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Nanomechanics of Engineered Articular Cartilage: Synergistic Influences of Transforming Growth Factor- β 3 and Oscillating Pressure

Arshan Nazempour[#], Chrystal R. Quisenberry[#], Bernard J. Van Wie, and Nehal I. Abu-Lail
Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, Washington

[#] These authors contributed equally to this work.

Abstract

Articular cartilage (AC), tissue with the lowest volumetric cellular density, is not supplied with blood and nerve resulting in limited ability for self-repair upon injury. Because there is no treatment capable of fully restoring damaged AC, tissue engineering is being investigated. The emphasis of this field is to engineer functional tissues *in vitro* in bioreactors capable of mimicking *in vivo* environments required for appropriate cellular growth and differentiation. In a step towards engineering AC, human adipose-derived stem cells were differentiated in a unique centrifugal bioreactor under oscillating hydrostatic pressure (OHP) and supply of transforming growth factor beta 3 (TGF- β 3) that mimic *in vivo* environments. Static micromass and pellet cultures were used as controls. Since withstanding and absorbing loads are among the main functions of an AC, mechanical properties of the engineered AC tissues were assayed using atomic force microscopy (AFM) under a controlled indentation depth of 100 nm. Young's moduli of elasticity were quantified by modeling AFM force-indentation data using the Hertz model of contact mechanics. We found exposure to OHP causes cartilage constructs to have 45-fold higher Young's moduli compared to static cultures. Addition of TGF- β 3 further increases Young's moduli in bioreactor samples by 1.9-fold bringing it within 70.6% of the values estimated for native cartilage. Our results imply that OHP and TGF- β 3 act synergistically to improve the mechanics of engineered tissues.

Keywords

Articular Cartilage; Tissue Engineering; Centrifugal Bioreactor; Oscillating Hydrostatic Pressure; TGF- β 3; AFM; Young's Modulus

1. INTRODUCTION

Articular cartilage (AC) covers the articulating surfaces of bones, and absorbs impact forces generated in diarthrodial joints. AC sustains these loads partly due to its biomechanical properties generated by the dense extracellular matrix (ECM) primarily composed of

glycosaminoglycans (GAGs) and type II collagen. Because AC is avascular, it has a poor intrinsic ability for self-repair. Osteoarthritis, caused by an AC defect, affects 200 million adults worldwide.^{1,2} Although current therapies for cartilage repair reduce the pain,³ they have unsatisfactory long-term results.⁴ Cartilage tissue engineering can lead to future remedies by developing cartilage replacement tissues that have similar structure, composition and function as native cartilage.

To engineer cartilage tissues, a cell source that is easily accessible with high chondrogenic potential is needed.⁵ Although chondrocytes, the dominant cell type in AC, result in constructs with superior mechanical functionality compared to cartilage tissues generated from stem cells,⁶ stem cells' use in tissue engineering is preferred because harvesting chondrocytes adversely impacts the cartilage at the donor site⁷ and monolayer *in vitro* expansion causes chondrocyte dedifferentiation.⁸ With the drawbacks of chondrocytes, multipotent stem cells such as bone marrow-derived stem cells (BMSCs) and adipose-derived stem cells (ASCs) are seen as promising alternatives. Isolation of ASCs through liposuction is less invasive and less painful, and leads to considerably more cells in comparison to BMSCs.⁹ Moreover, ASCs are more genetically stable in long-term culture and their limited chondrogenic potential can be overcome with the addition of growth factors.^{6,10} For these reasons, interest has increased in the use of ASCs to engineer AC.

In addition to cell source, growth factors and physical stimulation are important in cartilage tissue engineering.^{11–15} *In vivo*, AC experiences 3.9 times body weight for level walking and 8 times body weight for downhill walking corresponding to 2.5 MPa and 5 MPa for a 70 kg person, respectively.¹⁶ To provide cells with such physical environments, a disadvantage of common protocols is that cells must first be encapsulated in scaffolds, cultured and then transferred to the bioreactors where mechanical forces are applied.^{12,17,18} These types of transformations could increase the risk of bacterial contamination. Furthermore, mass transfer is limited by diffusion from the bulk medium to the construct surface despite the improvements caused by the physical stimulation.

To provide this mechanical stimulation and allow for simultaneous growth factor supplementation, we expand on a unique centrifugal bioreactor (CBR) concept.^{19,20} Our reactor is unique because cells are injected into a tapered chamber in a scaffold free mode, centrifuged to form a pellet, and fresh medium and OHP are provided in the same reactor. This reduces contamination risks and we postulate that enhanced convective mass transfer of growth factor through continuous medium flow and cyclic hydrostatic pressures leads to AC tissues with elasticity similar to native AC.

Once we have the construct, measuring the mechanical properties of the engineered cartilage is critical in checking the functionality of engineered tissues, especially if they will be used in repairing damaged sites in the joint. AFM can quantify these mechanical properties at the micro- and nanometer scales via modeling of force-indentation data. Because AFM allows for measurements under near native liquid conditions, it is ideal for probing mechanical properties of biological samples such as cartilage.^{21–30} In this paper, we will use AFM to test the hypothesis that the Young's modulus of elasticity of cartilage tissues grown with oscillating hydrostatic pressure in the CBR in concert with growth factor is advantageous in

promoting chondrogenesis with respect to resultant mechanical properties. Furthermore, we discuss how the CBR in concert with AFM can in future studies be used to test hypotheses and answer critical research questions related to the nature of cartilage produced and the physiological and biomolecular mechanisms responsible for the formation of healthy cartilage.

2. MATERIALS AND METHODS

Cell culture supplies were purchased from Invitrogen-Gibco®, Grand Island, NY, USA unless otherwise specified.

2.1. Cell Culture

Human adipose-derived stem cells (hASCs) isolated from a lipoaspirate tissue of a 33-year-old female were cultured in expansion medium (EM) containing high-glucose Dulbecco's modified Eagle's medium (HG-DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), and 5 µg/ml Gentamicin and maintained under standard conditions (37 °C in a humidified incubator with 5% CO₂). Medium was changed three times a week. Upon 80–90% confluency, cells were passaged using Gibco® TrypLE™ Select and used at passage seven for the following experiments.

2.2. Chondrogenic Differentiation

2.2.1. Micromass Culture—Cells were harvested and resuspended in EM at 1.6×10^7 cells/ml. Micromass cultures were started by carefully placing 10 µl droplet cell suspensions in the center of each well in a 24-well plastic plate. Cells cultured under standard conditions adhered after 2 hours. Then 500 µl of fresh EM was added. After a day, 250 µl EM was removed and replaced with either base medium consisting of DMEM/F12 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 5 µg/ml Gentamicin, 1% Insulin-Transferrin-Selenium, 50 µM L-proline (Alfa Aesar, Ward Hill, MA), and 1% penicillin-streptomycin (Sigma-Aldrich), or in chondrogenic medium consisting of base medium with 100 nM dexamethasone, 50 µg/ml L-ascorbic acid (both from Sigma-Aldrich), and 10 ng/ml TGF-β3 (PeproTech, Ward Hill, NJ) (Fig. 1(A(i))). Cells that received base medium were considered as negative controls (NC) and those that received chondrogenic medium as positive controls (PC).

2.2.2. Pellet Culture—Aliquots of 5×10^5 cells, suspended in 500 µl EM, were centrifuged at 600× g for 5 min in 15-ml polypropylene conical tubes. Pelleted cells were incubated at standard conditions with loose caps to permit gas exchange. After a day, when sedimented and cells formed a spherical aggregate, half the medium was replaced by either base or chondrogenic medium (Fig. 1(A(ii))).

2.2.3. Bioreactor Culture—Four 2.5 ml polycarbonate 3D funnel shaped CBRs were manufactured (Figs. 1(B(i and ii))) in the Washington State University (WSU) engineering shop. Figure 1(B(iii)) illustrates the cell pressurization process using a pancake tie rod air cylinder (McMaster-CARR, Los Angeles, CA). Compressed air moves the rod which in

sequence pressurizes hydraulic oil within a master cylinder that pushes on aluminum pistons connected by screws to polycarbonate pistons at the top of each bioreactor (Fig. 1(B(ii))) to pressurize liquid medium when the reactor inlet and outlet shut-off valves are closed (IDEX Health and Science, Oak Harbor, WA). For OHP, a directional control three-way solenoid valve (Parker, Cleveland, OH) in connection with an 8-channel USB relay card was used (Vellman, Fort Worth, TX). Pressure transducers (DJ Instrument, Billerica, MA) and a NI USB-6008 data acquisition device (National Instrument, Austin, TX) were used to read pressures.

The CBRs, connecting tubes, valves and pressure transducers were sterilized with 70% ethanol for 24 h. With reactor outlet ports plugged, 6×10^6 cells were injected into each reactor under sterile conditions in a biosafety cabinet. Inlet ports were plugged and CBRs mounted on a COBE Spectra™ Apheresis System (TERUMO BCT, Lakewood, CO) and centrifuged at 500 rpm for 15 minutes. Base or chondrogenic medium supplemented with 20 mM HEPES buffer on a daily bases was continuously pumped for one week into the reactors. Media flow was stopped in reactors for 2 hours a day, corresponding shut off valves closed, and cells exposed to OHP for a week. After pressurization, media pumping was re-started. Reactor samples were free of contamination. For micro-mass, pellet and bioreactor cultures, half the medium was exchanged three times a week.

2.3. AFM Sample Preparation

Polycarbonate isopore membrane filters with an 8 μ m pore size (Millipore, Darmstadt, Germany) trapped the bioreactor tissue constructs when medium containing constructs were allowed to pass through filters under vacuum pressure.³¹ Cartilage-containing filters were fixed to AFM metal specimen disks (Ted Pella, Redding, CA) by double-sided tape and placed on the sample stage for subsequent AFM studies.

2.4. AFM Force-Indentation Measurements

Colloidal probes (Fig. 2(A)) were chosen to quantify the mechanical properties of cartilage.³² Furthermore, the colloidal probe models a single asperity on one cartilage surface contacting an opposing cartilage surface.²³ All AFM force-indentation measurements were performed with a PicoForce scanning probe microscope with a Nanoscope IIIa controller and extender module (Bruker AXS Inc., Santa Barbara, CA). Prior to force measurements, the force constant of each cantilever was determined from the power spectral density of the thermal noise fluctuations in PBS.³³ Cantilevers with a manufacturer spring constant of 0.08 N/m had average deflection sensitivities of 83.5 ± 21.1 nm/V ($n = 3$). AFM force-indentation profiles were collected on the imaged areas of the cartilage sample with a trigger threshold of a 100 nm. Force-volume images were generated using a 16×16 grid of equally spaced indentation points (Fig. 2(B)) in contact mode with a 1 Hz scan speed and 10 μ m scan area. Each sample was scanned in PBS in at least 3 areas with at least 2 samples per treatment group. At each pixel, both approach and retraction curves were collected. Only approach curves are analyzed in this work.

2.5. Determination of Young's Moduli–Hertz Model

AFM approach position-deflection data files were converted to force-indentation files as described previously.³⁴ To quantify the Young's modulus, approach curves were fit to the Hertz contact model which assumes an infinitely hard sphere indenting a flat, deformable elastic substrate as described in Eq. (1):

$$F = \frac{4}{3} \times \frac{E_{\gamma} R^{1/2} \delta^{3/2}}{1 - \nu^2} \quad (1)$$

Where F is the applied force, E_{γ} is the Young's modulus, R is the relative radius, ν is Poisson's ratio, and δ is the indentation depth. Once all force-indentation profiles collected in a force-volume image were analyzed for their Young's moduli, a histogram that describes the heterogeneity in the data was generated (Fig. 3).

Although the Hertz model has limitations, we believe it is appropriate for our purposes for the following three reasons. First, the sample surface is continuous and non-conforming; second, the radius of the contacting bodies are large compared to the contact area so that surfaces can be approximated as an elastic half space;³⁵ and third, substrate effects can be ignored because the indentation depth (~900 nm) is much smaller than sample thickness (300 μm).³⁶ Only normal pressures are applied during indentation because there is a lubricated layer between the machine parts and the sample, so friction can be neglected and the indentation is on the nanometer scale, so strains will be small. This means that stresses and strains produced are not dependent on the sample or probe geometry.³⁷ Although cartilage is a viscoelastic material, we will only use the Hertz model to fit the elastic portion of the approach curve.

2.6. Statistical Analysis

Statistical analysis was performed using the SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) software package. One-way analysis of variance (ANOVA) was used with the Dunn test to determine whether significant differences existed between treatment groups, with statistical significance reported at the 99.9% confidence level ($P < 0.001$).

3. RESULTS

3.1. Heterogeneity of Young's Moduli of Cultures

All tissue constructs, micromass, pellet and bioreactor, were heterogeneous spanning a range of Young's moduli as shown by a log-normal dynamic peak function fit to histograms in Figure 3. CBR (OHP) samples supplemented with TGF- β 3 occupied an 87-fold wider range, 4 to 981 kPa, and an 84-fold higher mean Young's modulus, when compared to the highest range and highest modulus for the micromass and pellet free-swelling controls as shown in Table I. This implies that CBR samples are the most heterogeneous. In contrast, the free-swelling NCs occupied a 4.3-fold wider range and were therefore more heterogeneous compared to PCs. This demonstrates TGF- β 3 increases mechanical heterogeneity of cartilage samples and OHP has the most drastic effect on the mechanical heterogeneity.

3.2. Effects of TGF- β 3 on Static and CBR Cultures

Figure 3 and Table I show TGF- β 3 has different effects on CBR compared to free-swelling cultures. The most probable Young's modulus, determined with a log-normal fit to the histogram, for pellet and micromass culture is reduced from 3.61 (NC) to 0.769 (PC) kPa and from 1.10 to 0.318 kPa after TGF- β 3 supplementation, respectively. These trends are consistent with the mean Young's modulus (Table I) for micromass and pellet culture which decreased by 3.7- and 4.4-fold with TGF- β 3 supplementation, respectively. In contrast, an opposite trend was observed with TGF- β 3 supplementation in the CBR with OHP. Most probable values of 1120 kPa (NC) and 320 kPa (PC) were observed with means increasing by 1.85 fold.

3.3. Effects of OHP With and Without TGF- β 3

CBR constructs, in contrast to free-swelling controls, have visually stiffer force-indentation curves (Fig. 4) and have an 83-fold larger Young's modulus distribution and 88 times greater most probable Young's modulus (Figs. 3(e and f)). Consistent with the most probable Young's moduli, the mean modulus for the NC CBR in Figure 5 is 45-fold higher than the highest average value in the free-swelling samples, that for the pellet without TGF- β 3. This suggests OHP is capable of increasing the Young's modulus of elasticity compared to static controls. In samples with OHP, TGF- β 3 can further increase the Young's modulus of CBR samples by an additional 1.9 fold.

4. DISCUSSION

4.1. Heterogeneity of Young's Moduli

Similar to native cartilage and other biological tissues, the engineered tissues included a heterogeneous population of Young's moduli. Native human cartilage has Young's moduli ranging from 300 to 1000 kPa^{33,38} (Fig. 3(d)). Our bioreactor-grown constructs are nearing this range, spanning 2.17 to 981 kPa. Free-swelling samples do not have as wide a range, as shown by the 0.128–11.9 kPa range when considering all micromass and pellet cultures collectively, nor as high a Young's modulus as the native or bioreactor samples. AC is expected to have heterogeneous mechanical properties because of its wide variety of components including cells, collagen, and various proteoglycans, lipids, and glycoproteins.³⁹ The less heterogeneous samples (free-swelling NC) are presumably less differentiated, have fewer components, and therefore only represent the Young's modulus of those few components. This suggests TGF- β 3 induces expression of a larger number of cartilage components in our studies as is consistent with the literature.^{40,41} Also, because bioreactor samples had the most mechanically heterogeneous surface, results suggest OHP greatly increases expression of a fuller array of cartilage components. When used in concert, TGF- β 3 and OHP provide more heterogeneity than either alone.

4.2. Effects of TGF- β 3 on Young's Moduli of Tissues

TGF- β 3 had a negative impact on the Young's modulus of ASC-derived micromass and pellet constructs. Conversely, TGF- β 3 increased construct Young's moduli when OHP was applied during cartilage development, suggesting TGF- β 3's positive effect on ASCs requires

and is actuated by OHP. This agrees with theoretical work describing an increase in TGF- β 3 concentration by 2–3 fold when under dynamic loading compared to free-swelling environments.⁴² The micromass and pellet cultures rely on diffusion of TGF- β 3 to the cells, whereas the bioreactor samples may have an increased localized concentration of TGF- β 3 because OHP assist with mass transfer to the core of the cartilage construct. To test this hypothesis in future studies, one could measure the TGF- β 3 concentration within the construct in free-swelling and bioreactor conditions and determine parameters that will assist with developing theoretical models of concentration gradients in varied construct geometries. Using these concentrations, a TGF- β 3 dose-response experiment with ASCs could be performed to measure mechanical properties of 2-D AMSC cultures and/or 3-D hydrogel cultures in pellets of diminishing sizes to assess the impact of mass transfer limitations.

Rationale for the beneficial influence of TGF- β 3 on cartilage mechanical properties comes from its role in ECM synthesis¹² and its anabolic activity resulting from the induction of the expression of Sox9 transcription factor.⁴³ Sox9 is responsible for the expression of many key genes in chondrogenesis such as *Sox5*, *Sox6*,^{44,45} and collagen II $\alpha 1$ (*Col2a1*).^{46,47} In turn, Sox5 and Sox6 act as transcriptional enhancers for cartilage matrix genes.⁴⁴ In this way, TGF- β 3 activates Sox9 to cause the production of major ECM components, collagen and aggrecan. Increased production of these components improves the mechanical properties of TGF- β 3-supplemented cartilage constructs.

We note our results contradict those of Lima, et al. who described a negative effect of TGF- β 3 and dynamic loading when combined together.¹⁴ They surmise dynamic loading increases TGF- β 3 concentration, as we purport for the reason behind the apparent synergistic impact; however, they argue that too high of a concentration may elicit a negative response. Therefore, future studies should be focused on dose-response to determine if there is an optimum concentration.

Other studies have employed alternative growth factors, such as TGF- β 1, dexamethasone, and IGF-I in differentiation of human mesenchymal stem cells (hMSCs) into chondrocytes.^{40,48,49} Care is needed in weighing choices such as cell source, growth factor, and pattern of administration. For example, transient additions of TGF- β 3, supplementation for the first two weeks of culture, has positive impacts on the Young's modulus of bovine chondrocyte-laden disks,⁵⁰ but when MSCs are used, opposed to chondrocytes, the equilibrium modulus is cell density-dependent.⁵¹ Because ASCs are in some cases thought to have lower chondrogenic potential compared to BMSCs,^{52–54} further investigations in how to optimize ASC culture conditions must be performed. It has been found that early culture conditions such as the type of medium, cell density, and culture plastic can influence cells to move toward a specific phenotype.⁵⁵ Despite ASC's alleged lower chondrogenic potential compared to BMSCs, using a greater dose and a combination of growth factors has been shown to improve chondrogenesis to a level comparable to BMSCs.⁵⁶ ASC chondrogenic potential can be further increased by culturing in medium containing a combination of growth factors that includes BMP-6.⁵⁷ While we chose TGF- β 3 because it is the most commonly used TGF^{49–51} with more consistently positive results,^{40,58,59} in future

experiments medium should be supplemented with other growth factors with and without transient exposure to discern the impact of these application regimens.

4.3. Advantages of the CBR and Discussion of Effects of OHP With and Without TGF- β 3

The CBR represents a new and different process in which cells may be seeded in a scaffold free manner and held at a desired population density while the extracellular matrix is formed. It is a system in which mechanical stimulation can be performed without the requirement of transferring the cell-ECM construct to a separate chamber for OHP. In previous studies such as those done by Kaupp et al.¹⁷ and Toyoda et al.⁶⁰ cells were first encapsulated in scaffold and then cell-seeded scaffolds were transferred to another chamber to be exposed to mechanical stimuli. Transferring step has been bypassed in our newly developed bioreactor system and we believe doing so would reduce the risk of getting contamination. Moreover, it is a perfusion system in which growth factors can be introduced at varying flow rates and a suspension or a matrix can be held within the reactor by balancing outward-acting centrifugal forces and counteracting inward drag forces due to flow. The results in this study affirm the utility of the CBR and they even supersede those acquired by others when using static systems. Specifically, as stated earlier, we observed a 45-fold higher most probable Young's modulus up to an average value of 171 kPa when the CBR was used in OHP mode and a further increase by 1.9 times this value when the construct was held within the CBR and perfused with TGF- β . By contrast Mauck, Nicoll et al. 2000 only show an average unconfined compression modulus of 42 kPa after 28 days of culture for dynamically loaded disks.⁶¹ Further studies with the CBR are needed to affirm these differential gains and they should be combined with mRNA upregulation studies to compare results with those represented in the work by groups such as Miyanishi, Trindade et al. 2006 whose study showed intermittent hydrostatic pressure and TGF- β 3 can increase mRNA for Sox9, type II collagen, and aggrecan by 1.9-, 3.3-, and 1.6-fold, respectively.¹²

There are various ways in which the CBR system can be employed to test research hypotheses and answer critical questions about the nature of constructs formed as well as about the underlying mechanisms governing chondrogenesis. For example, ASCs on exposure to cyclic hydrostatic pressure and supplementation with TGF- β 3 and BMP-6 caused varying results. In one study, the combination of stimuli had no significant effect on DNA content or sulfated glycosaminoglycan (GAG) accumulation, but did increase the tissue's dynamic modulus.¹⁵ The improved mechanical properties may be because cyclic hydrostatic pressure leads to a more dense matrix,^{62–65} though there is variation from one donor to the next.⁶⁶ Another, explanation for the synergistic behavior of TGF- β 3 and OHP is that TGF- β 3 up-regulates collagen production while dynamic loading differentially enhances the production of proteoglycan and linkage molecules, such as cartilage oligomeric matrix protein (COMP), that assemble the ECM components forming a structural network within the tissue.^{61,67} Because the CBR system allows a number of chambers to be placed on the same rotor, varied conditions can be tested simultaneously on tissues formed from the same cell set, but divided into different reactors, which are easily disassembled after study completion for construct analysis.

Another explanation for why mechanical and bioactive factors work in concert is based on the theory of incompressible mixtures which says dynamic loading increases the convection of large molecules, such as growth factors, into a construct, increasing local concentrations.¹³ This implies that dynamic loading will further increase TGF- β 3 concentration within the construct compared to just increasing TGF- β 3 alone and prospectively improve mechanical properties beyond the use of either stimulus alone. Because CBR reactor chambers can be made of polycarbonate, which is able to withstand the high pressures needed for these types of studies, the see-through nature and visualization afforded by the use of a stroboscope allow studies with dye molecules that display whether or not large molecules are indeed entering into the construct.

The goal of cartilage tissue engineering is to mimic the native environment including supplying mechanical loading to influence stem cell differentiation. *In vitro*, dynamic loads have been applied at magnitudes ranging from 0.1–10 MPa^{62–64,66,68} and frequencies ranging from 0.01–1 Hz^{62,63,66,68–70} to stimulate cartilage matrix protein' expression and improve the engineered tissues' compressive properties.⁵⁸ These pressures simulate physiological loads in the joint during daily activities such as standing or descending stairs with average peak resultant forces of 107% and 346% body weight, respectively.⁷¹ Compared to static conditions, hydrostatic pressure increases the equilibrium modulus⁷² and improves the matrix composition of tissue engineered cartilage.⁶⁵

Others have found loading to be more effective when applied early in culture⁷³ and for multiple days.⁷⁴ Cyclic hydrostatic pressure initiates and enhances the chondrogenic differentiation of ASCs with or without TGF- β 1,⁷⁵ but hASCs express more than 40 times the amount of cartilage-specific matrix genes when the TGF- β 1 and cyclic hydrostatic pressure are combined compared to either cyclic hydrostatic pressure or TGF- β 1 alone.⁷⁶ Despite this, when cyclic hydrostatic pressure is applied to ASCs without any form of soluble chondrogenic inducing factors, the cells lose viability by day 21.⁷⁷ Also, human ASCs respond better to higher magnitudes of cyclic hydrostatic pressure such that constructs have improved matrix when 5 MPa is applied compared to 3 or 0.4 MPa of hydrostatic pressure.^{78,79} Mauck, Soltz et al. 2000 have shown dynamic loading can significantly increase the equilibrium aggregate modulus by 6-fold in comparison to free swelling controls,⁶¹ while hydrostatic pressure, in particular, causes a significant ($p < 0.002$) increase in equilibrium modulus for infrapatellar fat pad derived multipotent stromal cells.⁷² Still other studies have found that cells encapsulated in less concentrated agarose gels (2%) experience more mechanical stimulation and in 28 days increase their Young's modulus more than those grown in 3% gels.⁸⁰ The CBR system is adaptable for all such studies, and can be programmed to apply constant versus dynamic pressures, differing pressures, varied growth factor regimens, and different chambers can be used side-by-side to study scaffold free versus scaffolds that contain differing gel compositions.

4.4. Implications for Future Cartilage Tissue Engineering Studies

In addition to approaching the Young's moduli range and magnitudes present in native AC, other criteria must be met for successful engineered AC tissues including durability, integration capability with surrounding tissues, low friction coefficients, and abundant

lubrication. In this study we used ASCs because of ease of procurement with the prospect of making future autologous tissue engineering cheaper and easier. While ASCs form constructs having high concentrations of collagen⁶ with high levels of aggrecan gene expression,¹⁰ they have lower chondrogenic potential compared to BMSCs. It has been suggested that these results because of reduced expression of BMP-2, -4, and -6 mRNA and TGF β -receptor-1 protein, however, it has been shown that this limitation may be overcome with TGF- β and BMP-6 supplementation.¹⁰ Therefore, it is suggested that future investigations include the use of a wider variety of growth factors. Alternatively, other stem cells such as BMSCs could be used. BMSCs are well characterized and readily cultured in medium containing TGF- β 3 or TGF- β 1, dexamethasone, and BMP-6^{81–83} to induce differentiation.⁸³

When using growth factors and mechanical stimulation in cartilage tissue culture, the magnitude and pattern of physical stimulation must also be taken into account. For example, interstitial hydrostatic pressure magnitudes between 0.1 and 10 MPa have been shown to change the gene and macromolecular expression of chondrogenic markers.¹¹ Also, cyclic hydrostatic pressure in combination with TGF- β 3 was found to enhance the functional mechanical properties of fat pad derived multipotent stromal cells with respect to both their equilibrium and dynamic moduli.⁷² Another study shows that while cyclic hydrostatic pressure has no significant effect on DNA content or sulfated GAG accumulation, it still improves the tissue dynamic modulus measured by cyclic indentations.¹⁵ Variation of hydrostatic magnitudes and pulsation patterns are easily programmed in the CBR presented in this study and the ability to continuously perfuse with medium containing a variety of growth factors is an added bonus that will enhance the variety of studies that can be done in the future.

5. CONCLUSIONS

We successfully engineered a mechanically sound AC tissue starting from hASCs under OHP and TGF- β 3 in our unique bioreactor. The results presented show the capability of our newly developed system for inducing hASC chondrogenesis, and indicate hASCs responsiveness to OHP and TGF- β 3 in a way that Young's moduli increased to near native values and beyond those stimulated by either factor alone. Although the precise mechanisms remain unknown, data demonstrate the magnitude and frequency of OHP and the TGF- β 3 concentration used in this experiment results in beneficial mechanical properties of the engineered constructs. Future studies are needed on varied mechanical loading and growth factor regimens and on identifying intracellular and extracellular processes that lead to this result and the newly introduced CBR system not only affords these opportunities, but also may be used to test hypotheses in a number of studies that may be proposed.

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Figure 1.

Experimental design. (A(i)) A $10\ \mu\text{l}$ droplet of expanded hAMSCs at a density of 1.6×10^7 viable cells/ml was placed in the center of each of 15 wells in two 24-well culture plates, one serving as a negative control (NC) with base medium and one as a positive control (PC) with growth factor supplemented medium. (A(ii)) 5×10^5 hAMSCs in $500\ \mu\text{l}$ of EM were centrifuged in each of two microcentrifuge tube sets at 600 g for 5 minutes, four NC and four PC. (A(iii)) 6×10^6 hAMSCs in 2.5 ml of EM were injected into each reactor with two replicates at each condition, NC with and without oscillating pressure (OP) and PC with and without OP. (B(i)) Conical-shape bioreactor housed in a polycarbonate casing and topped with a pressurizing piston. (B(ii)) Schematic of conical-shape centrifugal bioreactor/pressurizing system. (B(iii)) Schematic process flow diagram for the bioreactor process system.

Figure 2.

(a) A colloidal probe approaching the sample surface, (b) A 16×16 force-volume image generated from indenting the sample at 256 points. The scale bar represents force in nN. Each pixel in the force-volume image consists of an approach and a retraction curve. The approach curves are converted into force-indentation profiles like that shown later in Figure 4. Retraction curves could be used for adhesion data, but this was not relevant to this paper.

Figure 3.

(a–c) Distribution of Young's moduli for each treatment group and (d and e) the log-normal probability distribution function for each histogram. (f) The distribution of Young's moduli and log-normal probability distribution function for bovine native articular cartilage.

Figure 4.
Example curves showing different force-indentation profiles for micromass, pellet, and bioreactor samples treated with TGF- β 3. Solid lines represent the Hertz fits to the data.

Figure 5.

Means of distributions of the Young's moduli for all 6 conditions. Samples listed as OP are from the CBR and samples listed as PC were positive control samples which are supplemented with TGF- β 3. NC stands for negative control which are samples without TGF- β 3 supplementation. Error bars represent the standard error of the mean. All treatment groups were statistically significant from each other within a 99.9% confidence limit as indicated by the *.

Table I

A summary of the mean, median, range, standard deviation and standard error of the mean for Young's moduli (E) resulting from Hertzian fits to force-indentation profiles measured for various treatment groups. The number of indentation events is indicated by n .

Treatment group	Mean of E (kPa) $\pm$ standard error (kPa)	Median of E (kPa)	Standard deviation of mean E (kPa)	Range of E (kPa)	r^2 for Hertz model
Micromass ($n = 338$)	1.850 $\pm$ 0.005	1.41	1.60	0.177–9.07	0.992
Micromass + TGF- β 3 ($n = 480$)	0.498 $\pm$ 0.001	0.392	0.276	0.128–2.22	0.988
Pellet ($n = 757$)	3.810 $\pm$ 0.002	3.60	1.69	0.621–11.9	0.990
Pellet + TGF- β 3 ($n = 733$)	0.871 $\pm$ 0.001	0.837	0.400	0.155–2.85	0.994
CBR ($n = 1090$)	171 $\pm$ 0.339	45.8	371	2.17–485	0.965
CBR + TGF- β 3 ($n = 1310$)	318 $\pm$ 0.359	185	470	4.09–981	0.951