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Research Paper

Hydrogels for nucleus replacement—Facing the biomechanical challenge

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ABSTRACT

Hydrogels are considered promising for disc regeneration strategies. However, it is currently unknown whether the destruction of the natural interface between nucleus and surrounding structures caused by nucleotomy and an inadequate annulus closure diminishes the mechanical competence of the disc. This *in vitro* study aimed to clarify these mechanisms and to evaluate whether hydrogels are able to restore the biomechanical behaviour of the disc.

Nucleus pressure in an ovine intervertebral disc was measured *in vivo* during day and night and adapted to an *in vitro* axial compressive diurnal (15min) and night (30min) load. Effects of different defects on disc height and nucleus pressure were subsequently measured *in vitro* using 30 ovine motion segments. Following cases were considered: intact; annulus incision repaired by suture and glue; annulus incision with removal and re-implantation of nucleus tissue; and two different hydrogels repaired by suture and glue.

The intradiscal pressure *in vivo* was 0.75 MPa during day and 0.5 MPa during night corresponding to an *in vitro* axial compressive force of 130 and 58 N, respectively. The compression test showed that neither the implantation of hydrogels nor the re-implantation of the natural nucleus, assumed as being the ideal implant, was able to restore the mechanical functionality of an intact disc.

Results indicate the importance of the natural anchorage of the nucleus with its surrounding structures and the relevance of an appropriate annulus closure. Therefore, hydrogels that are able to mimic the mechanical behaviour of the native nucleus may fail in restoring the mechanical behaviour of the disc.

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1. Introduction

The intervertebral disc is a complex structure that experiences both large deformations while providing spinal flexibility and high stresses as it resists and transmits loads along the spinal column. Due to degeneration, the disc suffers reduced mechanical function which is partly due to dehydration of the nucleus pulposus (Adams and Roughley, 2006). Loss of nucleus pressurisation is closely related to structural failure within the annulus fibrosus that may allow nucleus herniations (Krämer, 2006; Osti et al., 1992). Eventually, this could lead to impingement of nerves, which manifests as low back or leg pain.

Common surgical treatments for end-stage intervertebral disc degeneration include spinal fusion and total disc replacement. Despite the clinical success of these techniques in severe disc degeneration (Galbusera et al., 2008), they do not appear effective in early surgical interventions in mildly degenerated discs in preventing or slowing down the progression of degeneration. Replacing the nucleus alone is a potential alternative approach for patients with an almost intact annulus to restore the mechanical function of the altered disc.

The concept of replacing the nucleus alone raises some biomechanical questions regarding the aims of the treatment and how the replacement should be implemented. Initial solutions aimed only at the restoration of the disc height without mimicking the biomechanical properties of the nucleus. This may induce non-physiological loading on both the implant itself and the surrounding structures. More recent nucleus replacements consist of soft polymeric materials, such as hydrogels or silicone that are able to undergo larger deformations under compression. Ideally, this deformation should ensure tension of the annular fibres similar to the natural nucleus (Ahrens et al., 2009). An advantage of hydrogels over silicone is their potential ability to replicate a physiological fluid flow and to provide a micro-environment for disc cells to boost the regeneration of the intervertebral disc. Therefore, these implants are considered the most promising solution for the surgical treatment of low back pain in early diagnosed disc degeneration (Bergknut et al., 2010).

A natural nucleus is adnated with the neighbouring annulus and endplates and therefore tightly associated with these tissues. Through this anchorage, the interface between nucleus and surrounding tissue is able to support tensile and shear stresses. After nucleotomy and implantation of a nucleus replacement, this interface is destroyed. Furthermore, the replacement necessitates an annulus access. Regardless of the fact that the extrusion of the substitute is of major concern in nucleus replacement strategies, a closure of the entire access width cannot be guaranteed, in particular in the inaccessible, inner annulus regions. Therefore, the implant material may squeeze from the nucleus cavity into the inner defect and as a consequence cause a loss in nucleus pressurisation. Both, the destruction of the interface and the gaping of the inner annulus defect may cause a loss of the mechanical competence of the whole disc. Currently, it is not known whether both phenomena have a mechanical effect.

Therefore, this study aimed to investigate whether (1) a given annulus defect and the destruction of the interface have a mechanical effect on the intervertebral disc and (2) whether newly developed hydrogels for nucleus replacements have the ability to restore the biomechanical behaviour of the intervertebral disc using an *in vitro* approach on ovine lumbar specimen taking into account physiological loads determined previously *in vivo*.

2. Materials and methods

To consider physiological boundary conditions *in vitro*, the nucleus pressure in a healthy ovine intervertebral disc was first measured over 24 h *in vivo*. These pressure measurements were then adapted to a loading protocol on ovine lumbar motion segments *in vitro*. Different defects were simulated to investigate a possible mechanical effect caused by a destroyed interface. Subsequently, two different hydrogels were tested for their ability to restore the mechanical functionality of the intact disc after implantation.

2.1. *In vivo experiment*

Following the approval of the animal experiment by the local ethical committee (identification number 1032, Regierungspräsidium Tübingen, Germany), one 3-year-old merino sheep was operated according to international regulations for care and use of laboratory animals. The animal was sedated with an intramuscular injection of Rompun® 2% (xyalzine hydrochloride, 0.2 mg/kg body wt., Bayer, Leverkusen, Germany). For both peri- and postoperative care nonsteroidal analgesics (Rimadyl®, carprofen, 4 mg/kg body wt., Pfizer, Karlsruhe, Germany) and antibiotics (Veyxyl®, amoxicillin trihydrate, 7 mg/kg body wt., Veyx Pharma GmbH, Schwarzenborn, Germany) were administered prior to surgery and again until sacrifice. General anaesthesia was initiated by an intravenous injection of thiopental (Thiopental Inresa 0.5 g, 5 mg/kg body wt., Inresa GmbH, Freiburg, Germany) and continued as inhalational anaesthesia using isoflurane (Forene®, Abbott GmbH, Wiesbaden, Germany).

A retroperitoneal approach was performed with the animal lying in a right-sided lateral position. By blunt dissection of the abdominal muscles, the retroperitoneal cavity was followed in the direction of the lumbar spine. To reach the lateral part of the disc, the musculus psoas was split.

After puncturing the annulus with a wire (outer ∅ 1.50 mm) a hollow cannula (inner ∅ 1.50 mm) was introduced into the disc. Having removed the wire, the hollow cannula was used to insert a flexible pressure transducer (∅ 1.45 mm, FMSPEZ50, Mammendorfer Institut für Physik und Medizin GmbH, Mammendorf, Germany) in the centre of the nucleus of the L2–L3 segment from the left lateral side of the disc under fluoroscopic control. Keeping the pressure sensor in place, the cannula was pulled back and the sensor was sutured to the outer annulus layers. At the metal tip (∅ $1.45 \times 4.40\ mm^2$) of the transducer, a piezoresistant pressure sensor was located underneath a covering membrane (1.06×2.00 mm) that detected and transmitted pressure-dependent deformations of the membrane and subsequently induced the formation of an electrical voltage signal. The signal was amplified (DMCplus; HBM Hottinger Baldwin

Messtechnik, Darmstadt, Germany) and recorded at 50 Hz. Pressure data were transmitted using a telemetry system (BIOTEL 33 hybrid, Glonner Electronic GmbH, Martinsried, Germany) with the transmitter (14 × 6 cm, mass: 270 g) carried by the animal on its back so that it was able to move freely.

Measurements started as soon as the transducer was affixed to the disc and subsequently intradiscal pressure (IDP) was measured for 24 h. Throughout the surgery, no marked loss of nucleus pressure could be detected. After completion of the 24-hours-test-period, the animal was sacrificed by captive bolt stunning and subsequent exsanguination.

2.2. *Ex vivo experiment*

After sacrificing the animal, the lumbar spine of the sheep was extracted and prepared for further biomechanical testing. Subsequently, the motion segment L2–L3 was retrieved and excess muscle tissue was dissected so that ligaments, facet capsules and intervertebral discs could be preserved. Throughout the whole preparation process, the pressure sensor remained exactly in the position in which it was implanted during surgery. The cranial end of the upper vertebra L2 and the caudal end of the lower vertebra L3 were embedded in polymethylmethacrylate (PMMA) (Technovit 3040, Heraeus Kulzer, Werheim, Germany). To rigidly fix the vertebrae with the PMMA and to prevent micro-motion between both compartments, screws were additionally drilled in different directions into the ends of the vertebrae before casting them with the liquid plastic. The segment was embedded so that the disc midplane was horizontally aligned. The segment was kept moist with 0.9% saline solution throughout the entire preparation process. Specimens were fixed into an electro-mechanical material testing machine (Fig. 1a).

An axial compression test on the explanted segment was performed (Z010, Zwick GmbH & Co. KG, Ulm, Germany). After a preconditioning phase of 15 min at a constant axial force of 20 N a total of three loading cycles were driven up to

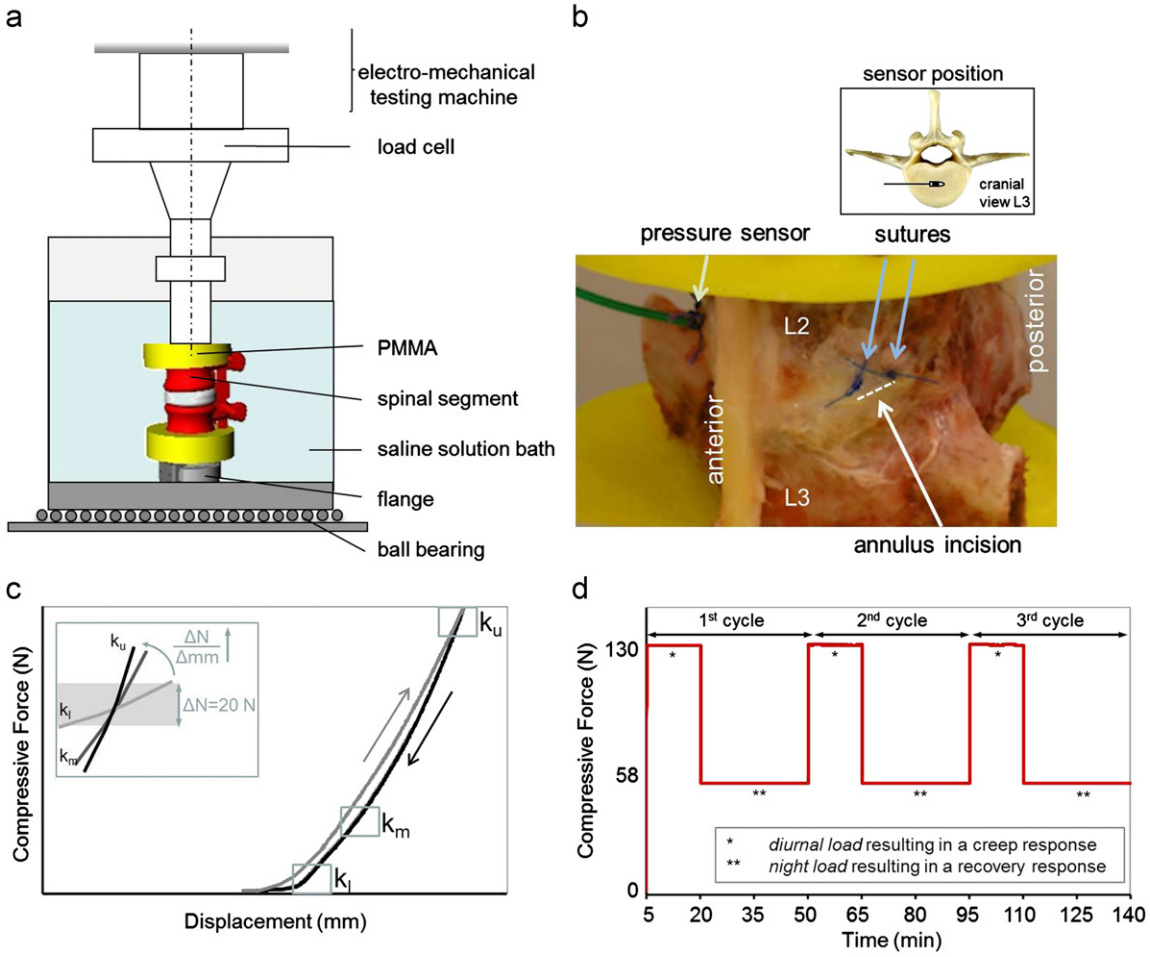

Fig. 1 – (a) The sketch illustrates the experimental setup for the stiffness and compression tests using an electro-mechanical material testing machine (Z010, Zwick GmbH & Co. KG). (b) For different defects and implant states, a standardized incision in the annulus was set at the left-lateral side. (c) Segmental stiffness was determined on the unloading part of the third loading cycle in the stiffness test (representative curve). The grey curve represents the loading path and the black curve shows the unloading path. Stiffness (ΔN/Δ mm) was determined at three different force intervals during unloading: k_u: 500–480 N, k_m: 130–110 N and k_l: 58–38 N. (d) The curve depicts the loading protocol of the creep-recovery experiment. Three loading cycles were performed, each consisting of a loading period of 15 min at 130 N and an unloading period of 30 min at 58 N.

500 N at a loading rate of 30 N/s. For data analyses only the last unloading period during retraction of the load cell was used.

2.3. *In vitro experiment*

For the *in vitro* tests lumbar spines from 22 skeletally mature merino sheep aged between 2 and 5 years were harvested and inspected visually to exclude spinal diseases and damages. Specimens were stored at −20 °C and thawed for 16 h at 0 °C prior to testing. Thirty motion segments (22 × L2–L3, 8 × L4–L5) were extracted from these lumbar spines.

According to the *ex vivo* experiment, the motion segments were prepared for the subsequent biomechanical tests. Prior to testing, an intradiscal pressure sensor of the same type used in the *in vivo* experiment (see Section 2.1. *In vivo experiment*) was inserted in the centre of the nucleus and remained in this position during the entire test.

The following five different defect and implant situations were investigated: (i) INTACT: the intact stage requires the unrestricted integrity and functionality of the nucleus and annulus, except for the puncture necessary for inserting the pressure transducer. (ii) DEF-ANN (Fig. 1b): a small oblique incision into the annulus, normally used for the surgical removal of the nucleus, was set at a left-lateral position (approximately 4 mm wide, 6 mm deep). This fissure was oriented along the outer annular fibre direction (Krismer et al., 1996). Besides the opening of the nucleus cavity, the nucleus tissue itself was not affected by the defect that was created by a safe and palpable penetration through the annulus layers with a scalpel blade. The access was closed by sealing the incision using a specialized more flexible cyanoacrylate-based glue (ADHBIO ADHESIVE®, Adhesion and Adhesives Laboratory, Department of Inorganic Chemistry, University of Alicante, Spain) and by stitching the outer fibrous annular layers. ADHBIO ADHESIVE® is a two-component glue consisting of high purity ethyl cyanoacrylate mixed with a long hydrocarbon chain acrylate; the addition of the acrylate decreases the stiffness of the cyanoacrylate facilitating the joining of curved defects. (iii) DEF-NUC: nucleus tissue was removed in fragments from the intervertebral disc (masses are given in Table 1) using rongeurs with straight and flexed jaws of 1.0 and 1.5 mm, respectively. In contrast to the gel-like nucleus of e.g. pigs or rabbits, the ovine nucleus is rather fibrous-like similar to the human one. Due to this consistency, it was not possible to remove the ovine nucleus by means of aspiration, but a nucleotomy with the opening of the annulus described before was necessary. In order to avoid any damage to the inner layers of the annulus on the opposite, anterior and/or posterior side of the defect, nucleotomy was terminated when by means of macroscopic assessment no further nucleus material could be removed from the disc. Without additional hydration the explanted tissue was collected in an airtight lockable tube for approximately 10 min and subsequently completely re-implanted into the disc. The annulus defect was closed as in DEF-ANN. In addition, two hydrogels were investigated as nucleus replacements: (iv) dodecyl-amide of hyaluronic acid (DDAHA) and (v) ionic-crosslinked methacrylated Gellan Gum (iGG-MA). In both cases, nucleus material was removed as in DEF-NUC, being replaced with the respective hydrogel. The annulus defect was again closed as in DEF-ANN after implantation.

Two different loading protocols were performed: (i) After pre-conditioning for 5 min at 2 N, three repeating cycles up to 500 N were applied at a loading rate of 30 N/s. The third loading cycle served to estimate the axial compressive stiffness of the intact, defect and implanted segments (further referred to as the stiffness test). The slope of the load–displacement response during the unloading portion of the third cycle was used to measure the compressive stiffness at three different force intervals: k_u: 500–480 N, k_m: 130–110 N and k_l: 58–38 N (Fig. 1c). (ii) Specimens were subjected to three loading cycles (further referred to as the compression test). While the first cycle was used for pre-conditioning, the second and third cycles were used for data analyses. Each loading cycle consisted of a loading period of 15 min at 130 N (further referred to as diurnal load) and a recovery period of 30 min at 58 N (further referred to as night load). Loading and unloading were performed at a loading rate of 30 N/s (Fig. 1d). Load and displacement of the crosshead of the material testing machine were recorded at 50 Hz. The vertical displacement of the crosshead was equivalent to the specimen height loss.

During mechanical testing, the specimens were placed in a bath with physiologic saline solution at room temperature. Due to the long duration of the experiments, each specimen was tested only once.

For the compression test, *in vitro* the specimen height loss was normalised to 58 N at the end of the first diurnal plus night load cycle. With this, the unloaded situation was perceived to correspond to a loading after night rest. Due to the limited sample size, data were assumed to be non-normally distributed. Therefore, differences between INTACT, DEF-ANN, DEF-NUC and DDAHA and iGG-MA were tested at the end of each loading and recovery phase using an unpaired, two-sample Wilcoxon signed rank test to a significance level of $p<0.05$, which is a general non-parametric equivalent test. The statistics were performed using Gnu R (R Development Core Team, 2011).

Table 1 – Ovine specimens used in different biomechanical tests.

State	Level	Removed NP mass	Implanted mass
INTACT	6 × L2-L3	–	–
DEF-ANN	6 × L2-L3	–	–
DEF-NUC	6 × L4-L5	0.14 g (0.10–0.15 g)	Equal removed mass
DDAHA	4 × L2-L3/2 × L4-L5	0.16 g (0.13–0.18 g)	0.15 g (0.13–0.17 g)
iGG-MA	6 × L2-L3	0.22 g (0.16–0.25 g)	0.22 g (0.19–0.31 g)

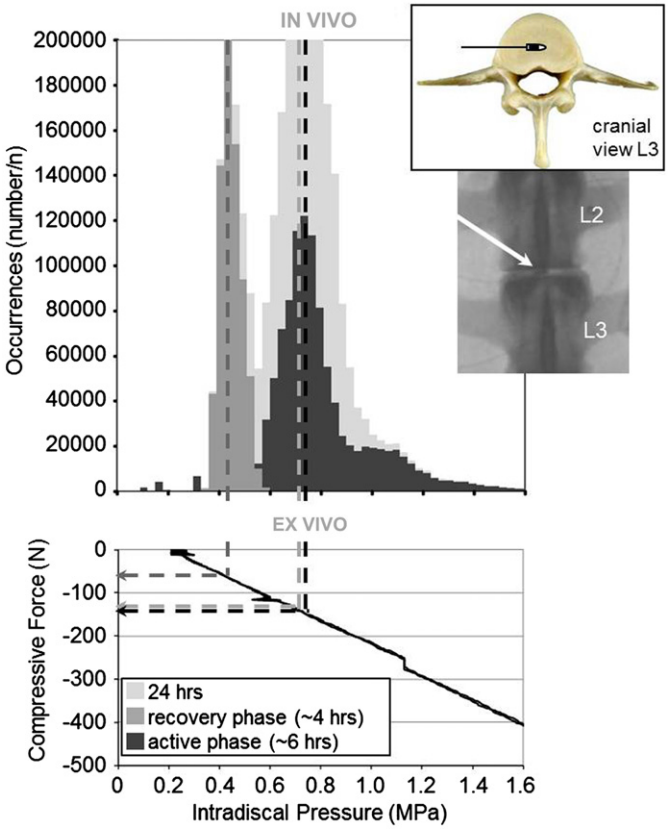

Fig. 2 – Occurrences distribution of the *in vivo* intradiscal pressure for the entire testing period measured in sheep. The total occurrences were divided into a recovery and an active phase. The dashed lines represent the mean pressure values for each single distribution. After sacrifice, an axial compression test on the explanted segment L2–L3 was performed in order to estimate the *ex vivo* compressive force which corresponds to the intradiscal pressures measured *in vivo*.

2.4. *Composition of the hydrogels*

DDAHA (Anika Therapeutics, Abano Therme, Italy) consists of an amidic hyaluronic acid derivate with lipophilic properties. Hydrated, it constitutes a viscous gel with an aggregate modulus of $H_0 = 0.2 \pm 0.04$ kPa, and a hydraulic permeability of $k_0 = 520 \pm 130 \times 10^{-13}\ m^4\ N^{-1}\ s^{-1}$, that is stable over time. DDAHA displayed advantageous features regarding (a) cellular approaches to regenerate diverse natural tissues both in clinical trials and in the animal model (Giannini et al., 2009; Hemmrich et al., 2008) and revealed promising results in a short term large animal in *in vivo* study (Revell et al., 2007).

iGG-MA (3 B's Research Group, University of Minho, Portugal) is an extracellular microbial polysaccharide from *Sphingomonas elodea* that has been proposed as a biomaterial for tissue engineering applications (Oliveira et al., 2010, 2009). It forms a colloidal, transparent gel in the presence of metallic ions with promising characteristics for application in IVD regeneration (Pereira et al., 2011). Gellan Gum is structurally described as a linear, anionic heteropolysaccharide that consists of glucose–glucuronic acid–glucose–rhamnose as monomer. For the purpose of this work, a chemically modified derivative of Gellan Gum was used, i.e. the ionic-crosslinked methacrylated Gellan Gum (iGG-MA) hydrogels (Silva-Correia et al., 2011). Its aggregate modulus is given as $H_0 = 10.5 \pm 3.1$ kPa, and its hydraulic permeability as $k_0 = 1.7 \pm 0.6 \times 10^{-13}\ m^4\ N^{-1} s^{-1}$. iGG-MA hydrogels adequately support the growth and ECM deposition of human articular chondrocytes both *in vitro* and *in vivo* (Oliveira et al., 2010, 2009) and possess adequate properties for being used as nucleus substitutes in acellular and cellular strategies, due to their ability to support cells encapsulation, their adequate mechanical properties, biostability and non-cytotoxicity *in vitro* and *in vivo* (Pereira et al., 2011; Silva-Correia et al., 2011). They are non-angiogenic, i.e. do not allow endothelial cell adhesion and ingrowth, and prevent blood vessel infiltration *in vivo* (Silva-Correia et al., 2012).

3. Results

3.1. *In vivo experiment*

The animal recovered gently and without complications from surgery showed a species-typical behaviour without any signs of constricted mobility due to the measuring unit. Good general condition of the sheep after the surgical intervention was concluded by the immediate and intuitive food intake upon awakening as a reliable indicator of wellness. At the exit location of the silicone cable of the sensor within the surgical wound no complications e.g. exsudations were visible throughout the test duration of 24 h until sacrifice.

The measured IDP was ~0.75 MPa during day and ~0.5 MPa during night (Fig. 2).

3.2. *Ex vivo experiment*

After sacrifice, a linear correlation between applied force and measured IDP was found in the axial compression test on the explanted segment L2–L3. IDP values of 0.75 and 0.5 MPa corresponded to an axial force of 130 and 58 N, respectively (Fig. 2).

3.3. *In vitro experiment*

3.3.1. *Stiffness test*

The load–deformation-curves of all tested segments showed a nonlinear behaviour characterised by a toe-region at the beginning of the loading (Fig. 1c). This was followed by a progressive region and ended with an almost linear response. Significant differences were found between the three testing locations k_u, k_m and k_l (Fig. 3). However, no significant differences were observed between the different testing groups, except between INTACT and DEF-NUC for k_l.

3.3.2. *Compression test*

For INTACT, the constant diurnal load caused a typical creep response, with a steady loss of specimen height (Fig. 4). The night load only led to a slight recovery. Equilibrium was almost reached under night load but not under diurnal load. The mean specimen height loss after the complete loading protocol was 0.03 mm. This shows that two intervals of night load did not compensate for the loss of height resulting from two intervals of diurnal load. The loss of height calculated over an interval of 15 min diurnal load and 30 min night load showed no significant differences between the second and third loading cycles (Fig. 5). Intact ovine intervertebral discs caused a nucleus pressure of ~0.3 MPa without any external load. In the first pre-conditional cycle, the application of 130 and 58 N initially caused an IDP of 0.75 and 0.5 MPa, respectively. These values slightly decreased with time over the three loading cycles (Fig. 6). During the diurnal load, IDP slightly decreased by ~3%, while it remained almost constant during the night load.

In comparison to INTACT, the annulus incision (DEF-ANN) caused no significant change in specimen height or IDP over the two loading cycles (Figs. 4–6).

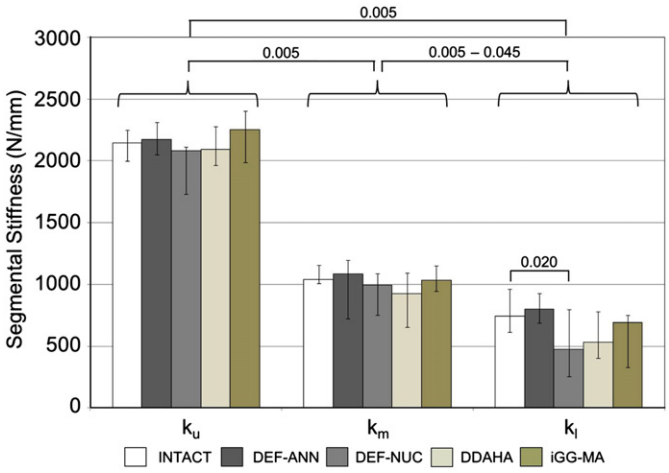

Fig. 3 – Median (min–max) axial compressive stiffness for the intact state (INTACT), a small annulus incision (DEF-ANN), a replaced nucleus (DEF-NUC) and two different hydrogels (DDAHA, iGG-MA) at three different force intervals: k_u: 500–480 N, k_m: 130–110 N and k_l 58–38 N (see Fig. 1c). Numerical values represent the statistically significant differences.

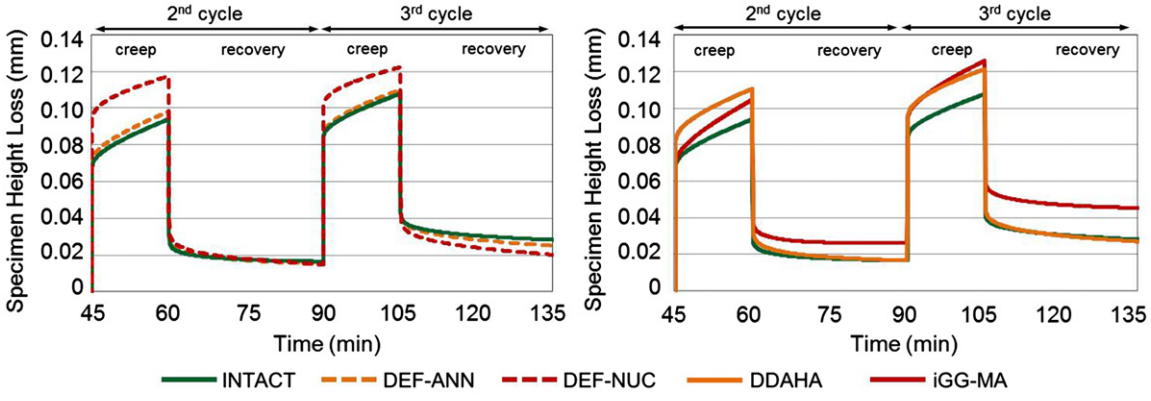

Fig. 4 – Temporal changes of specimen height loss for the intact state (INTACT), a small annulus incision (DEF-ANN), a replaced nucleus (DEF-NUC) and two different hydrogels (DDAHA, iGG-MA) for the second and third loading phases. Specimen height loss is presented relative to 58 N at the end of the first loading cycle.

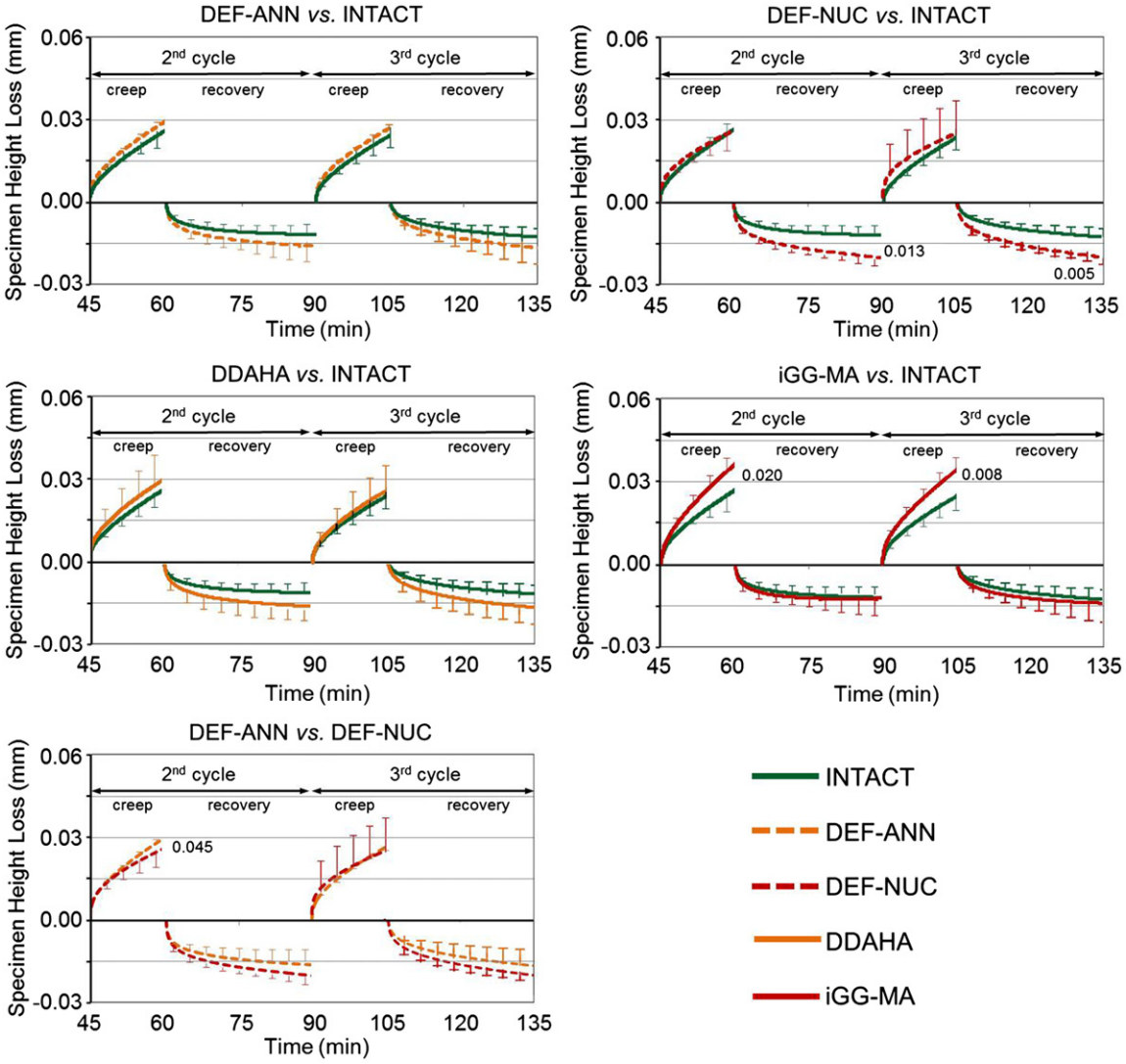

Fig. 5 – Temporal changes of specimen height loss during creeping and recovery (negative values) for the second and third loading cycles. The change of height was calculated relative to the height at the beginning of creep and recovery phases. Error bars indicate standard deviations. Numerical values represent the statistically significant differences.

DEF-NUC led to a stronger decrease in height immediately after load application compared to INTACT (Fig. 4). This height loss slightly increased over the two loading cycles, but at the end of the loading protocol the height loss was comparable to that found for INTACT. Comparing the creeping response between both groups, significant differences were found only during night load. Here, DEF-NUC showed some recovery (Fig. 5). The defect led to a significant reduction by 50% in IDP before applying the external load. In the course of the following loading cycles, pressure diverged on average ~26% between DEF-NUC and INTACT (Fig. 6).

When comparing the two defect states DEF-ANN and DEF-NUC, results did not differ significantly in terms of specimen height loss but, however, concerning the drop in IDP they did ($p=0.005$–0.01) (Figs. 4 and 6).

The implantation of the DDAHA hydrogel caused an increase in height loss, which was almost midway between INTACT and DEF-NUC directly after load application (Fig. 4). The implant displayed a comparable creeping and recovery behaviour during diurnal and night load as found for DEF-NUC. The difference in recovery between INTACT and DDAHA was not significant (Fig. 5). The implant showed a poorer pressure resistance compared to INTACT during the entire test with an average decrease of 34% (Fig. 6).

At the beginning of the second loading cycle, iGG-MA displayed a similar height loss as INTACT. In contrast to all other test groups, this height loss markedly increased over the following two loading cycles (Fig. 4). The creep response for iGG-MA differed significantly from INTACT during diurnal load, whereas no significant differences were observed during night load (Fig. 5). The behaviour of iGG-MA was substantially comparable to DDAHA regarding pressurisation, i.e. the implant showed a significant pressure loss compared to INTACT throughout the whole compression test (Fig. 6).

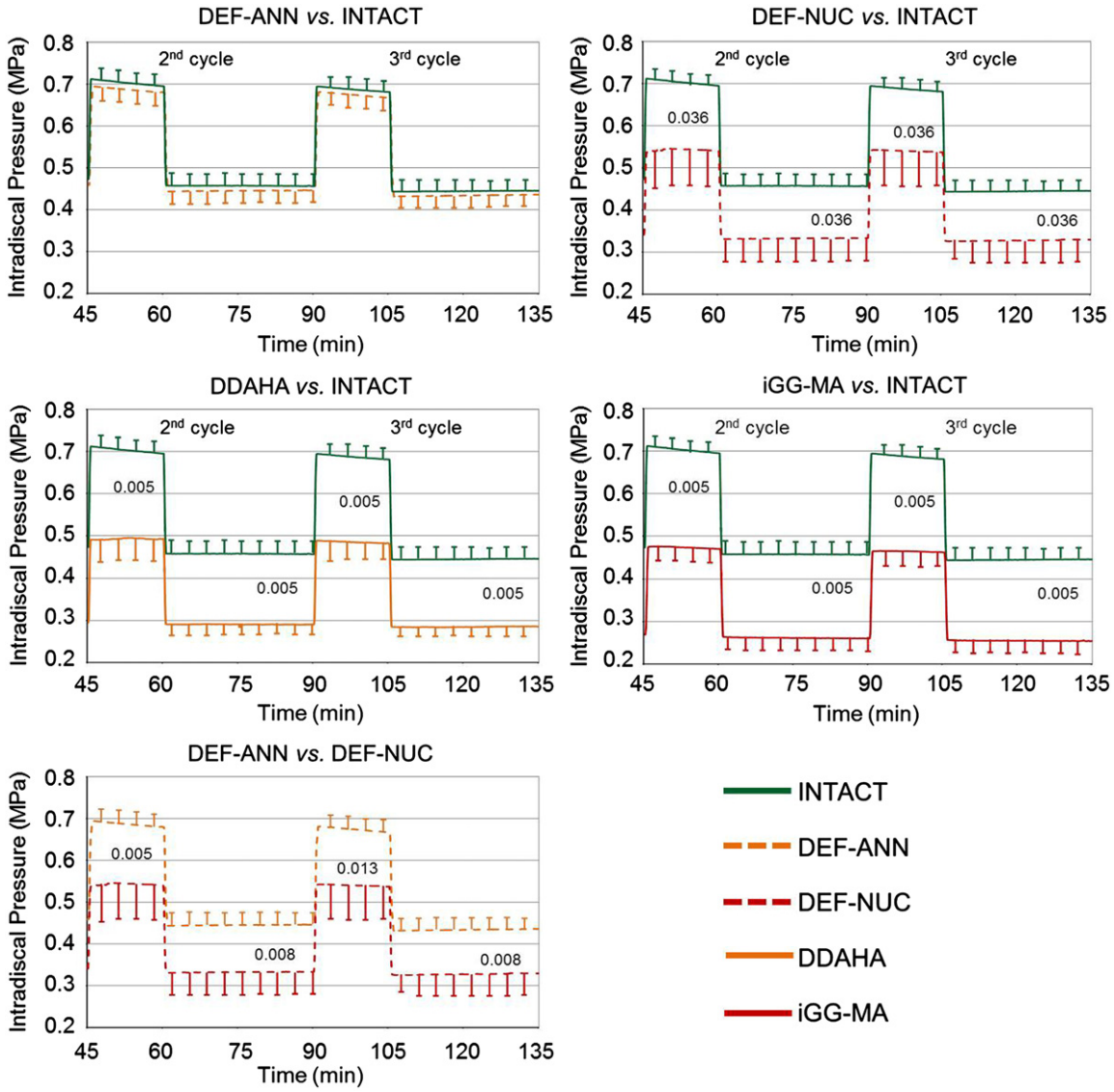

Fig. 6 – Temporal changes of intradiscal pressure over the second and third loading cycles. Error bars indicate standard deviations. Numerical values represent the statistically significant differences.

4. Discussion

The results of the current *in vitro* study demonstrate that a given annulus incision in combination with an intact nucleus (DEF-ANN) did not significantly influence axial segmental stiffness, specimen height-loss and fluid pressurisation. In addition, removal and re-implantation of the native nucleus (DEF-NUC) as well as the implantation of both hydrogels (DDAHA and iGG-MA) did not affect the axial segmental stiffness. However, DEF-NUC, DDAHA and iGG-MA did increase the specimen height loss and decrease the fluid pressurisation compared to INTACT. The destructions of both the nucleus compound itself and the interfaces with surrounding annulus and endplates (DEF-NUC) in comparison to DEF-ANN caused an increased specimen height loss and a significant decrease in IDP during compression.

An isolated annulus incision of 4 mm (DEF-ANN) did not affect the IDP. This seems to contradict the present state of knowledge that already 27G needles induce disc degeneration in the long term *in vivo*. The knowledge about inducible disc degeneration was widely used to specifically initiate degenerative cascades in a variety of animal models (Sobajima et al., 2005; Zhang et al., 2011) but also led to a general uncertainty in the spine research field, calling diagnostic procedures into question in which the disc is necessarily punctured (Carragee et al., 2009). Due to a degradation of the hydrophilic components of the matrix, which represents a slowly progressive active catabolic reaction of living cells, a drop of IDP in the course of degeneration was detected *in vivo* (Holm et al., 2007). All these mechanisms (e.g. release of pro-inflammatory cytokines, up-regulation of matrix degrading enzymes) following the injury *in vivo* are actually cellular responses that are no longer present *postmortem* and thus associated long-term effects cannot be expected in an experimental *in vitro* test set-up which only accounts for the damage that was manually set neglecting biological processes. From the

mechanical point of view the annulus defect has, under these simplified loading conditions, no effect on the overall load sharing between nucleus and annulus. Applying more complex loads (e.g. combining flexion and compression loads together) could influence the results by directly applying tension or shear on the annulus defect which then would cause an increase in the nucleus share of applied load.

After three cycles of loading and recovery phases, INTACT showed a net loss of height. This is consistent with earlier *in vitro* results on porcine specimens in which no or limited recovery was found (van der Veen et al., 2005; van Deursen et al., 2001). Among various reasons, this may be due to postmortem formation of blood clots within the endplates. *In vivo* measurements of the IDP in a human disc showed an increase in nucleus pressure (Wilke et al., 1999), water content (Malko et al., 2002) and disc height (McGill and Axler, 1996; Reilly et al., 1984) during 7 h rest. Even though the gain in height and pressurisation cannot be reproduced *in vitro*, the advantage of the compression test used is that it includes all other viscous effects of the disc. Furthermore, it constitutes a possible case of disc degeneration, since the state of the specimens used could mimic sclerotic endplates with a restricted fluid flow. Especially if the fluid flow is limited, the implants should nevertheless provide gain in height and pressure in order to meet the final destination of intervertebral disc regeneration. Therefore, the implant not only has to fulfil the native nucleus properties but also has to compensate a limited inflow caused by sclerotic endplates, e.g. by an increased osmotic potential. If this is not achieved, the concept of cell seeded hydrogels will not function because the cells will be insufficiently nourished. In the current study, DDAHA caused a net loss of height at the end of the compression test similar to INTACT, while iGG-MA produced an even greater loss. This indicates that the current hydrogel designs do not appear to be designed to compensate for the degenerative effects found during the early stages of disc degeneration.

Analysing the segmental stiffness in the compression test during load reduction (130→58 N) displayed no changes for INTACT and iGG-MA, but a decrease of 20% for DEF-NUC and 10% for DDAHA compared to the values determined in the stiffness test. The stiffness reduction of DEF-NUC and DDAHA is the reason for the abrupt decrease in height directly after the diurnal load application compared to INTACT. In contrast, iGG-MA showed a constant stiffness over time but a greater height loss during diurnal load. This caused the reduction in height at the end of the compression test.

With the nucleotomized material, confined compression tests were performed to asses if the material properties of the injected hydrogels are similar to native ovine nucleus tissue. The mechanical testing protocol was based on an earlier published work (Mizuno et al., 2006). The aggregate modulus was 13.8 kPa (min: 10.2, max: 17.5 kPa) and the hydraulic permeability was $9.2\times10^{-14}\,m^4\,N^{-1}\,s^{-1}$ (min: 6.4×10^{-14}, max: $1.3\times10^{-13}\,m^4\,N^{-1}\,s^{-1}$). The latter is similar to the early measurements of Mizuno et al. (2006). However, the aggregate modulus in our study is three times lower than the values published by Mizuno et al. (2006). iGG-MA therefore represents the ovine nucleus within an adequate range, while DDAHA does not. It therefore seems that DDAHA is not a good candidate for an immediate biomechanical restoration. However, despite the differences in their material properties, DDAHA and iGG-MA gained comparable results in the implanted state suggesting the material, when within a specific stiffness and permeability range, to play a subordinate role.

A recently published *in vitro* study showed that disc height and range of motion was restored to the intact level with increasing volumes of hydrogel implant (Arthur et al., 2010). In several preliminary trials we attempted to restore the intact IDPs by increasing the implant volume, but unfortunately the annulus closure made by suture and glue failed to withstand the ensuing high IDP. Arthur et al. (2010) prevented material extrusion by inserting a balloon into the nuclear cavity thereby inhibiting a natural interface between the hydrogel and the surrounding tissue which is of fundamental importance for disc regeneration processes which require ingrowth pathways for native local cells and a physiological fluid flow to nourish them.

To better compare the hydrogels with the native nucleus replacements (DEF-NUC), the same mass of hydrogel was thus re-implanted as nucleus tissue was previously removed (see Table 1). Assuming similar densities of both hydrogels and native nucleus tissue with a similar water content of about 80%, the hydrogels re-filled the entire cavity after nucleotomy. Even under the claim that the nucleus cavity was completely re-filled, neither of the hydrogels, irrespective of its material properties, seemed appropriate for an immediate restoration of disc function. This, indeed, also applied to DEF-NUC, where a similar loss of biomechanical competence occurred even after total replenishment of the nucleus cavity with the natural tissue.

Given the results of our current *in vitro* experiment, at first glance the used hydrogels do not seem appropriate for the immediate restoration of the disc function. Nevertheless, this does not necessarily mean that their potential application *in vivo* is inevitably inconceivable and hence not successful. A previous *in vivo* study indicated that in tissue-engineered discs, the aggregate modulus increases and the hydraulic permeability decreases in the course of 16 weeks *in vivo* so that both parameters at the end of the observation closely fitted the native tissue properties of the disc due to an adaptation of the scaffold to a physiological environment (Mizuno et al., 2006).

Interestingly, no differences in stiffness were found between DEF-NUC and INTACT in the stiffness test. Only after the first loading cycle of the compression test was a decrease in stiffness observed. This stiffness loss may be caused (1) by a destroyed interface between the nucleus and its surrounding structures, (2) by a mechanical destruction of the collagen–proteoglycan compound making up the native nucleus tissue during re-implantation and (3) by a displacement of nucleus material into the inner annulus defect. For the latter it could not be guaranteed that the glued inner annulus defect opens under compression which allows a squeezing of nucleus material into the inner defect. The present *in vitro* findings alone do not allow a distinct differentiation between these three effects as to which has a greater influence on the results.

Finite element analyses showed that a destroyed interface is one possible reason for the strong differences between DEF-NUC and INTACT (Reitmaier et al., 2012). The model predicted a 10% drop in IDP during the creep phases after

replacing the native nucleus material. Therefore, even when a given nucleus implant shows similar biomechanical properties as the native nucleus, it may not be able to provide proper fluid pressurisation due to the disturbed interface. To the authors' knowledge no studies currently exist that investigate the interface from a biomechanical point of view. This may be due to the fact that this zone cannot be detected as a sharp boundary, but rather as a smooth change between nucleus and annulus or endplate tissue. Anatomically, the transition zone is composed of dense fibrillar connective tissue intertwining the nucleus with the annulus (Roberts et al., 1993). This insertion could serve as a guide for the fluid flow and thus control re-pressurisation in addition to its probable ability to transmit normal and shear stresses. Therefore, the very useful properties of hydrogels such as their high water content, biocompatibility, ease of cell seeding and good mechanical properties (Ahmed et al., 2008; Lavik and Langer, 2004) should be optimised to restore the interface between the nucleus and the surrounding structures, otherwise nucleus implants may not be of use for the patient.

Further investigations to demonstrate the effect of the interface between implanted hydrogel and surrounding tissue on immediate restoration of biomechanical function are required. One possibility would be a comparison of hydrogel that can be either non-adhesive, or adhesive to the surrounding tissue.

In addition, the present results indicate that it is not sufficient to use a single annulus defect (DEF-ANN) as a model to mimic the defect in nucleus replacement experiments.

Ovine spines were used in this investigation because they show less degeneration and more uniform mechanical properties than elderly human spines. In the lumbar region they have a gross anatomy similar to the human spine (Wilke et al., 1997). The nucleus water and collagen content are comparable between ovine and human intervertebral discs (Leahy and Hukins, 2001; Lyons et al., 1981; Meakin and Hukins, 2000; Reid et al., 2002). Additionally, the stiffness values of the current study were in agreement with previous *in vitro* studies on human lumbar specimens (Edwards et al., 1987; Gardner-Morse and Stokes, 2004). Therefore, ovine specimens should have been a suitable model in the tests performed in the current study. However, one limitation could have been the overall smaller size of the sheep intervertebral discs and the curvature of the endplates in comparison to human discs.

The pressure value for night load of 0.5 MPa was actually evaluated when the sheep was lying at night. Sheep, unlike humans, do not show a continuous rest period over the whole night. The actual average night pressure is presumably higher than 0.5 MPa due to repetitive active phases, and therefore may be closer to the average daily load. *In vitro*, a greater night load would be even more restrictive to recovery. On the other hand, this could indicate that sheep only need very limited rest periods to completely recover the disc compared to human intervertebral discs.

5. Conclusions

This *in vitro* study clearly indicates the importance of the interface between the nucleus and its surrounding structures and the relevance of an appropriate annulus closure to avoid a displacement of implant material into the inner annulus defect. Therefore, hydrogels that are able to mimic the mechanical behaviour of the native nucleus may still fail in restoring the disc height and fluid pressurisation. In order to obtain a biomechanical equivalent of the natural nucleus, more attention needs to be paid to the anchoring of the nucleus and its replacement inside the disc.

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