude mice.

Keywords. BMP, noggin

(47.O1) POROUS POLY(METHYL METHACRYLATE) CONSTRUCTS FOR OSSEOUS SPACE MAINTENANCE AND INFECTION CONTROL

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1. Rice University; 2. University of Texas Health Science Center at Houston

Introduction. Towards the goal of treating traumatic craniofacial injuries with significant bone/tissue loss and active/latent infection, our laboratory is developing a two-stage regenerative medicine approach that initially uses an alloplastic implant to maintain the defect space and to prime the wound site for later, definitive reconstruction. To fulfill these functions, a porous space maintainer is favorable because fibrovascular and other soft tissues can potentially grow into the surface pores to promote wound/tissue healing and anchor the implant to the host tissues. In addition, the porous structure provides open paths for the release of incorporated antibiotic drugs, potentially creating a surrounding therapeutic drug level to address local infections.
Methods. In this study, a clinically available gel matrix derived from gelatin was selected for incorporation with a clinical poly(methyl methacrylate) (PMMA) bone cement product. The morphology and porosity of the resulting constructs was characterized by scanning electron microscopy and microcomputed tomography, respectively, and the release of the antibiotic drug colistin in vitro was characterized by high performance liquid chromatography.
Results. The porosity of PMMA constructs ranged between 6.3±1.5 and 22.9±2.2%, depending upon the swelling ratio of the gel matrix and the gel weight percent in the construct. The antibiotic drug colistin, which was loaded in the gel matrix, was released over a period of 10 or 14 days, with an average release rate above 10 μg/ml per day.
Discussion and Conclusion: The results demonstrated the capability of the clinically available gel matrix product to produce porosity and control antibiotic release in the PMMA-based constructs, which may serve as an effective strategy to maintain a bony defect space until definitive reconstruction may be achieved.

Acknowledgements. This work was supported by a grant from the Armed Forces Institute of Regenerative Medicine (W81XWH-08-2-0032).
Keywords. craniofacial bone tissue engineering, infection control, antibiotic release

(47.O2) MOBILIZATION OF BONE MARROW-DERIVED ENDOTHELIAL AND PERIVASCULAR PRECURSORS IN AN ECTOPIC MODEL OF BONE REGENERATION

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1. National Instistute for Cancer Resarch, Genova, Italy, and Department of Oncology, Biology, and Genetics, University of Genova, Genova, Italy

Introduction. The classical concept of tissue repair is progressively transforming into a new idea of regeneration, which deeply focuses in stimulating the endogenous mechanisms through which transplanted stem cells promote a host response to regenerate the damaged tissue. We previously demonstrated that, following implantation of porous ceramic cubes seeded with mouse MSC into syngenic mice, the newly-formed bone was of host origin. In particular, we isolated and

characterized different endogenous populations, with endothelial and pericytic features, migrating from the host to the MSC-seeded ceramic. The aim of the project is to identify the host compartmental origin of the recruited cells.

Methods. Combinations of wild-type (WT) MSC/scaffold were implanted in syngenic WT mice, that were previously lethally irradiated and reconstituted with a Green Fluorescent Protein-positive (GFP+) bone marrow (chimeric mice). Implants were extracted at different time points and cells, harvested through enzymatic digestions, were phenotipically characterized in order to evaluate their anatomic compartmental origin.

Results. Using the bone marrow transplantation model, we demonstrated that about 35% of the cells with endothelial features, recruited after 7 days, derived from the recipient GFP+ bone marrow, and they shared some characteristics with the Endothelial Progenitor Cells (EPC), being CD133+, VEGFR2+, CD34+. The rest of the cells mobilized within the first 7 days appeared mature endothelial cells, being CD31+, and derived from the host surrounding tissues. All the CD146+ pericyte-like cells recruited after 11 days derived from the recipient bone marrow.

Conclusions. Our present experiments demonstrate that a coexistence of angiogenic and vasculogenic phenomena are involved in the bone regenerative process. Moreover, bone marrow-derived precursors with pericytic characteristics contribute to the development of the engineered tissue.

A better comprehension of the interactions between the recruited cells and the regenerative microenvironment are mandatory to elucidate cellular and molecular mechanisms behind the bone formation/regeneration process.

Keywords. mesenchymal stem cells, endothelial progenitor cells, bone regeneration, host response

(47.O3) VEGF-EXPRESSING MSC FOR RAPID VASCULARIZATION OF TISSUE-ENGINEERED BONE GRAFTS

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1. University Hospital Basel - Department of Biomedicine; 2. University Hospital Basel - Department of Surgery and Biomedicine

Introduction. Rapid vascularisation of tissue-engineered grafts is a major obstacle in the development of regenerative medicine approaches. Vascular Endothelial Growth Factor (VEGF) is a powerful angiogenic factor. However its dose must be controlled in the microenvironment around each producing cell to avoid aberrant vascular growth. To achieve controlled expression in vivo, we developed a high-throughput method to rapidly purify genetically-engineered progenitors secreting a desired level, by linking VEGF expression to that of a FACS-quantifiable cell-surface marker (truncated CD8a). Mesenchymal stromal/stem cells (MSC) are rich in osteoprogenitors, but their differentiation potential is gradually lost during expansion. Therefore, we sought to genetically modify MSC with minimal expansion to generate bone grafts with intrinsic vascularisation potential.

Methods. Primary human MSC were retrovirally transduced to express VEGF-CD8 or just CD8. Transgene expression was verified by ELISA and by FACS. Proliferation and multilineage differentiation potential were assessed in vitro, while bone formation and vascularisation were determined histologically 8 weeks after in vivo implantation.

Results. An optimized protocol allowed MSC to be transduced with high efficiency (80-95%) during the first plating, so that no extra expansion was necessary. Neither CD8- nor VEGF-expression impaired MSC proliferation and differentiation potential in vitro. In 2 donors VEGF actually appeared to improve in vitro osteogenic differentiation. In vivo vascularization potential was significantly increased in VEGF-expressing MSC. Although VEGF expression was heterogeneous, no aberrant angiogenesis was observed. Bone formation is currently being determined.

Conclusion. Optimized transduction allowed the genetic modification of primary MSC with minimal manipulation and no loss of biological potential, leading to improved in vivo vascularisation.

Acknowledgements: This work was supported by the EU FP7 Project MAGISTER (CP-IP 214685).

Keywords. bone TE, human mesenchymal stem cells, angiogenesis, gene therapy, VEGF

(47.O4) MOLECULAR ACTORS INVOLVED IN TUBULAR-LIKE NETWORK FORMATION IN HUMAN BONE MARROW STROMAL CELL AND HUMAN UMBILICAL VEIN ENDOTHELIAL CELL COCULTURES

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Introduction. Angiogenesis or vascularization is very critical for bone tissue engineering: oxygen and nutrient supply will be insufficient due to the lack of blood vessel network, cell loss during the early post-implantational stage causes failure of bone engineering and subsequent bone repair. Our previous studies showed that the coculture of Human Bone Marrow Stromal Cells (HBMSCs) and Human Umbilical Vein Endothelial Cells (HUVECs) could induce the self-assembly of HUVECs to form capillary tubes, which are normally considered as prevasculars. The current study aimed to investigate the communication mode and the main molecular actors required for the formation of capillary tubes in a two-dimensional direct-contact coculture of HBMSCs and HUVECs.

Methods. Quantitative real time polymerase chain reaction, immunofluorescence, western blot, as well as functional studies were applied to perform the studies at both protein and gene levels. Besides normal methods for characterizing cells, time-lapse videomicroscopy have been used for monitoring cell migration and for detecting colocalization of cellular proteins, respectively. Functional studies were also performed in order to indentify the roles of key vascular molecules in the communication of HBMSCs and HUVECs.

Results. Results show that the cocultures stimulate the migration of HUVECs and the tubular-like network

formation. Expression of vascular endothelial growth factor (VEGF165) was upregulated in cocultured-HBMSCs. VEGF165-receptor2 (KDR) and urokinase-type plasminogen activator (uPA) were upregulated in cocultured-HUVECs. Neutralization of VEGF165 blocked the migration, the tubular-like network formation and downregulated the expression of uPA and its receptor. Vascular endothelial-cadherin (VE-cad) colocalized with KDR and beta-catenin in cocultured cells, while neutralization of VE-cad did not affect the migration of cocultured-HUVECs but impaired the tubular-like network formation.

Conclusion. Cocultures upregulated the expression of VEGF165 in cocultured-HBMSCs; VEGF165 then activated uPA in cocultured-HUVECs, which might be responsible for initiating the migration and the tubular-like network formation. VE-cad appears to be necessary for the tubular-like network formation although it has weak effects on the migration of cocultured-HUVECs. This multiple dialogue between endothelial cells and osteoprogenitors could enhance both osteogenesis and angiogenesis after their implantation in a 3D matrice for bone tissue engineering applications.

Keywords. endothelial cells, vascularization, vascular endothelial growth factor, VE-cadherin

(47.O5) VARIABILITY OF CD146 SURFACE EXPRESSION AND HETEROTOPIC BONE FORMATION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Introduction. The surface antigen CD146 is supposed to be a marker for osteogenic pre-committed self-renewing bone marrow-derived mesenchymal stem cells (BMSC) which are capable of transferring, upon transplantation, a haematopoietic microenvironment to heterotopic sites. Aim of this study was to assess whether the number of CD146-positive cells within BMSC cultures from different donors correlates with the capacity to undergo in vitro osteogenesis and to form heterotopic bone in vivo.

Methods. Human BMSC (n=11 donors) were isolated from fresh bone marrow aspirates. To test the culture medium-dependence, 4 populations were directly seeded into two different media. BMSC of 10 donors were cultivated in standard medium until passage 2 or 3, were seeded on β-TCP granules (1x106/10mg) and transplanted subcutaneously for 8 weeks into immunodeficient mice. The effect of long term-cultivation was assessed based on 4 BMSC populations transplanted at passage 1, 3 and 5. The percentage of CD146-positive BMSC was determined by FACS-analysis. Osteogenic in vitro differentiation was performed at each passage.

Results. Human BMSC showed a broad donor variability regarding CD146 surface expression which additionally differed depending on the expansion medium. The average percentage of CD146-positive BMSC dropped during expansion from 40.3% in passage 1 to 1.3% in passage 5. Downregulation of CD146 during culture correlated with a declining growth rate at increasing passage. There was no correlation between CD146-expression and osteogenesis in vitro. The number of CD146-positive BMSC at transplantation correlated positively to the amount of ectopically formed bone, but haematopoiesis never occurred.

Conclusions. The number of CD146-positive cells within BMSC populations varied with donor, culture conditions and conferred improved bone formation. The presence of heterotopic bone with absent haematopoietic tissue pointed out that even high numbers of CD146-positive cells in non-clonal BMSC populations were not sufficient to transfer a haematopoietic microenvironment to heterotopic sites within eight weeks.

Keywords. CD146, human mesenchymal stem cells, heterotopic bone formation

(47.O6) VASCULARIZED TISSUE ENGINEERED BONE USING DIRECTLY AUTO-TRANSPLANTED MESENCHYMAL STEM CELLS AND BMP-2 IN THE AV-LOOP SHEEP MODEL

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Introduction. Today the transplantation of a free vascularised bone flap is the only therapeutic option to treat bone defects where a non-vascularized graft is not sufficient. To solve the problem of the initial missing of vascularization in engineered constructs the AV-loop rat model was established. The successful transfer to the sheep model (A) to generate axially vascularized tissue in clinically relevant extent has been demonstrated previously by complete axial vascularization of an implanted ß-TCP / HA (ß-tricalcium phosphate / hydroxyapatite) bone matrix. The present study now aims at engineering axially vascularized bone tissue.

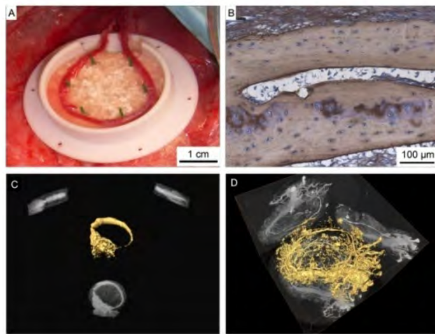
Methods. Directly auto-transplanted mesenchymal stem cells (MSC) with 60 µg/ml BMP-2 and without BMP-2 were implanted within a ß-TCP/HA-matrix into the AV-loop model. Axial vascularization was assessed by using micro-CT and magnetic resonance angiography (MRA) imaging. Standard histology and immunohistology staining were performed to evaluate bone generation.

Results. Histological evaluation revealed bone formation and osteoblastic cells in the newly formed bone parts close to the β-TCP / HA granules in both experimental groups (B). Trabecular-like bone in contact to the ß-TCP / HA granules and a higher amount of bone mass was detected in the BMP-2 group compared to the group without BMP-2. MRA demonstrated increased perfusion of the matrix within the chamber over time (C). Dense vascularisation was evidenced by micro-CT analyses and histology in both groups with no statistical difference between both groups (D).

Conclusions. Ectopic axially vascularized transplantable bone was engineered in this study with a clinically relevant volume in the sheep model without interim in vitro MSC expansion steps. In the future this concept could overcome the limitations of non-vascularized grafts in humans.

Acknowledgements. This study was funded by the University of Erlangen “ELAN Fonds”, “Xue Hong und Hans Georg Geis Foundation”and the Baxter Healthcare Corporation Inc., Vienna, Austria.

Keywords. AV-loop, BMP-2, bone tissue engineering, mesenchymal stem cells, sheep model, vascularization



(47.O8) NOGGIN RESISTANT PROTEIN N445T AT LOW DOSES MAINTAINED A HIGH REGENERATIVE CAPACITY

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Introduction. Bone morphogenetic proteins (BMP) and growth and differentiation factor (GDF) signaling is tightly regulated by BMP antagonists, (ie. Noggin) that can reduce the healing capacity of a BMP applied at the fracture site. A Noggin resistant GDF5 mutant (N445T) was identified and has shown promising healing capacity when administered at high doses. Using a lower dose, we hypothesized that N445T would improve healing compared to wtGDF5 and have a similar healing capacity to BMP2.

Methods. A 5mm segmental femoral bone defect was created in female Sprague Dawley rats (weight 236-298 g), stabilized using a unilateral external fixator. Within the defect, collagen scaffolds were loaded with 5 µg either N445T (n=5), wtGDF5 (n=8) or BMP2 (n=7). In vivo microcomputed tomography was performed at 2, 4, and 6 weeks postoperation. Outcome measures included mineralized callus volume (BV, mm3), total callus volume (TV, mm3), mineralized callus volume fraction (BV/TV), and bone mineral content (BMC, mg).

Results. BMP2 treated specimens (2wks: 6/7, 4wks: 7/7) achieved bony bridging earlier than N455T (4wks: 3/5, 6wks: 5/5) or wtGDF (4wks: 6/8, 6wks: 7/8). At 2 weeks, BV, TV and BMC of N455T specimens were significantly greater ($p<0.024$) (Table 1) than wtGDF5, but less than BMP2 ($p<0.008$). By 6wks, BV/TV of N445T was significantly greater than wtGDF5 ($p=0.0307$) or BMP2 ($p=0.0088$). However, BV, TV and BMC of N455T at 6wks were not significantly different than wtGDF5 or BMP2.

Conclusions. The data demonstrate that even at a low dose, Noggin resistant GDF5 (N445T) has an earlier regenerative capacity than wtGDF5, however bony bridging was achieved at similar time points. Defects with N445T achieved bony union at later time points than BMP2, although N455T had similar mineralized tissue volume and density at 6 wks. Even at low dosage antagonist resistant proteins are promising new drugs to enhance bone healing.

Keywords. Growth factors; bone healing; endochondral ossification

	2wks	4wks	6wks
GDF5			
BV	6.1 (3.7-7.9)	37.3 (19.1-51.4)	60.9 (53.4-80.4)
TV	15.5 (9.4-36.8)	56.3 (36.4-81.5)	74.5 (66.0-101.5)
BV/TV	0.44 (0.2-0.57)	0.66 (0.53-0.74)	0.78 (0.73-0.86)
BMC	3.6 (2.3-4.9)	24.0 (11.3-34.7)	44.2 (38.5-60.9)
N445T			
BV	12.7 (7.3-21.3)	55.3 (38.2-88.6)	77.8 (54.7-120.1)
TV	51.1 (33.8-63.3)	77.9 (56.1-128.8)	92.6 (60.5-149.6)
BV/TV	0.24 (0.15-0.34)	0.71 (0.65-0.77)	0.84 (0.80-0.90)
BMC	7.2 (4.1-12.2)	37.2 (26.1-54.8)	59.4 (41.0-86.7)
BMP2			
BV	40.8 (22.0-59.8)	67.3 (42.2-106.2)	80.9 (42.6-133.9)
TV	99.7 (76.4-132.6)	103.4 (76.6-139.5)	115.3 (84.7-178.8)
BV/TV	0.40 (0.28-0.52)	0.63 (0.53-0.76)	0.68 (0.50-0.80)
BMC	23.6 (12.8-35.0)	47.9 (26.9-75.1)	59.4 (41.0-86.7)

(47.O9) EFFICIENT BONE TISSUE ENGINEERING – COMPARISON OF DYNAMIC AND STATIC CULTURE CONDITIONS FOR SEVERAL TYPES OF BIOMATERIALS

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Bioreactors are expected to enhance efficiency of manufacturing tissue engineered products. In order to provide systematic data on the influence of dynamic culture in bioreactor on the cells seeded within 3D scaffolds, the same experimental system was used for observation of cells on several types of biomaterials.

Materials and Methods. Human bone derived cells (HBDCs) were cultured in differentiating medium under either dynamic or static conditions on six types of porous scaffolds: three various calcium-phosphates, calcite, corundum, polylactide/polyglicolides. Dynamic culture was performed in a Spinner Basket® bioreactor. Cell number (PicoGreen), viability (XTT), total protein content (BCA), cell differentiation/maturation (alkaline phosphatase activity or osteocalcin concentration) as well as cell localization within the scaffolds (Hoechst staining) and extracellular matrix presence (scanning electron microscope) were assessed in a five-weeks culture in vitro.

Results. Neither cell proliferation nor cell viability was effected by applying a bioreactor. The only exception was calcite, where significant medium alkalization resulted in almost 100% cell death under static conditions, while satisfactory results concerning cell proliferation, viability and function have been obtained in a dynamic culture. Cell differentiation/maturation reliance on the culture conditions was unequivocal, i.e. there were no differences, or more advanced differentiation in a population cultured in a bioreactor depending on the material used as a scaffold . The significant advantage which came from using a bioreactor was the uniform localization of the cells within the scaffolds. Additionally, the total protein content was much higher after the dynamic culture. Extracellular matrix (ECM) was appropriately distributed within the scaffolds.

Conclusion. Using a Spinner Basket® bioreactor promotes uniform cell and ECM distribution within 3D scaffolds and enhances total protein content in culture, while it does not influence cell proliferation or not necessarily - differentiation. In the case of scaffolds which change medium pH, application of a bioreactor may be indispensable.

Keywords. osteoblasts, scaffolds, culture conditions

(47.O10) IS THERE AN ALTERNATIVE TO ALLOGRAFT AND AUTOGRAFT IN BONE TISSUE ENGINEERING?

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Introduction. The healing of challenging bone defects can be problematic and often requires the use of allograft or autograft. However, problems can exist with the transmission of disease and limited supply, which poses the question, is there a synthetic alternative? This study will investigate the use of a synthetic bone graft substitute for use in bone tissue engineering. The fibre forming process of electrospinning has been used to fabricate a 3D scaffold composed of Poly(ε-caprolactone)(PCL) and an active moiety believed to enhance bone formation. The fibrous nature of the scaffold resembles the collagen fibres found in the extracellular matrix of bone thus providing a biomimetic structure for cell attachment and proliferation.

Materials and Methods. A 10% w/v polymer solution of PCL (Mn≈ 80,000) in acetone was electrospun using an applied voltage of 20kV, flow rate of 0.05ml/min and a needle-collector distance of 15cm. Fibres were collected on a grounded collector plate, eliciting a randomly orientated morphology. PCL scaffolds were functionalised with the active moiety. Human osteoblast cells were cultured under standard conditions on the prepared samples for up to 21-days. Cell morphology on the surfaces was evaluated using scanning electron microscopy (SEM). Samples were stained using DAPI, phalloidin and collagen I.

Results and Discussion. After 21 days, there was an overall increase in cell number on all substrates. Osteoblasts on the electrospun mats demonstrated superior spreading when compared to the control. DAPI/phalloidin staining of the cells demonstrated healthy cell structure with greater spreading of filopodia when compared to glass. The enhanced spreading of cells on the electrospun scaffolds can be owed to the microstructure of the scaffolds and the hydrophilicity. Electrospun mats mimic the natural extracellular matrix of bone.

Acknowledgments. The authors would like to thank Dr Zakikhani for his expertise, and the BBSRC and NIHR i4i for funding this research.

Keywords. Bone Tissue Engineering, 3D Scaffolds, Electrospinning, In vitro Cell Culture

(47.O11) REGENERATIVE FACIAL RECONSTRUCTION OF TERMINAL STAGE OSTEORADIONECROSIS AND OTHER ADVANCED CRANIOFACIAL DISEASES WITH ADULT CULTURED STEM AND PROGENITOR CELLS

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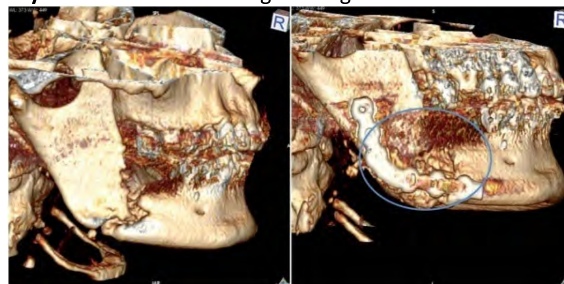
The application of multidisciplinary approaches to a deeper understanding of the underlying biological mechanisms involved in the healing process have been applied to the development of innovative techniques in craniofacial and long bone tissue regeneration and repair. Several surgical strategies have been developed by our group for craniofacial tissue regeneration. The procedures are based on the biology of wound healing enhanced by the use of platelet-derived growth factors in combination with bone marrow derived cultured cells. We present 3 cases of compassionate craniofacial treatments using Tissue Repair Cells (TRC, Aastrom Biosciences, SL), an autologous mixed population of bone marrow-derived stem and progenitor cells . TRCs are produced by culturing bone marrow mononuclear cells for 12 days under single-pass perfusion in an automated closed system. The TRCs contain mesenchymal, endothelial and monocyte/macrophage cells in significantly higher numbers than in native bone marrow.

- Case 1. Severe mandibular osteoradionecrosis grade IIIR with pathological fracture, oral and extraoral fistula and complete anaesthesia of the lower lip as well as severe facial nerve denervation treated with extensive bone excision, calcium triphosphate and TRC: Revascularization, arteriogenesis of facial and lingual vessels, high levels of bone formation, fracture consolidation and recovery of lip numbness. Electrophysiological evidence of facial nerve recovery. Implant insertion and fixed dental prosthesis in grafted area. Parotid gland reactivation.
- Case 2. Post-traumatic mandibular osteomyelitis with massive bone loss and total impairment of both mandibular nerves; nerve dissection with cell injection, grafting with TRC: complete nerve recovery and osteogenesis. Placement of multiple dental implants and fixed dental prosthesis in affected areas.
- Case 3. Severe maxillary atrophy. Treatment with TRC + calcium triphosphate for sinus elevation: early osteogenesis. Dental implants in grafted area; fixed prosthesis.

All three cases underwent multiple surgical treatments without success; the first two spent several months in hospital with large doses of antibiotics and other medical treatments. At the time of cell grafting, only one day of hospitalization was required for each one of the patients as well as 1 week antibiotics. There were no complications, swelling or pain. A second outpatient surgery was required for implant placement 4 months after the first surgery.

The results of this pilot study were published in the Journal of the American Association of Plastic Surgeons: Plast Reconstr Surg. 2010 Nov;126(5):1699-709.

Keywords. Bone Tissue Engineering



(47.O12) MODELS OF BONE TISSUE ENGINEERING: APPLICATION IN HUMANS

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The transplantation of autogenous bone is regarded as the gold standard in the surgical treatment of humans till today. Inevitable risks on the harvesting site as well as on the recipient site lead to critical consideration, especially in the treatment of multimorbid old patients. Here, the

new bone tissue engineering techniques offer alternatives.

Different models of bone regeneration will be presented (critical sized defects, vertical bone development).

Bone tissue engineering techniques require: 1. Matrices, 2. Cells, 3. Signaling molecules.

Platelets are the main regulators of the inflammatory phase and play an essential role in the proliferation and differentiation phase. Thus, a concentration of platelets (platelet-rich plasma – PRP) is used also in bone development. Furthermore, bone morphogenetic proteins (BMPs) are combined with modern scaffolds to improve the results of bone regeneration. In addition special surgical interventions have to be carried out to achieve optimal results.

Keywords. Osteogenesis, translational research, orthopedics, oral-maxillofacial surgery, growth factors, signal transduction

(47.O13) A CLINICAL STRATEGY TO CONCENTRATE ASPIRATED BONE MARROW FOR SKELETAL STEM CELLS TO ENHANCE SKELETAL REGENERATION

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Background. New bone to replace that lost as a consequence of trauma or disease is a considerable unmet clinical need and recent approaches have sought to harness the potential of stem cells for bone regeneration. Bone marrow aspirate (BMA) provides a promising autologous source of skeletal stem cells (SSCs) however previous studies have demonstrated that the concentration of SSCs required for robust bone regeneration is below that present in iliac crest BMA. Here we present a novel strategy to concentrate BMA for SSCs, clinically applicable for intra-operative orthopaedic use.

Methods. Iliac crest BMA was purchased from commercial suppliers and femoral canal BMA was obtained with informed consent from older patients undergoing total hip replacement. 5 to 40ml of BMA was processed via an acoustically-aided exclusion filtration process to obtain 2-8 fold volume reductions. SSC concentration and function was assessed by flow-cytometry and assays for fibroblastic colony-forming units (CFU-F) and multi-lineage differentiation. Seeding efficiency of concentrated and unconcentrated BMA (normalised to cell number) onto allograft was assessed.

Results. Iliac crest BMA from 15 patients was enriched for SSCs in a processing time of 15 minutes. Femoral BMA from 15 patients in the elderly cohort was concentrated up to 5-fold with a corresponding enrichment of viable and functional SSCs (Fig 1) confirmed by flow cytometry and assays for CFU-F. Enhanced osteogenic and chondrogenic differentiation was observed using concentrated aspirate and enhanced cell seeding efficiency onto allograft was observed as an effect of SSC concentration ml-1 aspirate confirming the utility of this approach for application to bone regeneration.

Conclusion. The ability to rapidly enrich BMA demonstrates potential for intra-operative application to enhance bone healing and offers immediate potential for clinical application to reduce morbidity in many scenarios associated with local bone stock loss. Further analysis in vivo is ongoing prior to clinical tests.

Keywords. Bone Marrow Aspirate, Skeletal Stem Cells, Bone Regeneration, Clinical Translation

(47.O14) UPSCALING IN BONE ENGINEERING: FROM RODENT TO LARGE ANIMAL TO HUMAN

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It is important to test tissue engineering approaches before they are transferred to the clinical arena. This testing is first performed in vitro. However, in vivo studies are necessary and demanded by federal institutions. The studies have to be performed according to animal welfare guidelines causing as least suffering as possible for the experimental animals involved.

Depending on the subject area of interest, different models have to be applied. For bone regeneration, several models are available. First, a simple drill hole model in the femur of rats is applicable for screening purposes. Having assessed the best concentration etc. the cells can be applied in an non-union femur defect model in the rat. In this model, a segment of the femur is removed and replaced by a silicon spacer for 4 weeks. After this period, the femur doesn't heal anymore. The stem cells are administered and first results are promising. In the last phase, a sheep segmental defect model in the tibia can be used. An intramedullary nail is inserted and locked in the tibia. A 2.5 cm segment is excised causing a non-union defect. The defect can be filled with substances and healing can be assessed by µCT and histology. Upon these studies, human applications might be considered. It is important to choose the optimal animal model for the hypothesis tested. It is also important to use sophisticated methods to obtain good quality data. Furthermore, one should have experience in applying the animal models to obtain consistent and reliable data.

Keywords. Osteogenesis, translational research, orthopedics, oral-maxillofacial surgery, growth factors, signal transduction

(47.O15) BIOMATERIAL IMPREGNATION WITH BONE MARROW ASPIRATE: DOES IT LIVE UP TO THE PROMISE?

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Introduction. The limited supply of autografts for spinal fusion has prompted extensive research on bone graft substitutes. So far, various biomaterials have been applied either stand alone or impregnated with blood or bone marrow aspirate to promote spinal fusion. Bone marrow aspirate harvested from the iliac crest is known to contain osteoprogenitor cells, which are supposed to differentiate into osteoblasts and form new bone at the desired fusion site. But according to literature, only 0,001% - 0,01% of bone marrow aspirate cells are in fact osteoprogenitor cells 1. So, if using bone marrow impregnated biomaterials according to manufacturer´s

specifications, are we really implanting what we think we are?

Experimental methods. Surplus material of bone marrow aspirate impregnated biomaterials remaining after cage and intervertebral space preparation for routine spinal fusion surgery was analyzed. Three different biomaterials (demineralised bone matrix - DBX, ChronOS® and HealOS® were impregnated according to specifications of the supplyer. Mesenchymal stem cell density was assessed after impregnation and the cell seeded biomaterials as well as the biomaterial-tissue interaction were investigated after implantation into the chick chorionallantoic membrane model using histomorphometrical methods.

Results and Discussion. Most of the cells in bone marrow aspirate were identified as erythrocytes while only a small fraction was identified as nucleated stem cells. After biomaterial impregnation, average stem cell density was 1,13 cells/mm2 (ChronOS®), 0,92 cells/mm2 (HealOS®) and 0,008 cells/mm2 (DBX). Only the marginal pores of the biomaterials were filled with cells (mostly erythrocytes) after biomaterial implantation. Demineralized bone matrix showed significantly lower cell seeding densities ($p<0,008$) and poor tissue integration in the HET-CAM test. ChronOS® and HealOS® showed good tissue integration and comparable but low cell densities. Despite the low cell counts, all cell impregnated biomaterials were able to stimulate angiogenesis at the implantation site.

Conclusion. Impregnation of biomaterials with bone marrow aspirate according to standard procedures for spinal surgery can only deliver very small amounts of osteoprogenitor cells to the implantation site. Although these constructs are able to stimulate angiogenesis, the number of osteoprogenotor cells delivered seems to small to have a significant effect on osteogenesis. Increasing cell density by centrifugation or expansion in culture might therefore be required to improve fusion results and prevent pseudarthrosis formation

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Keywords. bone marrow aspirate, stem cells, biomaterials, spinal surgery

(47.O16) CELL DIMENSION IS DIRECTLY CONTROLLED BY CELLULAR FUNCTION IN OSTEOGENESIS LINEAGE

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Introduction. Growth factors like BMP-2 are well known for their ability to induce osteogenesis (1). On the osteoblast (OB) lineage, mesenchymal stem cells (MSCs) gradually change morphology, clearly indicating their differentiation into OBs (2). However, the cell dimension during differentiation has yet to be examined. For this, we identified various states of cellular functions such as OB adhesion and differentiation and we studied the dimension of cells. We used functionalized surfaces with mimetic peptide of BMP-2 and those of adhesion (RGD) to obtain various levels of differentiation and adhesion. Then, we applied the optical 3D profiler system (OPS) approach to investigate the cell thickness and volume signature of different cell statuses. We focused on the characterization of induction status of OB compared with other cell statuses.

Materials and Methods. PET is a film (bi-oriented with thickness of 100 µm). This material was activated in two steps. RGD and BMP-2 mimetic peptides were dissolved in solution and covalently linked to the activated surface (3). For our study, MC3T3-E1 cells, a clonal pre-osteoblast mouse calvaria cell line, were cultured on grafted surfaces in α-MEM cell culture medium supplemented with 10% serum. Runx2 mRNA was quantified with the Syber green intercalating agent at different time points (12h and 24h). Cells were observed with scanning electron microscopy. Cell imaging with OPS involves raster scanning resulting in a profilometer image consisting of a tree-dimensional map of the apical cell surface.

Results. By targeting the serine/threonine kinase receptor Ia of BMPs with mimetic peptides, we observed an increase in osteoblast-essential genes, including the transcription factor required for in vivo bone formation Runx2. We further investigated the extracellular matrix thickness of induced OB and at rest. Our results reveal that cell dimension informs us on different statuses of OB adhesion, differentiation or induction (Figure 1).

Conclusion. The study of the OB dimension (thickness and volume) on various surfaces highlighted its direct correlation with OB function.

Acknowledgments. This work was supported in part by the "Région Aquitaine" as well as the "Agence Nationale pour la Recherche" (ANR) and Advanced Materials in Aquitaine (GIS).

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Keywords. Cell Dimension, Differentiation, BMPs, Extracellular matrix, surface modification

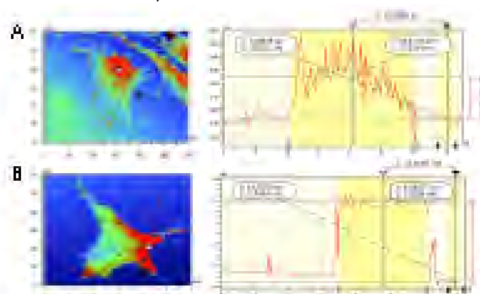

Figure 1. OPS micrographs showing single cell on different surfaces. (A) OB in rest cultured during 24h on PET and (B) induced OB cultured on PET grafted with BMP-2 mimetic peptide. In right, graph quantifies the cell height. Cell height varies with the OB function.

(47.O17) SCAFFOLD-GUIDED BONE REGENERATION OF CRITICALLY-SIZED DEFECTS IN SHEEP TIBIA

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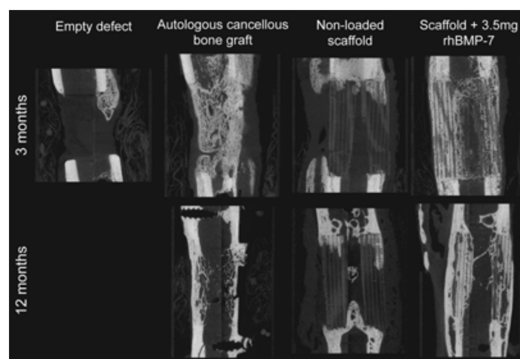
Introduction. Large bone defects can occur as a result of trauma, inflammation and tumour resection. The transplantation of autologous cancellous bone graft represents the current "gold standard" treatment, despite the harvesting co-morbidity and limited availability. An alternative scaffold-guided bone engineering approach is presented.

Methods. Composite scaffolds consisting of bioresorbable medical grade polycaprolactone with embedded beta tricalcium phosphate microparticles (80:20) were manufactured by fused deposition modeling, a rapid prototyping technology. The scaffolds were implanted in ovine, tibial, critical-sized segmental defects (3 cm). They were loaded with 3.5 mg of recombinant human bone morphogenetic protein 7 (rhBMP-7) and compared with (i) a non-loaded scaffold, with (ii) autologous cancellous bone graft and with (iii) an untreated defect (negative control). Torsional testing to failure, microcomputed tomography (microCT), histology, environmental SEM and small angle X-ray scattering (SAXS) were used to assess the regenerated bone after three and twelve months post surgery.

Results. The scaffold only group exhibited significantly lower torsional moment and stiffness, as well as bone volume and polar moment of inertia values measured by microCT, than the rhBMP-7 loaded scaffold treatment at both time points. The latter showed no significant difference compared to the current "gold standard" autograft at three months but increased significantly after twelve months. Histology analyses unveiled that scaffold structure provided a guide for fibroblasts and osteoblasts to assemble and arrange collagen fibers over distances beyond single cell sizes. The pre-aligned soft tissue guided lamellar bone formation. SAXS measurements confirmed the alignment of mineral particles in the proximity of the scaffold and lack of alignment in distant regions.

Conclusions. These results indicate that the rhBMP-7 loaded scaffolds are able to regenerate highly functional bone in a large segmental defect of the tibia. Further, it discusses the possibility of a structural benefit of scaffolds for soft tissue and mineralized tissue organization.

Keywords. biodegradable scaffold, bone morphogenetic protein 7 (BMP-7), critical segmental defect, sheep animal model



(47.O18) COLLAPAN IS A BIOCOMPOSITE MATERIAL FOR REPLACEMENT OF BONE DEFECTS AND ACTIVIZATION OF REPARATIVE OSTEOGENESIS IN BONE SURGERY

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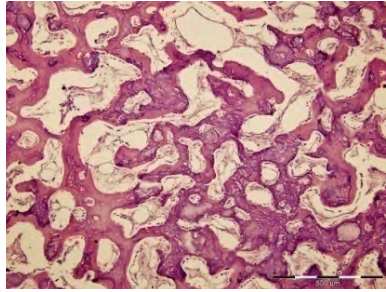
Methods. On the different kinds of animals (rats, rabbits, dogs) and experimental models a biocomposite material Collapan (synthetic nanostructured hydroxyapatite and collagen and immobilized antibiotics) had been implanted into the bone defect. Histological and microbiological techniques used.

Results. Has been shown that Collapan is a biocompatible, slowly resorbable, and produce antibiotic matrix, which is possessing osteoconductive and osteoinductive properties. At implantation in area of the fracture of long bones the Collapan has a pronounced effect on activization of processes osteogenesis. Most clearly it is shown in acceleration of formation, remodeling and maturing periosteal and intermediate callus. On the based of received the experimental data the Collapan has been applied in the clinic for more than 670 patients with complex treatment of the open and closed fractures, nonunions of long bones and also it was effectively used at the treatment of osteomyelitis, and in filling of defects of a bone after removal of benign tumors. It is necessary to note separately use of Collapan in a combination with PRP (Platelet-Rich Plasma) at 115 patients. It has allowed to reduce healing terms of slowly consolidating of fractures to 17 ± 2, 5 days, and nonunions of long bones to- 22 ± 3, 2 days in comparison with traditional methods of treatment.

Conclusion. Biocomposite material Collapan, which has osteoconductive and osteoinductive properties, is an ideal matrix for the immobilization of various growth factors and cytokines, biologically active substances, the cellular elements which contributes to enhance of reparative osteogenesis, and to prevention and to suppression of the infectious process.

Firm Intermedapatit making Kollapan, corresponds to the international standard ISO 13485-2008, number of the certificate is 2618

Keywords. biomaterials, reparative osteogenesis, hydroxyapatite



(fig.) Has been shown that Collapan is a biocompatible, slowly resorbable

(47.O19) NON-VIRAL GENE THERAPY (SONOPORATION) FOR ORTHOTOPIC BONE REGENERATION

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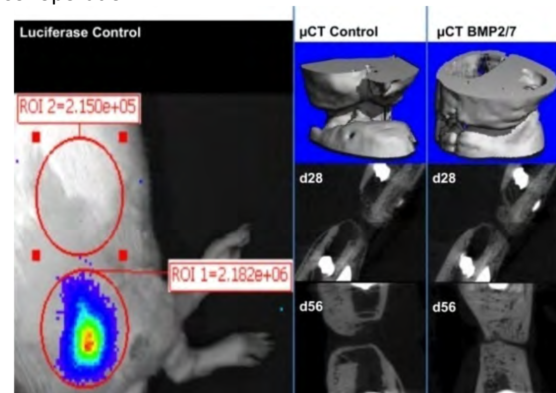
Non-viral transient gene transfer is a promising alternative treatment strategy in regenerative medicine. It has been shown that gene transfer of plasmid vectors encoding BMP growth factors mediate ectopic ossification in vivo. Furthermore, combination of BMP2 and BMP7 in a co-expression strategy has been proven highly osteoinductive, potentially through the in situ formation of BMP2/7 heterodimers. Sonoporative gene transfer, a minimal invasive non-viral gene transfer method using ultrasound in conjunction with contrast agents, has been shown to trigger expression of plasmid vectors in vivo circumventing adverse effects associated with viral gene transfer. Therefore, taking the above-mentioned advantages into account, the aim of this study was to design and test a BMP2/BMP7 co-expression plasmid for its regenerative potential in a femur non-union model through sonoporation.

Human BMP2 and BMP7 were cloned into a modified pVAX1 expression plasmid for co-expression. Induction of differentiation by the plasmid was tested in vitro in C2C12 cells. After successful in vitro testing, the plasmids were applied for bone regeneration in a rat femur non-union model (3mm defect, AO steel plate) 3 days post fracture using sonoporation. Animals received 5 treatments of therapeutic plasmid, contrast agent (Sonidel MB101) and ultrasound (Sonidel SP100) on 5 subsequent days. Control animals received luciferase plasmid instead of pVAX1-BMP2/BMP7, which allowed monitoring of gene transfer efficacy through bioluminescence imaging. All animals were imaged using an in vivo µCT on day 14, 28 and 56 post fracture. Bioluminescence imaging showed strong luciferase expression confined to the defect area. µCT images suggest enhanced bone regeneration with almost complete union in fractures sonoporated with BMP2/BMP7 co-expression plasmid. Therefore, we conclude, that sonoporative gene transfer is an effective non-viral method to mediate transient transgene expression in an orthotopic setting and that BMP2/BMP7 co-expression is a feasible method to enhance bone regeneration in non-union fractures.

Keywords gene therapy, bone regeneration, sonoporation



(47.O20) BIOMATERIALS RELEASING CALCIUM MODULATE OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS IN VITRO AND BONE FORMATION IN VIVO

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Introduction. Calcium is an important ion, mainly stored in the bone tissue. During bone remodeling, osteoclast's activity increases the extracellular Ca+2 concentrations. Ca+2 is a wellknown second messenger, capable of acting as a first messenger through the Calcium Sensing Receptor (CaSR). In bone tissue engineering, the use of biomaterials based on calcium phosphates is a successful strategy in bone tissue regeneration. Calcium phosphate glasses are bioactive and highly biodegradable. During degradation release Ca2+ and have osteogenic effects. This study aims to evaluate the calcium role in the osteoinductive effects of bioactive glasses.

Methods. Immunofluorescence: MSCs isolated from bone marrow's rat were stained with antibodies against Stro1, CD44, CD105, CD34, CD45, ALP, OPN and OC. qPCR: gene expression of ALP, BSP and Col I on MSCs was measured at 30min–21d. Migration Assay was evaluated against increasing Ca2+ concentrations using Boyden chambers. In vivo: PLA and PLA/G5 were implanted in a bone defect of rats for a month, Micro-CT and histological analyses were used to evaluate new bone formation after 15d and 30d of implantation.

Results. immunofluorescence demonstrated our MSCs have a characteristic expression profile for MSCs in control conditions: Stro1+, CD44+, CD105+, CD34- and CD45-. Additionally, MSCs were CaSR+. Afterwards, these cells were treated with Ctrl, Ca2+ and ostegenic medium for 7d and 15d. Results revealed Ca2+ induces the expression of ALP, OPN and OC. Concomitantly, the qPCR has shown overexpression of Col I, OC and BSP on cells treated with Ca2+. Migration assays demonstrated Ca2+ induces chemotaxis through CaSR's activation on MSCs. Micro-CT revealed mineralization of the newly formed tissues increases with the PLAG5 80% implantation.

Conclusions. our findings suggest the key role of calcium and CaSR on osteogenic differentiation of MSCs and in vivo bone formation elucidated a new target for developing new strategies for bone tissue engineering.

Keywords. Bone tissue engineering, Bone Formation, Bioactive glasses, Calcium

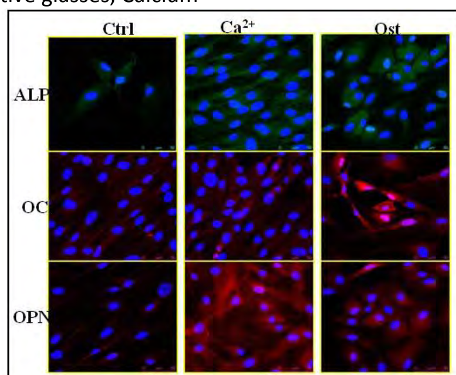

Fig.1: show the expression of markers of osteogenic cells (ALP, OC, and OPN) on MSC treated with Ctrl, Ca^{2+} and Ost mediums.

(47.O21) OSTEOGENIC PROPERTIES OF OSTEOBLAST-LIKE CELLS LOADED ON STARCH POLY(ε-CAPROLACTONE) FIBER MESHES IN A RAT CRITICAL-SIZED CRANIAL DEFECT

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Introduction. Mesenchymal stem cells are able to differentiate into the osteogenic lineage and together with a suitable scaffold, these cell-scaffold constructs can aid the bone regeneration process. A suitable scaffold could be starch poly(ε-caprolactone) (SPCL), which proved to be a biocompatible and biodegradable polymer in previous studies. Therefore, this study aims to assess the osteogenic properties of osteoblast-like cells loaded on SPCL fiber meshes in a critical-sized bone defect.

Methods. Bone marrow from 2 male Fisher rats was obtained and cultured in osteogenic medium for 7 days. Consequently, SPCL fiber meshes were loaded with 250.000 osteoblast-like cells and were implanted in a critical-sized cranial defect (of 8 mm) in male Fisher rats. SPCL fiber meshes without cells and empty defects were used as controls. Before implantation, samples were analyzed on morphology and early differentiation. After four and eight weeks of implantation, samples were analyzed on new bone formation by means of MicroCT, histological and histomorphometrical analysis.

Results. SPCL fiber meshes proved to have an average overall porosity of 62.5% with a mean pore diameter of 275 µm. Osteoblast-like cells adhered to the surface of the SPCL fibers and showed an alkaline phosphatase activity of 1.18 ± 0.47 nmol/ng/hr. MicroCT reconstructions showed that after 4 weeks of implantation, new bone formation in the implants loaded with osteoblast-like cells was 5.99 ± 4.09 mm3, while SPCL without cells showed 3.83 ± 1.70 mm3 of new bone formation. After 8 weeks of implantation new bone formation was 7.35 ± 3.15 mm3 in the implants containing osteoblast-like cells, while SPCL without cells showed 4.91 ± 2.51 mm3 new bone. Histological analyses are currently ongoing.

Conclusions. According to these results, besides an appropriate scaffold, osteoblast-like cells proved to be an important factor to aid the bone regeneration process in a critical-sized bone defect.

Keywords. osteoblast-like cells, starch poly(ε-caprolactone) (SPCL), critical-sized cranial defect

(47.P1) EFFECT OF ENDOTHELIAL CELLS ON OSTEOBLASTS IN CO-CULTURE

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Introduction. Osteoblast-endothelial cell co-cultures have been recently investigated for their potency in bone tissue engineering. Promising results have been already reported, but all the aspects of osteoblasts-endothelial cells interaction have not been fully recognized yet. The aim of this study was to evaluate the effect of endothelial cells on osteoblasts expansion and differentiation in co-culture.

Materials and Methods. Human bone derived cells (HBDCs) and human umbilical vein endothelial cells (HUVEC) were co-cultured in direct contact at the ratio of 1:1, 4:1 or 1:4 or were separated with an insert (ratio 1:1), on the tissue culture polystyrene. Cell number was determined by DNA measurement and by fluorescence-activated cell sorting. Cell cycle analysis was performed by propidium iodine staining. Alkaline phosphatase activity (ALP) was determined after 1, 4 and 7 days of culture. Expression of the ALP, collagen type I and osteocalcin genes was investigated by real-time PCR.

Results. Our observations confirmed the increasing ALP activity in direct co-cultures of osteoblasts-endothelial cells comparing to osteoblast monocultures and a lack of paracrine effect of endothelial cells on ALP activity of osteoblasts. Increasing ALP activity was found for all cell proportions although not to the same extent. In the presence of endothelial cells a significant enhancement of HBDCs' proliferation was observed, i.e. 12-fold growth of HBDCs number after a week comparing to the 4-fold one in a monoculture. Both HBDCs and HUVECs were still viable (found) after 7 days of co-culture, independently on the initial cell proportion. Both the ALP- and collagen type I- genes expression was up-regulated in co-culture, although no differentiating agents were added to the culture medium.

Conclusions. Our observations indicate that endothelial cells may support osteoblasts' expansion and up-regulation of the selected osteoblast differentiation markers.

Keywords. osteoblast, endothelial cells, co-culture, expansion

(47.P2) BONE REGENERATION INDUCED BY CHITOSAN rhBMP-2 ACTIVATED: A PRELIMINARY STUDY ON MAXILLARY DEFECTS ON PIGS

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Introduction. Previous work has been conducted to test chitosan film as coating of implantable materials and also

as carrier of recombinant human Bone Morphogenetic Protein 2 (rhBMP-2). We have reported the osteoconductive and osteoinductive properties of this coating on porous ceramic scaffolds implanted in rabbit calvaria delayed healing defect model (1). In order to test its performance in another anatomical site (suffering mechanical strength), we studied this coating in an animal model closer to human being (maxillary defects on pigs).

Materials and Methods. Porous ceramic materials (Bio-Oss®) were coated with chitosan/rhBMP-2. Both control and coated scaffolds were implanted in pig superior maxillary critical size defects (rhBMP-2 dosage: 1mg). Three months after surgery samples were harvested and studied by µCT and histology. All animal handling and experimental procedures were previously approved by the Animal Care and Use Committee of Hospital Clínico San Carlos, according to the EC guidelines for ethical care of experimental animals.

Results. Control non-implanted defects show lack of bone tissue due to the performed surgery and control non-coated scaffolds show low bone regeneration in the implant site. In contrast, chitosan/rhBMP-2 coated scaffolds show a high amount of newly formed bone. In addition, the histology shows how the newly-formed bone follows the porous structure of the tested material.

Conclusions. Results show that tested approach provides osteoinductive properties to clinically available porous implantable materials in a pig orthotopic implantation model which is quite similar to surgeries performed in humans. Thus data suggest the potential applicability of chitosan/rhBMP-2 film on clinically available materials.

Acknowledgements: Contract grant sponsors are Ministerio de Ciencia e Innovación, PET2008_0168_03 and CSD2009-00088.

References: 1- Abarrategi et al. Improvement of porous beta-TCP scaffolds with rhBMP-2 chitosan carrier film for bone tissue application. Tissue Engineering Part A. 2008;14(8):1305-19.

Keywords. Chitosan, rhBMP-2, Bone regeneration

(47.P3) DOSE DEPENDENT EFFECT OF GDF-5 ON PROLIFERATION AND OSTOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STROMAL CELLS

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Objectives. Bone morphogenetic proteins (BMPs) – with some of them also being referred to as growth-and-differentiation-factors (GDFs) – belong to the transforming growth factor-b (TGF-b) superfamily and have proven to be potent agents to induce bone formation. Currently, the range of growth factors being available for clinical bone regeneration applications is limited. Up to now for specific indications only BMP-2, BMP-7, and GDF-5 (BMP-14) are in clinical use. The aim of the present study was to investigate the dose dependent effects of GDF-5 on proliferation and osteogenic differentiation of human mesenchymal stromal cells (hMSCs).

Materials and Methods. HMSCs of 4 healthy donors were pooled and cultured with different amounts of GDF-5 for up to 28 days with and without osteogenic supplements. Proliferation was assessed by total DNA quantification. For evaluation of osteogenic differentiation cell-specific alkaline phosphatase (ALP) activity as well as calcium content was determined. Additionally, osteogenic differentiation was proofed qualitatively by fluorescent ALP staining and by Runx-2, ALP, and BSP-2 marker gene expression analysis using quantitative real-time RT-PCR.

Results. Compared to the control (cell cultures not conditioned with growth factor) a significantly ($p<0.05$) higher increase in cell number and specific ALP-activity could be observed for osteogenically stimulated cells that were cultured with 10, 50 and 200 ng/ml GDF-5, respectively. After 28 days of cultivation the expression of the osteogenic transcription factor Runx-2 was higher in non-stimulated cells treated with 100 ng/ml GDF-5 compared to controls. Additionally, GDF-5 significantly enhanced the expression of BSP-2 gene in osteogenically as well as in non-stimulated hMSCs and was even more effective than BMP-2.

Conclusions. We conclude that GDF-5 promotes osteogenic differentiation of hMSCs as shown by stimulatory effects on the expression levels of crucial bone differentiation markers. These findings make GDF-5 an attractive growth factor for clinical bone regeneration applications.

Keywords. mesenchymal stromal cell, GDF-5, osteogenic differentiation

(47.P4) THE EPC (ENDOTHELIAL PROGENITOR CELLS) FRACTION CONTAINED IN THE BMC (BONE MARROW MONOCYTE CELL) POPULATION IMPAIRS THE OSTEOGENIC DIFFERENTIATION

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1. AO Foundation

Introduction. Endothelial cells-osteoblast co-culture are known to induce a synergy of cell differentiation and activity1-2. Bone marrow is a rich source of mesemchymal stem cells (MSC), but EPC are also present. MSC can develop an osteogenic phenotype while EPC will differentiate into endothelial cells. The aim of our study was investigate the effect of the EPC present within the whole BMC population on the MSC osteogenic differentiation.

Methods. Human BMC of 5 donors (KEK Bern 126/03) were isolated by density gradient centrifugation (Ficoll). CD133+ and CD34+ cells were further depleted from the BMC using magnetic beads (Miltenyi), the resulting population was named MM. Identical numbers of BMC and MM cells were seeded and culture over 28 days in classical osteogenic medium (10nM Dexamethasone), or in autologous growth factor medium (PRGF). PRGF was prepared from thrombocyte concentrates resuspended in PBS (2x106platelets/mL). Cell growth was assessed by DNA quantification, osteogenic differentiation by real time RT-PCR, and ALP activity. Matrix mineralization was estimated by Ca45 incorporation.

Results. In both culture media, the full BMC grew faster than MM. However, if PRGF showed an overall superiority for both populations' cell growth, cell differentiation was

much higher in Dex+ medium, for both BMC and MM. MM showed high up-regulation of all tested osteogenic marker genes in both media. Cell differentiation was confirmed by ALP activity that was found higher in MM compared to BMC in both media, with higher values for Dex medium. Matrix mineralization analyses confirmed these results.

Discussion. The EPC present in full BMC may grow faster than the MSC (especially in PRGF3) and impair the proportion of cell with osteogenic potential. These 2 cells population also might be in early phase of differentiation to promote co-differentiation at this stage.

1-Villars et al., (2002), 2-Hofmann et al., (2008). 3-Lippross et al, (2011) submitted

Keywords. Platelet rich plasma, PRGF, bone marrow mesenchymal cells, endothelial progenitor cells

(47.P5) EFFECTS OF PLATELET RICH PLASMA ON BONE REGENERATION IN RABBIT CALVARIAL DEFECTS IN COMBINATION WITH A SILICON STABILIZED HYDROXYAPATITE TRICALCIUM PHOSPHATE SCAFFOLD (SKELITE®)

El Backly RM (1), Mastrogiacomo M (1), Zaky SH (2), Canciani B (1), Saad MM (3), Eweida A (4)

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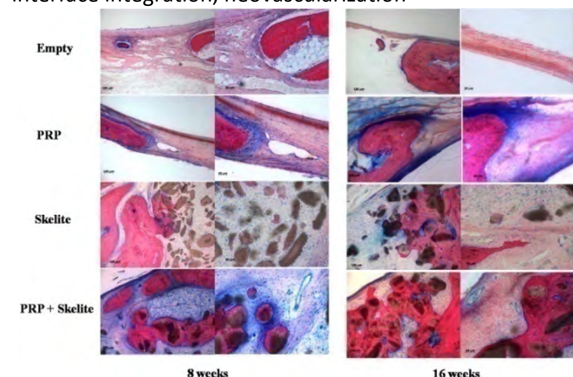
Introduction. The use of platelet rich plasma (PRP) in bone regeneration remains highly controversial. It has been shown that its association with different osteoconductive materials can greatly influence the effects of PRP on local tissue environments. Upon their activation, platelets release several chemokines and growth factors that trigger numerous cell processes at the site of injury. In the present work, we evaluated the effects of lyophilized PRP on bone regeneration of calvarial defects when associated with a silicon stabilized hydroxyapatite tricalcium phosphate scaffold (Skelite).

Materials and Methods. 20 New Zealand white rabbits were used in which 1cm defects in the calvaria were created. The periosteum was completely stripped from the immediate and far vicinity of the defect. Two parietal defects were made, 0.5 cm apart. Each defect was either packed with allogenic PRP gel or skelite particles, or a mixture of skelite and PRP gel or left empty. 5 Animals were sacrificed after 8 and 5 after 16 weeks. Specimens were processed histologically for undecalcified resin embedding after microCT analysis of all samples.

Results and Conclusions. By microCT analysis, bone regeneration in the defects containing skelite and those with skelite+PRP was shown to have enhanced from 8 to 16 weeks. The addition of PRP did not significantly enhance bone regeneration, yet in-depth analysis showed that the presence of PRP enhanced integration at the scaffold-bone margin interface. By histology, defects with both Skelite and PRP were noticeably well-vascularized, showed a higher amount of osteoid tissue throughout the defects and more regular collagen fibers where bone formation had not yet occurred. Results showed that the addition of PRP in conjunction with skelite did not directly contribute to bone regeneration, but could influence the local tissue microenvironment by enhancing neovascularization, collagen deposition, and by creating a bridging interface between the scaffold and the bone.

Keywords. platelet rich plasma, bone regeneration, interface integration, neovascularization



(47.P6) THREE-DIMENSIONAL CULTIVATION OF SINGLE-CELL-DERIVED HTERT-EXPRESSING HUMAN MESENCHYMAL STROMAL CELLS ON CANCELLOUS BONE ALLOGRAFT

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The combined use of bone marrow-derived mesenchymal stromal cells (MSCs) and cancellous bone allografts (CBA) denotes a promising strategy for bone regeneration. However, senescence and the demand for high cell numbers limit the applicability of primary MSCs. The lifespan of MSCs can be extended by ectopic expression of human telomerase reverse transcriptase (hTERT). In this study, we evaluated the suitability of single-cell-derived, hTERT-expressing MSCs for three-dimensional (3D) static cultivation on CBA with respect to cell adherence, proliferation and osteogenic differentiation.

Single-cell-derived hTERT-expressing MSCs were cultured in basic medium containing 10% fetal calf serum with or without osteogenic supplements (OS) for up to 21 days. 3D cultivation was performed on peracetic acid-ethanol sterilized CBA cubes (DIZG, edge length ~ 5mm). Two-dimensional (2D) cell monolayer cultivation served as control. Cell proliferation was assessed by measuring DNA content. Osteogenic differentiation was evaluated by cell-specific alkaline phosphatase (ALP) activity assay, expression analysis of the osteogenic marker genes ALP, osteopontin (OPN) and osteocalcin (OC) by real time RT-PCR, and by quantification of OPN and OC protein levels. Immunofluorescence microscopy was performed to demonstrate cellular adhesion and cellular viability on CBA. Overall statistical significance was calculated by use of 2way-ANOVA using Bonferroni correction with n=6.

3D-cultivation of hTERT-MSCs stimulated osteogenic differentiation as observed by increase of cell-specific ALP activity and OC protein level compared with 2D cultivation after 21 days ($p<0.05$). Combined use of OS and 3D-cultivation decreased proliferation of hTERT-MSCs ($p<0.001$). Osteogenic marker gene expression was

elevated in 3D versus 2D-cultivated hTERT-MSC in the presence of OS. Fluorescence microscopy demonstrated viability, adhesion and cellular distribution of hTERT-MSCs within the interconnected CBA pores. 3D-cultivation of single-cell-derived hTERT-MSCs on CBA stimulates osteogenic differentiation compared to 2D monolayer culture. Further studies will address the effects of additional biophysical differentiation stimuli by dynamic 3D-cultivation of hTERT-MSCs.

Keywords. mesenchymal stromal cell, immortalized, three-dimensional cultivation, human cancellous bone allograft

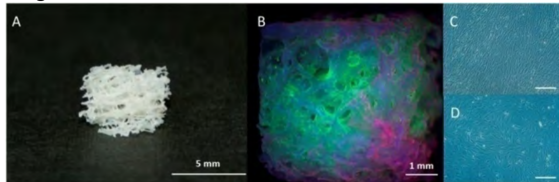

Fig. 1 Macrograph of human cancellous bone allograft, 3D Live-Dead-Stain of hTERT-MSCs, 2D hTERT-MSCs

(47.P7) COMPOSITE CHITOSAN-HYDROXIAPATITE WET-SPUN MICROFIBERS: STRUCTURAL, MECHANICAL AND BIOLOGICAL EVALUATION

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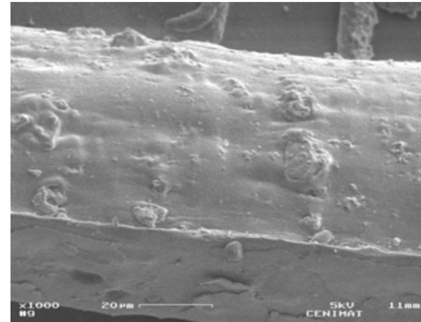
Introduction. Composite materials have the advantage of combining the useful properties of its constituents. This paper reports the production and characterization of composite microfibers of two biocompatible and biodegradable materials: chitosan – a linear polysaccharide with a known wound healing accelerating effect - and hydroxyapatite – an osteoconductive ceramic that resembles the most the composition of the mineral phase of human bone.

Methods. Using the Wet Spinning technique, five types of chitosan fibers containing different amounts of hydroxyapatite were produced. Different coagulation and drying baths were tested, yielding fibers that were characterized by tensile tests, scanning electron microscopy and diameter measurement. This analysis led us to choose a coagulation bath with 70% NaOH and 30% methanol and a drying bath of 100% methanol for subsequent studies. Then, scaffolds were constructed with the composite fibers (having 0%, 10%, 20%, 30%, 40% and 50% HAp). These fibers were subjected to tensile tests, SEM observation, cytotoxicity analysis using the MTT test and osteoblasts culture.

Results. Tensile tests revealed that composite chitosan-hydroxyapatite fibers have mechanical properties that deteriorate with increasing HAp content. The elastic modulus varies from 8 to 5 GPa, tensile strength from 130 to 60 MPa and tensile strain from 5 to 2%. Fiber diameter was 74±8 micrometer. Cytotoxicity tests revealed relative cell viabilities in excess of 100% for all fiber compositions. SEM observation of cells seeded on the scaffolds showed good cell growth and adhesion. Picture shows SEM micrograph of chitosan + 20% HAp microfiber.

Conclusions. Composite chitosan-hydroxyapatite microfibers have good mechanical properties and in vitro cell support. They can be assembled into 3D scaffolds of any desired shape, making them good candidates as bone substitutes.

Keywords. Chitosan, Hydroxyapatite, Microfibers, Scaffold, Bone



(47.P8) STRUCTURAL, MECHANICAL AND BIOLOGICAL EVALUATION OF NEW COMPOSITE SCAFFOLDS FOR BONE TISSUE ENGINEERING

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Introduction. A composite of poly(ε-caprolactone) (PCL) and hydroxyapatite (HAp) synergistically associates the elasticity of the polymer with the stiffness and osteoconductivity of the ceramic. We implemented a simple technique to produce 3D scaffolds with high porosity and interconnected pores of controllable size. Here, we analyse the influence of porosity and HAp content on their mechanical properties and in vitro biological performance as supports for bone regeneration.

Methods. PCL films are obtained through solvent (acetone) evaporation of solutions spread over a glass surface. HAp and NaCl may be incorporated in the solution. Multilayers of polymeric films and NaCl are hot pressed. Salt leaching leads to porous 3D structures. The amount of salt used controls porosity (P). Initial modulus (E) and critical point (σ) were determined from stress-compressive strain curves. After relaxation from the first compression, a second compression was performed. Osteoblast cultures were used for the MTT viability test and observed by SEM.

Results and Discussion. Results for first compressions are summarized in the Table. The NaCl to PCL mass ratio (xNaCl) is specified as the sum of the part incorporated in the film and the part interspersed between disks. Permanent deformations occur mainly during the first compression. E decreases with P due to the decrease of scaffold density. When HAp is absent, structure and mechanical properties benifit from the incorporation of salt in the film, however for a high HAp to PCL mass ratio

(yHAP) this is irrelevant. When yHAP is increased up to 0.30 the ceramic reinforcement is translated by an increase of E. The subsequent decrease is probably due to failure of the weak polymer-ceramic bond. MTT tests indicated relative viabilities around 100%. SEM revealed cells adherent to the scaffold.

Conclusion. Our technique is adapted to tune composition and structure of composite scaffolds for bone regeneration.

Keywords. Poly(ε-caprolactone) (PCL), Hydroxyapatite (HAp), Salt-leaching, hot-pressing, osteoblasts

	xNaCl	*yHAP*	*P*	*E/MPa*	*σ/kPa*
S1	5.0+5.7	0	84.2±1.5	1.10±0.15	65±5
S2	5.0+12.0	0	86.7±1.2	0.84±0.09	76±6
S3	5.0+30.9	0	91.5±0.3	0.32±0.02	84±5
S4	5.0+12.0	0.15	84.9±0.9	1.07±0.27	78±5
S5	5.0+12.0	0.30	81.8±3.7	1.56±0.37	69±4
S6	5.0+12.0	0.50	83.0±1.2	1.37±0.27	75±6
S7	0.0+17.0	0.50	79.2±2.6	1.35±0.18	69±9
S8	0.0+17.0	0.70	79.9±2.3	1.13±0.06	71±8
S9	0.0+17.0	1.0	78.9±1.0	0.98±0.04	78±8

(47.P9) NEUROTROPHIC POTENTIAL OF OSTEOBLAST LINEAGE: OSTEOBLAST AND SENSORY NEURONS COCULTURE

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Introduction. One of the aspects that support the neuro-osteogenic concept is the presence of nerve fibers, immunoreactive to different neuropeptides and neurotransmitters, in bone microenvironment at the embryonic stage and adulthood [1,2]. Therefore, a better understanding of the mechanisms underlying the involvement of bone innervation/re-innervation in bone homeostasis and bone regeneration will provide new insights to better control the morbidity associated with fracture healing. This study aims to establish a coculture system for osteoblasts and sensory neurons to explore the cellular mechanisms behind the functional relationships between bone and the peripheral nervous system, namely the neurotrophic potential of osteoblast lineage.

Methods. Since osteoblasts (MC3T3-E1 cell line) and sensory neuronal cells (ND7/23 cell line) are from different origins, prior to the cocultures we established the optimal conditions (cell density, serum concentration and time of coculture) to ensure viability of two cell types and the maintenance of their phenotypes in coculture. Afterward, the coculture system was assembled and the differentiation of sensory neurons was evaluated by the presence or absence of neurites as well as the measurement of neurites' length.

Results and Discussion. The optimization of the coculture showed that 1000 sensory neuronal cells/cm2, 10000 osteoblastic cells/cm2, 0.5% serum and 24h of coculture were the ideal conditions for the maintenance of the viability and phenotype of osteoblasts and sensory neurons in coculture. The results showed the sensory neuronal differentiation and neurites outgrowth was enhanced when cells were exposed to osteoblasts in early stages of osteogenic differentiation suggesting that osteoblasts might produce factors holding neurotrophic potential able to induce neurites outgrowth. In other hand, the percentage of differentiated sensory neuronal cells was promoted when the cells were exposed to osteoblasts at later stages of differentiation.

Acknowledgments. This work was funded by FCT project:PTDC/SAU-OSM/101469/2008

[1]Franquinho, F. et al. 2010.

[2]Sisask, G. et al. 1996.

Keywords. Bone innervation, Osteoblasts, Sensory neurons

(47.P10) FUNCTIONALIZED MESOPOROUS BIOMATERIAL PTHrP LOADED AS AN OSEOUS REPAIR INDUCTOR.

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Introduction. Parathyroid hormone-related protein (PTHrP) (107e111) (osteostatin) is an important endochondral bone development regulator; we loaded onto mesoporous silica biomaterial SBA-15. Our objective was asses this novel biomaterial C8 functionalized in bone repair when is implanted into a cavitary defect in the rabbit femur.

Methods. Cavitary defects are made in femurs of New Zealand rabbits (6 months of age; n=6). Where the samples are implanted biomaterials: SBA-15 without PTHrP (lateral right side), the SBA-15 with PTHrP (medial right side), and SBA-15 functionalized only with C8 (lateral left side) and SBA-15 functionalized with C8 and PTHrP loaded (medial left side). After 8 weeks implantation, the samples were studied in histology, immunohistochemistry (RunX2, Osteopontin OP, Osteocalcin OC) and micro-computerized tomography (µCT) analysis.

Results. In µCT and histological studies bone formation was more evident in PTHrP-loaded biomaterials. In SBA15-C8 group the abundant fibrous capsule formation were observed. The bone repair shows travecular bone formation and hypertrophic scarring cortical area to the outside of the implant. In the PTHrP-loaded capsule allow the osteoid tissue formation around of the biomaterial. RunX2 significant differences were found in PTHrP loading groups where this marker is increase. In a similar way to the expression of Runx2 the expression in the marker OC was observed increase in PTHrP loading groups.

Conclusions. SBA-15 biomaterial seems to be suitable as a bioactive peptide-releasing matrix; PTHrP loading improves bone repair in all groups. This finding could be these biomaterials as potential formulations for their use in regenerative medicine.

Acknowledgements. This work has been supported by CAM(S-0505/MAT/0324) and CAM(S-2009/MAT/1472)

Keywords. Parathyroid hormone-related protein (PTHrP), bone, biomaterial, mesoporous material, bone repair.

(47.P11) VISUALIZATION OF CELLULAR ECM PRODUCTION IN MICROPOROUS NANOCELLULOSE SCAFFOLDS BY NON LINEAR MICROSCOPY

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Introduction. Nano-cellulose biosynthesized by bacteria is an attractive 3D scaffold biomaterial for tissue engineering applications due to its biocompatibility and good mechanical properties. The objective of this study was to investigate microporous nano-cellulose as a potential scaffold for differentiation of osteoprogenitor cells and to visualize their differentiation behavior using new microscopy methods.

Materials and Methods. Wax porogens were introduced during the cultivation process of the bacteria. Upon culture completion the porogen was leached out leaving an interconnected microporous structure. Such structures are thought to facilitate osteoprogenitor cell ingrowth and formation of tissue specific extracellular matrix (ECM). ELISA was used to quantify the collagen type I production and alkaline phosphatase (ALP) activity was measured to verify osteoblastic differentiation. Coherent Anti-Stokes Raman Scattering (CARS) microscopy was used to visualize cell integration, proliferation and differentiation, while Second Harmonic Generation (SHG) microscopy was used for imaging cellulose and collagen fibers. Samples were also analyzed using immunohistology.

Results. The presence of collagen type I fibers were verified and quantified by ELISA, and the ALP activity verified osteoblastic differentiation. The results show that osteoprogenitor cells can proliferate and differentiate into microporous nano-cellulose scaffolds. CARS and SHG microscopy, in addition to visualize the cells in the pores also provide evidence of extracellular matrix production.

Conclusions. We have developed new bioimaging tools; CARS and SHG, enabling visualization of osteoblastic differentiation and thus contributed to the development of optimal 3D architecture of scaffolds

Keywords. Nano-cellulose, Cell Differentiation, Non Linear Microscopy

(47.P12) BMP SIGNALLING IN OSTEOBLAST PRECURSOR CELLS: INTEGRATION OF BIOCHEMICAL AND MECHANICAL STIMULI

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Introduction. Bone homeostasis and regeneration require differentiation of mesenchymal precursor cells, a process that is strongly influenced by mechanical and biochemical boundary conditions. One major pathway known to be associated with osteogenic differentiation is the bone morphogenetic protein (BMP) signalling cascade. BMPs are secreted growth factors that induce signal transduction by binding to two types of transmembrane serine/threonine kinase receptors. Subsequently, Smad and non-Smad pathways are triggered that regulate transcription of specific target genes. Beyond this biochemical influence, mechanical forces, such as loading- or shear forces, have been shown to enhance the osteogenic capacity of precursor cells. Our study aims for a molecular understanding of the crosstalk between BMP signalling and mechanotransduction pathways during osteogenic differentiation.

Methods. Human foetal osteoblasts (hFOB) were seeded on 3 dimensional collagen scaffolds and subjected to cyclic-compressive loading and BMP stimulation in a bioreactor device.

Results. Here we demonstrate that the BMP signalling cascade is directly addressed by both, ligand and mechanical stimuli in vitro. As expected, BMP2 induced Smad1/5/8 phosphorylation in hFOBs. In addition, in combination with mechanical loading early BMP signalling events were altered. In line with this, we also observed a regulation of several BMP target genes.

Conclusion. Taken together, we postulate a direct regulation of immediate early signalling events downstream of the BMP receptors by mechanical signals. These findings highlight the interplay of chemical and mechanical factors during cell differentiation. Answering the question how mechanosensitive signals are integrated into the BMP pathway is now subject of current research.

Keywords. mechanical loading, BMP signalling

(47.P13) IN VITRO AND IN VIVO BONE REGENERATION INDUCED BY ENCAPSULATED BMP-2

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Introduction. Commercially available formulations of BMP-2 are administered in a collagen sponge with burst release. High doses of this growth factor are needed due to short half-life. Therefore, we developed injectable silicate (Si) coated starch-poly-caprolactone (SPCL) microparticles for sustained BMP-2 delivery needing lower doses.

Methods. Si-SPCL microparticles loaded with BMP-2 were administered using fibrin glue to a 2 mm femoral drill hole defect in a rat. The observation times were 2 and 4 weeks, at which µCT and histology were performed.

Results. BMP-2 was rapidly released from the microparticles during the first 12 hours, followed by sustained release of 85.4ng/ml for up to 10 days. Administration of these Si-SPCL loaded BMP-2 particles to C2C12-cells showed increased alkaline phosphatase (ALP)

activity, mineralization and osteocalcin promoter activity. Moreover, human adipose stem cells exposed to the microparticles produced high amounts of ALP. Osteocalcin and osteopontin were immunohistochemically detected. Mineral deposits consist of calcium and phosphate as assessed by energy dispersive spectroscopy using scanning electron microscopy. RunX2, osteocalcin and osteopontin mRNA levels were significantly expressed. Injection of the Si-SPCL BMP-2-loaded particles intramuscularly using a fibrin hydrogel revealed bone formation at 4 and 8 weeks in rats. Without Si this formation was less. Administration to a rat femur drill hole revealed rapid closure at 2 weeks, whereas the empty ones or fibrin only ones were still open. Moreover, Si-coated particles are superior to those without Si as assessed by µCT.

Conclusion. The data suggest silicate-coated starch-poly-caprolactone microparticles are suitable carriers for the incorporation and controlled release of growth factors and induce osteogenesis. Specifically, they reduce the amount of BMP-2 needed and allow more sustained osteogenic effects in vitro and in vivo.

Keywords. SPCL microparticles, BMP-2, adipose derived stem cells, in vivo bone formation

(47.P14) WHARTON´S JELLY STEM CELLS AS A SOURCE FOR ARTIFICIAL BONE TISSUE FOR MAXILLOFACIAL TISSUE ENGINEERING

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Introduction. Several types of stem cells have been isolated from the human umbilical cord. Among them, Wharton´s jelly stem cells (WJCs) have been extensively studied due to their easy access to obtain and due to their high proliferative capability. In this context, the generation of an efficient substitute of bone tissue using autologous stem cells may be a useful tool for in vitro studies and for the generation of artificial bone in the laboratory. In this work we have successfully diferentiated WJCs into bone-like cells for use in tissue engineering protocols.

Materials and methods. Primary cultures of WJCs were generated by using small explants of the umbilical cord and cells were seeded in two-dimensional chamber slide culture systems. After this, WJCs were induced for 24 hours, 7, 14 and 21 days using osteogenic medium. Osteoblast-like cells were analyzed after all induction periods using histochemical and immunofluorescence protocols with alizarin red S stain and alkaline phosphatase antibodies, respectively.

Results. WJCs induced cultures showed the presence of calcic deposits after 21 days of induction at the extracellular matrix which were verified by histochemistry with alizarin red S stain. Immunofluorescence for alkaline phosphatase showed that WJCs had a high reactivity after 21 days of induction, although after 7 and 14 days of osteo-induction flourochrome signal was moderate.

Conclusions. Our results showed that WJCs can efficiently transdifferentiate into bone-like tissue after 21 days of induction. These results suggest that WJCs could be used for bone regeneration in maxillofacial surgery and regenerative medicine.

Keywords. Wharton´s jelly, transdifferentiation, bone

48. MESENCHYMAL STEM CELLS (MSC)

(48.O1) BIOCHEMICAL STUDIES ON THE COMPOSITION AND STRUCTURE OF THE EXTRACELLULAR MATRIX OF DEGENERATE INTERVERTEBRAL DISCS RECONSTITUTED BY THE INJECTION OF ALLOGENEIC BONE MARROW DERIVED STRO-3+ MESENCHYMAL PROGENITOR CELLS

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Introduction. Our previous radiological, MRI and histological studies had shown that a single intra-discal injection of STRO-3+ Mesenchymal Progenitor Cells (MPC) into degenerate ovine discs restored their integrity. In this study we sought to identify the composition and structure of the new extracellular matrix (ECM).

Methods and Materials. The lumbar L3L4, L4L5 and L5L6 discs of 6 adult sheep were injected with Chondroitinases-ABC (cABC) to induce degeneration. The lumber L2L3 discs were used as normal controls. As shown previously after 3 months discs were degenerate. MPC (0.5 million) + hyaluronan (HA) was then injected into the L3L4 discs. The L4L5 discs were not injected and L5L6 discs received HA alone. Six months later animals were sacrificed, spines removed and the individual lumbar discs cut in a horizontal plane. The nucleus pulposus (NP) and annulus fibrosis (AF) of each disc was dissected free of the vertebral bodies, diced and lyophilized. Aliquots were either digested and/or hydrolysied to determine the glycosaminoglycan (GAG), DNA and collagen content. Proteoglycan subunits (PGs) were isolated and subjected to Sepharose-CL2B gel exclusion chromatography.

Results. The GAG contents of cABC and HA injected disc NPs were less than the control disc values ($p < 0.05$). In contrast, the GAG of the NP and NP+AF of MPC injected discs were not significantly different (NSD) from control GAG levels. With respect to the MPC collagen and DNA, AF collagen was higher and DNA lower than cABC or HA disc AF ($p < 0.05$) but again NSD from controls. PGs isolated from the NPs of MPC injected discs consisted of several distinct populations both equivalent to and larger than the hydrodynamic size of control PGs ($p < 0.05$).

Conclusions. This study confirms that injection of MPC into degenerate discs restores the ECM by preserving both the resident PG and supporting the biosynthesis of new larger PG species.

Keywords. Regeneration, repair of degenerate discs, Mesenchymal stem cells

(48.O2) EXPRESSION OF IMMUNSUPPRESSIVE FACTORS BY JAW PERIOSTEAL CELLS

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Introduction. There is supporting evidence for the use of allogeneic mesenchymal stem cells (MSC) from both in vitro and in vivo studies showing that MSC avoid normal alloresponses. Jaw periosteal cells (JPC) may represent a suitable stem cell source for tissue engineering applications in the oral and maxillofacial surgery. For allogeneic implantation purposes it is essential that JPC elicit immunomodulatory functions being hypoimunogeneic. The goal of this study is to examine the expression of immunosuppressive factors by JPC.

Methods and Results. We detected a high expression of the soluble Galectin-1 und -3 (galactoside-binding lectin) in undifferentiated JPC by microarrays, quantitative RT-PCR and proteome arrays. The expression of both genes/proteins seemed to be further induced during JPC osteogenesis (1.5-2-fold). Microarray analysis revealed an upregulation of the hepatocyte growth factor receptor (Met) whereas the semi-quantitative proteome arrays detected an induction of the corresponding soluble protein (HGF) (2-3-fold). Additionally, a 200-400-fold elevation of the expression of prostaglandin E receptor 2, 3 and 4 was detected in osteogenic differentiated JPC versus the undifferentiated cells by microarrays. However, these results have to be validated by quantitative RT-PCR and on protein level.

Conclusions. Preliminary data show that JPC express immunosuppressive factors which may avoid putative alloresponses of cell-seeded scaffolds. Further analysis should elucidate the influence of JPC on the maturation of dendritic cells and proliferation of T-cells.

Keywords. jaw periosteal cells, immunosuppressive factors, soluble factors, immunomodulatory function, hypoimmunogeneic

(48.O4) FGF-2 MAINTAINS A NICHE-DEPENDENT POPULATION OF SELF-RENEWING HIGHLY POTENT NON-ADHERENT MESENCHYMAL PROGENITORS THROUGH FGFR2C

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Introduction. Bone marrow (BM)-derived mesenchymal stem/stromal cells (MSC) represent a fundamental tool in regenerative medicine, but they are rare and require significant in vitro expansion, which is accompanied by rapid loss of differentiation capacity, limiting their potential for clinical application.

Methods. Fresh human BM nucleated cells were plated at clonal density and cultured in alpha-MEM with 10% serum alone, FGF-2 (5 ng/ml) or PDGF-BB (10 ng/ml). The non-adherent fraction was serially replated and tested for the presence of clonogenic cells and their potential of in vitro multilineage differentiation and in vivo ectopic bone formation.

Results. We found that primary human BM cultures contain a population of non-adherent mesenchymal progenitors (NAMP), which are distinct from the initially adhering CFU-f and are discarded in current culture procedures. NAMP generated an adherent progeny with significantly greater proliferation and multilineage differentiation potential in vitro and 3-fold greater bone forming efficiency in vivo. NAMP progeny was enriched with cells expressing the early MSC markers CD146, SSEA-4 and SSEA-1. Upon serial replating, NAMP were able to expand in suspension as non-adherent clonogenic progenitors, while also giving rise to an adherent progeny. This took place at the cost of a gradual loss of proliferative potential, shown by a reduction in colony size, but it could be completely prevented when NAMP were expanded on the initial adherent fraction. Conditioned medium experiments showed that the adherent BM fraction positively regulates NAMP survival and proliferative potential at least in part through secreted signals. Mechanistically, we found that NAMP function specifically depends on signaling through FGFR2c, alterations of which have been described to affect osteoprogenitor self-renewal and differentiation.

Conclusions. Our data show that the adherent BM fraction can provide a niche function for NAMP and suggest potential strategies to overcome a crucial limitation in the use of MSC for regenerative medicine.

Keywords. Mesenchymal stem cells, regenerative medicine, cell therapy

(48.O5) UTILISING DYNAMIC SURFACE CHEMISTRIES TO UNLOCK THE POTENTIAL OF MATERIALS AS CELL ADHESION SUBSTRATES TO CONTROL CELL FUNCTION BY DESIGN

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Research has established that specific functional chemistries, -CH3, -NH2, -OH and –COOH can be used to induce adult mesenchymal stem cell (MSC) differentiation on an array of base substrates. Historically this approach has utilised silane modification techniques and resulted in heterogenous mixed cell populations across substrates; the levels of induced differentiation have not been sufficient for in vitro homogenous cell population expansion and further clinical use. A limiting factor has been the lack of characterisation of the modified surfaces and an understanding of how contextual presentation of chemical groups can affect initial cell adhesion and subsequent function. Specific material cell interactions can be exploited to direct cell populations by direct contact to deliver a homogenous cell populations but this requires further understanding of the exact initial cell binding mechanisms i.e. integrin binding and clustering.

This research presents experimental evidence of the potential for material induced cell responses in the purification, enrichment and expansion of MSCs utilising –CH3 functionalised nano-arrays produced by DPN®. Model surfaces were translated into bulk coatings to produce –CH3 modified surfaces with additional dynamic properties that were defined and controlled by varying chemical group chain length and the level of binding to the base substrate using silane chemistry. Changes in dynamic surface chemistry and contextual presentation of the -CH3 groups controlled integrin expression and clustering, induced changes in cell adhesion, proliferation and phenotype and stimulated the release of specific growth factors. Control of growth factor release provided a mechanism for the definition and therefore development of surfaces that had a role in enrichment of stem cell populations from primary human adult sources

and provided surfaces that significantly enhanced the MSC phenotype during cell population expansion phases all associated with the defined and controlled contextual presentation of the underlying chemistry.

Keywords. Stem cells, cell adhesion, nano-arrays and silane modification

(48.O6) CHONDROGENIC AND OSTEOGENIC DIFFERENTIATION OF HMSCS CULTURED ON 3D PEOT/PBT SCAFFOLDS

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Recent approaches to regenerate tissues involve the use of human mesenchymcal stem cells (hMSC) either alone or in combination with soluble factors and/or three-dimensional (3D) scaffolds. It is known that scaffold material properties can influence the behavior of hMSCs and their fate in vitro. Less is known about the influence of scaffolds on retaining the multipotency of hMSCs. This study focuses on the differentiation potential of hMSC cultured on rapid prototyped 3D scaffolds towards osteogenic and chondrogenic lineages. When cell fate can be controlled with soluble factors only, the culture system could be transferred to a perfusion bioreactor in which the metabolic activity of the different cell state and the resulting extra cellular matrix (ECM) formation can be monitored real-time. Ultimately, a better understanding of cell metabolic state and ECM production could lead to a model predicting tissue development and to the design of instructive properties in 3D scaffolds.

First hMSCs were seeded on PEOT/PBT scaffolds and cultured for 7 days in expansion medium. Subsequently the expansion medium was replaced with basic or osteogenic and chondrogenic differentiation medium. Biochemical, histological and molecular assays were performed after 7 days and 21 days of differentiation. hMSCs cultured in chondrogenic medium showed a significant increase in glycosaminoglycans (GAGs) production and a significant up-regulation of Sox9, collagen type II and aggrecan gene expression after 21 days. Cells cultured in osteogenic differentiation medium showed a significant increase in alkaline phosphatase (ALP) production and up-regulation of ALP and collagen type I gene expression after respectively 7 and 21 days. No considerable morphological differences were found between the cells of both groups. The produced ECM appeared of fibrillar nature as observed by scanning electron microscopy. Future studies will be performed to further understand ECM formation and development during hMSCs-based cartilage and bone regeneration in 3D scaffolds.

Keywords. Differentiation hMSCs chondrogenic osteogenic

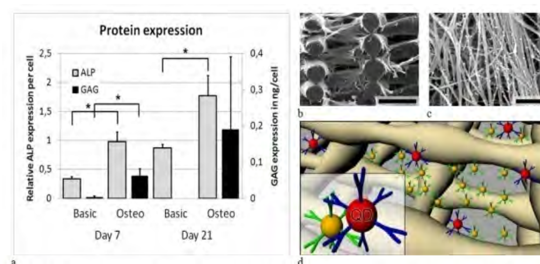

Figure 1 **a** Protein expression levels of respectively ALP for osteogenic and GAGs for chondrogenic differentiated hMSCs after 7 days and 21 days of culture are shown, in both cases there is an up-regulating trend observed, **b** and **c** show scanning electron microscope images of hMSCs on 3D rapid prototypes scaffolds (scale bars are respectively 500 µm and 1 µm), **d** when the metabolic states of hMSCs can be monitored, quantum dots will be one of the approaches to study the formation of extracellular matrix in a 3D culture system.

(48.O7) IMPROVEMENT OF VOCAL FOLD WOUND HEALING BY BONE MARROW-DERIVED STEM CELLS USING A XENOGRAFT ANIMAL MODEL

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Introduction. Vocal fold scarring can be caused by trauma, inflammation, or surgery and commonly result in severe dysphonia. Cell based therapies to minimize scarring and enhance healing have been growing interest. This study was aimed to investigate the effect of bone-marrow derived stem cells (BMSCs) for prevention of vocal fold scarring by the use of xenograft animal model.

Methods. Vocal fold scarring was induced in New Zealand white rabbits by a direct injury. BMSCs were isolated from bone marrow aspirates of GFP transgenic mice and were injected into the vocal folds of rabbits immediately after scarring. PBS was injected into the vocal folds in the same manner for the sham group. Endoscopic, histologic, and biomechanical evaluations of vocal folds were performed after 2 months of the injection.

Results. The BMSCs-treated vocal folds showed decreased collagen bundles compared to the sham group and the relative hyaluronic acid content in BMSCs-treated vocal folds was higher than the sham group. The BMSCs reduced expression of procollgen I. The mean dynamic viscosity in the BMSCs was improved compared to the sham group. A large number of the injected BMSCs were detected in the vocal folds of rabbits at 2 months after injection.

Conclusion. This study suggests that the BMSCs could prevent the scarring-induced dysphonia and be developed as a cell-based therapy for vocal folds regeneration. This xenograft animal model also might be a useful tool for research of vocal fold regeneration.

Keywords. vocal fold, scarring, mesenchymal stem cells

(48.O8) OPTIMISING EXTRACELLULAR MATRIX COATINGS FOR MSC ATTACHMENT AND PROLIFERATION IN SERUM-FREE CULTURE CONDITIONS

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Therapeutic protocols using human mesenchymal stem cells (MSC) are being tested clinically for a number of disorders including osteoarthritis, bone fractures,

cartilage defects, and graft-versus-host disease. However, current protocols for isolation and expansion of MSC for clinical application generally use fetal bovine serum-containing media. In an effort to move towards xenofree culture of cells, it is critical to devise protocols to enable serum-free propagation. One of the major issues associated with serum-free cellular processing is low cell adherence to tissue culture plastic. To overcome this issue, development of extracellular matrix (ECM) coatings for enhanced adhesion and subsequent growth is an important area of research.

A rational analysis of the MSC receptome identified a number of matrix receptors on the surface of MSC. Four cognate ECM ligands of these receptors, namely, fibronectin, vitronectin, hyaluronic acid and laminin were selected to examine MSC adherence and growth in serum-free conditions. MSC, isolated from bone marrow in the presence of serum were seeded at a density of 3,000 cells/cm2 on tissue culture plastic pre-coated with fibronectin, vitronectin, hyluronic acid at concentrations of 12µg/ml, 6µg/ml and 1µg/ml or laminin at concentrations of 1µg/ml, 0.5µg/ml and 0.1µg/ml in serum-free media containing 1ng/ml bFGF. Cells were seeded in the presence of 10% FBS or in serum-free media on tissue culture plastic as controls.

In all cases the MSC attached to the ECM coated plastic with no effect on morphology; 0.5□g/ml of vitronectin was the most favourable for MSC attachment after 24 hours, while optimal MSC proliferation was associated with culture on 12µg/ml and 6µg/ml fibronectin pre-coated plastic. Inclusion of EGF in the medium further increased attachment and activation of focal adhesion kinase. These data add to our understanding of conditions required for optimisation of serum free culture of MSC for clinical applications. (PurStem was supported by the EU Framework 7 programme under HEALTH-2007-B).

Keywords. Mesenchymal stem cells, extracellular matrix, serum-free

(48.P1) EFFECT OF CHRONIC KIDNEY DISEASE AND METABOLIC SYNDROME ON BONE MARROW MESENCHYMAL STEM CELLS PROLIFERATION POTENTIAL, IN VITRO STUDY

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Background. Transplantation of autologous Bone Marrow Mesenchymal Stem Cells (MSCs) is an interesting way to regenerate many organs, including kidney. The aim of this study was to check if chronic kidney disease and metabolic syndrome (induced by fructose) has influence on MSCs proliferation capacity.

Methods. 2 experimental group of animals was established. Right kidney and approximately 1/3 of the left kidney cortex were removed in 11 Wistar rats (CKD5/6). 6 animals in control were left intact. In each group metabolic syndrome was induced by fodder supplementation with <3% (standard diet), 10% and 60% fructose. Cells obtained from femurs were isolated and counted with trypan blue test. Number of isolated cells was presented as means with standard deviation. After 2nd passage to all cultures of MSCs the 0, 5 and 10 units of erytropoethin was added.

Results. Number of cells in cultures established from control animals was 41.8*103±5.53 on standard diet, on diet with 10% and 60% fructose content was 31.8*103±1.41 and 25.4*103±3.68, respectively. Number of cells isolated from animals with CKD5/6 on standard diet, on diet with 10% and 60% fructose content was 24.9*103±4.97, 30.1*103±7.39 and 41.1*103±3.40, respectively. Additionaly, with the increase of erytropoethin content, the number of MSCs was higher.

Conclusions. We speculate that induction of chronic kidney disease and metabolic syndrome causes the mobilization of bone marrow mesenchymal stem cells. In addition, the cell proliferation capacity can be improved by erytropoethin supplementation. Nevertheless, further studies are needed.

Keywords. bone marrow mesenchymal stem cells, chronic kidney disease, metabolic syndrome

(48.P2) POSSIBILITY OF STEM CELL THERAPY FOR AORTIC ANEURYSM

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Aim. The pathogenesis of aortic aneurysm (AA) is characterized by degradation of extracellular matrix with increased matrix metalloproteinases (MMPs) and inflammatory reaction. Mesenchymal stem cells (MSCs) are known to have a potential of anti-inflammation. To clarify whether MSCs might be effective as for aortic aneurysm cell therapy, we examined the effect of MSCs on vascular smooth muscle cells (SMCs) and macrophages in vitro, on aortic tissue ex vivo, and on aortic aneurysm in vivo.

Methods and Results. Murine bone marrow derived MSCs were co-cultured with SMCs, macrophages (in vitro) and aortic tissue (ex vivo). MSC sheet was implanted on an abdominal aorta which was induced aortic aneurysm in apolipoprotein E-deficient mice by administration of angiotensin (Ang) II continuously (in vivo). In the presence of MSCs, gene expression of MMP-2, -9 and TNF-α were significantly decreased in macrophages, and gene expression of elastin was significantly increased in SMCs compared to control group which was not co-cultured with MSCs. MSCs preserved elastin content (68.1 vs 40.3 mg/mg, $p<0.01$) and decreased active-MMP-2 (0.31 vs 0.61 U/ml, $p<0.05$) in aortic tissue ex vivo. An infra-renal aortic dilation was inhibited (0.73 vs 1.04 mm, $p<0.05$) and elastin content preserved (46.9 vs 25.6 mg/mg, $p<0.05$) at 4 wk in vivo compared to control group which was not implanted MSC sheet. Down-regulation of active-MMP-2 (0.10 vs 0.49 U/ml, $p<0.001$) active-MMP-9 (0.07 vs 0.25 U/ml, $p<0.001$) and inflammatory cytokines including IL-6 (1462 vs 3612 pg/ml, $p<0.01$), MCP-1 (425.1 vs 1374.7 pg/ml, $p<0.05$), and TNF-α (41.8 vs 53.8 pg/ml, $p<0.05$), and up-regulation of IGF-1 (13.6 vs 5.7 ng/ml, $p<0.01$) and TIMP-1 (2933.2 vs 1522.8 pg/ml, $p<0.01$) were demonstrated with MSC implantation in vivo.

Conclusion. Our results suggested that MSC implantation inhibits Ang II–induced aneurysm in apoE-/- mice through

elastin preservation and MMPs and inflammatory cytokines attenuation.

Keywords. aneurysm, Mesenchymal stem cells, Extracellular matrix, Elastin, Inflammatory cytokine

(48.P3) OPTIMISATION AND STANDARDIZATION OF MSC CULTURE: INTRA-LABORATORY VALIDATION OF "CURRENT BEST PRACTICE" MSC METHODS

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Introduction. Mesenchymal Stem Cells (MSCs) require significant in vitro expansion to yield sufficient cell numbers for clinical application. Unfortunately there are inconsistencies between MSC research centres in their isolation and culture methods**.** Hence there is an unmet need to generate a validated, standardised protocol for the culture of MSCs. We performed a validation study, as part of the PurStem initiative, to demonstrate that MSCs cultured from the same bone marrow donors using the same standard conditions across four partner laboratories could be comparable.

Methods. Samples from three bone marrow aspirates were distributed equally among four participating research institutions. Each partner adhered to a previously agreed isolation and expansion procedure. Cfu-f/105 MNC, cumulative population doublings (CPDs), doubling time and MSC morphology were compared between each partner. Post cryopreservation, samples were analysed for tri-lineage differentiation potential, cell surface antigen expression, as well as the karyotypic stability of the cells to further validate the standardisation of the procedures.

Results. Colony forming capacity was variable between partners for two of three marrows; however, variability was significantly reduced for the third marrow. CPDs were relatively reproducible between partners although some variations were observed, as was the case for doubling times. Cell surface phenotype analysis showed no significant differences between the partners. MSCs from all partners were shown to be tri-potential and cytogenetically stable.

Discussion. In conclusion, each partner was able to produce MSCs by adhering to the same protocol, as was subsequently demonstrated through analysis of the kinetics and characterisation of the MSCs. Therefore, we have validated a collaborative standardized protocol for the isolation and characterisation of in vitro MSCs. Furthermore, this exercise identified critical parameters that contributed to intra-laboratory variability, an understanding of which will result in additional refinement. (PurStem was supported by the EU Framework 7 programme under HEALTH-2007-B).

Keywords. Mesenchymal stem cells, validation, standardisation, isolation and expansion

(48.P4) HEDGEHOG MODULATION DURING HUMAN MESENCHYMAL STEM CELL ENDOCHONDRAL DIFFERENTIATION

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Introduction. Hedgehog (Hh) pathways play key role in chondrogenesis of mesenchymal stem cell (MSC) and in endochondral ossification. We have shown that human bone marrow MSC could recapitulate events occurring during endochondral process (Scotti, PNAS, 2010) and activate morphogenetic pathways, above all Indian Hh (Ihh). Here, we hypothesize that Hh modulators could regulate MSC chondrogenesis within early time sensitive window.

Methods. Expanded MSCs were 3D cultured in transwells up to 5 weeks in defined chondrogenic and hyperthrophic media. Cyclopamine (an Hh antagonist) and Purmorphamine (an Hh agonist) were added at specific time points during the differentiation culture. Resulting tissues were assessed histologically and biochemically (glicosaminoglycans, GAG) and by real time RT-PCR.

Results. Basal Hh activation was detected along the culture: 3D chondrogenesis up-regulates Ihh (2000-fold vs post-expansion cells), Patched, Ptch (5-fold) and Gli1 (4-fold) mRNA already after 3 days. Adding Cyclopamine to chondrogenic medium, cell differentiation was impaired; on the contrary, if added only when hyperthrophy was induced, no effect was noticed. Consequently, the sensitive window for Purmorphamine was defined at the beginning of chondrogenesis: two different incubation times were chosen (2 or 7 days, followed by withdrawn), and the shorter one gave significant improvements in terms of up regulated Hh gene expression (fold increase vs control for Ihh: 4; Ptch: 92; Gli1: 12) and GAG deposition. Supplementing Purmorphamine for 2 days at the beginning of hyperthrophic culture did not modulate extent of chondrogenesis, in analogy with the experiment with Cyclopamine.

Conclusions. MSC fate could be manipulated by means of individual cues in specific early temporal window during chondrogenesis. Further efforts are warranted to improve culture conditions by substituting conventional growth factors with specific pathway activators or suppressors.

Keywords. hedgehog chondrogenesis endochondral

(48.P5) OPTIMIZATION OF PROCESSING AND EXPANSION FOR MESENCHYMAL STEM CELLS FROM UMBILICAL CORD TISSUE

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The attraction of human umbilical cord as a donor tissue for regenerative medicine has been enhanced by the discovery of mesenchymal like stem cells in the cord suggesting cord blood banks may be able to expand their activities to provide cells for mesenchymal stem cell therapies such as cartilage, bone and muscle repair.

This project’s aims are driven by the opportunity to expand the therapeutic potential of a local cord blood bank, Future Health Technologies (FHT), which is based in Nottingham, UK, but operates internationally. They are

currently in the process of developing a product based on human mesenchymal stem cells (hMSC's) extracted from umbilical cord tissue. In order to deliver a viable medically regulated product, tight control and characterisation of process are critical. Consequently our research work is focused on optimization of processing and expansion of high quality hMSC's from umbilical cord tissue along with addressing a series of important questions:

Is it possible to get a consistent, characterized and therapeutically useful population of adherent stem cells from this source?

What are the most appropriate characterization methods to predict the functional potency of the population?

What are the derivation techniques that deliver the most consistent, multipotent and homogenous populations?

How distinct are the reported different adherent cell populations?

In answering these questions and accomplishing our objectives we are employing a systematic approach by using a process engineering method.

Current experimental plan is targeted towards finding an optimal isolation method of mesenchymal stem cells from the UC tissue, by means of digestion/explant culture, for both frozen and fresh tissue; establishing an optimum cryopreservation method for UC tissue and consequently for mesenchymal cells extracted from this and identify the expansion marker profile and potency differences based on diverse extraction and freezing methods of the cord tissue.

Keywords. Umbilical cord tissue; human mesenchymal stem cells

(48.P6) HUMAN SKIN DERIVED MESENCHYMAL STEM CELL CHARACTERISATION AND EFFECTS OF DMSO ON GROWTH HORMONE SECRETION AND MARKER PROFILE

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The aim of this study was to characterize human skin derived mesenchymal stem cells (MSC) from different donors and to evaluate the effect of dimethylsulfoxide (DMSO), clinical grade cryoprotectant and chemical with wide range of biological activities, on MSC surface marker, differentiation factor and growth hormone expression.

Skin-derived MSCs are easily accessible and could be used to develop cell therapy products. First, we introduced three-stage MSC cell bank system, including cell characterization and testing at certain stages of culture expansion. MSCs cultures reached 50 population doublings and showed no TERT expression and karyotype aberrations. We also determined that DMSO as cryoprotectant provides significantly higher cell viability compared to glycerol.

FACS analysis indicated that MSC cultures grown for 48 hours in medium conditioned with 1 to 3% DMSO preserve expression pattern of MSC markers CD73, CD90, C105. DMSO did not affect mesenchymal differentiation into adipogenic, chondrogenic and osteogenic lineages, as evaluated by cytochemical methods, whereas expression of mesenchymal lineage differentiation markers RUNX2, SPARC, PPARG, FASN was elevated more than 2-fold and HPLN1 decreased 4-fold by real-time PCR.

We performed ELISA to estimate levels of several secretion factors relevant for angiogenesis and regeneration processes. Our results indicate considerable variability of tested factor secretion basal levels amongst individual MSC cultures. DMSO presence had no effect on secretion of IL-1 and FGF-4. However, decrease of IL-8 secretion, up to 10-fold increase of VEGF and more than 2–fold increase of SDF secretion were detected after incubation with DMSO.

Our findings indicate that DMSO can exert profound effect on MSC gene expression patterns and growth hormone secretion, which may affect their biological activity. Therefore use of DMSO can be considered useful approach for pre-clinical investigations of somatic cell therapy products.

Keywords. skin derived mesenchymal stem cells, dimethyl sulfoxide, growth factors, FACS

(48.P7) DOES THE MESENCHYMAL STEM CELL AGING LIMIT THEIR POTENTIAL APPLICATION IN CLINICAL PRACTICE?

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Introduction. Mesenchymal stem cells (MSCs) are of great therapeutic potential because of their ability to self-renew and differentiate into multiple tissues. However the low frequency of this population in bone marrow necessitates their in vitro expansion prior to clinical use. The aim of this study was to evaluate the effect of long-term culture on the senescence of these cells.

Methods. MSCs were isolated from bone marrow and selected by plastic adherence. Their phenotype was characterized by flow cytometry and immunocytochemistry (CD44, CD34, CD90). The colony forming assay (CFA), osteogenic and adipogenic differentiation potential was performed. Senescent cells were identified by their ß-galactosidase activity in following passages.

Results. Isolated MSCs were CD44+, CD90+, CD34- and their clonogenity potential was 0.22%. The ß-galactosidase activity, which is the senescence marker, instantly increased after 4th passage. We further observed that cells morphology was changed, the size of MSCs was extending in following passages. These results indicate on consecutively aging of MSCs in in vitro culture.

Conclusions. MSCs enter senescence almost undetectably from the moment of in vitro culturing. It could be a limitation in clinical use. Therefore, it is much better to consider them for cell and gene therapy early on.

Keywords. mesenchymal stem cells, aging, ß-galactosidase

(48.P8) SECRETED ADIPONECTIN IS AN EARLY MARKER OF ADIPOGEINC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

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The pluripotency of mesenchymal stem cells (MSCs) is often assessed by the ability of MSC extracts to differentiate toward the osteogenic, chondrogenic, and adipogenic lineages *in vitro*. In particular, MSC adipogenesis is commonly evaluated by staining cell cultures with Oil Red O, which detects lipids that accumulate during adipogenic differentiation. While the Oil Red assay is simple and direct, it is problematic in that it requires approximately 3-4 weeks to detect adipogenic potential and necessitates the destruction of the specimen, inhibiting its use for further analysis and clinical application. Moreover, the oil red assay does not allow for the quantification of induction, preventing the determination of MSC potency. In this study, we investigated the possibility of assessing and quantifying MSC differentiation at earlier stages by monitoring adiponectin (ADPN) levels secreted into the induction medium. Adiponectin is a hormone secreted from adipose tissue that regulates several metabolic processes. MSCs were isolated from the iliac crests of patients and cultured. Cell supernatants were extracted at days 0, 3, 7, 10, 14, 17, and 21 after adipogenic induction and ADPN levels were determined by ELISA assay. Our results indicate that secreted ADPN levels steadily increase during adipogenesis and are definitively detectable at levels of 5.986 ng/mL ± 2.545 at day 10. Adipocyte-forming potential was confirmed by Oil Red staining. This study is relevant in that it demonstrates the ADPN assay is able to quantify MSC potency as well as shorten the time required to determine adipogenic potential. We therefore conclude that the ADPN assay is a fast, surrogate test that can be used to non-invasively qualify and quantify adipogenic differentiation and should be used to facilitate quality control in laboratory and clinical applications of MSCs in regenerative medicine.

Keywords. Mesenchymal stem cells, Differentiation, Adipogenic

49. GENERAL SESSION

(49.O1) IN VITRO RECONSTRUCTION OF A LIVER-LIKE TISSUE POSSESSING A BILE TRANSPORT SYSTEM

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Introduction. One of the major issues in liver tissue engineering is to recapitulate the well-organized tissue architecture and tissue polarity: specifically, reconstruction of the bile duct network besides hepatic parenchyma, and realization of bile transport. The objective of this study is to present a method for reconstructing a well-organized hepatic organoid composed of hepatocytes and biliary epithelial cells (BECs) in a bid to create a liver tissue possessing a bile drainage system. Here, we propose a novel 3D co-culture system exploiting in vitro-reconstructed bile ducts as a “scaffold”.

Methods. We isolated adult BECs from male Wistar rats and reconstructed bile ductular networks in a collagen gel sandwich configuration, following a previously reported method (Hashimoto et al., Am. J. Pathol. 2008). As hepatic progenitor cells, we used fetal liver cells (FLCs) isolated from embryonic day 17.5 Wistar rats. Prior to the onset of co-culture with FLCs, we isolated the bile duct fragments (BDFs) from the collagen gel configuration by collagenase digestion. Subsequently, we inoculated BDFs to 96-well low cell-adhesion plates (2-3 pieces/well), and added FLCs at 5×104 cells/well. 2 days after inoculation, the formed aggregates were transferred to non-treated 35 mm dishes and cultured under rotation.

Results. We observed the isolated BDFs under, and confirmed that the ductular networks were intact (Fig. 1A). Concomitantly, hematoxylin-eosin (HE) staining revealed that continuous lumens were preserved in these BDFs. Subsequently, HE staining of D 7 aggregates revealed the presence of cyst- and duct-like morphologies rimmed by epithelial cells besides hepatocyte-like morphologies (Fig. 1B). Detailed characterization of the structure and further assessment of the capacity to transport bile is now under investigation.

Conclusions. These results suggest that 3D co-culture of FLCs and BECs in the form of “BDFs” allowed formation of an organoid possessing hepatic parenchyma-like and bile duct-like structures.

Keywords. fetal liver cell, biliary epithelial cell, bile transport

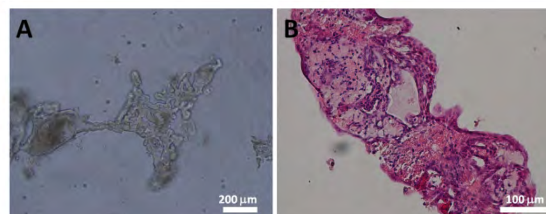

Fig. 1 (A) A phase contrast image of a isolated BDF. (B) HE staining image of D 7 aggregates.

(49.O2) HEPATOCYTE-EMBEDDED FUNCTIONAL HYDROGEL-FILLED SCAFFOLD SYSTEM FOR LIVER TISSUE ENGINEERING

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Introduction. Liver is a central organ for metabolism in our body and is complicated structure. Therefore, Liver Tissue Engineering (LTE) reconstructing a functional liver tissue is one of the most important and difficult themes in Tissue Engineering field. In this study, we developed a novel culture technology consisting of hepatocytes-embedded functional hydrogel-filled scaffold (CGS) culture for the creation of LTE.

Materials and methods. We used natural materials obtained from animals except macroporous scaffold for the development of CGS culture. Heparin-immobilized collagen and gelatin were made in this study. Primary rat hepatocytes were suspended in neutralized collagen or tissue transglutaminase (tTGase) containing gelatin

solution, and were inoculated into hydrophilic-treated scaffold. After the incubation for 30 minutes at 37°C for gelation, in vitro culture or transplantation was performed.

Results and discussion. Cytotoxicity of tTGase was not observed by using the evaluation of mitochondrial activity of hepatocytes. In addition, albumin production activity of hepatocytes in tTGase-gelatin gel culture was higher than that in monolayer culture on collagen-coated dish. Furthermore, the expression of liver-specific function was enhanced by incorporating hepatocyte growth factor with heparin in the gel.

Various types of growth factors could be immobilized to this functional hydrogel developed in this study. In addition, neo-vascularization could be induced by this hydrogel in vivo. Furthermore, not only neo-vascularization but also viability of transplanted hepatocyte could be enhanced by combining transplantation of this hydrogel and partial hepatectomy treatment.

Conclusion. Hepatocytes-embedded heparin-immobilized hydrogel-filled scaffold culture technology is a potential culture method to create LTE.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research (B): 22360348 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Keywords. Liver tissue engineering, Hydrogel, Heparin, Growth factor

(49.O3) EVALUATION OF HUMAN IMMUNE RESPONSE TO ACELLULAR LUNG SCAFFOLDS PRODUCED USING DIFFERENT METHODS

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Natural scaffolds consist of the extracellular matrix (ECM) secreted by the resident cells of the tissue or organ from which they are prepared. These scaffolds already possess the correct anatomical, chemical and morphological structure of the natural tissue and have been shown to facilitate the constructive remodeling of many different tissues in both preclinical animal studies and in human clinical applications. Procedures to decellularize tissues vary significantly in terms of detergents and methods to remove cell debris. The aim of this study was to investigate the human immune response to acellular (AC) human, rat or pig lung produced using a variety of currently established decellularization methods [1-5 below]. Evaluation of the human immune response to these AC lungs was determined after culturing with human peripheral blood mononuclear leukocytes (MNL). Cell death, immune activation (expression of immune activation markers CD69, MHC I and II), proliferation (CFSE), cytokine production (IL-1, TNF, IL-6) and apoptosis was examined for AC lung tissue produced from human and pig using currently described decellularization methods [1-5]. Responses were compared to those generated in response to coculture of MNL with the commercially available products BD Matrigel™ Basement Membrane Matrix, Gelfoam, AlloDerm and INTEGRA. The lowest levels of cell death, apoptosis and production of inflammatory cytokines (Fig. 1) were seen when method 5 below was used to produce acellular lung scaffold. Methods (1-5) below induced very different levels of activation (expression of immune activation markers CD69, MHC I and II), proliferation (CFSE), cytokine production and apoptosis.

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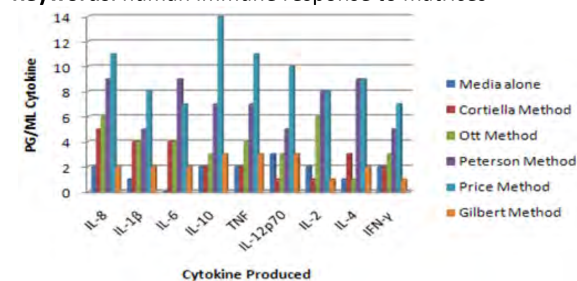
Keywords. human immune response to matrices



(49.O4) LUNG INJURY AND REPAIR IN INFLUENZA INFECTED MOUSE MODEL

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Influenza infection is one of the most common respiratory tract infections leading to annually 3-5 million severe cases and 250.000 – 500.000 deaths of high-risk population worldwide. So far, studies on influenza infections have been focused on innate and adaptive immunity and less attention has been given to the lung damage and repair, especially to the loss and recovery of bronchial and alveolar epithelial that play an essential role in maintaining lung homeostasis.

In this study, we established a mouse model of influenza induced lung damage and repair to study the mechanisms underlie these processes. Our results showed that influenza infection resulted in infiltration of immune cells leading to severe lung damage. Immunofluorescence staining of bronchial epithelial cells (clara cells) and alveolar epithelial cells (alveolar type II cells) of infected lungs showed that influenza infection caused depletion of both clara cells and alveolar type II cells. However, the recovery of clara cells took place earlier than alveolar type II cells. Quantification of lung epithelial cells using our novel quantification method showed that the number of clara cells was dramatically decreased at day 5 post infection (p.i.) followed by active proliferation and recovery of clara cells at day 14 p.i.. However, even though the number of alveolar type II cells also decreased at day 5 p.i., the recovery of type II cells is slower and less proliferating type II cells is observed. Interestingly, bone marrow transplantation experiment confirmed that the cells replenished lung epithelium were not bone marrow derived as many suggested.

In conclusion, our study provides a kinetics model of lung damage and repair after influenza infection and revealed epithelial cells dynamics in lung damage and repair

processes. In addition, we confirmed that the cells that reconstruct lung epithelium were resident lung epithelial or progenitor cells and not bone marrow derived progenitor/stem cells.

(49.O5) PORCINE ARTERIES DECELLULARIZATION

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Introduction. Decellularized tissues with preserved extracellular matrix in terms of biochemical composition, tissue ultrastructure and mechanical properties would represent optimal non-immunogenic scaffolds for tissue engineering. Different protocols have been investigated on various tissues, achieving different results in respect to the above stated features [1]. Following our positive clinical experience on engineered trachea transplantation [2], this study focuses on the translation of the decellularization protocol to a different application: arterial blood vessels.

Methods. For each cycle of the protocol, samples were treated under agitation with (a) antibiotic-antimicotic water solution (4°C), (b) sodium deoxycholate 4% (room temperature), (c) DNase-I in NaCl 1M (room temperature) using a custom-made device for the automatic exchange of fluids. Different trials were performed varying number of cycles, duration and exchange rate of the water phase, and volumes. As first stage of the study, porcine abdominal aortas (Ø 5÷7mm) were processed; currently, vessels from different anatomical districts are also treated (e.g., carotid, Ø < 5mm). Histological (hematoxylin and eosin staining), immunochemical (MHC-I and MHC-II) and mechanical (annular sample, uniaxial tensile test) characterizations are made.

Results. The protocol revealed to be effective also for blood vessels decellularization. In particular, histological results showed a complete decellularization after two cycles, with outcomes varying in relation to volumes of fluids after one cycle. The ultrastructure of the tissue appears to be preserved. Mechanical testing outcomes suggest that breaking stress and strain are preserved in relation to the native tissue while stiffness tends to rise in the decellularized matrices.

Conclusions. Based on these promising results, further investigations are currently ongoing towards the definition of the optimized protocol. A safe, rapid and effective decellularization process will be functional to the supply of non-immunogenic scaffolds with preserved native ECM for tissue engineering applications.

Acknowledgements. Supported by Regione Lombardia.

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Keywords. Extracellular matrix, decellularization, biological scaffold

(49.O6) ECTOPIC PREGNANCY CONCEPTUS: PROBABLY A NOVEL ETHICAL SOURCE OF EMBRYONIC / FETAL STEM CELL RESEARCH AND THERAPY IN ISLAMIC COUNTRIES

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Introduction: Regenerative medicine has a lon history. Several diseases are candidated to treat by different types of stem cell. Many sources of stem cell have been described such as bone marrow, and the embryo / fetus. Despite the potential benefit of embryonic / fetal stem cells, the utilization of this source remains controversial because, termination of pregnancy under a normal condition is forbidden by nearly all the major world religions such as Islam and laws in most of Muslim countries permit termination of pregnancy only when the life of mother is threatened or continuing pregnancy lead to birth of a malformed child. According to the mentioned rules, finding an ethical source for embryonic / fetal stem cell is too difficult. On the one had scientists have an ideal as finding a normal embryo / fetus as a source for research and therapy, but on the other hand, they should adhere to these rules in all parts of their activity. Our purpose is that Ectopic Pregnancy (EP) conceptus can meet these major priorities. Ectopic Pregnancy: The incidence of EP is around 2% of pregnancies. EP is the cause of 10% of pregnancy-related deaths that has to be terminated because of maternal life saving. Several factors are associated with risk of EP such as Pelvic inflammatory disease, history of previous EP, tubal ligation, intrauterine devices, ovulation induction, in vitro fertilization, Smoking and maternal age > 35years. As we described, EP is an ethical source because there are no cytogenetic or other etiologies that could affect the results of research and therapy.

Conclusion: Authors suggest that EP as a source of stem cell has some advantages. Termination of an EP is accepted by all of Islamic laws because of maternal life saving, and also EP is a valuable source for stem cell.

Keywords. Ectopic Pregnancy. Embryonic Stem Cell. Fetal Stem Cell. Ethical

(49.P1) OPTIMIZATION OF DECELLULARIZED CONDITIONS OF LIVER FOR THE DEVELOPMENT OF LIVER TISSUE ENGINEERING

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Introduction. Liver is an important organ because it has many functions. However, there is no cure for severe liver disease except transplantation. Therefore, we will create a novel method for the reconstruction of liver tissue equivalent by using decellularized organ and cell-embedded gel.

Material and method. Liver of rat was decellularized by using dodecyl sodium sulfate (SDS) or TritonX-100. In some cases, DNase and RNase treatment was performed

at 37 degrees C. Decellularized condition and a framework of blood vessels network in decellularized liver were evaluated by using scanning electron microscopy, hematoxylin-eosin staining and MERCOXⅡ. Furthermore, human umbilical vein endothelial cells (HUVEC) were seeded to decellularized liver via portal vein.

Results and discussion. Decellularizing ability of SDS was stronger than that of TritonX-100. However, a fine framework of blood vessels network was destroyed by using SDS. On the other hand, the network structure of decellularized liver treated by TritonX-100 could be remained. Furthermore, DNase and RNase treatment after decellularization with 4% TritonX-100 was the optimum condition to obtain complete decellularized liver with a fine framework of blood vessels. In addition, HUVEC was specifically adhered to the internal surface of a framework of blood vessels.

Conclusion. Decelluralized liver with a fine framework of blood vessels could be obtained. HUVEC could be adhered to the framework of blood vessels. This method has potential for the development of Liver Tissue Engineering.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research (B):22360348 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Keywords. Liver tissue engineering, Hydrogel, Decellularized organ, Vascular network

50. SPANISH SOCIETY OF HISTOLOGY AND TISSUE ENGINEERING (SEHIT SATELLITE MEETING)

50.1. HISTOTECHNOLOGY AND NOVEL METHODS IN HISTOLOGY AND TISSUE ENGINEERING

(50.1.O1) EVALUATION OF PIGMENTATION AND COLLAGEN FIBERS REORGANIZATION IN MELANOCYTIC LESIONS USING A NEW FONTANA MASSON-PICROSIRIUS HISTOCHEMICAL METHOD

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Introduction. For the histopathologic diagnosis of the melanocytic lesions, the degree of dermal sclerosis, the collagen reorganization, the neoplastic cells organization and pigmentation degree should be evaluated for establishing the diagnosis. In all these lesions, several techniques are currently used and evaluated in parallel.

In this work we describe a novel Fontana Masson-Picrosirius (FMPS) histochemical method that allowed us to evaluate the histological patterns, argentaffin reaction of the melanin pigment, and histochemical reaction of the stromal collagen fibers, simultaneously.

Materials and methods. The FMPS method was performed on 40 biopsies with several lesions of melanocytic origin, obtained from the Department of Pathology, University of Granada. All samples were fixed in 10% formalin in PBS 0.1M and embedded in paraffin. Sections of 5 µm thickness were stained with Haematoxylin-Eosin, Fontana-Masson and the novel Histochemical FMPS method.

Results. The FMPS method allowed us to properly evaluate the histological patterns, dermal sclerosis and the different degrees of pigmentation on a single tissue section. The identification of melanine and collagen fibers were highly specific with the FMPS method. This method allowed a correct identification of cell nest, establishing different degrees of dermal sclerosis and collagen reorganization at the tumor-stromal interface in all cases.

Discussion and conclusions. The FMPS method is significantly superior in the identification of collagen fibers, combining morphological and histochemical parameters that could be useful in the study of pigmented lesions of melanocytic origin.

Supported by CTS-115 Tissue Engineering Group

Keywords. melanin, collagen fibers, picrosirius, Fontana-masson, hematoxylin-eosin, melanocytic lesions

(50.1.O2) LECTIN HISTOCHEMICAL CHARACTERIZATION OF DIFFERENT POPULATIONS OF MUCOUS AND PARIETAL CELLS IN THE RAT GASTRIC EPITHELIUM

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Introduction. The gastric mucosa is covered by a mucous layer rich in oligosaccharides with sugar moieties, like Galactose (Gal). Our aim is the identification of Gal residues in the cells of the rat gastric mucosa.

Materials and methods. Four lectins were used: *Arachis hypogaea* lectin (PNA), *Ricinus communis* agglutinin-I (RCA-I), *Bandeiraeda simplicifolia*-I-B4 agglutinin (BSI-B4), and *Maackia amurensis*-I agglutinin (MAA-I), each recognizing different forms of galactosylated glycans. Removal of N- and O-linked oligosaccharides, sialic acid and sulphate groups were performed with PNGaseF, ß-elimination, acid hydrolysis and desulphation methods, respectively.

Results. The results are summarized in the Table 1. The surface epithelium, the gastric pit and the mucous neck cells secrete O-linked oligosaccharides containing terminal Gal residues. PNA labelling at the surface epithelium and gastric pits are not observed until some O-linked oligosaccharides are removed with a short ß-elimination procedure. Other Gal residues in the surface and gastric pit cells are sialylated since the lectins show a stronger labelling after removal of terminal sialic acid. The removal of sulphate groups increase the PNA labelling at the surface and gastric pit cells. BSI-B4 was almost exclusively positive in the parietal cells and showed the existence of sulphated Gal residues. This and other two lectins (PNA and MAA-I) showed the existence of two populations of parietal cells with different

oligosaccharidic composition; e.g. those of the upper region of the glands are stained with PNA showing Gal residues in O-linked oligosaccharides. The transitional and chief cells showed the presence of Gal residues in O-linked oligosaccharides in the cytoplasm (RCA-I), and in the luminal surface (RCA-I and PNA).

Conclusions. Two main conclusions can be spotlighted: 1) the mucous secretion of the surface and gastric pit cells has a different composition to that of mucous neck cells. 2) There are two populations of parietal cells located in the upper and lower region of the glands, with a different oligosaccharidic content.

Keywords. Gastric gland, oligosaccharides, lectin cytochemistry, glycoproteins

Table 1: Lectin binding intensities. The staining intensity was evaluated and classified into six categories: no labelling (0), very weak (1), weak (2), moderate (3), strong (4), and very strong (5).

	Lectin	No treatment	PNGaseF	β-ELIM (1d)	β-Elim. (1d) + PNGasaF	β-lim. (5d)	β-Elim. (5d) + PNGasaF	AH	Desul.
Surface epithelium	PNA	G 1	G 1	5	5	0-1	0-1	4	2 LS 5 G 4
	RCA-I	2	2-3	5	4-5	4	3-4	4	2
	MAA-I	2	2-3	4		0		3-4	
	BSI-B$_4$	0	0	0	0	0	0	0	3-4
Parietal cells	PNA	UR 5	UR 5	UR 1-2	UR 1-2	0	0	UR 5	UR 5
	RCA-I	4-5	4	5-4	4	4	4	4	4-5
	MAA-I	0	0	UR 0-1 LR 3		0		0	
	BSI-B$_4$	5	UR 4 LR 0-1	5	3	4	0	5	5
Mucous neck cells	PNA	5	4-5	5	5	0	0	4-5	5
	RCA-I	3	3	3	3	0	0	3	3
	MAA-I	2	2-3	2-3		0		2	
	BSI-B$_4$	0	0	0	0	0	0	0	1
Transitional and Chief cells	PNA	LS 3	LS 3	0	0	0	0	LS 3	LS 3
	RCA-I	LS 2	LS 2	2	2	0	0	LS 2	LS 2
	MAA-I	0	0	0		0		0	
	BSI-B$_4$	0	0	0	0	0	0	0	0

AH: acid hydrolysis. ß-elim. (1d): the sections were incubated for 1 day for the removal of O-linked oligosaccharides. ß-elim (5d): the sections were incubated for 5 days for the removal of O-linked oligosaccharides. Desul.: desulphation method. G: Golgi region of the cell. LR: parietal cells located at the lower region of the gland. LS: luminal surface of the cell. PNGaseF: the sections were incubated for the removal of N-linked oligosaccharides. UR: parietal cells located at the upper region of the gland.

(50.1.O3) IN VITRO ASSESSMENT OF TIME-RELATED CHANGES AND UVA MEDIATED BIOACTIVATION OF FUNCTIONALIZED PET SURFACES ON HUMAN OSTEOBLASTS

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Our group has recently described how scaffolds activation by plasma enhanced chemical vapour deposition of TiO2 appears to be an alternative to wet chemical treatments in bone tissue engineering. Ultraviolet (UV) light-mediated photofunctionalization of titanium has been described to improve osteoconductivity of titanium implants. The precise molecular mechanism of why and how chemical surface modification and increased hydrophilicity impacts on tissues is not yet well understood. In the present study, time related changes in bioactivity induced by uva irradiation of PET TiO2 activated surfaces were assessed in vitro.

Material and Methods. Very thin TiO2 (some tenths of nanometer) layers were prepared on PET by plasma enhanced chemical vapour deposition (PECVD). Functionalized and non functionalized samples were uva irradiated for 20m each side. Human normal osteoblasts were grown on TiO2 functionalized and non functionalized TiO2 PET samples in the following way: (a) on new samples immediately seeded with osteoblasts after irradiation or (b)ond “old” samples that were kept in darkness for 4 weeks after uva irradiation. The same protocol was followed for non functionalized PET samples. Rhodamine-phalloidine and antivinculin fluorescent labelled cells were analyzed after 24 and 48 h and 7 days in culture. Confocal laser scanning microscopy was used to examine cell morphology and cytoskeletal arrangement. Surface measurements were evaluated using atomic force microscopy and contact angle measurements were obtained using a goniometer equipped with a digital camera and image analysis software.

Results. Morphological and chemical characterization of the TiO2 functionalized PET shows that these surfaces are equivalent to those of this materials deposited by PECVD on other substrates.Cell attachment, proliferation, phenotipical changes and cytoskeletal arrangement of osteoblasts were substantially higher on the “new” TiO2 functionalized surfaces than on the 4-week-old functionalized and non functionalized surfaces. Thus, although UV treatment converted the titanium surface from hydrophobic to superhydrophilic, our present results demonstrate the suitability of PEVCD as an alternative for surface functionalization of polymers to be used in bony tissue regeneration.

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(50.1.O4) IMMUNOHISTOCHEMICAL METHODOLOGY AND ITS APPLICATION IN HISTOLOGY

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Immunohistochemistry is an essential tool in morphomicroscopic sciences that allows to “in situ” identify different molecules of interest in routinely processed samples using conventional microscopes. This procedure combines the capacity of antibody specific binding to a certain protein –antigen– and its later visualization by employing a histoenzymatic technique. Besides, the range of commercially available antibodies, both polyclonals and monoclonals, has increased considerably over the last few years, making the number of detectable molecules nearly illimited.

Immunohistochemistry is a technology that was born at the end of the sixties, which is in constant progress so as to optimize its results and generate a greater reliability. Thus, besides increasing the range and quality of the antibodies, more sensitive and less complex visualization kits have been developed which have led to considerable improvements, such as detection of low-quantity antigens and reduction unspecified background. Limitations deriving from routine processes have also been faced, especially in formol-fixing and paraffin-embedding, by applying heat-induced antigen retrieval techniques.

Likewise, other interesting technical challenges have been performed with magnificent results. Thus, in order to detect more than one molecule that is present either in different cells or in the same cell, even using antibodies developed in the same species, multiple immunolabeling techniques, that use similar visualization kits with different chromogenes, have been developed.

The success of this elegant and, apparently, simple methodology has been and will be one of the main stimuli

in the search for adequate answers to the new challenges improving its possibilities in research and histopathologic diagnosis.

(50.1.P1) A NEW METHOD FOR EVALUATING THE ULTRASTRUCTURE OF IMMOBILIZED HUMAN SPERM

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Introduction. Sperm immobilization is considered necessary for efficient fertilization to happen for ICSI. However, the sperm immobilization elicits changes in the plasma membrane and trigger acrosomal reaction. The aim of this study is to identify the acrosomal status and DNA damage by Electron Microscopy Transmission (TEM) and labeled with Pisum sativum lectin (PSA)-FITC and Tunel assay, respectively. Semen samples were obtained from 15 healthy normozoospermic voluntary donors of Instituto Bernabeu.

Materials and methods. To perform the study in the TEM, human oocytes that failed to fertilize in vitro were used as containers of immobilized sperm. ~80-100 immobilized sperm were aspirated into an ICSI needle and injected into the perivitelline space of oocytes. Sperm without treatment were used as control. Immediately after sperm injection, the oocytes were embedded in 2% agar and fixed in 2% glutaraldehyde in PBS and embedding in LRWhite resin. The samples were examined in a Philips TECNAI 12 electron microscopy.

Results. In the control group, the most sperm cells did not show alterations in the plasma membrane, acrosome and nucleus status assessed by TEM, PSA-FITC and Tunel assay respectively. Nevertheless we observed damages in the plasma membrane and sperm with reacted acrosome studied with TEM and PSA-FITC. We did not find DNA fragmentation in immobilized sperm evaluated by TEM and Tunel assay.

Conclusions. This is the first time that human oocytes are used for studying the ultrastructure of human sperm. This technique is an appropriate method for assess cell in small numbers.

Keywords. Human sperm, in vitro fertilization, oocytes

(50.1.P2) MICROSCOPIC EN FACE OBSERVATION OF WHOLE-MOUNT SPECIMENS OF VARIABLE THICKNESS USING A SIMPLIFIED PREPARATION SYSTEM

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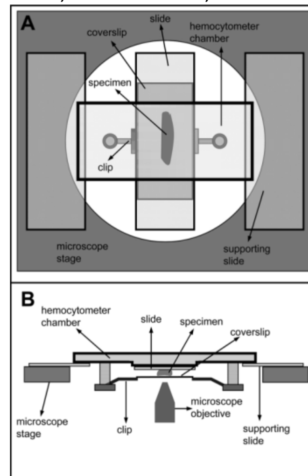
Introduction. En face techniques allow microscopic examination of the surface of tissues, as the endothelium of vascular vessels, but generally these procedures are limited to the observation of thin specimens and the use of low magnification objectives. We have developed a procedure to mount whole tissue samples of variable thickness by using a hemocytometer chamber with spring clips for fine tuning of the pressure exerted on the specimen.

Materials and methods. Figure depicts the lab-made system (A, top view; B, side view) developed to mount en face preparations. A microscope slide was placed transversely on the thick hemocytometer glass slide, as the latter is not an even surface. The specimen was placed on it and covered with a rectangular coverslip that was softly tightened with the hemocytometer spring clips. The preparation was placed upside down on the stage of an inverted microscope. As the hemocytometer slide was shorter than the diameter of the stage hole, 2 supporting microscope slides were placed across the stage to lay the edges of the hemocytometer chamber over them. Vascular vessel samples were opened longitudinally, pinned on a silicone rubber strip, and fixed with 3% paraformaldehyde. Metallic silver deposition was used to visualize endothelial cells perimeter; specific antigens were detected using immunohistochemical and immunofluorescence techniques.

Results. The setup allowed microscopic examination of thick (>1 mm) specimens. Endothelial cells stained after metallic silver deposition showed the characteristic morphology. Morphometric analysis of digital images allowed to calculate several cdell parameters, as the average endothelial cells luminal area. Specific detection of cells antigens and a low background was observed using both immunofluorescence and immunohistochemical methods.

Conclusions. We describe a mounting technique that allows en face microscopic examination of thick tissues and the morphological and antigenic characterization of the superficial cell layer.

Keywords. En face; whole-mount; inverted microscope



(50.1.P3) LECTIN HISTOCHEMISTRY. A USEFUL TECHNIQUE TO DETECT APOPTOSIS OF GERM CELLS IN SEMINIFEROUS EPHITELIUM DURING TESTICULAR REGRESSION IN SYRIAN HAMSTERS (M*esocricetus auratus*) SUBJECTED TO SHORT PHOTOPERIOD

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Introduction. In the Syrian hamster (Mesocricetus auratus) testicular regression due to short photoperiod involves a reduction of the germinal epithelium through apoptosis and a change in the expression pattern of glycoconjugates in the plasma membranes of spermatocytes. The purpose of this communication is to characterize the expression pattern of glycoconjugates

during the different phases of the testicular regression, using lectin histochemical techniques.

Materials and methods. For this, a total of 50 Syrian hamsters (45 treated, 5 controls) were used. The treated groups were submitted to an 8:16 light-dark photoperiod for 12 weeks. Three groups were establissed: middle (MR), strong (SR) and total (TR) regression. Removed testes were methacarn fixed to perform the lectin histochemistry techniques using peroxidase-conjugated lectins (PNA, SBA, WGA, HPA, UEA-1, LTA and DBA) and digoxigenin-conjugated lectins (GNA, MAA, AAA and SNA).

Results. It showed changes in the affinity of lectins in the plasma membranes of germ cells during testicular regression, especially in spermatocytes. Thereby, the lectins PNA, AAA and HPA gradually became more positive as regression progressed. At the same time, lectins PNA, AAA, GNA and LTA showed affinity for apoptotic cells. In the case of PNA and GNA they were both positive for spermatocytes and early spermatids, while LTA and AAA were only positive for spermatocytes.

Conclusion. During testicular regression there is a significant loss of germ cells through apoptosis, which involves a change in the lectins affinity pattern in these cells. Therefore, lectin histochemistry may be a new tool for detecting both germ cell apoptosis during testicular regression due to short photoperiod and in other similar situations. Supported by GERM 04542/07 and 05741/PI/07 from Fundación Seneca CARM.

Keywords. Lectin, apoptosis, regression, hamster

(50.1.P4) LECTIN HISTOCHEMICAL IDENTIFICATION OF FUCOSYLATED GLYCOCONJUGATES DURING *Xenopus laevis* SPERMIOGENESIS

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Introduction. Lectin histochemistry has been used for study of the carbohydrate composition of the tesitis and their role. The involvement of fucose (Fuc) residues in acrosome formation has been reported, as well as the presence of fucosylated glycans in the acrosome. However, the testis of *Xenopus laevis*, an animal model extensively employed in biochemical, cell and developmental research, has not yet been analyzed. Our aim was the histochemical characterization of Fuc-containing glycoconjugates in the sperm formation of *Xenopus* by means of lectins, combined with deglycosylation pre-treatments.

Materials and methods. Four Fuc-binding lectins were used: Aleuria aurantia lectin (AAL), Ulex europaeus agglutinin-I (UEA-I), Anguilla anguilla agglutinin (AAA), and Lotus tetragonolobus agglutinin (LTA), each recognizing different forms of fucosylated glycans.

Results. Labelling with UEA-I, which preferentially binds Fucα (1,2) containing oligosaccharides, did not show any appreciable staining. LTA, specific for Fucα (1,3), and AAA, which binds Fucα (1,2), labelled spermatocytes and spermatids, but no labelling was seen when the histochemical procedure was carried out after either β-elimination (which removes O-linked oligosaccharides) or PNGase F digestion (which removes N-linked oligosaccharides). AAL, which shows its highest affinity to Fucα (1,6), but also recognizes Fucα (1,2) and Fucα (1,3), labelled the whole testis, including spermatocytes and spermatids; the staining of this lectin remained when the histochemical method was performed after either β-elimination or incubation with PNGase F.

Conclusions. The unlabelling with LTA and AAA after either incubation with PNGase F or β-elimination, suggests that fucosylated glycans are of both N- and O-linked types in spermatocytes and spermatids of *Xenopus laevis*. Labelling with AAL could be explained by the fact that this lectin could be binding to diverse fucosylated glycans in N- and O-glycans, and even in glycolipids. This is supported by the finding of fucosylated glycolipids in mammalian testis.

Supported by UPV/EHU (Grant number 1/UPV00077.310-E-15927/2004 and GIU09/64) and Fundación Séneca (Grant number: 04542/GERM/06).

Keywords. Spermatogenesis, oligosaccharides, lectin histochemistry, glycolipids, glycoproteins

Table 1: Fuc-binding lectin labelling of *Xenopus laevis* testis.

	AAL[a]	LTA[b]	AAA[b]	UEA-I[c]
c1	2	1	1	0
es	3	2	1	0
ms	3	3	1	0
ls	3	1	0	0
sp	2	0	0	0

The staining intensity was evaluated and classified into five categories: no labelling (0), very weak (1), weak (2), moderate (3), strong (4), and very strong (5). a: labelling remained after either incubation with PNGase F or β-elimination; b: no labelling was seen after either β-elimination or incubation with PNGase F; c: cells remained unlabelled after any of the desglycosylative pre-treatments.
Abbreviations: c1: primary spermatocytes, es: early spermatids, ls: late spermatids, ms: midstage spermatids, sp: spermatozoa.

(50.1.P5) A NOVEL HISTOCHEMICAL METHOD FOR A SIMULTANEOUS IDENTIFICATION OF MYELIN AND COLLAGEN IN BIOENGINEERED PERIPEHRAL NERVES

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Peripheral nerves are complex histological structures whose main components are neuron axons and myelin sheaths synthesized by Schwann cells. These structures can be affected by a variety of neuropathies with different degree of axonal degeneration and demyelination. For the study of peripheral nerve regeneration in tissue engineering, it is necessary to evaluate the capability of regeneration, remyelination and extracellular matrix remodelation of the bioengineered neural tissues. Currently, different techniques must be used and interpreted in parallel, and a correlation among their findings should be further performed. For the evaluation of these parameters, the development of techniques that allow for a comprehensive assessment of the main histochemical properties using a single histological slide would be useful. In this work, we describe a new histochemical method for the evaluation of the morphology, myelin sheath positive reaction and extracellular matrix collagen histochemical reaction using a fibrin-agarose peripheral nerve regeneration model. For that purpose, paraffin-

embedded tissue sections were first stained with luxol fast-blue for myelin identification. Then, the same slides were stained using picrosirius for collagen staining and finally, tissues were counterstained with Harris haematoxylin. The results of this technique revealed that this new histochemical method allowed us to properly evaluate histological patterns, and to observe the colocalization of the histochemical reaction of the stromal collagen fibers and the histochemical reaction of the myelin sheath around the each axon.

In conclusion, this new method demonstrated high sensitivity and specificity for the identification of these components, and allowed us determine with high accuracy the degree of remyelination and stromal fibrosis. For all these reasons, we hypothesize that this new histochemical method could be useful in tissue engineering quality control.

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Keywords: collagen fibers, myelin sheath, histochemistry

(50.1.P6) MELATONIN RECEPTOR EXPRESSION IN HUMAN MAJOR AND MINOR SALIVARY GLANDS

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Introduction. Results of an immunoblotting study of MT1 and MT2 receptors in the parotid gland of rat suggested that melatonin may be related to salivary regulation via direct action on its receptors and via nitric oxide. However, no data have been published on the immunohistochemical expression of melatonin receptors in salivary glands. The objective of the present study was to analyze the expression of MT1 receptors in normal major and minor salivary glands from humans.

Methods. The study included samples of major salivary glands from 14 patients and minor salivary glands from 10 patients. Structures corresponding to the major (parotid, submaxillary, sublingual) and minor (palatal and labial) salivary glands were preserved in all cases and utilized in the study. We used a goat polyclonal antibody raised against a peptide mapping at the N-terminus of MEL-1A R of human origin; it was applied at a dilution of 1:500. Formalin-fixed, paraffin-embedded tissue blocks were sectioned at 4-µm thicknesses. Tissue sections were deparaffinised in xylene, processed through a graded series of alcohols, and rehydrated in distilled water.

Results. The serous cells that make up the acini showed focal intracytoplasmic positivity for MT1 in thick clumps, with no evidence of positivity in the cytoplasmic membrane. The myoepithelial cells surrounding the serous cells were not MIT-positive. The excretory ducts (lobar and lobular) demonstrated intense intracytoplasmic positivity but scant cytoplasmic membrane positivity for MT1.

Conclusion. Our study shows that MT1 may participate in the modulation of absorptive and/or secretory processes, preferentially those of ductal epithelial cells in the major and minor salivary glands. Further immunohistochemical studies are warranted to elucidate the role of melatonin and its MT1 and MT2 receptors in salivary secretion and their influence in inflammatory or tumour diseases of the oral cavity.

50.2. BIOPATHOLOGY

(50.2.O1) IDENTIFICATION OF NOVEL MOLECULAR TARGET FOR ENDOMETRIOSIS: QUANTIFICATION OF DOPAMINE RECEPTOR 2 ON ENDOMETRIOTIC LESIONS AND HISTOLOGICAL CHANGES AFTER ANTIANGIOGENIC TREATMENT

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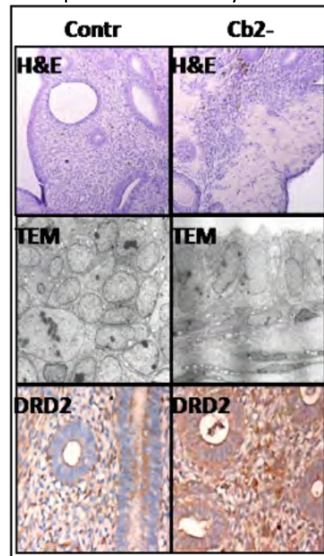
Introduction. Structural changes induced in endometriosis lesions by new therapies are important because the glandular cells are involved in the lesion growth and establishment. Previous studies in experimental endometriosis model have shown that the DA, cabergoline (Cb2), reduces angiogenesis and endometriotic lesions, hypothetically binding to the dopamine receptor type-2 (DRD2). To date, this has not been described in human endometrium and/or endometriotic lesions. Thus, we aimed to investigate the DRD2presence in said tissues and the tissular processes after Cb2 administration.

Materials and methods. Endometrium fragments were implanted in nude mice treated with different doses of Cb2. PCR-assays and immunohistochemistry were performed to analyze the DRD2, VEGF, and KDR gene and protein expressions. Optical microscopy, morphopetric and ultrastructural studies were carried out to evaluate the lesions tissular components. In addition, lesions and endometrium from mild/severe endometriosis women and healthy women endometrium were collected to analyze their gene expression profile.

Results. In experimental endometriosis, there was a significant decrease of glandular component and cellular density in Cb2-treated lesions as compared to controls (Figure). DRD2 was expressed at gene and protein levels (Figure) in all groups. KDR protein and VEGF protein and gene expressions were significantly lower in Cb2-treated lesions than in controls. In eutopic endometria, there was a DRD2 expression significant decrease and VEGF increase in women with mild/severe endometriosis with respect to healthy patients. KDR expression was significantly higher in red than in white and black lesions. VEGF expression was significantly lower in black than in red lesions.

Conclusions. DRD2 is present in the human eutopic/ectopic endometrium and is regulated by DA, which antiangiogenic action induces tissular disturbance characterized by glandular component disruption and stromal changes, inhibiting the cellular interactions necessary for the lesions development. These findings provide the rationale for pilot studies to explore its use in the endometriosis treatment.

Keywords. Endometriosis, dopamine agonists (DA), Vascular Endothelial Growth Factor Receptor-2 (KDR), structural and morphometrical study



(50.2.O2) ROLE OF ADRENOMEDULLIN DEFICIENCY IN THE PATHOGENESIS OF ELASTASE-INDUCED MURINE PULMONARY EMPHYSEMA

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Introduction. Chronic obstructive pulmonary disease is characterized by chronic bronchitis, emphysema and irreversible airflow limitation. The pathogenic pathways leading to emphysema are still a matter of debate. Adrenomedullin (AM) is a potent vasodilator and antioxidant peptide that plays a protective role in elastase-induced emphysema when provided via systemic treatment. In this setting, AM favors tissue regeneration through mobilization of bone marrow cells and attenuates ventilator-induced lung injury in mice. In addition, AM receptor expression in the basal cells of the airway epithelium and alveolar epithelial type 2 cells promotes epithelial regeneration. We have used AM conditional knockout mice to investigate whether AM improves lung structure and function in elastase-induced emphysema.

Methods. We have generated a lungconditional knockout of AM by crossing mutant “floxed” AM mice with transgenic animalsexpressing Cre recombinase under the Clara Cell Secretory Protein (CCSP) promoter.

To study the repair of pulmonary structures following an elastolytic injury, we examined the effect of different concentrations of porcine pancreatic elastase (PPE) on airspace enlargement and morphological features of the lung. Intranasal instilation of PPE was performed on 8 weeks old C57BL/6 male mice and on AM deficient mice. Histological analyses were performed 31 days after treatment.

Results. There was evidence of higher lung parenchyma destruction and focal enlargement of airspace in the peribronchiolar and perivascular areas in AM knockout mice than in their wild type counterparts. Picro-Syrius red revealed abnormal deposition of collagen in perivascular, peribronchiolar, and perialveolar areas in AM knockout mice.

Conclusions. These results suggest that AM plays a critical role on lung regeneration in elastase-induced pulmonary emphysema mouse models.

Keywords. Adrenomedullin; emphysema; elastase; mice

(50.2.O3) MORPHOMETRIC COMPARATIVE STUDY OF TWO DISTINCT TYPES OF BLOOD VESSELS AND LYMPHATIC VESSELS IN RENAL CELL CARCINOMA

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Introduction. Angiogenesis is an essential process for tumor growth and is related to blood-borne metastasis in Renal Cell Carcinoma (RCC). Microvascular density (MDV) and Lymph vessel density (LVD) is an often-quantified variable of tumor vasculature. Recently, three types of vessels have been valorated in RCC: CD31 + vessels (undifferentiated), CD34 + vessels (differentiated) and D2-40 + lymphatic vessels. It has been suggested that high microvascular density with undifferentiated vessels correlates with a high tumour grade and survival shortening.

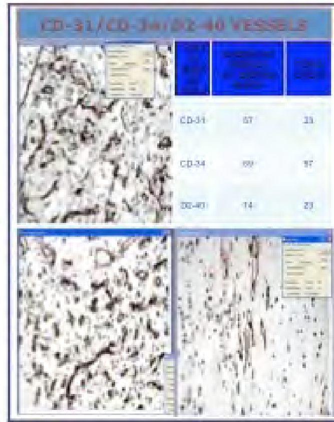
Material and methods. TNM stage and grade of 90 conventional RCC were reviewed. LVD and MVD were measured by quantitative immunohistochemistry using anti-D2-40 antibody, anti-CD31 and anti-CD34, respectively.

Results. D2-40 positive lymphatic vessels were detected mainly in the peritumoral area. No significant difference was found between LVD in peritumoral areas of the RCC tissues and the normal kidney (P= 0.238). Intratumoral D2-40 positive lymphatic vessels were detected in only five cases. The number of lymphatic vessels was less than the number of blood vessels (58,33 versus 247). The number of indiferentiated vessels CD31+ was greater that the number of differentiated vessels CD34+ ($P<0.001$). Not statistically significant correlation was found between of CD 31 vessels numbers and Fuhrman grade, size and behaviour of tumours. However we found that low-grade tumors have a higher number of CD34 that CD31 vessels. By contrast, high-grade tumors have a greater number of vessels CD31 that CD34 ($P<0.042$ and $P<0.003$ respectively).

Conclusions. Lymphyangiogenesis seems to play a minimal role in the progression of human RCC. The number of lymphatic vessels was less than the number of blood vessels. When tumor grade compared with the

number of vessels we found that low-grade tumors have a higher number of CD34 to CD31 vessels. By contrast, high-grade tumors are more undifferentiated CD31 vessels.

Keywords. Angiogenesis, microvascular density, lymph vessel density, renal cell carcinoma



(50.2.O4) EXPRESSION OF SMOOTHELIN IN THE SKIN

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Introduction. The objective of this study was to evaluate the expression of smoothelin and smooth muscle actin (SMA) in the skin in order to establish specific localizations of smoothelin in smooth muscle cells with high contractile capacity and in the epithelial component of cutaneous adnexal structures.

Methods. Samples were fixed in 10% buffered formalin for 24 hrs and embedded in paraffin. Paraffin-embedded 4-µm sections were dewaxed, hydrated, and heat-treated at 95°C for 20 min in 1 mM EDTA buffer pH 8 for antigenic unmasking. Sections were incubated for 30 min at room temperature with smoothelin.

Results. SMA was expressed by the vascular structures of superficial, deep, intermediate and adventitial plexuses, whereas smoothelin was specifically expressed in the cytoplasm of smooth muscle cells of the deepest vascular plexus and in no other plexus of the dermis. The hair erector muscle showed intense expression of smoothelin and SMA. Cells with nuclear expression of smoothelin and cytoplasmic expression of SMA were observed in the outer root sheath of the inferior portion of the hair follicles and intense cytoplasmic expression in cells of the dermal sheath to SMA.

Conclusion. We report the first study of the smoothelin expression in normal skin, which differentiates the superficial vascular plexus of the deep. The deep plexus comprises vessels with high contractile capacity, which is important for understanding dermal hemodynamics in normal skin and pathological processes. We suggest that the function of smoothelin in the outer root sheath may be to enhance the function of SMA, which has been related to mechanical stress. The smoothelin has not been studied in cutaneous pathology; however we believe may be a marker specific for the diagnosis of leiomyomas and leiomyosarcomas of the skin. Also, smoothelin would differentiate arteriovenous malformations of cavernous hemangioma of the skin.

(50.2.O5) ROLE OF CKIS P27 AND P18 IN THE PATHOGENESIS OF MULTIPLE ENDOCRINE NEOPLASIA TYPE 1

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Multiple Endocrine Neoplasia (MEN) is a dominant autosomic disorder with tumors in various endocrine glands. There are two main types of this syndrome, 1 and 2 with two subtypes, 2A and 2B. Affected endocrine glands differ in each type. Previous observations by members of our team in knock-out p27 -/- mice showed that they developed endocrine tumors. Recently some researchers have described p27 mutations in a very small number of patients with clinical MEN1. Other authors also related p18 with these type of tumors. In 1997, a germ line mutated recessive gene, MEN1 gene, was identified in MEN1 patients. This gene encodes a protein, menin, which molecular interactions are not totally understood. The aim of this research was to study the expression of p27 and p18 in endocrine tumors of the patients as a part of a broader scope objective, i.e. to find out the possible role of these proteins in human MEN syndrome. 46 paraffin-embeded biopsies of different endocrine glands, mostly parathyroid, from 39 patients of several Spanish hospitals were analyzed by means of immunohistochemistry. p18 is mostly negative (76,6%) so its absence might be involved in the development of MEN1 through a lack of cell proliferation inhibition. p27 is mostly positive (71,42%). Therefore this protein might not have a role in MEN1, might be mutated and non functioning, or might have a more complex role in the pathogenesis of MEN1. In conclusion, though lack of menin has shown to be a cornerstone in MEN1 pathogenesis, other cell cycle regulators such as p27 and p18 have presumably a role in the development of MEN1 or MEN1-like clinical syndromes.

(50.2.P1) PIT-1 TRANSCRIPTION FACTOR PROMOTES EPITHELIAL-MESENCHYMAL TRANSFORMATION OF HUMAN BREAST CANCER CELLS AND METASTASIS

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Introduction. Pit-1 is a transcription factor critical for cell differentiation of anterior pituitary gland and for gene transcription. Pit-1 is also expressed in nonpituitary cell

tissues (including breast) and it has been suggested that could be related to cell proliferation and tumorigenesis. Epithelial-mesenchymal transition (EMT) is a mechanism for carcinoma progression, inducing neoplastic epithelial cells to acquire mesenchymal malignant traits (motility and invasiveness) thereby contributing to metastatic development. The aim of this study was to analyze the effects of Pit-1 overexpression on the breast carcinogenic and metastatic process.

Materials and methods. Evaluation of the effect of Pit-1 overexpression was performed by using immunodeficient mouse tumor xenograft model and immunohistochemistry to analyze EMT and cell proliferation. For experimental metastasis assays, we generated primary tumors in mice by inoculation of MCF-7 cells stably transfected with either the pTRE2 control vector (n = 11) or the pTRE2-Pit-1–overexpressing vector (n = 12). Primary tumors and lung metastasis were analyzed in paraffin sections by H-E and immunohistochemistry.

Results. Immunohistochemical analysis showed that mouse tumors with MCF-7 cells overexpressing Pit-1 are involved in EMT, as demonstrated by decreased epithelial markers (CKAE1-AE3, CK19, E-cadherin and β-catenin) and increased mesenchymal markers (vimentin). We also found increased cell proliferation (Ki-67 higher). Nine out of the twelve mice injected with Pit-1–overexpressing cells developed metastasis in lungs, while 2/11 mice injected with control MCF-7 cells presented metastasis in lungs (P = 0.012). Metastatic cells showed immunopositivity for CK7, CK19 and vimentin.

Conclusion. Overexpression of Pit-1 in breast cancer cells increases tumor growth in immunodeficient mice, induces EMT and promotes metastasis to lung. The analysis of Pit-1 expression in human breast samples could contribute to improving recognition of patients with higher probability to develop distant metastasis.

Keywords. Pit-1, epithelial-mesenchymal transformation, breast cancer, immunohistochemistry

(50.2.P2) HISTOPATHOLOGY AND DISSEMINATION OF TOXOPLASMA GONDII'S JKF STRAIN IN LUNG TISSUE OF MICE

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Introduction. *Toxoplasma gondii*, Coccidia of the Phylum Apicomplexa is a parasite of worldwide distribution and the causal agent of toxoplasmosis. The JKF strain of *T. gondii* is described as of low pathogenicity as regards to survival of the intermediate host. Our objective was directed to the observation of histological changes in lung tissue of mice (*Mus musculus*) after experimental toxoplasmosis, using the immuno-enzymatic test Avidin-Biotin-Peroxidase (ABC) complex which permits the evidence of antigens in formalin fixed tissues1-3.

Materials and methods. We used 12 young male mice of the NMRI strain, distributed in two groups of 6 individuals each, to study dissemination and histopathology of JKF strain of *T. gondii*. Group 1: infected by intraperitoneal inoculum of 0.5 mL (2 x 103 parasites/mL). Group 2, not infected were the controls. At four and twelve weeks after infection, one individual from each group was sacrified under inhalational anesthesia and samples from lungs were taken for histological preparation. Once the specimens were processed, we observed by light microscopy the histopathological effects and number of parasites in lung tissue.

Results. Observation of lung tissue (1200 X) showed parasite dissemination (cysts and parasites inside of alveolar macrophages and hemorrhages). Parasite account at four weeks post infection (P.I.) was: 7 cysts, 28 groups of parasites and 20 isolated zoites. At twelve weeks p.i.: were 3 cysts, 34 groups of parasites and 3 isolated zoites. Lung specimens from individuals from the group control showed normal appearance and absence of parasites. As regards to the survival, individuals infected and controls were alive after 6 months of observation.

Conclusions. In this study we could appreciate that in experimental toxoplasmosis by the JKF strain of *T. gondii* the cyst formation in lung tissue is of early formation and the mice survival after infection does not seem to be greatly affected.

Keywords. *T. gondii*, toxoplasmosis, tissue dissemination

(50.2.P3) HISTOPATHOLOGY AND DISSEMINATION PRODUCED BY *Toxoplasma gondii*'S JKF STRAIN IN MICES'S LIVER TISSUE

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Introduction. *Toxoplasma gondii* Coccidia of the Phylum Apicomplexa parasite of worldwide distribution is responsible of toxoplasmosis. *T. gondii*'s JKF strain has been described as of low pathogenicity as regards to survival of the host. As the final diagnosis of toxoplasmosis is not always possible by serologic tests and parasite isolation, we suggested the application of the immunoenzymatic test Avidin-Biotin-Peroxidase (ABC) complex, technique created by Hsu et al1 that permits the evidence of antigens in formalin fixed tissues. Our aim was directed to the observation of dissemination and histopathology produced by *T. gondii* in hepatic tissue of mice.

Materials and methods. Included 12 young male mice of the NMRI strain, distributed in two groups of 6 individuals, to study dissemination and histopathology of JKF strain of *T. gondii*. Group 1: infected by intraperitoneal inoculum of 0.5 mL (2 x 103 parasites/mL). Group 2 were controls.

Results. At four and twelve weeks after infection, one individual from each group was sacrified under inhalational anesthesia and samples from liver were taken for histological preparation. Specimens were observed by light microscopy (1200 X) for histopathological effects and count of parasites in hepatic tissue (cysts, isolated and grouped parasites). At four weeks post infection (p.i.) no cysts were found, 24 groups of parasites and 0 isolated zoites. At twelve weeks p.i. count was 4 cysts, 56 groups of parasites and 15 isolated

zoites. Liver specimens from group 1 showed alterations in sinusoid in some areas and disorganized trabecula; liver tissue from individuals from group 2 (control) had normal appearance and were parasite free. As regards to survival, infected and control individuals remained alive after 6 months of observation.

Conclusions. In experimental toxoplasmosis by JKF strain of *T. gondii* cyst formation in liver tissue is of late formation and mice survival does not seem to be greatly affected.

Keywords. *T. gondii*, toxoplasmosis, tissue dissemination

(50.2.P4) LYSYL OXIDASES GENE AND PROTEIN EXPRESSION IN THE DEVELOPMENT OF HUMAN PTERYGIUM

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Introduction. A pterygium is a triangular growth of fibrovascular tissue from the conjunctiva onto the cornea. The pterygium may be the result of newly synthesized elastic fiber precursors and abnormal maturation of elastic fibers (elastodysplasia) that undergo secondary degeneration (elastodystrophy). The balance of tropoelastin, part of elastic fiber, and lysyl oxidases (LOX), essential enzymes for elastin polymerization, play an important role in elastic fiber stabilization. The aim of this study is to evaluate the role of LOX/LOXL-1 in pterygium pathogenesis.

Materials and methods. Specimens of pterygia (n=12) and normal conjunctival (n=12) were obtained from patients after written informed consent. Tissue was fixed in Bouin solution and embedded in paraffin. Tissue sections were used for immunohistochemical stains with anti-LOX and LOXL-1 antibodies. Real Time quantitative transcriptase polymerase chain reaction (qRT-PCR) technique was also used to analyze the LOX and LOXL-1 gene expression.

Results. Regardless of the age and gender factors, increased LOX and LOXL-1 immunostaining was observed in the pathological population. Increased expression for both proteins was observed in the conjunctivas of young patients compared to those older than 50 years. Within the older age group, the disease significantly increased the expression of both proteins. A significant increase in LOXL mRNA was observed in the pterygium samples compared to the conjunctiva. The age and gender factors of the population did not affect LOXL gene expression. No significant differences were found in LOX mRNA between the control conjunctiva and the pterygium. When patients were divided according to age, increased LOX gene expression could be observed in the pathological group over 50 years compared with the conjunctiva. Within the pathological population, women showed a significant decrease of the messenger compared to men.

Conclusions. Lysyl oxidases are overexpressed in the human pterygium tissue, supporting the hypothesis of a dysregulation in the synthesis and reticulation of the elastic components in this type of pathology.

Keywords. Lysyl oxidases, human pterygium, elastic components

(50.2.P5) AMITRIPTYLINE OXIDATIVE DAMAGE IN MICE TISSUES IS ATTENUATED BY ANTIOXIDANTS

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Introduction. Amitriptyline (Amit) is a tricyclic antidepressant widely used in the treatment of depression and chronic pain. We have already described that in vitro treatment with this drug causes an increase in oxidative stress and apoptosis. The aim of this work was to study the biochemical and histopathological changes related to oxidative stress in mice treated with Amit, and the protective role of two antioxidants, coenzyme Q10 (CoQ10) and N-acetylcysteine (NAC).

Material and Methods. 30 female C57BL-6 mice were divided into five groups: Control, Amit, Amit+CoQ, Amit+NAC, and Amit+CoQ+NAC. Mice were treated with a daily i.p. injection of Amit (20 mg/Kg b.w.) for 2 weeks. In some groups, antioxidants were also injected (CoQ at 10 mg/Kg b.w., and NAC at 20 mg/Kg b.w.). Mice were sacrificed and several organs were removed. Lipid peroxidation and antioxidants status (catalase and SOD) were determined by C11-Bodipy and ELISA, respectively. Histopathologic examination was performed by hematoxylin-eosin staining and caspase-3 immunostaining.

Results. Most tissues showed an increase of lipid peroxidation levels in mice treated with Amit, especially significant in liver and lung. Antioxidant levels were decreased in mice treated with Amit. Oxidative damage was not observed in mice co-treated with Amit and antioxidants. Histopathologic study revealed increased apoptosis rate in the liver of Amit-treated mice, absent in mice co-treated with antioxidants.

Conclusion. Amit treatment induces oxidative stress mainly in liver and lung, leading to increased lipid peroxidation, an important consequence of disproportionate free radicals, and decreasing the antioxidant machinery. CoQ10 and NAC supplementation attenuated oxidative stress in these organs.

This study was partially supported by IV Plan Propio de Investigación (Universidad de Sevilla, Ref. 2010/00000453).

Keywords. Amitriptyline, oxidative stress, antioxidants

(50.2.P6) NEW APPROACH TO DIAGNOSTIC OF PLEOMORPHIC SARCOMA AND DEDIFFERENTIATED LIPOSARCOMAS

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Introduction. The term liposarcoma refers to an array of cancerous tumors and the behaviour of any liposarcoma

is dependent on its histological subtype. It has three principal forms: atypical lipomatous tumour: well-differentiated (WDLPS) or dedifferentiated (DDLPS), myxoid or round cells LPS, and pleomorphic LPS. Differential diagnostic between benign lipomas and liposarcomas is one of the most frequent problems encountered. Advantages in molecular biology and cytogenetic techniques lead us new point of view respect conceptual and classification of these neoplasm. Currently, it is known that WDLPA and DDLPS show similar features with one or two supernumerary rings or giant rod chromosomes with amplified sequences of various regions, It is notary that CDK4 and MDM2 genes present a strong correlation between its amplification and expression in WDLPS and DDLPS. In contrast, MDM2-CDK4 amplification is not usually observed in benign adipose tumour.

Methods and results. The aim of this research is the determination of MDM2-CDK4 amplification by fluorescence in situ hybridization (FISH) and quantitative RT-PCR compared to immunohistochemical techniques, in order to distinguish WDLPS and DDLPS from benign adipose tumour. Results showed that FISH and QRT-PCR methods gave concordant results and were equally informative in most cases.

Conclusions. FISH was more specific and sensitive than QRT-PCR and IHC, by allowing visualization of individual cells. The evaluation of MDM2-CDK4 amplification using FISH or QRT-PCR be used to supplement IHC analysis when diagnosis of adipose tissues tumours in not possible based on clinical and histological information alone. Furthermore we proposed the independent or synergistic MDM2 and CDK4 amplification may have prognostic value in adipose tissue tumours.

Keywords. Pleiomorphic sarcoma; liposarcoma

(50.2.P7) COMPARATIVE STUDY OF INFARCT SIZE AND THE LYMPHOCYTE POPULATION IN A PORCINE MODEL OF MYOCARDIAL INFARCTION TREATED WITH ANTI-TNF-ALPHA

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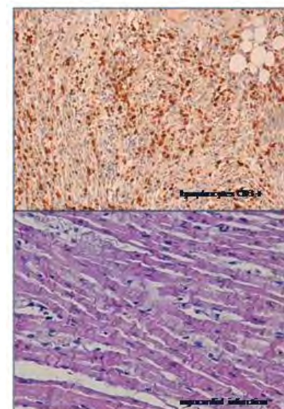
Introduction. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine with pleiotropic biological effects. It is released in response to acute myocardial infarction from macrophages, monocytes and cardiomyocytes within minutes. The precise impact of TNF- α signalling on myocardial ischemic injury remains controversial but the inhibition of TNF- α signalling via quenching antibodies has been reported to protect against myocardial ischemia/reperfusion injury. In this study we aim carry out a preliminary evaluation of the effect of "ethanercept" an anti-TNF- α soluble receptor, to reduce infarct size and microvascular obstruction in a porcine model of myocardial infarction.

Materials and methods. We have studied infarct size, histological structure and the number of T lymphocytes in 16 pigs that had previously been induced myocardial infarction by means of mid left anterior descending artery occlusion using percutaneously introduced angioplasty balloon. Six pigs were given anti-inflammatory treatment with TNF- α and 10 pigs served as controls. We have also studied microvascular obstruction detected by intracoronary injection of thioflavin S (4% solution) to define the region of microvascular obstruction.

Results. The mean infart size and mean microvascular obstruction marked by Tetrazolium (TTC) and Thioflavin S on postmorten examination and measured by morphometry in 6 pigs treated with TNF- α was the 2±2% (p=0.02 vs. controls) and 7±13% (p=0.2) respectively. The mean infart size and microvascular obstruction in 9 pigs treated byTNF- α was the 4,32 and 1,32 respectively. We also studied the amount of CD3 positive lymphocytes T measured by morphometry. In the control group we found that 17020 lymphocytes T CD3+, whereas in the group trated by TNF- α was 5275. We found that in all cases but one, the number of lymphocyte was much lower than that found in the control group.

Conclusions. In a porcine model of myocardial infarction we found that the infarct size, microvascular obstruction and the number of lymphocytes T was higher in the control group than in the group treated with TNF- α. Further studies are needed to confirm this promising preliminary data.

Keywords. Myocardial infarction, microvascular obstruction, infarct size, lymphocytes T, TNF-α



(50.2.P8) MELATONIN RECEPTOR EXPRESSION IN WARTHIN'S TUMOR

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Introduction. Melatonin (N-acetyl-5-methoxytryptamine) is a lipophilic hormone that is primarily synthesized and secreted by the pineal gland and is widely distributed

throughout the human body. The first melatonin receptors to be cloned were located in the cell membrane and designated Mel1a and Mel1b. These were later renamed MT1 and MT2 receptors. The objective of this study is to analyze the expression of MT1 melatonin receptor in Warthin's tumor and normal parotid gland.

Methods. The study included samples of Warthin's tumors from 14 patients. All surgical procedures were carried out between 9 am and 1 pm. We used a goat polyclonal antibody raised against a peptide mapping at the N-terminus of MEL-1A R of human origin; it was applied at a dilution of 1:500. Formalin-fixed, paraffin-embedded tissue blocks were sectioned at 4-µm thicknesses. Tissue sections were deparaffinised in xylene, processed through a graded series of alcohols, and rehydrated in distilled water.

Results. All 14 Warthin's tumors studied showed intense cytoplasmic positivity for MT1 receptor in all cylindrical epithelial cells lining spaces and a less intense positivity in basal cells. The lymphoid component accompanying the tumor was always negative for MT1 receptor. The parotid structure surrounding the tumor showed intense cytoplasmic positivity in all cells lining excretory ducts (lobar and lobulillar), with a lesser and focal positivity in cells of the acinar component.

Conclusion. These findings indicate that the MT1 receptor may participate in synthesizing secretions contained in the spaces demarcating tumor cells and in synthesizing cytokeratin filaments. The biological activity of MT1 receptor in epithelial cells lining parotid excretory ducts may resemble its activity in Warthin's tumor cells. Hence, we propose Warthin's tumor as a useful positive control in immunohistochemical studies of MT1 melatonin receptor.

(50.2.P9) MORPHOLOGICAL CHANGES INDUCED BY EXPERIMENTAL GLAUCOMA IN THE RAT OPTIC NERVE. EFFECT OF LATANOPROST

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Introduction. Glaucoma is a chronic neuropathy associated with elevated intraocular pressure *(*IOP). A rat model of glaucoma, in which there is chronic, moderately elevated IOP and loss of retinal ganglion cells *(*RGC), has been established to study pharmacological agents that have the potential to be neuroprotective. In this model, the pharmacological use of latanoprost, a prostaglandin F2α analogue, significantly prevents the loss of RGC. The aim of the present work was to evaluate the effect of chronic elevated IOP on the optic nerve and examine nerve fibre survival and glial reactivity after treatment with latanoprost.

Materials and methods. Male Wistar rats were divided in two groups: an untreated experimental group and an experimental group treated with latanoprost. IOP was elevated by cauterization of three episcleral veins in the right eye. The left eye served as a sham control. The hypotensive drug was administered 15 days after IOP elevation and continued for 3 months. The IOP was measured every two weeks. The optic nerves *(*ON) were removed 3 mm posterior to the eye and processed for electron microscopy.

Results. Experimental eyes showed morphological changes in the RGC axons compared with fellow untreated eyes. Axonal changes included degenerating axon profiles with shrinkage, swelling and fragmentation. Astrocytes became reactive with hypertrophic morphological changes. An apparent reduction of the degenerating fibres was observed after treatment with latanoprost. Intense glial reactivity persisted, including hypertrophic astrocytic processes and mitosis. The quantitative study showed an increase in the density and total number of axons, and in the surface density of astrocytes in the ON treated with latanoprost.

Conclusions. Chronic elevated IOP produces changes in ON consistent with axonic degeneration and loss of optic nerve axons. Our findings suggest that latanoprost is able to provide neuroprotection of the optic nerve fibres after IOP elevation.

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Keywords. Axonal degeneration, Glaucoma, Latanoprost, Optic nerve

(50.2.P10) INTRATISULAR PERCUTANEOUS ELECTROLYSIS IN THE TREATMENT OF CHRONIC TENDINOUS LESIONS. A EXPERIMENTAL STUDY

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Introduction. The intratisular percutaneous electrolysis (IPE) was introduced as a physiotherapy tool for treat chronic tendinous and musculosqueletical lesions. In first time (1895) was used an acupuncture needle that was inserted near the lesion (cathode) and other needle (anode) placed in the other part of the lesion. The cathode was connected to a galvanic current of low intensity and diverse time.

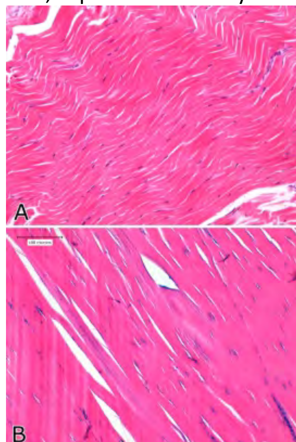
Materials and Methods.Five rotulian tendon of rabbits were used. A galvanic current was applied with diverse intensity (mA) at different time (1 to 5 minutes). The pieces of the all animals were removed and fixed in formalin 4% for one week. The material was included in paraffin, and the sections obtained were colored with hematoxylin eosin and trichrome staining. Each case was photographed and lesions were sized in length and depth.

Results. All five experimental tendons showed different length and depth in correspondence with the intensity and the time that are exposed. The major lesion appeared when 2 mA of intensity and 5 minutes of duration was applied. This lesion implicates a destruction of all fibroblast situated within the cathode and the anode as well as the peritenon tissue.

Conclusions.We show here a new therapeutic treatment, no invasive and whit very low cost for chronic lesions that permits obtain good results with restoration of the lesion and the tissues. Although this is a experimental model and no restorative tissues were appreciated in a future

we want to develop this method to appreciate the evolution of the lesions induced by IPE.

Keywords. Intratisular Percutaneous Electrolysis, Chronic Tendinous Lesions, Experimental Study



(50.2.P11) CELL CYCLE CONTROL PROTEINS AS MARKERS IN LARYNX PRECANCEROUS LESIONS

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Introduction. A hallmark of malignant tumors is an uncontrolled cell proliferation. Normal proliferation is controlled by the interaction of different proteins, enhancing and inhibiting progression along the cell cycle. Some of these proteins are altered in cancer cells and could be useful as markers of diagnosis and prognosis of premalignant and cancerous lesions. The objective of this research was to determine the expression of several cell proliferation inhibitors in laryngeal preneoplastic lesions and its possible clinical application.

Material and methods. A multicentric retrospective study was performed on patients with a diagnosis of laryngeal preneoplastic lesion (mild, intermediate, severe dysplasia). Paraffin-embedded biopsies were immunohistochemically stained with antibodies against the tumor suppressors p53, p73 and pRb, and against the cyclin dependent kinase inhibitors (CKIs) p21, p27, p57, p15, p16 and p18.

Results. pRb was expressed in almost all samples, including those progressing to carcinoma. This result was unexpected taking into account the role of this protein as a cell cycle main brake. P53 was expressed in most dysplasias, particularly those intermediate and, more outstandingly, in the severe ones, a result in accordance with the probable gene mutation in the case of protein detection. P21, also a cell proliferation inhibitor, was particularly expressed in mild and intermediate dysplasias. P27 was detected very occasionally, as expected for a tumor suppressor. P57 was negative in all cases. P15 was expressed only in a few cases of mild dysplasia and was absent in the severe ones. P16 was positive in low grade dysplasia and negative in most severe forms. P18 was negative in all grade dysplasias.

Conclusions. To study the expression of cell cycle markers could be helpful in understanding laryngeal cancer genesis and useful in predicting the severity of the precancerous lesions. International networks allowing the analysis of wider number of cases should be promoted.

(50.2.P12) DELETION OF H-RAS DECREASES RENAL FIBROSIS AND MYOFIBROBLAST ACTIVATION FOLLOWING URETERAL OBSTRUCTION IN MICE

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Introduction. Tubulointerstitial fibrosis is characterized by the presence of myofibroblasts, responsible for extracellular matrix accumulation. Myofibroblasts can originate from the activation and proliferation of resident fibroblasts, from bone marrow-derived cells and from renal epithelial cells upon undergoing epithelial to mesenchymal transition (EMT). RasGTPases are activated during renal fibrosis and, as they play crucial roles in regulating both cell proliferation and TGF-beta-induced EMT, we set out to assess the contribution of Ras to the fibrosis induced by unilateral ureteral obstruction (UOO), a well-established experimental model of renal fibrosis.

Materials and methods. All studies were performed in parallel in H-ras-/- and H-ras+/+ littermates male.

mice aged 8 weeks. Left ureter of all animals was ligated and the obstruction was maintained for 15 days. Functional, morphological and statistical studies were performed in kidneys from the ureteral obstructed animals.

Results. Here we show that fifteen days after UUO, both fibroblast proliferation and the expression of EMT inducers were lower in obstructed kidneys of H-ras-/- compared with H-ras+/+ mice and in the fibroblast cell lines obtained from them. Interestingly, interstitial fibrosis, fibronectin, collagen I accumulation and the myofibroblast population were also lower in H-ras-/- than in H-ras+/+ mice. As expected, lower levels of activated Akt were found in the kidneys of H-ras-/- than in those from H-ras+/+mice and cultured fibroblasts.

Conclusions. lower fibrosis is observed in obstructed kidneys from H-ras-/- mice due to a reduction in the proliferation of resident fibroblasts and to a reduced EMT. Thus, our data suggest that the inhibition of Ras may be a useful approach to prevent the progression of renal fibrosis.

Keywords. H-Ras, Renal fibrosis, myofibroblasts

(50.2.P13) METHYLATION OF RARB GENE: POSSIBLE DIANOSTIC MARKER FOR PROSTATE CANCER

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Intoduction. DNA methylation is an epigenetic event that affects gen expression. Aberrant methylation is a relative frequent event in neoplasic processes. The aim of this work was to analyze methylation patterns of CpG-rich promoter regions of the RARbeta gene in samples from normal and prostate cancer (high, medium and low Gleason) diagnosed patients. We discuss the use of this method as a preventive strategy for possible biochemical relapses of the disease.

Materials and methods. This study was carried out in 5 patients with non pathologic prostate, 30 patients diagnosed of prostate cancer (10 with low, 10 with medium and 10 with high Gleason grades). After extraction and purification from paraffin embedded sections, DNA was bisulfite-modificated, and amplified by PCR using both methylated and non-methylated primers (Invitrogen). The obtained products were visualized after electrophoresis.

Results. 100% of samples from the low and high Gleason grades groups shown RARbeta methylation, whereas only 40% of medium samples from medium Gleason patients shown methylation. To the non-methylated RARbetta was detected in 20% low Gleason samples and 40% medium Gleason samples.

Conclusion: Previous reports suggest that methylation reach near 80% of patients. The importance of this fact lies in gene silencing of tumor suppressor genes. Our results suggest that RARbeta may be a useful marker, together with other genes, for prostate cancer diagnosis and prognosis.

Supported by grants from the "University of Alcalá" (CCG10-UAH/BIO-5985) and the "Foundation Mutua Madrileña" (AP76182010), Spain.

Keywords. RARB, prostate cancer, methylation

(50.2.P14) PROSTATE-SPECIFIC ANTIGEN (PSA), NF-KB AND PATHOLOGY PROSTATE (BENIGN HYPERPLASIA AND CANCER): RELATION WITH THE MALIGNANCE

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Introduction. NF-kB (p50/p65) is a transcription factor involved in TNF-a-induced cell death resistance by promoting several antiapoptotic genes. The aim of this study was to relate the expression of NF-kB (p50 and p65) with serum levels of prostate-specific antigen (PSA) in normal (no pathological samples) as in pathological samples (hyperplasia and cancer), in order to elucidate their possible role in tumour progression. We are also discussing the possible usefulness of NF-kB as a potential therapeutic target.

Methods. This study was carried out normal, benign prostatic hyperplastic (BPH) and prostate cancer (PC) human prostates. Immunohistochemical and Western blot analysis were performed. Serum levels of PSA were assayed by PSA DPC immulite assays (Diagnostics Products Corporation, Los Angeles, CA).

Results. p65 was not found in NP. P50 was scantly detected in the cytoplasm of epithelial cells in 60% of NP samples. In BPH, p50 and p65, were expressed in 62.5% of samples and in the three groups of PSA serum levels patients: 0-4 ng/ml, 4-20 ng/ml and >20 ng/ml. In cancer both subunits (p50 and p65) were expressed in 63.15% of patients (also in the nucleus). p50 and p65 increased its frequency of expression with PSA serum levels.

Conclusions. Activation of NF-kB, by its nuclear translocation in prostate cancer could be related with cancer progression and elevated serum levels of PSA. A better understanding of the biologic mechanism by which circulating PSA levels increase and its relation with NF-kB expression could help to understand the evolution of this disease. Thus, NF-kB blockage could counteract the excessive proliferation index typical of prostate cancer.

Acknowledgement. Supported by grants from the "University of Alcala" (CCG10-UAH/BIO-5985) and the "Foundation Mutua Madrileña" (AP76182010), Spain.

Keywords. NF-kB/p50, NF-kB/p65, PSA and prostate

(50.2.P15) IL-6: ROLE NO PROLIFERATIVE IN LNCaP AND PC3 POSTATE CANCER CELLS

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Introduction. Emergence of androgen-refractory tumors is the main problem for prostate cancer treatment. ndrogen-independent growth could be directed by, among other mechanisms, the presence of signals within the tumor milieu which fulfill the lost of proliferative stimuli of androgens. IL-6 production has been correlated with tumor progression in a number of human malignances. Specifically, serum levels of IL-6 has been shown to be enhanced in hormone-refractory prostate cancer patients, because of this IL-6 has been proposed as mediator of androgen-independent tumor growth. In the present work we analyzed the effect of IL-6 in the growth of LNCaP and PC3 cells upon serum-starved conditions.

Methods. LNCaP and PC3 cells growing in medium supplemented with FBS 10% were harvested and seeded in 96-well adherent plates at 70-80% confluence. Cells were or not pre-incubated in FBS-starved medium for 24 hours. Then, cells were exposed to different IL-6 concentrations (10, 25, 50, 100 and 150 ng/ml) for 72 hours upon serum-starved conditions. At the end of treatment, cells were incubated with MTT for 3 hours, MTT precipitates were dissolved by adding SDS, and absorbance at 570 nm was measured as an estimation of the number of viable cells.

Results. No changes were found between control and treated cells, at any condition, in any of the two experimental conditions neither cell line.

Conclusion. IL-6 it is not able to promote in vitro androgen-independent growth. Other cytokines as EGF or IL-1 could contribute to tumor growth at low levels of androgens.

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Keywords. IL-6, proliferation, prostate cancer

(50.2.P16) CD11C AND MHCII DECREASE IN MOUSE THYMUS AFTER INHALED VANADIUM EXPOSURE. IMMUNOHISTOCHEMISTRY AND FLOW CYTOMETRY STUDY

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Metals are transported in the air adhered into suspended particles (SP) which are the final product of the combustion of oil derivates such as gasoline; also the metallurgic industry is a source for the presence of metals

in the air. One of the recurrent compounds find on these particles is vanadium pentoxide (V_2O_5), and by inhalation enters into the respiratory system and to the main blood stream. Dendritic Thymic Cells (DC) are regularly distributed into the thymus medulla and are responsible for the negative selection of auto-reactive T cells. There are some reports that indicate the changes in the cortex-medulla thymic distribution as a consequence of V_2O_5 exposure as well as changes in cell populations' arrangement in a mice model. These changes suggest a modification in the morpho-physiology of CD with possible health repercussion. With this in mind we decide to explore the presence and the expression of CD11c (Dendritic's cell biomarker) and MHCII in the thymus of mouse exposed to inhaled V_2O_5.

Mice inhaled V_2O_5 [0.02M] twice a week for a 4-week time period. Control and exposed mice were sacrificed; the thymus was extracted and processed for regular immunohistochemistry for CD11c and flow cytometry for CD11c and MHCII. Five fields from each mouse thymus were photographed and analyzed with Image Pro Plus 2.0 software. Differences in color density (pixels) were compared (ANOVA Tukey's $p<0.05$). Results indicated a decrease in color density for CD11c, as the time of exposure increases; also the expression of CD11c and MHCII determined by flow cytometry decreased. It is possible to consider that the presence of CD11c decreased as a consequence of the oxidative potential of vanadium, which generates Reactive Oxygen Species (ROS); reports mention that ROS may modify transcriptional factors associated with the inflammatory response.

Keywords. dendritic cells, thymus, vanadium pentoxide

(50.2.P18) TOOTH SURFACE MICROWEAR AND ROUGHNESS SHOW TROPHIC ECOLOGY IN TELEOSTS

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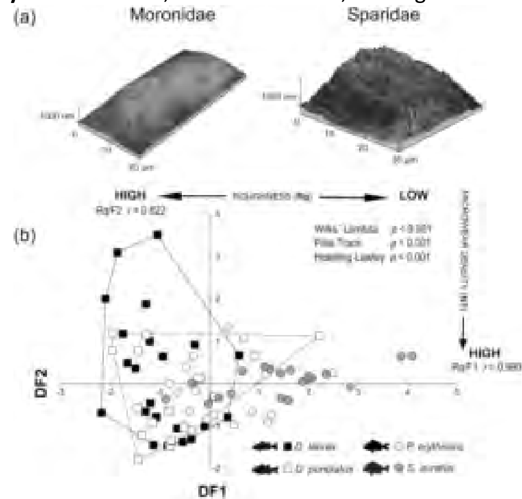
Teleosts are members of a large and extremely diverse group of ray-finned fishes, constituting more than 50% of vertebrate species. The role of trophic ecology in fishes can be tested through direct observations of structures in use. Since the effects of feeding on the tooth surface are still not well understood, the aim of this work was to compare tooth surface microwear textures and predatory behavior in two families of teleosts. Premaxillae teeth of wild-caught specimens of Moronidae (Dicentrarchus labrax, n=22 and Dicentrarchus punctatus, n=18) and Sparidae families (Sparus auratus, n=19 and Pagellus erythrinus, n=17) were selected and grouped into more and less predatory behavior respectively based on previous studies. Atomic force microscopy (AFM) was used to obtain 3D root mean square roughness (Rq) (~30×30µm areas) and, 2D microwear density (NT) was measured using Scanning Electron Microscopy (SEM) at 1000X (0.01mm2 areas) from the labial-tooth surfaces. Univariate and multivariate statistical test ($p<0.05$) were used to evaluate interspecific differences. Least-squares regression results of log10(NT) and log10(Rq) (independent variable) reveal significant negative correlation ($r = -0.483$; $p<0.000$) and a covariance analysis showed that the equality of slopes can be rejected ($p = 0.01$) within taxon. We found that the surface Rq showed significant higher values in predatory species (Moronidae family) than in less predatory (Sparidae family) ones. Likewise, Moronidae species tend to have less microwear density (NT: $p<0.01$) and anisotropic tooth surface textures, dominated by homogeneous scratch pattern (Fig. 1). Species with low values of Rq tend to have high tooth-microwear density, showing that isotropic surface texture for the Sparidae teeth indicates a preference for abrasive benthic feeding and minor predatory behavior. These results indicate that tooth surface microwear textures can detect interspecific differences in trophic ecology and structural surface topography role in fishes as a driver of speciation.

Keywords. Teleost, tooth-microwear, feeding



(50.2.P19) BIOMARKERS OF CELL PROLIFERATION AND APOPTOSIS IN PATIENTS WITH SJÖGREN SYNDROME

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Introduction. Sjögren's syndrome occurs associated with parotid neoplasm, non-Hodgkin's B cell lymphoma, which could impair the condition or be life-threatening of patients. The aim of this work was to analyze the expression of biomarkers of cell proliferation and apoptosis modifications in morphological areas of salivary glands in patients with Sjögren Syndrome, keratoconjunctivitis or stomatitis sicca or in healthy subjects, to establish parameters that indicate the likelihood of malignancy of the disease in populations at risk.

Methods: A study was performed of histological samples of lower lip salivary gland (SG) from patients diagnosed with SS (n=20), SICCA (n=20) and control individuals –clinical health- (n=18) attending the Hospital Complex of Jaén (Spain) between 2003 and 2008 and the study was

approved by the hospital ethics committee. The SS or SICCA were diagnosed according to the criteria of the European-American Consensus Group. Ki67 and Caspase-3 and p53 immunolabeling were performed. The exploration of patterns of association among proliferation and apoptosis and different study groups was performed by correspondence analysis.

Results: The correspondence analysis evaluated the relationship between immunomarkers and studied groups. A remarkable association was observed between strong and mild percentages of capase-3 and Ki67, p53 with the SS group. The most important result was significant differences between the three study groups in Ki67 and Caspase-3 markers ($p<0.0001$) in infiltrated lymphocytes. On contrary, p53 did not mark any region of the gland. Conclusion: The results of this work are indicative of a high degree of proliferation (85%) in infiltrated lymphocytes (IL) associated with SS which, according the literature, could be considered a risk. Furthermore, the markers used in this work are widely known and represent a lower cost than others, and can be used to determine risk groups within the population of SS patients, enabling their follow-up.

Keywords. Sjögren's syndrome, Biomarkers, caspase -3, Ki-67, p53

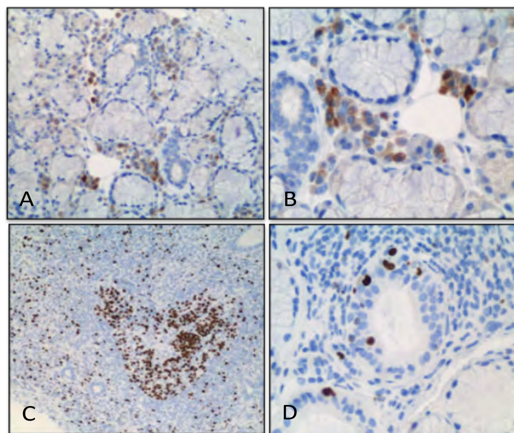

Figure 1. A) Intense expression of caspase-3, predominantly in infiltrating lymphocytes, in a patient with Sjögren's syndrome. This marker is also observed in scant ductal cells (immunohistochemical technique; 20X, Scale bar= 100 µm). B) Expression of caspase-3 in lymphocytes clustered in a single focus in a patient with Sjögren's syndrome (immunohistochemical technique; 40X, Scale bar= 100 µm). C) Intense expression of Ki-67 in foci of lymphocyte infiltration in a patient with Sjögren's syndrome (immunohistochemical technique; 10X, Scale bar= 100 µm). D) Ki-67 expression in ductal cells and scant lymphocytes in a patient with Sjögren's syndrome (immunohistochemical technique; 40X, Scale bar= 100 µm).

(50.2.P20) CYTOPLASMATIC PRESENCE OF TWIST AS EPITHELIAL TO MESENCHYMAL TRANSITION MARKER, AND ITS ASSOCIATION WITH VEGF IN MALIGNANT AND NON MALIGNANT PROSTATE TISSUE

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Introduction. Prostate cancer is a type of malignancy with high prevalence in Chile and the world. Eventhough principal screening methods have helped in early detection, still have very low specificity and sensitivity, so scientist remain searching for new diagnostic and pronostic markers.

The epithelial-mesenchymal transition program (EMT), wich plays a physiological role during embryonic development, becomes aggressive and destructive in most epithelial tumors, since it is pathologically activated, leading malignant cells to release from epithelium through down-expression of E-cadherins, and become able to migrate and settle in distant tissues. The cell-cell and cell-extracellular matrix interactions are also altered, what helps migration. TWIST as a transcription factor represses E-cadherin, tumor progression, and metastasis. Also, TWIST inhibits apoptosis and promotes synthesis of vascular endothelial growth factor (VEGF) inducing tumoral angiogenesis. Our objective was to identify TWIST and VEGF in malignant and non malignant prostate tissue samples and compare them with control prostates.

Methods. 40 tissue samples from patients with recently diagnosed prostate cancer and 40 non malignant controls were analized with immunohistochemical staining for TWIST and VEGF. Proportion of stained cells were counted and compared. Correlation index was analized between both factors.

Results. We found that TWIST and VEGF were highly present in citoplasm of glandular cells in prostate cancer, and significantly different than non malignant controls. Additionally, proportion of stained cells for both proteins (TWIST and VEGF) are highly correlated. No association was found with Gleason score, prostatic specific antigen nor recurrence of cancer.

Discussion. Proportion of cells stained for VEGF and TWIST is significantly different in glandular prostatic cells of malignant and non malignant tissue samples and both proteins are highly correlated, what suggests that presence of VEGF, besides tumor angiogenesis, may be related to activation of signal pathways of EMT program.

KEYWORDS. Prostatic cáncer, benign prostatic hiperplasia, TWIST, VEGF, epitheliat to mesenchymal transition EMT

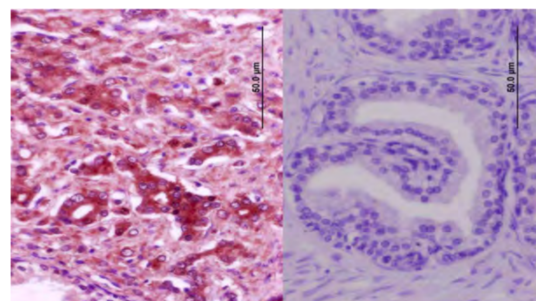

Figure: Immunohistochemical demonstration of TWIST in prostate cancer and
benign hyperplasia. Left: Prostate Cancer Right: benign hyperplasia.

(50.2.P21) PATHOLOGICAL FINDINGS OF THE FIRST CASE EVER OF FATAL FAMILIAL IMSOMNIA IN ASTURIAS

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Introduction: All Spanish regions have experience in the diagnosis of spongiform encephalopathies, specially the most common one CJD. From the Basque Country FFI cluster diagnosis, the neighbouring regions have had the opportunity to diagnose some cases from the same region. In Asturias there has been just one confirmed case of FFI. Aim: To review the pathology of one case of FFI, studying in depth specially the thalamic lesions and comparing them with other two cases of patients with sleep sever pathology.

Materials and Methods: Serial sections of the thalamus were studied in the three cases with myelin histochemical technique and glia, prionic protein, stress proteins and apolipoprotein D immunostaining. The rest of the structures were studied with conventional hematoxylin-eosin.

Results: The only FFI isolated case is linked to the Basque Country cluster. The pathological changes are the ones described for the classical forms, with minimal cortical spongiform changes and a predominance of the thalamic, cerebella and olives damage. The ASP178ASN mutation on PRP gen was confirmed, being the patient heterozygote for VAL129MET. In the two cases of severe insomnia these changes were not observed, being Alzheimer's type primary changes the predominant ones with senile plaques in the nuclear groups of the thalamus. Detected gliosis was better observed with microglia and astroglia staining, being also remarkable the presence of stress proteins and apolipoprotein D. No PrP deposits were observed.

Conclusions: The FFI case detected in Asturias has the anatomopathological characteristics of the classical FFI form. Its lesions are better detected with special techniques of routine use as well as the apolipoprotein D staining a lipocalin family member that has been described by our group in the senile plaques of Alzheimer's disease.

Keywords. spongiform encephalopathy, insomnia

(50.2.P22) CASPASES EXPRESSION IN NORMAL AND PATHOLOGICAL HUMAN PROSTATE: POSSIBLE ROLE AS BIOMARKERS

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Introduction. In the prostate gland, homeostasis is a balance between proliferation and apoptosis. Caspases are a family of highly conserved cysteine-dependent-aspartate-specific proteases that play a crucial role in the apoptosis regulation. This study focuses to analyze caspase 8, caspase 3, caspase 7 and caspase 9 expressions in normal prostate, benign prostate hyperplasia (BPH), prostate intraepithelial neoplasia (PIN) and prostate cancer (PC) in order to know its relationship with apoptosis levels.

Methods. Immunohistochemical and Western blot were performed in normal prostates, BPH, PIN (low- and high-grade PIN) and PC (low, medium and high Gleason) samples.

Results. In normal prostates samples immunoreactivity to all caspases studies was found in cytoplasm of epithelial cells. In BPH all antibodies were detected with the same intensity that in normal prostate, except procaspase 8 that its immunoexpression increased. In PC, immunoexpression to procaspase 3 caspase 7 and caspase 9 were similar than normal prostate and BPH; immunoexpression to procaspase 8 and caspase 8 increased in comparison with BPH; while immunoexpression to caspase 3 decreased significantly. Although, the percentage of positive samples decreased in BPH compared with normal samples and, more markedly, in high grade PIN and PC (increasing with Gleason grade).

Conclusion. We conclude that caspases expression in prostate malignant cells is downregulated in a substantial number of patients. Such an alteration occurs early in prostate cancer development, at pre-malignant phase. In some cases, caspases downregulation has been linked to promoter hypermethylation of genes encoding both caspases and transcription factors that enhance caspases expression. This has led to propose demethylating agents to target caspases for cancer treatment.

Acknowledgement. Supported by grants from the "University of Alcala" (CCG10-UAH/BIO-5985) and the "Foundation Mutua Madrileña" (AP76182010), Spain.

Keywords. caspases, pro-caspases, apoptosis, prostate cancer

(50.2.P23) IL-6/ERK/NF-KB TRANSDUCTION PATHWAY: A STUDY IN NORMAL AND PATHOLOGICAL HUMAN PROSTATE

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Background. This study focuses on IL-6/ERK transduction pathway, its relations with NF-kB and the consequences of this deregulation in the development of prostate pathologies such as benign prostate hyperplasia (BPH) and prostate cancer (PC).

Methods. Immunohistochemical and Western blot analyses to IL-6, gp-130, Raf-1, MEK-1, ERK-1, p-MEK, ERK-2, p-ERK, NF-kB/p-50 and NF-kB/p-65 were carried out in normal prostates (NP), BPH, and PC (low, medium and high Gleason) samples.

Results. Immunoreaction in NP samples to IL-6, gp-130, ERK-1, ERK-2, p-ERK and NF-kB/p50 was found in the cytoplasm of epithelial cells; p-MEK was found in the nucleus of epithelial cells; but not expression to Raf-1, MEK-1 and NF-kB/p65 were found. In BPH, all proteins were immunoexpresed, while increased

immunoexpression to IL-6, gp-130, p-MEK, ERK-1, ERK-2 and NF-kB/p50 (cytoplasm location). In PC, immunoexpression to IL-6 and gp-130 were similar than BPH; while immunoexpression to Raf-1, MEK-1, p-MEK, ERK-1, ERK-2, p-ERK, NF-kB/p50 (nucleus and cytoplasm) and NF-kB/p65 (nucleus and cytoplasm) was higher than BPH.

Conclusions. Translocation of NF-kB to the nucleus in PC could be slightly stimulated by IL-6/ERK transduction pathway, but could be stimulate by other transduction pathway, such as TNF-a/NIK, TNF/p38, IL-1/NIK or IL-1/p38. Activation of NF-KB in PC could be regulating IL-6 expression. These transduction pathways are also related to activation of other transcription factors such as Elk-1, ATF-2 or c-myc (also involved in cell proliferation and survival). PC is a heterogeneous disease where multiple transduction pathways altering the apoptosis/proliferation balance. Significant attention should be directed to combination of novel agents directed toward inactivation of pro-inflammatory cytokines because could be disrupt tumour cell growth.

Acknowledgement. Supported by grants from the "University of Alcala" (CCG10-UAH/BIO-5985) and the "Foundation Mutua Madrileña" (AP76182010), Spain.

Keywords. IL-6, ERK, NF-kB, prostate cancer

(50.2.P24) CELL PROLIFERATION AND APOPTOSIS DETERMINATION IN PROSTATE CANCER

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Purpose. Prostate Cancer (PCa), in Chile and worldwide, represents one of the most important causes of death. Widely used indicators of cellular proliferation and apoptosis are Ki-67 and DNA fragmentation respectively. Actually, there is a controversy concerning the correlation between these markers and diagnosis or prognosis of PCa; this is where this study points at.

Material and methods. This case control study was performed with patients diagnosed with PCa or Benign Prostatic Hyperplasia (BPH), operated by prostatectomy. The prostate biopsies were analyzed for Ki-67 by immunohistochemistry and DNA fragmentation by TUNEL in glandular cell tissue. A proliferation proportion (PP), apoptosis proportion (AP) and a proliferation apoptosis radio (PAR) were calculated and correlated with PCa diagnosis and its recurrence after one year.

Results. A statistically significant correlation was neither observed between PP, AP and PAR with diagnosis (p=0.2469, p=0.6327, p=0.5331 respectively), nor with recurrence after one year (p=0.715, p=0.279, p=0.357 respectively). Both PCa and BPH present high proliferation proportions, so this condition is essential but not specific for cancer. In the AP, a difference between PCa and BPH was not found; however, this proportion does not necessarily reflect the number of apoptotic cells, due to the increase of the total number of cells because of the high proliferation rate. As both Ki-67 and DNA fragmentation, showed no difference between cancer and hyperplasia, we conclude that these techniques, very useful in other malignancies, have no usefulness in prostatic cancer, and other prognostic factors have to be found for diagnosis and prognosis.

Keywords. Prostatic cáncer, Ki-67, TUNEL, apoptosis, benign prostatic hyperplasia

Tabla 1. PP, AP, PAR in prostatic cancer and benign hyperplasia.

	Benign Pros Hyperplas n=40	Prostatic Ca n=40	p*
Age	70 (65-75	65 (60-69	0.0005
Gleason	-	6 (6-7)	-
PSA pre sur	3.8 (2.8-5.	7.5 (6-10	p<0.000
PSA 12 mor	-	1.1 (0.065-1	-
Proliferative pro PP	0.449(0.362-(	0.474(0.406-(	0.2469
Apoptotic propo	0.0027(0.0016-	0.0039(0.0019-	0.6327
Proliferation/ap radio PA	140.641(65. 265.212	119.913 (38. 275.161	0.5331

*Wilcoxon ranksum Mann-Withney test

(50.2.P25) ARCHITECTURE OF AN INTRANET DATABASE TO SUPPORT TRANSLATIONAL RESEARCH IN TUMORS

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Introduction. To understand the influence of the genome, transcriptome, proteome and interactome in the behaviour of cancer, it is important to study large activity-based structures, or hyperstructures, in which a variety of types of cells and molecules are brought together to perform a function. The combination of clinical, histopathological and biological data is necessary to understand the course of the disease. The correct link of data for interpretation of microscopy images is an essential action in this process. The images visualized in the light microscopes must be acquired with a digital camera and incorporate in the database. The objective was to design a unique database for neuroblastic tumors (NeuPAT). This database must hold the retrospective information and images available in different clinical, histopathological and genetic databases; and provide for optimal input, management and reporting of extensive neuroblastic tumor-related data for scientific research.

Methods. NeuPAT provides importation features for the process of integrating retrospective information and images. The application uses the HTTPS protocol for communication and access is protected by the use of encrypted transmissions, digital certificates and passwords. It is implemented and deployed using open-source tools. In particular, it is written in Java Server Page (JSP), deployed with Apache Web Services using the JavaTM 2 Platform, and uses Apache Jakarta Tomcat 5 as the served container server.

Results. The current database holds the information from almost 1000 cases, with more than 2200 tumor entries, and is used in neuroblastoma diagnostic and translational research.

Conclusion. NeuPAT has been designed to store and integrate clinical, histopathological and molecular resources of patients with neuroblastoma, including images, in order to support basic scientific and translational research. The complete architecture of this database can be generalized for other tumors.

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Keywords. intranet database; translational research; tumors

(50.2.P26) RELATIONSHIP BETWEEN TNF-A, P53 AND P21 IN HUMAN BENIGN BREAST LESION AND TUMORS (IN SITU AND INFILTRATIVE)

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Introduction. The aim of this study was to characterize the expression pattern of TNF-α, its receptors, p53 and p21 in breast samples (benign diseases and, in situ carcinomas, and infiltrating carcinomas) in order to elucidate the effects of these cytokines on the proliferation/apoptosis equilibrium.

Methods. Immunoexpressions of TNF-α, its receptors (TNFRI and TNFRII), p53 and p21 were studied by Western bloting and immunohistochemistry.

Results. The percentages of positive samples to TNF-α and TNFRII were higher in in situ carcinoma than in benign breast diseases, and TNFRII was even higher in infiltrating tumors. The percentages of positive samples to TNFRI were similar in the three groups. For the three proteins and in the three patient groups, immunoreactions appeared in the peripheral cytoplasm. In the positive samples, immunostaining to TNF-α was more intense in infiltrating tumors than in the other two patient groups; whereas immunostaining to both receptors was higher in in situ carcinoma than in benign breast diseases, and even higher in infiltrating tumors. Comparing the TNFα results with previous results for mp53, p21 and IL-6, we found an association between the expression of these four proteins and increasing malignancy.

Conclusions. TNF-α might be an important factor in breast cancer promotion since its proliferation/survival effects seems to be enhanced through the increased expression of TNFRII. Also, the pro-apoptotic pathway of TNFRI could be inhibited by p21 (which appeared increased in breast cancer) altering TNFRI effects towards promote the expression of several factors such IL-6, which contributes to tumor promotion.

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Keywords. TNF-a, p21, p53, breast cancer

(50.2.P27) ROLE AND MODULATION OF NF-κB-RELATED PROTEINS DURING TNF-α-PROVOKED APOPTOSIS IN PROSTATECANCER CELLS

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Introduction. The involvement TNF-α in cancer development is controversial, since this cytokine was reported to act either as tumour promoter or suppressor. TNF-α may activate signalling pathways critical for life/death decisions, such as mitogen-activated protein kinases (MAPKs) and the anti-apoptotic NF-κB pathway. In a basal state, NF-κB is retained in the cytoplasm by binding to the inhibitor of kappaB (IκB). Binding of TNF-α to TNFR1 results in IκB kinase (IKK) phosphorylation/activation, which in turn phosphorylates IκB, targeting it for proteosomal degradation. Once released from IκB, NF-κB translocates to the nucleus, where it promotes the transcription of anti-apoptotic genes and inflammation-related genes. In this work we sudy the alterations in the NF-κB pathway in relation to cell death in TNF-α-treated LNCaP (androgen-independent) and PC3 (androgen-dependent) prostate cancer cell lines.

Methods. The toxicity of TNF-α in LNCaP and PC3 cells, with or without p38-kinase and NF-κB chemical inhibitors (SB203580 and PS1145 respectively), was assessed by changes on viability (MTT assay) and apoptosis (loss of DNA, annexin-V binding, caspase activation). Expression of NF-κB-related proteins (IKK-α/β, p38-MAPK, IκB-α and NF-κB p50/p65) in these cell lines was analyzed by Western blot.

Results. TNF-α caused apoptosis with higher efficacy in LNCaP cells. This response was potentiated by p38-MAPK inhibitor (LNCaP cells) and IKK-β inhibitor (both cell lines). TNF-α induced IKK-α/β and p38-MAPK activation, and IκB-α degradation in both cell lines, NF-κB p50/p65 nuclear translocation was only reported in LNCaP cells. IKK-β mediated its protective action through NF-κB in LNCaP cells but not in PC3 cells.

Conclusions. IKK-β mediates both NF-κB-dependent and -independent anti-apoptotic functions in prostate cancerous epithelium. IKK-β and p38-MAPK may represent useful therapeutic targets against prostate cancer.

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Keywords. IKK, NF-kB, IkB, prostate cancer

(50.2.P28) CpG ISLANDS HYPERMETHYLATION IN HUMAN GLIOMA TUMORS AS A DIAGNOSTIC MARKER

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Human gliomas are common and aggressive primary brain tumors in adults. Its prognosis is very poor despite multimodal management including surgery, radiotherapy and chemotherapy. However, the specific epigenetic modifications that take place in human gliomas are not well understood.

In this work, we have analyzed the CpG island hypermethylation status of several key genes in a group of human gliomas and we correlated this status with the patients prognosis.
The results showed that several genes with a role in development and cell metabolism were hypermethylated in a high percentage of the tumors analyzed. In addition, we demonstrated that the hypermethylation of some specific genes was associated to impaired prognosis. These results suggest that a number of relevant genes could tightly regulate tumor generation and tumor progression by epigenetic mechanisms, and their epigenetic status could have diagnostic value and guide treatment decisions.
Supported by grant SAS PI2007/135 by Junta de Andalucia, Spain.
Keywords: glioma, epigenetics

(50.2.P29) MEK AND JNK PROTECTS LNCaP and PC3 FROM TNF-α TUMOR SUPRESSIVE EFFECTS

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Introduction. JNK and ERK are signal transducers involved on a broad range of cell functions. Whereas JNK has been generally linked to cell death and tumor suppression, ERK is accepted to play a prominent role in cell survival and tumor promotion in response to a broad range of stimuli, including cytokines as TNF-α. However, there is a growing body of evidence supporting that JNK, besides ERK, contribute to the development of a number of malignances. In this work we analyze the role of these kinases in TNF-α-induced cell death in LNCaP (androgen dependent) and PC3 (androgen-independent) prostate cancer cells.
Methods. LNCaP and PC3 cells were pre-treated with JNK and MEK (the upstream kinase of ERK) inhibitors (SP600125 and PD98059, respectively) and then exposed to TNF-α. After treatment we assessed changes on cell viability by MTT assay, apoptosis by estimating sub-G1 cell fraction and phosphatidylserine exposure, and plasma membrane permeation (as indicator of primary or secondary necrosis) by measuring propidium iodide free entrance.
Results. In spite of JNK and MEK inhibitors enhanced TNF-α–induced toxicity in both LNCaP and PC cell lines, they increased apoptosis only in LNCaP cells. In those cases in which toxicity was not linked to apoptosis induction, no changes in plasma membrane permeability were reported, suggesting a negative effect in cell proliferation; and dismissing a switch to a necrotic mode of death.
Conclusions. MEK and JNK have a protective role in TNF-α-induced toxicity in LNCaP and PC3 prostate cancer cells. Their selective inhibition could be an effective strategy for prostate cancer treatment.
Acknowledgements. Supported by grants from the “University of Alcala” (CCG10-UAH/BIO-5985) and the “Foundation Mutua Madrileña” (AP76182010), Spain.
Keywords. MEK, ERK, JNK, prostate cancer

50.3. NEUROSCIENCES

(50.3.O1) MECHANISM OF ENDOCANNABINOIDS AEA AND 2AG ON APOPTOSIS IN PERINATAL HYPOXIC-ISCHEMIC INJURY: NEUROPROTECTION OR NEUROPROLIFERATION?

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Introduction. Neurons of the central nervous system are the most vulnerable elements after a hypoxic-ischemic [HI] event. Different molecular mechanisms are enhanced whose final result is promoting cellular death. The aim of this study was to analyse the protector mechanism of anandamide [AEA] and 2- Arachidonoylglycerol [2AG], related to apoptosis in perinatal HI model.
Materials and methods. The Rice-Vannucci experimental procedure was used to cause HI injury in 7-day-old Wistar rats. Immediately after the HI an intraperitoneal injection of endocannabinoids, AEA or 2AG, was administrated. Brains were analysed 24h, 72h and 7 days after the insult using histologic and cytometric techniques. Cerebral slices were stained with Nissl, TUNEL method and Bromodeuxiridine [BrdU] to test the number of healthy, dead cells and proliferation, respectively. For cytometric studies we used non-fixed tissue to evaluate the cell membrane asymmetry with Annexin V.
Results. The study with Annexin V showed an increment of apoptotic cells in HI group in different points of time studied. 72h after HI treated groups showed no statistical differences respect to Sham group. The quantification of TUNEL positive cells, in the parietal cortex and hippocampus, 7 days after injury did not show differences in the treated groups when compared with Sham group. Neuronal density of healthy cells with Nissl stain, showed a decrease of 34% in the HI group while treated groups showed no differences with Sham one. BrdU positive cells were present in all the experimental groups, although no differences were observed.
Conclusion. Our results suggest that the way of action of this both endocanabinoids, AEA and 2AG, after HI injury in perinatal rats could promote the neuroprotection effect instead of enhanced proliferation.
Supported by FIS (PS09/02326), Basque Government (GCI-07/79, IT-287-07) and Saiotek (SA-2010-00186).
Keywords. hypoxia-ischemia, apoptosis, neuroprotection, neuroproliferation

(50.3.O2) MELATONIN ADMINISTRATION REDUCES HYPOXIC-ISCHEMIC INDUCED CELL DEATH AND GLIAL INJURY

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Introduction. Melatonin, with its high antioxidant efficiency, NMDA-blocking, anti-inflammatory properties, both water and lipid solubility and lack of toxicity, makes it a good candidate for its use as a strong neuroprotectant against experimental brain injury. The aim was to evaluate the effect of melatonin after hypoxic-ischemic (HI) brain injury in neonatal rats determining necrosis, apoptosis and oligodendroglial injury.
Methods. Twenty four Wistar rats at postnatal day 7 (P7) were randomly assigned to: two hypoxic-ischemic groups: pups with the left common carotid artery ligated and then asphyxiated for 2 hours with 8% O2 (HI, n=8), one of them receiving 15 mg/kg melatonin just after HI event and repeated twice with an interval of 24 hours (HI+Mel, n=8). Pups without ischemia or asphyxia were used as controls (Control, n=8). Seven days after surgery (P14), animals were sacrificed, brains collected and coronal sections were Nissl-stained, TUNEL-labelled or MBP-immunolabelled prior to determine early neuronal necrosis, apoptosis or oligodendroglial injury, respectively. One-factor ANOVA was performed ($p<0.05$).
Results. The number of surviving neurons showing a well preserved architecture in HI+Mel group was similar to that observed in Control group. Moreover, apoptotic cells only appear in the HI group. Ratio of left-to-right hemispheric MBP showed a significant decrease in the HI group in comparison with the Control, which was restored after melatonin administration.
Conclusion. Our results suggest that treatment with melatonin led to a neuroprotective effect reducing both apoptosis and necrosis, and preserving oligodendroglial viability.
Grants. Fondo de Investigación Sanitaria of Spanish Ministry of Health (PS09/02326) and from the Basque Government (GCI-07/79, IT-287-07).
Keywords. Hypoxia-ischemia; brain injury; melatonin; neuroprotection

(50.3.O3) AMYLOID β IMPAIRS cGMP PATHWAY, SYNAPTIC EXPRESSION OF AMPA RECEPTORS AND LONG-TERM POTENTIATION IN HIPPOCAMPUS. PROTTECTIVE ROLE OF IBUPROFEN

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Introduction. Amyloid beta plays a main role in the pathogenesis of Alzheimer's disease and contributes to cognitive impairment. Administration of amyloid beta to rats rapidly disrupts cognitive function in the absence of neurodegeneration, suggesting a direct interference on the mechanisms of learning and memory. Long-term potentiation (LTP) in hippocampus is a form of synaptic plasticity considered the bases of some forms of learning and memory. By other hand, brains of patients with Alzheimer's disease show neuroinflammation. Epidemiological studies have shown that chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk for Alzheimer's disease. By other hand, hyperammonemia (considered the main factor of hepatic encephalopathy) and inflammation cooperate to induce neurological alterations. Recent studies in animal models suggest that chronic hyperammonemia and neuroinflammation impair learning and memory ability by the same mechanism.
Methods. The aims of this work were to assess, through electrophysiological experiments, whether ibuprofen prevents amyloid beta induced LTP impairment in hippocampal slices and analyze, by biochemical methods, the role of altered NO-cGMP-protein kinase G pathway and AMPA receptors phosphorylation and synaptic expression in the mechanisms by which amyloid beta impairs and ibuprofen restores LTP. Also immunohistochemestry assays, using brain slices, and the following morphological analysis of microglial activation corroborate the protective role of ibuprofen.
Results. These results indicate that amyloid β impairs LTP by impairing the NO-cGMP pathway and that ibuprofen restores LTP by restoring this pathway. On other hand, we found that hyperammonemia itself induces neuroinflammation. Expression analysis of major histocompatibility complex class II, a marker of microglia activation, indicate that chronic moderate hyperammonemia induce microglial activation, which is reversed by treatment with ibuprofen.
Conclusions. These data suggest that use of NSAIDs may have therapeutic utility to improve cognitive function in neurological pathogenesis such as Alzheimer's disease and hepatic encephalopathy.
Key words: Amyloid beta, ibuprofen, neuroinflammation

(50.3.O4) AUTOMATED IMAGE ANALYSIS OF STEM CELL-RELATED GENE SOX2 EXPRESSION IN NEUROBLASTOMA

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Introduction. Cancer Stem Cells (CSCs) denominate the small percentage of cells within a solid tumor capable of unregulated self-renewal, leading to continued tumor growth. Heterogeneity of human neuroblastic tumors (NBT) makes this disease a candidate for a malignancy arising from CSCs of the neural crest. SOX2 is a transcription factor that regulates embryonic stem cell pluripotency. In several cancers, immunohistochemical expression of SOX2 has been associated with a stemness phenotype predicting poor outcome. Automated image analysis programs (AIAP), combined with digital images, are able to classify immunostained cells based on colour, size, and shape of these cells. We explored the expression and clinical significance of the stem cell marker SOX2 in NBT using digitized images and AIAP.
Methods. Seventy-six samples from primary NBT were included in 2 tissue microarrays (TMAs). Immunohistochemistry (IHQ) was performed with automated staining method (Autostainer, Dako) using anti-SOX2 primary antibody from Abcam (Cambridge, UK) at 1:50 dilution. Stained TMAs were captured through the 40x objective to obtain digitized images with high resolution using Aperio ScanScope. To score SOX2 expression level, we developed specific algorithms with ImageProPlus software.

Results. SOX2 positive staining was observed in more than a half of the analyzed NBTs. In addition, SOX2 expression was more frequent in MYCN amplified tumors, progressive disease and older patients.
Conclusion. The identification of CSCs may allow the targeting of CSCs for therapeutic approaches. AIAP combined with digitized image have improved the levels of sensibility, precision, reproducibility and standardization of quantification methods in IHQ. Our study revealed that NBT expressed the stem cell-related gene SOX2 and that high protein levels correlated with aggressive disease. Thus, SOX2 may be involved in the process of invasion and metastasis.
Acknowledgments. This study was supported by grants from ISCIII & ERDF, Madrid, Spain (RTICC RD06/0020/0102; FIS PI10/15), and from FAECC.
Keywords. neuroblastoma, SOX2, cancer stem cell, automated image analysis programs

(50.3.O5) MICROSCOPIC, MULTILOCUS AND PANGENOMIC STUDIES: ADVANTAGES IN NEUROBLASTOMA PROGNOSIS

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Introduction. Tumor development results in the accumulation of a set of genetic aberrations (point mutations, chromosomal rearrangements, copy number variations) and/or epigenetic changes. Different biological approaches are presently available to study the molecular basis of the complex cancerous phenotypes; some are more suitable for particular applications than others. In neuroblastoma (NB), the prognostic impact of known copy number chromosomal aberrations determines the therapeutic approaches.
Methods. Genomic DNA from frozen and paraffin embedded tissue (FPPE) was isolated in 36 NB. Fluorescence in situ hybridization (FISH) was performed to evaluate the status of at least two chromosome regions (MYCN and/or 1p36 and/or 11q and/or 17q) using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). Multiplex ligation-dependent probe amplification (MLPA) and 250K single nucleotide polymorphism (SNP) arrays were carried out to detect multi or pangenomic alterations.
Results. FISH technique detected amplification (MYCN), gains (2p and 17q) and losses (1p36 and 11q23). In addition, other simple segmental chromosome aberrations (SCA) were observed by MLPA and SNP arrays; the main loci affected being located in chromosome 3, 4, 9, or 19. Complex rearrangements and copy neutral loss of heterozygosity (CN-LOH) in several loci were also confirmed by SNP arrays.
Conclusions. FISH examines individual cells and enables the detection of alterations present in a low percentage of tumor cells; consequently FISH is essential for the diagnosis of MYCN status. MLPA is also useful for routine diagnosis of the common genetic alterations in NB with implications at prognostic and therapeutic levels. SNP arrays provide gene dosage information at higher resolution and allele specific status. Taken together, the data generated from these techniques can provide useful information with important clinical applications.
Acknowledgements. Grants: ISCIII (RD06/0020/0102) Ministerio de Ciencia e Innovación, FEDER and FAECC (396/2009).
Keywords. neuroblastoma, SNP array, MLPA, FISH

(50.3.O6) CHANGES IN GLIAL IMMUNOSTAINING IN THE COCHLEAR NUCLEUS AFTER BILATERAL COCHLEAR ABLATION IN ADULT RATS

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Loss of cochlear integrity bilaterally has been shown to result in a variety of morphological, biochemical, and metabolic changes throughout the auditory brainstem. However, it is still unknown whether such deprivation of presynaptic activity results in alterations in glial phenotypes that may contribute to the recovery of neuronal function and synaptic plasticity. Accordingly, the goal of the present study was to determine whether or not bilateral cochlear ablation in adult rats results in changes in glial-immunostaining in the cochlear nucleus, the first relay station in the auditory pathway, at 1, 10, 30 y 60 days post-ablation. Our data suggest that glial activation occurs as early as 24 hours after the bilateral cochlear ablation and that such activation is still present at least 2 months after the lesion in comparison to control animals. The present results provide evidence of a persistent upregulation in glial cells that may reflect a neuroprotective mechanism in order to reestablish the loss of primary afferents from the ablated cochlea.
Keywords. cochlear ablation, glia, cochlear nucleus

(50.3.O7) EXPERIENCE AND SENSORY ACTIVITY-DEPENDENT MODIFICATIONS IN SYNAPTIC STRUCTURE: LESSONS FROM AUDITORY NEURONS

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Activity-dependent dynamic changes in synaptic structure, linked to experience acquisition through sensory activity, are one of the foundations of learning and behaviour. Such structural modifications are an integral part of brain response mechanisms to changing environmental conditions in normal and pathological situations. Knowledge of such structural modifications, their mechanisms and functional or pathological implications is still limited. We will show an illustrative example of how interfering with normal activity in a neuronal population, driven by peripheral auditory input, leads to changes in synaptic structure, likely of adaptive nature.
Calyces of Held are large synaptic endings in the medial nucleus of the trapezoid body, an auditory region of the brainstem. They are adapted to transmit highly precise timing signals, essential for accurate sound localization. To accomplish their functions, the calyces of Held are supplied with a specialized molecular machinery which allows a tight regulation of membrane excitability and neurotransmitter release. Several classes of voltage-

dependent potassium channels seem to participate in such mechanisms. One type, designated as KCNQ5 or Kv7.5, is intimately associated with the synaptic ending and its membrane, as seen with high resolution immunocytochemistry. It has been suggested that the slow, persistent kinetics of this channel shortens the duration of successive depolarizations which otherwise would summate, thus limiting time precision at this synapse. Globular/bushy cells in the cochlear nucleus originate the calyces of Held. These neurons are activated by peripheral auditory signals through excitatory synaptic inputs from auditory nerve axons. Limiting sound-evoked activity in these neurons by removing the cochlea and auditory nerve through experimental deafness, quickly leads to disappearance or attenuation of Kv7.5 labelling in calyces of Held. A similar result is obtained by blocking nerve activity with tetrodotoxin. This supports the notion that structural localization of Kv7.5 in the calyx of Held depends on neuronal activity driven by acoustic stimulation. When normal activity is lost in the parent neurons, the synapses at the calyx of Held modify their structure, in this case changing the distribution of an ion channel, likely adapting to new conditions of diminished activity. We will discuss different mechanisms which may lead to this structural adaptation to changing auditory experience and its consequences.

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(50.3.P1) ANATOMICAL BASES OF BRAIN´S SOCIAL BEHAVIOR NETWORK IN A WEAKLY PULSE-TYPE ELECTRIC FISH

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In vertebrates, the social behavior network (SBN) is an evolutionarily conserved core of forebrain and midbrain nuclei that regulates different social behaviors. These behaviors are activated by steroid hormones (androgens, in particular) and hypothalamic neuropeptides (AVT, in particular) acting on brain regions that belong to the SBN. Brachyhypopomus gauderio is a weakly pulse-type electric fish that emits conspicuous electric signals during courtship behavior. This species is gregarious and displays seasonal intrasexual agonistic behavior.

We focused our exploration on one region of the SBN: the preoptic area (POA) of the hypothalamus. Our aims were to do an anatomical description of the POA in B. gauderio, to identify the populations of AVTergic neurons, and to determine the presence of androgen receptors (AR) in the neurons of the POA. Serial sections of the POA were stained with toluidine blue, and a three dimensional reconstruction was performed. We used immunohistochemistry to identify vasotocine neurons and androgen receptors. Morphometrical measures were done to determine different AVT neuron populations. This neuroanatomical description of the POA is the first one done in a weakly electric fish. AVT neurons were immunodetected along the rostral caudal axis of the POA. As in other teleosts, we identified three different populations of these neurons: parvocells, magnocells and gigantocells; AR expression was found in different neurons of the POA. Our results strongly suggest that the POA acts as a node of the SBN in this species. We expect to continue this study looking for differences in the expression of AVT in different social contexts.

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Keywords. Preoptic area, social behavior network, vasotocine, androgen receptor

(50.3.P2) IMMUNOLOCALIZATION OF DIFFERENT HYPOTHALAMIC REGULATORY PEPTIDES IN THE THYROID GLAND OF SEVERAL MAMMAL SPECIES

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Introduction. Thyroid hormone synthesis is controlled by the hypothalamic-pituitary-thyroid axis, through the liberation of thyrotropin-releasing hormone (TRH) and thyroid-stimulating hormone (TSH), respectively. Besides TRH, hypothalamus produces other regulatory peptides (RP) such as “cocaine and amphetamine-regulated transcript” (CART), somatostatin (SS), neuropeptide Y (NPY). Some of those RP have also been demonstrated in different extrahypothalamic tissues, Specifically, in the thyroid gland, several RP have been localized in C cells, where they probably exert a paracrine control of the adjacent follicular cells. The aim of the present work is to identify the presence and distribution of some of those hypothalamic peptides in normal and neoplastic thyroid gland belonging to different mammal species.

Material and methods. Normal thyroid glands from rats, pigs, rabbits, guinea-pigs and humans and C-cell carcinomas from rats (CCC), medullary thyroid carcinomas from humans (MTC) were subjeted to standard procedures and immunostained for CART, TRH, GHS, SS and CT.

Results. All CT-immunopositive cells were also immunoreactive for TRH; most C cells showed also positivity for CART, with the exception of human C-cells. In relation to SS immunoreactivity, it varied considerably among species: few SS-positive C-cells were only found in rats, pigs and humans thyroid glands, but they represented most of C-cells in rabbits and guinea-pigs. Eventually, GHS was only identified in rat C-cells. In regard to the immunohistochemical pattern displayed by C-cell derived tumours, rat CCC showed a similar pattern that the normal thyroid gland. Nevertheless, neoplastic C-cells from MTC only expressed TRH and, very scarcely, SS.

Conclusions. Our results suggest that C cells, in coordination with the hypothalamic-pituitary-thyroid axis, would exert a local modulation of thyroid hormone biosynthesis.

Keywords. Thyroid gland; C-cells; Hypothalamus; Regulatory peptides

(50.3.P3) DOSE-DEPENDENT EFFECTS OF MELATONIN IN THE THYROID FOLLICULAR CELLS BIOSYNTHETIC ACTIVITY

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Objectives. Melatonin, besides playing an important role as a transmitter of photoperiodic information, has antioxidant, anti-aging, antiproliferative, and, potentially, anticancerogenic activities, including suppressing effects on secretory and growth processes of the thyroid gland. The thyroid gland has two different endocrine cell populations: follicular cells, the most abundant cells and responsible for secreting T3 and T4; and C cells or parafollicular cells, which produce calcitonin, a hormone involved in calcium homeostasis. C cells also secrete other regulatory peptides implicated in the local fine-tuning of follicular-cell activity. In fact, melatonin has been detected in thyroid C-cells. Many effects of melatonin on the thyroid gland have been also described. In rodents, high doses of melatonin inhibit basal and TSH-stimulated mitotic activity of thyroid follicular cells in vivo and in primary culture. Melatonin has a direct inhibitory effect on T4 secretion and depresses the response of the thyroid to TSH. Furthermore, melatonin has protective role against oxidative stress in the thyroid gland. In the present study, we have analyzed the mRNA expression level of thyroperoxidase (TPO), thyroglobulin (TGB) and melatonin receptor (MT1) at five different doses of melatonin in the rat follicular PC-Cl3 cell line.

Materials and methods. PC-Cl3 cells were treated with melatonin at low (0.1, 1, 10 µM) and high concentrations (0.2, 0.5, 1 mM). TPO, TGB and MT1 mRNA expression was studied by quantitative RT-PCR in PC-Cl3 cells treated with melatonin during 24 h. TGB and MT1 proteins expression were analyzed by immunofluorescence.

Results. At high doses of melatonin, MT1 expression in PC-Cl3 cells was significantly higher than in untreated controls, meanwhile TGB expression decreased. On the other hand, at low doses of melatonin, TGB expression was increased. No significant changes were observed in TPO expression after melatonin treatment.

Conclusions. In this study we have demonstrated that melatonin has a dose-dependent effect on TGB and MT1 expression. Although more studies must be performed, it seems that melatonin directly affect TGB synthesis.

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Keywords. Melatonin, thyroid follifular cell

(50.3.P4) IMMUNOHISTOCHEMICAL EVIDENCE OF THE PRESENCE OF MELATONIN, SEROTONIN AND AANAT ACTIVITY IN RAINBOW TROUT GASTROINTESTINAL TRACT

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In vertebrates including fish, the neurohormone melatonin is mainly synthesized in the pineal gland and the retina. However, melatonin synthesis has been also reported at peripheral tissues, and the gastrointestinal tract (GIT) has received a particular attention. Recently, we have described in trout GIT the existence of aralkylamine N-acetyltransferase (AANAT) gene expression and activity, which is the limiting enzymatic step in melatonin formation. However, no studies have been addressed to the specific localization of this enzyme in fish GIT, neither for its role in acetylating serotonin to form melatonin. In the present study we studied by immunohistochemial techniques the distribution of melatonin and its precursor serotonin, in the GIT of the rainbow trout (Oncorhynchus mykiss). In addition, the presence of AANAT was also assayed.

Immunohistochemical tests detected melatonin- and serotonin-containing cells in all segments of the GIT, particularly in stomach and middle intestine. The tissue localization showed that melatonin was present mainly in the mucosa and the muscular layers. In addition, serotonin immunoreactivity was noted in elements running in the lamina propia of the mucosal folds, and the submucosa as well. The immunoreactivity for AANAT was localized mainly in enterocytes of the mucosal folds. All these data support the existence of a local synthesis of melatonin in trout GIT, which could be involved in regulation of digestive activity or contributing to maintain melatonin levels in plasma.

Keywords. Fish, immunohistochemistry, melatonin

(50.3.P5) ROLE OF C-CELL SECRETED TRH ON THE PARACRINE CONTROL OF THYROID ACTIVITY

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Introduction. Thyrotropin-releasing hormone (TRH), originally characterized and extensively studied as a hypothalamic tripeptide (pGlu–His– ProNH2), plays a key role in the regulation of thyroid axis, inducing the release of thyroid-stimulating hormone (TSH) from the anterior pituitary. In addition to its hypothalamic origin, TRH has been identified in many systemic organs including the thyroid. It is widely known that C-cells (CC), classically known for secreting calcitonin (CT), synthesize and release a number of different regulatory peptides (RP), such as somatostatin, CGRP, Ghrelin (GHS), CART and TRH, which could locally regulate follicular-cell activity and modulate thyroid function. Furthermore, our research group has described the expression of one of the two subtypes of TRH receptors (TRH-R) in thyroid follicular cells (FC) which are adjacent to C-cells. On the other hand, our group has also recently described new data supporting the idea that CC are somehow involved in the hypothalamic-pituitary-thyroid axis. The main objective of the present research is to study the role played by C-cell secreted TRH on the follicular-cell activity.

Materials and methods. PreproTRH, TRH-R1 and TRH-R2 functional expression has been analyzed at mRNA and

protein levels by qRT-PCR, Western blot and immunofluorescence, in both cell lines and thyroid tissue. Moreover, we have studied the putative role played by TRH on the synthesis of thyroid hormones by analyzing its effect on the expression of specific-thyroid genes and proliferation rates in follicular cells.

Results. Differences in the expression of genes involved in thyroid-hormone synthesis and TSH-induced cell-proliferation rate by qRT-PCR and BrdU incorporation have been observed in TRH-treated follicular-cell lines

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Conclusions. Our results suggest that C-cell secreted TRH could regulate thyroid function of follicular cells at both, hormone-synthesis and cell-number levels.

Keywords. Thyroid, C-cells, TRH, TRH receptor

(50.3.P6) INTRACELLULAR ROUTES AND DEVELOPMENTAL REGULATION OF GROUP I MGLUR ACTIVITY IN NEURONS OF THE RAT INFERIOR COLLICULUS

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Introduction. Group I metabotropic glutamate receptors (mGluRs) are widely expressed in the central nervous system playing multiple roles in normal and pathological situations. Group I mGluRs are linked to intracellular Ca2+ signalling and they are composed of mGluR1 and mGluR5 subtypes. Although they have been reported in cochlear nuclei and superior olivary complex, little is known about the abundance and role of these receptors in other nuclei of the auditory brainstem.

Materials and methods. By using optical imaging of the fluorescent Ca2+-sensitive dye Fura-2, in postnatal midbrain slices, and real-time quantitative RT-PCR, we have determined: 1) the signalling routes trigger by group I mGluR activation in the central nucleus of the inferior colliculus (CNIC) and 2) the influence of postnatal development on the group I mGluR fenotype and associated CNIC neuronal Ca2+ dynamics.

Results. Application of DHPG, an specific agonist of group I mGluRs, on postnatal day 10 (P10) slices, elicited large [Ca2+]i responses in most of the CNIC neurons. They consisted of an initial Ca2+ peak followed by a plateu phase. Intracellular Ca2+ stores were necessary for the initial peak of the Ca2+ responses, and surprisingly, a store-operated Ca2+ entry mechanism was also present in CNIC neurons.

Conclusions. During postnatal development, [Ca2+]i dynamics elicited by DHPG were different among different stages, reaching early, at P5, the highest Ca2+ responses, whereas the lowest levels of mGluR activation were found at P14, just around the hearing onset. RT-PCR analysis during development also revealed important differences in the expression of both mGluR1 and mGluR5 subtypes, with respect to the developmental stage studied.

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Keywords. Inferior colliculus, glutamate, slices, calcium

(50.3.P7) CHARACTERIZATION OF THE POTASSIUM CHANNEL KCNQ5 IN RETINA

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Introduction. KCNQ5/Kv7.5, a low-threshold non-inactivating voltage-gated potassium channel, is targeted to excitatory endings of auditory neurons in the rat brainstem. No member of the KCNQ channel family has been studied yet in the visual system. In this study, we intend to seek KCNQ5 in retina because the response to light in the inner segment of rods is composed of voltage-sensitive conductances whose kinetic properties are very similar to those of KCNQ5. Just as there are variations in the KCNQ5 distribution in animals with hearing problems, it can be a key molecule in retina with degenerative problems. Due to this, we study the distribution and mechanisms of action of KCNQ5, and we want to analyze possible expression changes of the channel during degenerative processes of the retina.

Materials and methods. To achieve the objectives, we used techniques of Western blot, immunohistochemistry and detection of protein interactions (proximity ligation assay).

Results. Preliminary results from rat retina extracts indicate the presence of a specific band of 140 kDa, corresponding to KCNQ5. We find that KCNQ5 is mainly distributed in the segment of photoreceptors and outer plexiform layer (OPL), where there is colocalization of KCNQ5 and the glutamate transporter VGluT1. In addition, some bipolar cells are also target of the channel.

Conclusions. Considering the presence and particular distribution of KCNQ5 in the healthy retina and taking into account the redistribution of the channel in the auditory system of deaf animals, we consider future experimentation guidelines in animals with hearing and visual problems simultaneously (characteristics of Usher Syndrome).

Supported by grants from the Consejería de Educación y Ciencia de la Junta de Comunidades de Castilla-La Mancha (Ref. # PPII10-0139-6156).

Keywords. Immunocytochemistry, Kv7.5, PLA, potassium channel, retina, rodents, visual system

(50.3.P8) MARKERS OF NEURAL CREST CELLS IN THE ADULT ENTERIC NERVOUS SYSTEM

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Introduction. An increasing body of evidence has accumulated in recent years supporting the existence of multipotent neural progenitor cells in the adult mammalian gut. The repair of the intestinal enteric plexuses should occur, in the adult, from poorly

differentiated or undifferentiated cells capable of becoming neurons under the influence of certain stimuli. Presumably these cells are similar to neural crest cells, which, during embryonic development will be differentiated, to the stage of mature neurons. The aim of our study is to make a escreening of intestinal nerve plexus, using a battery of neural crest markers, in order to locate the niche of neural stem cells.

Material and methods. Four to 3 month old rat Wistar (Jackson Laboratoires) were used in accordance with institutional guidelines. (CEICA Acta nº CP 06/05/2009). To carry out immunocytochemical techniques we used: 1) the method in Vision. 2) Technical fluorescence double labeling. The antibodies used were: C-Ret, sox 10, nestin and doublecortin, combined with proliferation markers (BrdU and Ki 67).

Results. All neural crest markers studied showed positivity around or inside the enteric ganglia. Some of the nestin positive cells mark the periphery of the enteric ganglia and in Inside of these colocalize with GFAP. Sox 10, p75 and doublecortin mark some cells within the intestinal ganglia. C-Ret colocalizes with PGP9.5 (pan-neuronal marker), but it does not with GRAP (glial marker) or with c-Kit (specific marker of ICCs). The few cell divisions observed are located on the periphery of the enteric ganglia.

Conclusions. The neural progenitors of the enteric nervous system of rat intestine express markers of neural crest cells.

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Keywords. Neural stem cells, intestinal adult stem cells, neural crest progenitors

(50.3.P9) INTERSTICIAL CAJAL CELL: THE FATE OF DEDIFFERENTIATED SMOOTH MUSCLE CELLS IN THE GASTROINTESTINAL TRACT

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Introduction. The mature cell dedifferentiation together with compensatory hyperplasia, and the activation of reserve adult stem cells, are the three ways for renewal of smooth intestinal muscle (SMC). The dedifferentiation is a process of loss of phenotypic specialization and could convert dedifferentiated cells into adult stem cells. Intestitial cells of Cajal (ICC) of the myenteric plexus (ICC-MY) and longitudinal smooth muscle cells, develop from the same Kit-positive mesenchymal precursors, and it is possible that ICCs located in the deep muscle plexus (ICC-DMP) and the thin layer of circular muscle beetwen the DPM and the submucosa share a common origin. The factors in the microenvironment determine whether a kit-positive precursor develops into an ICC or a smooth muscle cell and these factors are not fully understood.

Materials and methods. Human biopsies and different mammalian vertebrate specimens were analyzed by electron microscopy.

Results. In all species, we observed a cellular dedifferentiation physiological process, characterized by the loss of intercellular coupling, the appearance of vesicles in the perinuclear zone that fuse together until the vesicle membrane breaks, the cytoplasmic fragments are then totally detached from their own muscle cell. The dedifferentiated muscle cell shows a very small cytoplasm that expands in fine prolongations. The nucleus aspect is maintained compatible with high transcriptional activity.

Conclusions. We propose dedifferentiation of smooth muscle cells as a natural repair process. SMC display as well as ICCs, a high degree of plasticity. The dedifferentiation process observed causes the cell regress to an embryonic state. We suggest that the fate of the dedifferentiated SMCs could be an ICC in the presence of certain microenvironmental factors.

Support from Aragon Institute of Health Sciences (I+CS) (PIPAMER 001/11) and the European Social Fund (ESF), DGA (B83).

Keywords. Dedifferentiation; intestinal smooth muscle cell; ultrastructure; Interstitial Cajal Cell

(50.3.P10) INTERSTITIAL CELLS OF CAJAL IN THE LAMINA PROPRIA OF THE INTESTINAL MUCOSA

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Introduction. Interstitial cells of Cajal (ICCs) have been described extensively in intramuscular location and in relation to intestinal nerve plexus.

Cajal proposed the existence of ICCs in the lamina propria but, to date, there has been no ultrastructural study of these cells.We performed a study using transmission electron microscopy, analyzing morphological and ultrastructural features of ICCs located immediately beneath the mucous glands.

Material and methods. Four adult Wistar rats, three months old, (Jackson Laboratoires) were used in accordance with institutional guidelines (PI 22/08). Rats were anesthezized with pentobarbital sodium and perfused intracardially with 2.5% glutaraldehyde and 2 % paraformaldehyde. The samples are processed conventionally

The samples were washed in Palade tampon, post-fixed with 2% osmium, rinsed, dehydrated in graded acetones, cleared in propylene oxide and embedded in araldite. Serial semithin sections were stained lightly with 1% toluidine blue. Ultrathin sections were counterstained with 1% uranyl acetate and for 10 minutes with Reynold's lead citrate and examined under a FEI Tecnai G2 Spirit transmission electron microscopy.

Results. In semithin sections observed two different morphologies: monopolar spindle cells and bipolar cells with triangular nuclei morphology.

The cells have scant perinuclear cytoplasm and long cytoplasmic processes.

The cytoplasmic processes contacted with other ICCs through gap junctions or small punctate contacts.These prolongations establish relationships with endocrine cells, nerve trunks and capillaries.

These cells have characteristics of immature cells.

Conclusions. By their ultrastructural characteristics we consider these cells as a particular type of ICCs.

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Keywords. Interstitial cells of Cajal, lamina propia, enteric plexus, ultrastructure

(50.3.P11) APOLIPOPROTEIN D DECREASE IN DEMYELINATION PLAQUES IN MULTIPLE SCLEROSIS

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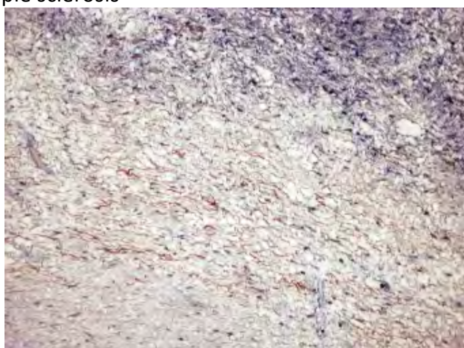
Introduction: Apolipoprotein D (apo D) is a lipocalin synthesized in the CNS, mainly by oligodendrocytes and astrocytes. It has been observed an increase in tis expression levels in several neurodegenerative diseases. In multiple sclerosis (MS) it has been described an increase in its intrathecal synthesis as well as in cerebrospinal fluid. Morphologically, MS is characterized by the presence of demyelination and remyelination plaques. It has been suggested that apo D could parcipate in the transport of molecules during maintenance and regeneration processes in the nervous parenchyma. As the demyelination plaques present inflammation, myelin and oligodendrocytes loss, astrocytosis and axonal loss in different grades, the aim of this study is the correlation, in different types of MS plaques, between apo D expression en the different demyelination and remyelination grade.

Methods: Twelve MS samples and 9 controls of the cortex and periventricular areas from several brain banks were examined. Histochemical techniques for myelin were used to define demyelination areas. Double immunohistochemistry for apo D and NT200 neurofilament (to check axonal damage) was performed.

Results: Apo D expression in MS plaques, independently of their activity grade, is considerably lower than in the surrounding white matter (WM)or than in the WM of an equivalent control area. In controls, apo D is expressed in oligodendrocytes but also in some protoplasmatic astrocytes. In MS plaques, a progressive decrease of apo D expression in these glial cells, from the external edge to the centre of the plaque, is corresponded with progressive axonal loss.

Keywords. Apolipoprotein D, demyelination plaques, Multiple sclerosis



(50.3.P12) MAGNESIUM SULFATE REDUCES THE INITIAL CEREBRAL DAMAGE AFTER PERINATAL ASPHYXIA

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Introduction. Magnesium sulfate administration has been proposed as a neuroprotective agent to the brain damage produced during the hypoxic-ischemic event in newborns. The aim was to evaluate the effects of $MgSO_4$ on cerebral cell death fate in a hypoxia-ischemia event in fetal lambs.

Materials and methods. 20 preterm lambs were assigned to: one healthy group (sacrificed 3 hour after delivery) and three hypoxic-ischemic groups (one sacrificed immediately after HI event, and two others receiving or not 400 mg/kg of $MgSO_4$ during 20 minutes and sacrificed 3 hours after HI event). Injury was induced by partial occlusion of umbilical cord during 60 minutes. Brains were fixed, divided and embedded in paraffin wax for hematoxylin-eosin studies and apoptosis detection by TUNEL. One-factor ANOVA was performed ($p<0.05$).

Results. In both 0h and 3h HI groups, the number of necrotic cells observed was similar to $MgSO_4$ group. Respect to apoptosis study, animals sacrificed immediately after the injury (0h HI group) did not show differences in comparison to healthy one. However, apoptosis index was significantly increased in the 3h HI group in comparison to healthy and 0h HI groups. The administration of $MgSO_4$ gave rise to apoptotic values similar both to healthy and 0h HI groups.

Conclusions. Our results show that $MgSO_4$ treatment after the HI event does not avoid the death by necrosis but maintains apoptotic index similar to healthy group, suggesting that $MgSO_4$ treatment may have potential therapeutic benefits after the HI event.

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Keywords. Hypoxia-ischemia; brain injury; magnesium sulfate; neuroprotection

(50.3.P13) MICROSCOPICAL PATTERNS OF CORTICAL LESIONS IN CJD AND THEIR CORRELATION WITH PRP 129 CODON, EEG AND NEUROIMAGE

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Introduction. Cortical spongiform pattern helps to establish the differential diagnosis between classical CJD form and the new variant, since in the last one the vacuoles appear around of plaques being named “flowered”. Among the different patterns of prionic protein distribution it has been described accumulation in plaque, that in the classic CJD form it is predominant in the deepest layers of the cortex but all along the cortical thickness in the new form. Aim: To review the last correlative ten cases of CJD diagnosed in Asturias and to correlate the cortical pattern of vacuoles and prionic

protein distribution with the metabolic activity loss through neuroimage and with the EEG pattern.

Material and Methods. Hippocampus, neocortex and cerebellar cortex sections were studied with glia, prionic protein and apolipoprotein D staining. Distribution pattern of vacuoles, gliosis and prionic protein by layers, as well as the staining intensity were evaluated, the last one using a semicuantitative image analysis method. The abnormality grade of the periodic complexes was evaluated as present or absent, separating both hemispheres. Cortical pathological levels were established in three grades.

Results and Conclusions. There is an association between the established levels of damage, the mitochondrial activation and the cortical metabolic activity. Although the pathology tends to be similar in both hemispheres, one of the cases revealed hemispheric asymmetry regarding to the distribution of the neuroradiologic cortical affectation, associated to hemispheric differences in the cortical pathological grade. The association between certain 129 codon polymorphisms and the intensity and distribution of PrP staining is confirmed.

Keywords. Creutzfeldt–Jakob disease, prionic protein, trasmissible spongiform encephalopathy

(50.3.P14) UPREGULATION OF CALRETININ IMMUNOSTAINING IN THE COCHLEAR NUCLEUS OCCURS DURING THE NOISE-INDUCED TOUGHENING EFFECT

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The toughening effect is defined as a progressive threshold shift reduction following an interrupted noise exposure paradigm. Although it has been suggested that this effect helps to protect the auditory system from a subsequent traumatic noise exposure, the mechanisms of this protective effect are not fully understood yet. It has been suggested however, that during the toughening effect there is an increase in the level of metabolic activity and/or in the neurotransmitter release in auditory structures such as the cochlea. Accordingly, the goal of the present study is to determine the relationship between calretinin levels, a calcium-binding protein used as a maker of neuronal activity, and the toughening effect in the cochlear nucleus. To do that, auditory brainstem responses (ABR) were evaluated in Wistar rats (6-11 month) before and after they were exposed to a broad-band white noise of 118 dB SPL at 1h every 72h, 4 times. After the last ABR evaluation, animals were perfused, their brains removed and the cochlear nucleus processed for calretinin immunohistochemistry. Our findings demonstrate that there is a toughening effect which is expressed as a faster and progressive recovery of the threshold shift and waves amplitude of the ABR in the latest noise exposure compared to that in the initial ones. Additionally, evaluation of calretinin immunostaining shows an upregulation of the levels of calretinin in the cochlear nucleus in experimental rats compared to that in control non-exposed rats. Overall, these results suggest that calretinin is related to the toughening effect perhaps, participating in a protective mechanism such as buffering the calcium overloading induced by the noise overexposure.

Keywords. noise, toughening, cochlear nucleus, calretinin

(50.3.P15) COMPREHENSIVE GENE EXPRESSION ANALYSIS OF EMP3 NATIVE AND HYPERMETHYLATED HUMAN NERVE SYSTEM TUMORS

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Introduction: Previous reports suggest that the gene *EMP3* (epithelial membrane protein 3) could be epigenetically silenced by CpG island hypermethylation in human nerve system tumors, especially in children neuroblastomas and adult gliomas. However, the genetic pathways that could be associated to this silencing have not been previously studied.

Methods: First, the *EMP3* hypermethylation status was analyzed by methylation-specific PCR (MSP). Then, we have analyzed 99 human nerve system tumors using Affymetrix Human U95v2 oligonucleotide RNA expression microarrays. The expression of all genes/EST was correlated with that of the gene *EMP3* using Pearson correlation tests, and all genes with an absolute correlation coeficient >0.6 were selected. Genes functions were identified using Cytoscape-BiNGO software.

Results: 39.02% of all analyzed gliomas and 24% of the neuroblastomas showed *EMP3* hypermethylation by MSP. The expression of *EMP3* was positively correlated with 1065 genes/EST and negatively correlated with 7 genes/EST. Negatively correlated genes mainly corresponded to nervous system development gene functions, weres positively correlated genes played a role in numerous gene functions, including defense, immune response, MHC expression and extracellular matrix synthesis.

Conclusions: Our results suggest that *EMP3* silencing by hypermethylation could be associated to a decreased immune response to human nerve system tumors and therefore, to an increased tumor survival and decreased patient survival.

Acknowledgements: This work was supported by grant SAS PI2007/135 by Junta de Andalucia, Spain.

Keywords: nerve system tumors, *EMP3*, hypermethylation

(50.3.P16) MORPHOLOGICAL AND NEUROCHEMICAL CHARACTERIZATION OF AQP4-IMMUNOREACTIVE CELL POPULATIONS IN THE TELEOST FISH RETINA

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Purpose. Aquaporins (AQPs) are membrane proteins that facilitate water transport across biological membranes, being essential for the correct function of the visual system. Despite knowledge of AQPs expression in the mammalian retina, little is known of its presence in teleost fish retinas. In turn AQP4, expressed in mammalian Müller cells, has an important role in the

maintenance of extracellular potassium homeostasis. We characterized morphologically and neurochemically AQP4-immunoreactive cell populations in the teleost fish retina of Sparus aurata, and whether its expression is altered during light/dark adaptation.

Methods. The study was performed in adult giltheads (Sparus aurata L.). Animals were maintained on a daily 12 h light/dark cycle. Fish were light- and dark-adapted at least 2 hours prior to sacrifice. Dark-adapted animals were sacrificed under dim red light. The expression pattern and the identity of AQP4-immunoreactive cells was studied by immunocytochemistry.

Results. AQP4 immunoreactivity was present in Müller cells somas at the INL and in end-feet processes abutting the vitretinal border. Moreover, AQP4 was also evident in cone photoreceptor cells, and in a GABaergic subpopulation of amacrine cells (AQP4-ACs). Four different types of AQP4-ACs were characterized based on their morphology and dendrite stratification. Finally, a greater expression of AQP4 at the INL occurred during dark adaptation, also accompanied by a significant increment in the cell size of AQP4-ACs.

Conclusion. The presence of AQP4 in the teleost retina could be indicative of water redistribution through different retinal cells and its implication in potassium regulation at the inner retina. In addition, the direct relation between AQP4 expression and the cell size of AQP4-ACs, could suggest a role in volume regulation in certain neuronal populations during light/dark adaptation.

Keywords. retina, amacrine cell, aquaporin-4, teleost, inmunoreactivity

(50.3.P17) APOLIPOPROTEIN D EXPRESSION IS UP-REGULATED IN RESPONSE TO AMYLOID Β-PEPTIDE-INDUCED NEUROTOXICITY IN HT22 HIPPOCAMPAL CELLS

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Introduction. Apolipoprotein D (Apo D) is a secreted glycoprotein that is markedly induced in several pathological and stressful conditions to the nervous system where oxidative stress may be the initiating event. In the central nervous system Apo D expression is up-regulated during aging, after traumatic brain injury and in several human neuropathologies such as Alzheimer's disease (AD). Recent studies showed a neuroprotective and antioxidant function of mammalian Apo D against oxidative stress. The aim of this work is to study the effect of Amyloid β-peptide (Aβ), which is believed to play a major role in the neurodegenerative process of AD, in Apo D expression in a HT22 mouse hippocampal cell line.

Methods. HT22 neuronal cells were exposed to growing concentrations of Aβ (10-30µM) for 24 hours. The cytotoxic effect of Aβ was studied by using MTT and trypan blue exclusion assay and by flow cytometry. The levels of expression of Apo D after treatments were measured by immunocytochemistry and western-blot.

Results. Preliminary data showed that Aβ induces senescence-like growth arrest in hippocampal cells. This effect was apparent from the mentioned assays and by the examination of cell morphology with a phase contrast microscopy. Interestingly, a clear tendency to the increment of Apo D protein level with Aβ treatments was observed, specifically at those Aβ concentrations that caused growth arrest.

Conclusion. The data obtained in the present study suggest that Aβ induces Apo D expression in hippocampal cells, which would explain the elevated levels of Apo D found in AD brain.

Keywords. apolipoprotein D, amyloid β-peptide, growth arrest, neurotoxicity, Alzheimer disease

(50.3.P18) ENDOTHELIAL NITRIC OXIDE SYNTHASE INHIBITOR L-NIO REDUCES PROTEIN NITRATION IN HYPOXIC RAT STRIATUM

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Introduction. Nitric oxide (NO) is an inorganic free radical involved in hypoxia processes in the striatum, the most vulnerable brain basal ganglion to low oxygen situations. NO is formed from L-arginine by NO synthases (NOS): endothelial, inducible and neuronal NOS (eNOS, iNOS, nNOS). Peroxynitrite, formed by the reaction of NO and superoxide, can alter protein function by nitrating phenolic rings, including tyrosine to form the stable product 3-nitro-L-tyrosine, which can be used as a marker of the potentially cytotoxic effect of NO. All NOS isoforms have been reported to lead to nitrosative chemistry. However, the implication of eNOS-derived NO on hypoxia-induced nitrosative stress has not yet been investigated in the striatum, and constitutes the aim of this study.

Methods. Wistar rats were submitted for 30 min to acute hypobaric hypoxia, with or without a previous treatment with the eNOS inhibitor L-NIO (20 mg/kg). In situ NOS activity (NADPH-diaphorase histochemistry), as well as expression and location of NOS isoforms (nNOS and iNOS) and nitrated proteins were analyzed in stiatum of adult rats by means of immunohistochemical and image processing techniques.

Results. In situ NOS activity, nNOS, iNOS and nitrated proteins were located in some neurons in the striatum of all experimental groups. NADPH-diaphorase and nNOS expression significantly decreased in both hypoxic groups. In situ NOS activity was reduced in groups treated with L-NIO vs. untreated groups. Contrarily, iNOS was not altered in any group. Nitrated protein expression significantly raised in hypoxic groups vs. control groups. After L-NIO administration, protein nitration diminished in the hypoxic group in comparison with the untreated group.

Conclusions. The inhibition of eNOS activity by L-NIO decreased nitrotyrosine formation in hypoxic striatum, indicating an involvement of eNOS-derived NO in the nitrosative stress that occurs in this brain basal ganglion under hypoxia.

Keywords. nitric oxide; hypoxia; L-NIO; striatum; nitrotyrosine

(50.3.P19) INMUNOHISTOCHEMICAL PROFILE OF SOLID CELL NEST OF THYROID GLAND

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It is widely held that solid cell nests (SCN) of the thyroid are ultimobranchial body remnants. SCNs are composed of main cells and C cells. It has been suggested that main cells might be pluripotent cells contributing to the histogenesis of C cells and follicular cells, as well as to the formation of certain thyroid tumors. The present study sought to analyze the immunohistochemical profile of SCN and to investigate the potential stem-cell stem cell role of SCN main cells. Tissue sections from ten cases of nodular hyperplasia (non-tumor goiter) with SCNs were retrieved from the files of the Hospital Infanta Luisa (Seville, Spain). Parathormone (PTH), calcitonin (CT), thyroglobulin (TG), thyroid transcription factor (TTF-1), galectin 3 (GAL3), cytokeratin 19 (CK 19), p63, bcl-2,OCT4, and SALL4 expression were evaluated by immunohistochemistry. Patient clinical data were collected, and tissue sections were stained with hematoxylin–eosin for histological examination. Most cells stained negative for PTH,CT,TG, and TTF-1. Some cells staining positive for TTF-1 and CT required discussion. However, bcl-2,p63, GAL3 GAL3, and CK 19 protein expression was detected in main cells. OCT4 protein expression was detected in only two cases, and SALL4 expression in none. Positive staining for bcl-2 and p63, and negative staining for PTH,CT, and TG in SCN main cells are both consistent with the widely-accepted widely accepted minimalist definition of stem cells, thus supporting the hypothesis that they may play a stem-cell stem cell role in the thyroid gland, although further research will be required into stem cell markers. Furthermore, p63 and GAL-3 staining provides a much more sensitive means of detecting SCNs than staining for carcinoembryonic antigen, calcitonin, or other markers; this may help to distinguish SCNs from their mimics.

Keywords. Solid cell nests, Main cells, Stem cells, p63, Galectin-3

(50.3.P20) IMMUNOHISTOCHEMICAL LOCALIZATION OF CEREBRAL APOLIPOPROTEINS D AND J IN ALZHEIMER'S DISEASE. STUDY OF THEIR INTERRELATIONSHIPS

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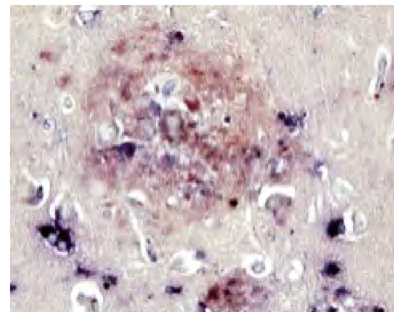
Introduction. Among apolipoproteins group apo D, apo E and apo J stand out to be synthesized in the nervous system. Apart from the classical functions common to all the apolipoproteins these three have been proposed as markers in several neuropathologies. Certain polymorphisms of apo E and apo J are implicated in the development of different Alzheimer's disease (AD) types. Although there are studies about the relationship of apo E with apo J and apo D in AD, there are no studies about the possible relationship between apo J and apo D in this disease so far. In this work we have studied the colocalization of apo D and apo J at cellular and extracellular level in AD, as well as their relationship with amyloid beta protein.

Methods. Seventeen hippocampus and cerebral cortex samples of AD brains from different brain banks have been used. To show apolipoproteins presence simple and double immunohistochemical methods have been used. Beta amyloid protein localization has been studied with the Congo Red histochemistry method.

Results. Most of the senile plaques are positive for both proteins, with the exception of those in the first stages of development that only show apo J positive signal. The main protein of the plaque is apo J that colocalizes with the amyloid core. All the neurons are apo J positive but only a few of them show apo D signal. However, apo D is mainly located in the glial cells, mostly in astrocytes and microglia in close relationship with the senile plaque.

Keywords. Apolipoprotein D, Apolipoprotein J, Alzheimer disease



(50.3.P21) LOCALIZATION OF APOLIPOPROTEIN D AND MUTATED HUNTINGTIN IN THE STRIATE NUCLEUS OF HUNTINGTON DISEASE

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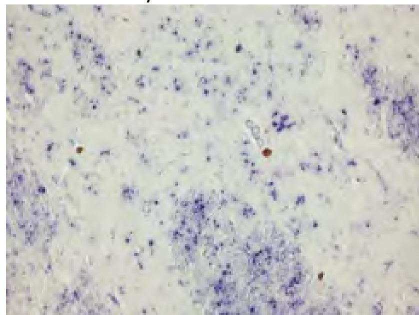
Introduction. Mutated huntingtin (mHnt) inclusions are charateristic of certain brain regions of Huntington's (H) patients. Being ubiquitin positive, it is considered that proteasome has difficulties to eliminate these inclusions and that is the reason why they accumulate. However, neurons containing inclusions are not the ones that suffer degeneration. These aggregates contain fibres with a beta sheet structure, a characteristic that they seem to share with the amyloid presents in Alzheimer's disease. Due to the apolipoprotein D synthesis in the nervous under other neuropathological conditions and its localization in the senile plaques around the extracellular amyloid core, in this study we characterised the presence of this apolipoprotein in the brain areas with a higher neuronal loss and a notorious accumulation of huntingtin inclusions in Huntington's patients.

Methods. We used 11 samples of Huntington's disease patients. The samples obtained were from the striate nucleus with Vonsattel grades ranging from II to IV. A double immunohistochemistry was performed to localize apo D and mHnt.

Results. In most of the samples there is no colocalization of apo D and mHnt. Apo D staining, as expected, is located in reactive astrocites and fagocitic cells, being almost negative in the neurons of this area nucleus. The observation of the samples indicates that in most of the

cases mHnt positive inclusions tend to be located close to the blood vessels with no macrophage or astrocitic activity detected around them. This reinforces the hypothesis about the lack of reactivity of the mHnt inclusions in the nervous parenchyma.

Keywords. Apolipoprotein D, mutated huntingtin, immunohistochemistry



50.4. TISSUE ENGINEERING

(50.4.O1) SKIN KERATINOCYTE DIFFERENTIATION POTENTIALITY OF ADIPOSE TISSUE, DENTAL PULP AND UMBILICAL CORD WHARTON´S JELLY MSCS. A COMPARATIVE STUDY

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Introduction. Numerous reports have demonstrated that adipose tissue, tooth pulp, and umbilical cord wharton´s jelly contain a subset of MSCs cells that not only are capable of self-renewal, but also can differentiate into multiple mesenchymal cell lineages. In this study, we have analyzed the potential of MSCs to transdifferentiate into tissues of ectodermic origin by means of epithelial-mesenchymal interactions.

Methods. First, we established primary cultures of human skin fibroblasts, and three MSCs populations: Dental pulp stem cells (DPSC), adipose tissue stem cells (ADSC) and Wharton´s Jelly stem cells (WJSC). Subsequently, we validated the presence of stem cell markers in all MSCs populations by flow cytometry. Then, we generated an artificial skin substitute using nanostructured fibrin-agarose scaffolds with skin fibroblasts immersed within and DPSC, ADSC, and WHJSC cultured on top during 7, 14, 21 and 28 days of *in vitro* development. For *in vivo* studies, these artificial tissues were implanted on the skin of nude mice during 28 days. Histological analyses were performed by H&E staining.

Results. Our results demonstrated that the three MSCs populations used in this study were positive for typical stem cell markers such us CD105, CD90, and negative for CD45. The *in vitro* analysis demonstrated that ADSC were able to transdifferentiate into skin epithelial tissue after 21 days *in vitro*. Moreover, WHJSC were able to differentiate into keratinocyte-like cells after 14 days. In addition, DPSC became transdifferentiated very early after 7 days of culture. At the *in vivo* levels, we did not find significant histological differences on the transdifferentiation process along the three types of cell MSCs cells.

Conclusions. These results suggest the possibility that DPSC and WHJSC can differentiate more efficiently into skin-like epithelial structures at the *in vitro* levels. However, *in vivo* analysis showed that all skin substitutes were efficiently transdifferentiated to the epithelial lineage by the influence of epithelial-mesenchymal interactions.

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Keywords: Transdifferentiation, Dental Pulp, stem cells, Wharton’s Jelly, Adipose

(50.4.O2) CELL THERAPY INHIBITS CONTRACTION DURING REPAIRING PROCESS IN CUTANEUOS DEFECTS

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Introduction. The combination of tissue engineering and cell therapy with adult mesenchymal stem cells could represent an effective tool to repair damaged tissues. The aim of this work was to evaluate the applicability of muscle stem cells in the tissue repair and regeneration of excisional skin defects.

Materials and methods. White New Zealand rabbits underwent 2 and 2.3 cm diameter rounded defects in the dorsal side of both ears (n=12 per group). Stem cells from dorsal muscle were isolated from each animal, cultured and marked with the fluorescent cell linker PKH26. Autotransplantation of cells was developed on the right ear defect of the specimens (3.5x106 cel/defect), while the left ear defect was used as control. Animals were sacrificed after 14 days. Histological and ultrastructural analyzes were carried out. Contraction and reepithelialization percentages were measured. Statistical analyzes were developed by calculating U-Mann-Whitney and ANOVA tests.

Results. The presence of inflammatory granulation tissue and disepithelialized areas was higher in control than in treated defects. Regarding the size of the defect, the most relevant difference was found in 2 cm control defects, which showed a contraction percentage significantly higher compared with 2.3 cm control defects (50.83±12.84 vs 23.63±9.42). Morphometric assays showed that, regardless of the size of the defect, reepithelialization percentage was significantly higher in treated than in control defects (2cm: 96.33±3.09 vs 70.91±6.94; 2.3cm: 91.39±5.57 vs 76.03±13.78). Interestingly, contraction was significantly reduced in 2cm treated defects comparing with control (22.25±9.56 vs 50.83±12.84).

Conclusions. Contraction is inversely proportional to defect size. Cell therapy can inhibit contraction events and increases reepithelialization.

This work has been supported by SAF 2009-13240-C02-02.

Keywords. Cell therapy, wound healing, muscle stem cells, contraction, reepithelialization

(50.4.O3) SATELLITE CELLS AND CAPILLARIES: TOPOLOGICAL RELATIONS IN REGENERATING SKELETAL MUSCLE

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Introduction. In normal skeletal muscle a significant majority of satellite cell niches are found in close proximity to muscle microvasculature. With the restoration of blood supply during skeletal muscle regeneration, the resident satellite cells become active, proliferate, and differentiate into myoblast; these cells fuse to form myotubes that gradually increase in size and mature into myofibers. The aim of this study was to examine the morphological relations between the capillary supply and satellite cell population in regenerating muscle fibers.

Material and methods. Male Wistar rats received a single intramuscular injection of mepivacaine to induce degeneration-regeneration in the anterior tibial muscle. Five animals each were sacrificed 4, 7, and 30 days after the injection; a further group of rats served as control. Samples were processed for transmission electron microscopy. Semithin sections were stained with 1% toluidine blue for light microscopy, where the capillaries and satellite cells were easily identified. The number of capillaries around each muscle fiber, the number of satellite cells associated to the same one, and the number of satellite cell-capillar units were recorder. Diffusion distances between satellite cells and capillaries were measured in electronmicrographs.

Results. During the growth of regenerating muscle fibers associated satellite cells population and the surrounding capillaries undergoes quantitative changes. The quantitative study indicated a greater capillary supply of individual regenerating muscle fibers besides a high number of satellite cells at 4 days; at 7 days the number of satellite cells and capillaries decreased and returning to normal values at 30 days.

Conclusions. Our results provide morphological evidence that the topological relations capillary- satellite cells are stable during the growth and development of regenerating muscle fibers. This stable histological behaviour can be the background for understanding the recent evidences that suggests that cells residing within the vascular niche may participate in skeletal muscle regeneration of and to replenish the satellite cells population.

Keywords. Skeletal muscle, satellite cells, muscle capillaries, muscle regeneration

(50.4.O4) NEUROPHYSIOLOGICAL NERVE REGENERATION USING THREE-DIMENSIONAL NEURAL CONSTRUCTS WITH ADIPOSE TISSUE-DERIVED STEM CELLS

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Introduction. Extensive nerve damage with the existence of long tissue gaps must be treated by using a sensory nerve autograft or a synthetic nerve conduit. The aim of this study is to evaluate the electromyographic signs of nerve regeneration in a model of nerve repair using fibrin-agarose hydrogels with adipose tissue-derived stem cells in experimental animals.

Material and Methods. In this work we used 40 12 weeks-old Wistar rats weighting 250 - 300 g. First, a nerve injury was induced by surgical excision of one centimeter of the left sciatic nerve. The animals were divided in four groups according to the type of nerve defect repair. Group A (n = 10), nerve damage, without surgical repair; Group B (n = 10), repair using a commercial lyophilized collagen conduit (NeuraGen ®); Group C (n = 10), repair using a commercial lyophilized collagen tube containing a hydrogel of fibrin and agarose; Group D (n = 10), collagen conduits with a fibrin-agarose hydrogel with adipose tissue-derived stem cells within. All animals were followed for 12 weeks after the intervention. In each animal, specific neuropsychological variables were collected. We determined the signs of denervation and reinnervation for each of the muscle groups distal to the nerve injury produced.

Results. Our results showed that the degree of denervation of the biceps femoris and gastrocnemius muscles was lower in the group of animals grafted with fibrin-agarose neural constructs with adipose tissue-derived stem cells than in the rest of the animals. Signs of reinnervation were more important for the muscle groups proximally to the injury site of the animals grafted with stem cells and biomaterials.

Conclusions. The application of fibrin-agarose biomaterials is able to improve the degree of denervation of the biceps femoris and gastrocnemius muscle groups. The use of stem cells significantly reduced the signs of denervation in comparison to other groups. In addition, the use of biomaterials with adipose tissue-derived stem cells was associated to relevant signs of reinnervation in each of the muscle groups studied, with the exception of distal muscles (Pedi muscle/ intrinsic muscles foot).

(50.4.O5) QUALITY CONTROL STUDY OF GLASS IONOMER CEMENT IN AN "IN VITRO" EXPERIMENTAL MODEL OF HUMAN GINGIVAL FIBROBLASTS

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Introduction. Glass ionomer cements are biomaterials used to protection of pulp dentin complex. For this

reason, the biological quality control analysis should be important to stablish a correct clinical utilization. In this work, we are evaluating the cell viability of human gingival fibroblast submitted to glass ionomer cement contact by DNA quantification method and morphological analysis.

Materials and Methods. Primary culture of human gingival fibroblasts was cultured using 24 wells in a cell density of $2X10^5$ cells/500 ul of DMEM culture medium. Then, the cells were exposed to 2 mm disks of diameter and 1 mm of thickness of glass ionomer GC FujiCem (GC), during 72 hours. To analyze possible morphologic alterations, cells were examined under light microscopy. Finally, to quantify DNA released into the cell culture, spectrofotometry technique was used. As negative controls, fibroblasts were cultured with DMEM culture medium (CM) and fibroblast incubated in 2% triton X (CT) were used as a positive controls.

Results. Light microscopic findings showed a greater morphologic alteration in cells exposed to GC characterized by a rounded morphology and lower cells number than CM controls. The DNA quantification demostrated that GC exposed cells released a 78% of free-DNA into supernantant in comparison with CM.

Conclusion. Morphological analysis and DNA quantification showed that GC produced several alterations on human gingival fibroblasts at 72h characterized by a citotoxic effect on nuclear membrane. According to this, GC should be limited used in deep dentin or exposed pulp or even in Tissue Engineering protocols.

Keywords: Glass ionomer cement, human gingival fibroblast, quality control

(50.4.P1) CELL SEEDING ON TITANIUM DEVICES FOR DENTAL IMPLANTS COATED WITH BONE MORPHOGENETIC PROTEIN-2 (BMP-2): AN IN VITRO MODEL

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Introduction. BMP-2 plays an important role in the development of bone and cartilage. This protein is being used in several surgical therapies such as implant dentistry. Our aim was to determine the effects of BMP-2 on cultured fibroblasts and stem cells seeded on titanium screws designed as devices for dental implants.

Materials and methods. Titanium screws (n=24) were coated with BMP-2 (1μg/mL) and seeded with rat stem cells from adipose tissue, MSCat (n=12) and dermal fibroblasts, Fb (n=12). Both cell types were also seeded on non-coated screws as control (n=12 each). Samples were cultured in Amniomax medium and collected at days 1, 3, 7, 14, 21 and 28 for ultrastructural analysis. For migration assays, MSCat and Fb were separately seeded on the central well of a 3-well glass slide, with a collagen sponge coated with BMP-2 on the left well and a non-coated sponge on the right. Histological evaluation was developed at days 1, 3, 7 and 14.

Results. After 24h, non-coated screws showed a lower performance than experimental groups; this difference increased over time, especially with MSCat. Cells seeded on BMP-2 coated screws displayed healthy morphology and a proliferative status. Both Fb and MSCat formed a monolayer that fully covered the screw at days 14 and 21, respectively. At day 28, there were almost no cells in control groups, and the number of cells in coated screws was significantly lower. Interestingly, Fb morphology from coated screws was healthier than MSCat. Migration assays showed a slightly chemotactic response to BMP-2 on both Fb and MSCat cultures.

Conclusions. BMP-2 seems to increase the cell colonization on the surface of the screws, and has a chemotactic effect on cultured cells. Due to the fibroblastic response observed with these experiments, BMP-2 could facilitate the tissue integration of dental implants seeded with these cells.

Keywords. BMP-2; dental implant, titanium screw; cell seeding

(50.4.P2) DEGREE OF HISTOLOGICAL RECOVERY IN SKELETAL MUSCLE AFTER RECONSTRUCTION BY ADIPOSE TISSUE IMPLANTATION

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Introduction. The muscle regeneration process allows, in general, that muscle histology returns to normality, and only regenerated muscle fibers with central nuclei can be observed. However after muscle resection scars are commonly seen replacing the ablated tissue. We used adipose tissue transplantation to repair a volume deficit after muscle ablation in order to examine the recovery degree in skeletal muscle histoarchitecture.

Material and methods. A cylindrical fragment in the central portion of the rat anterior tibial muscle was removed. In the hole, inguinal subcutaneous adipose tissue of the same rat was inserted; finally skin was sutured and desinfected. A reference group was transplanted with freeze-thawed adipose tissue three times. Normal rats were used as control. The animals were sacrificied at 90 days after trasplantation and the muscles were analyzed by light microscopy using histological, histochemical and inmunohistochemical techniques. The following parameters were recorder: muscle fiber size, number of fibers abnormally oriented, number of fibers showing cytoarchitectural changes, and fibrosis.

Results. Though the host muscle contributes to provide vascularization and innervation, in reference muscle the core is substituted by reparative conective tissue infiltrated by mononucleated cells. On the contrary, experimental muscle showed an almost complete reconstruction of the muscle tissue in the implantation area. However, quantitative and qualitative changes in the histoarchitecture of new muscle were observed: spatial desorganization of the muscle fibers and centronucleated fibers with irregular shape and diameters. Also, histochemical and inmunohistochemical analysis showed ring fibers and targetoids fibers, which

indicated inadequate innervation and mechanical reintegration impaired.

Conclusions. Our results confirm that adipose tissue transplantation form new skeletal muscle. Nevertheless, though these results could have putative clinical applications, the histoarchitectural changes can contribute to the deterioration of his function and biochemical properties.

Keywords. Skeletal muscle, muscle regeneration, adipose tissue, transplantation

(50.4.P3) DIFFERENT MYOGENIC CAPACITY IN SLOW AND FAST SKELETAL MUSCLES

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Introduction. Satellite cells provide new myonuclei required for growth, hypertrophy and regeneration of damaged myofibers.The frequency of satellite cells appears to be much higher in slow compared with fast muscles. It has been reported that myofibers with large numbers of associated satellite have a higher myogenic capacity compared to myofibers with fewer satellite cells. We tested the hypothesis that the myogenic reponse of soleus (SOL) muscle to myotrophic stimuli (in a denervated muscle environment) should be higher compared to extensor digitorum longus (EDL) muscle.

Material and methods. Normal Wistar rats were treated with denervated muscle extract (DmEx) during 10 days. Soleus and EDL muscles were then dissected and processed for light and electron microscopic analysis. Activated satellite cells were dentified immunohistochemically, using muscle-specific marker molecules, and ultrastructurally. Quantitative studies were performed.

Results. Histological examination and inmunostaining for MyoD and desmin revealed which satellite cells were activated; also we observed mononucleated cells and small muscle fibers desmin (+) in the interstitial space. The quantitative analysis of these changes showed significant differences between both muscles (SOL>EDL).

Conclusions. Our results clearly indicate that in the presence of an intact and functional satellite cell population, derived factors from denervated muscle can promote the myogenic response in normal skeletal muscle. However differences among myogenic capacity from fast and slow muscles were detected. These results may be important to muscle repair after trauma and in pathological situations.

Keywords. Skeletal muscle, denervated muscle extract, myogenic capacity

(50.4.P4) GENE EXPRESSION ANALYSIS OF ASCS TRANSDIFFERENTIATED TO SKELETAL TISSUES FOR MAXILLOFACIAL PROCEDURES

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Introduction. The search of novel techniques based on tissue engineering and cell therapy to employ in maxillofacial procedures supports transdifferentiation processes of single cell sources. In this work we succesfully transdifferentiated ASCs into osteoblast and chondroblast–like cells. The transdifferentiation process was evaluated by histochemistry, immunofluorescence and by gene expression as determined by microarray.

Methods. Human ASCs primary cultures were obtained, expanded and seeded in a two-dimensional culture system. After this, cells were induced to the osteogenic and chondrogenic lineages for 20 days using conditioning media. Then, samples were obtained after 24 hours; 10 and 20 days and processed for histological and immunofluorescence analyses. Microarray expression analysis was carried out by using total RNA corresponding to control non-induced ASCs and induced ASCs for 20 days.

Results. Gene expression analysis of ASCs induced to the osteogenic lineage for 20 days showed significant upregulation of several genes related to osteogenic differentiation and bone function in comparison with controls including ALPL, BGLAP, BMP15, BMP3, BMP6, BMP7, BMP8B and BMPR1B (Fig. 1). Whereas ASCs induced to the chondrogenic lineage after the same time period showed upregulation of the genes BGN, CHAD, CHSY1, CHSY3, CILP, CILP2, COL10A1, COL11A1, COL2A1, CSGALNACT2, HAS1 and HAS2. The histological analysis and immunofluorescence confirmed that the transdifferentiation of ASCs was succesfully completed after 20 days of inductions respectively.

Conclusions. These results suggest that the transdifferentiation process of ASCs to both the osteogenic and chondrogenic lineages was efficiently carried out using the methods described here which implies that these cells could have clinical potential in maxillofacial procedures.

Acknowledgments. Supported by CTS-115, Tissue Engineering Group.

Keywords. Transdifferentiation, gene expression, skeletal

(50.4.P6) PROADRENOMEDULLIN N-TERMINAL 20 PEPTIDE (PAMP) INCREASES KINESIN SPEED

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Introduction. Vesicles and other orgenelles move inside the cell through a well orchestrated cargo transport system based on microtubules and motor proteins such as kinesins and dyneins. Nowadays we have ample knowledge of the mechanisms by which motor proteins hydrolyze ATP and change their conformations to propel themselves along the microtubules, but little is known about intracellular factors that regulate motor speed. Proadrenomedullin N-terminal 20 peptide (PAMP) is usually secreted to the extracellular milieu where it functions as an autocrine, paracrine, or endocrine hormone acting through specific cell surface receptors,

but here we show it is also an intracellular peptide that regulates kinesin speed.

Material and Methods. Kinesin ATPase activity, microtubule polymerization, and gliding assays were performed with the “Kinesin ELIPA Biochem Kit”, the “tubulin polymerization assay kit”, and the “Kinesin Motility Assay” (Cytoskeleton), respectively.

Results and Discussion. PAMP increases kinesin speed and ATPase activity in a dose-dependent manner. The terminal amide group of PAMP is required for this activity and the smallest peptide fragment retaining a kinesin modulatory role is PAMP(12-20). The peptides were active on a variety of kinesins, including kinesin-1, chromokinesin, CENP-E, and Eg5. PAMP increased the speed of microtubules gliding on immobilized kinesins, thus identifying PAMP as an intracellular peptide that regulates kinesin speed. Since many of the current cancer chemotherapies target the microtubules and their associated proteins PAMP may constitute a new molecular target for cell division regulation. In addition, these results may be also relevant for the rapidly evolving discipline of nanotechnology since PAMP may be used as an accelerator of nanodevices based on microtubules and motor proteins.

Conclusion. PAMP is the first peptide shown to be involved in the regulation of kinesin speed. The implications of this observation for basic biology and nanotechnology warrant future studies in this field.

Keywords. PAMP, kinesin speed, amidation, nanodevices

(50.4.P7) EXPANSION UNDER HYPOXIC CONDITIONS ENHANCES THE CHONDROGENIC POTENTIAL OF EQUINE MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW

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Introduction. Oxygen is a biochemical molecule involved in numerous biological processes. The cultivation of mesenchymal stem cells (MSCs) is usually performed under 20% O2, contrasting with the physiological oxygen tension inside the bone marrow, which ranges between 1 and 7%. MSCs are currently being used for the treatment of equine orthopedic injuries. The cultivation of equine MSCs under hypoxic conditions might help to understand their behaviour in an environment closer to the physiological environment.

Methods. Cells isolated from bone marrow were cultured at 20% O2 (normoxia) and 5% O2 (hypoxia). Cell doubling time for each experimental condition was calculated for ten passages. At third passage, equine MSCs were culture in osteogenic, adipogenic and chondrogenic inductive media. To determine osteogenic differentiation the alkaline phosphatase activity and mRNA expression of RUNX2 and ALP were quantified. To assess adipogenic differentiation oil red O staining and mRNA expression level of LPL and PPARγ quantifications were carried out. To evaluate chondrogenesis, sGAG and mRNA expression level of COL2A, LUM, ACAN, COMP and BGN were analysed. Specific staining for each tri-lineage differentiation was performed.

Results. Cell doubling time was lower in MSCs expanded under hypoxic from passage 2 to 6 than in normoxic condition. MSCs slowed down their growth from passage 8 and 9 for hypoxic and normoxic conditions, respectively. Normoxic and hypoxic cells did not show differences in osteogenic and adipogenic differentiation. However, chondrogenic related quantifications were significantly higher in hypoxic than in normoxic MSCs. These results were confirmed by alcian blue staining.

Conclusions. Equine MSCs under hypoxic and normoxic conditions had similar cell doubling time. The cells expanded under both experimental conditions did not display differences in osteogenic and adipogenic potentials. In contrast, chondrogenesis was found enhanced by hypoxia.

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Keywords. Equine MSC Hypoxia

(50.4.P8) BIOCOMPATIBILITY OF BONE SUSBTITUTES USED IN DENTAL PRACTICE

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In dental practice, many situations such as periodontal bone defects, alveolar ridge augmentation, and dental implant placement, may require bone substitute materials. In the “Laboratorio de Hemoderivados” of the “Universidad Nacional de Córdoba”, Argentina, it has been developed a Bone Matrix (Matriz Osea-UNC). It is also recognized that Platelet-rich plasma (PRP) is widely used in situations of bone regeneration. Experimental studies on animal models are necessary to obtain accurate data on bone substitutes and cientific evidenced of their action upon sourronding tissue. This study was carried out to evaluate the biological behaviour of a particulate bone matrix (the “Matriz Osea-UNC”) and platelet-rich plasma (PRP) in the subcutaneous tissue.

Surgical procedures were performed in twenty Wistar rats. One ml of blood was extracted by intra-cardiac puncture to obtain platelet-rich plasma (PRP). Sterile silicone tubes were implanted as follows: a)empty, (control group, CT) and filled with the experimental materials: b) Matriz Osea-UNC (MO group), c) platelet rich plasma (PRP group) and c) Matriz Osea-UNC in association with PRP (MO+PRP group). Samples were taken at 30 and 60 days post-implantation and biopsies were processed by routine techniques for observation in optical microscope. In al cases National Institute of Health guidelines for care and use of laboratory animals (NIH publication Nº 85-23, revised 1985) were taken into account.

Results: Observations of the silicone tubes in the sub-cutaneous tissue showed no inflammatory phenomena associated with particles of Bone Matrix alone (MO group) or with PRP. Both materials showed biocompatible properties, however, osteoinduction was not demostrated in this extra-osseous site. In this experimental conditions the particles of “Matriz Osea-UNC” and PRP behave as biocompatible materials.

Keywords. Freeze dried bone allograft; Bone induction, Biocompatibility

(50.4.P9) A COMPARATIVE STUDY OF TWO COLLAGEN-BASED NEURAL CONDUITS IN PERIPHERAL NERVE REGENERATION

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Introduction: The complex histological structure of the peripheral nerves can be affected by several neuropathies with important clinical consequences. Although several models of bioengineered peripheral nerve have been described, an efficient substitute of this structure is still needed. In this work, we describe a fibrin-agarose peripheral nerve regeneration (FAPN) model using a combination of fibrin-agarose within a commercially available collagen conduit (CC) and we evaluate the nerve regeneration capacity using morphological methods.

Materials and methods: 24 Wistar rats were anesthetized using ketamine and 10mm of the right sciatic nerve were surgically removed. Then, in 12 rats we implanted the CC between both ends of the nerve following the same technique currently available for the clinical treatment of human nerve lesions (controls). In the other animals, the CC filled with fibrin-agarose biomaterials were implanted following the same technique. The implants were extracted after three months, fixed in 10% buffered formalin and paraffin embedded. Tissue sections were stained with haematoxylin-eosin. The presence of a myelin sheath and collagen fibers in the implants were analyzed by histochemistry. Laminin, S-100 and neurofilament were analyzed by immunohistochemical methods.

Results: Our results revealed that the FAPN model had significantly higher levels of nerve regeneration in the implant, with positive reaction for collagen and myelin, and immnostaining was positive and intense for all markers (laminin, S-100 and neurofilament). The control CC group showed lower levels of nerve regeneration, which was limited to the proximal portion of the implant, with lower reaction for collagen. The inmunostaining was positive for all markers only in the proximal portion of the implant.

Conclusion: The FAPN model could allow a more efficient axonal regeneration, Schwann cells proliferation and cellular migration of the regeneration front as compared to the control group. The fibrin-agarose biomaterial has promising results, although further analyses are in need to identify the clinical usefulness of this model from a molecular point of view.

Acknowledgements: This work was supported by grant SAS PI2007/135 by Junta de Andalucia, Spain.

Keywords: peripheral nerve, neural conduit

(50.4.P10) EVALUATION OF TWO LOCAL ANAESTHETICS ON HUMAN ORAL MUCOSA FIBROBLASTS. A COMPARATIVE STUDY

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Introduction. Lidocaine 1% with epinephrine (1:100.000) (LE) and mepivacaine 3% without vasoconstrictor (M) have been globally used as a rutine anaesthetic in many of the dental procedures. In this context, many studies have been carried out to evaluate their toxicity effect at the systemic levels. However, the cytotoxic effects of these anaesthetics on human oral mucosa fibroblasts have not been deeply described until now.

Methods. First, human oral mucosa fibroblasts were cultured in a density of 10.000 cells per well under standard cell culture conditions. Subsequently, cells were exposed to increasing concentrations of (LE) and (M) diluted in DMEM free of fetal bovine serum (FBS) during 6 and 24 h. For cytotoxic analysis, lactate dehydrogenase (LDH) assay were developed in (M) and (LE) treated cells. The controls groups were cells that did not receive any treatment (CM) and cells were treated with Triton X-100 1% solution (CT). Statistical analysis was performed by using t-student test.

Results. The results of LDH analysis revealed significant differences between both experimental times (6h and 24h). The short terms of exposure (6h) to (LE) and (ME) demonstrated to be significant (p value=0.000). Moreover, large terms of exposure (24h) also showed to be significant (p value=0.015) after cytotoxicity testing. Apart from this, short terms (6 h) of exposure to (LE) and (ME) indicated a higher cell toxicity in (M) compared with (LE) exposed fibroblasts. However, large terms (24 h) showed elevated cell toxicity levels in (LE) treated cells.

Conclusion. In conclusion we could suggest (M) in large and (LE) for short exposure times especially for non-dental anestethics procedures. For future studies we recommend deep analysis focused in epinephirine effects on cell toxicity.

Keywords. lidocaine, mepivacaine, cytotoxicity, fibroblasts, lactate dehydrogenase (LDH)

(50.4.P11) QUALITY CONTROL OF BIENGINEERED HUMAN UROTHELIUM USING TRANSMISSION ELECTRON MICROSCOPY

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Introduction. In the urinary tract exists several diseases that often require tissue replacement. Tissue engineering is new area which permits to build artificial tissues with the objective to be useful therapeutically. Transmission electron microscopy can be fundamental to evaluate these artificial tissues.

Material and Methods. Urothelial cell cultures generated by enzymatic treatment of normal human bladder biopsies, were seeded on top of the stromal substitute. The susbstitue of the bladder stroma was constructed using fibrin- agarose sacaffolds with fibroblasts within. These specimens were processed for quality control analysis using transmission electron microscopy.

Results. The analysis of the different specimens suggest that both control and bioengineered samples had an

immature urothelium in which, in some areas, the basal membrane is joined to the epithelium by the existence of union systems. This work also shows the presence of intercellular junction complexes, which are fundamental for an efficient barrier function.

Conclusions. The final result after transmission electron microscopic evaluation revealed that the urothelial cells obtained from biopsies, and after the construction of the artificial bladder showed the characteristics of an immature urothelium, which might need longer time of maturation, although the typical cytokeratines of the epithelial cells are well expressed.

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50.5. LEARNING AND TEACHING HISTOLOGY AND TISSUE ENGINEERING

(50.5.O1) NEW MULTIMEDIA RESOURCES FOR LEARNING HISTOLOGY AND ITS USE IN E-LEARNING

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Introduction. The EEES model has provoked deep changes in learning methodology, mainly derived from the greater protagonism of the student regarding its self-training. It has motivated the authors to develop tools such as the presented in this work, which improve the self-training using TIC resources. We have elaborated different learning-modules integrated by an informative iconographic nucleus, as well as tests of self-evaluation and hyperbonds leading to other resources. Both the student and the professor can know the obtained results in real-time by means of the resources of an e-learning platform.

Method. The modules have the file format "interactive flash" and they can be visualized from the platform using an Internet navigator. The graphical content consists of drawings performed by means of several computer applications, whose components appear according to rules of animation controlled by the user. They show a three-dimensional aspect, so that the student becomes an idea of the complex histological structures, since in the preparations and microphotographies they can only contemplate these structures in a plane. They are also accompanied by explanatory texts and allusive microphotographies. The final assembly has been performed with two applications of e-doceo, which combine informative contents and tests for the self-evaluation of different abilities and competitions.

Results. With these applications is obtained a SCORM package that, integrated in the virtual platform, allows its interactive execution by the users, as well as the monitoring of the learning process by the professor. The modules have been organized following rules of an animal and vegetable histology program.

Conclusions. The development of didactic resources of this type will help the students to acquire competitions of difficult acquisition. This is essential for studies including histological-type contents (i.e. Biomedical Degrees).

Supported by a Project of Innovation of the University of Jaén.

Keywords. Histology, Multimedia resources, E-learning

(50.5.O2) STRENGTHENING THINKING ABILITIES; A MULTI-MEDIA EXERCISE OF MEANINGFUL EVALUATION AND FEEDBACK LEARNING. "EPITHELIAL TISSUE IN MY PATIENT"

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Introduction. The younger generation seems to have mutated genes that give them special information technology abilities. Multi-media is used for meaningful assessment and feedback for their current learning. Simultaneously, acquire and / or reaffirm thinking skills (for analysis and problem solving). This, in turn, enables the subsequent achievement of competence in our Health Science students. This is reflected in the medium term during their contact with clinical subjects and in the longer term with their inclusion in a professional world.

Material and methods. We used multi-media material for the analysis and resolution of a situation medical (evaluation and interactive feedback) as a reinforcement of previous knowledge regarding epithelial tissue. It was applied to 20 students in their second semester of medicine, with an average age of 17, in the subject of "Fundamentals of basic medical sciences", in the "University del Norte" in Barranquilla, Colombia.

Results. Six students were required to perform the full activity a second time, because 83% of them had not attended theoretical classroom activity. Of the remaining fourteen, six chose the ideal resolution in each situation. Eight had made mistakes in various choices and needed a first or second check-over to correct them. Two students made written recommendations and one of those was taken up. 95% of the students felt comfortable with the methodology.

Conclusions. To design multi-media materials with situation evaluation characteristics (for analysis and problem solving), conducive to development and strengthening of basic thinking skills for students of health programmes. The coherence between the material with the characteristic of the students helps to achieve our educational goals. The Multi-media material is important as strategy and to complement task work. However, the prior acquisition of the theoretical concept was and will remain fundamental. These methodologies facilitate integration into curriculas for systems.

Keywords. Thinking abilities, multi-media materials, analyze situations

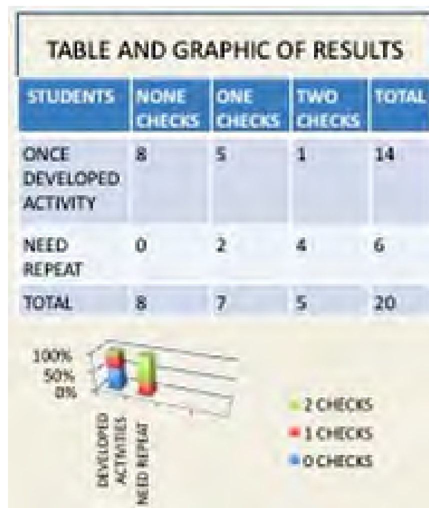
TABLE AND GRAPHIC OF RESULTS

STUDENTS	NONE CHECKS	ONE CHECKS	TWO CHECKS	TOTAL
ONCE DEVELOPED ACTIVITY	8	5	1	14
NEED REPEAT	0	2	4	6
TOTAL	8	7	5	20

(50.5.O3) USEFULNESS OF "SYLLABUS OF HISTOLOGY" TO IMPROVE THEORETICAL TEACHING IN HISTOLOGY

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Introduction. The students of Histology in the Faculty of Medicine have free access (available in "Aula Virtual") to many documents and archives from the beginning of Histology course; between these documents we include the so called "Syllabus of Histology". This didactic material includes the basic knowledge of the theme who students must know, selected images and schemes, and several questions that must be solved consulting Histology books.

Objective. We claim that in the theoretical lecture, students are attentive to the teacher explanations and take part actively asking, answering and debating and do not lose time taking notes. Moreover, we pretend that this tool facilitate Histology learning and promote self-learning in the students.

Material & methods. This study included 122 students of the subject "Histology" from the Faculty of Medicine of Córdoba (Spain). By means of an anonymous survey we asked them about 8 items (scored 0 to 10) related with "Syllabus of Histology".

Results and conclusions. The students believe that Syllabus is a useful tool for Histology learning. Moreover, they consider that this Syllabus promote the use of Histology books and consequently self-learning (7. 52 ± 0. 22). On the other hand, they have that Syllabus before therotical lectures which favour that they do not take notes (8. 51 ± 0. 21); this fact allows, under our point of view, a better participation in class.

Finally, the students consider that Syllabus improve their comprehension (9. 27 ± 0. 10).

Keywords. Histology, self-learning, histology teaching

(50.5.O4) STUDENTS OBJECTIVES FOR PRACTICAL HISTOLOGY IN HEALTH SCIENCES CURRICULA

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Introduction. Health science students need practical observation of histological preparations as one of the main instruments for their learning period. However, the potential objectives to reach professional competences of these students have not been studied to date. In this work, we have compared the expectations of Medicine and Dentistry students in relation to histological practical learning.

Methods. In this study, we have analyzed a total of 80 first-year students enrolled in Medicine and Dentistry studies by using a specific survey including several questions focused on the main interest topics that the students expect concerning their histological practical learning. Each item was evaluated by the students using a Likert-scale ranging from 1 to 5. We compared the results obtained between both degrees using the T-test.

Results. Results showed statistically significant differences between Medicine and Dentistry students ($p<0.05$) regarding some specific analyzed items. Medical students expected to identify the histological structures that they studied in theoretical lectures more than Dentistry students. However, the expectations of Dentistry students concerning the observation of stem cells and the success in their practical learning period are higher than those of Medical students. No differences were found for other items: increasing the knowledge about the inside of the human body, direct learning without books or notes, low lecturer-student ratio, adequate exam training, entertain learning and decreasing boring learning.

Conclusions. Our data show different expectations between Medicine and Dentistry students regarding the practical learning period of the Histology module. While Medical students´ practical learning objectives are to complete the theoretical concepts for being applied to the microscopic diagnosis, Dentistry students´ objectives range from the pragmatic improvement of academic commitment and innovative findings linked to the research of Histology.

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(50.5.O5) VIRTUAL HISTOLOGY TEACHING: IMPACT AND PERSPECTIVES

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The application of new ICTs to the field of education has increased in recent years, particularly in the field of Health Sciences and by extension in the Histology. These technologies provide us information, tools for processing and communication channels. The use of the computer as a standard tool in the teaching-learning today is an established fact. The virtual education provides flexibility in space and time, access to content, materials and activities at any time, allowing an adjustment to time,

space, form and learning needs the student may serve as support for classroom teaching and can be integrated in the use of formal teaching. Virtual education to improve the quality of the educational process. In the university context, there is no university, which has not developed virtual spaces for teaching.
The implementation of the Degree within the framework of the EHEA, claims that the axis around which turn the whole educational activity is the student and he demands the acquisition of a range of transferable skills which enable it to further integration employment. In our subject, Histology, the observation and interpretation of microscopic images are skills to be learned and perfected in the practical classes. Any methodology that brings students to the microscopic image is good for Histology and we think that the use of "virtual campus", and the vast field of internet, can be a tool to significantly improve the teaching practice and serve as a complement to comprehensive education student.
In short, we think the virtual teaching of histology is a support and supplement traditional teaching. Provide flexibility in the teaching-learning process and is a useful and helpful to meet the demands of the EHEA. But keep in mind that no substitute for actual teaching and training required and excessive time and labor for the teacher, and a certain maturity and responsibility of the students in their learning.

(50.5.O6) BOLOGNA AND HISTOLOGY

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We analyze the evolution of education in the light of the Medicine curriculum and Bologna process. It reviewed the evolution of access to teachers, the entry of students to the School of Medicine, the intervention in the educational systems, and methods of evaluation of the different educational actors.
The authors refer personal experiences on each of these matters that have led to the creation of the Bologna Process. In Bologna it has been tried to improve the quality of education, avoid absenteeism, promote teamwork and the use of new technologies and to facilitate mobility of students and teachers. To do so, it has been prepared guidelines which are reflected in a myriad of regulations, which, in our point of view, blocked the University. The University has been the center of creation and dissemination of thought, philosophy and science; functions that can only be fully developed in freedom and bureaucracy are antagonistic. As the bureaucracy grows, creativity diminishes. The free-thinking and creative teacher is not subject to the rigid rules that are doomed to the university today.
It is paradoxical that in Paris and Bologna were born the first universities and that in these places it has been incubated its decline. The implementation of the Bologna process through jobs, copy-paste, obligation to attend class (asking and evaluating during class or a roll), extensive practices, "The busier, the lesser think,". The student evaluation: continued, theoretical, practical, skills, competencies, mainstreaming ... are having a tremendous influence in our subject: Histology. In our medical school, the staff of Human Histology Unit have been taken a number of decisions to avoid the deterioration of education such as:
- A single theoretical group, which prevents discrimination against education, based on good lectures.
- Freedom of teaching: Every teacher teaches their best, with or without new technologies.
- Freedom for the student to attend lectures: Students go to school because they consider useful for learning, not by coercion. No roll call in class.
- Real practical classes (microscope electronic and photographs). Not a theoretical extension of the lectures content.
- No asking students to realize copy and paste jobs. Histology is about learning, not layout and graphic design.
- Assess the students only for their expertise in histology. The assessments of competencies, skills, understanding, etc. are matters of high school.

Through this, we do not coerce the teachers, not stun the students and give them a great teaching that we can be proud and presume in our area.

(50.5.O7) USING MOODLE AS A TOOL TO SUPPORT TEACHING HISTOLOGY

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Moodle is an electronic learning platform used by a increasing number of universities and institutions in Spain. The platform offers a wide range of tools and resources that can be used in teaching in a broad variety of applications, ranging from support face-to-face teaching to curses fully online. The present talk seeks to provide some examples of the many and wide-ranging resources the platform has to offer, and aims to illustratehow these tools are used at the University of Valladolid to support and further complement onsite histology teaching. By way of examples, we will show how we use resources such as Latest News and Announcements, Classroom Documents, which include lessons with texts and photographs as well as class-room presentations used by teaching staff, Questionnaires to be completed by students, and students' Grades. Moodle also includes tools which greatly enhance communication between teaching staff and students.

(50.5.O8) MEANING OF THE LECTURE IN THE BOLONIA PROCESS. "A SPECIES IN DANGER OF EXTINCTION"

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The lecture is a teaching method focused on the teacher, who tries to transmit a series of knowledge, previously acquired through continuous exposure to them. It was born with the university.
Expository method is a teacher's active participation and passive involvement of students. It says it is a didactic one-way communication process, which produced a series of "vices" in modern education (EHEA) must be corrected for the benefit of listeners, in this case of students in the teaching of Cytology and Histology and Tissue Engineering. For this reason the number of hours of theory of a subject, or lectures, has been declining in higher education and specifically in the medical

curriculum. In the Faculty of Medicine, Manchester Cytology and Histology has gone from 180 hours (old Plan) to 102 (in the Bologna Process). Outlined below the major criticisms that have led to a decrease in the number of hours of lectures are discussed.
However, as noted by Professor Carrascal the lecture is worthless especially when taking into account three factors: the one who teaches (professor), the learner (student) and aids the University offers. For this and according to Brown the lecture should be properly planned, in close contact with students as recommended by Bologna, and always in small groups. Finally we show in defense of the lecture their strengths clearer showing the inestimable value of this teaching method. We note that to adapt to the EHEA all participants must change the "chip" as indicated by Professor Gallardo, in order to improve the learning process. In short the lecture can transmit knowledge, provides a critical approach to the subject, try to make students reflect and form a critical mind.

(50.5.P1) STUDENTS THEORETICAL BACKGROUND FOR PRACTICAL LEARNING HISTOLOGY

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Introduction: Histology module is taught in different health science degrees applying similar communication process and contents in the practical learning period. Nevertheless, the theoretical concepts taught, not only in the Histology module but also in other degree modules, are different. In this work, we have compared the theoretical background priorities that Medicine and Dentistry students established in order to success in the practical learning period of their Histology module.
Methods: In this study, we have analyzed a total of 80 first-year students undertaking Medicine and Dentistry by using a specific survey including several questions focused on the theoretical backgrounds that they think they need to success in their histological practical learning period. Each item was evaluated by the students using a Likert-scale ranging from 1 to 5. We compared the results obtained between both degrees using the T-test.
Results: Results showed significant statistical differences between Medicine and Dentistry degrees ($p<0.05$) in relation to some specific analyzed items. The theoretical background areas needed to success in the practical learning period showed significant differences between students with higher scores for Dentistry students for embryology, microscopic measurements and units, and histological staining techniques.
Conclusions: Our data show that the priorities of Medicine and Dentistry students are different regarding the need to success in their practical learning period in the Histology module. These results suggest that Dentistry students link the practical learning Histology to the preparation of histological samples and to the histogenetic development of the tissues and organs, while both degrees link it to the diagnostic identification of the human tissue structures.

(50.5.P2) CATALOG OF TISSUE STRUCTURES TO SYSTEMATIZE THE HISTOLOGY SELF-LEARNING IN INTERNET

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Introduction. The tissue concept is a theoretical construct although a very useful tool to knowing the microscopic structure of the organism. The traditional study to identify histological images (HI) has been their routine observation. This method is boring and is very time consuming. To reduce time and enhance learning, we proposed a conceptual systematization of the tissue structures (TS), in three levels according to their complexity. The aim of this work was created a virtual catalog of complex TS for to use in virtually self-learning of Histology.
Materials and methods. To carry out this project, we elaborated an exhaustive Catalogue of TS (CTS), together its related HI. The CTS and HI were implemented, like Virtual Self-Learning Modules, and carried out by means of Internet where instructional materials (systematized CTS and HI) were presented like a self-instruction models. The model was applied to students for experimental evaluation.
Results. With this method, students learn easily to recognize and remember the HI on their own. Preliminary data indicate a strong effectiveness of use of systematized CTS, and its application to Virtual Self-Learning of Histology.
Conclusions. Systematized CTS permit to develop basic criteria for tissue understanding and infer their functional and pathological significance.
Keywords. Human histology, self learning, tissue structure

(50.5.P3) WHAT IS THE OPINION OF MEDICAL SCHOOL STUDENTS OF THE TOOLS USED FOR HISTOLOGY LEARNING?

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Introduction. We use a variety of tools to achieve our aim that students should be able to apply their acquired knowledge of tissular organization in the recognition of body structures at the microscopic level. In order to improve our learning objectives, we present the results of the student's opinion polls in the last three courses.
Methods. An anonymous survey with 25 items was conducted in which students were asked about the use, satisfaction and usefulness of different learning tools, besides microscopy slide sessions (68 slides in 12 sessions). The simple objective of tissue structure recognition was provided by video explanations of each microscopy slide, available from our website. More complex objectives were analyzed, such as the

development of a notebook with drawings of tissues observed under microscope, as well as the recognition of 50 new microscopy images. The ultimate objective corresponded to the collection, synthesis and oral presentation of histological topics using data from published studies, encouraging them to take responsibility for their own learning.

Results. We analyzed 593 inquiries. Video microscopy tool was used by 82% of polled, most of them with more than 8 sessions view. Sketching tissues in a notebook was done by 96% of them, investing more than 8h in completing it, which was considered a moderately difficult task by 50% polls. Seventy-five percent thought that both activities generated an increment in their learning. Most (80-100%) of the new microscopic images were analyzed and recognized by 89%, with a subjective 61% increase in their learning. Oral presentation provided the highest degree of satisfaction, which reported an increase in both theory and practice learning.

Conclusions. Our teaching methods and techniques are working out. Students are free to learn more if they wish so, but our specific previously-designed learning objectives should define the 'bottom line'.

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Keywords. Teaching tools, medical students, opinion poll

(50.5.P4) CAN VIRTUAL TOOLS IMPROVE STUDENT PERFORMANCE IN A COURSE OF PRACTICAL HISTOLOGY?

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Introduction. Advances in computer and related technologies have facilitated the management of electronic resources, making student-centered alternatives both possible and feasible. They provide interactive and complementary activities that enable individuals to address unique learning interests and needs, study multiple levels of complexity, and deepen understanding. Our aim was to improve Histology teaching using these technologies, with the following purposes:

1. To optimize the use of didactic resources (microscopes and histological preparations) in classroom activities.
2. To increase student motivation through the use of educational tools that promote basic science-clinical integration.

Materials and methods. A virtual course was generated using the Moodle environment. Optative activities were designed involving: access to "virtual microscopes", self-assessment questionnaires and discussion forums with topics of basic science-clinical integration. The total enrollment was divided into 48 groups of 20-25 students. The activities were assessed by a survey at the end of the course. The performance in practical examination was compared between students with low compromise to virtual activity (n = 164) and those who showed high commitment to it (n = 158).

Results. About 60- 90% of students performed self-assessments and less than 15% attended to the forums. Almost 70% of the students reported that self-assessments were helpful or very helpful. Many students attributed their poor involvement to the limited incidence of the virtual experience in the Histology course as a whole, and to the low consideration of the discipline compared to the rest of the courses taken at the same time. Seventy-eight percent of students with high involvement in virtual activities obtained the approval of the practical examination while only 47% achieved it in the group with weak commitment.

Conclusions. Our results provide evidence that new patterns of communication and offered educational tools contribute effectively to teaching medical histology and may enhance the learning experience.

Keywords. Virtual tools, practical histology, performance

(50.5.P5) DENTAL HISTOLOGY KNOWLEDGE BY MEDICAL STUDENTS. A DIAGNOSTIC EVALUATION

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Introduction. The dental histology and bucodental embryology have not been considered as a priority in the learning of histology for medical students. However, these subjects should be considered as key points into the histology learning processes because the oral pathology represents a significant percentage of human pathology at local and systemic levels. For this reason, the knowledge of the oral structures should be a complementary goal in medical education. The aim of this study was to evaluate the knowledge of medical students about dental histology and bucodental embryology that have previously completed the basic level of histology.

Methods. A specific questionnaire was performed on fifty medical students who have previously completed a basic cycle of histology. The questionnaire included twenty questions related with general histology knowledge, soft and hard dental tissues, dental development and salivary glands knowledge were the evaluated topics. All those items let us to assessment the students knowledge related to the bucodental structures. The assessment was done in a scale that range from 1(lowest) to 10 (highest).

Results. Our results showed that medical students who have previously completed the basic level of histology have a general topographic and terminology knowledge of the constitutive elements of the oral cavity (4,45). Although, these students do not have a specific knowledge of the structural patterns forming the oral cavity (1,65) that are considered absolutely necessary in many cases to establish the etiology of the oral cavity associated pathology. Our results showed that students have more knowledge of the general histological structures applicable to the region, such as salivary glands (5,16) in comparison to hard tissues.

Conclusions. Due to the prevalence of oral pathology and its relation with the systemic pathology specific dental histological knowledge should be enhanced in preclinical education of medical students. To solve this, the options include the increasing of contents in dental histology or the establishment of an elective course that already exists

in the medical faculty of the University of Granada in which converges the basic teachings of the oral cavity and head and neck region, which serves for future pre-clinical learning in medical surgical pathology.

(50.5.P6) KNOWLEDGE INTEGRATION DERIVED FROM CURRICULAR CONTENT RESEARCH

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Nowadays, the university activity quality implies the joint development of research and didactical tasks, together with its translation to the knowledge productive sector. In order to incorporate such knowledge derived from research to degree teaching, the development of adaptive changes to make this knowledge apt for teaching content should be performed. The transition from an accurate knowledge content to a didactical version of such knowledge object is known as didactical transposition.

This paper intends to show a pedagogical strategy enabling to integrate such knowledge derived from research on dental enamel microstructure and its functional implication to curricular contents of the Subject Histology and Embryology of the Facultad de Odontología de La Plata. The methodology used was the one known as Problem- Based Learning (PBL), where such knowledge emerged from research is submitted to the students being it faced against the conceptual contents of the bibliography suggested, in order to generate problems and give rise to cognitive discussion. The students attend classes with their knowledge previously obtained from text books and, organized in groups they work with research published material. The teacher proposes the following working instructions: reading, observation of dental enamel with the aid of a magnifying glass, electronic and optical microphotographs; group discussion and some material achievement, basically a concept comparative chart according to the definitions of the various authors, followed by a final consensus.

Problem-Based Learning (PBL) appears as a resource enabling the didactical transposition and, at the same time, challenging the students to a knowledge research, so as to find answers not only to such questions raised either by a text book or a teacher but also to their own questions.

Keywords. Didactics, transposition, problem-based learning

(50.5.P7) PREVIOUS KNOWLEDGE OF HISTOLOGICAL SKIN STRUCTURES BY PREGRADUATE DERMATOLOGY STUDENTS

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Introduction. Histology and histopathology are basic subjects needed to understand dermatology diseases. Clinico-pathological correlation is essential for the diagnosis of many skin problems. The objective of this study was to evaluate histology and histopathology concepts, learned through the first year of the degree, in dermatology students (fifth year) during the first day of class.

Methods. This cross-sectional study included 65 medical students between 21 and 23 yrs from the School of Medicine of Granada. Dermatology students completed the survey which contained a total of 30 questions and two information blocks on: knowledge of histology and histopathology (8-question test) and about motivation and satisfaction with the degree. The statistical analyses were performed with the SPSS/PC software (Version 15.0 for windows). Two samples student's t test was used to compare mean values of quantitative variables. Correlations among variables were studied by using the Pearson coefficient.

Results. A total of 61 students fulfilled the survey (response rate 93.8%), 70.5% of the students were females and the mean age was 22.7. Histology (42.5%) and histopathology subjects (39.3%) were considered as the most important basic subjects for the study of dermatology. However students did not displayed a very high level of knowledge of histology and histopathology in the test (mean value 1.6 points over 8) without differences between males and females (1.82 vs. 1.48 points, p=0.22 respectively). The highest punctuation was achieved in embryology questions (91.8%). There was a positive significant correlation between the score of this test and the punctuation (mean value 8.87 over ten) of the students to start the degree (r=0.28, P=0.036).

Conclusions. Dermatology students are aware of the importance of histology and histopathology for the study of dermatology; however their previous knowledge of these subjects should be enhanced with histology and histopathology concepts of the skin to promote a better learning of dermatology.

(50.5.P8) UPDATING OF THE NEUROHISTOLOGY IN TEACHING/LEARNING

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We built a teaching material on the histology of the Nervous Tissue for the subject of "Cellular Biology and Animal and Plant Histology" included in the new Degree in Biology at the Complutense University of Madrid (CUM). It is consist of a file with 100 presentations with texts, drawings, microphotographies, animations, bibliography and web links to neuro-histological documents. The included materials have been selected from the best publications on this field and Internet, or from materials elaborated by the own authors of this communication. This didactical material then aim to update the teaching/learning process on the histology of the nervous tissue.

This file, in pdf format, can be loaded in the Web of the CUM Virtual Campus to be offered to the students. Its

content represents a support script for both the professors lectures and the particular study of each student. We aim to establish a bridge between the teaching of the professor and the learning of the students. The final purpose of this work is therefore to support the theoretical learning. The use of these new technologies has shown that the students increase their personal learning compared to the classic credits of each discipline. With this file, the students can privately prolong their study if necessary, thanks to the resources used or recommended by their professor in the theoretical lectures. This support material therefore fully covers the new european credits included in the European Higher Education Area, from the lectures to the more personal study or in groups.

Keywords. nervous tissue, neuron, glial cell

50.6. REPRODUCTIVE BIOLOGY AND DEVELOPMENT

(50.6.O1) MORPHOLOGICAL ANALYSIS OF THE SPERM FROM AFRICAN PYGMY MICE, SUBGENUS *Nannomys* (RODENTIA, MURINAE, *Mus*)

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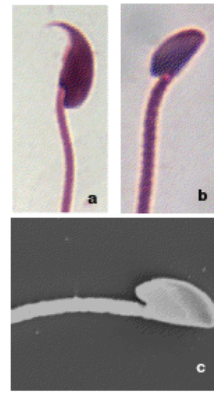
It was previously showed that the sperm morphology varies depending on the species studied. The reason of these changes is still unknown but it seems that it is related to the phylogeny, mode of fertilization and post-copulatory sexual selection (sperm competition). A unique characteristic of the rodent sperm is the presence of a falciform head with an apical hook. However, in few species it is observed that this form is absent. In the present study, the sperm from *Mus mattheyi* (subgenus *Nannomys*) from West Africa is analyzed by mean of light microscopy and scanning electron microscopy. *Mus musculus* was used as control species. Animals were killed by cervical dislocation and the epidydimis was obtained. The caudal epidydimis was minced in buffer to allow the sperm got out. Sperm were fixed in 10% formalin and 2% glutaraldehyde for light and scanning electron microscopy analysis respectively. Sperm from *Mus musculus* show a typical falciform head with an apical hook (Fig. 1a). However, the sperm head of *Mus mattheyi* lacks an apical hook (Fig. 1b,c) showing a morphology similar to the one of an other murin rodent: *Tokudaia*.

The length of the sperm is 78.54±0.79 and 115.5±0.32 µm in *M. mattheyi* and *M. musculus* respectively. Both the head (5.92 ±0.05 vs 7.9±0.05 µm) and the tail are shorter in *M. mattheyi* than in *M. musculus*. Future analyses are necessary to investigate the sperm ultrastructure of *M. mattheyi*. Additionally, this result is surprising because sperm morphology observed in *M. mattheyi* is similar to the one of *Tokudaia* which belongs to a phylogenetic group different from the *Mus* group.

This study was supported by a grant from Fundación Séneca de la Región de Murcia (0452/GERM/06).

Keywords. Sperm, ultrastructure, morphology



(50.6.O2) PROGRAMMED DEATH OF SERTOLI CELLS DURING TESTICULAR REGRESSION IN SYRIAN HAMSTER (*Mesocricetus auratus*) SUBJECTED TO SHORT PHOTOPERIOD

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Introduction. In seasonal breeding animals, gonadal regression occurs during non-reproductive periods. In the Syrian hamster (*Mesocricetus auratus*) this regression occurs when days are short, during which time germ cells are lost though apoptosis. Sertoli cells, which are essential for maintaining structural and functional integrity of the testis, are usually a fairly constant cell population and resistant to changes in the seminiferous epithelium. The aim of this communication was to investigate whether the testicular regression process due to short photoperiod produces a loss of Sertoli cells and if so whether it occurs through apoptosis or necrosis mechanisms.

Materials and methods. For this, a total of 50 Syrian hamsters (45 treated, 5 controls) were used. The treated groups were submitted to an 8:16 light-dark photoperiod for 12 weeks. Three groups were established: middle (MR), strong (SR) and total (TR) regression. The extracted testes were submitted to different protocols, for light microscopy (TUNEL technique) and for transmission electron microscopy (TEM).

Results show that after application of the TUNEL technique to testicular sections of the three regression groups abundant TUNEL-positive germ cells were found. Next to these germ cells but more isolated and in the basal part of the seminiferous tubule, some TUNEL-positive cells with a similar morphology to Sertoli cells were observed. In addition, TEM pointed to changes in the nucleus and cytoplasm of Sertoli cells typical of apoptotic cells.

Conclusion. during testicular regression when germ cells are lost through apoptosis, there is also a loss of Sertoli cells through the same mechanism. It seems, then, that the population of Sertoli cells probably remains unchanged throughout testicular regression in hamsters exposed to a short photoperiod and during the later period of resurgence.

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Keywords. Sertoli Cell, Apoptosis, Hamster, Regression

(50.6.O3) POSTNATAL EVOLUTION OF THE ULTIMOBRANCHIAL FOLLICLES IN THE RAT THYROID GLAND

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Introduction. In mammals, the thyroid gland exhibits a double embryologic origin: most of endocrrine cells -follicular cells- derives from the medial anlage arising from the pharinx floor, while C cells derive from the ultimobranchial bodies (UBB). In the adult thyroid gland, this complex structure remains as embryonic remnants known as "ultimobranchial follicles" (UBF), in rats, or "solid cell nests" (SCN), in humans. The fate of the cells that constitute the UBB walls has been an unsettled question for many years. The aim of the present research is to follow up the evolution of the UBB in the postnatal rat, from birth to senile-aged animals.

Materials and methods. Thyroid glands ranging from birth to 24-months old Wistar rats, of both sexes, were fixed in 10% buffered formalin and embedded in paraffin by a standard procedure. Five equidistant levels of the thyroid gland per rat were immunostained for calcitonin (CT) and thyroglobulin (TG), using LSAB system (Dako) and 3,3'-diaminobenzidine. Part of the sections were also stained with the PAS-technique and counterstained with Harris haematoxylin. Morhometrycal analysis of area and perimeter of UBF and normal follicles was also performed.

Results. UBFs were observed in 68% of young rats (0- to 30-days old rats) and over 30% of adult animals (3- to 24-months old rats). Differences in frequency of UBF between male (18%) and female rats (48%). were also observed. Depending on the age of the rat considered, three kinds of UBF could be distinguished: "inmature UBF", "mature UBF" and "cystadenomata", which exhibited characteristical appearances and measures.

Conclusion. In relation to the presence and evolution of the UUB remnants in the postnatal thyroid gland of the rat, a conspicuous sexual dimorphism was manifested.

This work was supported by grants from the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía, Spain (refs. CTS-439/2009 and P08-CVI-03598).

Keywords. Thyroid, ultimobranchial, C-cells

(50.6.O4) IMMUNOHISTOCHEMICAL EXPRESSION OF THERAPEUTIC TARGETS IN ADULT AND FOETAL UTERINE TISSUES

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Introduction. Cellular receptors c-kit and c-erbB2 (Her2/Neu) are useful therapeutic targets in tumors such as sarcomas and breast and gastric carcinomas (GIST). To confirm the clinical significance of the expression of these receptors in a group of uterine mesenchymal and epithelial neoplasm, firstly we need to know the expression of these receptors in control samples. Normal and foetal tissue expression of c-kit and c-erb2 (Her2) was analysed, and the results were related to numerical status of some chromosomes.

Methods. Uterine tissue samples (endometrium, endometrial stroma, and/or myometrium) were assembled in two tissue microarrays (TMAs) using a manual tissue arrayer (Beecher Instruments). Adult (n=13) and foetal (n=14) biopsies were including in TMAs. Immunohistochemical staining was performed using c-kit (CD117) and erbB2 (Herceptest) primary antibodies. UroVysion kit (Abbot Inc, USA) including specific probes for chromosomes was used for fluorescent in situ hybridization (FISH).

Results. We found similar c-kit expression in both mature and foetal tissue. Negative expression was observed in endometrial stroma and myometrium, and a generalized cytoplasmic expression in the endometrium. Unspecific cytoplasmic expression of ErbB2 (Her2/Neu) protein was observed in mature endometrial tissue, whereas it was negative in endometrial stroma and myometrium. In foetal uterine tissue, we found a high focal positivity in the endometrial stroma and heterogeneous expression in endometrial epithelium. The myometrium was negative. All tissues were disomic for chromosomes 3, 7, 9 and 17 by FISH.

Conclusions. The comparison between different expression in normal uterine tissue as well as chromosomal copy number with the expression in tumors could be the first step towards understanding the pathogenesis of endometrial adenocarcinomas, but will probably not be useful for uterine sarcomas. Other therapeutic targets should be studied in uterine tumors as alternative strategies for treatment.

Acknowledgments. Grants from Conselleria de Sanitat (CS2010-AP-125).

Keywords. uterine mesenchymal and epithelial neoplasm, c-kit, c-erbB2 (Her2/NEU), FISH, IHQ, therapeutic targets

(50.6.O5) NORMOBARIC HYPOXIA EFFECT ON THE TESTIS: VASCULARIZATION, PROLIFERATION AND STEROIDOGENESIS

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Introduction. Exposure to high-altitude generates spermiogram impairment as a consequence of germinal epithelium, Leydig cells, sperm and seminal plasma alterations. Physiological and molecular mechanisms involved are not precisely known. The objective of this work was to analyze the effect of normobaric hypoxia on the morphology of the testicular interstitium and on some associated molecules and hormones as well as on cell proliferation.

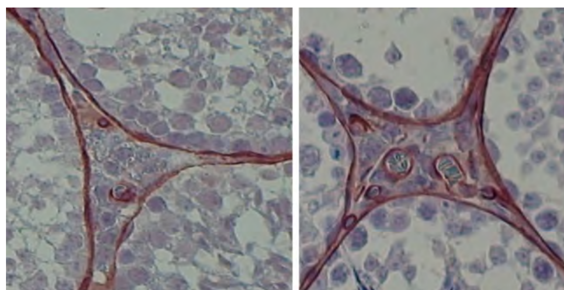
Methodology. Mice were exposed to an atmosphere with an 8.1% FiO2 (fraction of inspired oxygen) for various days. Effects on microvasculature were studied with immunohistochemistry,, plasmatic and intratesticular testosterone by radioimmunoassay, and HIF1-α, VEGF were analyzed through Western Blot. Cell proliferation studied by immunohistochemistry anti Ki-67. All variables

were registered at 1, 5, 10, 15 and 20 days of exposure and compared to controls.

Results. Blood vessels total number and their diameter as well as intratesticular and plasmatic testosterone were raised significantly after 24 hours. Plasmatic testosterone levels decreased the second day while testicular testosteron remains raised until the third day. Interstitial area occupied by seminiferous tubules suffers a significant decrease after one day of exposure followed by a recovery from fifth day and another decrease at the end of the experiment.

Discussion. The most important early effects were testosterone level increase and testicular edema. By the end of the exposure period, both angiogenesis and androgenic decrease take place. Normobaric hypoxia generates similar effects to those induced by hypobaric hypoxia. Based on this point, our results can be applied to understand side effects of pathologies involving a severe hypoxia component at sea level such as lung emphysema, COPD and cancer.

Keywords. testis, hipoxia, steroidogenesis, angiogenesis



Left: Normal rat testis interstitium, where interstitial area is occupied by some capilars and Leydig cells. Right: After 24 hours exposure to hypoxia, interstitial area shows many more capillars than normal testis.

(50.6.O6) MORPHOLOGICAL AND COMPARATIVE ASPECTS OF DYSFUNCTIONAL PLACENTA

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Introduction: The placenta plays an essential role in the maintenance of pregnancy and foetal growth. The morphogenesis and development of this organ are common among species that have haemochorial placentation, such as rodents, non-human primates and humans. Pathologies during the pregnancy are related with morbidity of offspring in human and animal models, with an increased risk of fetal death, growth disruption and several morphological changes in the placenta.

Methods: To analyze the effect of pathologies during the pregnancy in placental development, rats with experimental induced diabetes or malnutrition during the pregnancy were used. Placentas were collected on different days of pregnancy. We used morphological and immunoperoxidase and in situ hybridization techniques to identify several molecules relevant to normal placental development.

Results: Placentas from women with pathological pregnancies have been reported to have an abnormal trophoblasts layers, focal thickening of trophoblastic basement membranes, narrowing of small vessels, and changes in the cell proliferation or apoptosis. In animal models, alterations of morphology, extracellular matrix protein distribution and cell proliferation were found in the placenta.

Conclusion: These observations suggest that pathological conditions during the pregnancy may induce alteration in cellular function of human and animal models placentas that may be alter the normal interchange between the mother and fetus.

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(50.6.P1) EFFECTS OF CRYOPRESERVATION ON SPERM MOTILITY AND TUBULIN DISTRIBUTION OF HUMAN SPERMATOZOA FROM NORMOZOOSPERMIC, ASTHENOZOOSPERMIC AND OLIGOZOOSPERMIC PATIENTS

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Human semen cryopreservation is an extensively performed routine technique in fertility clinics. Cryostorage of spermatozoa or testicular tissue is becoming more important because of novel clinical needs and current clinical practice: assisted reproduction, preservation of fertility following chemotherapy, radiotherapy or surgical procedures. Although semen cryopreservation has proven to be very valuable, the quality of frozen sperm is highly affected during the process. Some studies have revealed that the freezing can cause tail defects. α and β tubulin subunits are the major components of sperm tail microtubules and centrosomes. They are therefore implicated in sperm motility, as well as in the early fertilization events.

The purpose of this study was to investigate the effect of freeze-thawing procedure on motility (according WHO) and the modifications in the pattern distribution of α-tubulin from 5 normozoospermic, 5 asthenozoospermic and 5 oligozoospermic patients of Hospital Universitario La Fe. Motility of spermatozoa was evaluated using the Makler chamber, according to the WHO guidelines .The distribution of tubulin was assessed by immunofluorescence using an anti-α-tubulin antibody. The samples were evaluated with a Leica DM IRBE2 confocal microscopy. We observed that the percentage of progressive motility in fresh and thawed were 59% vs 35% in normozoospermic, 35% vs 12% in asthenozoospermic and 33% vs 11% oligozoospermic samples. In relation to the tubulin distribution, we found in all groups three patterns in the tail, as fresh as thawed

sperm (pattern 1 (P1): homogenous labeling in whole tail; pattern 2 (P2): discontinuous fluorescence along the tail; pattern 3 (P3): labeling only at the end of the tail). Before and after sperm cryopreservation, we observed that the pattern most frequent was P1. P2 was the second more abundant in fresh samples otherwise in frozen spermatozoa was P3 ($p<0.05$). These changes were observed in sperm cells from normozoospermic, asthenozoospermic and oligozoospermic patients.
Keywords. Human sperm; cryopreservation; tubulin

(50.6.P2) DIFFERENCES IN FLAGELLAR α-TUBULIN EXPRESSION IN FRESH, CAPACITATED, AND ACROSOME-REACTED HUMAN SPERM

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Introduction. Sperm microtubules are composed of a heterodimer of the α- and β-tubulin. The tubulin and actin are the two most important flagellum proteins involved in sperm motility. During capacitation and acrosome reaction it has been shown that there are changes in the organization of cytoskeletal proteins. The aim of this work is to determine the relationship between events that prepare the sperm to fertilize the egg and the reorganization of α-tubulin.
Materials and methods. Normozoospermic semen samples were collected from healthy donors (aged 25-35 years). Samples were capacitated ("swim up") and acrosome reaction was induced (calcium ionophore A23187). The expression of α-tubulin was detected by immunofluorescence using mouse anti-α-tubulin antibody. Samples were examined by both, confocal and fluorescent microscope. Then, was measured the area labeled α-tubulin and the whole area of the flagellum for the three sperm groups: freshly ejaculated, capacitated and acrosome-reacted. Statistical analysis was performed using an ANOVA.
Results. A statistically significant difference ($p<0.05$) in the ratio of the labeled α-tubulin/flagellum area, was found between freshly ejaculated, capacitated, and acrosome-reacted sperm groups. Acrosome-reacted group had a significant decrease in α-tubulin/flagellum area compared to the capacitated group ($p<0.05$). However, here was no clear correlation between freshly ejaculated group with capacitated and acrosome-reacted groups, respectively.
Conclusions. As others, many sperm proteins, α-tubulin undergoes relevant changes in different physiological stages: fresh ejaculated, capacitated and acrosome-reacted spermatozoa. Data strongly suggest that there was a decrease tubulin expression during sperm acrosome reaction.
Keywords. Tubulin sperm reproduction immunofluorescence

(50.6.P3) AUTOPHAGIC PROCESSES ALONG THE ESTROUS CYCLE OF THE GOLDEN HAMSTER

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The Harderian glands of Syrian hamsters exhibit marked sexual dimorphism in porphyrin production and cell types. Damage derived from this production of porphyrins, forces the gland to develop survival strategies, which are mainly based on autophagic processes. In the female hamster, both gland histology and activity are known to be under hormonal influence, so the present study was undertaken to examine autophagic activity over the estrous cycle in the female hamster. Syrian hamsters were divided into two groups according to the estrus (n=4) and diestrus (n=4) phase of the sexual cycle. In each experimental group, main autophagic markers were assayed. Beclin 1, LC3-II and p62 expressions were evaluated and Mtor phosphorylation at Ser-2448, as a key regulator of autophagy, was localizated into nuclei of glandular cells by inmunohistochemistry.
Our results showed different degrees of autophagy in Syrian hamsters HG depending on phase of reproductive cycle. We observed more Beclin 1, LC3-II and p62 expression in estrous than diestrus phase. Likewise, positive staining to mTOR phosphorylated was observed in nuclei from estrous phase. These data could indicate a important role of autophagy in hormonal changes along estrous cycle and corroborates the hormonal influence in female histology of HG.
Keywords. autophagy, Harderian gland, Syrian hamster, estrous cycle

(50.6.P4) CELL PROLIFERATION IN THE DEVELOPING FOREBRAIN OF THE SEA LAMPREY

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Neurogenesis is a common feature of vertebrate central nervous system (CNS), but the rate and places of neuron generation vary between species and depend on the stage of development. Anamniote vertebrates exhibit the ability of producing new neurons throughout life and this process is initially responsible of the remarkable enlargement of the neural tube at early developmental stages. We analysed the spatiotemporal pattern of cell proliferation in the forebrain of prolarval and larval sea lampreys (*Petromyzon marinus*) by using different pulse-labelling (4h to 25 days) of 5-bromo-2´-deoxyuridine (BrdU). Short incubation times with BrdU revealed a number of labeled cells only in periventricular areas. Differences in proliferation were observed between ventricular zones, being higher in the subpallium, in the proximities of the lamina terminalis and close to the preoptic recess, in the primordium of the prethalamic eminence, and in the habenula. Prolonged BrdU incorporation times resulted in larger numbers of labeled cells in all the proliferating zones, but no additional areas containing proliferating cells were revealed. Moreover, in larvae exposed 25 days to BrdU virtually all the periventricular cells were labeled, with thicker proliferation strata in those regions where more staining was already present with shorter exposition times. In

addition, a considerable number of labeled cells were located far away from the ventricular surface. These migrating postmitotic cells were particularly abundant in the evaginated cerebral hemispheres. The continuous generation and addition of new cells may be partially responsible for the substantial increase in size of these areas of the larval lamprey. Furthermore, these results are in agreement with previous observations on lampreys but show remarkable differences as compared with those observed in other vertebrates, which could be related to the peculiar life cycle of these animals.

Supported by the Spanish MICINN-FEDER (BFU2009-13369)

Keywords. Development, cell proliferation, cyclostomes

(50.6.P5) P27KIP1 MUTANT MICE EXHIBIT INCREASE IN NUMBER OF MATURING FOLLICLES AND PROFOUND INCREASE IN MULTIOOCYTE FOLLICLES (MOFS)

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Introduction. P27 is an inhibitor of cell proliferation which belongs to ckis group (Cyclin-dependent Kinase Inhibitors). P27-/- female mice are sterile but the exact mechanism by which p27 affects ovary function is not yet established. The aim of this research was: a.To look for structural differences between ovaries from p27-/- and wt mice. B. To find out the molecular mechanisms through which p27 acts in ovary development and function.

Material & methods. Ovaries from wt and p27-/- juvenile (4 weeks) and young adult (12 weeks) mice were fixed in paraformaldehyde, paraffin embedded and serially sectioned. Immunohistochemical staining with VASA antibody was performed. Subsequently, the slides were scanned and digital images were analysed using Aperio scanner and analysis software. The following data were obtained: a. Number of VASA-positive germ cells, i.e. Total number of oocytes. B. Number of follicles at different stages of development: "small" (primordial and monolayered primary), "large" (multilayered primary, secondary and Graafian). C. Number of multioocyte follicles (mofs).

Results. Our results showed an increase in the number of oocytes per ovary (twofold greater) and in the number of "large follicles", i.e. Multilayered primary and secondary (5.4 times greater) in p27-/- mice compared to wt. The ratio "small follicles" vs. "large follicles" was also increased in p27 mutants (twice) and is an important parameter to distinguish them from wt mice. A most striking finding was the presence of an increased number of mofs (multioocyte follicles) in mutant mice (32.2 times greater).

Conclusions. Our results indicate a role for p27 in oogenesis and ovary development. The strikingly increased presence of mofs in mice lacking p27 suggests that this protein is involved in the formation and assembly of ovarian follicles. This work helps to a better understanding of the ovarian cellular dynamics and could contribute to the knowledge of ovarian-related diseases.

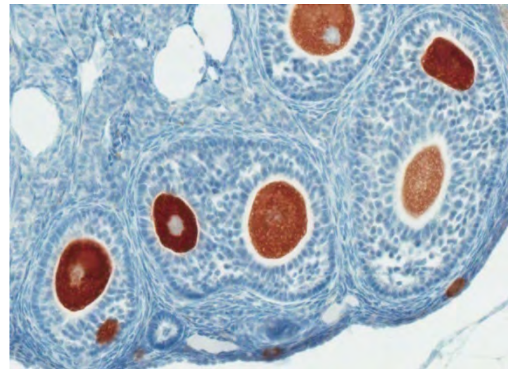

Fig. 1. Multioocyte follicles in the ovary of p27-/- mice

(50.6.P6) HSP-47 AND COLLAGEN IV IN LEYDIG CELLS OF SYRIAN HAMSTER (*Mesocricetus auratus*) DURING AGING AND PHOTOINHIBITED TESTIS

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Introduction. In recent years evidence has accumulated that the functionality of Leydig cells appears to be regulated by the testicular interstitial extracellular matrix, more specifically by collagen IV. HSP-47 is a protein that acts in the new collagen synthesis, to be a good marker of such cells. In this study, we propose to obtain the index of Leydig cells that express HSP-47 and collagen IV during aging and in photoinhibited testis to determine whether the expression of both proteins changes.

Materials and methods. For the analysis of these proteins, 32 animals were used, 9 of them (6 months old) kept in short photoperiod of 8 hours light and 16 hours darkness for 8 weeks and the remaining animals kept under long photoperiod with 16 hours light and 8 hours darkness. The animals had different ages: 8 of them 6 months, 8 of them 12 months and 7 of them 24 months old. The testis samples were fixed in methacarn. Immunohistochemistry was performed for detection of HSP-47 and collagen IV (α5).

Results. We counted HSP-47 positive and collagen IV positive Leydig cells with respect to the total number of them. Tests were performed by ANOVA, DMS, Bonferroni. The index of Leydig cells positive to HSP-47 was higher in animals with photoinhibited testis than in the corresponding control animals and aged animals (12 and 24 months old). The percentage of positive Leydig cells to collagen IV was significantly lower in animals with photoinbited testis compared to animals 6, 12 and 24 months.

Conclusion, the increased expression of HSP-47 in Leydig cells in photoinhibited testis could be related with the need for an increase in the expression of collagen proteins necessary to its re-differentiate during testicular recrudescence, while the decrease in cellular expression of collagen IV probably reflects the inactive state of Leydig cell in photoinhibited testis.

Supported by GERM 04543/07 from Fundacion Seneca. CARM.

Keywords. HSP-47, collagen and Leydig cells

(50.6.P7) CHANGES IN CONNECTIVE TISSUE VOLUME IN THE TESTIS OF SYRIAN HAMSTER (Mesocricetus auratus) DURING AGING AND IN PHOTOINHIBITED TESTIS

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The connective tissue, in addition to being the testicular stroma, influences the functionality of the testicular interstitium and peritubular lamina propia. Therefore, our aim was to determine whether aging and short photoperiod affect in the volume of interstitial and peritubular connective tissue. Thirty-two animals were used, 9 of them kept in short photoperiod of 8 hours light and 16 hours darkness for 8 weeks and the remaining animals were kept under long photoperiod with 16 hours light and 8 hours darkness. As regards the age of the animals, 8 of them, were 6 months, 8 were 12 months and 7 were 24 months old. After Masson Trichrome staining in sections of the study groups, morphometric analyse were performed. Images were captured with an Olympus DP25 camera mounted on an Olympus BX51 microscope using the specific morphometric program, cell D. The volume density (VD) of connective tissue in the interstitium and the peritubular lamina propia was calculated. The total volume of connective tissue in both locations was calculated (VD x Testis Volume). For this purpose, 3 random sections were taken from each animal and studied (15 fields / section with a 20x objective). Tests were performed by ANOVA, DMS and Bonferroni. The volume of interstitial connective tissue was not affected by aging, while a decrease in the same was observed in testes subjected to short photoperiod. On the other hand, the connective peritubular tissue was not affected in photoinhibited testis while in aging testis it increased in volume. In conclusion, the decrease of interstitial connective tissue in photoinhibited testis may be related with the decrease of interstitial function during this process, while the increase in peritubular connective tissue in aged testes could be associated with atrophy of the seminiferous epithelium that occurs during aging.

Funded by GERM 04543/07 from Fundacion Seneca. CARM.

Keywords. Connective tissue, aging and photoinhibited testis

(50.6.P8) HPA IS NOT A UNIVERSAL MARKER FOR AMPHIBIAN ACROSOME

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Introduction. Glycan characterization in amphibian testis has shown the existence of N-acetilgalactosamine (GalNAc)-containing carbohydrates. Labelling of the sperm acrosome with *Helix pomatia* agglutinin (HPA), a GalNAc-binding lectin, has allowed the identification of GalNAc-containing glycans in this organelle. Futhermore, this specific labelling of the acrosome has allowed the study of the acrosomal biogenesis by lectin histochemistry. However, the testis of *Xenopus laevis* has never been analyzed by HPA lectin histochemistry to locate GalNAc-containing glycoconjugates. The aim of this work was to elucidate the expression of GalNAc in glycoconjugates of *Xenopus* testis using *Helix pomatia* lectins.

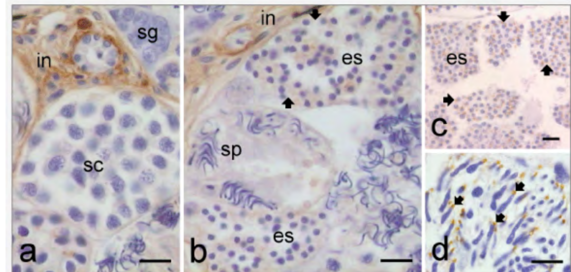
Materials and methods. Biotin-labelled lectin from *Helix pomatia* (HPA) was used to identify GalNAc in testis of *Xenopus laevis*. The testis were fixed in Bouin's fixative and embedded in paraffin wax by routine procedures. After incubation with Streptavidin-biotin-peroxidase (ABC) complex, which binds to biotin-labelled lectin, the peroxidase was developed by diaminobenzidine and H2O2. The results were compared with those previously obtained by our group in the amphibian *Pleurodeles waltl*.

Results. HPA labelled spermatids, but neither spermatogonia nor spermatocytes. No specific labelling of the acrosomal content was seen. Previous results from our group in another amphibian, *Pleurodeles waltl*, have shown an intense labelling of the acrosome in spermatids (figure 1).

Conclusions. HPA, which has been used as an acrosomal marker in some amphibians cannot be used in *Xenopus* testis, suggesting that acrosomal glycoconjugates in amphibians are species-specific.

Supported by the UPV/EHU (1/UPV00077.310-E-15927/2004 and GIU09/64) and Fundación Séneca (04542/GERM/06).

Keywords. HPA, Lectin histochemistry, acrosome, spermatids, glycoconjugates, oligosaccharides, spermatogenesis



HPA histochemstry in amphibian testis. a: In *Xenopus laevis*, neither spermatogonia (sg) nor spermatocytes (sc) are labelled. b: The early spermatids (es) of *Xenopus* are labelled, but not the acrosome (arrows). c and d: In *Pleurodeles waltl* testis, both early (es) and late spermatids (d) show the acrosome strongly labelled (arrows). Scale bars: 20 µm.

(50.6.P9) NOTCH PATHWAY IN SALIVARY GLAND DEVELOPMENT

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The process of organogenesis of the salivary gland begins with a signaling cascade where we can emphasize the epithelium-mesenchyme interactions. Lots of different signaling cascades that are related with mammalian organogenesis have been described, such as Notch signaling pathway. To better understand the role of Notch in salivary gland development, our study focused on the different parameters of the embrionary development E13 mouse submandibular salivary glands (SMGs). We used organ culture to evaluate the effect of DAPT (N-[N- (3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl

ester), an inhibitor of γ-secretasa that specifically disrupts Notch pathway, on SMGs morphogenesis.
We carried out in vitro culture of E13 SMGs for 48h in the presence or M DAPT. We noticed that treated SMGs had less spoonerμabsence of 20 ratio (acini number) when compared to control, as a consequence of an inhibition of branching morphogenesis, which could be produced because of the abrogated Notch signaling. Cell proliferation was similar in treated and control cultured SMGs. A moderate number of apoptotic cells were detected in tubules and acini of treated SMGs. Histology of SMGs cultured in the presence of DAPT showed that acini were not completely developed and branching clefting were not always formed.
In a complementary set of experiments, we used the transmission electron microscopy to ensure DAPT is affecting the ultra-structural morphogenesis of the SMGs. In control sample we could notice some epithelial cell layers in the acini, whereas in treated SMGs some apoptotic cells were detected in the inner layers of the acini. In addition, in treated SMGs we found a cellular continuity of the basal epithelial cells, which indicated the absence of formation of new acini. In conclusion, Notch pathway seems to be important during first stages of salivary gland development.
Supported by UPV/EHU and Fundación Gangoiti.
Keywords. Submandibular salivary glands, Notch pathway, DAPT

(50.6.P10) EFFECTS OF 6-BROMOINDIRUBIN-3'-OXIME (BIO) ON TOOTH DEVELOPMENT

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Regulation of intercellular signalling pathways is a key question in dental organogenesis. The aim of our work is to know the importance of Wnt (Wingless) signaling in each particular stage of dental development. We obtained the overactivation of the Wnt patthway by 6-bromoindirubin-3'-oxime (BIO), a specific inhibitor of glycogen synthase kinase 3 (GSK-3). The subject of this study is to evaluate the concentration of BIO for cell and tooth cultures, and to asses the effects of the drug when administered for a long time in vitro, on dental morphogenesis and development of the dental mesenchyme and the inner dental epithelium.
We cultured first molars of 14.5 days of mouse embryonic development (E14.5) for 6, 10 or 12 days, in the presence of BIO 10um, 20um, 40um and 100uM. The rate of apoptosis, proliferation and cell differentiation was analyzed by immunohistochemistry for caspase-3, H3P and Nestin, respectively. Following the results we first conclude that the concentration of BIO 20uM is suitable to study the overactivation of Wnt / beta-catenin pathway from tooth morphogenesis to cell differentiation stage, as we found morphological differences in tooth cultures without affecting cell survival. In addition, cultures of dental germs in the presence of the drug for a long time, and subsequent histological and immunohistochemical analysis showed that the effect of BIO on teeth treated in vitro for 6 days, is reversible and we could rescue normal tooth development when drug was removed from the culture. However, longer treatments irreversibly inhibited the formation of typical dental structures during tooth development.
All these results can attest that BIO treatment is effective on the Wnt signaling pathway during tooth development. In the future, it would be interesting to know the molecular targets that are affected by the overactivation of the Wnt / beta-catenin on odontogenesis.
Supported by UPV/EHU and Fundación Gangoiti.
Keywords. odontogenesis, GSK-3, BIO, Wnt pathway

(50.6.P11) HSP-47 AND COLLAGEN IV IN LEYDIG CELLS OF SYRIAN HAMSTER (Mesocricetus auratus) DURING AGING AND PHOTOINHIBITED TESTIS

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In recent years evidence has accumulated that the functionality of Leydig cells appears to be regulated by the testicular interstitial extracellular matrix, more specifically by collagen IV. HSP-47 is a protein that acts in the new collagen synthesis, to be a good marker of such cells. In this study, we propose to obtain the index of Leydig cells that express HSP-47 and collagen IV during aging and in photoinhibited testis to determine whether the expression of both proteins changes. For the analysis of these proteins, 32 animals were used, 9 of them (6 months old) kept in short photoperiod of 8 hours light and 16 hours darkness for 8 weeks and the remaining animals kept under long photoperiod with 16 hours light and 8 hours darkness. The animals had different ages: 8 of them 6 months, 8 of them 12 months and 7 of them 24 months old. The testis samples were fixed in methacarn. Immunohistochemistry was performed for detection of HSP-47 and collagen IV (α5). We counted HSP-47 positive and collagen IV positive Leydig cells with respect to the total number of them. Tests were performed by ANOVA, DMS, Bonferroni. The index of Leydig cells positive to HSP-47 was higher in animals with photoinhibited testis than in the corresponding control animals and aged animals (12 and 24 months old). The percentage of positive Leydig cells to collagen IV was significantly lower in animals with photoinbited testis compared to animals 6, 12 and 24 months. In conclusion, the increased expression of HSP-47 in Leydig cells in photoinhibited testis could be related with the need for an increase in the expression of collagen proteins necessary to its re-differentiate during testicular recrudescence, while the decrease in cellular expression of collagen IV probably reflects the inactive state of Leydig cell in photoinhibited testis.
Keywords. HSP-47, collagen IV, Leydig cells

(50.6.P12) HUNTER SCHREGER BANDS IN TEMPORARY DENTITION

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Introduction. The tooth enamel is a very hard but fragile tissue with two phylogenetic solutions to this problem, a continuous replacement of teeth and the specialization enamel microstructure. Prisms intercrosses as enamel with bands of Hunter Schreger (HSB) is an effective mechanism to increase resistance to fracture in mammals that employ high occlusal forces during the chewing. The purpose of this study was to determine the layout and microhardness of the bands in the temporary dentition.

Materials and methods. 5 anterior and 5 posterior teeth were included in epoxy resin, grinded with sandpaper of decreasing granulation, polished with powder aluminum oxide, and etched with 37% phosphoric acid during 3”, cleaned both with flowing water and ultrasonically, metallized and observed under Scanning Electron Microscopy (SEM). Microhardness was determined by micro durometer Shimadzu with penetrators Vickers and loads of 25 grams for 5".

Results. Bands anterior teeth were observed in incisal zone as alternating layers groups of prisms in longitudinal and transverse section and in the medium and cervical third free face. In molars, in cervical and medial thirds of free faces, in the form of arcs from the connection to the dentin occupying two-thirds internal enamel thickness. The outer third of the enamel represents radial enamel with parallel prisms. Light bands to the SEM related to prisms in cross section and dark bands to prims in longitudinal section. Microhardness (arithmetic mean) was: light bands Hv25 = 227,5 Vk and dark bands HV25 = 174, 5 Vk.

Conclusions. As recorded in permanent teeth the HSB combine with radial enamel in the external portion. Microhardness variation between light and dark bands confirms the research that shows differences in wear and acid engraving. Location and layout relationship with functional areas and different crystalline orientation determines its physical properties.

Keywords. Bands, temporary, SEM, microhardness

(50.6.P13) ONTOGENESIS OF THE RUMEN: A COMPARATIVE ANALYSIS OF THE MERINO SHEEP AND IBERIAN RED DEER

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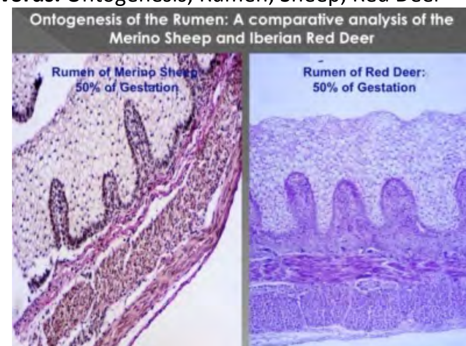
Introduction. The aim of this study is to describe differences in the ontogenesis of the rumen in the sheep (domestic ruminant) and deer (wild ruminant).

Material and methods. A total of 50 embryos and fetuses of Merino sheep and 50 of Iberian deer were used, from the first stages of prenatal life until birth. For the study, the animals were divided into five experimental groups according to the most relevant histological characteristics.

Results. The appearance of the rumen from the primitive gastric tube was earlier in the sheep (22% gestation, 33 days) than in the deer (25% gestation, 66 days). In both cases it displayed a primitive epithelium of a stratified, cylindrical, non-ciliary type. At around 28% gestation in the sheep (42 days) and 26% (67 days) in the deer, the rumen was configured of three clearly-differentiated layers: internal or mucosal, middle or muscular and external or serosal. In both species the stratification of the epithelial layer was accompanied by modifications in its structure with the appearance of the ruminal pillars and papillae. The pillars appeared before the papillae and the appearance of both structures was always earlier in the deer (pillars: 70 days, 27% gestation; papillae: 97 days, 36% gestation) than in the sheep (pillars: 42 days, 28% gestation; papillae: 57 days, 38% gestation). The outlines of the ruminal papillae appeared as evaginations of the basal zone toward the ruminal lumen, dragging in their formation the basal membrane, the lamina propria and the submucosa.

Conclusions. The tegumentary mucosa of the rumen was without secretion capability in the first embryonic phases. From 67 days (26% gestation) the neutral mucopolysaccharides appeared in the deer and at 46 days (30% gestation) in the sheep. In both cases they continued to decrease until birth, this diminution being more pronounced in the deer. Finally, the presence of neuroendocrine and glial cells was detected in the deer at earlier stages than in the sheep.

Keywords. Ontogenesis; Rumen; Sheep; Red Deer



(50.6.P14) ONTOGENESIS OF THE ABOMASUM IN THE IBERIAN RED DEER

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1. Histology. Fac. Vet. Med. University of Extremadura; 2. Anatomy. Fac. Vet. Med. University of Extremadura

Introduction.Histomorphometric and immunohistochemical analyses were carried out on 50 embryos and fetuses of red deer from the initial stages of prenatal life until birth.

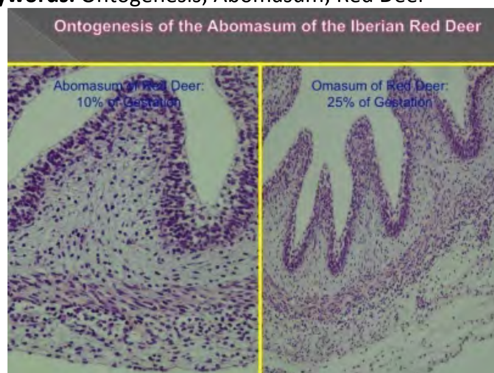
Material and methods. The animals were divided for test purposes into five experimental groups: GROUP I (1.4 to 3.6, CRL Length cm; 30-60 Days, 1-25% of gestation); GROUP II (4.5 to 7.2, CRL Length cm; 67-90 Days, 25-35% of gestation) GROUP III (8 to 19, CRL Length cm; 97-135 Days, 35-50% of gestation) GROUP IV (21 o 33, CRL Length cm; 142-191 Days, 50-70% of gestation) GROUP V (36 to 40, CRL Length cm; 205-235 Days, 75-100% of gestation).

Results. In the organogenesis of the primitive gastric tube of red deer, the differentiation of the abomasum, took place at 67 days, forming a three-layered structure: epithelial layer (pseudostratified), pluripotential blastemic tissue and serosa. The abomasal wall displayed the primitive folds of the abomasum and by 97 days villi were observed on the fold surface. At 135 days the abomasal surface is had by single mucous cylindrical epithelium and gastric pits were observed in the spaces between villi. At the bottom of these pits the first outlines of glands could be observed. The histodifferentiation of the lamina propia-submucosa, tunica muscularis and

serosa showed patterns of behaviour similar to those referenced for the forestomach of red deer. The abomasum of red deer during prenatal life, above all from 67 days of gestation, was shown to be an active structure with full secretory capacity. Its histological development; its secretory capacity, detected by the presence of neutral mucopolysaccharides, and its neuroendocrine nature, detected by the presence of positive non-neuronal enolase cells and neuropeptides vasoactive intestinal peptide and neuropeptide Y, were parallel to the development of the rumen, reticulum and omasum. Gastrin immunoreactive cells first appeared in the abomasum at 142 days, and the number of positive cells increased during development. As in the case of the number of gastrin cells, plasma gastrin concentrations increased throughout prenatal life.

Conclusions . However, its prenatal development was later than that of the abomasum in sheep, goat and cow.

Keywords. Ontogenesis; Abomasum; Red Deer



(50.6.P15) ONTOGENESIS OF THE OMASUM: A COMPARATIVE ANALYSIS OF THE MERINO SHEEP AND IBERIAN RED DEER

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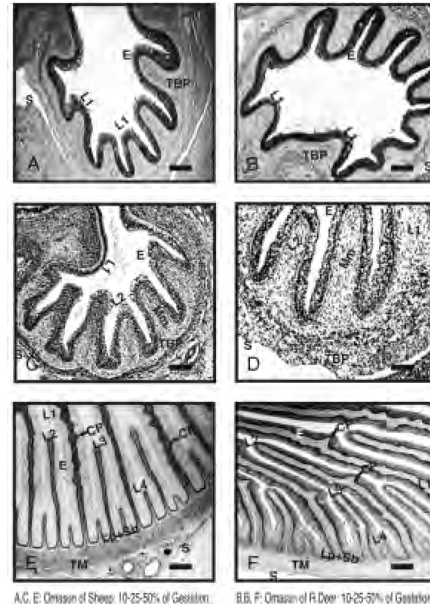
Introduction. The aim of this study is to describe differences in the ontogenesis of the omasum in sheep (domestic ruminant) and deer (wild ruminant).

Materials and methods. A total of 50 embryos and fetuses of Merino sheep and 50 Iberian deer were used, from the first stages of prenatal life until birth. For the study, the animals were divided into five experimental groups according to the most relevant histological characteristics. The appearance of the omasum from the primitive gastric tube was earlier in sheep (22% gestation, 33 days) than in deer (25% gestation, 66 days). In both cases it displayed a primitive epithelium of a stratified, cylindrical, non-ciliary type.

Results. The appearance of four laminae of different sizes was always earlier in sheep than deer. At around 36% gestation in sheep (53 days) and 36% (97 days) in deer, the omasum consisted of 4 clearly-differentiated layers: mucosa (with epithelial layer and lamina propria), submucosa, tunica muscularis and serosa. The temporal order of appearance of the four order laminae and omasal papillae was always earlier in sheep than deer.

Conclusions. The tegumentary mucosa of the omasum was without secretion capability in the first embryonic phases. From 67 days (26% gestation) the neutral mucopolysaccharides appeared in deer and at 46 days (30% gestation) in sheep. In both cases they continued to decrease until birth, this decrease being more pronounced in deer. Finally, the presence of neuroendocrine and glial cells was detected in deer at earlier stages than in sheep.

Keywords. Ontogenesis; Omasum; Sheep; Red Deer



A,C, E: Omasum of Sheep: 10-25-50% of Gestation. B,B, F: Omasum of R.Deer: 10-25-50% of Gestation

(50.6.P16) ONTOGENESIS OF THE RETICULUM: A COMPARATIVE ANALYSIS OF THE MERINO SHEEP AND IBERIAN RED DEER

Franco A (1), Masot AJ (2), Gázquez A (2), García A (2), Redondo E (2)

1. Anatomy. Fac. Vet. Med. University of Extremadura; 2. Histology. Fac. Vet. Med. University of Extremadura

Introduction. The aim of this study is to describe differences in the ontogenesis of the reticulum in the sheep (domestic ruminant) and deer (wild ruminant).

Materials and methods. A total of 50 embryos and fetuses of Merino sheep and 50 of Iberian deer were used, from the first stages of prenatal life until birth. For the study, the animals were divided into five experimental groups according to the most relevant histological characteristics: phase I, gastric outline and compartmental individualization; phase II, primary parietal stratification; phase III, primitive epithelial stratification and appearance of primary ribs; phase IV, definitive epithelial stratification and appearance of reticularis cells; phase V, parietal stabilisation and maturity of the mucosa.

Results. The appearance of the reticulum from the primitive gastric tube was earlier in the sheep (22% gestation, 33 days) than in the deer (25% gestation, 66 days). In both cases it displayed a primitive epithelium of a stratified, cylindrical, non-ciliary type. At around 48% gestation in the sheep (72 days) and 36% (97 days) in the deer, the reticulum was configured of 4 clearly-differentiated layers: mucosa (with epithelial layer and lamina propria), submucosa, tunica muscularis and serosa. In both species the stratification of the epithelial layer was accompanied by modifications in its structure with the appearance of the primitive reticular ribs. The primary ribs began to be formed first in the deer, at 117

days of prenatal life (40 % gestation) and later in the sheep (79 days, 53 % gestation). The differentiation of the corneum papillae in the primary ribs coincided with the appearance of secondary reticular rib. These structures began to be formed first in the deer, at 142 days of prenatal life (51 % gestation) and later in the sheep (83 days, 55 % gestation).

Conclusions The tegumentary mucosa of the reticulum was without secretion capability in the first embryonic phases. From 67 days (26% gestation) the neutral mucopolysaccharides appeared in the deer and at 46 days (30% gestation) in the sheep. In both cases they continued to decrease until birth, this diminution being more pronounced in the deer. Finally, the presence of neuroendocrine and glial cells was detected in the deer at earlier stages than in the sheep.

Keywords. Ontogenesis; Reticulum; Sheep; Red Deer

INDUSTRY DAY: CELLS AND TISSUES AS THERAPEUTIC TOOLS

INDUSTRY DAY: TRANSLATING RESEARCH AND INNOVATION INTO THE CLINICS

Yves Bayon (1)*, Miguel Alaminos (2)*, Antonio Campos (2)*
1. Covidien - Sofradim Production, Trevoux, France; 2. Tissue Engineering Group, Dept of Histology, University of Granada, Granada, Spain
** Industry Day co-organizers*

After the successful experience during the last TERMIS-EU2010 meeting held in Galway, Ireland, an Industry Day session will again be held at the TERMIS-EU2011 meeting in Granada, Spain. The main goal of the Industry Day is to reach a common language and perspectives among different partners in the objective of translating research and innovation into the clinics for the advancement of the practice of medicine and for better patient outcomes, for the most challenging clinical indications.
Key-note speakers from a variety of areas, including academia, regulatory agencies, health systems, etc. will participate in the forum. Several companies will also share their experience and points of view on topical issues. In addition, high level panel sessions will focus on topics which will be very useful for both the academic and entrepreneurial communities, shedding light on critical issues for the successful development and commercialization of regenerative medicine products:
1) "Translating academic research into commercial products" with experts sharing their experience on the management of an academic concept to a clinical stage company, the potential of academic / industry research collaborations, interactions with patient advocacy groups / associations, the management of intellectual property issues, the manufacturing of cells therapy products;
2) "Regulation of advanced therapies: Overcoming regulatory hurdles" with US and European representatives;
3) "Regulatory issues and Clinical trial Management of cell based products" with companies sharing their experience on cell products, tissue-engineered products and on cell delivered gene therapy.
This forum will give without any doubt high level information and answers to critical questions around the development of commercial regenerative medicine products. It may further nurture entrepreneurial initiatives from academics, wishing to translate smart and promising regenerative medicine projects into clinical products, raising new hopes for disease patients waiting for effective treatments.

REGENERATIVE MEDICINE: ACADEMIC PERSPECTIVE ON TRANSLATIONAL RESEARCH

James J. Yoo
Wake Forest Institute for Regenerative Medicine, Winston-Salem, North Carolina, U.S.A.

Recent advances in tissue engineering and regenerative medicine have provided new therapeutic opportunities for repairing damaged tissues and organs. While therapeutic paradigms that utilize the principles of regenerative medicine have shown to be effective, only a limited number of technologies have been successfully translated to patients. This is largely due to various scientific challenges encountered in the tissue building process, and delayed translation by the limited understanding of regulatory processes involved in the development of clinical therapies. To effectively develop and translate regenerative medicine therapies in a timely manner, a new strategy has to be designed and implemented. This session will focus on the approaches that would lead to rapid translation in the academic setting.

CELL THERAPY INDUSTRY - TRANSLATING RESEARCH INTO COMMERCIALLY SUCCESSFUL THERAPEUTICS - FROM BENCH TO BEDSIDE

Chris Mason
Advanced Centre for Biochemical Engineering, University College London, UK

There is a great deal of uncertainty as to what exactly is *'regenerative medicine'*. To many it is any technology (pharmaceutical, biologic, medical device or cell therapy) that replaces or regenerates tissue or organs, to restore or establish normal function. To others, itimplies only cell-based therapies including tissue engineering (cells + scaffold). However, what is universally agreed is that deploying living cell as medicines is undoubtedly a disruptive technology - a step-change in patient care by providing cures or advanced treatments for a wide spectrum of previously unmet medical needs. The range of clinical indications for cell therapies is thus far broader thanjust regeneration and includes: permanent cell replacement, tissue engineering, transient cell therapies that disrupt natural diseaseprogression, immunomodulatory cell therapies, cell therapies that protect tissues at risk, and cell cancer vaccines*.In 2009 the potential US market for all cell therapies was estimated to have been well in excess of 100 million patients.
The cell therapy industry (CTI), as opposed to regenerative medicine, had global sales of $410M in 2008, and is predicted to grow to $2.7B by 2012 and $5.1B in 2014. With even greater growth expected to follow. Currently there are 8 FDA/EMA approved cell therapies including Provenge (Dendreon), a cell-based prostate cancer vaccine with reimbursement in the USA set at $93,000, and expected through Medicare sales alone to generate revenues of $1.7B p.a. by 2014*. However, it is also very important to acknowledge that the emerging CTI, with its living cell-based products and services, has its own unique set of requirements and challenges that are totally different to those of pharma, biotech and the medical device industry. However, once resolved, the CTI will become a new multibillion-pound industry establishing cell therapy as the fourth and final therapeutic pillar of healthcare.
*Cell therapy industry: billion dollar global business with unlimited potential. Mason C. et al. Regen. Med. (2011) 6(3), 265–272.

TRANSLATING RESEARCH INTO VIABLE CLINICAL TREATMENTS. HOW TO BUILD ON 60 YEARS OF PATIENT FOCUSED CLINICAL DELIVERY

Simon Ellison
National Health Service (NHS) Blood and Transplant, UK

Clinical Translation Partnerships enables organisations to utilise the manufacturing and supply chain skills within NHS Blood and Transplant to speed the translation of their treatments into viable therapies. Delivery of cellular therapies presents many challenges to the NHS as with any other healthcare provider. However there are opportunities, in the UK, to learn from the past and build on current delivery mechanisms to speed the translation of research into the delivery of life changing therapies.

If the discussion moves from identifying the challenges and disruptive technologies to a gaps analysis based on current bone marrow, organ and blood based cellular therapies. It could be said that the NHS already manages the delivery and reimbursement of over 2 million cellular therapies every year, and therefore has the infrastructure to manage increased provision. Making the question less about inventing the wheel but more about making it turn faster.

This presentation will identify the potential to build on current manufacturing, cold supply chain and clinical delivery skills that already exist within the NHS to produce a viable delivery model that all cellular therapy organisations can access.

Key Points:

- The criticality of managing the process from consent to patient.
- Utilising open innovation partnerships can deliver treatments and revenue.
- How to generate patient focused manufacturing and scale up.
- Accessing validated national cold supply chains.

PALIFERMIN (KEPIVANCE/KGF) IS A GROWTH FACTOR WITH MANY POTENTIAL USES, BUT STILL ONLY APPROVED FOR TREATMENT OF ORAL MUCOSITIS IN A SMALL SUBSET OF PATIENTS. WHY?

Maarten de Château
Medical Program Director, Swedish Orphan Biovitrum (SOBI), Stockholm, Sweden

Palifermin is a truncated form of human Keratinocyte Growth Factor that was approved by the FDA and the EMA in 2005 for the treatment of oral mucositis (OM) in patients undergoing hematopoietic stem cell transplantation (HSCT). It is the first and only approved drug for OM. The approval was based primarily on very convincing phase III data in patients with hematological malignancies undergoing chemoradiotherapy prior to HSCT. The approval was on condition that several post marketing trials were to be performed to study effects and safety in chemotherapy settings, children, long-term and in combination with heparin. Several studies to prevent OM in patients with solid tumors have also been conducted. Two phase III studies in patients with head and neck cancer came up with positive results. A recent study in sarcoma patients undergoing high-dose chemotherapy showed good results on OM. These solid tumor studies still have to translate into regulatory filings and approvals. However, a study mandated as a follow-up measure in EU failed to show efficacy in multiple myeloma patients undergoing HSCT, most likely due to an issue with the timing of palifermin in this setting. Nevertheless, this study resulted in a restriction in the indication in the EU. OM is a common and debilitating side effect of many cancer treatments. The underlying mechanisms are not fully understood, although the understanding is that OM pathophysiology is the same irrespective of cause. The incidence and severity of OM varies between treatment settings. The fact that patients with OM are spread over many different diseases and treatment settings poses a challenge as the dose and timing of administration has to be worked out for each and every treatment setting. To conclude, it is a challenge to gain widespread use in OM of this potent and promising drug. Still other indications, such as lung disease and immune reconstitution also await exploration.

CASE STUDY: ReN001 STEM CELL THERAPY FOR THE TREATMENT OF PATIENTS LEFT DISABLED BY THE EFFECTS OF A STROKE

John Sinden
Chief Scientific Officer, ReNeuron Ltd, Guildford, Surrey, U.K.

The recent start of the ReN001 ***PISCES*** (Pilot Investigation of Stem Cells in Stroke) clinical trial at Glasgow Southern General Hospital in Scotland (ClinicalTrials.gov Identifier: NCT01151124) is the first fully regulated allogeneic stem cell trial in the U.K. and the first regulated neural stem cell trial for stable stroke disability worldwide.

Twelve patients are being enrolled with moderate to severe hemiparesis following an ischaemic stroke 6 - 24 months prior to recruitment. Following a 2 month baseline testing phase to ensure patient stability, each patient will be treated with ReNeuron's conditionally immortalised *CTX0E03* drug product at 4 escalating doses (2, 5, 10 & 20 million cells). Each dose cohort of 3 patients will be evaluated for safety by an independent DSMB at 3 months post-cell implant to recommend dose escalation if appropriate.

Primary endpoints are safety related, but along with measures of motor function, sensory neglect, cognition and quality of life, extensive functional MRI data will be used to evaluate potential biomarkers of stem cell-produced efficacy in stroke patients which could be followed up in later trials.

REGENERATIVE MEDICINES FOR SPINAL CORD INJURED PATIENTS – THE EXPERIENCE OF A PRINCIPAL CLINICAL INVESTIGATOR

Michael G. Fehlings
University of Toronto

Spinal cord injury (SCI) is a devastating clinical event with deleterious consequences for the patient and family and significant costs for society. SCI occurs as a result of a primary mechanical injury which is usually due to compression/contusion and a series of secondary injury events, including ischemia, inflammation, apoptosis, free-radical mediated cell death and excitoxicity. The chronic injury is characterized by a central cyst (myelomalacia), surrounded by a glial scar and often some spared, thinly myelinated axons. The complexity of SCI pathophysiology

will require therapeutic strategies which address the secondary injury cascade, bridge gaps in the oord, replace lost cells, enhance endogenous plasticity and attenuate the inhibitory effects of the glial scar. In this talk, I will summarize the clinically relevant aspects of SCI pathophysiology , discuss preclinical experience with novel bioengineered strategies including the use of self-assembling nanofibres which attenuate glial scar formation and provide perspectives on experiences with recent clinical trials using bioengineered compounds including Cethrin, a recombinant protein Rho inhibitor.

REGULATION OF ADVANCED THERAPIES IN THE EUROPEAN UNION

Sol Ruiz

Spanish Medicines Agency

In 2007 the European Commission published a new regulation for medicinal products based on gene therapy, cell therapy and tissue engineering that were defined as advanced therapy medicinal products (ATMP). The main objective of this new legislation (Regulation (EC) no 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products) that is applicable from 30 December 2008 is to improve patients' safe access to advanced therapies by increasing the research, development and authorisation of these products and more specifically: to guarantee a high level of health protection for European patients treated with ATMP; to harmonise market access and ensure the free movement of these products by establishing a tailored and comprehensive regulatory framework for their authorisation, supervision and post-authorisation vigilance; to foster the competitiveness of European undertakings operating in this field, in particular small and medium-sized enterprises; and to provide overall legal certainty, while allowing for sufficient flexibility at the technical level, in order to keep pace with the evolution of science and technology. The key measures included in the regulation are:

· A centralised marketing authorisation procedure for all ATMP coordinated by the European Medicines Agency (EMA);

· A new and multidisciplinary expert committee (Committee for Advanced Therapies, CAT), within the EMA, gathering European experts in many different areas to assess the quality, safety and efficacy of ATMP and follow scientific developments in the field;

· Tailored technical requirements adapted to the particular characteristics of these products;

· Strengthened requirements for risk management and traceability;

· A system of low-cost, top-quality scientific advice provided by the EMEA;

· Special financial and administrative incentives for small and medium-sized enterprises.

The CAT had its first meeting in January 2009. An update on the ongoing activities of the CAT and the implementation of the new regulation will be presented.

REALIZING THE POTENTIAL OF ACADEMIC/INDUSTRY RESEARCH COLLABORATIONS --- THE MultiStem® STORY

Robert W. Mays

Senior Director of Regenerative Medicine and Head of CNS Research: Athersys, Inc.; Cleveland, Ohio USA

Athersys is a clinical stage biopharmaceutical company engaged in the discovery and development of therapeutic product candidates designed to extend and enhance the quality of human life. Athersys has forged several key strategic alliances and collaborations with leading pharmaceutical and biotechnology companies, as well as world-renowned research institutions in the United States and Europe to further develop its platform technologies and products. The lead technology at Athersys is MultiStem®, a patented and proprietary product consisting of a clinical grade preparation of adult (non-embryonic) stem cells obtained from bone marrow that have the potential ability to produce a range of factors and form multiple cell types. MultiStem appears to work through several mechanisms, but a primary mechanism appears to be the production of therapeutic proteins and other molecules produced in response to inflammation and tissue damage. Athersys believes that MultiStem represents a unique “off-the-shelf” stem cell product based on its apparent ability to be used without tissue matching or immunosuppression and its capacity for large-scale production. MultiStem has received regulatory allowance from the FDA for testing in man and is currently in Phase I or Phase I/II clinical trials in the US for treatment of myocardial infarction, bone marrow transplantation and oncology treatment support, ischemic stroke, and inflammatory bowel disease. The translation of the initial academic discovery and the subsequent pre-clinical development and subsequent clinical testing of MultiStem has been the result of successful academic-industry collaborations at each and every stage of development.

JVS-100, A NOVEL GENE-THERAPY STRATEGY FOR REGENERATIVE MEDICINE

Rahul Aras

Juventas Therapeutics, Inc., Cleveland, OH, USA

Juventas Therapeutics is a clinical-stage regenerative medicine company developing novel therapies for ischemic disease. The company’s lead product, JVS-100, is a non-viral DNA plasmid encoding Stromal Cell-derived Factor-1 (SDF-1). Several groups have shown that SDF-1 significantly increases end-organ function following tissue injury through promoting cell survival, recruiting endogenous stem cells to the damaged region and increasing new blood vessel formation. The SDF-1 repair pathway is well-conserved throughout end-organ systems, including the heart, brain, kidney, dermis, vasculature and nervous system providing the opportunity for one product to impact a broad range of clinical indications.

The first generation of proposed regenerative therapies focused on harvesting a person’s cells from one part of the body and re-delivering them to the damaged organ. While the strategy is clinically compelling, it has proven commercially challenging. Cell-based cardiovascular therapies are projected to cost upwards of $20,000 per treatment and will only be available through hospitals with the most advanced, state-of-the art clinical facilities, making them largely unattainable to the general population.

Focused on the second generation of regenerative therapies, Juventas will develop biopharmaceuticals that activate the body's natural repair process. This strategy distills cell therapy down to the specific factors that have a therapeutic effect. A biopharmaceutical approach to regenerative medicine provides an more cost-effective and broadly accessible alternative to current stem cell-based therapies currently in the clinic.
Juventas recently completed a Phase I clinical trial evaluating the safety and preliminary efficacy for JVS-100 in treatment of patients with critical limb heart failure. Also, the FDA recentlyauthorized initiation of a Phase II clinical trial evaluating the efficacy and safety of JVS-100 for treatment of patients with critical limb ischemia. This presentation will focus steps taken to develop JVS-100 to its current phase.

INNOVATIVE ANIMAL COMPONENT-FREE SURFACE FOR THE CULTIVATION OF HUMAN EMBRYONIC STEM CELLS

Stelzer T (2), Neeley C (1), Marwood K (3)
1. Thermo Fisher Scientific, Langenselbold, Germany; 2. Thermo Fisher Scientific, Rochester, NY, USA; 3. Thermo Fisher Scientific, Roskilde, Denmark

The concerns over contaminants from animal components and batch-to-batch variability of the coating matrices for human stem cells culture have significantly hindered the usage of this type of surface for translational and clinical applications. The Thermo Scientific Nunclon Vita is an energy-treated polystyrene surface free of animal components. It enables culture of human stem cells without matrix or feeder layers. Human embryonic stem (ES) cells are grown directly on the surface in conditioned media containing ROCK-inhibitor, and can be sustained for more than ten passages without signs of differentiation. During this presentation, we will present data that demonstrate karyotypic normality, pluripotent status, and induced differentiation to embryoid body formation of H1 and H9 human ES cells cultured on Nunclon Vita surface.

COMMERCIAL DEVELOPMENT OF REGENERATIVE MEDICINE THERAPEUTICS - IP ISSUES

Roke Iñaki Oruezabal, Arturo Argüello, Eduardo Suárez, Gustavo Fúster
1. Iniciativa Andaluza en Terapias Avanzadas, Sevilla, Spain; 2. Oficina de Transferencia de Tecnología, Andalusian Public Health System, Junta de Andalusia, Spain

In most cases the intellectual property of classic pharmaceutical companies, mainly those specialized on small-molecules, is based on a few patents that protect the final product that is placed on the market. These patents constitute crucial assets for the overall strategy of the company. On the other hand, the strategy of protection in the majority of inventions in the field of regenerative medicine might follow other pathways. Particularly, if we take notice that most final products in this field of technology are of a superior complexity in comparison with conventional pharmaceutical drugs, due to the great deal of know-how associated with the manufacturing process of the biological product. In this sense, we can exemplify the following biological products in the field of regenerative medicine which complexity will became apparent to the reader: cell lines and culture media, animal cells or tissues, the culture conditions to differentiate cells, different types of scaffold material, polypeptides, growth factors, enzymes or antibodies. Based on these premises and the fact that some countries or even regions share some legal exceptions in terms of obtaining intellectual property rights for biological products, we shall make an in-depth comparison, between the way of protecting products, processes or uses in the field of regenerative medicine with the way conventional pharmaceutical products are currently dealt with. The latter reflection shall be made with a clear focus on the marketability of these products and how these intangible assets can help us ensure the broadest possible sales on a country-by-country basis.

MANUFACTURING OF CELL THERAPY PRODUCTS – FROM BENCH TO BEDSIDE

José Castillo (1,2)
1. ATMI LifeSciences, Brussels, Belgium; 2. Cardio3 BioSciences, Mont-Saint-Guibert, Belgium

Expansion of adherent stem cell use traditional polystyrene T-flasks or multitray stacks. Such culture methods are not suitable for large-scale production since they involve large numbers of T-flasks or stacked trays, large incubation rooms and multiple manual sterile operations. Safety of the patient is a concern when thousands of aseptic operations are implemented into a production facility, as well as consistency. Harvesting cells is a major concern, as each multitray stack is to be harvested individually, before pooling. Capital and operating costs for large-scale production are prohibitive. This is especially the case for autologous treatments that need lots of cells.
In order to implement a process able to be "scaled-out", Artelis has developed together with Cardio3 a set of solutions made of:

- A new 2D bioreactor, Xpansion, which is composed of multiple, polystyrene plates stacked in a closed bioreactor which enables controlled media flow for dissolved oxygen and pH control. Due to its large surface area of up to 125 000 cm² and multi-plate design, Xpansion enables production of large amounts of cells in a process easily adapted from traditional T-flask or stacked tray methods.
- A new harvesting system to allow cells recovery, concentration and washing in one operation.
- A completely closed system, incorporating the two previous elements, to avoid any single contact between the cells and outer environment.

The presentation will be about the impact of the overall solution on manufacturing, as well as some insight into the Xpansion bioreactor, especially the small scale Xpansion One, the bench top testing of the system.

FINANCIAL AND CLINICAL DEVELOPMENT STRATEGIC ISSUES IN DEVELOPING A MATRIX-EMBEDDED CELL THERAPY PRODUCT FOR VASCULAR INJURY REPAIR; CASE STUDY: PERVASIS

Frédéric Chéreau
Pervsasis

Pervasis Therapeutics is a venture capital backed company based in Cambridge – Ma, founded out of MIT in 2004. Pervasis is developing a tissue engineered

endothelial cell (TEEC) therapy technology, Vascugel®, with broad platform potential. In September 2009, the company raised a Series C financing of $17M. At the time, Pervasis had successfully completed Ph. 2 t trials in arteriovenous (AV) access procedures for hemodialysis patients and was in discussions with FDA about a pivotal Ph.3 trial design. Additionally, the company had recently received positive pre-clinical data in a new, larger indication, Peripheral Artery Disease (PAD), for which a slightly different formulation of the technology (PVS-10200) was used (enabling percutaneous, minimally invasive delivery). Furthermore, there was emerging preliminary pre-clinical evidence that the TEEC technology had potential efficacy in non-vascular indications such as oncology, orthopedics and inflammation. Upon closing the financing, Pervasis faced a strategic decision about how to allocate its new cash resources to its late-stage (Vascugel for Vascular Access), mid-stage (PVS-10200 for PAD) and early-stage (non-vascular) programs.

THE ANDALUSIAN INITIATIVE FOR ADVANCED THERAPIES: PARTNERING RESEARCHERS AND COMPANIES IN THE TRANSLATION OF RESEARCH INTO COMMERCIAL BREAKTHROUGH THERAPEUTICS

Natividad Cuende
Executive Director. Andalusian Initiative for Advanced Therapies

As a publicly funded organization, in 2008 the Andalusian Government -having in 2003 pioneered embryonic stem cell legislation in Spain- created what is now known as the Andalusian Initiative for Advanced Therapies. The Initiative draws up plans and tailors resources to promote research in the field of advanced therapies in Andalusia and to transfer basic research into clinical practice through the forging of alliances among academia, research institutions, hospitals, patient associations, and the biotech and pharmaceutical industries.

Our work comprises all the steps between the generation of knowledge and knowledge transfer with a special emphasis on the development of new therapies. For that reason, over the last few years we have pushed forward the building of a cluster of research centers, biobanks, and GMP facilities. We have implemented a human resources policy that includes direct recruitment of researchers through competitive calls, training and return programs, and mobility programs. We have also designed our own training program for manufacturing and clinical research on advanced therapies that includes practical training modules carried out under real conditions at a GMP facility built for training purposes.

Moreover, the Andalusian Initiative offers a distinct, major translational focus. That is, in addition to building research centers and funding basic research grants for advanced therapies, as other regional administrations have done elsewhere in the world, our organization provides a comprehensive support hub to develop clinical research, and in particular to promote the development of new advanced therapies not only out of commercial interests but also purely as a service. Support comes not only in the form of financing, but also with implementation of complementary expertise. To that end, we act as sponsors, providing the support that our researchers and clinicians need and that would usually be provided by the pharmaceutical industry. Our organization has built 10 GMP facilities in multiple research centers and hospitals. Most of the clinicians work for our health service, but an increasing number of clinicians based at other health services collaborate with us in multicenter clinical trials. In summary, we currently support the construction and accreditation of a network of clean rooms, the development of investigational medicinal products, and the design and implementation of clinical trials. In the case of the latter, our support begins with a regulatory assessment during preclinical development, followed by promotion of collaborations between basic and clinical researchers, and extends all the way to monitoring the safety of the resulting investigational medicinal products.

The success of this organizational model, designed with the intention to facilitate clinical research and innovation within the public health system, is evidenced by the authorization of 13 Phase I/II noncommercial clinical trials in the last 3 years by Spanish regulatory authorities in the areas of cardiology, neurology, immunology, peripheral vascular disease and hepatic regeneration (more information available at http://www.juntadeandalucia.es/terapiasavanzadas/).

Nevertheless, there are some areas in which further progress depends on collaboration with biotechnological and pharmaceutical companies. For that reason, we have started to sign several collaboration agreements with different companies and we consider them as strategic partners. Some of them have been included on our steering committee and advisory board.

Andalusia has been designated until 2013 as one of the few convergence regions in Western Europe making it a particularly attractive area to invest in, as the maximum financial incentives coming from the European Union are available here. In addition, further financial support is offered by our Regional Government in order to attract new enterprises or to make them more competitive.

THE CHONDROCELECT EXPERIENCE: DURABLE AND EFFECTIVE TREATMENTS FOR CARTILAGE DAMAGE IN THE KNEE

Gil Beyen, Wilfried Dalemans
TiGenix

ChondroCelect is the first cell therapy product approved as Advanced Therapy Medicinal Product (ATMP) under the central European regulatory framework. ChondroCelect is a cell therapy product indicated for the repair of cartilage lesions in the knee and consists of a cell suspension of autologous chondrocytes expanded by in vitro cell culture. This cell product is produced in a strictly controlled GMP manufacturing process. Its safety and efficacy has first been demonstrated in a series of preclinical models. In a subsequent randomized controlled clinical trial, ChondroCelect was shown to provide structural superiority over the control treatment microfracture, and demonstrated clinical benefit over time. Based on these development results, the product was granted a central market authorization by the European Commission in 2009.

ALLOGENEIC ADIPOSE DERIVED STEM CELLS FOR THE TREATMENT OF AUTOIMMUNE MEDIATED INFLAMMATORY DISEASES

Eduardo Bravo
Cellerix, Madrid, Spain

Stem cell therapy, widely utilised within the regenerative medicines field is currently establishing itself as a new modality in the treatment of autoimmune and inflammatory disorders. Cellerix is leading the development of a new generation of cellular therapies by using expanded adult stem cells from adipose tissue (eASCs). Adipose stem cells have an established anti-inflammatory and immunomodulatory mechanism of action that make them an attractive candidate for the treatment of a broad range of indications.

Cellerix primary focus is the development of therapies for the treatment of autoimmune mediated inflammatory diseases and the initial aim was to establish proof of principle in orphan indications with local autologous eASC therapy, before targeting further indications with allogeneic eASCs. The progression from personalized therapies to allogeneic eASCs will enable Cellerix to develop "off-the-shelf" cellular therapies to fulfill the needs of high incidence inflammatory and immune indications.

Accordingly, having established the safety and promising efficacy of autologous eASCs in Phase I and II trials in complex perianal fistula, allogeneic clinical development is progressing rapidly. A Phase II trial in complex perianal fistula concluded in late 2010, with a Phase III trial planned to commence in 2011. Development of therapies to address the broader autoimmune market will start with a Phase I/II trial in rheumatoid arthritis (planned to start patient recruitment in the first quarter of 2011). The Cellerix pipeline is further bolstered with pre-clinical studies that are on-going in both autoimmune inflammatory indications (IBD; colitis; rheumatoid arthritis second generation product) as well as degenerative disorders (osteoarthritis). Having one of the most advanced pipelines in the stem cells field, Cellerix is well positioned to exploit the therapeutic potential of stem cells.

TISSUE ENGINEERING BY SELF-ASSEMBLY: A NEW PARADIGM IN CARDIOVASCULAR REPAIR

Nicolas L'Heureux
CSO at Cytograft Tissue Engineering, Novato, California, USA

A series of spectacular financial failures have led to a "nuclear winter" for the commercialization of tissue engineering. Enthusiasm for stem cells has revived the interest of the investment community for cell-based therapies but, are we going to witness history repeat itself?

Cytograft has followed a development strategy stemming form the often forgotten reality that development timelines of tissue-based therapies have historically stretched over 20 years. From this sobering fact, two guiding principles have steered our development. First, from an R&D perspective, we have focused on factors that will promote product effectiveness rather than on *initial* economical feasibility. As the company, the technology and the product approach maturity, the focus can be progressively shifted to cost-effectiveness issues. Second, from a financial point of view, we have emphasized the need to maintain a low burn rate. Elements of this spartan approach include planning for funding rounds, production facilities and QC/QA levels that are commensurate to the stage of product development.

Cytograft was founded in 2000 to develop a completely biological and autologous human tissue-engineered blood vessel that. This was done despite the prevailing dogmatic view that synthetic scaffolds were needed to built strong grafts and that allogeneic approaches would be the only economically viable approaches. We reached First-in-Man on less than $6M of private equity and completed a Phase I/II trial on about $25M. Outstanding clinical efficacy already supports predictions of cost-effectiveness. Early clinical results using devitalized and allogeneic grafts suggest the possibility of using such simplified product.

Homerun scenarios so often touted in the world of Pharma and Device are not relevant to tissue-engineered and a paradigm shift is needed in development strategies to build a stable and productive field of Tissue Engineering.

JUNTA DE ANDALUCIA

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WHO ARE WE?

We are an Initiative promoted by the Regional Government of Andalusia (Junta de Andalucía) through its Ministry of Health and Ministry of Economy, Innovation and Science. Our main objectives are **THE DEVELOPMENT AND TRANSLATION** into new therapies of the results obtained in the three Andalusian Research Programmes related to Advanced Therapies:

> The **Cell Therapy** and **Regenerative Medicine Programme**
> The **Clinical Genetics** and **Genomic Medicine Programme**
> The **Nanomedicine Programme**

CLINICAL TRIALS

Clinical Trials promoted by Andalusian Inititative for Advanced Therapies

CLINICAL TRIALS IN ADVANCED THERAPIES 2011. PHASE I/II	PENDING AUTHORIZATION	START UP PHASE	RECRUITMENT PHASE	FOLLOW-UP PHASE	TRIAL FINISHED
>> CARDIOLOGY:					
Dilated Myocardiopathy				1	
Myocardial Infarction				1	
Chronic Ischemic Cardiopathy			1		
>> NEUROLOGY:					
Multiple Sclerosis	1			1	
Stroke			1		
>> IMMUNOLOGY:					
Graft vs Host Disease			1		
>> PERIPHERAL VASCULAR DISEASES:					
CCI in lower limbs in diabetic patients	1		1	1	1
CCI in lower limbs in NON-diabetic patients			2		
>> DIGESTIVE:					
Hepatic Regeneration		1			
>> OPHTHALMOLOGY:					
Corneal Ulcus	1				
>> GASTROENTEROLOGY:					
Faecal Incontinence	1				

TRAINING

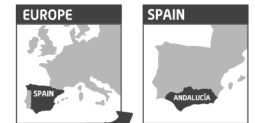
Master Programme in Manufacturing Advanced Therapy Medicinal Products organized together with the University of Granada and the collaboration of Agencia Española de Medicamentos y Productos Sanitarios and Fundación Iavante.

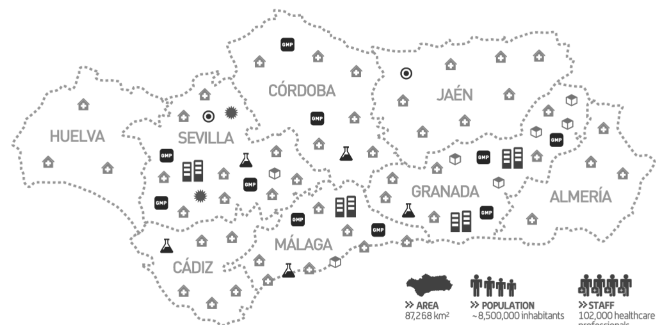
MISSION STATEMENT

Our **mission** is to promote the development of new therapies in order to improve the population's health and to incorporate innovative advanced therapies in the healthcare and progress of our region, by seeking alliances between the academic world, research institutions, health centres, patients' associations, SMEs and the pharmaceutical industry.

FACILITIES MAP

Andalusian Biomedical Reseach and Healthcare **Facilities Map**:





SPECIALIZED RESEARCH CENTRES

- **CABIMER** Andalusian Centre for Molecular Biology and Regenerative Medicine (Sevilla)
- **GENYO** Pfizer - Universidad de Granada - Junta de Andalucía Centre for Genomics and Oncological Research (Granada)
- **BIONAND** Andalusian Centre for Nanomedicine and Biotechnology (Málaga)
- **MEDINA** Research Centre in Innovative Medicines (Granada)

NETWORK RESEARCH CENTRES

- **CEAS** Excellence Centre in Research about Olive Oil and Health (Jaén)
- **DESAFIA** Andalusian Centre of Research Excellence for Sport, Health and Physical Activity (Sevilla)

HEALTH RESEARCH INSTITUTES

- **IMIBIC** Maimonides Institute for Biomedical Research (Córdoba)
- **IBIS** Biomedical Research Institute (Sevilla)
- **IBIMA** Biomedical Research Institute (Málaga)

PLANNED:

- **IBIGRA** Biomedical Research Institute (Granada)
- **IBICA** Biomedical Research Institute (Cádiz)

ANDALUSIAN PUBLIC HEALTH SYSTEM BIOBANK

(Healthcare and Research)

- **DNA** (network centre, central node Granada)
- **STEM CELL** (Granada)
- **TUMOURS** (network centre, central node Granada)
- **BLOOD CORD** (Málaga)
- **TISSUE** (network centre, central node Granada)
- **BIOLOGICAL FLUIDS OF HUMAN NEUROLOGICAL DISEASES** (Sevilla)
- **BLOOD AND ITS DERIVATIVES** (network centre, central node Granada)

HEALTHCARE CENTRES

HOSPITALS > 47
PRIMARY CARE CENTRES > 1,500 (Spread around Andalusia)

CENTRES WITH GMP ROOMS

- **2** in Córdoba
- **3** in Granada
- **2** in Málaga
- **3** in Sevilla

SPECIALIZED LABORATORIES

- **CASEGH** Andalusian Human Genome Sequencing Centre (Sevilla)
- **LARCEL** Andalusian Cellular Reprogramming Laboratory (Sevilla)

SILVER SPONSOR:

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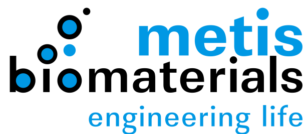

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inc.

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TISSUE ENGINEERING GROUP

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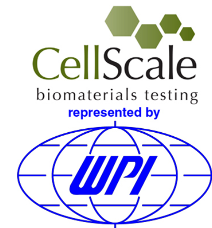
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