

Perfusion and cyclic compression of mesenchymal cell-loaded and clinically applicable osteochondral grafts

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Abstract Osteochondral lesions are often seen in orthopedics, but the available treatment strategies are limited in success. Regenerative medicine provides novel concepts for curing them. The purpose of this study was to test the effects of perfusion and cyclic compression on cell differentiation and mechanical properties using a custom-made biomechanoreactor in a recently established system of human bone marrow stromal cells (hBMSC) cultured in a 3-D collagen I-bone hybrid matrix out of commercially available and separately in human-certified products. Seeded hBMSC were viable for $88 \pm 8.9\%$ during the entire experimental period in the constructs. GAG and DNA levels did not change. Perfusion induced collagen II and cyclic compression increased collagen X expression. Matrix stiffness was significantly increased after 28 days of

cyclic compression. Cyclic compression of cell-loaded hybrid constructs enhanced chondrocyte differentiation and matrix stiffness. This system is a promising tool with a view to a later clinical application.

Keywords Cartilage · Bone · Stromal cells · Osteochondral lesion · Bioreactor · Joint disease

Introduction

Traumatic and degenerative osteochondral defects in weight-bearing areas of joints represent a frequent problem in orthopedic surgery. These lesions are a major healthcare issue, resulting in a high economic burden [5]. Despite the availability of cell- and tissue-based therapies, such as microfracturing, or osteochondral and chondrocyte transplantation with or without matrices, the cure of articular cartilage defects is still challenging and prosthetic joint replacements remain often the terminal treatment [10, 21].

Generation of osteochondral constructs is a promising multidisciplinary approach in regenerative medicine aimed at providing new concepts in the treatment of patients with osteochondral lesions [1, 4, 17, 18, 23, 29]. The combination of biomaterials of either biological or synthetic origin and human bone marrow stromal cells (hBMSC) is a widely used approach in generating osteochondral constructs [11]. Autologous hBMSC can be easily obtained by bone marrow aspiration and expanded by in vitro culture [7]. hBMSC are thought to be located early in the differentiation cascade, and able to give rise to different cell types of the mesenchymal lineage including osteoblasts, adipocytes, myocytes, and chondrocytes [8]. A homogeneous distribution of hBMSC, their regulated differentiation, and sufficient scaffold stability remain key issues in

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producing osteochondral constructs. Mechanical stability of hBMSC-loaded scaffolds has been shown to be improved by different types of mechanical stimulation, such as fluid-shear stress, compression, and cyclic axial strain [26]. Although it is not clear yet what the specific differences or similarities are between different stimuli, all types of mechanostimulation have been shown to activate a divergent array of key anabolic events and are known to stimulate the differentiation of hBMSC [13]. Thus, mechanical stimulation plays a key role in concepts of tissue repair of cartilage and bone [4, 18, 22].

A recently established custom-made biomechanoreactor system was used in this study focusing a later clinical application [12]. Effects of perfusion and cyclic compression on a 3-D osteochondral matrix loaded with hBMSC were evaluated. Next to testing this in vitro system over a longer period, we wanted to test different hypotheses according to tissue engineering principles in combining cells, scaffolds, and culture conditions. We proposed to demonstrate affection on cells and their differentiation over time and different stress patterns focusing immunohistology and proliferation. Changes in the extracellular matrix by quantitative assays and biomechanical properties should be observed, too in case of distinct cell differentiation pathways.

Methods

Isolation and expansion of hBMSC

The institutional ethical committee had approved all procedures and written informed consent had been obtained from all subjects. Bone marrow aspirates were harvested from seven healthy donors (4 males, 3 females, 33 ± 7.1 years) by iliac crest puncture during routine orthopedic procedures. Isolation and cultivation of hBMSC were performed according to a modified protocol as previously described [16]. Briefly, density gradient centrifugation was used to enrich hBMSC. Cells were then resuspended in tissue culture medium (DMEM/Ham's F12 1:1, Biochrom, Berlin, Germany including 10% fetal calf serum) supplemented with 200 U/ml penicillin/streptomycin (Gibco, Karlsruhe, Germany), 2.5 mg/ml amphotericin B (Biochrom), 2.5 $\mu\text{g/ml}$ ascorbic acid (Loges, Winsen, Germany) and 3 ng/ml FGF-2 (Pepro Tech, Offenbach, Germany). hBMSC's were subsequently plated in 75-cm² tissue culture flasks (Nunc, Berlin, Germany) and incubated at 37°C and 5% CO₂ in a humidified atmosphere. Medium was changed twice a week completely. After reaching 90% confluency between days 14 and 21, hBMSC's were enzymatically released with 0.25% trypsin (Gibco) and subcultured. Experiments

were performed with a single cell pool of all donors after four passages.

Construct preparation

As a basis for the cell-loaded construct, a bovine acellular cancellous bone cylinder (Tutobone[®], Tutogen Medical GmbH, Neunkirchen a. Br., Germany) was used. The porosity and mineral density of the cancellous bone cylinder is homogeneous according to the manufacturer. To facilitate cyclic compression of the construct, the cancellous bone matrix was partially decalcified (Decal[®], Decal Corporation, Tallman, NY, USA) for 40 min. After decalcification, samples were compressible for $10 \pm 0.5\%$ of their total height in response to a pressure of 3.18 kPa (0.318 N/cm²). The matrix was rinsed with deionised distilled water until the pH was 7.4.

A total of 10^7 hBMSC were homogeneously resuspended in GNL media (Arthrokinetics AG, Esslingen, Germany; HEPES-buffered and double concentrated DMEM) and then mixed at a 1:1 ratio with a 6 mg/mL rat collagen type I stock solution which was adjusted with a purity of 98% by the manufacturer (CaReS[®], Arthrokinetics) at a temperature of 5°C and supplemented with 20% FCS. The mixture was then transferred to a compression device (Fig. 1). The cell containing gel was filled on top of the partially decalcified bone matrix in a glass cylinder. After incubation for 60 min at 37°C in a humidified chamber, a glass plunger was inserted into the compression device. The weight of the glass plunger caused a pressure of 0.104 N/cm², which was combined with a vacuum force of 0.667 N/cm² targeted at the bottom of the constructs. Both forces were required to consolidate the bi-phasic construct. After 24 h, constructs were transferred either to the biomechanoreactor (Fig. 2) or to a static and unloaded cell culture system. The static control was placed in petri dishes and completely rinsed in the same media which was inside the reactor system. Additionally constructs were prepared without cells and stimulated for 3 and 4 weeks. The dishes were placed next to reactor in same humidified chamber. The construct had a diameter of 22 mm and a height of less than 11 mm.

Biomechanoreactor

Based on its design, the biomechanoreactor allows for both the application of constant peristaltic perfusion and additionally cyclic axial compression (Fig. 2). Both stimuli were permanently monitored during the experiments. Cyclic compression was adjusted to 10% of the total height of the construct and performed at a frequency of 0.5 Hz. The perfusion rate was maintained at 12 ml/min causing a low-pressure quasi hydrostatic condition inside the reactor

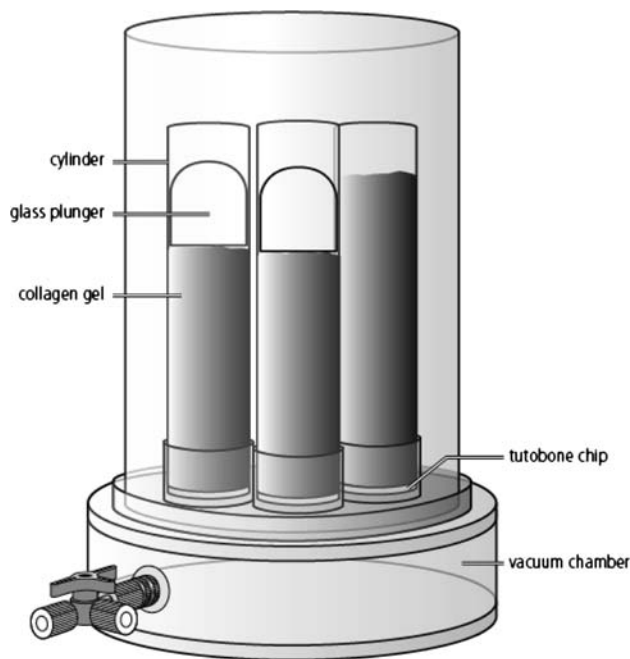


Fig. 1 Schematic drawing of the compression device. The cylinders were filled up with cell containing collagen gel on top of the Tubone chip. After 30 min of initial polymerization, pressure was applied above via plungers and a vacuum from below for 24 h. Afterward the constructs were stable enough to be transferred to the different stress protocols

chamber via a roller pump. Constructs that were only initially compressed and not further stimulated under static cell culture conditions served as unloaded controls. Inside the reactor a chamber hosts the construct radially and allows perfusion via a porous glass plate below the construct. The biomechanoreactor was kept inside a tissue culture incubator during the experiments. Cell culture medium was the same as described above with the exception that FGF was replaced by 1.3×10^{-7} M dexamethasone [22]. Medium was partially (25% of the entire amount of medium) changed

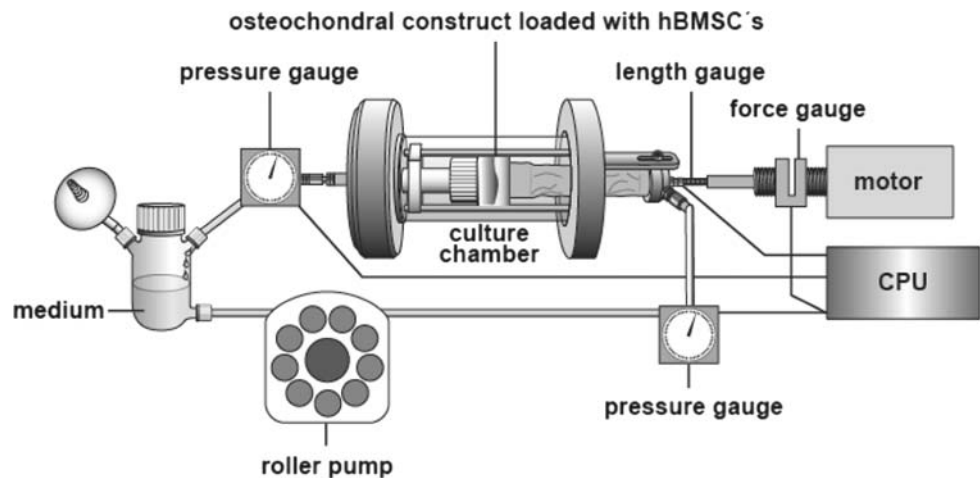
twice a week. Tissue samples were harvested and split vertically into thirds for histological, immunohistochemical and biomechanical analysis on days 7, 14, 21, and 28. Experiments were done at least in triplicate.

Histological and immunohistochemical analysis

Samples were analyzed by light microscopy using standard staining like hematoxylin & eosin (H&E) and toluidine blue to determine the distribution of collagen I fibers and hBMSC's as previously described (14). Immunofluorescence was performed using antibodies against the chondrocyte markers collagen II (Acris, polyclonal—biotin, R1038B, Hiddenhausen, Germany) and collagen X (Sigma, monoclonal anti-collagen Type X clone Col-10, C7974, Taufkirchen, Germany), at a 1:100 dilution. Prior to antibody incubation protein blocking was performed for 30 min in a protein blocking solution (DakoCytomation, Glostrup, Denmark). As negative controls served cells from the supernatants of the density centrifugation with a hematopoietic fate. To exclude unspecific immunofluorescence, IgG1 (non-immunized mouse; DakoCytomation Glostrup, Denmark) staining were performed additionally on sections out of testing system. Cell viability was assessed using a live/dead assay (Molecular Probes, Leiden, Netherlands) and analyzed by sigma scan (Systat Software Inc., San Jose, CA, USA) according to the manufacturer's guidelines. A Dapi staining was added with an incubation of 15 min to visualize the cell nuclei. Three individual sets of experiments were performed in triplicate.

Alteration of the extracellular matrix was evaluated by quantification of glycosaminoglycan (GAG) production (Blyscan Glycosaminoglycan, Biocolor, Newtonabbey, UK). Cell proliferation was determined by DNA quantification (DNA Quantification Kit, Sigma-Aldrich, Taufkirchen, Germany). Both parameters were quantified using these standard kits according to the manufacturer's

Fig. 2 Schematic drawing of the bioreactor. The reactor contains a chamber that hosts the construct radially. A peristaltic pump steers the flow of medium and an electric engine drives a gauge to provide cyclic mechanical loads. Each force is monitored and steered by a control panel on a personal computer



instructions for photometry. Intercalating fluorescents were used which was bisbenzimidazole for the DNA and Blyscan for GAG. The transmission was standardized by stock probes delivered by the manufacturer. The contents were normalized to the wet weight of the substrates. Controls were performed at every single step of the experiments to validate the methods (data not shown). Mean values of three read outs were quantified on two different days.

Mechanical testing

Characterization of mechanical properties was conducted using a confined compression quasi-static loading setup at 0.1 mm/s, by which a complete push-out was performed. This testing system was established and validated on human osteochondral fragments before [12]. The size (length/diameter) of the harvested cylinders (nominal diameter: 6 mm) was measured using a contactless laser micrometer. A press-fit technique using a special drilling chisel (OATS; Arthrex; Naples, FL, USA) was used to prepare cylindrical specimens from the construct, which was taken from the biomechanoreactor. This allowed for an accurate and reproducible alignment of the construct with both the chamber plunger of 5.5 mm diameter, and a 4.5-mm hole centered below the support chamber. A computer-controlled micro-stepper motor displaced the plunger (Zwick Universal Testing Machine 1484 200 KN, Zwick GmbH & Co KG, Ulm, Germany) at a temperature of 37°C with a semipermeable indenter (diameter 3 mm). A load-sensing detector was attached to the plunger to measure the actual push-out force. For each specimen, load measurements were monitored. Stiffness at 20% strain of the constructs was calculated from the maximum force measured during the failure process for the matrix constructs. Strain was computed based on the length of the entire construct under a nominal load of 0.1 N.

Statistical analysis

Statistical analysis was performed by comparing results between groups. It was carried out by multivariate analysis of variance (ANOVA), followed by univariate ANOVA and the Scheffe test (Version 11.0, SPSS Inc., Chicago, IL, USA). All results are shown as mean and standard error of the mean. Statistical significance was assumed if $P < 0.05$.

Results

Histology

The hybrid construct with the cell-containing collagen I gel (upper phase) and the cancellous bone slice (lower phase)

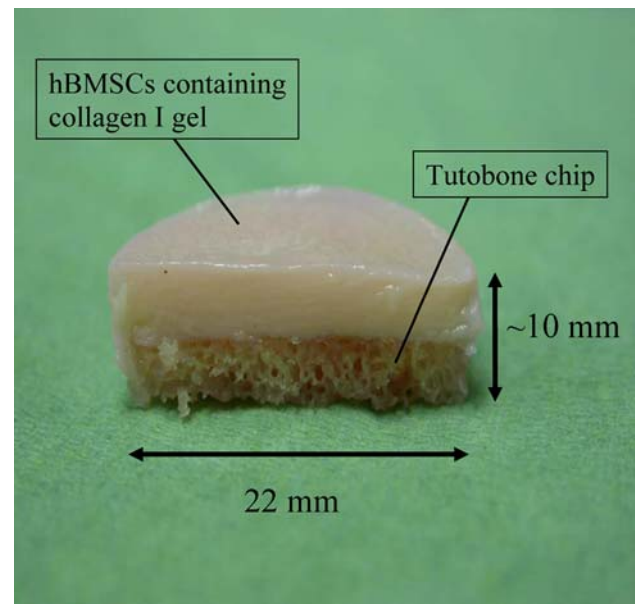


Fig. 3 Harvested sample construct with a diameter of 22 mm. The lower phase of the constructs represents the cancellous bone chip on which the cell containing collagen I gel (upper phase) was hybridized in a customized compression device

is shown in Fig. 3. Sample analysis by hematoxylin & eosin (H&E) staining revealed that mixing the cells with the gel successfully resulted in a homogeneous distribution of hBMSC within the gel layer (data not shown). In H&E-stained samples, the interconnection of the collagen fibers was denser after initial compression compared to non-compressed controls, most likely due to the water that was pressed out. The high-collagen I content of the matrix and relatively low extracellular production of the cells was the most likely explanation. By day 21, the collagen II staining was upregulated only by perfusion (Fig. 4a–c). The abundance of collagen X was slightly upregulated by perfusion but was most prominently increased by cyclic compression (Fig. 4d–f). Although perfusion also increased collagen II expression by day 28 (Fig. 5a–c), it prevented the upregulation of collagen X, as was seen for the unloaded control samples and the cyclically compressed constructs (Fig. 5a–c). There was no obvious difference in collagen X staining by day 28 between the cyclically compressed constructs and the unloaded control samples (Fig. 5d, e). Analysis of cell viability revealed that cells were highly viable at a rate of $88 \pm 8.9\%$ at all points of analysis and independent of the type of stimulation.

Glycosaminoglycan and DNA quantification

In order to determine modification of the extracellular matrix and cell proliferation, quantitative glycosaminoglycan (GAG) and DNA assays were performed, since both

Fig. 4 *Top panel* Collagen II immunohistology (black) on day 21 (H&E). *Lower panel* Collagen X immunohistology (green) on day 21. **a/d** Unloaded static culture; **b/e** hydrodynamic stress; **c/f** mechanical stress. The bar represents 50 μ m. In the *lower panel* the blue Dapi staining indicates cell nuclei

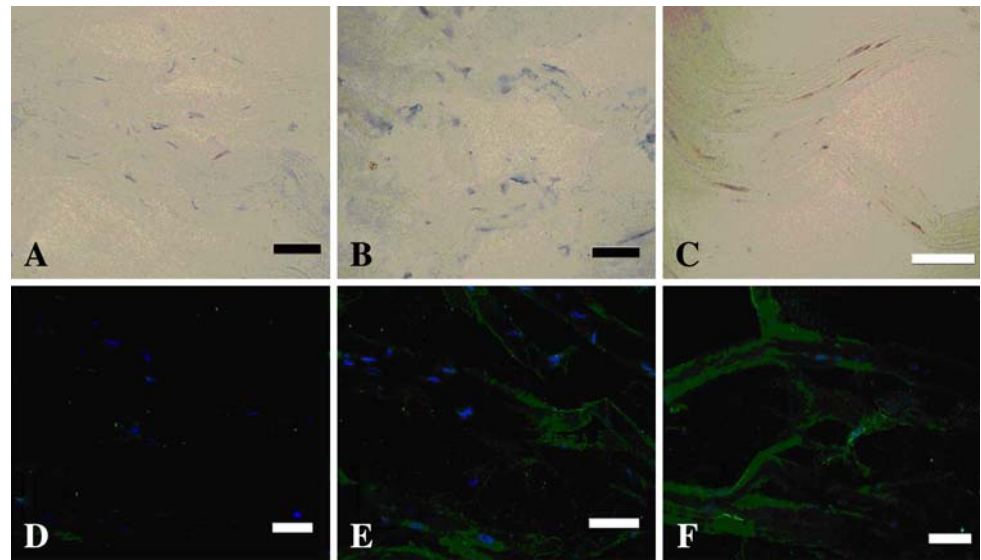
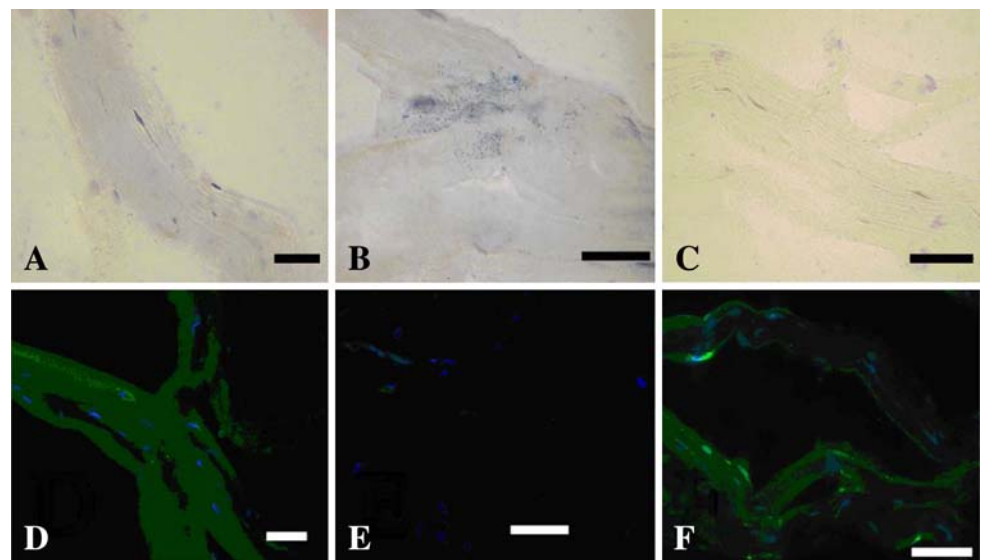


Fig. 5 *Top panel* Collagen II immunohistology (black) on day 28 (H&E). *Lower panel* Collagen X immunohistology (green) on day 28. **a/d** Unloaded static culture; **b/e** hydrodynamic stress; **c/f** mechanical stress. A positive reaction was found for collagen II in the hydrodynamically stimulated group (**b**) and for collagen X in the mechanically stimulated group (**c**). The bar represents 50 μ m. In the *lower panel* the blue Dapi staining indicates cell nuclei



alteration of the extracellular matrix and cell proliferation are influenced by mechanical stress and other forces [31]. GAG is a common marker for extracellular matrix production by chondrocytes. No statistically significant differences in the synthesis of either GAG or DNA were found in any group during the investigation (Figs. 6, 7).

Biomechanical testing

Samples were fitted in a custom-made cutting tool and inserted in a biomechanical testing machine at a temperature of 37°C with a semipermeable indenter (diameter 3 mm). During the initial compression that was applied to unite both phases, the collagen I-cell loaded upper phase was compressed to approximately 1/10 of its original height. The construct height did not change significantly over time or upon application of different kinds of stimulation, except for

an increase observed in the unloaded static control group after 28 days (Fig. 8). However, the construct was swollen due to the lack of stimulation and a subsequent water influx. Mechanical tests revealed a significant enhancement of matrix stiffness in constructs that were primarily compressed for the first 24 h and then stimulated by cyclic compression for 28 days compared to the unloaded static compressed controls (Fig. 9). Additionally this increase was not observed in case of constructs prepared without cells and stimulated mechanically. Stimulation by continuous perfusion did not change the construct stiffness significantly.

Discussion

This study was aimed at developing a 3-D stimulation system to determine the effects of continuous medium

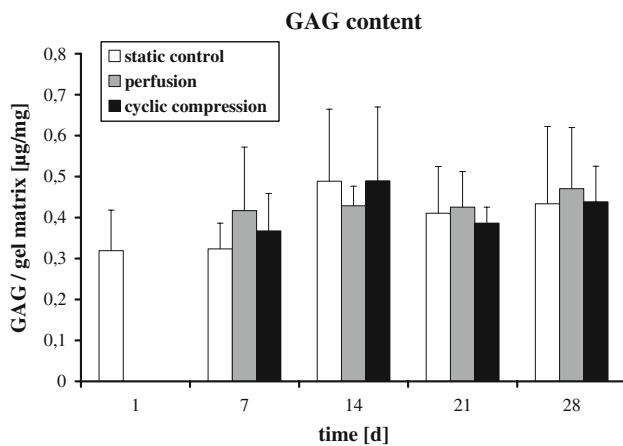


Fig. 6 GAG quantification in ng/mg probe (wet weight) as mean values including standard deviations. The unloaded static and mechanical groups show very similar expression patterns on all days of harvest, but the perfusion group shows higher amounts except on day 14

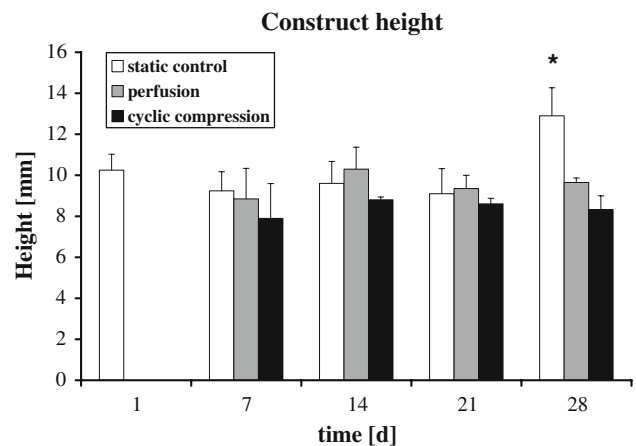


Fig. 8 Construct height for the different groups investigated. *Significant change ($P < 0.05$)

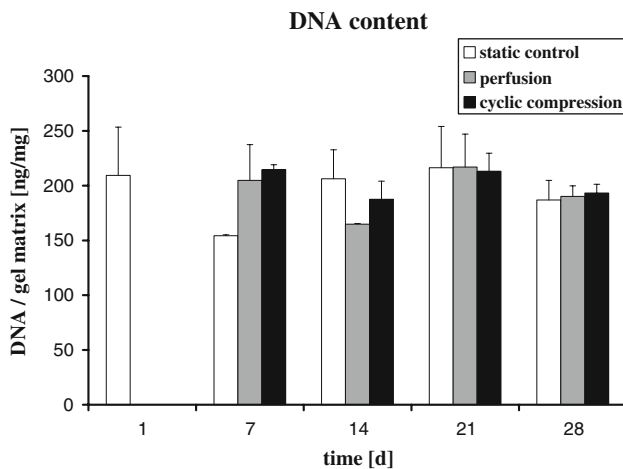


Fig. 7 DNA quantification in ng/mg probe (wet weight) as mean values including standard deviations. The unloaded controls show the lowest amount of DNA among all groups on day 7. By day 21, the average concentration of DNA per tissue is the highest for all groups during the entire experiment. At days 21 and 28 the amount is similar in all groups

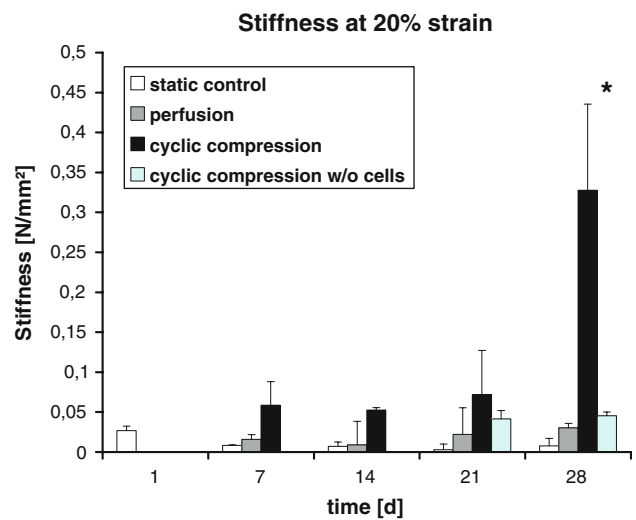


Fig. 9 Mechanical loading at 20% compression as mean values and standard deviations. Significant differences were observed on day 28 in the mechanically stimulated group ($*P < 0.05$)

perfusion and cyclic compression on the mechanical properties of a mesenchymal cell-loaded osteochondral construct [12]. In our system collagen I-entrapped hBMSC were highly viable and differentiated as they were modulated by the specific type of stimulus applied over up to 4 weeks successfully. Cyclic compression but not perfusion only was found to increase the stiffness of the construct after 4 weeks of stimulation, thereby improving the overall biomechanical properties of the material.

It is well established that hBMSC are multipotent precursor cells that can differentiate into adipocytes, myocytes, osteoblasts, and chondrocytes [8]. This diverse

differentiation capacity, the fact that hBMSC are reasonably easy to harvest without major ethical concern and the lack of immunological problems in an autologous context have made hBMSC the premier cellular source for approaches aimed at generating artificial grafts for musculoskeletal and osteochondral regeneration [19]. However, a well-known major limitation in using hBMSC is their heterogeneity. These cells belong to a lineage hierarchy in which only some of the cells are multipotent precursor cells or primitive progenitors whereas others are more restricted [3]. Primary bone marrow stroma cells vary in terms of phenotype, functional, and differentiation stage, both between donors and within the same culture, in comparison to the more-established and well-characterized mesenchymal or osteoblastic cell lines used in other studies [6, 15, 30]. However, in this study were primary cells of

human origin used, because intentionally this would be the preferred material in a future therapeutic context. It avoids murine cell lines, which have their own limitations as a model system, too. To minimize assay variations caused by cell heterogeneity, we combined cells derived from several subjects. Based on earlier studies [14], FGF-2 was added to the cell culture medium during the period of cell expansion to obtain the best proliferation rate. To avoid undesired side effects in the assays, FGF-2 was omitted from the culture medium after hBMSC were plated for experiments. Although the suitability of hBMSC as an experimental cellular source is debatable, these cells are widely used in regenerative medicine, especially in combination with degradable matrices for the repair of bony or osteochondral lesions [2, 19].

Grafts for the repair of osteochondral lesions are preferentially composed of two layers: a cancellous bone-like layer that serves as an interface for integrating the construct into bone and a cell-loaded gel-like layer prefiguring the future cartilage articular surface [27]. With a view to a later clinical application in humans in the present study a commercially available and clinically established cancellous bone combined with a collagen I gel matrix with which hBMSC were homogeneously mixed. In preparing this hybrid construct constant pressure was initially required to consolidate both layers and to stabilize the entire construct. If this initial compression was not applied, the construct disintegrated within a few days [12].

Analysis of the effects of continuous perfusion or additionally cyclic compression of the constructs on chondrogenic differentiation of hBMSC revealed distinct differences. Continuous perfusion upregulated the expression of collagen II by days 21 and 28 but cyclic compression did not. Additional cyclic compression increased the expression of collagen X by days 21 and 28. Interestingly, we observed that by day 28 the abundance of collagen X was remarkably decreased after perfusion compared to both the cyclically compressed samples and to controls. These findings suggest that depending on what kind and combination of physical stimulus was applied, molecularly distinct genetic programs were activated. It is well established that within the framework of chondrogenesis and endochondral ossification chondrocytes express differentiation stage-specific lineage markers [31]. At an early stage mesenchymal cells proliferate and start to differentiate into chondrocytes, a process accompanied by the expression of collagen II, an early chondrocyte lineage marker. As chondrocytes undergo maturation they become withdrawn from the cell cycle and proliferation ceases. Prehypertrophic and hypertrophic terminally differentiated chondrocytes express collagen X, a late marker of chondrogenesis, and eventually mineralize their surrounding matrix.

We hypothesize that although continuous perfusion of gel-entrapped hBMSC promoted the entry of mesenchymal precursor cells into the chondrocyte differentiation program, this mode of stimulation also retained chondrocytes at an early differentiation stage. In contrast, additional cyclic compression forwarded the immature cells through several checkpoints in the differentiation cascade up to a terminal differentiation stage at which the cells started to strengthen the surrounding matrix. This hypothesis is further supported by the observation that additional cyclic compression improved the stiffness of the constructs by day 28, and perfusion had no beneficial effect on the mechanical properties. The increase in stiffness could be a direct stimulation-dependent result of the terminally differentiated hBMSC that had begun a cell-based rigidifying of the surrounding matrix. The finding that cyclic mechanical stimulation activates a specific cell differentiation program more efficiently than perfusion has been described in other studies [2, 24, 28].

Cyclic mechanical stimulation is also known to cause osteogenic differentiation of hBMSC as has been reported by our group and by others [9, 13, 16]. Although we cannot completely exclude that some cells of the heterogeneous progenitor pool adopted an osteoblastic fate. A calcification or ossification could explain the increase in matrix stiffness additionally to the lack of GAG change. However, others reported an increase first after 4 weeks in mature cell culture [20]. In this study the hBMSC were homogeneously embedded in a gel matrix. The entrapped cells were exposed to reduced oxygen tension. Hypoxia is a strong and essential inducer of chondrogenesis [25], which could have been sufficient to cause a shift from osteoblast to chondrocyte differentiation. In addition, the collagen I gel might have also been contributed to secure the chondrocyte phenotype since the use of biomaterials seeded with autologous cells for the treatment of osteochondral defects is known to prevent dedifferentiation of *in vitro* pre-differentiated chondrogenic cells [24].

Cell proliferation is an integral part of early stage chondrocyte differentiation, and although both types of stimuli had distinct effects on the cell fate, none of the stimulation modes had any additional effect on cell proliferation. The amount of DNA isolated from the cell-loaded constructs did not vary suggesting that cell proliferation and apoptosis or cell death was well balanced. On the other hand, physical stimulation did also not diminish cell viability since cells of all groups were highly viable with little variation.

In summary, we have established a custom-made 3-D stimulation system for the long-term culture of xenogenic osteochondral grafts consisting of already in humans-used matrices. Loaded cells were viable and began to undergo an overall maybe chondrogenic differentiation, which was

specifically modulated by the type of stimuli applied. This study thus demonstrates the applicability of the established system and the usefulness of a defined cyclic compression program for the *in vitro* generation of stable osteochondral grafts with improved biomechanical properties. In the long-term, our system could be used to broaden the spectrum of therapeutic options for the treatment of osteochondral lesions. This study lacks a detailed expression analysis to prove a specific differentiation pathway of the cells. This has to be completed with a future study. However, to our knowledge, we were reporting for the first time the use of a system combining autologous cells in for human-use certified matrices with a potentially clinical application. Future studies are needed to determine the superiority of the stimulated grafts *in vivo*, in both animal models and carefully selected patients.

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