REGULAR ARTICLE

Effect of reduced oxygen tension and long-term mechanical stimulation on chondrocyte-polymer constructs

Ellen Wernike · Zhen Li · Mauro Alini · Sibylle Grad

Abstract We have investigated the influence of long-term confined dynamic compression and surface motion under low oxygen tension on tissue-engineered cell-scaffold constructs. Porous polyurethane scaffolds (8 mm × 4 mm) were seeded with bovine articular chondrocytes and cultured under normoxic (21% O_2) or hypoxic (5% O_2) conditions for up to 4 weeks. By means of our joint-simulating bioreactor, cyclic axial compression (10–20%; 0.5 Hz) was applied for 1 h daily with a ceramic ball, which simultaneously oscillated over the construct surface (±25°; 0.5 Hz). Culture under reduced oxygen tension resulted in an increase in mRNA levels of type II collagen and aggrecan, whereas the expression of type I collagen was down-regulated at early time points. A higher glycosaminoglycan content was found in hypoxic than in normoxic constructs. Immunohistochemical analysis showed more intense type II and weaker type I collagen staining in hypoxic than in normoxic cultures. Type II collagen gene expression was slightly elevated after short-term loading, whereas aggrecan mRNA levels were not influenced by the applied mechanical stimuli. Of importance, the combination of loading and low oxygen tension resulted in a further down-regulation of collagen type I mRNA expression, contributing to the stabilization of the chondrocytic phenotype. Histological results confirmed the beneficial effect of mechanical loading on chondrocyte matrix synthesis. Thus, mechanical stimulation combined with low oxygen tension is an effective tool for modulating the chondrocytic phenotype and should be considered when chondrocytes or mesenchymal stem cells are cultured and differentiated with the aim of generating cartilage-like tissue in vitro.

Keywords Chondrocytes · Mechanical loading · Oxygen tension · Cartilage tissue engineering · Collagen type I · Bovine

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Introduction

Hyaline cartilage is the dense tissue that covers articulating surfaces and confers low friction and durable wear resistance. Furthermore, it minimizes the stress on subchondral bone during peak loading. These properties are based on the composition of glycosaminoglycan (GAG) and collagen (primarily type II), which are arranged in a three-dimensional (3D) matrix/network. Chondrocytes represent the cell type of cartilaginous tissue. They form,

maintain, and remodel the tissue and its structure through their biosynthetic activities.

Cartilage is a hypocellular, avascular, alymphatic tissue of minimal healing capacity. Degradation may result from various factors including metabolic or genetic disorders, mechanical stress, or trauma. Common surgical treatment methods, such as mosaic-plasty, autologous chondrocyte implantation, or matrix-induced autologous chondrocyte implantation, generally fail to entail complete restoration of hyaline cartilage. Therefore, recent research has focused on new tissue-engineering approaches for clinical application (Freed and Vunjak-Novakovic 2000).

Exposure to mechanical forces that mimic physiological conditions can enhance the biochemical and mechanical properties of engineered cartilage-like tissue and has shown promising results (Lee et al. 2004). The most appropriate type of stimulation is dynamic loading. Contrary to static compression, which has shown suppressive effects, dynamic loading can stimulate cartilage matrix synthesis (Lee et al. 2004). For instance, application of dynamic compressive loading at levels of 2%–10% strain and physiological frequencies (0.01 to 1 Hz) can enhance collagen and proteoglycan levels (Wong et al. 1999; Larsson et al. 1991). Furthermore, fluid flow is one of the factors that can stimulate cell metabolism through shear stress or can increase the nutrition supply through compression (Vunjak-Novakovic et al. 1999; Saini and Wick 2003; Raimondi et al. 2002; Waldman et al. 2003). Moreover, static hydrostatic pressure can increase aggrecan and collagen II mRNA levels of gel-embedded bovine chondrocytes (Toyoda et al. 2003a), although proteoglycan synthesis is inhibited in bovine explants (Hall et al. 1991). Cyclic hydrostatic pressure has been shown to increase the proteoglycan content in bovine and human explants and in bovine and human monolayer cultures (Hall et al. 1991; Parkkinen et al. 1993; Scherer et al. 2004; Ikenoue et al. 2003; Smith et al. 1996, 2000).

The effect of physical cell stimulation depends on a variety of factors, including short- or long-term study, type and parameters of loading, the biomaterial used, and the source of cells (Lee et al. 2004). Articular cartilage in vivo is exposed to a complex interaction of physical forces, and thus the loading experienced by natural joints is more complicated than simple compression, shear, or pressurization. To address this combined interplay of joint-level compression, shear, and articular motion, we have developed a cartilage bioreactor that allows for simultaneous compression, shear, and articular fluid transport of developing constructs (Wimmer et al. 2004; Grad et al. 2005, 2006).

The oxygen concentration is an additional factor that could influence the outcome of engineered cartilage. Physiologically, oxygen diffuses from the synovial fluid through the tissue and reaches tensions of around 10% in the superficial zone and less than 5% in the deep zone (Zhou et al. 2004). Recent studies have shown a beneficial effect of low oxygen tension, which mimics the in vivo environment, on the development of tissue-engineered cartilage. Generally, low oxygen culture has been recognized as an effective means to control oxidative stress and to increase the proliferation potential and biosynthesis of chondrocytes (Kurz et al. 2004; Saini and Wick 2004; Scherer et al. 2004; Moussavi-Harami et al. 2004).

The feasibility of biodegradable porous polyurethane scaffolds as 3D carriers to support chondrocyte adhesion, growth, and matrix production has previously been reported (Grad et al. 2003a). Biodegradable polyurethanes have the advantage that their porosity, hydrophobicity, degradation time, and mechanical properties can be varied in order to meet the requirements of diverse clinical applications (Gorna and Gogolewski 2000). The particular interest of biodegradable polyurethanes with respect to cartilage tissue-engineering studies is their deformation capacity, elasticity, and resilience, which allow the application of mechanical forces to stimulate incorporated cells. In short-term studies, we have shown the beneficial effect of joint-specific motion regimes on chondrocyte-seeded scaffolds (Grad et al. 2005, 2006). Reducing the oxygen tension may have an additional advantageous effect on the proliferation and metabolism of chondrocytes (Moussavi-Harami et al. 2004; Malda et al. 2003; Murphy and Sambanis 2001). In this study, we have therefore investigated the influence of decreased oxygen tension on chondrocytes in free-swelling 3D cultures and the influence of long-term mechanical stimulation under various oxygen tensions in a 3D confined compression system. The aim of the confined compression design is to limit the fluid flow out of the construct and to build up a pressure gradient inside the construct. Furthermore, a confined system is more likely to retain the synthesized matrix molecules inside the scaffold during culture than is an unconfined setup.

Bovine articular chondrocytes were seeded into biodegradable porous polyurethane scaffolds and cultured in vitro for up to 34 days. The effect of mechanical loading and reduced oxygen tension was assessed by analyzing DNA and GAG content, gene expression, and histological preparations.

Materials and methods

Polyurethane scaffold

Cylindrical (8 mm×4 mm) porous polyurethane scaffolds were prepared as described elsewhere (Gorna and Gogolewski 2000). The scaffolds with interconnected pores had an average pore size of 90–300 μm, with interconnection sizes of 57.1±22.9 μm, and a pore-to-volume ratio of

85%. The polymers used for scaffold preparation were synthesized with hexamethylene diisocyanate, poly (ε-caprolactone)-diol with a molecular mass of 530 Da, and isosorbide-diol (1,4:3,6-dianhydro-D-sorbitol) as a chain extender. The hydraulic permeability of the resulting material was approximately 7.8×10^{-11} m^4/N per second and the compression modulus was 2.5 ± 0.15 MPa (Gorna and Gogolewski 2002, 2006). The scaffolds were sterilized in a cold-cycle (37°C) ethylene oxide process and subsequently evacuated at 45°C and 150 mbar for 3–4 days. Before cell seeding, the scaffolds were evacuated in the presence of growth media for 1 h in order to wet the hydrophobic polymer. Samples for loading were cemented into a centering holder with common bone cement (Norian SRS, Norian, Cupertino, Calif., USA) to ensure an axial application of load.

Chondrocyte isolation, seeding, and culture conditions

Chondrocytes were isolated from full-thickness metacarpal joint cartilage of 4– to 8-month-old calves by using sequential pronase and collagenase digestion (Grad et al. 2003b). Isolated chondrocytes were suspended in fibrinogen solution and then mixed with thrombin solution immediately prior to being seeded into the polyurethane scaffolds at a cell density of 5×10^6 per scaffold for oxygen experiments or 10×10^6 per scaffold for loading experiments.

Embedding the cells in fibrin gel has previously been shown to allow a homogeneous distribution of the cells throughout the scaffold (Lee et al. 2005a). The fibrin components were provided by Baxter Biosurgery (Vienna, Austria). The final concentrations of the fibrin gel were 17 mg/ml fibrinogen, 0.5 U/ml thrombin, and 665 kIU/ml aprotinin (Lee et al. 2005a). Constructs were incubated for 1 h (37°C, 5% CO_2, 95% humidity) to permit fibrin gelation.

In oxygen experiments, culture medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with antibiotics, 10% fetal calf serum (FCS), 50 µg/ml ascorbic acid, 40 µg/ml L-proline, non-essential amino acids, and 500 kIU/ml aprotinin) was added, and constructs were cultured under free-swelling conditions for 2, 14, or 28 days at 37°C, 5% CO_2, 95% humidity and either 5% or 21% O_2 in a CO_2-O_2 incubator (Cytoperm2, Thermo Scientific, Waltham, Mass.). In loading experiments, bovine nasal cartilage rings (3 mm thick) surrounding the scaffolds were added to the samples to provide a confined system (Fig. 1). Cells of the nasal cartilage had previously been lysed by repeated freezing-thawing cycles. The rings were cut co-planar according to the height of the scaffold. To prevent fluid flow out of the constructs during loading, possible gaps between the cartilage ring and centering holder and between the cartilage ring and construct were closed with fibrin. Culture media was then added. After a 6-day pre-
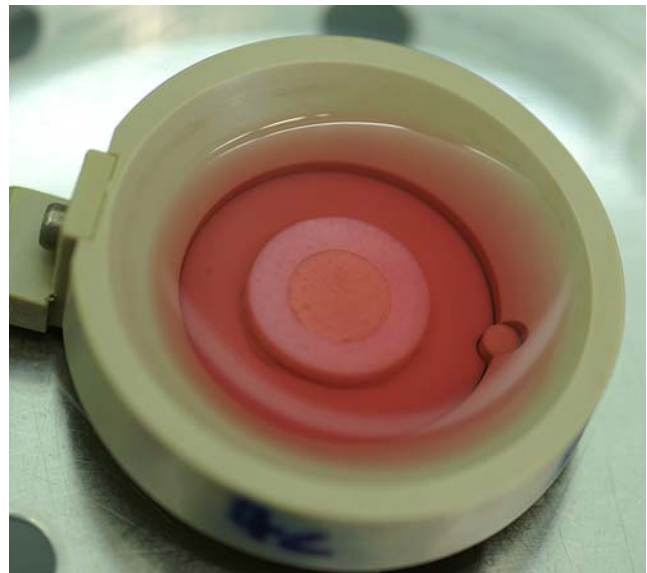

Fig. 1 Confined compression setup consisting of the cemented scaffold surrounded by a cartilage ring in the specimen holder

incubation, the samples were exposed to mechanical loading as described below. Loaded and free-swelling control constructs were cultured for another 2, 14, or 28 days at 37°C, 5% CO_2, 95% humidity and either 5% or 21% O_2, resulting in total culture periods of 8, 20, or 34 days.
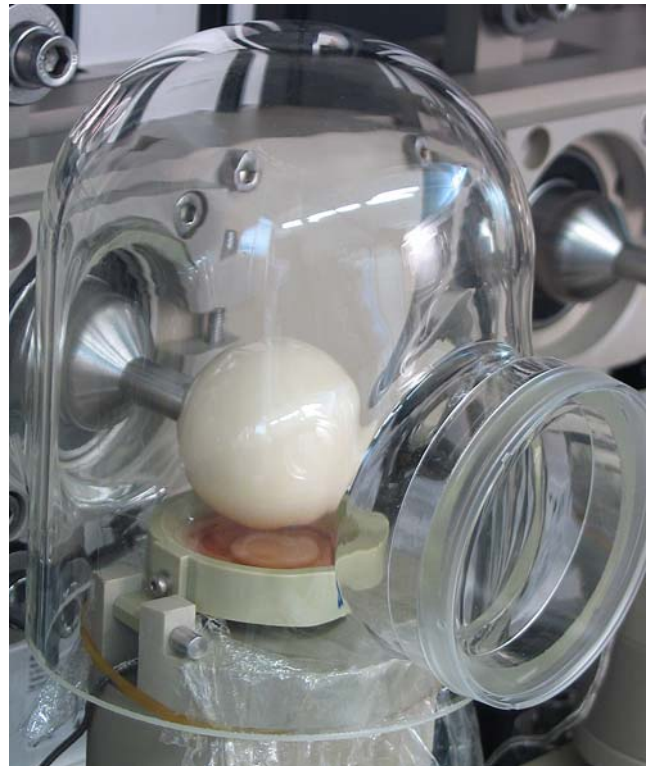

Fig. 2 One station of the four-station bioreactor that allows for application of joint-specific biomechanical stimuli to cell-seeded scaffolds

Mechanical loading

Mechanical conditioning of the cell-scaffold constructs was performed by using our bioreactor system, which was installed in a Cytoperm2 incubator at 37°C, 5% CO_2, 85% humidity, and either 5% or 21% oxygen tension (Fig. 2; Wimmer et al. 2004). Briefly, a commercially available ceramic hip ball (32 mm in diameter) was pressed onto the cell-seeded scaffold. Interface motion was generated by oscillation of the ball about an axis perpendicular to the scaffold axis. Superimposed compressive strain was applied along the cylindrical axis of the scaffold.

For each experiment, samples were assigned in duplicates to one of two groups. The loaded group was exposed to 1 h of mechanical conditioning daily over 6 days per week for an overall period of 4 weeks. Mechanical stimulation was applied in a displacement controlled manner with the starting point at the ball-scaffold contact. Accordingly, all displacements were related to the center of the scaffold. After application of a preload of 0.4 mm (i.e., 10% of the scaffold height), the ceramic ball sinusoidally oscillated between 0.4 mm and 0.8 mm at a frequency of 0.5 Hz. Simultaneously, the ball oscillated, around an axis perpendicular to the scaffold axis, over the scaffold surface at ±25° and 0.5 Hz. Between loading cycles, the constructs were kept without surface contact with the ball. The group of unloaded constructs served as controls.

Biochemical analysis

For biochemical analysis, samples were digested with 0.5 mg/ml proteinase K at 56°C overnight. DNA content was measured spectrofluorometrically by using Hoechst 33258 dye (Polysciences, Warrington, Pa.) and purified calf thymus DNA as a standard (Labarca and Paigen 1980). The amount of GAG was determined by the dimethylmethylene blue dye method, with bovine chondroitin sulfate as the standard (Farndale et al. 1986). The total GAG content of the culture media was also measured to assess the release of matrix molecules from the constructs into the media. Conditioned media of each sample were pooled before analysis.

Gene expression analysis

Total RNA was extracted from homogenized constructs (TissueLyser Quiagen, Retsch, Germany) with TRI Reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's specifications, by using the modified precipitation method with a high salt precipitation solution (Molecular Research Center). Reverse transcription was performed with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, Calif.) with random hexamer primers and 1 μg total RNA sample.

Table 1 Oligonucleotide primers and probes used for real time polymerase chain reaction

Gene, database accession number (Acc.)	Primer/probe	Sequence
Procollagen 1A2, Acc. NM_174520	Primer fw (5′–3′)	TGC AGT AAC TTC GTG CCT AGC A
	Primer rev (5′–3′)	CGC GTG GTC CTC TAT CTC CA
	Probe (5′–3′)	CAT GCC AAT CCT TAC AAG AGG CAA CTG C
Procollagen 2A1, Acc. X02420	Primer fw (5′–3′)	AAG AAA CAC ATC TGG TTT GGA GAA A
	Primer rev (5′–3′)	TGG GAG CCA GGT TGT CAT C
	Probe (5′–3′)	CAA CGG TGG CTT CCA CTT CAG CTA TGG
Aggrecan, Acc. NM_173981	Primer fw (5′–3′)	CCA ACG AAA CCT ATG ACG TGT ACT
	Primer rev (5′–3′)	GCA CTC GTT GGC TGC CTC
	Probe (5′–3′)	ATG TTG CAT AGA AGA CCT CGC CCT CCA T

Probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5′-end and with the quencher dye TAMRA (6-carboxy-N, N, N′, N′-tetramethylrhodamine) at the 3′-end (*fw* forward, *rev* reverse).

The polymerase chain reaction (PCR) was performed on a 7500 Real Time PCR System (Applied Biosystems). Oligonucleotide primers and TaqMan probes (Table 1; all from Microsynth, Balgach, Switzerland) were designed with Primer Express Oligo Design software, versions 1.5/2.0 (Applied Biosystems). Probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5′-end and with the quencher dye TAMRA (6-carboxy-N, N, N′, N′-tetramethylrhodamine) at the 3′-end. To exclude amplification of genomic DNA, the probe or one of the primers was selected to overlap an exon-exon junction. The primers and the probe for the amplification of 18S ribosomal RNA, which was used as an endogenous control, were from Applied Biosystems. PCR was carried out under standard thermal conditions with TaqMan Universal PCR master mix (Applied Biosystems), 900 nM primers (forward and reverse), and 250 nM TaqMan probe. Relative quantification of target mRNA was performed according to the comparative C_T method with 18S ribosomal RNA as the endogenous control (Perkin Elmer Applied Biosystems 1997; Lee et al. 2005b).

Histology and immunohistochemistry

Histology samples were fixed in 4% formaldehyde, dehydrated in graded ethanols, and embedded in methylmethacrylate. Sections (6 μm thick) were stained with

toluidine blue to visualize the cell distribution and morphology and extracellular matrix accumulation, or with safranin O/fast green to visualize the proteoglycan and collagen deposition. Immunohistochemical samples were fixed in 100% methanol at 4°C and incubated in 5% D (+) sucrose solution in phosphate-buffered saline (PBS) for 12 h at 4°C before being cryo-sectioned at a thickness of 12 μm. Immunohistochemical staining was performed by using the Vectastain Elite ABC Kit (Vector Laboratories). Horse serum was used for blocking non-specific sites. The sections were then probed with primary antibodies against collagen type I (Sigma-Aldrich, Saint Louis, Mo., USA), collagen type II, or aggrecan (both from Development Studies Hybridoma Bank, University of Iowa, Iowa City, USA). Primary antibody was applied for 30 min at room temperature, followed by biotinylated secondary antibody (30 min, room temperature) and the preformed avidin-biotin-peroxidase complex (30 min, room temperature). Color was developed by using 3, 3'-diaminobenzidine monomer (DAB). Bovine articular cartilage tissue was employed as a positive control. For negative controls, the primary antibody was replaced by PBS.
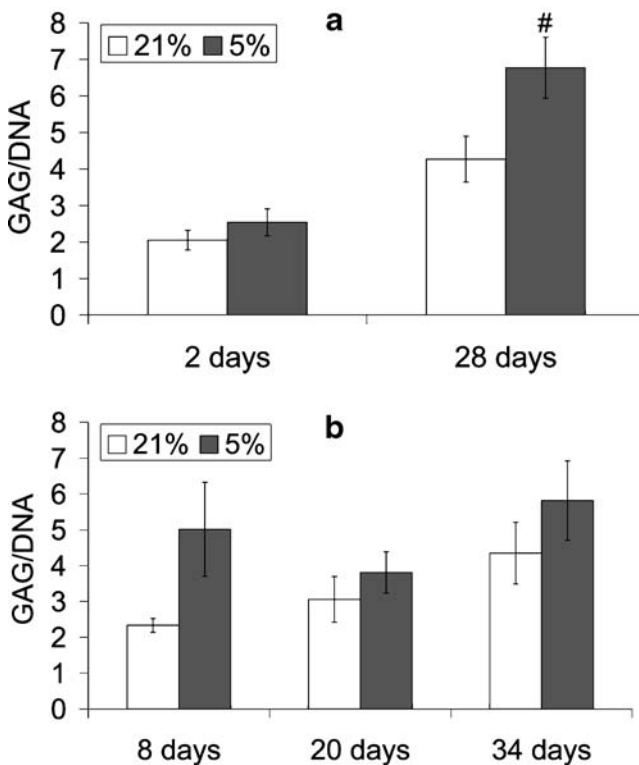

Fig. 3 a GAG/DNA content of bovine articular chondrocytes-polyurethane 3D constructs cultured at 5% O_2 and 21% O_2 after 2 days or 28 days of free-swelling culture. b GAG/DNA content of bovine articular chondrocytes-polyurethane loaded 3D constructs cultured at 5% O_2 and 21% O_2 after 8 days, 20 days, or 34 days of culture. Starting at day 7 of culture, constructs were exposed to 1 h of mechanical loading (dynamic confined compression and surface motion) daily. Mean±SEM, $n=4$, #$P=0.053$ for 5% vs. 21% O_2 at 28 days (independent samples, t-test)

Results

Biochemical analysis

Oxygen experiments

Under free-swelling conditions, the DNA content per construct increased on average from 53±5.5 μg (day 2) to 69±5.6 μg (day 28) per construct, whereas no difference occurred in the DNA content between samples cultured at different oxygen tensions (data not shown). However, higher GAG/DNA amounts were observed in hypoxic than in normoxic cultures after 28 days of incubation (Fig. 3a). The total amounts of GAG incorporated in constructs plus that released into the media during 28 days of incubation were also higher in constructs cultured at 5% (3.5±0.60 mg) than in those at 21% (0.97±0.23 mg) oxygen tension.

Loading experiments

No major difference was detected in DNA content between loaded and control samples at either oxygen tension. Similar to free-swelling cultures, loaded constructs at 5% oxygen showed a trend toward higher GAG/DNA levels than those at 21% oxygen at all time points (Fig. 3b).

Gene expression

Oxygen experiments

The gene expression levels of the cell-scaffold constructs cultured at 5% oxygen tension were compared with the levels at 21% oxygen tension after 2 days, 14 days, and 28 days of free-swelling culture. Collagen type I showed a lower expression level at 5% oxygen tension until day 14, whereas by day 28, no difference was observed between 5% and 21% oxygen tension. For collagen type II and aggrecan, the expression ratio of 5% versus 21% continuously increased during the incubation period, and after 28 days, higher aggrecan mRNA levels were found in constructs cultured at 5% oxygen tension (Fig. 4a).

Loading experiments

Gene expression levels of loaded constructs were normalized to the values of the unloaded constructs at the respective time points and oxygen tensions. After 34 days of culture, the relative collagen type I gene expression was increased at 21% oxygen, whereas at 5% oxygen, its expression consistently remained down-regulated. Collagen type II expression was slightly elevated after 8 days at 21% oxygen, whereas aggrecan mRNA levels were not influenced by mechanical loading (Fig. 4b).
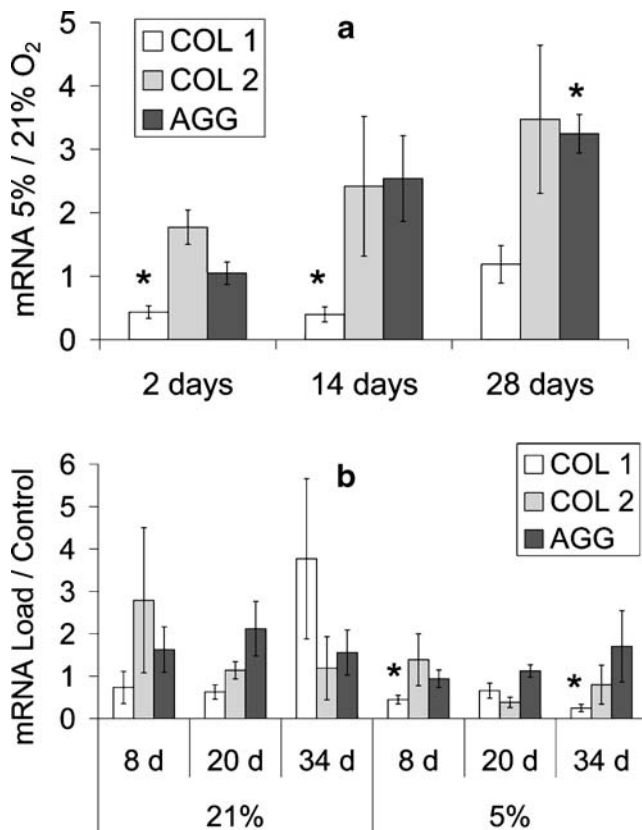
Fig. 4 a Relative mRNA expression of bovine articular chondrocytes cultured at 5% O_2 and normalized to chondrocytes cultured at 21% O_2 after 2 days, 14 days, or 28 days of 3D free-swelling culture. b Relative mRNA expression of bovine articular chondrocytes of loaded constructs normalized to unloaded constructs after 8 days, 20 days, or 34 days of 3D culture at 21% or 5% O_2 (d days). Starting at day 7 of culture, constructs were exposed to 1 h of mechanical loading (dynamic confined compression and surface motion) daily. Mean±SEM, n=4, *P<0.05 5% vs. 21% O_2 or load vs. control, respectively (one sample t-test)

Histology and immunohistochemistry

Oxygen experiments

Sections of cell-scaffold constructs at day 28 of culture were stained with toluidine blue and safranin O/fast green (Fig. 5). Constructs cultured at 5% oxygen tension (Fig. 5a, b) showed more intense toluidine blue staining than constructs cultured at 21% oxygen tension (Fig. 5e,f) in both edge and center areas. At both oxygen concentrations, the extracellular matrix was more enriched toward the edges of the constructs (Fig. 5a,e), whereas generally less extracellular matrix was present in the central areas (Fig. 5b,f). Similar results were found for sections stained with safranin O/fast green (Fig. 5c,d,g,h). Staining for proteoglycan (safranin O) was similar to the toluidine blue staining, whereas no difference was observed in the collagen (fast green) staining between 5% and 21% oxygen tension. Generally, the seeded chondrocytes maintained

their rounded morphology, representing the chondrocytic phenotype, throughout culture, although cells with a spindle-shaped appearance were occasionally noted at the edges (Fig. 5i).

Immunostaining for collagen types I and II and aggrecan at day 14 of culture is shown in Fig. 6. Collagen type I staining was mainly present as thin layers at the edges of the constructs (Fig. 6 arrows). Furthermore, constructs cultured at 5% oxygen showed less intense collagen type I reaction than constructs cultured at 21% oxygen (Fig. 6a,i). The central zones of the constructs at 5% and 21% oxygen were comparable and barely stained (Fig. 6b,j). Collagen type II staining was noted at the edges and also in the center around the cells (Fig. 6c,d,k,l). No evident differences were observed between normoxic and hypoxic conditions. For both oxygen tensions, the aggrecan immunostaining was weak and mainly noticeable at the edges of the constructs (Fig. 6e,f,m,n). Negative control sections showed no staining throughout the constructs (Fig. 6g,h,o,p). Positive control sections showed strong staining for type II collagen and less intense staining for aggrecan throughout the tissue, whereas only faint staining for collagen type I was noted at the cartilage surface (not shown).

Loading experiments

Sections of chondrocytes seeded in polyurethane scaffolds with or without mechanical loading at day 34 of culture were stained with toluidine blue (Fig. 7). All samples demonstrated a homogeneous distribution of cells throughout the construct. Generally, within 34 days of incubation, the chondrocytes maintained their rounded morphology. The accumulation of matrix was more pronounced at the edges of the constructs; fewer cells and less matrix were noticed in the central areas. In the border area, intense staining was also observed around the cells at both oxygen concentrations. Unloaded samples showed weaker staining intensities than loaded samples with regard to both edge and center areas.

Sections were also stained with safranin O/fast green to reveal proteoglycan and collagen deposition (Fig. 8). Loaded samples cultured at 21% oxygen tension showed a thick layer of intensely stained matrix toward the edge (Fig. 8e). This layer was stained for proteoglycan (reddish) and for collagen (dark green). The center of these sections showed less-intense green staining (Fig. 8f). Control samples at 21% oxygen tension showed weaker staining intensities than loaded samples in both edge and center areas. Under hypoxic conditions, sections were stained for collagen but not for proteoglycan throughout the constructs (Fig. 8a–d). No difference in safranin O/fast green staining was noted between loaded and control samples at 5% oxygen tension.
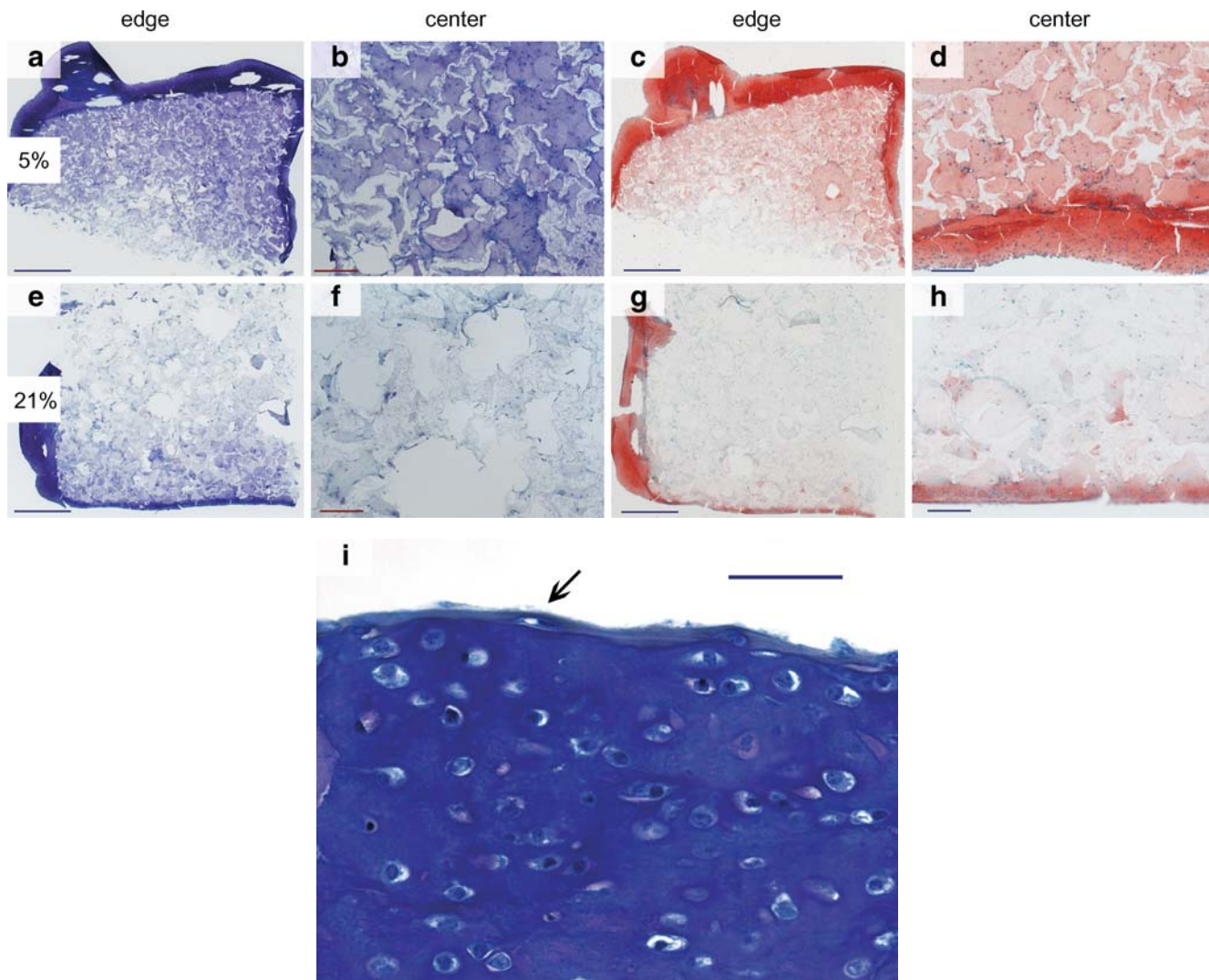
Fig. 5 Representative methylmethacrylate-embedded sections (6 μm thick), from the edges and center of chondrocyte-polyurethane 3D constructs cultured at 5% O₂ and 21% O₂ for 28 days, stained with toluidine blue (**a**, **b**, **e**, **f**, **i**) and safranin O/fast green (**c**, **d**, **g**, **h**). A higher magnification image (**i**) shows the appearance of spindle-shaped cells (*arrow*) at the edge of a construct cultured at 5% O₂ for 28 days. *Bar* 1,000 μm (**a**, **c**, **e**, **g**), 200 μm (**b**, **d**, **f**, **h**), 50 μm (**i**)

Discussion

While an applicable approach to engineering cartilage-like tissue involves a wide range of parameters that could be decisive, this study's aim has been to investigate two of them. Specifically, the potentially beneficial effect of long-term mechanical stimulation has been evaluated in combination with a different environmental influence, viz., hypoxic versus normoxic conditions. Several studies have described various biochemical and biomechanical stimuli used to encourage chondrocyte metabolism with respect to extracellular matrix production, gene expression, and tissue functionality (Lee et al. 2004). We have previously investigated the short-term effect of applied cyclic axial compression and surface motion and observed positive outcomes (Grad et al. 2005, 2006). Based on these results, this study has been directed at determining the effect of long-term dynamic confined compression. Furthermore, the influence of reduced oxygen tension has widely been studied, and most investigations have shown that chondrocytes cultured under low oxygen tension (usually 5% O₂) better maintain their phenotype and synthesize more cartilage-specific molecules compared with chondrocytes cultured under normoxic conditions (21% O₂; Kurz et al. 2004; Saini and Wick 2004; Scherer et al. 2004; Moussavi-Harami et al. 2004; Hansen et al. 2001). We therefore anticipated that the combination of both mechanical stimulation and low oxygen tension would be a useful tool to preserve or specifically to induce the chondrocytic phenotype and enhance matrix production.

In agreement with previous literature (Kurz et al. 2004; Saini and Wick 2004; Scherer et al. 2004; Moussavi-Harami et al. 2004; Hansen et al. 2001), culture of chondrocytes at low oxygen tension appears to be favorable: (1) by day 28, chondrocytes cultured under 3D free-swelling conditions
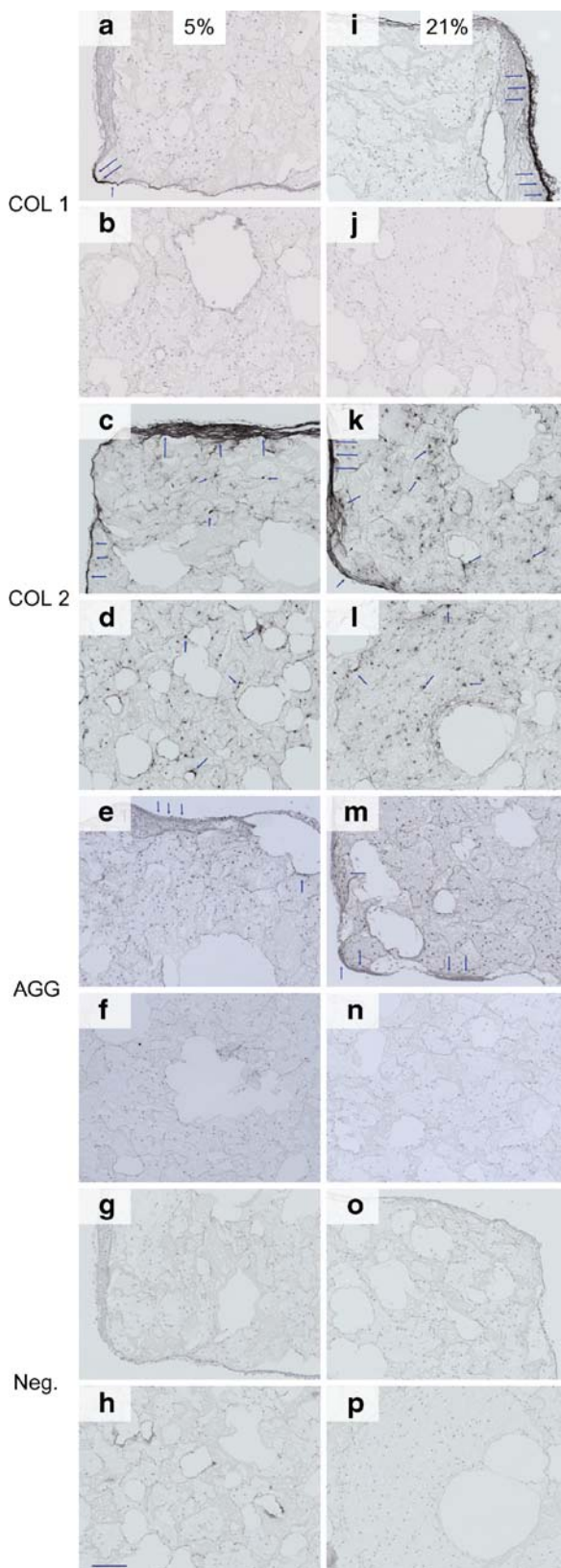
Fig. 6 Immunohistochemical staining (*arrows*) for collagen types I and II (*COL 1*, *COL 2*, respectively), aggrecan (*AGG*), and the negative control (*Neg.*) from the edges (**a, c, e, g, i, k, m, o**) and the center (**b, d, f, h, j, l, n, p**) of chondrocyte-polyurethane 3D constructs cultured at 5% O$_2$ and 21% O$_2$ for 14 days. *Bar* 200 μm

show higher GAG synthesis in hypoxic than in normoxic culture; (2) the gene expression ratio of collagen type II and aggrecan at 5% versus 21% oxygen continuously increases during 28 days of culture, whereas collagen type I gene expression is generally lower in chondrocytes cultured at 5% compared with 21% oxygen; (3) histological and immunohistochemical results also illustrate the enhanced accumulation of cartilage matrix molecules (collagen type II and aggrecan) and weaker collagen type I staining in constructs cultured at reduced oxygen tension. Moreover, the additional suppressive effect of mechanical loading on type I collagen gene expression is evident up to day 34 of culture only at 5% oxygen, and not at 21% oxygen, indicating that low oxygen and mechanical stimuli may act synergistically with respect to the stabilization of the cell phenotype. Thus, our results further corroborate that low oxygen culture can be an effective tool for cartilage tissue engineering.

Whereas long-term loading under confined compression is able to suppress type I collagen expression constantly over 34 days, the only potentially stimulating effect on collagen type II gene expression is seen after a short-term stimulation of 2 days; any further loading shows no advantage. One reason for this might be that insufficient amounts of extracellular matrix are present in the scaffolds during load application. Mechanical stimulation has been shown to induce matrix synthesis more effectively in systems in which denser extracellular matrix is present (Waldman et al. 2003; Buschmann et al. 1995). A pre-existing extracellular matrix may also facilitate the trans-duction of mechanical stimuli in polyurethane constructs. Therefore, a longer pre-incubation period to achieve more extracellular matrix prior to loading might further improve the outcome of long-term loading experiments. Previous investigations have shown that pre-incubation of 5–7 days is sufficient for an up-regulation of cartilage-related genes (such as superficial zone protein, hyaluronan synthase, cartilage oligomeric matrix protein, collagen type II, aggrecan) when a short-term load is applied (Grad et al. 2005, 2006). In the present study, constructs have been pre-incubated for 6 days, and an early up-regulation of collagen type II gene expression after 2 days of loading would thus be consistent with previous results. Furthermore, the cells might adapt to the applied stimulus during long-term mechanical loading. The loading protocol used in this study is limited to one 1-h loading/day to avoid this phenomenon of "mechanical saturation" and to prevent "overuse" stimulation that may lead to increased matrix catabolism. Various regimes have been used for the long-term mechanical conditioning of chondrocytes in tissue-engineering studies. One (Seidel et al. 2004) or three (Mauck et al. 2000) 1-hour periods of dynamic loading have shown positive effects, whereas other studies have
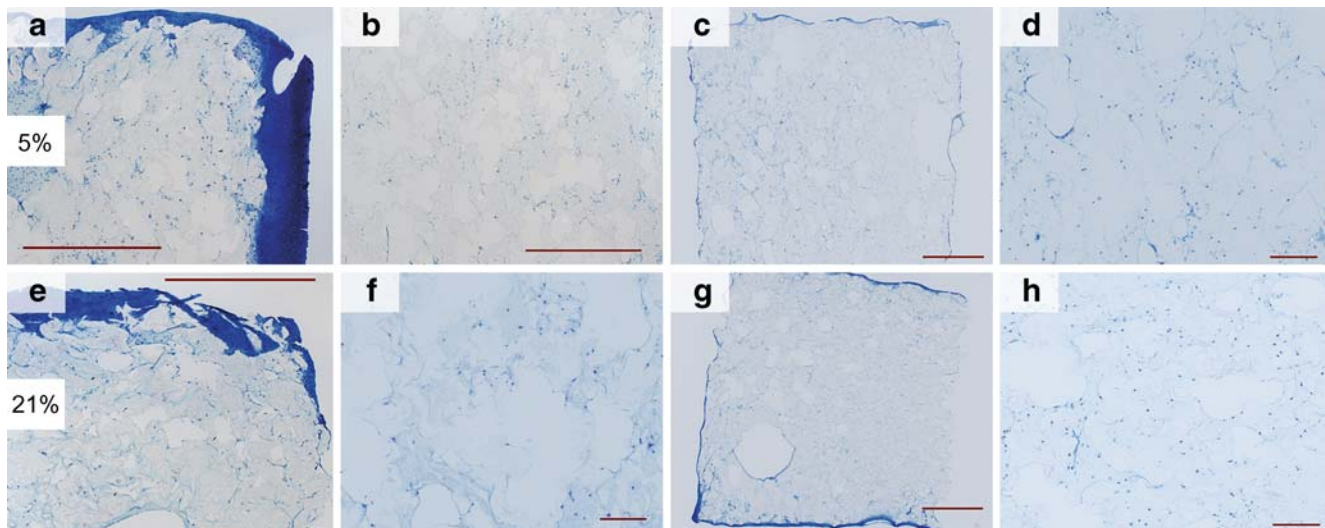
Fig. 7 Representative methylmethacrylate-embedded sections (6 μm thick), from the edges (**a**, **c**, **e**, **g**) and center (**b**, **d**, **f**, **h**) of loaded (**a**, **b**, **e**, **f**) and unloaded (**c**, **d**, **g**, **h**) constructs cultured at 5% O_2 and 21% O_2 for 34 days, stained with toluidine blue. *Bar* 1,000 μm (**a**, **b**, **c**, **e**, **g**), 200 μm (**d**, **f**, **h**)

also demonstrated the benefits of alternate day loading (Kisiday et al. 2004; Waldman et al. 2003, 2004). Interestingly, Waldman et al. (2003, 2004) have reported that only 6 min of dynamic compression or shear stimulation every other day significantly affect the quality of tissue-engineered constructs. From these findings, we can envisage that an alternate change in the loading parameters (i.e., frequency, amplitude, and duration) and/or longer intervals between loading cycles during the course of a long-term study might be necessary to maintain the responsiveness of the cells.

Our histological observations generally verify the preservation of the rounded morphology of chondrocytic cells. The sections also demonstrate the homogeneous distribution of

fibrin-embedded cells throughout the polyurethane construct. Loaded samples show more intense staining for toluidine blue and safranin O/fast green in both edge and center areas than control samples, confirming that the applied mechanical stimuli are able to enhance matrix accumulation.

In spite of the many positive results, this study suffers from certain limitations. The bovine nasal cartilage that surrounds the cell-seeded scaffold has been used to increase the fluid pressure throughout the construct during loading, while not being completely impermeable. However, we cannot exclude any fluid flow out of the construct, since we have not been able to control the complete tightness of the system. Several studies have reported the benefits of
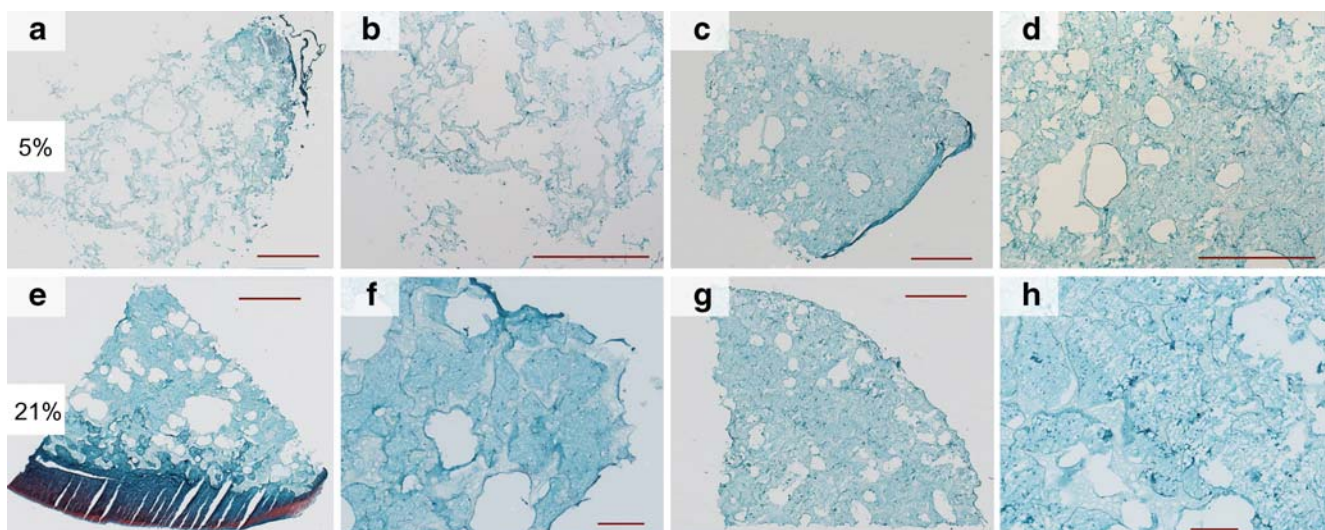

Fig. 8 Representative methylmethacrylate-embedded sections (6 μm thick), from the edges (**a**, **c**, **e**, **g**) and center (**b**, **d**, **f**, **h**) of loaded (**a**, **b**, **e**, **f**) and unloaded (**c**, **d**, **g**, **h**) constructs cultured at 5% O_2 and 21% O_2 for 34 days, stained with safranin O/fast green. *Bar* 1,000 μm (**a**, **b**, **c**, **d**, **e**, **g**), 200 μm (**f**, **h**)

increasing pressure with regard to the synthesis of cartilage-like tissue (Toyoda et al. 2003a,b; Carter et al. 2004; Carver and Heath 1999). Furthermore, using finite element modeling, we have found that a confined compression system is necessary to achieve a significant pressure gradient within the highly permeable and elastic porous polyurethane scaffold during load application (Goerke et al. 2004). Therefore, the present sub-optimal confined system will need to be re-designed for further studies in order to allow in complete fluid containment. In addition, the ongoing finite element analysis that specifically takes into account the material parameters of the scaffold and the applied load may also help to identify potentially beneficial loading protocols (Goerke et al. 2004).

In conclusion, although this investigation has to be considered as preliminary, the results obtained clearly demonstrate that mechanical stimuli simulating natural joint movements and combined with low oxygen tension are important regulators of the chondrocytic phenotype. This should be considered in future investigations if chondrocytes and/or mesenchymal stem cells are to be cultured and differentiated in order to generate cartilage-like tissue in vitro.

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