Effects of Confinement on the Mechanical Properties of Self-Assembled Articular Cartilage Constructs in the Direction Orthogonal to the Confinement Surface

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ABSTRACT: This study examined the effects of radial confinement and passive axial compression-induced vertical confinement, on the biomechanical, biochemical, and histological properties of self-assembled chondrocyte constructs. The self-assembled constructs, engineered without the use of an exogenous scaffold, exhibited significant increases in stiffness in the direction orthogonal to that of the confinement surface. With radial confinement, the significantly increased aggregate modulus was accompanied by increased collagen organization in the direction perpendicular to the articular surface, with no change in collagen or glycosaminoglycan (GAG) content. Additionally, radial confinement was most beneficial when applied before 2 weeks. With passive axial compression, the significantly increased Young's modulus and ultimate tensile strength were accompanied by a significant increase in collagen production. This study is the first to demonstrate the beneficial effects of confinement on tissue engineered constructs in the direction orthogonal to that of the confinement surface. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 26:238–246, 2008

Keywords: articular cartilage; self-assembling process; tissue engineering; mechanical stimulation; extracellular matrix

INTRODUCTION

Cartilage degeneration, from injury or osteoarthritis, is an important problem in current orthopedic practice. Articular cartilage is unable to repair itself, resulting in a permanent defect or the formation of mechanically inferior fibrocartilage.[1] Tissue engineering is a promising approach for the treatment of cartilage injuries, as this approach may eventually allow for production of engineered tissue indistinguishable from native cartilage.

A chondrocyte self-assembling process for tissue engineering articular cartilage was recently developed[2] that allowed constructs to reach one-third the stiffness of native cartilage. The self-assembling process avoids many of the problems associated with scaffold use, namely concerns over stress shielding, biocompatibility, and biodegradation. Intermittent hydrostatic pressure applied at 10 MPa and 1 Hz, for 4 h per day and 5 days per week was shown to increase collagen production further with this process.[3]

Articular cartilage is exposed to compression, shear, and hydrostatic pressure. Mechanical stimulation is vital for maintaining tissue integrity; articular cartilage demonstrates changes representative of a loss of function when immobilized.[4,5] Therefore, mechanical intervention is likely necessary for refinement of tissue engineering techniques. Although the signaling pathways involved in cartilage mechanotransduction have not been fully elucidated, several studies showed promising results with dynamic compression,[6–12] shear,[10,13] and hydrostatic pressure.[14–17]

Coupling mechanical stimulation with the self-assembling process for tissue engineering articular cartilage represents a promising solution for treatment of injuries, but several questions remain concerning this approach. Studies comparing the effects of passive confinement on the anisotropy of articular cartilage are lacking, and few studies have involved the effects of mechanical intervention at different times.[18]

The purpose of this study was to examine the effects of construct confinement in different directions and at different times on mechanical properties using radial confinement and passive axial compression-induced vertical confinement of self-assembled constructs. We hypothesized that confinement would enhance mechanical properties in

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the orthogonal direction. We further hypothesized that confinement at different timepoints would significantly affect construct properties. To test these hypotheses, three experiments were performed (Fig. 1). First, self-assembled constructs were radially confined in agarose wells for 1, 2, 3, or 4 weeks, after which the constructs were cultured unconfined for the remainder of the 4-week study; the effects of confinement on compressive stiffness were investigated. Second, the constructs were cultured in the same wells used in the first experiment for 2 weeks, after which they were transferred to incrementally larger wells for the 3rd and 4th week of culture. Finally, the effects of vertical confinement, in the form of passive axial compression, on the tensile stiffness were examined.

METHODS

Chondrocyte Isolation and Seeding

Chondrocytes were isolated from the distal femur of week-old male calves[19–21] (Research 87 Inc., Boston, MA)

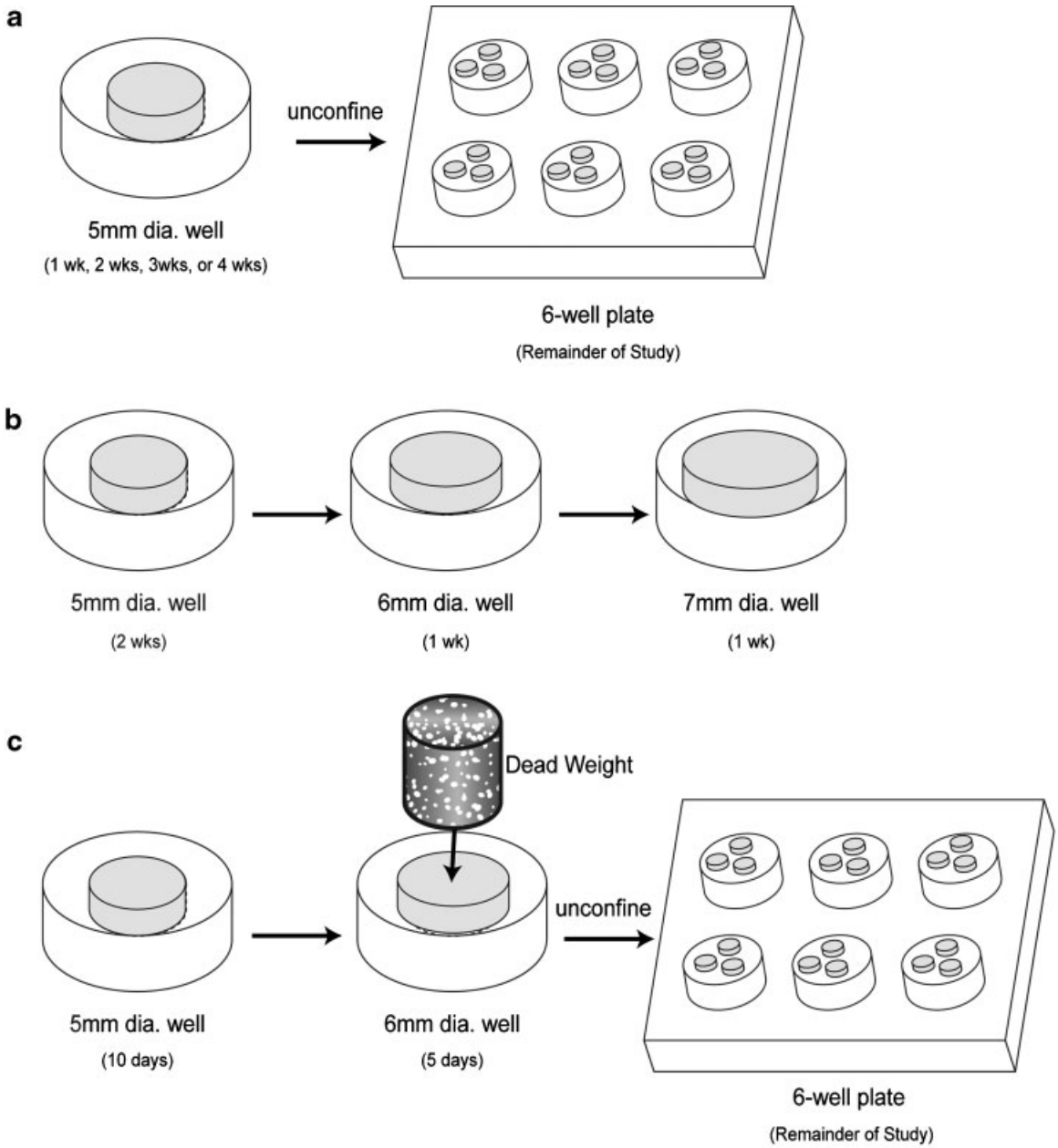

Figure 1. The experimental design. (a) 1st study: Radial confinement of self-assembled constructs; (b) 2nd study: Maintenance of radial confinement of self-assembled constructs; (c) 3rd study: Passive axial compression of self-assembled constructs.

less than 36 h after slaughter, with collagenase type 2 (Worthington, Lakewood, NJ) in the chemically-defined culture medium (DMEM with 4.5 g/L-glucose and L-glutamine (Biowhittaker/Cambrex, Walkersville, MD), 100 nM dexamethasone, 1% fungizone, 1% penicillin/streptomycin (BD Biosciences, Bedford, MA), 1% ITS+, 50 μg/mL ascorbate-2-phosphate, 40 μg/mL L-proline, and 100 μg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA)). Each femur came from a different animal and yielded roughly 150 million chondrocytes. To reduce variability among animals, cells were pooled together to yield a mixture of chondrocytes; a mixture of cells from eight legs was used in the 1st study, while a mixture from six legs was used in the 2nd and 3rd studies (described below). The pooled cells were counted on a hemocytometer, and viability (>99% for the pooled cells) was assessed using a trypan blue exclusion test. Chondrocytes were frozen in culture medium supplemented with 20% FBS and 10% DMSO at −80°C for 2 weeks to a month before use. After thawing, viability remained >75%. A polysulfone die consisting of 5-mm diameter × 10-mm long cylindrical prongs was constructed to fit into 6 wells of a 48-well plate. Additional polysulfone die consisting of 6-mm diameter × 10-mm long cylindrical prongs and 7-mm diameter × 10-mm long cylindrical prongs were fabricated. To construct each agarose mold, sterile, molten 2% agarose was introduced into a well fitted with the die. The agarose was allowed to gel at room temperature for 1 h. The agarose mold was then separated from the die and submerged into two exchanges of culture medium to saturate the agarose well by the time of cell seeding. To each agarose well, 5.5×10^6 cells were added in 150 μl of medium. The cells self-assembled within 24 h in the agarose wells and were maintained in the same well for a specified amount of time; t = 0 was defined as 24 h after seeding.

1st Study: Radial Confinement of Self-Assembled Constructs

At 1, 2, or 3 weeks, self-assembled constructs ($n = 6$) were removed from confinement in the 5-mm diameter agarose well, and placed in a well coated with 2% agarose (Fig. 1a). Each agarose-coated well contained three to four constructs, and 500 μl of medium per construct were changed daily (1.5–2 ml per well). At 4 weeks, all samples were tested for morphological, histological, biochemical, and biomechanical properties.

2nd Study: Maintenance of Radial Confinement of Self-Assembled Constructs

At 2 weeks, self-assembled constructs ($n = 5$) were removed from confinement in the 5-mm diameter agarose wells, and transferred to 6-mm diameter agarose wells (Fig. 1b). At 3 weeks, these constructs were removed from confinement and transferred to 7-mm diameter agarose wells. A control consisted of constructs confined in 5-mm diameter agarose wells for 2 weeks, then maintained in agarose-coated wells, as described above. Each day, 500 μl of medium were changed. At 4 weeks, all samples were tested for morphological, histological, biochemical, and biomechanical properties.

3rd Study: Passive Axial Compression of Self-Assembled Constructs

At 10 days, self-assembled constructs ($n = 5$) were removed from confinement in 5-mm diameter agarose wells and transferred to 6-mm diameter agarose wells. Vertical confinement, in the form of passive axial compression with a dead weight, was applied by placing a 5-mm diameter × 1-cm long, 1-g, porous, sintered steel cylinder on top of each construct (Fig. 1c). The weight corresponded to a 0.5 kPa stress. At 2 weeks, the cylinders were removed, and the constructs were transferred to agarose-coated wells for the remainder of the study. A control consisted of constructs cultured in 5-mm diameter agarose wells, then transferred to 6-mm diameter agarose wells at 10 days, and finally maintained in agarose-coated wells for the remainder of the study.

Histology and Immunohistochemistry

Samples were frozen and sectioned at 14 μm. Safranin-O and fast green staining were used to examine glycosaminoglycan (GAG) distribution.[22,23] Picrosirius red was used for qualitative examination of collagen content. Polarized light microscopy of picrosirius red-stained sections was used to examine the collagen organization of the constructs. Slides were also processed with immunohistochemistry (IHC) to test for the presence of collagen types I (COL1) and II (COL2) on a Biogenex (San Ramon, CA) i6000 autostainer. After fixing in chilled acetone, the slides were rinsed with IHC buffer (Biogenex), quenched of peroxidase activity with hydrogen peroxide/methanol, and blocked with horse serum (Vectastain ABC kit, Vector Labs, Burlingame, CA). The slides were then incubated with either mouse anti-COL1 (Accurate Chemicals, Westbury, NY) or mouse anti-COL2 (Chondrex, Redmond, WA) antibodies. The secondary antibody (mouse IgG, Vectastain ABC kit) was then applied, and color was developed using the Vectastain ABC reagent and DAB (Vector Labs).

Quantitative Biochemistry

Samples were digested with 125 μg/ml papain (Sigma) in 50 mM phosphate buffer (pH = 6.5) containing 2 mM N-acetyl cysteine (Sigma) and 2 mM EDTA (Sigma) at 65°C overnight. Total DNA content was measured by Picogreen® Cell Proliferation Assay Kit (Molecular Probes). Total sulfated GAG was then quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor), based on 1,9-dimethylmethylene blue binding.[24,25] After being hydrolyzed by 2 N NaOH for 20 min at 110°C, samples were assayed for total collagen content by a chloramine-T hydroxyproline assay.[26]

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Indentation Testing

Samples were evaluated with an automated indentation apparatus.[27] A step mass of 0.7 g (0.007 N) was applied with a 1-mm flat-ended, porous indenter tip, and the specimens were allowed to creep until equilibrium, as described elsewhere.[2] Preliminary estimations of Young's moduli of the samples were obtained using the analytical solution for the axisymmetric Boussinesq problem with Papkovich potential functions.[28,29] The intrinsic mechanical properties of the samples were then determined using the linear biphasic theory.[30]

Tensile Testing

Samples cut 500-μm thick were tested under incremental stress relaxation, using a uniaxial materials testing machine (Instron 5565).[31] Following preconditioning, specimens were exposed to incremental stress relaxation, whereby they were exposed to 15 min constant strain increments until failure. A maximum of 11 increments were allowed (4, 8, 12, 16, 20, 25, 35, 50, 70, 100, and 130% strain). A strain rate of 6 mm/min was employed between constant-strain increments. Stress vs. strain plots were constructed from the peak stress and relaxed stress corresponding to the strain at each increment. Material properties were calculated from the plots. The modulus was the slope of the linear region, the tensile strength equal to the maximum stress, the maximum strain was the strain corresponding to the maximum stress, and the energy was the area under the curve (trapezoid rule) from zero strain to maximum strain.

Statistical Analysis

A single factor ANOVA was used with Tukey's post hoc test when warranted. Significance was defined as $p < 0.05$.

RESULTS

Gross Appearance and Histology

No differences in gross morphology were observed among the groups. After 2 weeks, all constructs reached a diameter slightly <6 mm, by 3 weeks constructs reached a diameter slightly <7 mm, and by 4 weeks constructs reached a diameter approaching 7.5 mm. In the confinement study, no significant thickness differences were found among the 2-week confinement group and the 1-, 3-, or 4-week groups (thickness $= 1.05 \pm 0.05$, 1.02 ± 0.06, 1.07 ± 0.17, and 1.10 ± 0.14 mm, respectively. In the passive axial compression study, there were no significant thickness differences among treatment groups (compressed group, thickness $= 0.73 \pm 0.09$ mm; control group, thickness $= 0.81 \pm 0.07$ mm). In the follow-up confinement study, there was no significant thickness differences among the 2-week confinement group and the groups confined for 2 weeks in 5-mm diameter wells, 1 week in 6-mm diameter wells, and 1 week in 7-mm diameter wells, with values of 0.58 ± 0.09 and 0.51 ± 0.04 mm, respectively.

At 4 weeks, all constructs stained positive for collagen throughout the thickness of the construct (Fig. 2b). Safranin-O staining for GAG was observed throughout the constructs (Fig. 2c), as was COL2 immunostaining, with no differences in production among the treatment groups (Fig. 2d). Based on IHC, there was no COL1 production for any construct (Fig. 2e).

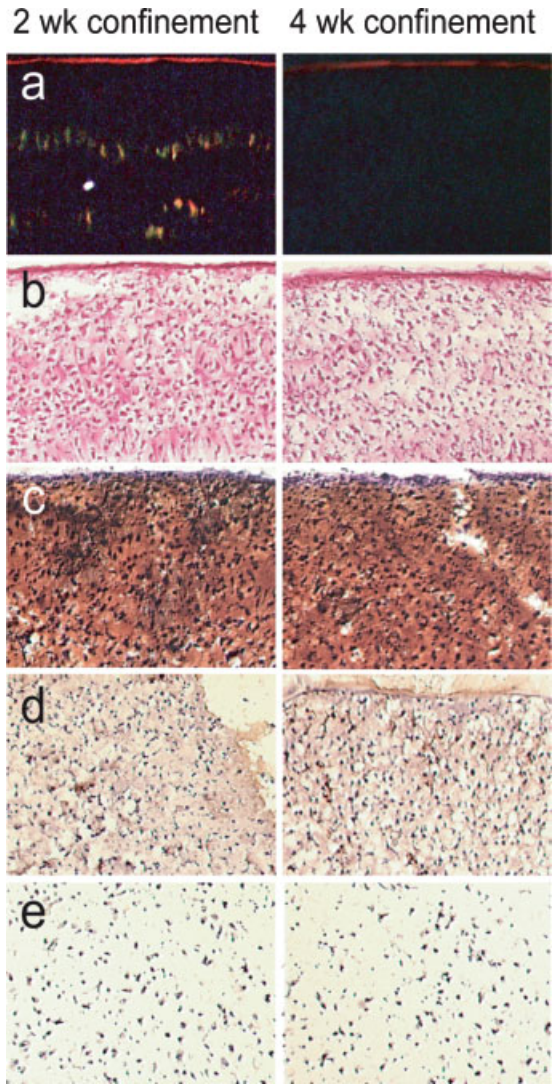

Figure 2. Histology of 2-week and 4-week confined constructs at 4 weeks. Original magnification, ×10. (a) Polarized light microscopy images with the construct surface at the top. Two-week confined group demonstrated organization of collagen fibrils perpendicular to the surface. (b) Picrosirius-red. (c) Safranin-O. (d) Collagen 2 IHC. (e) Collagen 1 IHC.

Polarized Light Microscopy

In the confinement study, constructs confined in 5-mm diameter wells for 2 weeks and then unconfined and cultured in agarose-coated wells for the remaining 2 weeks exhibited small-fiber collagen organization perpendicular to the construct surface and larger-fiber collagen organization in the direction parallel to the surface (Fig. 2a). The small collagen fiber alignment resembled struts. Small-fiber collagen organization was minimally observed in the other treatment groups (Fig. 2a), namely confinement in 5-mm diameter wells for 1, 3, or 4 weeks. The increased collagen organization was not observed in the passive axial compression study.

Quantitative Biochemistry

No significant differences were found in WW/construct, DNA/construct, GAG/WW, and collagen/WW among the 2-week confinement group and the 1, 3, or 4-week groups. The 2-week group had a WW of 39 ± 4 mg, while the 1-week, 3-week, and 4-week groups had a WW of 44 ± 3, 37 ± 4, and 41 ± 2 mg, respectively. The 2-week confinement group had a DNA/construct of 49 ± 2 μg, while the 1-week, 3-week, and 4-week groups had a DNA/construct of 44 ± 11, 46 ± 13, and 47 ± 5, respectively. The 2-week confinement group had a GAG/WW of 0.061 ± 0.009 mg/mg, while the 1-week, 3-week, and 4-week groups had a GAG/WW of 0.067 ± 0.014, 0.055 ± 0.003, and 0.050 ± 0.004 mg/mg, respectively. The 2-week confinement group had a collagen/WW of 0.039 ± 0.006 mg/mg, while the 1-week, 3-week, and 4-week groups had a collagen/WW of 0.032 ± 0.005, 0.041 ± 0.009, and 0.036 ± 0.008 mg/mg, respectively.

In the follow-up confinement study, there was no significant difference in WW/construct, DNA/construct, GAG/WW or collagen/WW between the 2-week confinement group and the group confined for 2 weeks in 5-mm diameter wells, 1 week in 6-mm diameter wells, and 1 week in 7-mm diameter wells. These groups had WW values of 16 ± 1 and 14 ± 2 mg, DNA/construct values of 31 ± 2 and 31 ± 4 μg, GAG/WW values of 0.074 ± 0.008 and 0.065 ± 0.006 mg/mg, and collagen/WW values of 0.071 ± 0.019 and 0.088 ± 0.011 mg/mg, respectively. The collagen/WW for the passive axial compression group at 0.067 ± 0.009 mg/mg was significantly higher than the unloaded control group, which had a collagen/WW of 0.044 ± 0.008 mg/mg. There was no significant difference in GAG/WW between the passive axial compression group and the control, with values of 0.070 ± 0.006 and 0.064 ± 0.007 mg/mg, respectively. Finally, the passive axial compression group had WW values of 20 ± 1 and 29 ± 3 mg, respectively; there was no difference in DNA/construct between the passive axial compression group and the control group, with values of 41 ± 7 and 40 ± 2 μg, respectively.

Mechanical Evaluation

The aggregate modulus of the 2-week confinement group reached 225 ± 32 kPa, and was significantly higher than the aggregate moduli of the 1, 3, or 4-week groups, with values of 120 ± 43, 126 ± 56, and 94 ± 52 kPa, respectively (Fig. 3). In the follow-up confinement study, the aggregate modulus of the 2-week group at 214 ± 110 kPa was insignificantly higher than that of the group confined for 2 weeks in 5-mm diameter wells, 1 week in 6-mm diameter wells, and 1 week in 7-mm diameter wells, at 177 ± 96 kPa.

The Young's modulus of the passive axial compression group at 1.4 ± 0.3 MPa was significantly higher than the tensile modulus of the control group at 1.0 ± 0.1 MPa (Fig. 4a). The ultimate tensile strength of the passive axial compression group at 339 ± 86 kPa was significantly higher than that of the control group at 200 ± 71 kPa (Fig. 4b). However, there was no significant difference between the aggregate modulus of the passive axial compression group and control group, with values of 101 ± 48 and 111 ± 52 kPa, respectively.

DISCUSSION

This study was designed to assess the effects of radial confinement and, separately, to determine the effects of passive axial compression-induced

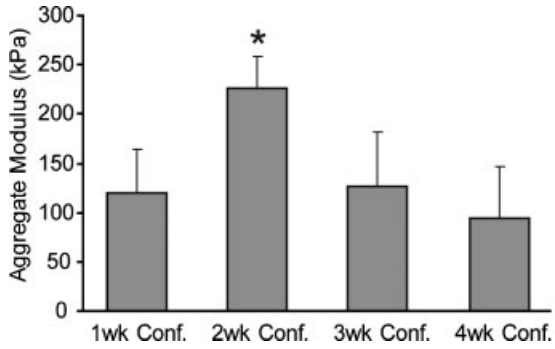

Figure 3. Mechanical properties of constructs in radial confinement study. Constructs confined for 2 weeks demonstrated significantly higher aggregate modulus than the other groups. Means and standard deviations.

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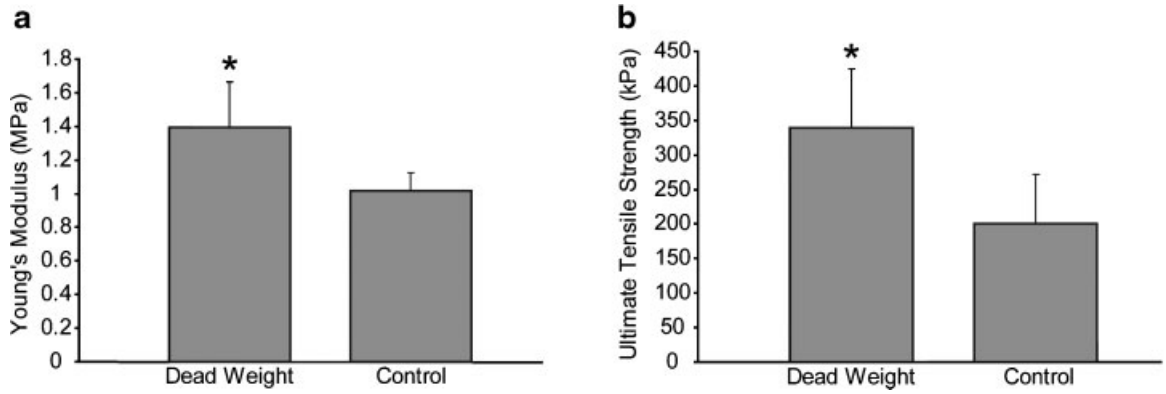

Figure 4. Mechanical properties of constructs in passive axial compression study. (a) Passive axial compression group exhibited significantly higher Young's modulus than control group. (b) Passive axial compression group exhibited significantly higher ultimate tensile strength than control group. Means and standard deviations.

vertical confinement, on the mechanical properties of 3-D self-assembled articular cartilage constructs over a 4-week culture period. Confining constructs for 2 weeks in 5-mm diameter agarose wells led to a significantly increased aggregate modulus. This increased compressive stiffness was accompanied by increased collagen organization without a change in GAG or collagen content. However, confinement in 5-mm diameter wells for 2 weeks, followed by confinement in 6-mm diameter wells for 1 week and confinement in 7-mm diameter wells for 1 week did not enhance the compressive properties of the constructs, and trended towards a decrease in aggregate modulus. The application of a 0.01 N dead weight to the constructs, corresponding to 0.5 kPa of stress, resulted in significant increases in tensile modulus and ultimate tensile strength, as well as total collagen per wet weight. These results support our hypothesis, as changes in mechanical properties were identified in a direction orthogonal to the confinement surface in tissue-engineered articular chondrocyte constructs. Additionally, this study demonstrates further refinement and characterization of the self-assembling process.

Radial confinement in 5-mm diameter agarose wells for 2 weeks led to about a twofold increase in aggregate modulus at 4 weeks, relative to confinement for 1, 3, or 4 weeks. There was no difference in extracellular matrix (ECM) content among the confinement groups; however, increased collagen organization in the direction parallel to that of the compression testing and orthogonal to the confinement surface was observed only in the group confined for 2 weeks. The organized collagen fibers appeared to form struts that may have helped increase compressive stiffness. These results were unexpected as the collagen of articular cartilage is typically responsible for the tensile stiffness, while the GAG content is responsible for the compressive stiffness. However, these results agree with those found in previous studies[32,33] that have demonstrated that total GAG and collagen content may not be an ideal indicator of mechanical properties; rather, ECM organization may play a significant role in predicting mechanical properties. A possible explanation is that the organization of the collagen fibers aids the proteoglycans in resisting compressive forces.

Confinement during the self-assembly process may lead to radial construct compression. At 10 days, the constructs reached the wall of the 5-mm diameter wells. At 2 weeks, constructs slightly <6-mm diameter were unconfined from the 5-mm diameter wells. Therefore, we hypothesized that the constructs confined for 2 weeks exhibited a higher aggregate modulus and increased collagen organization due to the effects of a low-magnitude radial compression and contact with the agarose well. This radial compression would neither be constant strain nor constant stress, since the constructs continued to grow radially while confined, thus resulting in potentially increasing strain and increasing stress with construct growth. This could account for the results in the other three groups, as the constructs confined for 1 week did not contact the walls of the well and therefore may not have been radially compressed. Additionally, at 3 weeks, constructs slightly <7-mm diameter were unconfined from the 5-mm diameter wells, and at 4 weeks, constructs approaching 7.5-mm diameter were unconfined from the 5-mm diameter wells; therefore, they may have experienced higher magnitudes of radial

compression, negating the positive effects of the lower-magnitude compression.

Since the aforementioned radial confinement-induced stress could not be quantified, the constructs could have been merely radially confined rather than radially compressed. Perhaps, confinement diminished the nutrient supply through the lateral surface, becoming more detrimental over periods longer than 2 weeks. However, the wells were constructed of agarose, with a 98% fluid phase to allow for adequate nutrient diffusion to the edges. Additionally, confinement did not affect cellularity, as there was no difference in histological images and DNA/construct between the 2-week confinement group and the other groups.

Ten to 14 days was the most beneficial time for constructs to be confined by the agarose well. To maintain radial confinement similar to that experienced by the 2-week confinement group during 10–14 days for a longer period, constructs were confined in incrementally larger agarose wells to mimic the radial growth of the constructs with time and allow constructs to contact the edges from 1.5 to 4 weeks. Constructs confined only for 2 weeks in 5-mm diameter wells and then unconfined were used as controls. Maintaining confinement for 4 weeks caused the aggregate modulus to trend lower than the 2-week confined control, from 214 ± 110 to 177 ± 96 kPa. These results demonstrate that application of radial confinement between 1 and 2 weeks was more beneficial than maintaining confinement longer, which may actually be detrimental. However, we were unable to apply confinement before approximately 1.5 weeks, so applying radial confinement at even earlier timepoints may be more beneficial.

The application of a passive axial stress from 10 to 14 days increased the tensile properties of the constructs. To eliminate the effects of radial confinement, the control constructs were placed in incrementally larger agarose wells. At 4 weeks, the passive axial compression group demonstrated a 1.4-fold increase in Young's modulus and a 1.7-fold increase in ultimate tensile strength relative to the control group, confirming our hypothesis that confinement of self-assembled constructs affects mechanical properties orthogonal to the confinement surface. The increased tensile strength was accompanied by a significantly higher collagen/WW, with minimal small-fiber collagen organization for either group. Vertical confinement led to different changes in the construct ECM than found in radial confinement, suggesting that different mechanotransduction pathways may exist for radial confinement and passive axial compression; future studies should be performed to elucidate these potential differences. Finally, application of a dead weight did not affect cellularity, as there was no difference in histological images or DNA/construct between the passive axial compression group and the control group.

To our knowledge, this study provides the first evidence of the benefits of confinement on mechanical properties in the direction orthogonal to the confinement surface. It shows that an increased construct aggregate modulus can be accounted for by increased collagen organization in the direction orthogonal to the construct surface. Previous studies also demonstrated a relationship between cartilage mechanical properties and collagen organization. For example, Kiviranta et al.[34] found a significant correlation between Poisson's ratio and collagen organization in bovine knee osteochondral plugs. Kelly et al.[33] found that dynamic deformational loading of chondrocyte-seeded agarose hydrogels led to an increased bulk Young's modulus with increased collagen organization in the radial direction relative to the free-swelling control.

Several prior studies investigated the use of dynamic compression[9,12,13] and/or shear[10,13] on the ECM of tissue-engineered cartilage constructs. These studies demonstrated 1.5–2.8-fold GAG increases, and 1.4-fold collagen increases with mechanical stimulation, which differ from our confinement results, which demonstrated no change in ECM content, and the passive axial compression results, which demonstrated a 1.5-fold increase in collagen without a GAG increase. Perhaps simultaneous GAG and collagen increases only occur under dynamic stimulation as a result of the increased nutrient diffusion.[33] However, Waldman et al.[12] found that dynamic compression of 5% at 1 Hz, for 400 cycles every other day for 1 week, resulted in increased collagen with no change in GAG content, matching our passive axial compression results.

Many studies examined the relationship between mechanical stimulation and construct properties, but to our knowledge, this is the first tissue engineering study to indicate the benefit of short term passive confinement. Confinement either results in increased collagen organization as seen in the radial confinement experiment, or increased matrix synthesis as seen in the passive axial compression experiment. Although using immature bovine cartilage explants, Boustany et al.[35] also found that static compression of $<25\%$ strain for 60 h increased the biosynthetic rate of

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GAG and collagen production, although the mechanical properties were not examined.

Additional studies should be performed to track construct development using electron microscopy to elucidate the mechanism leading to strut-like collagen organization. Also, future studies should investigate combining confinement with other mechanical stimulation modalities to determine synergistic effects. Finally, future work should examine effects of adding growth factors to the culture medium, before and after the application of confinement, to determine synergistic effects.

ACKNOWLEDGMENTS

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