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Chondrogenic phenotype of different cells encapsulated in κ-carrageenan hydrogels for cartilage regeneration strategies

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Abstract.

Engineering articular cartilage substitutes using hydrogels with encapsulated cells is an approach that has received increasing attention in recent years. Hydrogels based on κ-carrageenan (κC), a thermoreversible natural-origin polymer, have been recently proposed as new cell/growth factor delivery vehicles for regenerative medicine. In this work, we report the potential of such hydrogels encapsulating either human-adipose-derived stem cells (hASCs), human nasal chondrocytes (hNCs), or a chondrocytic cell line (ATDC5) for cartilage regeneration strategies. The *in vitro* cellular behavior of the encapsulated cells within κC hydrogel was analyzed after different culturing periods using biochemical assays and histological and real-time reverse-transcription PCR analysis. The three types of cells encapsulated in κC hydrogels showed good cellular viability and proliferation up to 21 days of culture, and the cell-laden hydrogels were positive for specific cartilage markers. In summary, the results demonstrate that hASCs embedded in κC hydrogels proliferate faster and exhibit higher expression levels of typical cartilage markers as compared with hNCs or ATDC5 cells. Based on these data, it is possible to conclude that κC hydrogel provides a good support for culture and differentiation of encapsulated cells and that hASCs may provide an advantageous alternative to primary chondrocytes, currently used in clinical treatments of cartilage defects/diseases.

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1. Introduction

The limited capacity of adult articular cartilage for self-repair is well recognized and is explained by its being an avascular and aneural tissue that consists of relatively few cells that cannot migrate; specifically, mesenchymal stem cells cannot recruit or get easy access to damaged areas [1],[2]. Currently, clinically used therapies involve either surgical technique or autologous chondrocyte transplantation, which are generally successful in terms of pain relief and improved function but do not completely restore the articular cartilage function [3]. Although the use of autologous chondrocytes has the advantages of avoiding possible immune rejection or disease transmission, their wider application presents several limitations associated with the harvesting and *in vitro* proliferation procedures. The isolation of autologous chondrocytes requires an additional surgical intervention to harvest cartilage tissue, which often results in donor-site morbidity. Moreover, the amount of articular cartilage tissue is limited, even if retrieved from a different site location such as the nasal septum [4]. In addition, the *in vitro* chondrocyte proliferation must be narrowed because these cells easily dedifferentiate in monolayer cultures and because of their low capacity to produce stable cartilage upon *in vivo* implantation with a tendency to form fibrous cartilage [5]. Because adult stem cells can be isolated from various tissue sources and have the ability to proliferate and differentiate along multiple lineage pathways in a reproducible manner, they have received great attention as an alternative resource to autologous chondrocytes, overcoming the limited number of primary chondrocytes and proliferation capacity [6],[7]. Thus, many studies have been conducted to evaluate the use of adult stem cells in cartilage tissue engineering, most of them focusing on the use of bone marrow stem cells. Recently, adipose-derived stem cells (ASCs) have been regarded as good candidates for the repair and regeneration of articular hyaline cartilage because of their availability, long-term cell viability, and multilineage differentiation potential,

Abbreviations: hASCs, human-adipose-derived stem cells; hNCs, human nasal chondrocytes; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; DMEM, Dulbecco's modified Eagle's medium; A/B, antibiotic-antimycotic; GAGs, glycosaminoglycans; PBS, phosphate buffered saline; κC, κ-carrageenan.

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including differentiation into the chondrogenic phenotype [8]. In fact, estimated yields of stem cells are in the range of 400,000 stem cells per milliliter of adipose tissue [9], resulting in up to a 300-fold increase in cell number from 100 g of adipose tissue as compared with 100 mL of bone marrow aspirate [10]. Nevertheless, in approaches consisting of the direct implantation of cells into the injured cartilage area, there are some challenging issues such as leakage of transplanted cells from the cartilage defects, leading to low cell retention and donor site morbidity [11]. Thus, three-dimensional (3D) vehicles such as hydrogels have been proposed to deliver high amounts of cells with a well-maintained phenotype to facilitate cell retention and assist in mechanical stability of the transplantation site [12]. In addition, the use of hydrogels might provide important features for cell functionality because of their high tissue-like water content, closely mimicking the natural environment in the body, enabling efficient transport of nutrients and waste [13], and the possibility to control the release pattern of signaling molecules by incorporating them into the hydrogel by physical and/or chemical means. Various studies have demonstrated the successful use of natural polymeric hydrogels for cell encapsulation and *in vitro* culture purposes such as hyaluronic acid [14], agarose [15], collagen, alginate [16],[17], and chitosan [18]. However, their mechanical properties are often insufficient for the stability of the tissue-like construct transplantation. Furthermore, hyaluronic acid promotes early inflammation, which is then rapidly metabolized *in vivo* by free radicals and hyaluronidase [19]. Alginate has high molecular weight and therefore cannot be readily eliminated by the body [20], and chitosan induce rapid bone regeneration at initial stages [21]. κ-Carrageenan (κC), an ionic hydrogel proposed recently for cartilage regeneration approaches, which forms a gel with potassium ions but also shows gelation under salt-free conditions, has demonstrated advantageous properties compared with other systems currently proposed for cartilage tissue repair. In this study, we evaluated the ability of κC hydrogels to support cellular functionalities, comparing simultaneously the potential of using human-adipose-derived stem cells (hASCs) versus primary human chondrocytes, in cartilage regeneration strategies. Additionally, ATDC5, a murine, well-characterized chondrogenic cell line, routinely used as a model system to study the chondrogenic process, was used as a control. The three different cell types were encapsulated in κC hydrogels and cultured for up to 21 days. The cells' response was evaluated in terms of metabolic activity (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium [MTS] assay), proliferation (DNA), presence of glycosaminoglycans (GAGs; typical histological staining—Alcian blue and Safranin O), and by the expression of specific cartilage markers (namely, Sox9, aggrecan, collagen type I, COL2, and collagen type X) using real-time quantitative reverse-transcription PCR (qRT-PCR) analysis. The results obtained suggest that κC hydrogel enables beneficial cellular response supporting extracellular formation and sustaining the chondrogenic phenotype. Moreover, encapsulated hASCs exhibit the highest proliferation rates and highest levels of chondrogenic marker expression, encouraging the use of these cells and the κC hydrogels as cell delivery carriers for applications in the regeneration of articular cartilage defects.

2. Experimental

2.1. Materials

κ-Carrageenan powder (22048), potassium chloride (KCl, P5405), phosphate buffered saline (PBS) tablets (P4417), Dulbecco's PBS (D5652), Dulbecco's modified Eagle's Medium (DMEM)—low glucose (D5523), sodium bicarbonate ($NAHCO_3$, S5761), and dexamethasone (D8893) were purchased from Sigma–Aldrich (Munich, Germany). Fetal bovine serum (FBS, 10270-106) and antibiotic–antimycotic (A/B, 15240-062) were purchased from Invitrogen (Karlsruhe, Germany). MTS reagent was purchased from Promega (Southampton, UK).

2.2. κC hydrogel preparation

An aqueous solution was prepared by dissolving the κC powder (herein referred as κC) in distilled water and heating at 60°C while stirring constantly from 30 Min until complete and homogeneous dispersion of the material was observed. The 1.5% (w/v) hydrogel solution was sterilized by steam power for 30 Min at 120°C. The samples were produced in the form of discs using cylinder moulds, and a complete gelation was achieved with KCl immersion for 10–15 Min at room temperature to stabilize the 3D structure. Afterward, the gels were washed with PBS to remove the excess of ions. Water content, degradation rate, and cell culture experiments were all performed using hydrogel discs with $Ø\,5 \pm 0.01 \times 2.5 \pm 0.46$ mm^2 height.

2.3. Cell isolation and expansion

Human-adipose-derived stem cells were obtained from liposuction procedures, under protocols established with the Plastic Surgery Department, Hospital da Prelada (Porto, Portugal), and isolated by enzymatic digestion, as previously described [22]. Briefly, the adipose tissue samples were digested with 0.2% collagenase type II (C6885; Sigma–Aldrich) in PBS for 45 Min at 37°C under gentle stirring. The digested tissue was filtered with a 100-μm filter mesh (Sigma–Aldrich) centrifuged at 290*g* for 10 Min at 20°C, and the cell suspension solution was washed 5 Min with lysis buffer to remove the erythrocytes. The adherent hASCs were expanded in minimum essential medium (MEM) alpha medium (12000-063; Invitrogen, Grand Island, NY, USA) with 10% (v/v) FBS, 1% A/B, and sodium bicarbonate with media changes every 3 days. Nasal cartilage was harvested from the nasal septum of adult patients undergoing reconstructive surgery under a protocol established with Hospital de São Marcos (Braga, Portugal). The chondrocyte isolation procedure followed a protocol presented elsewhere [23]. Briefly, the human nasal septum cartilage tissue was cut into pieces, washed in sterile PBS solution, and incubated in 20 mL of trypsin–ethylenediaminetetraacetic acid (DMEM) solution for 30 Min at 37°C on agitation. Then, trypsin was removed and 20 mL of filter-sterilized collagenase type II solution (2 mg/mL) in basic medium was added, and the mixture was incubated for 12 H. The digested tissue and cell suspension solution was centrifuged at 200*g* for 7 Min and the supernatant was removed. The cell pellet was washed with lysis buffer, centrifuged several times, and finally resuspended in expansion medium consisting of DMEM—high glucose (5671; Sigma, St. Louis, MO,

USA), containing 10 mM HEPES buffer (H403475, pH 7.4; Sigma), 1% A/B, 20 mM L-alanyl glutamine (G8541; Sigma), 1× MEM nonessential amino acids (11140-035; Sigma), and 10% (v/v) FBS supplemented with 10 ng/mL basic fibroblast growth factor (AF100-18B; PeproTech, London, UK). ATDC5 (cell line mouse 129 teratocarcinoma AT805 derived, ECACC, Porton Down, Wiltshire, UK) cells were expanded in Ham's F-12 cell culture medium (D-MEM/F-12 [1:1], 42400-028; Gibco, UK) supplemented with 10% FBS, 2 mM L-glutamine (G854; Sigma), and 1% antibiotic solution until obtaining the necessary number of cells for the experiments.

2.4. Hydrogel characterization

2.4.1. Water content and degradation rate

Hydrogel samples (111.15 ± 12.66 mg) prepared according to the procedure described above and accurately weighed (w_s) were incubated for 7, 14, and 21 days in fresh culture medium and PBS buffer. Simultaneously, samples of hydrogels loaded with hASCs, human nasal chondrocytes (hNCs), and ATDC5 (prepared as described in the section below) were immersed in specific culture medium for each type of cell and incubated at 37°C for the same time periods. At predetermined time intervals, the medium/PBS was removed from the samples, the hydrogels were lyophilized, and dry weights were measured (w_d). The water content of hydrogels was calculated from the equation $(w_s - w_d)/w_s \times 100$. The degradation rate was defined as the time needed for the gel to degrade, and for calculation, we considered Eq. (1). The medium was replaced twice a week and the studies were performed in triplicate.

$$\text{Degradation rate (\%)} = \frac{\text{Final weight}}{\text{Initial weight}} \times 100 \qquad (1)$$

2.4.2. Cell encapsulation into κC hydrogels

The κC hydrogels were produced using the ionotropic gelation method described above, and cells—namely, ATDC5, hNCs, and hASCs—were encapsulated at a density of 5×10^6 cells/mL and cultured for 7, 14, and 21 days. The mixture composed of the κC aqueous solution, and the cell suspension was resuspended until complete homogenization. Hydrogel samples loaded with different cell types were prepared using sterile polystyrene cylinder moulds. Discs with encapsulated hASCs were cultured in chondrogenic differentiation medium composed of DMEM—low glucose (D5523-DMEM; Sigma), supplemented with 1% A/B, ITS + 1 liquid media supplement (I2521-insulin–transferrin–selenium; Sigma), 17 mM L-ascorbic acid (A4544; Sigma), 0.1 M sodium pyruvate (P4562; Sigma), 35 mM L-proline (P5607; Sigma), 1 mM dexamethasone, and 10 ng/mL of human transforming growth factor-β1 (TGF-β1, 14-8348; eBioscience, Ireland, UK). Encapsulated hNCs and ATDC5 were cultured in the previously mentioned culture mediums. Additional controls consisted of κC hydrogel samples without cells, kept in the same culturing conditions for the selected time periods. At the end of each time of culture, the samples were retrieved, washed with PBS solution, and further processed according to the characterization assays to be performed, as described below.

3. Biological evaluation

3.1. Cell morphology and viability [calcein acetoxymethyl ester (AM) assay]

The morphology of the different cells before encapsulation was examined using an inverted light microscope (Axiovert 40 CFL, 459306; Zeiss, Jena, Germany), and images were obtained using a digital photo camera (PowerShot G6; Canon, Tokyo, Japan). The viability of the cells encapsulated in the κC hydrogel along the culture period was assessed using the Calcein AM assay. For this purpose, Calcein AM dye (C3099; Invitrogen) solution of 1/1,000 was prepared in the culture medium. At the end of each time point of the study, the κC hydrogel samples with encapsulated cells were collected from the culturing plates and incubated in the calcein AM solution for 15–30 Min at 37°C and afterwards washed in sterile PBS. The stained samples were placed on a microscope slide and observed under a fluorescence microscope with a 485 ± 10 nm optical filter (reflected/transmitted light microscope; Zeiss).

3.2. Metabolic activity: MTS assay

The metabolic activity of hASCs, hNCs, and ATDC5 cells encapsulated in κC hydrogel and cultured for the predetermined time points was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (MTS; Promega, Madison, WI, USA), which is based on bioreduction of the substrate into a brown formazan product by dehydrogenase enzymes in metabolically active cells and is commonly used for cell viability evaluation. Briefly, cell–hydrogel constructs ($n = 9$) were washed in PBS and placed in a mixture containing serum-free cell culture medium DMEM (without phenol red) and MTS reagent in a 5:1 ratio and incubated for 3 H at 37°C in a humidified atmosphere containing 5% CO_2, at the end of which 100 μL was transferred to 96-well plates and the optical density was determined at 490 nm.

3.3. Cell proliferation: DNA quantification

The proliferation of the encapsulated cells in the κC hydrogels was assessed using a fluorimetric double-strand DNA (dsDNA) quantification kit (P7589-PicoGreen, Molecular Probes; Invitrogen, Paisley, UK). For this purpose, samples collected on 0, 7, 14, and 21 days of culturing were properly washed in PBS and then transferred into 1.5-mL microtubes containing 1 mL of ultrapure water. Prior to dsDNA quantification, cell–hydrogel constructs and the sample controls (hydrogel samples without cells) were thawed and sonicated for 15 Min. Samples and standards (ranging from 0 to 2 μg/mL) were prepared and mixed with a PicoGreen solution (Invitrogen) in a 200:1 ratio and were added to a 96-well opaque white plate. Each sample or standard was made in triplicate. The procedure followed can be found elsewhere [24] and was based on manufacture instructions. The plate was incubated for 10 Min in the dark, and fluorescence was measured on a microplate reader (Synergy HT;

BioTek, Winooski, VT, USA) with an excitation of 485/20 nm and an emission of 528/20 nm. A standard curve was created, and sample DNA values were read from the standard graph.

3.4. Typical proteoglycans staining—Alcian blue and Safranin O

Alcian blue and Safranin O staining were used to evaluate cartilage extracellular matrix component deposition—namely, GAGs—on samples that were collected at the end of the experiment (21 days of culture). Alcian blue (A3157; Sigma) staining was performed by incubating the monolayer cells or the cell-laden hydrogels for 30 Min. After that, the stain was poured off and the samples were washed and dehydrated. The Safranin O staining consisted of staining the cells with Weigert's iron hematoxylin working solution for 7 Min and 0.1% Safranin O for 5 Min. Samples were washed after each staining step, left to air dry, and then rinsed in absolute alcohol. Stained cells were observed under a light microscope (reflected/transmitted light microscope; Zeiss), and images were captured using a camera (Axion MRc5; Zeiss).

3.5. RNA isolation and real-time qRT-PCR analysis of chondrogenic markers

The messenger RNA (mRNA) expression of chondrogenic genes (Sox9, aggrecan, collagen type I, COL2, and collagen X) by the different cell types studied encapsulated in κC hydrogels was quantified by real-time qRT-PCR analysis. Total RNA was extracted using TRI Reagent® RNA isolation reagent (T9424; Sigma) following the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 2 μg of RNA of each sample reverse transcribed with qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) in a 40 μL reaction using a MJ Mini™ Personal Thermal Cycler (Bio-Rad Laboratories, Berkeley, CA, USA) machine. Real-time qRT-PCR was performed to detect amplification variations using PerfeCTa® SYBR® Green FastMix®, (Quanta Biosciences) on Eppendorf Mastercycler® ep realplex gradient S machine according to manufacturer's instructions in a 20 μL reaction containing 2.5 μL of each primer. The relative quantification of the gene expression was calculated using the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ method [25]. The mRNA expression levels of target genes were normalized to the average expression of endogenous housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) value. Each quantitative real-time PCR run was carried out with an initial incubation at 95°C for 2 Min, followed by 45 cycles of denaturation (95°C, 30 Sec), corresponding annealing temperature for 30 Sec and extension step at 72°C for 30 Sec. A melting curve of 21 Min and a hold step at 5°C was performed at the end.

3.6. Statistical analysis

Sets of triplicates were expressed in mean ± SD for water content and degradation rate ($n = 3$), MTS, DNA ($n = 9$), and qRT-PCR data ($n = 3$). First, a Shapiro–Wilk test was used to ascertain the data normality. The results indicated that at the 0.05 level, the data were significantly drawn from a normally distributed population, and one-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significant differences between groups and conditions. The difference was considered significant when $P < 0.05$ [26].

4. Results and discussion

Tissue engineering is an emerging approach for the regeneration of damaged cartilage tissue due to disease or trauma [27] and because cartilage lacks self-regenerative ability, it is essential to develop methodologies for delivery of clinically relevant number of cells and/or cytokines and growth factors to the defect site. It is well known that in many situations, cells alone are not able to efficiently initiate a regeneration process once implanted, due to the dispersion or immune system rejection. Cell encapsulation techniques through hydrogel systems confer the means to overcome some of these problems [28]. The motivation of using a κC hydrogel as a cell support was to take advantage of its intrinsic properties and composition, being a hydrophilic polysaccharide with high swelling properties, characterized by its sulfate group's content, similar to GAG constituents. Additionally, it is a thermoreversible and an ionic gel with the ability to form hydrogels at conditions that allow *in situ* gelation and direct encapsulation of cells. These hydrogels have been investigated as drug delivery or growth factor delivery systems [29–31] for immobilization of enzymes [32], but have been very little explored until the present time for cartilage restoration [33]. In this study, we aimed at analyzing the behavior of κC hydrogels as encapsulation systems for adipose stem cells and primary chondrocytes.

4.1. Water content and degradation rate

In addition to the increase in the mass of the hydrogels, high water content ability may influence the biological behavior of the cells. The matrix environment, in which cells are grown, influences the type and extent of cellular response, which in turn affects cell viability and proliferation. Therefore, the water content/degradation rate of the developed hydrogels was evaluated in simulated physiological conditions, as the diffusion, exchange of nutrients, and waste throughout the entire hydrogel are related to the level of water being substituted with the surrounding medium. κC has an abundant number of hydrophilic groups, such as sulfate groups, which can promote a high water uptake in the structure. No previous dehydration of the hydrogels was performed, envisioning a better simulation of their application at the *in vivo* scenario. Significantly higher water content was observed for the hydrogels immersed in PBS as compared with the hydrogels dipped in DMEM/10% FBS (Fig. 1A) after 21 days, possibly due to the ionic composition of the solutions. Previous research studies have shown that the swelling behavior of hydrogels depends on the external environment such as ionic strength, temperature, and pH [34]. The high water content of the hydrogels immersed in PBS could relate to the higher exchange between the ions from the medium and the potassium ions needed for the cross-linking of

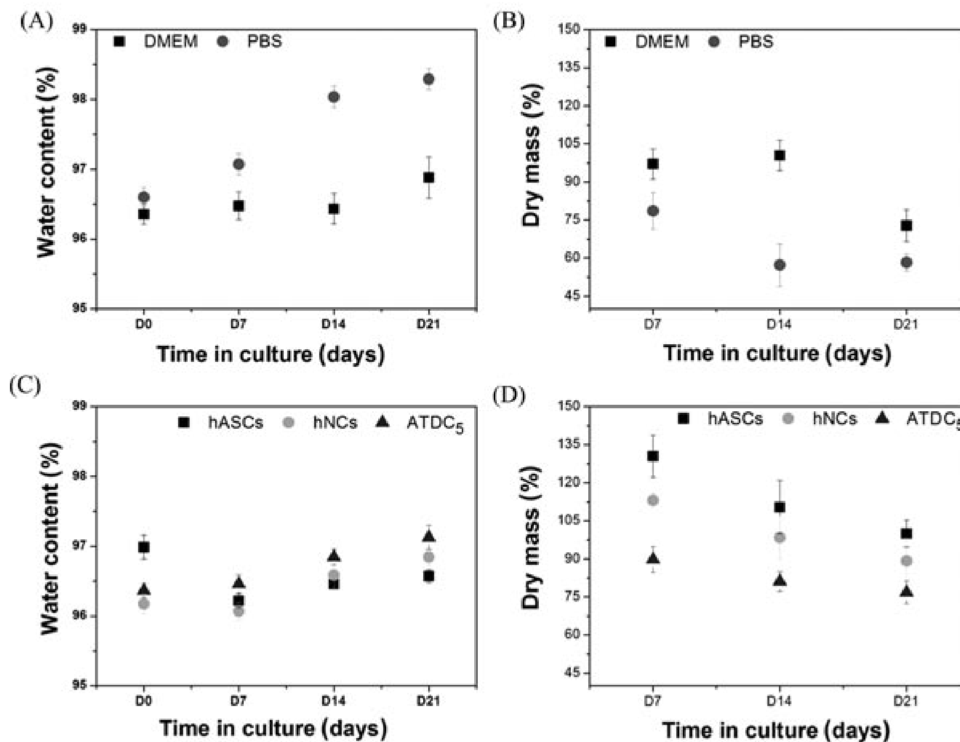

Fig. 1. (A) Water content and (B) degradation rate of κC hydrogels as a function of time, registered after immersion in DMEM/10%FBS and in PBS solution at 37°C and pH 7.4. (C) Water content and (D) degradation rate of κC hydrogels with encapsulated cells (hASCs, hNCs, and ATDC5) versus immersion time in culture medium (specific for each type of cell). Values reported as averages ($n = 3$) ± SD.

κC hydrogel. Thus, the lower water content registered for the hydrogels exposed to DMEM/10%FBS can be explained by the lower ionic exchange, but also due to possible binding of the FBS proteins present in the culture medium to the κC hydrogel sites. Nevertheless, all the hydrogel samples exhibited water content superior to 98% either in DMEM/10% FBS or in PBS medium. The degradation experiments were performed at neutral pH, resembling physiological conditions, (Fig. 1B) and, in general, the results show a higher decrease in dry mass for hydrogels in PBS as compared with hydrogels incubated in DMEM/10% FBS, in agreement with the water uptake results. Water content and degradation of the hydrogels with encapsulated cells were also evaluated after culturing for 7, 14, and 21 days at 37°C (Figs. 1C and 1D). The highest water uptake was registered for κC laden with ATDC5 cells, followed by hydrogels loaded with hNCs and hASCs. The mass-loss difference found between the different cell types could be associated with the particular stage of their growth cycle at each time point analyzed. For hASCs, this behavior could be linked to a higher extracellular matrix formation because the stem cells undergo differentiation stimulated by the culturing media. In fact, cell encapsulation and extracellular matrix deposition may result in progressive increase in the mass or in the mechanical properties for the hydrogels, as shown before for other systems [35]. In this study, this was corroborated with the qRT-PCR data (see below), as we observe an increase in the aggrecan and collagen gene expression for hASCs and hNCs [demonstrating higher extracellular matrix (ECM) deposition] and the lowest expression for ATDC5 cells.

4.2. Cell morphology and distribution

Adult stem cells have recently received great attention as an alternative resource to overcome the limited supply, as well as the dedifferentiation process often observed in primary chondrocytes *in vitro* expanded in monolayer. The use of cell lines in cell-based therapies is not considered safe, as these cells are often derived from tumors and typically accumulate genetic changes with increasing passage levels [36]. Optical microscopy was used to analyze hASCs, hNCs, and ATDC5 cell morphology in monolayer after 7 days in culture (Fig. 2A). Images depict the morphology of the different cell types—hASCs and hNCs presenting a fibroblast-like appearance due to chondrogenic phenotype instability, and ATDC5 cells exhibiting an extended, polygonal shape [37]. In monolayer culture, ATDC5 cells seemed to achieve confluence faster than hASCs and hNCs. Once different cells were encapsulated in the same hydrogel, the cell behavior was expected to be different, independently of additional influence of the hydrogel matrix. Fluorescence microscopy images of the cell-laden hydrogels are presented in Fig. 2B. The viability of encapsulated cells within the polysaccharide hydrogel was confirmed by the positive cell staining with calcein AM. The fluorescence images also show homogeneous distribution

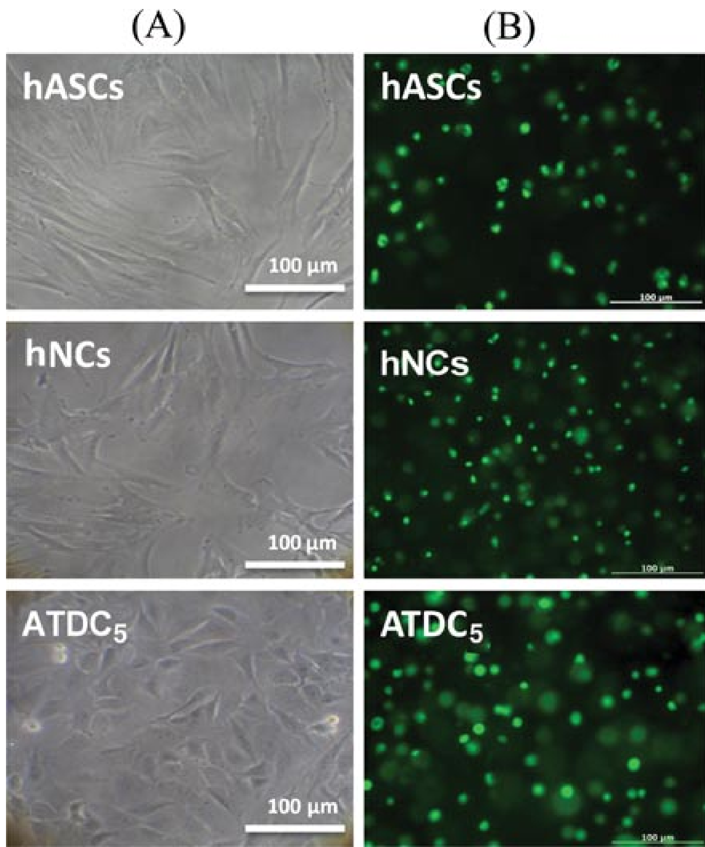

Fig. 2. (A) Micrograph images obtained from 2D-cultured cell monolayer of hASCs, hNCs, and chondrogenic cell line after 7 days in expansion; magnification 20 × with 100 μm scale bar. (B) Calcein AM staining of hASCs, hNCs, and ATDC5 encapsulated in κC hydrogels on day 7 of culture; magnification 20 × with 100 μm scale bar.

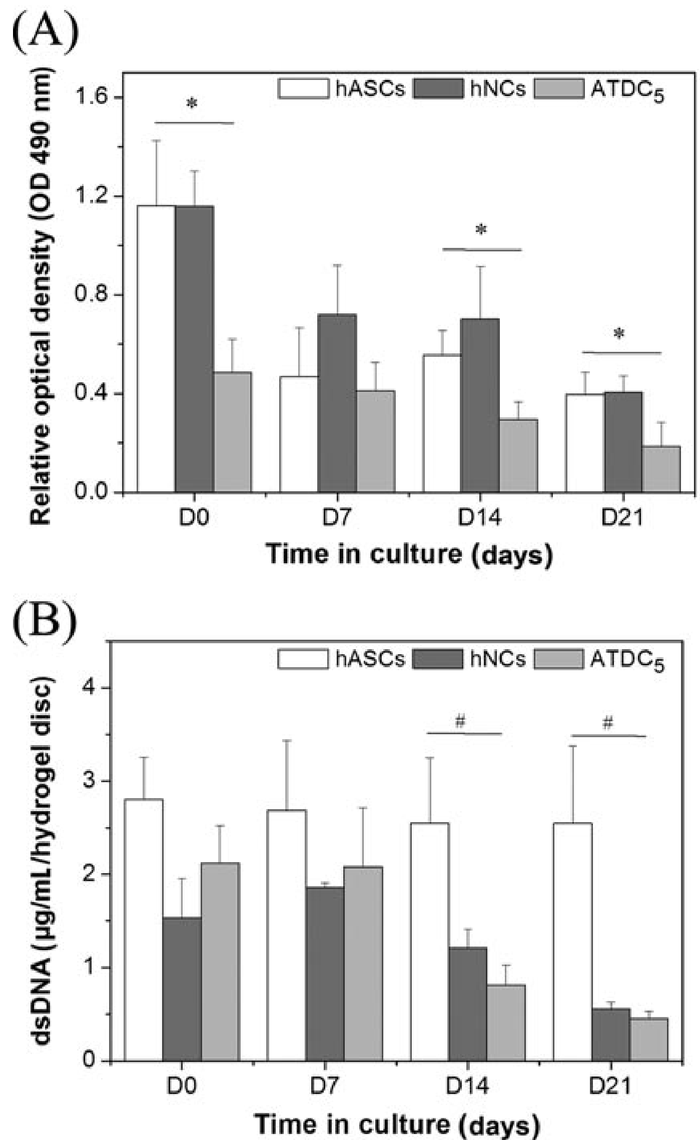

Fig. 3. (A) Metabolic activity results based on MTS test performed on 0, 7, 14, and 21 days of culture. (B) Cell content obtained from by DNA quantification of hASCs, hNCs, and ATDC5 encapsulated in κC hydrogels and cultured for 21 days in specific mediums. Error bars represent means ± SD ($n = 9$). The statistics using one-way ANOVA indicate that the mean difference is significant at the 0.05 level ($P < 0.05$). The symbol * indicates statistical significance between hASCs or hNCs and ATDC5 cell type in terms of metabolic activity response, and # implies statistical significance on day 14 and 21 between hASCs and hNCs or ATDC5 in the proliferation rate.

of all cells encapsulated in the hydrogels, whereas hASCs depicted a tendency to form cell clusters.

4.3. Metabolic activity and proliferation of encapsulated cells

Viability of encapsulated cells within κC hydrogels during 3 weeks of culture was assessed using the MTS assay. In general, a decreased viability was observed over time for all types of cells encapsulated in κC hydrogels (Fig. 3A), as it is typically observed in cells encapsulated in hydrogels [38–40]. This drop in cell proliferation could be due to, at least partially, the fact that cells are being released from the hydrogel systems during culturing, owing to degradation of the hydrogels. Furthermore, studies report that ions needed for hydrogel formation may influence the cell viability/proliferation and that low concentration has higher cell viability [41],[42]. Thus, remaining potassium ions could have also contributed to the decrease in cell viability. The effect of hydrogel concentration and thus its stiffness is also thought to affect encapsulated cells because the inverse dependency between permeability and stiffness is known [43]. The data obtained also demonstrated that hASCs and nasal chondrocytes, once encapsulated within the biodegradable hydrogel, are more metabolically active than ATDC5 cells ($P < 0.05$). DNA quantification (Fig. 3B) shows that hASCs encapsulated in κC hydrogels exhibit higher cell content than hydrogels laden with hNCs and ATDC5. This difference was found to be statistically significant ($P < 0.05$) at 14 and 21 days of

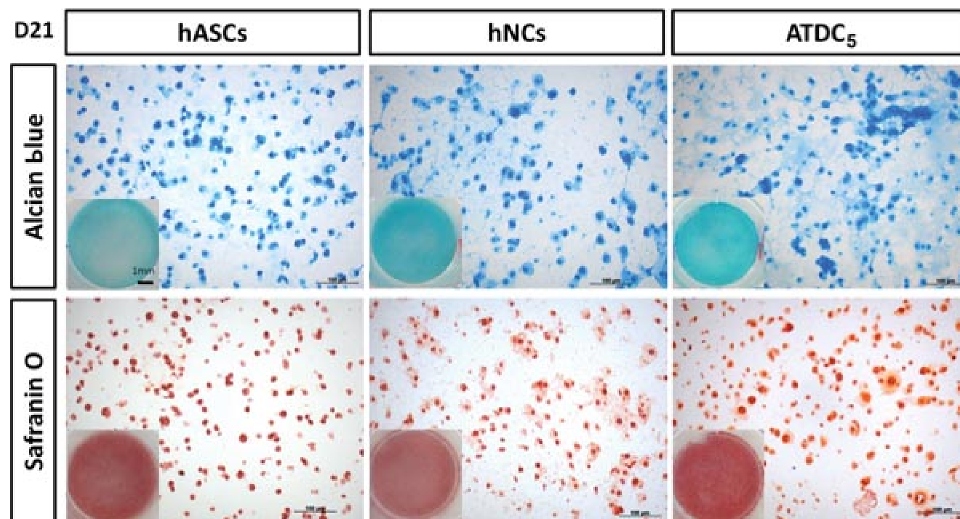

Fig. 4. Optical microscopic images of histological sections obtained from κC hydrogels with encapsulated cells, collected after 21 days of culture and stained with Alcian blue and Safranin O. The scale bar corresponds to 100 μm and the magnification used was 20 ×. Embedded pictures correspond to light microscopy images of stained hASCs, hNCs, and ATDC5 cell monolayers after culturing for 3 weeks.

culture. There are several hypotheses for the observed differences among the cells used. Each cell type was incubated in a different medium; therefore, we cannot rule out the effect of the cell culture medium and added supplements. This is probably related to the different growth profile of the different cells analyzed. Cell metabolic activity and proliferation data clearly indicate that κC, an easy-to-handle, simple, and nontoxic hydrogel formed under mild conditions with *in situ* gelation properties, sustains cell growth and proliferation for a long time in culture using different cell types.

4.4. GAG deposition

The GAG deposition in the hydrogels laden with the different cell types was evaluated by histological staining. Light microscopic images of sample sections obtained from hydrogels with encapsulated hASCs, hNCs, or ATDC5 cells stained with Safranin O and Alcian blue revealed positive staining for both cartilage ECM markers, confirming the extracellular matrix secretion by all the cell types embedded in the κC hydrogel, after 3 weeks of culture (Fig. 4). It is known that the extracellular matrix is not a static structure, but a dynamic network of molecules secreted by cells [44],[45], and thus the staining indicated a progressive increase in intensity over the period of culture (data not presented). The cell concentration chosen for the cell experiments was 5×10^6 cells/mL, based on previous studies [46], [47] and was the same for all the cell types investigated, to allow a more straightforward comparison among the outcomes produced by the different cell types [48]. Nevertheless, cell density has an important role in extracellular formation and may affect differently the different cell types used herein. It was noteworthy that the distribution of the cartilage matrix in the hydrogels was homogeneous for all cell types. Furthermore, ASCs exhibited stronger staining intensity, clearly demonstrating the chondrogenic differentiation undergone by these cells embedded in κC and stimulated by the chondrogenic supplements in the culture media. The round-shape cell morphology observed is a further indication of chondrocyte phenotype achieved by hASCs [49]. These results show that the κC hydrogel is not only acting as a cell vehicle but is also supporting cell functionality—namely, the chondrogenic differentiation—suggesting that this material can actually mimic ECM properties to a certain range, rendering it attractive for cartilage regeneration strategies.

4.5. Real-time qRT-PCR

The results obtained from the genotypic quantification of cartilage-related genes for hASCs, hNCs, and ATDC5 after 7, 14, and 21 days of culture within κC hydrogels are presented in Fig. 5. The relative expression of the cartilage-specific genes was normalized against the housekeeping gene *GAPDH* and compared with the initial culture time. A constitutive expression of all mRNA transcripts [*i.e.*, SOX9, aggrecan (AGCR), collagen type I (COL1), COL2 (COL2), and collagen type X (COLX)] was found during the 21 days of culture. In general, hASCs encapsulated in κC showed higher levels of all markers analyzed, except collagen X, as compared with hNCs and ATDC5 cells. Specifically, the mRNA levels in hASC cells for day 21 in culture were found to be upregulated for the following molecules: SOX9 (39.72-fold; $P < 0.05$), AGCR (53.57-fold; $P < 0.05$), type I collagen (10.35-fold; $P < 0.05$), type II collagen (94.23-fold; $P < 0.05$), and type X collagen (5.7-fold; $P < 0.05$). SOX9 followed a particular high expression pattern, increasing until the last day (Fig. 5A). It has been demonstrated that a highly expressed SOX9 stimulates the production of AGCR and COL2 [50]. In fact, the results obtained from statistical analysis of the AGCR expression by hASCs showed a significant increase ($P < 0.05$; Fig. 5B) between 14 and 21 days of culture. Along with aggrecan [51], COL2 is a major structural component of cartilage, particularly articular

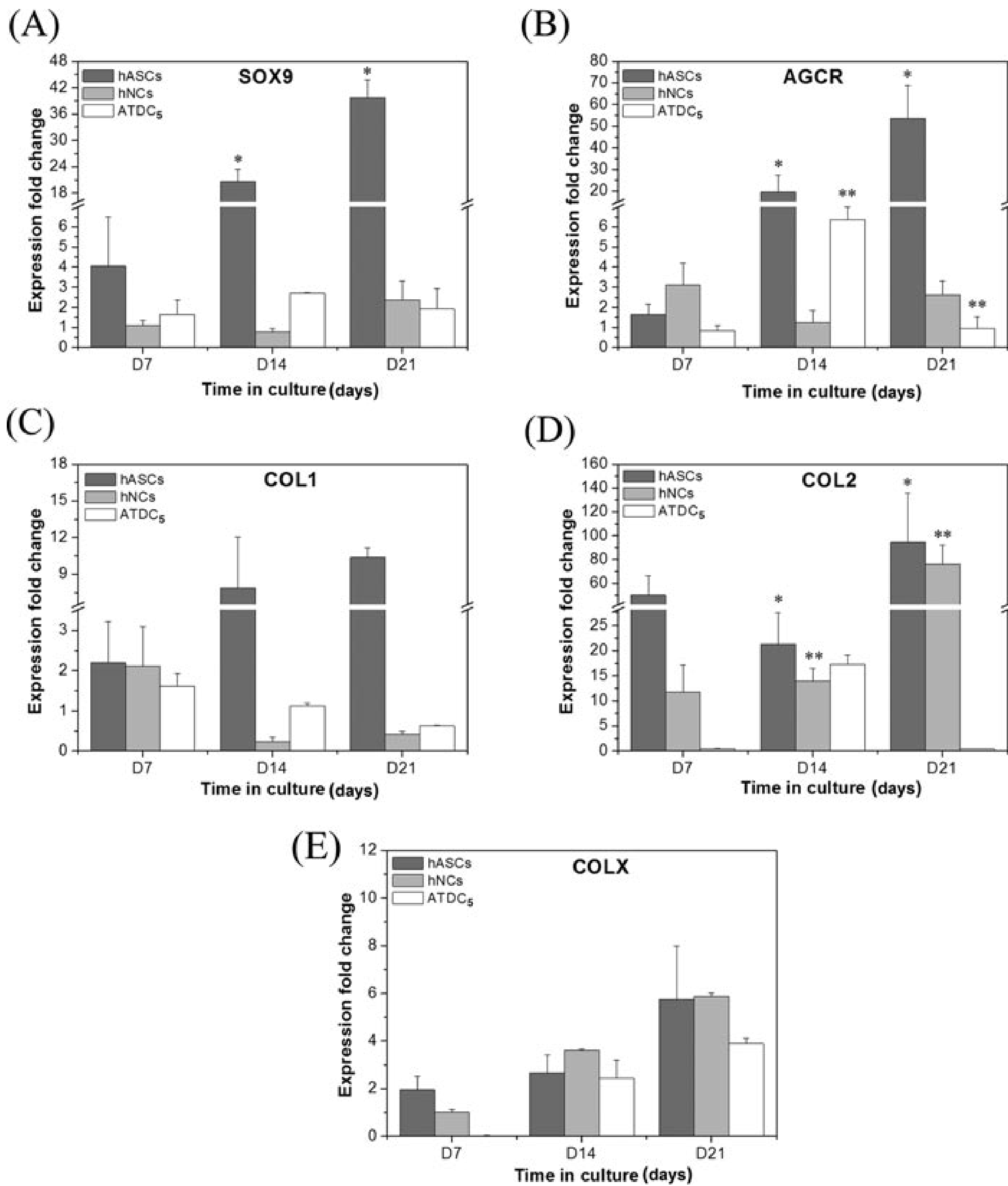

Fig. 5. Real-time analysis of chondrogenic-specific gene expressions—namely, (A) SOX9, (B) aggrecan, (C) collagen type I, (D) COL2, and (E) collagen type X—based on the mRNA produced by the encapsulated cells after 7, 14, and 21 days of culture. The expression of these genes was normalized against the housekeeping gene *GAPDH* and calculated by the Livak method. Error bars represent means $\pm$ SD. Statistical analyses were conducted using one-way ANOVA for $n = 3$; $P < 0.05$.

cartilage [52]. COL2 is the most important protein produced by chondrocytes, and the tensile property of cartilage depends on that, whereas aggrecan is responsible for the compressive properties [53]. The expression of the COL2 transcript was detected for all time points, with low values for day 14 and a significant increase for day 21 ($P < 0.05$; Fig. 5D). Even if COL2 exhibits a decreased expression on day 14, aggrecan gene expression has an increase, as these chondrogenic markers interplaying together are being expressed differently during chondrogenic differentiation. In contrast, for hASCs, collagen type I expression showed no significant increases between day 14 and 21 of culture (Fig. 5C), suggesting that an elastic cartilage-like tissue was formed (characterized by high expression of COL2) instead of fibrocartilage, usually characterized by the expression of collagen type I. In this system, hASCs were likely stimulated down the chondrogenic pathway by TGF-β1 growth factor present in the chondrogenic medium [54]. A significant increase in the mRNA levels of *COL2* gene was registered for hNCs during the 21 days of culture ($P < 0.05$), although at lower levels as compared with the increase in expression for the stem cells (Fig. 5D).

The ATDC5 cells showed a high expression for *COL2* gene and a low upregulation for AGCR on day 14, but no significant levels of the remaining genes analyzed were recorded (Figs. 5D and 5B). Interactions with κC matrix may have subsequently guided cell behavior favoring chondrogenic induction, thus explaining the regulation in *COL2* gene expression among different cell types, as suggested by other work [55]. Collagen type X is expressed in identical level values for all types of cultures and all time points (Fig. 5E). All cell types show an increased expression marker of this gene, frequently associated with the dedifferentiation of chondrocytes, suggesting that increasing the culturing time may compromise the functionality of these cells [56],[57]. ATDC5 culture produces chondrocyte maturation and apoptosis, indicating that this cell line is also suitable to study *in vitro* the mineralization process [58]. In summary, the data gathered from real-time RT-PCR analysis demonstrated that hASCs and nasal chondrocytes lead to better results in terms of chondrogenic potential when compared with ATDC5 cells. Moreover, hASCs showed higher phenotypic and functional characteristics against hNCs, demonstrating that these cells could be an alternative to the autologous approach already used in clinical applications. The obtained results also indicate that κC hydrogels are able to stimulate specific cellular responses at the molecular level when the encapsulated cells present a different growth profile with respect to early and late cellular events in the growth profile of cells.

5. Conclusion

The κC system enabled the viability and proliferation of different cells during long-term cell culture and showed its efficiency to support the production of an organized extracellular matrix and other chondrogenic features, as well as its ability to support the chondrogenic differentiation of human adipose stem cells. In fact, hASCs showed a better cellular response when encapsulated in κC hydrogels than primary chondrocytes obtained from nasal septum cartilage. Taking into account these findings, together with their wider availability and easier harvesting, it is possible to conclude that hASCs could serve as an alternative cell source to chondrocyte transplantation therapy. Altogether, the results obtained from this study clearly indicate the great potential for the application of κC laden with hASCs in cartilage regeneration.

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