Hypoxia and Flow Perfusion Modulate Proliferation and Gene Expression of Articular Chondrocytes on Porous Scaffolds

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The combination of reduced oxygen tension and flow perfusion bioreactor culture is investigated for its effect on the proliferation, glycosaminoglycan production, and chondrogenic gene expression of bovine articular chondrocytes on porous polymer scaffolds. It was hypothesized that the combination of such factors would more closely replicate the in situ *environment of these cells, leading to improvements in the cell phenotype. Chondrocytes were seeded onto electrospun poly(ε-caprolactone) scaffolds and cultured in static or perfusion culture in either normoxic or hypoxic conditions for 6 days. Results demonstrated that the combination of hypoxic and perfusion culture led to an increase in chondrocyte proliferation and glycosaminoglycan production, as well as an improvement in the ratio of collagen II/I gene expression over perfusion culture alone. The results demonstrate the need to combine multiple signals* in vitro*, in order to improve tissue growth by more closely replicating the native environment of cells.* © 2013 American Institute of Chemical Engineers *AIChE J*, 59: 3158-3166, 2013
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Introduction

Articular cartilage lesions, the result of trauma or disease, have a limited healing ability and often progress to osteoarthritis.[1] Osteoarthritis was the main contributor to the 450,000 cases of total knee arthroplasty in the United States in 2004, totaling $14.6 billion in healthcare costs, and this number is expected to rise to an astounding 3,500,000 cases by 2030.[2] There are currently several treatments that attempt to repair articular cartilage defects;[1,3] however, these approaches suffer from inconsistency[3] and usually result in the formation of fibrocartilage, which often fails due to inferior mechanical properties.[4] Thus, improved options to treat articular cartilage defects are needed, and tissue engineering provides a potential solution to this problem. This work focuses on investigating methods for the production of tissue-engineered cartilage with emphasis on the application of flow perfusion bioreactor culture and controlled oxygen tension on chondrocyte-seeded scaffolds *in vitro*.

Chondrocytes are a common cell source for cartilage tissue engineering and are currently used in the clinic during autologous chondrocyte transplantation; however there are several limitations to their use.[5] Chondrocytes can be obtained from articular cartilage biopsies and expanded *in vitro*.[4] However, clinically the isolation of chondrocytes causes donor site morbidity,[6] and, thus, isolation of sufficient numbers of cells, which are relatively sparse in articular cartilage,[7] presents a problem. Furthermore, upon *in vitro* expansion, chondrocytes dedifferentiate and express a fibroblastic phenotype,[8] which leads to the production of an inferior tissue engineered construct with significantly less collagen type II than native tissue.[9] For this reason, technologies that allow the expansion of articular chondrocytes without the loss of phenotype could be instrumental to tissue engineers.

In articular cartilage engineering, a significant volume of work has focused on the application of mechanical stimulation to cells in order to enhance the cell phenotype and matrix production.[10] Several forms of mechanical stimulation have been employed, such as hydrostatic pressure, direct compression, and shear.[11,12] Flow perfusion bioreactors are one method of applying mechanical stimulation to cells in a three-dimensional (3-D) scaffold, and are commonly used in many areas of tissue engineering.[13,14] These culture systems are used to apply shear stress to cells and to enhance mass transport through the scaffold.[15] In cartilage engineering, perfusion bioreactors have primarily been shown to enhance articular chondrocyte proliferation, matrix production, and infiltration into the scaffold porosity.[16–18]

In vivo, chondrocytes are continuously exposed to a gradient of hypoxic conditions,[19,20] and low-oxygen conditions are thought to play a vital role in cartilage differentiation and endochondral bone development.[21,22] For these reasons, the role of oxygen tension has been investigated in the regulation of chondroprogenitor cells. In chondrocytes, low-oxygen tension has been shown to promote both the production of cartilage-specific extracellular matrix (ECM) components and restoration of the chondrocyte

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phenotype,[23,24] as well as the production of mechanically superior constructs compared to chondrocytes cultured in normoxic conditions.[22] Enhancements in chondrocyte matrix production on both gene and protein levels can be seen with as little as 1 day of hypoxic exposure.[25]

The beneficial effects of perfusion and hypoxia in isolation have been demonstrated by others; however the combined effects of these two factors on articular chondrocytes have not previously been evaluated. In this study, it was hypothesized that hypoxia in combination with flow perfusion culture would more closely mimic the *in situ* environment of chondrocytes, and, thus, enhance the chondrogenic phenotype of these cells as detected by the production of cartilage-like extracellular matrix and expression of chondrogenic genes.

Materials and Methods

Experimental design

Bovine articular chondrocytes were seeded onto electrospun poly (ε-caprolactone) (PCL) scaffolds, and cultured in static or flow perfusion culture in both normoxic and hypoxic conditions in order to investigate the combined effects of perfusion culture and hypoxia. A flow perfusion bioreactor was used, the design of which has previously been described in detail.[26] Time points were taken after 2, 4, and 6 days of culture, as well as day 0 (after the overnight adhesion period, and just prior to the start of perfusion culture). At each time point, 3 bioreactor units and an equivalent number of static samples were harvested and samples were stored for biochemical analysis (n = 9), real-time reverse transcriptase polymerase chain reaction (RT-PCR) (n = 6), and histology (n = 6).

Scaffold fabrication and characterization

Nonwoven PCL microfiber mats were fabricated with an average fiber diameter of approximately 10 μm (9.30 μm $\pm$ 1.7 μm, n = 90) as previously described.[27,28] Mats were electrospun with a horizontal electrospinning setup using PCL (inherent viscosity range 1.0–1.3 dL/g in $CHCl_3$; Durect Corp., Cupertino, CA) with a number-average molecular weight (M_n) of 71,000 $\pm$ 2,300 Da, and a polydispersity index (M_w/M_n) of 2.2 $\pm$ 0.07, as determined by gel permeation chromatography (Phenogel Linear Column with 5 -μm particles, Phenomenex, Torrance, CA; Differential Refractometer 410, Waters, Milford, MA, n = 3), and a calibration curve generated from polystyrene standards (Fluka, Switzerland). An 18 wt % polymer solution in a 5:1 volume ratio of chloroform to methanol was pumped at a flow rate of 40 mL/h through a blunt 16 G needle. The needle was charged with a voltage of 30 kV and directed toward a grounded collecting plate 33 cm from the tip of the needle. Mats were electrospun to the desired thickness, and fiber diameter and morphology were inspected using a scanning electron microscope (FEI Quanta 400 Environmental; FEI, Hillsboro, OR). Scaffolds were determined to have an average pore size of 45 μm and a porosity of 87% as measured by mercury porosimetry (Quantachrome PoreMaster; Quantachrome Instruments, Boynton Beach, FL), and gravimetric analysis, respectively, which is consistent with previous characterization of electrospun PCL scaffolds with approximately the same fiber diameter.[27,29]

Scaffolds were prepared by die-punching 3 mm dia. discs from the electrospun mats using dermal biopsy punches. Scaffolds 1.2 to 1.3 mm thick were press-fit into custom-made polycarbonate scaffold holders designed to confine the cell seeding solution and support the scaffolds during perfusion culture. The scaffolds and scaffold holders were then sterilized by exposure to ethylene oxide (Anderson Sterilizers, Haw River, NC) for 14 h. Scaffolds were then prewetted by soaking in a graded series of ethanol (100 to 25%) followed by three rinses in phosphate buffered saline (PBS), and incubation in general culture medium (high-glucose DMEM, 10% fetal bovine serum (FBS) (BenchMark; Gemini Bio-Products, West Sacramento, CA), penicillin/streptomycin/fungizone (PSF)) for 3 days.[28]

Cell Isolation and culture

Bovine articular chondrocytes were isolated from 7 to 10 day old calves (Research 87, Bolyston, MA) less than 24 h after slaughter using previously established methods.[27,28] Briefly, articular cartilage was collected from the femoral condyle, minced to 1 $\times$ 1 $\times$ 1 mm pieces, washed with PBS and digested in chondrocyte growth medium (DMEM, 10% FBS, 1% nonessential amino acids, 50 μg/mL ascorbic acid, 46 μg/mL L-proline, 20 mM HEPES, 1% PSF) containing 2 mg/mL collagenase type II (Worthington biochemical corporation, Lakewood, NJ). Digestions were incubated on a shaker table at 37°C for 16 h. Cells were isolated from 4 legs, pooled, aliquoted and cryopreserved in freezing medium (DMEM containing 20% FBS and 10% dimethyl sulfoxide.)

Prior to use cells were thawed, removed from freezing medium, plated at 2 million cells per T-225 flask in chondrocyte growth medium, and expanded for one passage. Cells were then trypsinized using 0.05% trypsin-EDTA and counted using a hemocytometer. Chondrocytes were seeded onto each scaffold at a density of 40,000 cells/scaffold in a 30 μL cell solution, and incubated overnight for cell attachment.[27]

Static cultures were removed from the scaffold cassettes and placed in ultralow attachment 24 well plates with 1 mL of chondrocyte growth medium. Dynamic cultures were performed in a flow perfusion bioreactor as previously described[26] with 50 mL of medium, 10 scaffolds per bioreactor unit, and a flow rate of 10 μL/min through each 3 mm scaffold. Oxygen tension was controlled and monitored using a heat jacketed CO_2 incubator with oxygen sensing and control (HeraCell 150i; ThermoScientific).[30] Hypoxic cultures were maintained at 5% O_2 throughout the duration of the culture. Normoxic cultures were maintained at 20% O_2. At the conclusion of each culture, samples were rinsed with PBS before being stored until analysis.

Biochemical analysis

Samples were stored at −20°C until analysis. Thawed samples were digested in 500 μL of proteinase K digestion buffer (50 mM Tris-HCl (pH 7.6), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM iodoacetamide, 10 μg/mL pepstatin A, and 1 mg/mL proteinase K) in a water bath at 56 °C for 16 h. Following digestion, samples underwent three rapid freeze/thaw cycles (10 min in liquid nitrogen/10 min in 36 °C water bath) followed by 20 min of sonication to ensure complete extraction of DNA and matrix components from the scaffolds into the supernatant.

The concentration of double stranded DNA in the supernatant was quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR) according to the

manufacturer's instructions.[27,31] Cell lysate, assay buffer and dye solution were pipetted into an opaque 96 well plate in duplicates and fluorescence was measured using excitation and emission wavelengths of 485 nm and 528 nm (FL × 800 Fluorescence Microplate Reader; BioTek Instruments, Winooski, VT). A lambda DNA standard curve was used to calculate DNA concentrations.

Sulfated glycosaminoglycans were quantified using the colorimetric dimethylmethylene blue dye (DMMB) assay and a chondroitin sulfate standard curve.[27,32] Cell lysate and DMMB reagent were combined in a transparent 96-well plate in duplicates. Absorbance at 520 nm was measured (Powerwave x340 Microplate Reader; BioTek Instrumets). These results were converted to total sulfated GAGs per scaffold and normalized to the number of cells present in each scaffold.

RT-PCR

After rinsing with PBS, two scaffolds were pooled together for each RNA isolate sample (n = 6 samples). Scaffolds were placed in 600 μL of lysis buffer and vortexed before storing at − 80 °C until further processing. RNA isolation was performed using an RNeasy mini kit (Qiagen, Valencia, CA).[27,28] Cell lysate was passed through a QIAshredder homogenization column and combined with an equal volume of 70% ethanol. RNA isolation was then performed following the manufacturer's instructions for the isolation of RNA from animal cells. Reverse transcription was performed using Oligo(dT) primers (Promega, San Luis Obispo, CA), and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR (Applied Biosystems 7300 Real-Time PCR System, Foster City, CA) was performed on cDNA samples using SYBR green detection (PerfeCTa SYBR Green FastMix, ROX; Quanta Biosciences, Gaithersburg, MD) with previously established primer sequences (Integrated DNA Technologies, Coralville, IA).[28] β_2-microglobulin (B2M) was used as a housekeeping gene, as it has previously been shown to be stable under hypoxic conditions.[33]

Target gene expression was first normalized to the expression of the housekeeping gene B2M in the same sample (ΔCt), then to the average baseline expression of that target gene measured in the cell stock used to seed the scaffolds ($\Delta\Delta$Ct). The $2^{-\Delta\Delta Ct}$ method was used to convert normalized gene expression levels to fold differences,[34] and statistical analysis was performed on these values. Similarly, $2^{-\Delta Ct}$ was used to calculate the ratios of collagen II/collagen I. The sequences of primers used in this analysis were[28]: Collagen type I: 5′- CGGGTCTTGCTGGTCATCAT-3′, 5′-TGCACCAGGCTGTCCAATG-3′; Collagen type II: 5′-AG TGGAAGAGCGGAGACTACTG-3′; 5′-GTTGGGAGCCA GGTTGTCAT-3′; Aggrecan: 5′-AGAGAGCCAAACAGCC GACA-3′; 5′-TAGTCCTGGGCATTGTTGTTGA-3′; B2M: 5′-CCGGATAGTTAAGTGGGATCG-3′; 5′-CATGGACATG TAGCACCCAAG-3′.

Histological analysis

Harvested samples were fixed using 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Samples were dehydrated in 70% ethanol before embedding in HistoPrep freezing medium (Fisher Scientific). Scaffolds were cut into 5 μm thick sections using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany). Sections were then stained using safranin O and fast green to visualize the presence and distribution of cartilaginous matrix and cells, respectively. Images were obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).

Statistical analysis

Results are reported as means + standard deviation. Statistical analysis was performed using JMP 10 software package (SAS Institute, Cary, NC). One-way ANOVA and Tukey-Kramer multiple comparison tests were used to determine significant differences ($p<0.05$).

Results and Discussion

In previous studies, flow perfusion culture and controlled oxygen tension have been employed individually in order to enhance the production of cartilage-like ECM and maintain cell phenotype.[24,35] Here, it was hypothesized that the combination of flow-induced shear stress and hypoxia, external stimuli that are normally encountered by chondrocytes in the physiological environment, would further enhance cartilage-like ECM production and promote the chondrocyte phenotype of cells cultured *in vitro*. In order to test this hypothesis, the effects of flow perfusion and hypoxia were investigated individually and in combination on the ECM deposition and gene expression profiles of bovine chondrocytes *in vitro*.

The first advantage that was obtained by the combination of hypoxia and perfusion cultures was observed in the DNA content of the scaffolds. As seen in Figure 1, the amount of DNA per scaffold had increased from the initial value after 2 days in culture for each experimental group, with perfusion having an additional positive effect over static samples at all-time points. The amount of DNA in each group followed an increasing trend throughout the timeframe of the study, with the level of DNA at day 6 in samples exposed to perfusion far exceeding the amount of DNA in those samples cultured under static conditions. The combination of hypoxia and perfusion demonstrated its beneficial effects on cellular proliferation as the highest level of DNA at day 6 occurred in hypoxia perfusion. As observed by others, the increased proliferation in perfusion culture could be the result of improved mass transport into the interior of the scaffold, the application of shear stress to the cells, or a combination of both factors.[36–38] Here, in static cultures hypoxia had no effect on the cellularity of the constructs, which is consistent with previous studies[19,39]; however, in perfusion culture, hypoxia led to a significant increase in cellularity over all other groups at day 6, demonstrating a potential benefit of combined hypoxic and perfusion cultures. Although the mechanism for this effect is not understood, it has previously been demonstrated with other cell types, as hypoxia and mechanical stimulation can have a synergistic effect on proliferation of bladder smooth muscle cells.[40]

Furthermore, the quantity of GAGs in each scaffold increased from day 2 to day 6 although only slight changes were observed by day 4. As seen in Figure 2a the amount of GAGs at day 6 in samples exposed to perfusion exceeded the amount of GAGs in samples cultured under static conditions, and the highest level of GAGs at day 6 occurred in hypoxia perfusion. Normalizing the total GAG content to

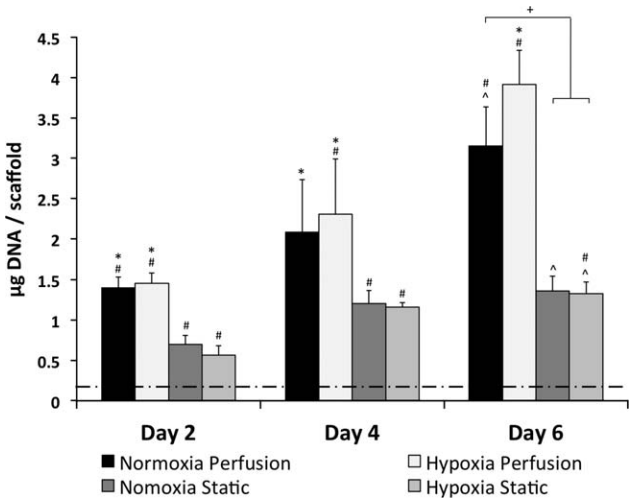

Figure 1. DNA content of scaffolds cultured in each condition after 2, 4, and 6 days of culture.

DNA content after the 24 h adhesion period (day 0) is represented by a dashed line. Data are presented as mean + standard deviation. * represents a statistically significant difference from all other groups at the same time point that are not marked ($p < 0.05$). # represents a statistically significant difference from the previous time point in the same group ($p < 0.05$), and ~ represents a statically significant difference from the day 2 time point in the same group ($p < 0.05$). + represents a statically significant difference between the groups indicated ($p < 0.05$).

DNA content in the scaffolds can provide additional information about the GAG synthesis. The ratio of the amount of GAG to DNA is regarded as a measure of the ECM production of a cell population on a cell equivalent basis; however, particularly in perfusion culture, this measure can be confounded by the loss of GAGs from the construct.[41] As shown in Figure 2b, in this study, GAG/DNA at day 2 was higher for samples cultured under static conditions compared to perfusion conditions. This difference could be the result of a high percentage of the GAGs being washed out of scaffolds in perfusion, particularly at the early stages of culture, when only low levels of ECM are present to increase the GAG retention[41] or a result of the increased proliferation in perfusion compared to static by day 2. A decrease in GAG/DNA was observed in all groups, from day 2 to day 4, followed by an increase from day 4 to day 6. This trend, along with the DNA content of the scaffolds, seems to indicate a period of proliferation, followed by GAG synthesis over the course of the study. Normoxia static saw the highest level of GAG/DNA at day 6 compared to all other groups, including hypoxia static. Although the GAG/DNA was lower in perfusion culture at day 6 compared to normoxia static, this could be a result of the high levels of proliferation in those cultures, and the overall result for perfusion in general was a construct with much higher levels of cartilage ECM. However, this may indicate that the increased level of GAGs in the perfusion scaffolds is not a result of increased GAG synthesis per cell, rather an increase in cell number.

Analysis of the histological sections, shown in Figure 3, corroborated the results of the biochemical assays. In Figure 3a and 3b sections are stained with fast green and safranin O, respectively. The images reveal the same trend as the biochemical assays, as it is evident that perfusion cultures led to higher amounts of cells and cartilaginous matrix. Perfusion cultures typically lead to improved cellular infiltration compared to static cultures, due to the improved mass transport into the interior of the construct.[15] In this study, the quantity of cells was significantly improved in perfusion, yet the cellular infiltration did not appear to be significantly affected. However, if this culture was conducted over a longer period of time, a more noticeable improvement in cellular infiltration may have been observed in perfusion cultures, as more ECM would be produced, potentially limiting the transport of nutrients into the interior of the static scaffolds.

The gene expression was quantified relative to a baseline value, which was established as the gene expression level of the cell population at the beginning of the study.[27,28] As

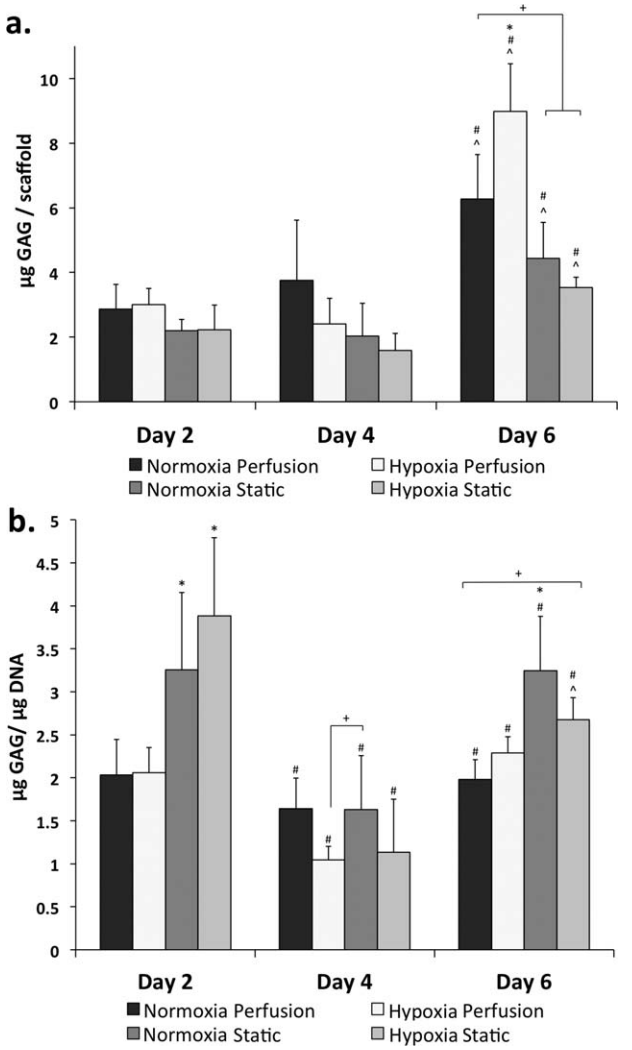

Figure 2. (a) GAG content per scaffold, and (b) µg of GAG normalized to µg DNA in each scaffold in each culture condition after 2, 4, and 6 days of culture.

Data are presented as mean + standard deviation. * represents a statistically significant difference from all other groups at the same time point that are not marked ($p < 0.05$). # represents a statistically significant difference from the previous time point in the same group ($p < 0.05$). ~ represents a statically significant difference from the day 2 time point in the same group ($p < 0.05$). + represents a statically significant difference between the groups indicated ($p < 0.05$).

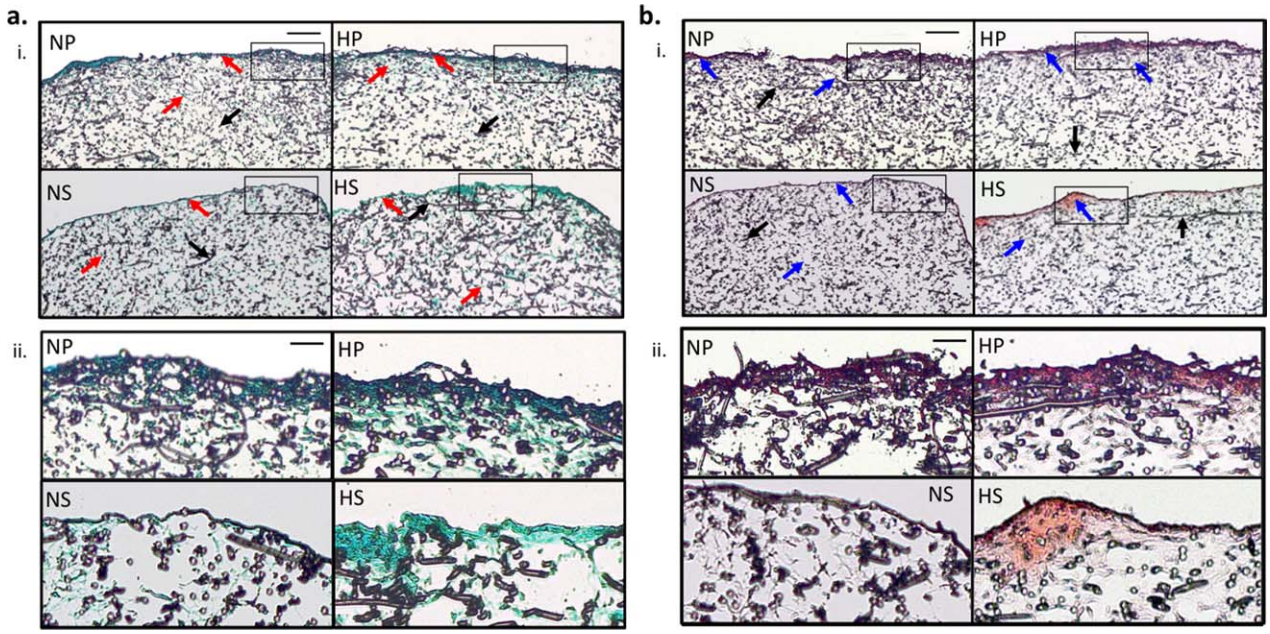

Figure 3. Representative histological sections of day 6 samples cultured in a flow perfusion bioreactor under normoxic conditions (NP), in a flow perfusion bioreactor under hypoxic conditions (HP), in static culture in normoxic conditions (NS), and in static under hypoxic conditions (HS).

Regions pictured in the magnified images (ii) are indicated with black rectangles in the corresponding image (i). Electrospun PCL scaffolds (representative examples of PCL fibers are indicated with black arrows) are stained with (a) fast green to visualize the distribution of cells (representative examples indicated with red arrows), and (b) Safranin O to visualize the distribution of cartilaginous matrix (representative examples indicated with blue arrows). Scale bars represent 200 μm and 50 μm in (i) and (ii) images, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

shown in Figure 4a, the expression of collagen I in static cultures did not change from day 2 to day 6. In perfusion, the collagen I expression showed a statistically significant increase from day 2 to day 4, but did not change from day 4 to 6. Comparing perfusion to static cultures, static cultures had higher levels of collagen I expression at day 2; however, by day 4 there was no statistical difference between the groups. These results demonstrate a delay in the up regulation of collagen I gene expression in perfusion cultures, which is beneficial as collagen I expression is indicative of the dedifferentiation of chondrocytes into a fibroblastic phenotype, a transition that normally occurs very early in the cell expansion process. The subsequent fibrocartilage ECM produced by these cells is inferior to the normal articular cartilage, and, therefore, not the desired type in cartilage tissue engineering.[42] By day 6, hypoxia static demonstrated an increased level of collagen I expression relative to hypoxia perfusion and normoxia static.

In Figure 4b, comparing the expression of collagen II at day 2 and day 6 showed a decrease in expression for all groups, except hypoxia perfusion. Collagen II expression was higher in static samples at day 2 and 4 compared to perfusion samples. At day 6, hypoxia static showed the highest level of expression. Normoxia static showed a higher level than normoxia perfusion, however, it was not statistically higher than hypoxia perfusion. These results indicate that by itself perfusion culture led to a decrease in collagen II expression below the levels of static cultures; however hypoxia led to an increase in collagen II expression above the levels of normoxic cultures. When combined, while perfusion still leads to a decrease in collagen II expression compared to hypoxia static, it did not decrease the expression below the levels of normoxia static. The predominant type of collagen found in cartilage tissue is type II, thus, high levels of collagen II expression by chondrocytes is indicative of the chondrogenic phenotype.[43] Here, perfusion led to an undesirable reduction in collagen II expression compared to static cultures; however, when combined with hypoxia, the collagen II expression is equivalent to the levels seen in static cultures, without the hypoxic treatment.

As shown in Figure 4c, aggrecan gene expression decreased comparing day 2 to day 6 for static cultures. Hypoxia perfusion exhibited aggrecan expression that peaked at day 4 before returning to its day 2 values, and normoxia perfusion followed the same trend, although it was not statistically significant. Aggrecan expression was higher for static groups at day 2 compared to perfusion. Higher levels of aggrecan expression were observed for hypoxia perfusion at day 4 compared to normoxia static. Aggrecan expression was higher for hypoxia static at day 6 compared to all other conditions. It is interesting to note that aggrecan expression peaked at day 4 for both perfusion groups; however, only the hypoxia perfusion was statically significant. This result is consistent with the levels of GAGs detected, as a large increase in GAG content is seen in the perfusion groups between days 4 and 6. Transient peaks in aggrecan gene expression as a result of compressive loading have previously been observed by others; however, these peaks occurred over a much shorter time period.[44] The decline in gene expression from day 4 to 6 could also be attributed to the change in the shear stress experienced by the cells as a result of the change in pore structure resulting from ECM production.[45]

In Figure 5, the collagen II/I gene expression ratio decreased in all groups from the day 0 value of 2.65, as well as from day 2 to day 6. Exposure to hypoxic conditions

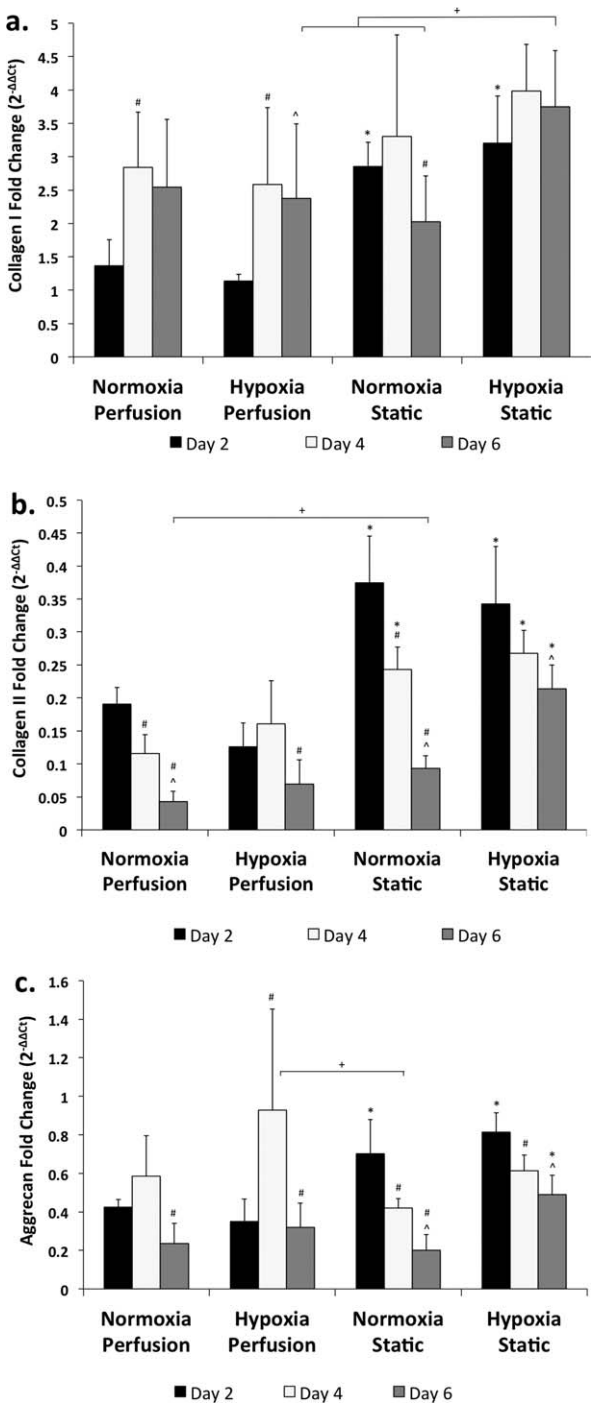

Figure 4. Quantitative gene expression of (a) collagen type I, (b) collagen type II, and (c) aggrecan for samples (n = 6) cultured in each condition after 2, 4, and 6 days of culture. Data are presented as mean + standard deviation.

*** represents a statistically significant difference from all other groups at the same time point that are not marked ($p < 0.05$). # represents a statistically significant difference from the previous time point in the same group ($p < 0.05$). ^ represents a statically significant difference from the day 2 time point in the same group ($p < 0.05$). + represents a statically significant difference between the groups indicated ($p < 0.05$).**

delayed the decrease in collagen II/I in samples exposed to perfusion at day 4, as normoxia perfusion exhibited a lower collagen II/I ratio compared to normoxia static, but at the same time point, hypoxia perfusion was equal to hypoxia static. By day 6, the collagen II/collagen I ratio in the static samples exceeded that of the perfusion samples. However, in the perfusion groups, hypoxia perfusion was found to have a higher collagen II/I expression than normoxia perfusion.

The decrease in the ratio of collagen II/I expression over the course of this study likely demonstrates a decrease in chondrogenic phenotype of the cells in these samples.[46] Multiple factors have been shown to lead to chondrocyte dedifferentiation, many of which are associated with the *in vitro* expansion of these cells.[42] Alternatively suboptimal levels of shear stress could lead to a reduction in chondrocyte specific gene expression compared to static cultures.[11,47] However, using a cylindrical pore model for the approximation of the scaffold geometry[13,48,49] the initial levels of shear stress used in this study were estimated to be approximately 5 mPa, which was previously found to lead to higher GAG/DNA ratios than other shear levels investigated[50] and is on the same level of shear stresses used by other investigators.[51,52] Furthermore, the reduction in collagen I expression that was also observed in the perfusion cultures, compared to static, may be further evidence that the levels of shear stress were not too high, as chondrocytes have been shown to increase production of collagen type I in response to high shear.[11] High levels of chondrocyte proliferation have been correlated with chondrocyte dedifferentiation,[42,53,54] as high levels of proliferation are not characteristic of differentiated chondrocytes,[55] and the high levels of proliferation seen in perfusion culture may be tied to the further reduction in collagen II/I expression in the perfusion groups compared to static. Interestingly, although hypoxia perfusion samples saw the

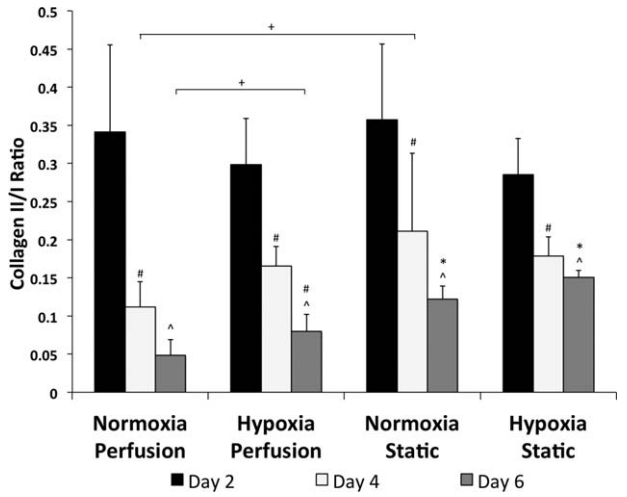

Figure 5. The ratio of collagen II to collagen I expression for samples cultured in each condition after 2, 4, and 6 days of culture. Data are presented as mean + standard deviation.

*** represents a statistically significant difference from all other groups at the same time point that are not marked ($p < 0.05$). # represents a statistically significant difference from the previous time point in the same group ($p < 0.05$). ^ represents a statically significant difference from the day 2 time point in the same group ($p < 0.05$). + represents a statically significant difference between the groups indicated ($p < 0.05$).**

greatest increase in cellularity, the reduction in collagen II/I ratio was delayed, compared to the normoxia perfusion and although the proliferation was much higher in hypoxia perfusion at Day 6, the collagen II/I ratio was not lower than that of normoxia perfusion, demonstrating the benefit of hypoxia in perfusion culture.

Many approaches have been taken in order to prevent dedifferentiation of chondrocytes, such as the use of growth factors,[56] or optimizing scaffold structure.[57] In this study, chondrocytes were cultured in FBS-containing, growth medium without any added growth factors. These conditions are likely to lead to high levels of chondrocyte proliferation, and a loss in chondrocyte phenotype, as was observed in this study. While hypoxia in combination with flow perfusion was effective in enhancing the cell phenotype compared to perfusion alone (as observed by the ratio of collagen II/I expression), it was not sufficient to maintain the cell phenotype throughout the study, which was demonstrated by the decrease in the collagen II/I ratio over time. Future work could investigate the use of additional factors to further enhance the chondrogenic phenotype over prolonged culture times.

Hypoxia in combination with mechanical stimulation (mostly hydrostatic compression[58,59]) has been investigated in several studies. One such study evaluated the effects of intermittant hydrostatic pressure in combination with hypoxia. Similar to the results of this study, mechanical stimulation was found to reduce type II collagen production; however in combination with hypoxia, the production was increased compared to compression alone, although not to the level of hypoxia without mechanical stimulation.[59] Similarly, it has been shown that hydrostatic pressure in combination with hypoxia can lead to increases in collagen production and enhance the phenotypic stability of chondrocytes cultured in monolayer; however, the response varied based on duration and magnitude of the loading regime.[58] Furthermore, the combined effects of such factors for chondrogeneis have been investigated with other cell types. Using human neonatal fibroblasts cultured on alginate beads, it was found that mechanical stimulation in combination with hypoxic conditions can lead to enhanced chondrogenic gene expression when combined with the addition of BMP-2.[60]

While the use of reduced oxygen tension in combination with other forms of mechancial stimulation have been evaluated, the use of flow perfusion bioreactors in combination with controlled oxygen tension presents a unique advantage. Flow perfusion culture can be used to increase the mass transport through the pores of scaffolds,[13] which includes the transport of oxygen and nutrients into the scaffold, as well as the transport of waste products out of the scaffold. Improved transport into the scaffold, in some cases may lead to higher levels of oxygen compared to static cultures, which in the case of chondrogenic cultures may not be beneficial and could contribute to the reduction in chondrogenic gene expression that was observed in perfusion culture. Thus, controlled oxygen tension in combination with flow perfusion can optimize the level of oxygen delivered to the cells, while still benefiting from the improved transport of other nutrients into and waste products out of the scaffold. Additionally, since static cultures rely on diffusion alone for the transport of oxygen, flow perfusion bioreactors are potentially a unique tool to study the effects of controlled oxygen tension in 3-D scaffolds, as they allow one to homogeneously control the level of oxygen in the scaffold.

In this study, flow perfusion culture in normoxic conditions led to similar effects reported by many in the literature, namely increased cellular proliferation, and extracellular matrix production,[35,37,50] as well as a delay in the increase in collagen I expression compared to static cultures; however, a reduction in the collagen II/I expression ratio was also observed. The addition of hypoxia to the perfusion culture led to significant increases in cellularity and GAG content of the scaffolds above the levels of perfusion alone. Additionally, the ratio of collagen II/I expression was increased in cells exposed to both hypoxia and perfusion compared to only perfusion. While others have investigated the effects of hypoxia combined with other factors, such as growth factors[22] and 3-D scaffolds,[61] these are the first results to demonstrate the benefits of hypoxia when combined with flow perfusion culture. As this study was the first to evaluate the combination of these factors on articular chondrocytes, and to demonstrate the beneficial effects of hypoxia and flow perfusion culture, there are several areas that could be further investigated. While this study quantified the expression of genes associated with the chondrogenic phenotype, its corresponding protein production was not evaluated. Future studies could evaluate the production of proteins, such as collagen type II. Additionally, this study employed cells from young calves. Adult human chondrocytes may exhibit a different response from the animals observed here, so future studies could evaluate the effect of these factors in human chondrocytes. Furthermore, the mechanism behind the combined effects of hypoxia and flow perfusion was not investigated in this study. Low-oxygen tension can lead to a variety of responses on systemic and cellular levels, and the mechanism of these effects is often attributed to the activation of the HIF-1α transcription factor.[21,25,62] Future studies could investigate interactions between cell signaling pathways activated by mechanoreceptors and the HIF-1α transcription factor. Finally, this study was conducted over a relatively short period of culture, as chondrocytes are thought to be more receptive to mechanical stimulation at early times, prior to the development of a pericellular matrix.[12] Future studies could be conducted to evaluate the effects over a much longer time frame.

Conclusion

In this work, the effect of hypoxia in combination with flow perfusion culture was investigated for its effect on the proliferation, GAG production, and chondrogenic gene expression in bovine articular chondrocytes. Here, it was concluded that flow perfusion culture led to an enhancement in chondrocyte proliferation and GAG production, and while compared to static cultures, it delayed the increase in collagen I gene expression, it also led to a reduction in collagen II and the ratio of collagen II/I expression. However, when combined with hypoxia, flow perfusion cultures had even higher levels of chondrocyte proliferation and GAG production along with higher collagen II/I ratios compared to perfusion in normoxic conditions after 6 days. This work demonstrates the benefits of both hypoxic and perfusion cultures, particularly in combination, and the need to combine multiple signals *in vitro* in order to more closely replicate the *in situ* environment of these cells. These effects could be leveraged by tissue engineers in order to achieve more robust *in vitro* methods of cartilage tissue fabrication.

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