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Use of a Centrifugal Bioreactor for Cartilaginous Tissue Formation from Isolated Chondrocytes

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Abstract

Although a centrifugal bioreactor (CCBR) supports high-density mammalian suspension cell cultures by balancing drag, buoyancy, and centrifugal forces, to date anchorage-dependent cultures have not been tried. Also, steady or intermittent hydrostatic pressures of 8 to 500 kPa, and shears of 0.02 to 1.4 N/m² can be simultaneously applied in the CCBR. This article demonstrates the use of a CCBR to stimulate chondrogenesis in a high-density culture. At 3 weeks, histological results show even distribution of glycosaminoglycan (GAG) and collagen, with $1,890 \pm 270$ cells/mm² cell densities that exceed those of $1,470 \pm 270$ in pellet cultures. Analysis of collagen content reveals similar levels for all treatment groups; 6.8 ± 3.5 and 5.0 ± 0.4 μg collagen/ μg DNA for 0.07 and 0.26 MPa CCBR cultures, respectively, in contrast to 6.6 ± 1.9 values for control pellet cultures. GAG levels of 5.6 ± 1.5 and 4.1 ± 0.9 μg GAG/ μg DNA are present for cultures stressed at 0.07 and 0.26 MPa, respectively, in comparison to control pellet cultures at the 8.4 ± 0.9 level. Although results to date have not revealed mechanical stress combinations that stimulate chondrogenesis over unstressed controls, system advantages include continuous culture at cell densities above those in the pellet, precise medium control, the ability to independently vary multiple mechanical stresses over a broad range, and the flexibility for integration of scaffold features for future chondrogenesis stimulation studies.

Keywords

chondrocyte; hydrostatic pressure; shear force; centrifugal bioreactor; high density; cartilage

Introduction

The limited ability of articular cartilage to heal following damage due to trauma or disease has spurred tissue engineering approaches to create an artificial construct mimicking native tissue in composition, function, and structure.^{1,2} Successful functional tissue engineering requires the use of engineering approaches to simulate both in vivo biochemical and biophysical aspects of the native environment in vitro. For cartilage, biochemical factors encompass a myriad of bioactive molecules including but not limited to growth factors and nutrient concentrations, whereas biophysical influences include a necessary three-dimensional (3D) growth environment as well as mechanical stimulation.

A 3D culture environment prevents chondrocyte dedifferentiation,³ and is accomplished through a variety of methods including gel encapsulation,^{1,4} porous scaffolds,^{5,6} or spontaneous chondrocyte aggregation during high-density suspension culture.^{7–9}

Additionally, mechanical stimuli such as shear, hydrostatic pressure, and direct compression have all been shown to improve tissue functionality through increased extracellular matrix (ECM) incorporation of collagen and glycosaminoglycan (GAG).^{6,10–12} The ability to apply precise combinations of multiple stimuli is crucial in promoting generation of functional artificial tissue and can be used to study chondrogenesis, degeneration, and regeneration in vitro; helping to drive the development of novel therapies.

Bioreactors systems proven to stimulate cartilage tissue development from isolated chondrocytes through the application of various stimuli include rotating wall bioreactors (RWBs),^{4,8,13} concentric cylinder bioreactors (CBs),^{14,15} scaffold perfusion bioreactors (SPBs),^{5,16} and various systems facilitating nutrient distribution through efficient mixing.¹⁷ SPBs, CBs, and well mixed reactors rely on the use of scaffolds and increased nutrient transport through fluid flow, providing shear forces proven to increase ECM development. RWBs seeded at densities above 5×10^5 cells/mL have been shown to result in spontaneous tissue development through spontaneous cell aggregation in a low shear, low-gravity environment.⁸

Presently novel bioreactors are being developed to investigate the possible synergistic effect of the simultaneous application of multiple mechanical forces. For example, systems are being used to investigate the simultaneous application of shear and hydrostatic pressure using both direct perfusion and system pressurization,^{18,19} whereas others simultaneously apply direct compression and shear.^{6,20} Another novel system, heretofore, only used for suspension cultures of yeast and mammalian cells, is the continuous centrifugal bioreactor (CCBR).^{21,22} In addition to maintaining high-density cultures at high-perfusion rates, the reactor provides simultaneous application of both hydrostatic pressure and shear, a combination of characteristics appearing attractive for stimulating chondrogenesis.

Cells are retained in the CCBR in a high-density fluidized bed due to a unique method of immobilization relying on counter-flow centrifugation.^{22,23} The balance of centrifugal force with opposing drag and buoyant forces results in cell immobilization and multiple mechanical forces. Centrifugal acceleration increases the weight of the liquid head above the cell suspension, thereby, increasing hydrostatic pressure within the reactor chamber. Enhanced shear forces result because increased culture medium perfusion rates, and the resultant drag forces, are necessary to maintain fluidized cell suspensions in the presence of increased gravitational forces. Both forces are constantly applied to cells in culture during reactor operation, but the magnitudes of each are individually variable through changes in rotation rate and fluid velocity.

In this article, we present studies showing the capability of the CCBR system to stimulate chondrogenesis. Our hypothesis is that the CCBR can stimulate chondrogenesis through the application of multiple mechanical stimuli to a high-cell density chondrocyte suspension. We present the theoretical framework for understanding the relationship between the pressure and shear forces applied during CCBR operation. In this preliminary study, chondrogenesis is assessed through histological staining and total protein assays. Results are compared with chondrocyte pellet culture, a high-cell density system lacking any mechanical stimulation. Results show chondrogenesis in multi-week CCBR cultures as evidenced through collagen and GAG production. However, most importantly, we validate a novel system capable of high-density chondrocyte culture over a range of hydrostatic and shear conditions. A highly variable set of perfusion rates can be maintained making the system amenable to future studies on infusion of biochemical factors while at the same time invoking multiple stressors. We not only expect the CCBR to become a valuable tool for elucidating the impact of variable conditions on chondrogenesis but also on a variety of other anchorage dependent cell cultures.

Materials and Methods

Cell isolation

Full thickness cartilage tissue was harvested from the carpal joint (front knee) of skeletally mature bovine animals (Potlach Pack, Potlach, ID, and the Washington State University/University of Idaho Center for Reproductive Biology, Pullman, WA) through joint disarticulation under aseptic conditions. Chondrocytes were released from the tissue matrix by treatment with pronase (Sigma, St. Louis, MO) for 1 h and collagenase (Roche, Indianapolis, IN) for an additional 16 h at 37°C using respective enzyme concentrations of 20 and 200 U/mL.¹ Enzymes were dissolved in digestion medium consisting of high-glucose Dulbecco's Modified Eagle Medium supplemented with 5% FBS (Atlanta Biologicals, Lawrenceville, GA), nonessential amino acids, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.4 mM proline (Sigma, St. Louis, MO). All medium ingredients were purchased from Invitrogen (Carlsbad, CA) unless otherwise specified. Following digestion the cells were filtered through a 100-µm cell strainer (BD Biosciences, Franklin Lakes, NJ), and centrifuged, washed in phosphate-buffered saline, resuspended in culture medium, and then counted using a Trypan Blue exclusion assay and a hemocytometer. Culture medium is similar to digestion medium except additionally supplemented with 10% FBS and 25 µg/mL ascorbic acid (Sigma, St. Louis, MO).

Pellet culture

Chondrocyte pellet cultures served as experimental controls as they are a high-density culture lacking the presence of any mechanical stimuli. Pellet culture controls were prepared and run concurrently with every CCBR experiment. Pellet cultures were prepared by adding 1 mL of a 1×10^6 cells/mL suspension in culture medium to a 1.5 mL microcentrifuge tube. This suspension was centrifuged at 500 g for 5 min resulting in a pellet of cells at the bottom of the tube.⁹ Pellets were then cultured in a 37°C incubator with 5% CO₂. Medium was replaced twice per week throughout the entirety of each study, after which pellets were harvested for biochemical and histological analysis.

CCBR culture

The CCBR consists of a smooth-walled polycarbonate conical chamber (11.4 mL) machined in the WSU Engineering shops. This chamber is integrated into the cell purging tubing set (Caridian BCT, Lakewood, CO) and connected to a COBEV© Spectra™ Apheresis System (Caridian BCT, Lakewood, CO). Each tubing set is supplied sterile from the manufacturer, but after modification of the tubing lines to reflect that shown in Figure 1, the system is resanitized by pumping 70% ethanol through the system for 24 h preceding reactor start-up. Fluid is supplied and removed from the rotating reactor chamber through a flexible tubing bundle and the use of an anti-twister mechanism on the COBEV© Spectra™ similar to that demonstrated by Van Wie et al.²⁴ Following sanitization, the system is purged of ethanol, rinsed with culture medium, and then filled with culture medium taking care to ensure no air bubbles remain in the reactor lines, as the presence of air will lead to culture failure.

Before inoculation, rotor speed, and the inlet volumetric flow rate are set at 600 rpm and 5 mL/min, respectively. A total of $2.0\text{--}2.5 \times 10^8$ chondrocytes (based on the yield of the cell isolation procedure) suspended in 10 mL of culture medium is then introduced into the system using the valve and inoculation line shown in Figure 1. Inoculation cell density and initial reactor settings were chosen based on previous experiments with hybridoma cells resulting in stable cell fluidization.²² Reactor settings remain unchanged for the initial 7 days of culture, after which specific cultures were harvested for evaluation while others continued development in the CCBR for an additional 2 weeks.

Over the final 14 days of a 3-week culture, the inlet volu-metric flow rate was constant at 2.5 mL/min, whereas rotor speeds were varied intermittently providing periods of increased hydrostatic pressure. Intermittent stressing regimens were carried out during four 1-h cycles per day in which rotor speed was increased to either 900 or 1,650 rpm for 30 min and all rotation and pumping are stopped for the subsequent 30 min of the 1-h cycle. During the remaining 20 h per day, rotor speed was 300 rpm. During the entire 3-week culture, 200 mL of medium was removed using the waste pump and replaced with fresh culture medium.

Histological analysis

Pellet or CCBP cultures harvested at the 1 and 3 week time points were immediately fixed in neutral buffered formalin for 1 h. The tissue was then embedded in paraffin and cut into 4- μ m sections. These sections were stained with either Toluidine blue for analysis of GAG distribution or a Trichrome stain for collagen. All sectioning and staining was performed by the Washington Animal Disease Diagnostic Laboratory (WADDL) Histology Lab at Washington State University. Sections were visualized with an Olympus BH-2 optical microscope (Olympus; Center Valley, PA) and recorded digitally with a ProgRes C12plus digital microscope camera (JENOPTIK Laser, Optik, Systeme GmbH; Jena, Germany).

Biochemical analysis

Samples for colorimetric biochemical analysis were digested overnight with proteinase K (Roche) in TE buffer at a concentration of 500 μ g/mL. Sulfated GAG content was determined for each sample using a dimethylmethylene blue assay,²⁵ whereas an assay to determine hydroxyproline content²⁶ and the assumption of a ratio of 7.4 grams of collagen per gram of hydroxyproline was used to estimate total collagen content. Finally, DNA content was estimated using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) allowing sample GAG and collagen measurements to be normalized to total DNA content.

Statistical analysis

Average values of GAG/DNA (μ g/ μ g) and Collagen/DNA (μ g/ μ g) were calculated as arithmetic means for pellet cultures ($n = 7$), for bioreactor cultures with low-intermittent stress (900 rpm cultures, $n = 2$), and for bioreactor cultures with high-intermittent stresses (1,650 rpm cultures, $n = 2$). Reported error bars indicate standard error of the mean calculated for each group. Statistically significant differences were established at the $P < 0.05$ (*) level.

Theory

Mechanical force calculations

During continuous operation, centrifugal force provides an increase in hydrostatic pressure within the reactor chamber to levels, which can be varied from 8 kPa at 300 rpm to 0.5 MPa (about 5 atm) at 2,300 rpm under the current configuration, corresponding to magnitudes of pressure above atmospheric. The values of hydrostatic pressure are calculated by integrating the centrifugal acceleration over the radius of rotation, this is necessary as the centrifugal force, and, thus, pressure increases with increasing distance from the center of rotation. The formula used for calculation of hydrostatic pressure is shown in Eq. 1.

$$\Delta P = \int \rho a(r) dr = \int_{r_1}^{r_2} \rho \omega^2 r dr = \rho \omega^2 \frac{r^2}{2} \quad (1)$$

where ΔP is the pressure increase above atmospheric at any point in the reactor, ρ is the density of the fluid, and r is the radius from the center of rotation. The term $a(r)$ is the

system acceleration as a function of r , which for a rotating system is the square of angular velocity (ω) (or $\text{rpm} \times 2\pi$) multiplied by r . Integration from the center of rotation $r_1 = 0$ to the point of interest $r_1 = r$ will result in the hydrostatic pressure at a particular distance, r , from the center of rotation. All reported pressures are determined relative to the radial distance of interest for a given analysis, for example, a pressure calculated at $r_1 = 13.1$ cm will correspond to the maximum pressure exerted or that which exists at the point in the CCBP where fluid enters the reactor chamber.

Also present during continuous operation is a shear force due to the flow of medium through the reactor. By equating the pressure loss per unit length for flow through a cylindrical tube (left-hand-side of Eq. 2) with the pressure loss through a fluidized bed (right-hand-side of Eq. 2), and assuming R is the hydraulic radius corresponding to the ratio of cross-sectional area available for flow to the wetted perimeter, the shear stress can be defined as shown in Eq. 2.

$$\tau 2\pi R = 150 \left(\frac{\mu V_0}{D_p^2} \right) \frac{(1 - \varepsilon)^2}{\varepsilon^2} \frac{R}{2} \quad (2)$$

where τ is the shear stress, R is the hydraulic radius $R = \frac{\varepsilon D_p}{(1 - \varepsilon) 6}$, μ is the fluid viscosity, V_0 is the superficial velocity through the chamber, D_p is the particle diameter, and ε is the fluidized bed porosity. Substitution of the equation for hydraulic radius into Eq. 2 results in an equality for the shear stress in a fluidized bed, Eq. 3.

$$\tau = \frac{150}{12} \left(\frac{\mu V_0}{D_p} \right) \frac{(1 - \varepsilon)}{\varepsilon^2} \quad (3)$$

From Eq. 3, it can be seen that shear stress is a function of both superficial velocity and porosity. Superficial velocity is directly proportional to inlet fluid velocity, a key operational parameter, while porosity, or cell population density, is specifically tunable based on adjustments in either rpm or inlet velocity. For an inlet volumetric flow rate ranging from 1 to 20 mL/min, shear stress will range from 0.1 to 2.6 N/m² at a porosity of 0.85. However, reducing porosity to 0.7 will increase shear stress to 0.3–7.8 N/m² over the same range of inlet fluid velocities. These values of shear stress are calculated using a void velocity found at the midpoint (lengthwise) of a typical cartilage construct, corresponding to a CCBP diameter of 0.64 cm. As porosity is a function of the balance between shear stresses, surface drag on a cell, and centrifugal force; it can be seen that reactor settings must be carefully chosen to deliver appropriate force magnitudes during cell culture.

Results and Discussion

Mechanical force magnitude during culture

As outlined in the Theory section hydrostatic pressure and shear stress can be calculated for specific reactor settings. For the conditions used during the first week of all CCBP cultures the maximum hydrostatic pressure and shear stress are constant at 0.03 MPa and 0.51 N/m², respectively. These forces correspond to operating conditions of 600 rpm and an inlet flow rate of 5 mL/min, which have previously been shown to maintain a cell bed with a porosity of 0.85.

Over the final 2 weeks of culture reactor settings correspond to a rotor speed of 300 rpm and an inlet flow rate of 2.5 mL/min corresponding to a hydrostatic pressure of 8 kPa and a shear

stress of 0.25 N/m^2 . These conditions remain unchanged over the final 2 weeks of culture except for four 1-h periods throughout the day when the hydrostatic pressure is cyclically increased to either 0.07 or 0.26 MPa ($\sim 2.5 \text{ atm}$) by increasing rpm to 900 or 1,650 as described in the “Materials and Methods” section. The magnitude of mechanical stimulation is reduced for much of the culture over the final 2 weeks allowing the culture to rest following periods of cyclically increased hydrostatic pressure.

We note that the pressure will vary spatially along the height dimension of the resultant cartilage construct, Figure 2, as this dimension is aligned with the radial distance from the center of rotation. However, the small size of the cartilage construct measuring 1.5 cm, cone height along axis aligned with the center of rotation, results in small variations in pressure (Eq. 1). At a particular rpm, the pressure will decrease by about 20% along the height of the conical construct. For instance at 1,650 rpm, the tip of the cartilage construct will experience a pressure of 0.25 MPa while the base, or the section of the construct with the widest cross-section, will experience a pressure of 0.20 MPa. Although the low pressures investigated here result in a relatively small pressure gradient across the construct at higher rpms a 20% gradient may result in MPa differences between the top and bottom of the construct. This is a significant characteristic to keep in mind during future studies in which mechanical forces are investigated in the CCB. Regarding shear (Eq. 3), fluidized bed-suspended species will adjust their spatial arrangement or porosity, so shear forces exactly compensate for the settling forces on the particles, thus eliminating any spatial variation in shear throughout the initial cell suspension as long as the suspension has a homogeneous porosity. A homogeneous cell suspension porosity has been confirmed by our group for CCB culture through extensive numerical and experimental analyses.²⁷ Following construct formation spatial variations in shear will exist, and this will be addressed in the Observations of bioreactor tissue formation section.

Observations of bioreactor tissue formation

Following inoculation in the CCB chondrocytes are suspended in the reactor chamber as a fluidized bed of cells. Assuming a void volume fraction near 0.85, a value based on previous numerical modeling and experimental work, and an average chondrocyte diameter of $13 \mu\text{m}$ ²⁸ the cell bed occupies approximately 2 mL of the total reactor volume (11.4 mL). The cell suspension remains unchanged during an initial 48- to 72-h lag phase, as cells adjust to the reactor's unique environment. A lag phase of this duration is typical of mammalian cells adjusting to a new environment and has been previously quantified in the CCB during hybridoma kinetic growth studies.²² Following the lag phase, cell flocculation occurs as a result of ECM component secretion causing cell aggregation. Consequently, the effective particle size is increased disrupting the equilibrium between drag and settling forces. As a result, the larger particles settle further in the centrifugal field and the volume of the reactor chamber occupied by the cells decreases by approximately 30–50% and remains unchanged for the duration of the culture. The initial volume of the reactor chamber occupied by the cells is similar to that previously shown for hybridoma cells cultured in the CCB at the same conditions.²⁷ At the conclusion of a 3-week culture, a cartilaginous tissue results, shown both before, Figure 2A, and after removal from the reactor chamber, Figure 2B. As gross tissue observations cannot differentiate the specific hydrostatic pressure culture conditions, the tissue shown in Figure 2 is representative of both stressing regimens.

Following removal from the reactor observations show the construct surface near the wall of the reactor is uniform and smooth, providing the most resistance to compression based on individual qualitative observations. The middle of the construct, on the other hand, is much softer with numerous observable macropores, roughly 1 mm in diameter. Immediately following inoculation, bed porosity is 0.85 as cells are individually suspended in the reactor chamber. However, at the time of tissue construct removal, the ECM has undergone

extensive transformation resulting in an ECM that blocks liquid passage through much of the matrix resulting in the formation of numerous macropores or channels allowing for fluid passage throughout the interior of the construct.

Because of tissue construct and macropore formation, the previous analysis of shear stress is no longer applicable. Consequently, shear stress must be calculated at the wall of the macropore assuming flow through a bundle of cylindrical pipes, approximately 1 mm in diameter. In this instance, shear stress at the wall of a macropore, or pipe, is a function of the pressure differential across the pipe; this will be affected by the pressure gradient along the height dimension of the construct as previously discussed. Consequently, the shear would be significantly increased to 31 N/m² at operating conditions of 300 rpm and a flow rate of 2.5 mL/min for flow through five macropores; increasing with increased flow rate and rpm, while decreasing with the presence of additional pores. However, we do not anticipate the increased shear to play a significant role in the current study as the majority of chondrocytes lie deep within the construct, some distance away from a channel, and consequently are not subjected to significant shear. Therefore, in this current study, the enhanced hydrostatic pressure is the primary focus as all cells regardless of position in the construct will be affected by this stimulus. However, a study investigating the effects of shear and ECM deposition as a function of radial distance from the observed macropores would be of significant interest.

Histological observations

Histological analysis gives a quantitative assessment comparing the cell density of pellet and CCBR cultures, as well as qualitative insight into GAG and collagen distribution throughout the construct. However, we note that relative cell density should be taken into consideration when drawing qualitative conclusions. For example, a construct lighter in coloration may actually have more GAG or collagen on a per cell basis than another construct darker in color, but which has a higher cell density. For this distinction, a more quantitative set of assays was performed on a per DNA basis and will be presented in the discussion surrounding Figures 4 and 5. We also note that there is no observable difference in histological staining between bioreactor cultures stressed at 900 or 1,650 rpm. Therefore, sections from a single 900 rpm culture are presented in Figure 3, and are considered representative of both bioreactor treatment groups.

Observation of multiple tissue sections shows pellet cultures have a significantly lower cell density, $1,450 \pm 120$ cells/mm², than bioreactor constructs, $2,300 \pm 300$ cells/mm², at the 1-week time point. The same analysis after 3 weeks again shows a significant difference between pellet and bioreactor culture cell density, $1,480 \pm 270$ cells/mm² and $1,890 \pm 60$ cells/mm², respectively. When comparing 1- and 3-week constructs from the same treatment group, pellet culture density remained unchanged, whereas CCBR construct cell density significantly decreased over time. Cell morphology observations show round and individually embedded cells within their respective ECMs for both culture techniques, an indication of chondrocyte differentiation.

Each histological section in Figure 3A indicates the presence of GAG in the ECM as evidenced by the purple color preferentially taken up by GAGs. At the Week 1 time point, the pellet culture ECM is stained with greater intensity than the CCBR culture indicating increased expression of GAG protein. Comparison of the Week 1 and Week 3 time points for pellet cultures does not appear to show an increase in intensity, whereas the staining intensity of the ECM has clearly increased over the course of the CCBR study. Collagen content as evidenced by blue staining of the ECM in Figure 3B shows the staining intensity has increased in both culture systems as the construct matures from the Week 1 to Week 3 time point. It must be kept in mind that staining intensity is representative of total protein

content and says nothing about per cell protein production, the focus of the biochemical studies on the tissue constructs in the Biochemical results section.

Biochemical results

Normalization of total collagen and GAG concentration to the amount of DNA in a sample allows protein production to be compared on a per cell basis. As each cell has a similar amount of DNA, this type of analysis will account for differences in cell density between samples; per cell comparisons are indicated as collagen/DNA and GAG/DNA. Comparison of the two culture systems shows similar levels of collagen/DNA at both the 1- and 3-week time points; results are shown in Figure 4. There is no statistical difference in the collagen/DNA content between pellet cultures, $2.8 \pm 0.6 \mu\text{g}/\mu\text{g}$, and bioreactor cultures, $1.8 \pm 0.4 \mu\text{g}/\mu\text{g}$, after 1 week of culture. Similarly, there is no statistical difference at 3 weeks between pellet cultures containing $6.6 \pm 1.9 \mu\text{g}/\mu\text{g}$ of collagen/DNA and bioreactor constructs containing 6.8 ± 3.5 and $5.1 \pm 0.4 \mu\text{g}/\mu\text{g}$ for 900 and 1,650 rpm culture conditions, respectively. Although there is not a statistical difference between treatment groups at individual time points, a significant increase in collagen/DNA content between the 1- and 3-week time points is observed for both pellet cultures and CCBR constructs stressed at 1,650 rpm.

Results of assays to determine GAG content are shown in Figure 5. Between the 1- and 3-week time points, the average amount of GAG/DNA in pellet cultures increases from 7.0 ± 1.1 to $8.4 \pm 0.9 \mu\text{g}/\mu\text{g}$, although not statistically significant when evaluated using a *t*-test at the $P < 0.05$ level. Bioreactor tissue analyzed following the initial week of culture under constant shear and hydrostatic pressure contains $1.4 \pm 0.1 \mu\text{g}/\mu\text{g}$ GAG/DNA, which is significantly lower when compared with pellet cultures at the same time point. The same analysis following 3 weeks of culture indicate GAG/DNA has increased in both CCBR construct treatment groups to 6.8 ± 3.5 and $5.1 \pm 0.4 \mu\text{g}/\mu\text{g}$ for the 900 and 1,650 rpm culture conditions, respectively. Here, there is no statistical difference between the pellet and 900 rpm CCBR; however, the 1,650 rpm values are significantly less than those found in pellet cultures by about 40%.

We note that *t*-tests work best when comparing means from normal distributions and this is increasingly more difficult with smaller numbers of data points. However, in our studies, the differences in means are often large, such as in the two to six order changes observed, and the standard deviations are often much smaller than the means, by as much as an order of magnitude. Therefore, in those instances, there is more likelihood the average and standard deviations of the two values reported are indeed representative of what one might expect for a larger number of replicates. Thus, the *t*-test results at least have some merit though it certainly will be advantageous to include larger numbers of replicates in future studies.

Implications for CCBR tissue culture

This study lays the groundwork for an entirely new system that allows the study of multiple factors on the growth and differentiation of mammalian cells including anchorage dependent cultures. Our hypothesis that the CCBR can be used to stimulate chondrogenesis in high-density chondrocyte cultures in the presence of multiple mechanical stimuli has been confirmed through studies involving 3-week cultures. Results show chondrogenesis following multi-week cell culture at a 0.11 N/m^2 shear stress in concert with the application of variable hydrostatic pressures in the 0.008 to 0.26 MPa (above atmospheric pressure) range. In this particular study, CCBR cell densities were 60% higher than corresponding values in pellet cultures at Week 1 and 30% at Week 3.

Similar collagen levels coupled with the statistically lower GAG levels do not yet support a more advanced hypothesis that multiple stressors, when applied simultaneously within the CCBR, will enhance chondrogenesis over unstressed controls. However, what we have shown is a system and methodology for more detailed studies that can now be added to the tool chest of cartilage researchers. As shown by Elder et al. (2006), the ability to investigate the affect of chondrocyte density in concert with hydrostatic pressure is important to the understanding of how to improve stimulation of chondrogenesis. The group showed when using pellet and alginate bead encapsulation cultures that high-density pellet cultures are more sensitive to hydrostatic pressure leading to ECM synthesis inhibition, whereas the lower density alginate bead cultures showed increased ECM synthesis.²⁹ As our cell densities in CCBR constructs are increased over pellet cultures, the findings by Elder et al. suggest chondrocyte hypersensitivity to hydrostatic pressure when cultured at high density in the CCBR, which may explain the deficit in GAG content in comparison to pellet cultures. In another study, Hunter et al. (2004) showed oscillatory dynamic compression of fibrin gels inhibits tissue development, whereas similar studies using agarose gel scaffolds stimulated tissue production;^{30,31} these studies underscore the importance of system design in stimulating chondrogenesis. Although the CCBR appears to be ideally suited for further studies on simultaneous stressors within different matrices, care must be taken to adjust the cell density as well as mechanical forces to realize the full potential of the CCBR in tissue engineering.

We expect the CCBR will prove to be a valuable system to study cartilage tissue development following modifications to exploit its unique properties and exploration of its full range of operating conditions. First, unlike the majority of culture systems relying on batch replacement of medium leading to nonuniform concentration gradients within the culture medium, the CCBR is a continuous system allowing precise control of metabolite and substrate concentrations. This may prove to be advantageous based on the myriad of studies proving the significant role that growth factors play in stimulating chondrogenesis in both chondrocytes and mesenchymal stem cells^{32–34} Another possible method for exploiting the mechanical environment is to immobilize scaffolds in the CCBR chamber. Scaffolds seeded at precise densities could then be subjected to hydrostatic pressure and shear while continuously perfused during culture. However, scaffold seeding density must be delicately controlled as Mauck et al. (2002) have shown increasing cell seeding density in agarose hydrogels from 20 to 60×10^6 cells/mL is beneficial to both the Young's Modulus and GAG content in free-swelling cultures, but inclusion of deformational loading is only beneficial to the lower density cultures.³⁵ Scaffold seeding density would be difficult to control in situ, but it would be possible to seed scaffolds at specific densities and transfer them to the CCBR for perfusion and hydrostatic pressure stimulation allowing cartilage development to be studied.

Future directions

As already stated the CCBR holds promise for understanding the influences of multiple biomechanical stimuli, individually and synergistically, not only on chondrogenesis but in a variety of other tissue and suspension culture applications. The need for inclusion of several new dimensions is obvious for future studies. With regard to cartilage tissue, follow-on studies will ascertain whether the CCBR is stimulating the production of Type I collagen (indicative of fibrocartilage) or the preferential production of Type II collagen found in articular cartilage in an effort to determine if the chondrocytes remain differentiated during CCBR culture. To date our focus has been to prove the utility of the system through analysis of total collagen and GAG content and chondrocyte population density and uniformity. This focus should now shift to analyses to differentiate between fibro and articular cartilage.

To fully understand the effect of the CCBR and variable stresses on tissue development, additional studies must be carried out to where mechanical forces are studied in a systematic manner. A series of important studies should be conducted to dissect the individual impact of each mechanical force and their combined synergistic effects on a developing tissue. This may likely require reactor modifications to eliminate spatial variations in mechanical forces. Spatial variations in mechanical force could possibly be eliminated by seeding scaffolds positioned within a static CCBR chamber, then varying shear across the scaffold surface and by keeping scaffold size small to minimize any hydrostatic pressure gradients. Immobilization of cells onto a scaffold would also allow the synergistic contributions of hydrostatic pressure and shear to be decoupled and their individual effect on tissue growth to be determined. Scaffold immobilization simplifies the decoupling of shear and hydrostatic pressure because these forces could be individually applied, something now possible when cells are immobilized using counter-flow centrifugation during normal CCBR operation. Preliminary AFM studies initiated in our labs also show promise for characterizing the mechanical properties of resulting cartilage. In summary, the authors believe the CCBR system may result as an important research tool for multivariate analysis of culture systems, especially, as this applies to the complex biomechanical milieu apparent in cell and tissue differentiation.

Conclusions

Various bioreactors have been developed to stimulate chondrogenesis from isolated chondrocytes with the objective of creating functional cartilage tissue. The individual application of mechanical forces such as shear stress, hydrostatic pressure, and direct compression has all been shown to stimulate chondrogenesis. Current research has begun to investigate the possibility of a synergistic effect on cartilage development from the simultaneous application of multiple mechanical forces. A novel use of the CCBR to stimulate chondrogenesis is presented in this article. Results are definitive in terms of showing utility of the device for eliciting chondrogenesis. When compared with pellet cultures, the ECM formed in the CCBR contains similar levels of collagen/DNA, but less GAG/DNA at both the 1- and 3-week time points. Histological results similarly confirm the development of a cartilaginous tissue due to positive staining for both GAG and collagen content at the 1- and 3-week culture time points. Although results indicate mechanical stimulation, the particular CCBR settings in this study do not significantly improve tissue quality over high-density culture alone; therefore a future study over a wider range of force magnitudes is in order.

It is likely through future work, the CCBR utility in tissue engineering will extend beyond that presented in this article given advantages that include application of multiples stressors that can be independently varied. Furthermore, the unique nature of the CCBR environment is advantageous allowing for well-mixed precise control of metabolite, substrate, and other soluble environmental factors. Also, it is expected that reactor chamber modifications will allow scaffolds to be cultured in a continuous environment at varying levels of hydrostatic pressure and medium perfusion. Further studies are warranted to fully realize the potential of this system to produce a quality cartilage tissue construct.

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Figure 1. Cell purging tubing sets were modified to reflect the configuration shown in the schematic of the CCBP and fluid lines

Fresh medium was supplied to the reactor chamber during centrifugation with the use of flexible tubing and an anti-twister device. During the 3-week culture, the waste pump was set to remove 200 mL/day, which was replaced with fresh culture medium from the fresh feed (FF) tank.

Figure 2. After 3 weeks of culture in the CCB, a cartilage construct has formed from the original 2.5×10^8 isolated chondrocytes seeded into the bioreactor. The construct is observed before (A) and after (B) removal from the reactor chamber. The reactor chamber containing the tissue construct (A) is viewed from above with direction of rotation to the left; with reference to this photo the axis of rotation is perpendicular to the plane of the page.

Figure 3. Histological results show cells are round and evenly distributed throughout the ECM of both the pellet and CCBR culture constructs, although the cell density is higher in the CCBR construct

Toluidine blue stain for GAG content is shown in (A) and an increase in staining intensity is difficult to discern in the pellet culture when comparing Week 1 and Week 3 sections, but an increase in intensity is clearly seen in sections of the CCBR construct. A trichrome stain (B) for collagen shows collagen incorporation is increased into the ECM of both constructs over time. (Bar represents 50 μm).

Figure 4. Collagen content normalized to DNA shows significant increases in collagen content from Week 1 to Week 3 for both the pellet and bioreactor culture systems

At Week 1, pellet cultures contain $2.8 \pm 0.6 \mu\text{g}/\mu\text{g}$ collagen/DNA while bioreactor cultures contain $1.8 \pm 0.4 \mu\text{g}/\mu\text{g}$ collagen/DNA at the same time point. These values increase to $6.6 \pm 1.9 \mu\text{g}/\mu\text{g}$ for pellet cultures at 3 weeks while bioreactor cultures increase to 6.8 ± 3.5 and $5.1 \pm 0.4 \mu\text{g}/\mu\text{g}$ for the 900 and 1650 rpm procedures, respectively. (* indicates $P < 0.05$)

Figure 5. Biochemical results of GAG content are normalized to DNA content for comparison of CCBR cultures as cell density varies between culture systems

GAG/DNA content is significantly higher in pellet cultures ($n = 7$) at the 1-week time point, with a value of $7.0 \pm 1.1 \mu\text{g}/\mu\text{g}$, when compared to the value for CCBR constructs ($n = 2$), $1.4 \pm 0.1 \mu\text{g}/\mu\text{g}$. The GAG/DNA content increases from Week 1 to Week 3 in all treatment groups, with pellet cultures containing, $8.4 \pm 0.9 \mu\text{g}/\mu\text{g}$ GAG/DNA, and CCBR constructs containing; $5.6 \pm 1.5 \mu\text{g}/\mu\text{g}$ at 900 rpm ($n = 2$) and $4.1 \pm 0.9 \mu\text{g}/\mu\text{g}$ for the 1650 rpm ($n = 2$) culture condition. (* indicates $P < 0.05$)