

---

# Effects of hydrostatic pressure on leporine meniscus cell-seeded PLLA scaffolds

---

Najmuddin J. Gunja, Kyriacos A. Athanasiou

Department of Bioengineering, Rice University, Houston, Texas 77251

Received 5 June 2008; revised 24 October 2008; accepted 9 December 2008

Published online 12 March 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.32451

**Abstract:** Hydrostatic pressure (HP) is an important component of the loading environment of the knee joint. Studies with articular chondrocytes and TMJ disc fibrochondrocytes have identified certain benefits of HP for tissue engineering purposes. However, similar studies with meniscus cells are lacking. Thus, in this experiment, the effects of applying 10 MPa of HP at three different frequencies (0, 0.1, and 1 Hz) to leporine meniscus cell-seeded PLLA scaffolds were examined. HP was applied once every 3 days for 1 h for a period of 28 days. Constructs were analyzed for cellular, biochemical, and biomechanical properties. At  $t = 4$  weeks, total collagen/scaffold was found to be significantly higher in the 10 MPa, 0 Hz group when compared with other groups. This despite the fact that the cell

numbers/scaffold were found to be lower in all HP groups when compared with the culture control. Additionally, the total GAG/scaffold, instantaneous modulus, and relaxation modulus were significantly increased in the 10 MPa, 0 Hz group when compared with the culture control. In summary, this experiment provides evidence for the benefit of a 10 MPa, 0 Hz stimulus, on both biochemical and biomechanical aspects, for the purposes of meniscus tissue engineering using PLLA scaffolds. © 2009 Wiley Periodicals, Inc. *J Biomed Mater Res* 92A: 896–905, 2010

**Key words:** tissue engineering; knee meniscus; hydrostatic pressure; PLLA; TGF- $\beta$ 1

---

## INTRODUCTION

The knee menisci, fibrocartilaginous tissues that lie on the tibial plateau, are involved in several important biomechanical processes, including load transmission, shock absorption, and lubrication of the knee joint.<sup>1,2</sup> The menisci are subjected to a variety of forces *in vivo*, including tension, compression, shear, and hydrostatic pressure (HP).<sup>1,3,4</sup> The ability of the meniscus to withstand these forces may, to a large extent, be attributed to the unique collagen fiber alignment and orientation within the structure.<sup>3,5</sup> Abnormalities or complications in the structure of the meniscus can lead to degeneration of the tissue as well as precipitate osteoarthritis.<sup>6,7</sup> A promising modality to overcome this problem is functional tissue engineering which serves to replace a damaged meniscus with an engineered construct with desired biomechanical and biochemical properties.

The past decade has seen a marked increase in efforts to engineer the meniscus using classical tissue engineering strategies involving cells, growth factors, and scaffolds. The concept of using mechanical stimulation as an additional element to tissue engineer the meniscus is now gaining popularity.<sup>3</sup> The importance of the mechanical environment for developing musculoskeletal tissues cannot be understated, as mechanical forces are known to influence these tissues' performance. For example, numerous experiments on disuse of musculoskeletal tissues like cartilage, muscle, and bone have been correlated to apoptosis within the tissues, followed by their subsequent atrophy.<sup>8–10</sup> Additionally, *in vitro* studies with meniscus explants and cells have shown that mechanical stimuli such as direct compression, tension, and HP can increase extracellular matrix (ECM) expression and synthesis.<sup>4,11–18</sup> The use of HP is of particular interest as it causes no macro-scale deformation to the construct, yet is responsible in stimulating cells to increase ECM synthesis, possibly by altering intracellular ion flux.<sup>19</sup> Such a stimulus may be favorable in the early stages of a tissue engineering study where cells seeded on scaffolds are still in the proliferation and migration stages and are vulnerable to external stimuli.

Correspondence to: K. A. Athanasiou; e-mail: athanasiou@rice.edu

Contract grant sponsor: NIAMS; contract grant number: RO1 AR 47839-2

HP studies can be broadly divided into three categories, (a) 2D monolayer studies, (b) 3D explant studies, and (c) 3D tissue engineering studies. Results from experiments in the first category are often reported in terms of gene expression. Studies on articular chondrocytes and fibrocartilaginous metaplasia of Achilles tendon fibroblasts have shown an up-regulation of aggrecan and collagen II expression in cells immediately after intermittent hydrostatic pressurization.<sup>14,20–22</sup> Interestingly, a further up-regulation of collagen and aggrecan expression was observed in the fibrocartilaginous metaplasia study when the samples were tested 24 h post stimulation suggesting that rest time might be an important factor to consider in long-term tissue engineering studies. TMJ disc cells have been shown to increase collagen I expression under static HP of 10 MPa and increase collagen II expression during intermittent HP stimulation of 10 MPa, 1 Hz.<sup>23</sup> Studies with explants have investigated the effects of HP on proteoglycan synthesis as well as MMP regulation. An experiment with articular cartilage explants showed that cyclic HP at physiological magnitudes caused an up-regulation of sulfate incorporation into the explants.<sup>24</sup> Cyclic HP on meniscus explants has been shown to inhibit upregulation of potent effector molecules such as MMP-1, MMP-3, and COX-2.<sup>4</sup> 3D studies for tissue engineering purposes have been performed by encapsulating cells in alginate beads, using pellet cultures, seeding cells on scaffolds, or using scaffoldless self-assembly techniques.<sup>23,25–29</sup> Results of these studies have varied significantly between research groups, often even when using similar cell sources. For example, although intermittent HP application has been shown to consistently increase GAG production by chondrocytes on PGA and PLGA scaffolds, as well as in pellet cultures, this has not been observed in self-assembled articular chondrocyte constructs.<sup>25,27,28</sup> Static HP has been shown to increase both collagen and aggrecan synthesis in intervertebral disc cells while only increasing collagen content in TMJ disc cells.<sup>23,26</sup> Thus, specific regimens of both static and intermittent HP stimuli exhibit beneficial effects for tissue engineering purposes, even though the underlying mechanisms are still unclear.

In this experiment, a traditional tissue engineering approach was employed using meniscus cells seeded on PLLA scaffolds. Nonwoven meshes of high molecular weight PLLA (>100 kDa) degrade slowly over time and are, thus, advantageous for long-term applications over the extensively studied PGA, which degrades rapidly in aqueous environments.<sup>30–33</sup> In addition to the longer half-life of the polymer, data from our laboratory using meniscus cells and TMJ disc fibrochondrocytes suggest that PLLA scaffolds adequately promote cell proliferation and ECM syn-

thesis and are comparable with data published with PGA scaffolds.<sup>23,30,34</sup> Thus, PLLA was chosen as the scaffold material for the experiment.

The objective of this study was to determine the effects of periodic and intermittent HP on leporine meniscus cell-seeded PLLA scaffolds. Several different loading regimens were tested where the frequency of loading and the pressure applied were controlled. The hypothesis was that both intermittent and static HP would increase the production of collagen and GAG molecules on the scaffolds as well as enhance the mechanical integrity of the scaffolds as a result of matrix deposition.

## MATERIALS AND METHODS

### Cell harvesting

Medial and lateral menisci were isolated under aseptic conditions from 1- to 2-year-old New Zealand white rabbits, sacrificed by a local rabbit breeder on the day of harvest. Each meniscus was taken to a cell culture hood, washed with autoclaved phosphate buffered saline (PBS), and transferred to culture media containing 2% penicillin-streptomycin-fungizone (PSF) (Cambrex). The culture media contained 50:50 Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen), 10% fetal bovine serum (FBS) (Mediatech), 1% nonessential amino acids (NEAA) (Invitrogen), 25 µg of l-ascorbic acid (Sigma) and 1% PSF. The menisci were minced into small fragments (<1 mm<sup>3</sup>) and digested overnight at 37°C in 2 mg/mL collagenase type II (Worthington Biochemical). Vascular portions of the meniscus were discarded prior to mincing. Postdigestion, an equal volume of PBS was added to the collagenase digest and the mixture was centrifuged at 200g. The bulk of the supernatant was removed, more PBS was added, and the mixture was centrifuged again to wash the pellet of collagenase. This process was repeated twice leaving behind a white pellet of meniscus cells. Cell counts were obtained using a hemocytometer. Cell viability was assessed using a Trypan blue exclusion test and was found to be greater than 95%. The cells were then resuspended in culture medium supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO) and frozen at -80°C for up to a month.

### Cell culture and passage

Cells from four rabbits were pooled together after thawing, and the viability was found to be over 90%. The cells were then plated on T-225 flasks at 25% confluence and allowed to expand. At full confluence, the cells were passaged using trypsin/EDTA (Invitrogen) and counted with a hemocytometer.

### Scaffold, spinner flask preparation, and cell seeding

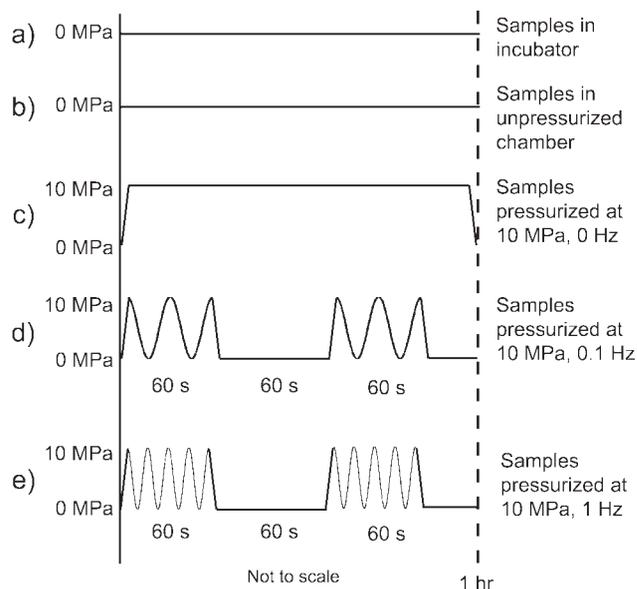
High molecular weight (100 kDa) nonwoven PLLA scaffold sheets (~90% porosity) (biomedical structures) were cut into cylinders 1.5 mm in thickness and 3 mm in diameter using a 3 mm dermal punch. Each scaffold was strung on a stainless steel wire, alternating with beads to avoid scaffold-scaffold contact. The scaffolds were sterilized using ethylene oxide. These were then prewetted in 70% isopropyl alcohol, washed twice with PBS, and placed in an autoclaved spinner flask filled with regular culture media. Cells, passaged once, were seeded into the spinner flask at a density of 0.7 million cells/scaffold corresponding to ~50,000 cells/mm<sup>3</sup> of scaffold by volume. Stirrer bars placed at the bottom of the spinner flask were rotated at 60 RPM post-seeding for 3 days. The scaffolds were left in the spinner flask for an additional 4 days without stirring to allow cells to fully adhere to the polymer. After 1 week, the steel wire was removed and the cell-seeded constructs were transferred to agarose-coated six-well plates. The agarose coatings on the wells were prepared by adding 1.5 mL of 2% sterile molten agarose to each well. Each well-housed six cell-seeded scaffolds.

### Experimental groups

Post static culture, the scaffolds were randomly assigned into HP or control groups ( $n = 6$  per group). Three different HP groups [Fig. 1(c,d,e)] were tested. Scaffolds in the first group were exposed to static HP of 10 MPa for 1 h every 3 days for 4 weeks. Semantically, a static HP regimen implies one continuous application throughout the entire culture duration; however, since the application occurred repeatedly over a period of 4 weeks with nonpressurized rest in between, the term periodic HP will be used throughout the results and the discussion section to describe this group. The second and third groups were intermittent HP groups where 10 MPa at 0.1 Hz or 10 MPa at 1 Hz of HP was applied for the same period as the periodic HP group. These particular regimens were chosen based on their benefits observed in prior HP experiments.<sup>14,23,35</sup> Additionally, during the 1 h of stimulation, a duty cycle of 60 s on, 60 s off was employed for the intermittent HP groups. This was employed based on a study with dynamically stimulated articular cartilage explants where increased radiolabeled sulfate uptake was observed if a rest time of 100 s or less was employed between each stimulation.<sup>36</sup> Two control groups were utilized in this experiment [Fig. 1(a,b)]. Scaffolds in the first control group remained in six-well plates for the duration of the experiment (culture control). The second control group consisted of unpressurized scaffolds that underwent the same manipulation as pressurized scaffolds including loading into the pressure chamber, except that no pressure was applied (HP control).

### HP preparation and application

The cell-seeded scaffolds in the HP groups were placed in histology cassettes (Fisher Scientific) that were previ-



**Figure 1.** Experimental groups. (a) Culture controls (b) HP controls (c) Periodic HP (10 MPa, 0 Hz) (d) Intermittent HP (10 MPa, 0.1 Hz) (e) Intermittent HP (10 MPa, 1 Hz). Samples were stimulated once every three days for 1 h for a period of 28 days.

ously cleaned with 70% isopropyl alcohol and sterilized with ethylene oxide. The cartridges were then transferred to individual heat sealable sterile bags (Ampac). To each bag, 30 mL of culture medium with 1% FBS (supplemented with 10 mM HEPES) was added, and the bag was tapped lightly to remove air bubbles from the media and from the cartridges. The bag was then heat-sealed without any bubbles inside.

The HP set-up used in this experiment has been described previously.<sup>23,28</sup> Briefly, the control specimens were placed in an open unpressurized chamber while the pressurized specimens were placed in a water-filled stainless steel chamber (Parr Instrument Company) that connected to an Instron 8871 via a water-driven stainless steel piston (PHD Inc.). Both chambers rested in a water bath set at 37°C. Displacement was controlled by an Instron WaveMaker program that also recorded pressures. Post stimulation, the bags containing the scaffolds were cleaned with 70% isopropyl alcohol and transferred back into the culture hood and unsealed. The scaffolds were then removed from the cartridges and placed back into six-well plates with fresh media.

### Histology

Post stimulation ( $t = 4$  weeks), one sample from each group was frozen and sectioned at 12  $\mu$ m and placed on adhesive slides (Instrumedics). Ultraviolet light was used to crosslink the section to the slide. Harris hematoxylin and eosin (H&E) stains were performed to confirm presence of ECM. Safranin-O fast green (Saf O/fast green) stains were performed to examine the distribution of

glycosaminoglycans in the section.<sup>37</sup> Picro-sirius red was used to determine the presence of collagen.<sup>38</sup>

### Biomechanics

The viscoelastic compressive properties of samples from each group ( $n = 5$ ) were tested poststimulation ( $t = 4$  weeks) using an Instron 5565 set-up described previously.<sup>39</sup> Incremental stress relaxation curves were obtained at 10, 20, and 30% strain for 5 min each. Data were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid.<sup>40</sup> Fitted parameters obtained were converted to instantaneous modulus ( $E_i$ ), relaxation modulus ( $E_r$ ) and coefficient of viscosity ( $\mu$ ).

### Biochemistry

Biochemical tests were performed on samples at  $t = 0$  weeks and  $t = 4$  weeks. The 4-week samples were obtained post-biomechanical testing. All samples were digested at 65°C overnight with 125  $\mu\text{g}/\text{mL}$  papain (Sigma) in 50 mM phosphate buffer (pH = 6.5) containing 2 mM *N*-acetyl cysteine (Sigma) and 2 mM EDTA. A picogreen cell proliferation assay kit (Molecular Probes) was used to determine total DNA content in each sample. It was assumed that each cell contains 7 picograms of DNA content, as reported in the literature.<sup>41</sup> Samples were also tested for total GAG present by a dimethylmethylene blue (DMMB) assay.<sup>42</sup> A modified hydroxyproline assay was used to determine total collagen in the scaffold.<sup>43</sup>

### Statistical analysis

The quantitative biochemical and biomechanical data were compared using a one-way analysis of variance (ANOVA) where HP was the single factor. If a statistical difference was observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. A significance level of 95% with a  $p$  value of 0.05 was used in all statistical tests performed. All values are reported as mean  $\pm$  standard deviation (SD).

## RESULTS

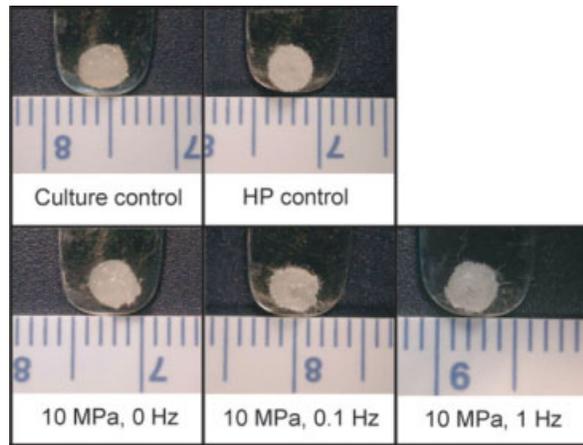
### Gross morphology and histology

Post-seeding, cells on the PLLA scaffold appeared elongated with multiple protrusions attached to the PLLA fibers. In addition, clusters of rounded cells were also observed lodged between fibers of the non-woven PLLA mesh. At  $t = 4$  weeks, the presence of translucent tissue was observable in all groups (Fig. 2); however, this was more prominent in the controls and the periodic HP group (10 MPa, 0 Hz) where holes created by the stainless steel wire

through the centers of scaffolds prior to seeding were filled with ECM. The holes were still partially visible in the intermittent HP groups. Although structural integrity was maintained over 4 weeks in all constructs, the scaffolds exposed to intermittent HP (10 MPa, 0.1 and 1 Hz) were less uniform around the periphery; that is, strands of single or multiple fibers projected outwards when compared with the scaffolds in the other groups. At  $t = 4$  weeks, construct diameter in the culture control and the intermittent HP groups was  $3.0 \pm 0.1$  mm while in the HP control and the HP 10 MPa 0 Hz groups the diameter was higher,  $3.1 \pm 0.1$  mm and  $3.1 \pm 0.8$  mm, respectively. The thickness of the constructs varied from  $1.4 \pm 0.2$  mm for the 10 MPa, 1 Hz group to  $1.7 \pm 0.2$  mm for the 10 MPa, 0 Hz group. No significant differences, however, were observed among groups for the construct diameter ( $p = 0.71$ ) and thickness ( $p = 0.27$ ). H&E stains confirmed the presence of ECM in all groups (Fig. 3). Saf O/fast-green stains exhibited little staining for proteoglycans, correlating with low GAG production observed in all groups (Fig. 3). Picro-sirius red staining was positive, with stronger staining observed on the periphery of the scaffolds (Fig. 3).

### Biochemistry

Dry and wet weights of the scaffolds at  $t = 4$  wks are shown in Table I. The dry weight of scaffolds exposed to a pressure of 10 MPa at 1 Hz was  $\sim 1.3 \pm 0.3$  mg, half the weight of scaffolds in other groups ( $p = 0.009$ ). The wet weight of the scaffolds ranged from  $12.4 \pm 0.5$  to  $15.7 \pm 2.9$  mg; however, no significant difference was observed among groups ( $p = 0.37$ ). A significant difference was observed in cell number/scaffold among groups ( $p < 0.0001$ ) (Table I). Cells were originally seeded at 0.7 million cells/scaffold. At  $t = 0$ , the cell count was measured at  $0.62 \pm 0.17$  million cells/scaffold (efficiency  $>80\%$ ) and by the fourth week, the number of cells on the control scaffolds increased to 0.8 million cells/scaffold, although increase over  $t = 0$  was not significant. However, an intergroup comparison showed that the cell numbers on scaffolds in all HP groups ( $\leq 0.5$  million cells/scaffold) were fewer than in the culture control at  $t = 4$  weeks. No detectable collagen or GAG was observed at  $t = 0$ . At  $t = 4$  weeks [Fig. 4(a)], the total collagen/scaffold was found to be two times higher in the periodic HP group ( $27 \mu\text{g} \pm 5 \mu\text{g}$ ) than in all other HP groups and controls ( $p = 0.0006$ ). At  $t = 4$  weeks, low amounts of GAG [Fig. 4(b)] were detected in all groups ( $<8 \mu\text{g}/\text{scaffold}$ ,  $p = 0.03$ ). A significant difference in GAG/scaffold was only observed between the 10 MPa, 0 Hz group, and the culture control ( $\sim 1.8$  times higher).

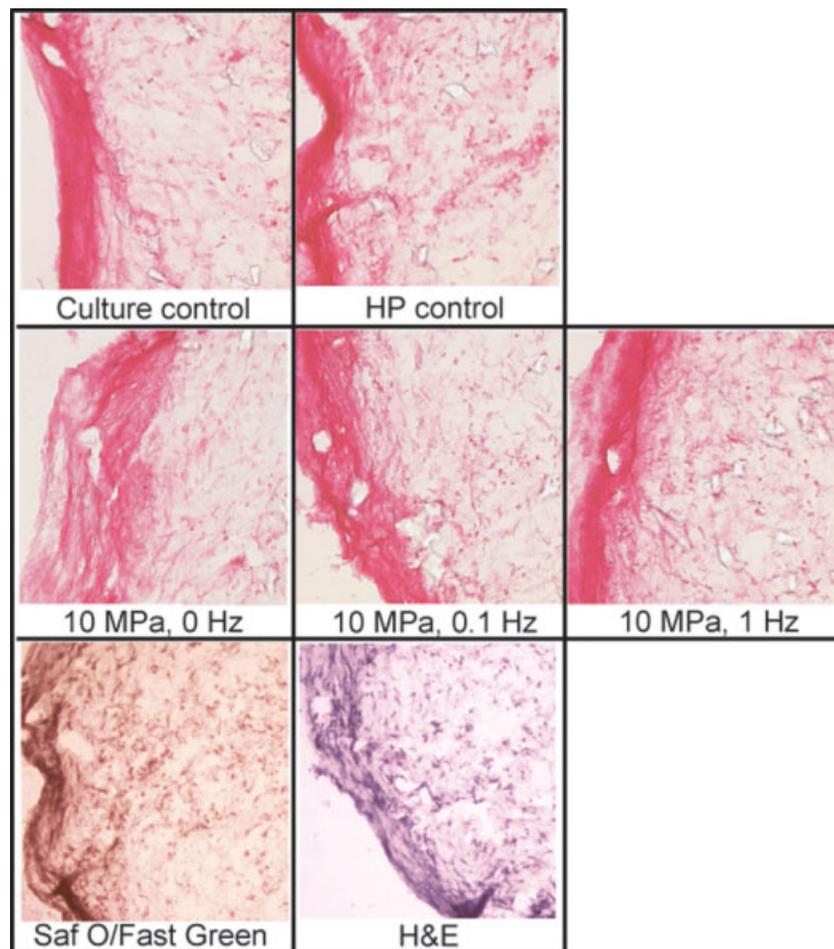


**Figure 2.** Gross morphology of pressurized and unpressurized scaffolds at 4 weeks. Top left and right pictures represent unpressurized controls. Bottom from left to right: constructs pressurized at 10 MPa and 0 Hz, 10 MPa and 0.1 Hz, and 10 MPa and 1 Hz. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

Collagen and GAG data were also normalized to cell number [Fig. 4(c,d)]. In both cases, all three HP groups (10 MPa 0, 0.1, and 1 Hz) were found to exhibit significantly higher collagen levels/cell when compared with the controls. The highest collagen content/cell ( $p = 0.003$ ) was exhibited in the 10 MPa, 0 Hz group with collagen values three times greater than the culture control. For GAG content/cell ( $p = 0.008$ ), a three times greater GAG accumulation was observed in 10 MPa, 0 Hz and 10 MPa, 1 Hz groups when compared with the culture control.

### Biomechanics

The viscoelastic parameters,  $E_r$ ,  $E_i$ , and  $\mu$ , were determined at 10, 20, and 30% strain via incremental stress relaxation tests. No apparent strain dependencies were identified for any of the parameters. Values of  $E_r$ ,  $E_i$ , and  $\mu$  at 30% strain are shown in



**Figure 3.** Histological stains of constructs at  $t = 4$  weeks: Top from left to right: Picrosirius red stains of unpressurized constructs (original magnification,  $\times 200$ ). Middle from left to right: Picrosirius red stains of constructs pressurized at 10 MPa and 0 Hz, 10 MPa and 0.1 Hz, and 10 MPa and 1 Hz. Bottom from left to right: Representative images of constructs stained with Saf O/fast green and H&E. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

**TABLE I**  
**Dry Weight, Wet Weight, and Cell Number of Scaffolds**  
**at  $t = 4$  Weeks from Different Treatment Groups**

Treatment	Dry Weight (mg)	Wet Weight (mg)	Cell Number (millions)
Culture Control	2.2 ± 1.1	15.7 ± 2.9	0.8 ± 0.1
HP Control	2.8 ± 0.2	12.4 ± 0.5	0.5 ± 0.1
10 MPa, 0 Hz	2.3 ± 0.5	13.8 ± 1.4	0.5 ± 0.1
10 MPa, 0.1 Hz	1.3 ± 0.3	13.1 ± 2.7	0.3 ± 0.1
10 MPa, 1 Hz	2.9 ± 0.8	13.7 ± 1.4	0.4 ± 0.1
<i>p</i> value	<i>p</i> = 0.009	<i>p</i> = 0.37	<i>p</i> < 0.0001

All values reported as mean ± SD. A *p* < 0.05 was considered significant.

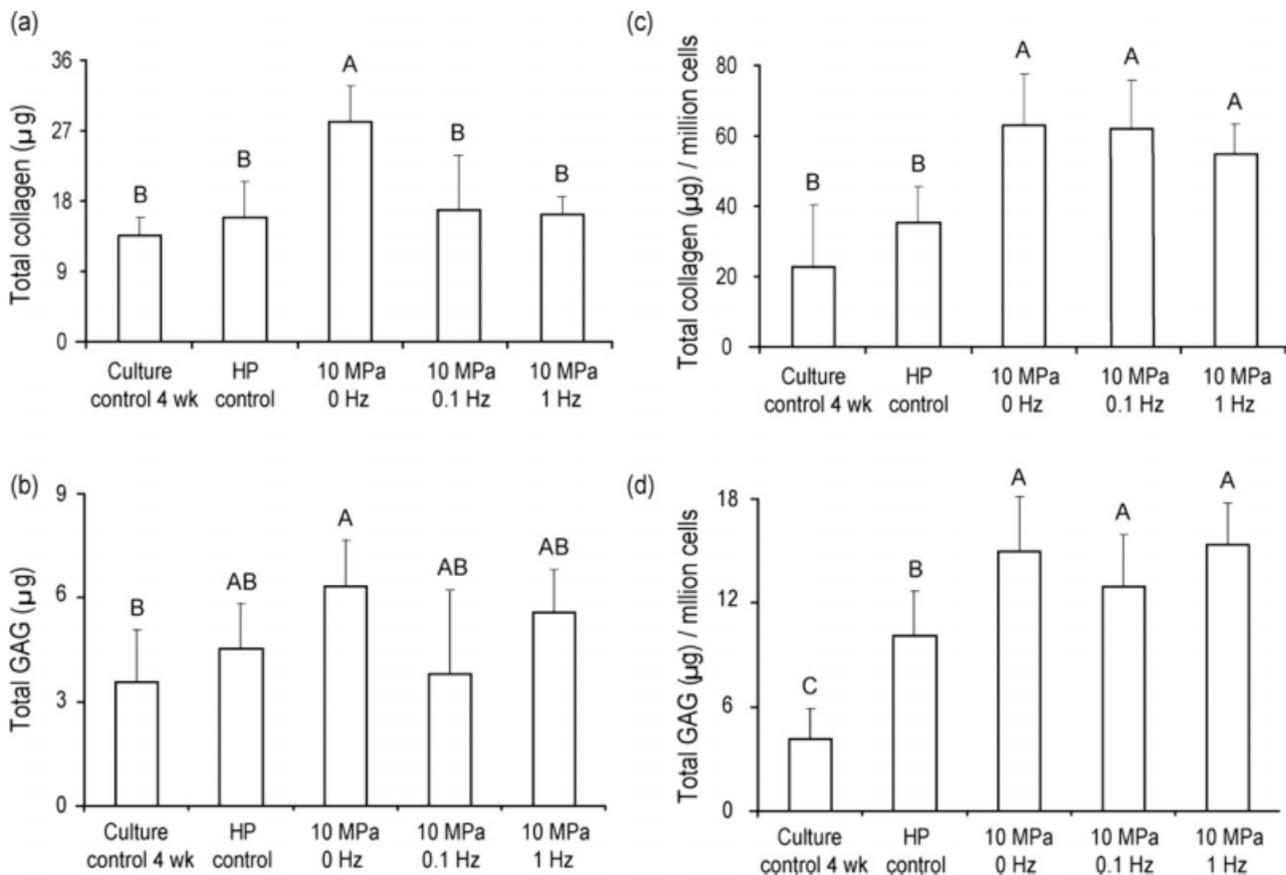
Figure 5. In general, the instantaneous modulus ranged from 31 to 88 kPa while the relaxation modulus ranged from 8 to 30 kPa among groups. The periodic HP group was found to be significantly stiffer than the culture control and 10 MPa, 1 Hz groups for the instantaneous moduli (~1.5 times stiffer, *p* = 0.005) and relaxation moduli values (~1.75 times stiffer, *p* = 0.02). The coefficient of viscosity (*p* = 0.05) was found to be greatest for the periodic HP

group (730 ± 167 Pa s) and lowest for the culture control (241 ± 190 Pa s). Similar differences were also observed for these parameters at 10% and 20% strain.

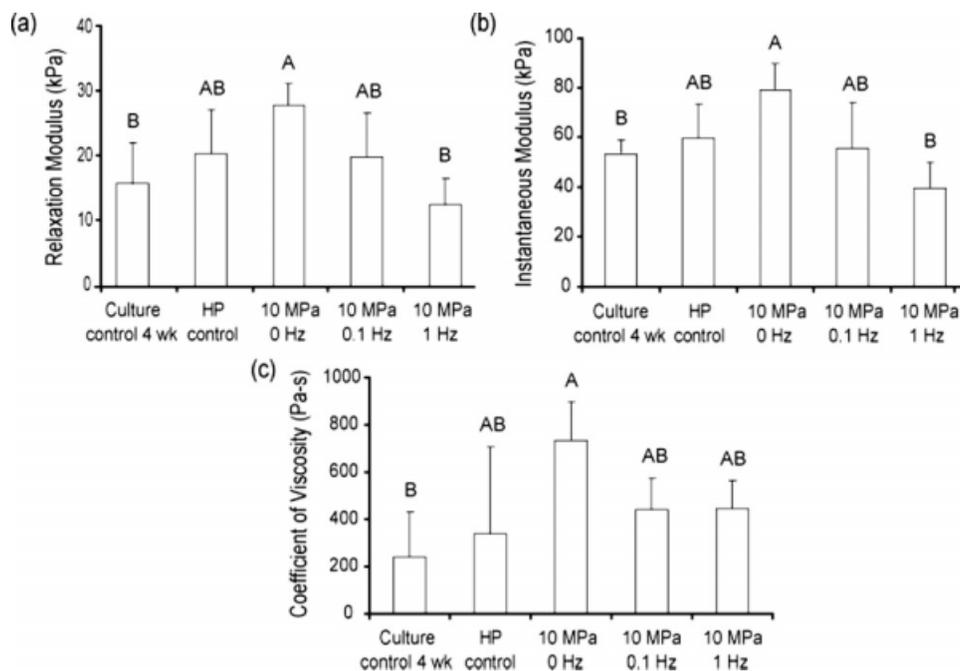
**DISCUSSION**

HP was used as a mechanical stimulus to coax meniscus cells seeded on PLLA constructs to up-regulate relevant ECM matrix *in vitro*, and to enhance the biomechanical properties of the constructs. The results of this study show that HP does, indeed, have a beneficial effect for meniscus tissue engineering purposes. Specifically, we found that the application of periodic HP (10 MPa, 0 Hz) for 1 h every 3 days resulted in constructs with greater collagen deposition when compared with the other examined groups. In addition, the compressive mechanical properties of scaffolds in this group were found to be significantly higher than scaffolds in the culture control and the 10 MPa, 1 Hz group.

Physiologic pressures in the knee joint range from 3 to 18 MPa.<sup>44,45</sup> To our knowledge, there is scant



**Figure 4.** Biochemical data at  $t = 4$  weeks: (a) Collagen/scaffold (*p* = 0.0006) (b) GAG/scaffold (*p* = 0.03). (c) Collagen/million cells (*p* = 0.003). (d) GAG/million cells (*p* = 0.008). All values reported as mean ± SD. Groups with the same letter are not significantly different from each other.



**Figure 5.** Biomechanical data (30% strain) at  $t = 4$  weeks: (a) Relaxation modulus ( $p = 0.02$ ) (b) Instantaneous modulus ( $p = 0.005$ ) (c) Coefficient of viscosity ( $p = 0.05$ ). All values reported as mean  $\pm$  SD. Groups with the same letter are not significantly different from each other.

information about pressures experienced by fibrochondrocytes and chondrocytes. Several mechanical tests have shown that chondrocytes in articular cartilage experience 7 to 10 MPa of hydrostatic pressure during normal activities.<sup>46</sup> In addition, a frequency ranging from 0.6 to 1.09 Hz is observed during normal walking rhythm of an adult human.<sup>47</sup> Thus, the goal in this experiment was to apply physiologically relevant pressures to knee meniscus constructs. Several tissue engineering studies with articular chondrocytes and TMJ disc fibrochondrocytes have shown beneficial effects for a loading regimen of 10 MPa<sup>21,23,48</sup> and for frequencies between 0 and 1 Hz<sup>23,28,49</sup> As a first step, we sought to use these beneficial regimens for a meniscus tissue engineering strategy. Thus, a pressure of 10 MPa at three frequencies (0, 0.1, and 1 Hz) was investigated with clear benefits for the 10 MPa, 0 Hz group. In future studies, we will examine alternative loading regimens and frequencies to further optimize and enhance the biochemical and biomechanical properties of meniscus cell-seeded constructs.

The cell numbers per scaffold in each group were analyzed to determine whether a particular HP stimulus resulted in cell proliferation on the scaffold. Contrary to our hypothesis that HP would increase cell proliferation, cells numbers/scaffold in all HP groups, including the HP controls, were found to be significantly lower than those of the unpressurized culture control at  $t = 4$  weeks, as well as lower than the original seeding density of  $\sim 0.7$  million cells/

scaffold. These results are consistent with a previous study using TMJ disc fibrochondrocytes seeded on PGA scaffolds where the average cell number/scaffold in pressurized groups was four times lower than the day 0 control.<sup>23</sup> However, unlike that study, we did not observe a drop in cell number in our unpressurized culture control. This leads us to believe that the manipulation involved in bagging the constructs was a major factor in inducing cell loss. Furthermore, our experiment was conducted over a long duration (4 weeks), with samples pressurized once every 3 days, thereby increasing the probability of cell dislodgment from the scaffolds. In addition to manipulation of the constructs for prolonged periods, the HP stimulation itself might have contributed to cell dislodgment from the scaffold. Although HP stimulation should not cause volumetric changes to the construct, theoretical models for the effect of HP on composite polymers, such as PLLA, have shown that increasing HP can increase the bulk modulus and Poisson's ratio of the polymer slightly while decreasing its specific volume.<sup>50</sup> Thus, these effects might result in extremely small displacements on the scaffold causing cells to dislodge. Despite observations of cell loss, however, HP was still shown to be beneficial for constructs in terms of their biochemical and biomechanical composition.

Meniscus cells were pooled from medial and lateral menisci which may have introduced some variability in our experiment. The meniscus contains two major cell types, fibroblast-like cells

and fibrochondrocytes, which are capable of producing different types of collagens *in vivo* and *in vitro*.<sup>5,51–53</sup> Although specific collagens present in our constructs were not examined, it is expected that both collagen I and collagen II would be present. A study in the literature has shown that meniscus cells seeded on scaffolds produced both collagen I and collagen II as seen by immunohistochemical analysis.<sup>52</sup> The focus of this experiment was to identify a hydrostatic pressure regimen that enhanced the total collagen and GAG content and improved the mechanical properties of the cell-seeded PLLA constructs. In future studies, we will investigate whether hydrostatic pressure influences specific collagen levels, akin to a study conducted with inner and outer fibrous cells where significant differences were observed in collagen I and collagen II content between inner and outer cells exposed to hydrostatic pressure.<sup>29</sup>

Biochemical tests were conducted after 4 weeks of pressurization to determine 10 MPa HP effects at varying frequencies (0, 0.1, and 1 Hz). HP was found to be a significant factor, and the 10 MPa, 0 Hz group showed the greatest collagen content/scaffold. This result is in agreement with a previous study using TMJ disc cells that showed significantly higher levels of collagen/construct under a 10 MPa static HP regimen than under an intermittent regimen of 10 MPa, 1 Hz.<sup>23</sup> If total collagen and GAG content are normalized to cell number, constructs in all three HP groups (10 MPa 0, 0.1, and 1 Hz) result in significantly higher collagen and GAG deposition per cell than in the controls, suggesting that both intermittent and periodic HP increase the cells' capability to synthesize ECM. This finding is consistent with gene expression studies on articular chondrocytes where intermittent HP results in up-regulation of collagen II and aggrecan expression.<sup>14,20,21,54</sup> Although the mechanism to explain these results remains elusive, several theories have been proposed in the literature. In chondrocytes, intracellular ionic and osmotic variations as a result of HP stimulus have been shown to alter the activity of membrane transport pathways that affect matrix synthesis.<sup>19</sup> In cultured mouse calvariae and periosteal cells, intermittent HP has been shown to stimulate the release of activated TGF- $\beta$ .<sup>55</sup> TGF- $\beta$ 1 has been shown in several experiments to up-regulate collagen expression and synthesis *in vitro* in meniscus cells.<sup>32,56</sup> HP has also been shown to regulate matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) in meniscus cells, which would affect matrix breakdown and accumulation. A recent study showed that removing rabbit menisci from the knee joint results in an up-regulation of MMP-1 and MMP-3 in the explant, which could then be down-regulated by HP.<sup>4</sup> Further, meniscus cells embedded in alginate beads down-regulated MMP-1 and MMP-

13 when exposed to static HP and up-regulated TIMP-1 and TIMP-2 when exposed to intermittent HP.<sup>57</sup> Thus, we believe that collagen regulation may be affected both directly due to HP mechanotransduction on the cells, as well as indirectly via the regulation of TGF- $\beta$  and MMP pathways which in turn may enhance collagen synthesis or prevent collagen breakdown.

Some interesting observations can be made about the compressive mechanical properties of the scaffolds in relation to their biochemical properties. Constructs in the 10 MPa, 0 Hz group were significantly stiffer in compression than the culture control and the 10 MPa, 1 Hz group. In addition, the intermittent HP regimens were neither detrimental nor beneficial for the formation of robust tissue when compared with the controls. *In vivo*, articular cartilage and, to a lesser extent, the knee meniscus exhibit high compressive properties because of the presence of highly negatively charged GAGs that attract water and hydrate the tissue.<sup>58</sup> A significant increase in GAG/scaffold at  $t = 4$  weeks for the periodic HP group over the culture control correlated well with the biomechanical data and suggested that periodic HP stimulation resulted in mechanically superior scaffolds.

Rest time between HP stimulation may be an important factor to consider in tissue engineering studies. A short-term gene expression study with fibrochondrocytes showed that ECM gene profiles were up-regulated to a greater extent 24 h post HP stimulation as opposed to directly after pressurization.<sup>22</sup> A delayed gene expression response for ECM molecules would likely translate to delays in ECM synthesis as well. On the basis of this working hypothesis, we utilized a rest time of 2 days after each HP stimulus to allow cells seeded on scaffold to up-regulate gene expression of important ECM components and provide sufficient time for matrix synthesis and organization prior to the next pressurization cycle. Other loading regimens of 5 day on, 2 days off or 2 days on, 1 day off have been employed in the past by researchers conducting HP tissue engineering studies on articular chondrocytes and TMJ disc cells.<sup>23,28</sup> However, these studies and ours examined only one rest time and so conclusions about their beneficial effect may be premature. Nonetheless, it will be prudent in future experiments to examine rest time as an experimental factor to determine an optimal condition that favors greater ECM synthesis such as the study on dynamically compressed articular cartilage explants where the authors discovered that a rest time of 100 s or less between stimulation increased the radiolabeled sulfate uptake in the explants.<sup>36</sup>

In conclusion, the experimental data provide significant evidence for the benefit of periodic HP (10 MPa, 0 Hz) for the purposes of meniscus tissue

engineering using PLLA scaffolds. The increase in total collagen in the periodic HP constructs, despite the drop in cell number, is a significant finding and an investigation on the influence of HP on the metabolic pathways of meniscus cells might shed some light on the underlying mechanism for this observation. The compressive stiffness of our constructs was found to increase concomitantly with increases in GAG deposition; however, the values (31–88 kPa) are still below those of native tissue (100–400 kPa).<sup>59</sup> To further improve the structural integrity, the use of growth factors can be employed. Previous research has shown that growth factors, such as TGF- $\beta$ 1 and b-FGF, are responsible for up-regulating collagen I, collagen II, and proteoglycan synthesis in cartilaginous cell cultures, explants, and cell-seeded scaffolds.<sup>32,56,60–62</sup> Further, static HP alone has been shown to up-regulate TGF- $\beta$ 1 expression in a characterized human chondrocyte cell-line.<sup>63</sup> Thus, a myriad of possibilities can be investigated to better understand the effects of HP at the cellular and tissue level, as well as create cartilaginous tissue engineered constructs with biochemical and biomechanical properties approaching those of native tissue.

The authors thank Dr. Jerry Hu for aiding with the revision of the manuscript.

## References

- Ahmed AM. The load-bearing role of the knee meniscus. In: Mow VC, Arnoczky SP, Jackson DW, editors. *Knee Meniscus: Basic and Clinical Foundations*. New York: Raven Press; 1992. p 59–73.
- Walker PS, Erkman MJ. The role of the menisci in force transmission across the knee. *Clin Orthop* 1975;109:184–192.
- AufderHeide AC, Athanasiou KA. Mechanical stimulation toward tissue engineering of the knee meniscus. *Ann Biomed Eng* 2004;32:1161–1174.
- Natsu-Ume T, Majima T, Reno C, Shrive NG, Frank CB, Hart DA. Menisci of the rabbit knee require mechanical loading to maintain homeostasis: Cyclic hydrostatic compression in vitro prevents derepression of catabolic genes. *J Orthop Sci* 2005; 10:396–405.
- Sweigart MA, Athanasiou KA. Toward tissue engineering of the knee meniscus. *Tissue Eng* 2001;7:111–129.
- Rangger C, Klestil T, Gloetzer W, Kemmler G, Benedetto KP. Osteoarthritis after arthroscopic partial meniscectomy. *Am J Sports Med* 1995;23:240–244.
- LeRoux MA, Arokoski J, Vail TP, Guilak F, Hyttinen MM, Kiviranta I, Setton LA. Simultaneous changes in the mechanical properties, quantitative collagen organization, and proteoglycan concentration of articular cartilage following canine meniscectomy. *J Orthop Res* 2000;18:383–392.
- Rittweger J, Frost HM, Schiessl H, Ohshima H, Alkner B, Tesch P, Felsenberg D. Muscle atrophy and bone loss after 90 days' bed rest and the effects of flywheel resistive exercise and pamidronate: Results from the LTBR study. *Bone* 2005; 36:1019–1029.
- Sevastik JA, Larsson SE, Mattson S. Bone atrophy by disuse in adult rats. *Calcif Tissue Res* 1968;2(Suppl):97.
- Ochi M, Kanda T, Sumen Y, Ikuta Y. Changes in the permeability and histologic findings of rabbit menisci after immobilization. *Clin Orthop Relat Res* 1997;334:305–315.
- Shin SJ, Fermor B, Weinberg JB, Pisetsky DS, Guilak F. Regulation of matrix turnover in meniscal explants: Role of mechanical stress, interleukin-1, and nitric oxide. *J Appl Physiol* 2003;95:308–313.
- Imler SM, Doshi AN, Levenston ME. Combined effects of growth factors and static mechanical compression on meniscus explant biosynthesis. *Osteoarthritis Cartilage* 2004;12:736–744.
- Vanderploeg EJ, Imler SM, Brodtkin KR, Garcia AJ, Levenston ME. Oscillatory tension differentially modulates matrix metabolism and cytoskeletal organization in chondrocytes and fibrochondrocytes. *J Biomech* 2004;37:1941–1952.
- Smith RL, Lin J, Trindade MC, Shida J, Kajiyama G, Vu T, Hoffman AR, van der Meulen MC, Goodman SB, Schurman DJ, Carter DR. Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression. *J Rehabil Res Dev* 2000;37:153–161.
- Jin M, Frank EH, Quinn TM, Hunziker EB, Grodzinsky AJ. Tissue shear deformation stimulates proteoglycan and protein biosynthesis in bovine cartilage explants. *Arch Biochem Biophys* 2001;395:41–48.
- Ragan PM, Chin VI, Hung HH, Masuda K, Thonar EJ, Arner EC, Grodzinsky AJ, Sandy JD. Chondrocyte extracellular matrix synthesis and turnover are influenced by static compression in a new alginate disk culture system. *Arch Biochem Biophys* 2000;383:256–264.
- Jin M, Emkey GR, Siparsky P, Trippel SB, Grodzinsky AJ. Combined effects of dynamic tissue shear deformation and insulin-like growth factor I on chondrocyte biosynthesis in cartilage explants. *Arch Biochem Biophys* 2003;414:223–231.
- Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. *J Orthop Res* 1989;7:619–636.
- Hall AC. Differential effects of hydrostatic pressure on cation transport pathways of isolated articular chondrocytes. *J Cell Physiol* 1999;178:197–204.
- Ikenoue T, Trindade MC, Lee MS, Lin EY, Schurman DJ, Goodman SB, Smith RL. Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. *J Orthop Res* 2003;21:110–116.
- Smith RL, Rusk SF, Ellison BE, Wessells P, Tsuchiya K, Carter DR, Caler WE, Sandell LJ, Schurman DJ. In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure. *J Orthop Res* 1996;14:53–60.
- Shim JW, Elder SH. Influence of cyclic hydrostatic pressure on fibrocartilaginous metaplasia of Achilles tendon fibroblasts. *Biomech Model Mechanobiol* 2006;5:247–252.
- Almaraz AJ, Athanasiou KA. Effects of hydrostatic pressure on TMJ disc cells. *Tissue Eng* 2006;12:1285–1294.
- Parkkinen JJ, Ikonen J, Lammi MJ, Laakkonen J, Tammi M, Helminen HJ. Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants. *Arch Biochem Biophys* 1993;300:458–465.
- Shin HJ, Lee CH, Cho IH, Kim YJ, Lee YJ, Kim IA, Park KD, Yui N, Shin JW. Electrospun PLGA nanofiber scaffolds for articular cartilage reconstruction: Mechanical stability, degradation and cellular responses under mechanical stimulation in vitro. *J Biomater Sci Polym Ed* 2006;17(1/2):103–119.
- Hutton WC, Elmer WA, Boden SD, Hyon S, Toribatake Y, Tomita K, Hair GA. The effect of hydrostatic pressure on intervertebral disc metabolism. *Spine* 1999;24:1507–1515.
- Elder SH, Sanders SW, McCulley WR, Marr ML, Shim JW, Hasty KA. Chondrocyte response to cyclic hydrostatic pres-

- sure in alginate versus pellet culture. *J Orthop Res* 2006;24:740–747.
28. Hu JC, Athanasiou KA. The effects of intermittent hydrostatic pressure on self-assembled articular cartilage constructs. *Tissue Eng* 2006;12:1337–1344.
  29. Reza AT, Nicoll SB. Hydrostatic pressure differentially regulates outer and inner annulus fibrosus cell matrix production in 3D scaffolds. *Ann Biomed Eng* 2008;36:204–213.
  30. Aufderheide AC, Athanasiou KA. Comparison of scaffolds and culture conditions for tissue engineering of the knee meniscus. *Tissue Eng* 2005;11(7/8):1095–1104.
  31. Ibarra C, Jannetta C, Vacanti CA, Cao Y, Kim TH, Upton J, Vacanti JP. Tissue engineered meniscus: A potential new alternative to allogeneic meniscus transplantation. *Transplant Proc* 1997;29(1/2):986–988.
  32. Pangborn CA, Athanasiou KA. Growth factors and fibrochondrocytes in scaffolds. *J Orthop Res* 2005;23:1184–1190.
  33. Gunja NJ, Athanasiou KA. Biodegradable materials in arthroscopy. *Sports Med Arthrosc* 2006;14:112–119.
  34. Allen K. Mechanical characterization, gene expression, and biosynthesis of the porcine TMJ disc for the purposes of tissue engineering [Ph.D.]. Houston: Rice University; 2006.
  35. Neidlinger-Wilke C, Wurtz K, Liedert A, Schmidt C, Borm W, Ignatius A, Wilke HJ, Claes L. A three-dimensional collagen matrix as a suitable culture system for the comparison of cyclic strain and hydrostatic pressure effects on intervertebral disc cells. *J Neurosurg Spine* 2005;2:457–465.
  36. Sauerland K, Raiss RX, Steinmeyer J. Proteoglycan metabolism and viability of articular cartilage explants as modulated by the frequency of intermittent loading. *Osteoarthritis Cartilage* 2003;11:343–350.
  37. Rosenberg L. Chemical basis for the histological use of safranin O in the study of articular cartilage. *J Bone Joint Surg Am* 1971;53:69–82.
  38. Battlehner CN, Carneiro Filho M, Ferreira Junior JM, Saldiva PH, Montes GS. Histochemical and ultrastructural study of the extracellular matrix fibers in patellar tendon donor site scars and normal controls. *J Submicrosc Cytol Pathol* 1996;28:175–186.
  39. Allen KD, Athanasiou KA. Viscoelastic characterization of the porcine temporomandibular joint disc under unconfined compression. *J Biomech* 2006;39:312–322.
  40. Allen KD, Athanasiou KA. A surface-regional and freeze-thaw characterization of the porcine temporomandibular joint disc. *Ann Biomed Eng* 2005;33:951–962.
  41. Rada JA, Matthews AL. Visual deprivation upregulates extracellular matrix synthesis by chick scleral chondrocytes. *Invest Ophthalmol Vis Sci* 1994;35:2436–2447.
  42. Pietila K, Kantomaa T, Pirttiniemi P, Poikela A. Comparison of amounts and properties of collagen and proteoglycans in condylar, costal and nasal cartilages. *Cells Tissues Organs* 1999;164:30–36.
  43. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961;93:440–447.
  44. Afoke NY, Byers PD, Hutton WC. Contact pressures in the human hip joint. *J Bone Joint Surg Br* 1987;69:536–541.
  45. Hodge WA, Fijan RS, Carlson KL, Burgess RG, Harris WH, Mann RW. Contact pressures in the human hip joint measured in vivo. *Proc Natl Acad Sci USA* 1986;83:2879–2883.
  46. Hall AC, Horwitz ER, Wilkins RJ. The cellular physiology of articular cartilage. *Exp Physiol* 1996;81:535–545.
  47. Waters RL, Lunsford BR, Perry J, Byrd R. Energy-speed relationship of walking: Standard tables. *J Orthop Res* 1988;6:215–222.
  48. Elder BD, Athanasiou KA. Effects of temporal hydrostatic pressure on tissue-engineered bovine articular cartilage constructs. *Tissue Eng Part A* 2009;15:1151–1158.
  49. Toyoda T, Seedhom BB, Kirkham J, Bonass WA. Upregulation of aggrecan and type II collagen mRNA expression in bovine chondrocytes by the application of hydrostatic pressure. *Biorheology* 2003;40(1–3):79–85.
  50. Ainbinder SB. Effect of hydrostatic pressure on the deformation properties of polymer materials. *Mech Compos Mater* 1968;4(4–6):787–791.
  51. Gunja NJ, Athanasiou KA. Passage and reversal effects on gene expression of bovine meniscal fibrochondrocytes. *Arthritis Res Ther* 2007;9(5):R93.
  52. Kang SW, Son SM, Lee JS, Lee ES, Lee KY, Park SG, Park JH, Kim BS. Regeneration of whole meniscus using meniscal cells and polymer scaffolds in a rabbit total meniscectomy model. *J Biomed Mater Res A* 2006;78:659–671.
  53. Hellio Le Graverand MP, Reno C, Hart DA. Gene expression in menisci from the knees of skeletally immature and mature female rabbits. *J Orthop Res* 1999;17:738–744.
  54. Hansen U, Schunke M, Domm C, Ioannidis N, Hassenpflug J, Gehrke T, Kurz B. Combination of reduced oxygen tension and intermittent hydrostatic pressure: A useful tool in articular cartilage tissue engineering. *J Biomech* 2001;34:941–949.
  55. Klein-Nulend J, Roelofsens J, Sterck JG, Semeins CM, Burger EH. Mechanical loading stimulates the release of transforming growth factor-beta activity by cultured mouse calvariae and periosteal cells. *J Cell Physiol* 1995;163:115–119.
  56. Pangborn CA, Athanasiou KA. Effects of growth factors on meniscal fibrochondrocytes. *Tissue Eng* 2005;11(7/8):1141–1148.
  57. Suzuki T, Toyoda T, Suzuki H, Hisamori N, Matsumoto H, Toyama Y. Hydrostatic pressure modulates mRNA expressions for matrix proteins in human meniscal cells. *Biorheology* 2006;43:611–622.
  58. Almarza AJ, Athanasiou KA. Design characteristics for the tissue engineering of cartilaginous tissues. *Ann Biomed Eng* 2004;32:2–17.
  59. Sweigart MA, Zhu CF, Burt DM, DeHoll PD, Agrawal CM, Clanton TO, Athanasiou KA. Intraspecies and interspecies comparison of the compressive properties of the medial meniscus. *Ann Biomed Eng* 2004;32:1569–1579.
  60. Collier S, Ghosh P. Effects of transforming growth factor beta on proteoglycan synthesis by cell and explant cultures derived from the knee joint meniscus. *Osteoarthritis Cartilage* 1995;3:127–138.
  61. Adesida AB, Grady LM, Khan WS, Hardingham TE. The matrix-forming phenotype of cultured human meniscus cells is enhanced after culture with fibroblast growth factor 2 and is further stimulated by hypoxia. *Arthritis Res Ther* 2006;8:R61.
  62. Fu Q, Lu M, Shen T. Experimental study of bFGF modulating rabbit articular chondrocytes cultured in vitro and seeded onto polylactic acid scaffold coated with different materials. *Zhonghua Wai Ke Za Zhi* 2005;43:1590–1593.
  63. Gunther M, Haubeck HD, van de Leur E, Blaser J, Bender S, Gutgemann I, Fischer DC, Tschesche H, Greiling H, Heinrich PC, Graeve L. Transforming growth factor beta 1 regulates tissue inhibitor of metalloproteinases-1 expression in differentiated human articular chondrocytes. *Arthritis Rheum* 1994;37:395–405.