Mechanical Properties and Structure-Function Relationships of Human Chondrocyte-Seeded Cartilage Constructs After In Vitro Culture

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ABSTRACT: Autologous Chondrocyte Implantation (ACI) is a widely recognized method for the repair of focal cartilage defects. Despite the accepted use, problems with this technique still exist, including graft hypertrophy, damage to surrounding tissue by sutures, uneven cell distribution, and delamination. Modified ACI techniques overcome these challenges by seeding autologous chondrocytes onto a 3D scaffold and securing the graft into the defect. Many studies on these tissue engineered grafts have identified the compressive properties, but few have examined frictional and shear properties as suggested by FDA guidance. This study is the first to perform three mechanical tests (compressive, frictional, and shear) on human tissue engineered cartilage. The objective was to understand the complex mechanical behavior, function, and changes that occur with time in these constructs grown in vitro using compression, friction, and shear tests. Safranin-O histology and a DMMB assay both revealed increased sulfated glycosaminoglycan (sGAG) content in the scaffolds with increased maturity. Similarly, immunohistochemistry revealed increased lubricin localization on the construct surface. Confined compression and friction tests both revealed improved properties with increased construct maturity. Compressive properties correlated with the sGAG content, while improved friction coefficients were attributed to increased lubricin localization on the construct surfaces. In contrast, shear properties did not improve with increased culture time. This study suggests the various mechanical and biological properties of tissue engineered cartilage improve at different rates, indicating thorough mechanical evaluation of tissue engineered cartilage is critical to understanding the performance of repaired cartilage. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

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In the last 20 years, a variety of tissue engineered strategies have shown promise in repair of focal cartilage defects.[1] Autologous chondrocyte implantation (ACI) is a widely recognized method for articular cartilage repair as evidenced by the vast number of scientific contributions in the fields of experimental and clinical research.[1,2] ACI delivers autologous chondrocytes into a defect and secures them under a periosteal flap. This technique has shown, through histology and integration measures, the potential to repair full-thickness cartilage defects.[3] Despite this promise, problems persist with graft hypertrophy, damage to surrounding tissue by sutures, uneven cell distribution, and delamination.

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The use of modified ACI techniques aims to overcome these technical disadvantages.[3] Modified ACI techniques create tissue engineered grafts by seeding autologous chondrocytes onto a 3D scaffold and securing the graft into the defect. Such techniques have been shown to promote integration between the graft and the surrounding tissue and allow for more even distribution of cells.[4] Many studies on these tissue engineered grafts have identified compressive tissue properties, to augment data on biological markers such as sGAG and collagen content. However, other metrics of mechanical performance, such as frictional and shear properties, have not been studied in detail. These metrics have been identified by the FDA as critical for assessing implant performance.[5] Additionally, low frictional properties and the appropriate shear properties provide load support and reduce chondrocyte apoptosis, protecting the joint from damage and wear.[6,7] Despite the importance of frictional and shear properties to cartilage function, only six studies on tissue engineered cartilage have identified shear properties, while even fewer (two studies) have identified frictional properties, of articular cartilage implants.[8–14]

The current study was motivated by the lack of data on the mechanical properties of human tissue engineered cartilage constructs. Although the frictional properties of tissue engineered cartilage constructs with human MSC has been documented,[15] until now, no published papers have documented the frictional and shear properties of cartilage tissue engineered from human articular chondrocytes. Similarly, no human

tissue engineered cartilage studies have examined all three mechanical properties examined in this paper (compressive, friction, and shear).

We studied a tissue engineered cartilage implant made using the same cell source, scaffold and hypoxic culture conditions used to produce NeoCart®, which is currently in phase III clinical trials.[16,17] In human trials, these implants have shown good integration, good defect filling, progressive maturation of collagen, and organized collagen formation. Therefore, the objective of this study was to understand the complex mechanical behavior, function, and changes that occur in human chondrocyte seeded collagen constructs during in vitro culture using multiple mechanical tests, which measure the compressive, frictional, and shear properties of the constructs.

METHODS

Tissue Construct Preparation

Constructs were prepared using a modified established technique.[16,17] Briefly, cadaveric normal human articular cartilage tissue of a 28-year-old male was obtained under protocol from National Disease Research Interchange (NDRI, Philadelphia, Pa), then processed by enzymatic digestion with collagenase (Worthington Biochemical, Lakewood, NJ) to yield chondrocytes. Chondrocytes were isolated, expanded in DMEM/F12 culture medium containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA) through passage 1 at 37°C under 5% CO_2, seeded using 3 mg/ml type I collagen solution (PureCol, Advanced Biomatrix, San Diego, CA), at a concentration of 5×10^6 cells/ml, and pipetted onto 6 mm diameter by 1.5 mm thick type 1 collagen honeycomb scaffolds (Koken, Tokyo, Japan). Both the scaffold and the collagen solution were produced from extracts of bovine dermis. Day 0 constructs consisted of the honeycomb scaffold infused with 3 mg/ml type I collagen solution without cells and incubated in culture medium overnight at 37°C. Cell seeded constructs at the remaining time points (1, 3, 5, and 7 weeks) were incubated under low oxygen (<5%) conditions at 37°C and 5% CO_2 in static culture with media changes at regular intervals. Constructs were removed from culture at multiple stages of development (1, 3, 5, and 7 weeks post seeding) and frozen at −20°C. A total of 40 constructs were allocated for each mechanical test described below, with eight constructs tested at each time point (0, 1, 3, 5, and 7 weeks). Additionally, human articular cartilage from cadavers was examined using each of the following methods to provide mature tissue reference data.

Histology

Constructs were prepared for histology according to standard procedure in that they were fixed in 10% neutral buffered formalin for 24–48 h, embedded into paraffin blocks, and sectioned at a thickness of 10 μm. Slide mounted constructs were cleared using xylene and a series of progressively stronger ethanol baths. To detect the presence of sulfated glycosaminoglycan (sGAG), sections were stained using Safranin-O/Fast Green.

Immunohistochemistry

Immunohistochemistry was conducted in a similar manner to methods previously described.[18] Slide mounted 5 μm sections were stained for localized lubricin using a modified Vectastain ABC kit protocol (Vector Laboratories, Burlingame, CA). Slides were cleared using xylene and dehydrated in ethanol, then incubated for 20 min in a citrate antigen retrieval buffer (10 mM citric acid, pH 6.0) at 90°C. Slides were washed twice for 5 min in TRIS buffered saline with 0.5% polysorbate 20 and incubated for 30 min in 3% hydrogen peroxide. A blocking solution containing normal serum, bovine serum albumin, triton X-100, and TWEEN-20 was applied for 60 min. The primary antibody (ab28484, Vectastain ABC) was applied overnight in a humidity chamber. A secondary antibody was applied for 30 min. Staining was then carried out by a peroxidase substrate (ImmPACT DAB, Vector) for up to 10 min.

Biochemistry

After confined compression testing, constructs were analyzed for biochemical content. Constructs were lyophilized and weighed to obtain construct dry weight. Then, constructs were papain digested at 60°C for 14 h. sGAG content was measured using a Dimethylmethylene Blue (DMMB) assay.[19]

Confined Compression

Constructs were tested in confined compression to determine the equilibrium modulus as previously described.[20,21] Briefly, constructs were thawed in a bath of phosphate buffered saline (PBS) with protease inhibitors. Constructs were cut into 4 mm circles using a dermal punch and placed in a 4 mm diameter confining chamber, covered with a porous plug and PBS with protease inhibitors, and mounted in a Bose EnduraTEC ELF 3200 (Eden Prairie, MN) for stress relaxation testing. Prior to testing, construct heights were measured with a caliper. A series of 5% steps in strain were imposed on each construct up to a maximum of 40% strain. After each step, the resultant load (and hence stress) was measured for 20 min. The stress relaxation curves were fit to a poroelastic model of material behavior to calculate equilibrium modulus (HA) and hydraulic permeability (k) using a custom MATLAB code.

Friction Testing

Friction testing was performed on a previously described, custom tribometer.[20,22] Cartilage constructs were thawed in a bath of PBS with protease inhibitors at 37°C for 10 min. Constructs were then mated against a flat polished glass counterface while bathed in PBS with protease inhibitors. Before friction testing, constructs were compressed to 45% axial strain and allowed to depressurize over the course of 40 min resulting in average normal loads of 80 g. After fluid pressure dropped to ambient pressure, the glass counterface was reciprocated at 0.1 mm/s. This combination of normal load and sliding speed has been shown to produce boundary mode lubrication.[20] These studies measured boundary friction coefficient in the absence of interstitial fluid pressurization. Although interstitial fluid pressurization is important throughout the gait cycle,[23] we chose to measure boundary friction coefficient because it represents the potential upper bound on friction and hence may be an important predictor of graft damage. Friction coefficients were recorded as the ratio of shear load to the normal load at the end of sliding, when friction had reached an equilibrium value, and averaged for both the forward and reverse sliding directions.

After measuring the friction coefficient, constructs were placed in a ADE Phase Shift MicroXAM Optical interferometric profilometer to measure surface roughness. The profilometer

uses non-contact white light profilometry to measure the 3D surface roughness of constructs.

Shear Confocal Strain Mapping

The shear modulus of constructs was obtained using previously established protocols.[24–26] Briefly, constructs were bisected longitudinally into hemi-cylinders then exposed to 14 μg/ml 5-dichlorotriazinyl-aminofluorescein (5-DTAF) (Molecular Probes1, Grand Island, NY) for 30 min followed by a 20 min rinse in PBS with protease inhibitors. Constructs were mounted between two plates on a tissue deformation imaging stage (TDIS) and placed on an inverted Zeiss LSM 510 5 live confocal microscope, then imaged using a 488 nm laser. Before shear testing, constructs were compressed to a 10% axial strain and allowed to undergo stress relaxation for 40 min. After stabilization, constructs were subject to a 1% oscillatory shear strain at a frequency of 1 Hz. The stress was calculated by measuring the force on the back plate of the TDIS and measured construct cross-sectional area. The shear strain on the system was measured by subtracting the transverse displacement of the back plate from the front plate and dividing by the construct depth. The shear modulus of the construct was then calculated by dividing the measured stress amplitude over the measured strain amplitude.

Statistical Analysis

The Kolmogorov–Smirnov goodness-of-fit hypothesis test was used to determine the distribution of constructs. Since data for time points was non-parametric, a two-way ANOVA based on ranks and a post-hoc analysis for pairwise comparison was performed using Dunn-Sidak difference criterion to determine statistical significance ($p < 0.05$). Each time point was then compared to the human cartilage control using a two sided Wilcoxon rank sum test. All statistical analyzes were carried out in MATLAB and expressed as a mean ± SD. To determine the relationship between composition and mechanical properties, the presence of sGAG on HA and k was determined by linear regression. Regressions were considered significant for $p < 0.05$.

RESULTS

Histology and Immunohistochemistry

Slide-mounted constructs stained with Safranin-O revealed a progressive increase in the proteoglycan content on the surface of the collagen scaffold with time. Signs of proteoglycans lining the inside of the collagen scaffold began after 1 week in culture and increased with increased culture time (Fig. 1A). By 7 weeks there was considerable proteoglycan deposition on the outside of the construct with a small amount of proteoglycan within the scaffold pores.

Similarly, constructs stained with lubricin antibodies revealed increased localization of lubricin coinciding with sGAG content on the construct surface and lining of the scaffold pores. After 1 week in culture constructs showed little lubricin on the surface. After 7 weeks, staining revealed increased lubricin near the construct surface and lining of the collagen scaffold (Fig. 1B). High magnification images revealed that cells on the construct surfaces were positive for lubricin at 1 and 3 weeks, but there was little lubricin present in the scaffold. Cells remained positive for lubricin at 5 and 7 weeks with considerable ECM staining at 7 weeks.

sGAG

A DMMB assay revealed sGAG content continuously increased from 2.5 ± 1.1 μg sGAG per mg construct dry weight at 1 week, to 212.3 ± 58.8 μg/mg at 7 weeks ($p < 0.001$). Constructs grown for 7 weeks in culture reached values not significantly different from human cartilage (285.0 ± 122.6 μg/mg, $p = 0.38$, Fig. 2A).

Confined Compression

Confined compression tests revealed a significant difference between the aggregate modulus at 0 week (0.10 ± 0.04 MPa) to 7 weeks (0.30 ± 0.14 MPa, $p < 0.05$, Fig. 3A). After 7 weeks in culture, the aggregate modulus reached values approximately 50% of human cartilage (0.59 ± 0.18 MPa) with constructs incubated for 7 weeks remaining significantly different than human cartilage ($p < 0.05$). The hydraulic permeability of engineered constructs decreased from 0 weeks ($55 \times 10^{-15} \pm 12 \times 10^{-15}$ m^2/Pa-s) to 7 weeks in culture ($23 \times 10^{-15} \pm 7.1 \times 10^{-15}$ m^2/Pa-s, $p < 0.05$, Fig. 3B). After 7 weeks in culture, the construct hydraulic permeability was significantly different than human cartilage ($p < 0.05$) with construct values approximately 51% more than human cartilage ($12 \times 10^{-15} \pm 2.5 \times 10^{-15}$ m^2/Pa-s).

Coefficient of Friction

A custom built cartilage on glass tribometer revealed a decrease in friction coefficient from 0 week constructs (0.40 ± 0.04) to 7 week constructs (0.24 ± 0.093, $p < 0.005$, Fig. 4A). After 3 weeks of culture, friction coefficients stabilized to values not significantly different than human cartilage (0.22 ± 0.016, $p = 0.38$). A white light profilometer showed surface roughness values continuously decreasing throughout culture from 122 ± 8.77 μm at 0 weeks to 3.41 ± 1.02 μm at 7 weeks ($p < 0.001$). Surface roughness of constructs grown for 7 weeks in culture were not significantly different than human cartilage (2.27 ± 1.00 μm, $p < 0.05$, Fig. 4B and C). Friction coefficients were positively correlated with construct roughness ($R^2 = 0.85$, $p = 0.014$, Fig. 4D).

Confocal Strain Mapping

Confocal imaging of the constructs under oscillatory shear strain revealed no significant difference in the shear modulus of engineered constructs between 0 weeks (0.06 ± 0.07 MPa) and 7 weeks (0.15 ± 0.10 MPa, $p = 0.44$, Fig. 5). The shear modulus measured at all time points remained 4–16 times less than human cartilage (0.95 ± 0.36 MPa, $p < 0.05$).

DISCUSSION

The goal of this study was to characterize changes in the compressive, frictional, and shear mechanical

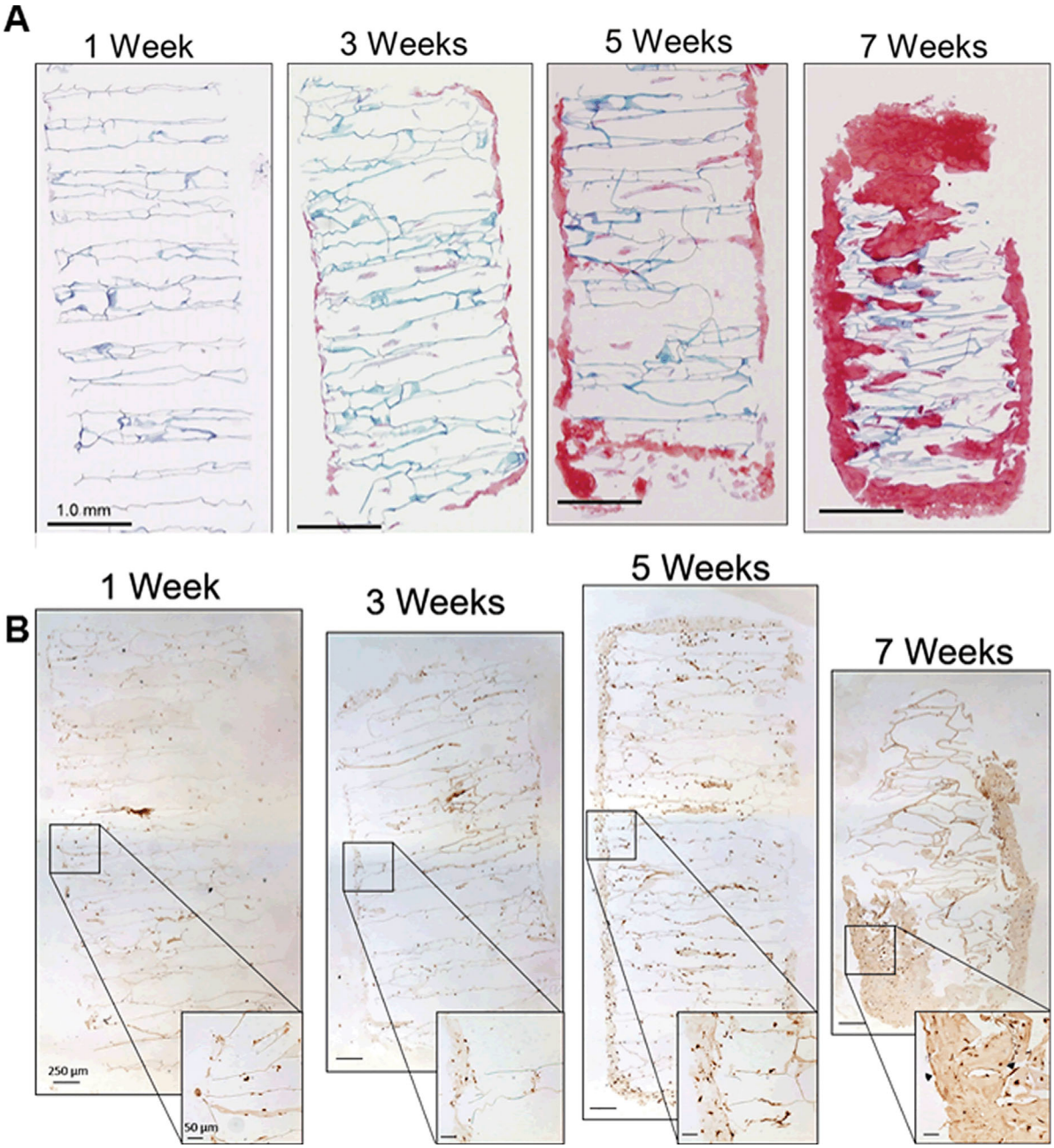

Figure 1. (A) Safranin-O staining revealed increased sGAG deposition on the construct surface and collagen fibers with increased culture time. (B) Lubricin staining of constructs revealed little to no lubricin on the construct surface after 1 week in culture. The amount of lubricin staining on the surface of constructs and lining the inside of constructs increases over the 7 weeks of culture.

properties of human chondrocyte seeded collagen constructs during in vitro culture. In this study, we showed improvement in the compressive and frictional properties of the constructs but no change in the shear properties. While a few studies have characterized the frictional and shear properties of tissue engineered articular cartilage constructs cultured with bovine or porcine cells,[8–10,27,28] this study is the first to perform such analysis on human chondrocyte-seeded implants. Additionally, only a few previous studies have characterized the compressive properties of human chondrocyte seeded implants.[29–32] Overall, the results of this study provide a deeper understanding of the spectrum of mechanical behavior exhibited by tissue engineered human articular cartilage. Specifically, we found that while all mechanical properties of human tissue engineered cartilage constructs nominally improved with time, frictional properties approached native values by 3 weeks and compressive properties by 7 weeks, while shear properties remained constant and did not reach values similar to native tissue over this time frame. This pattern of differential recovery of mechanical properties has been noted previously in animal studies.[27] Specifically, a previous 1 year in vivo

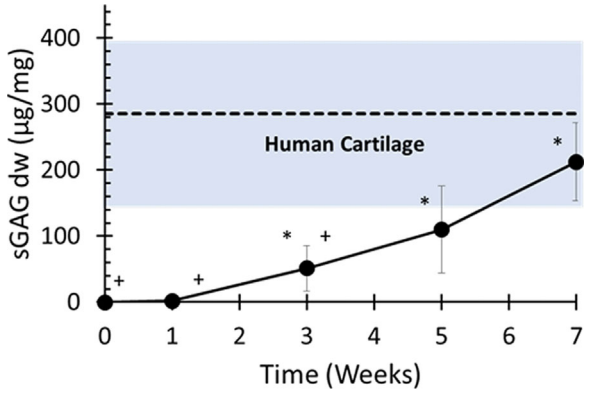

Figure 2. Changes in the biochemical composition of the constructs with increased culture time. sGAG content, normalized to dry weight, increased continuously during the 7 weeks of culture time. After 7 weeks in culture, construct sGAG content was not significantly different than human cartilage. ($^{*}p < 0.05$ vs. 0 day constructs, $+p < 0.05$ vs. human cartilage, $N = 3$ for human cartilage).

horse study that implanted a matrix induced autologous chondrocyte implant (MACI®), a collagen scaffold seeded with chondrocytes, found that frictional properties reached values similar to native tissue, compressive properties reach 70% of native tissue, and shear properties remained 4–10 times less than native tissue.

The compressive properties of our constructs follow similar temporal patterns to previous studies on tissue engineered cartilage. Tissue engineered studies using human articular chondrocytes grown in vitro produced compressive values about 50% of native tissue.[30,31] In contrast, previous studies using animal derived chondrocytes (porcine, bovine, and canine) have shown compressive properties that range from 14 to 90% of native cartilage.[8,33–36] The incorporation of growth factors and mechanical stimulation is known to enhance cartilage production and contributes to the improved compressive properties seen in some of these studies.[37,38] Without dynamic culture conditions, our construct compressive properties reached values (50% of native tissue) similar to previous studies on human chondrocyte seeded constructs and some animal chondrocyte seeded constructs. These results provide evidence that additional stimulation such as growth factors or mechanical loading may be necessary to improve the compressive properties.

Since the optimal goal of tissue engineered cartilage is to reproduce mechanical function in vivo, previous in vivo animal studies provide a benchmark for functional compressive properties. The use of both ACI and MACI techniques in vivo have reported compressive properties at or below the properties of native tissue. In an 8 month equine model, the ACI technique reported an aggregate modulus 12% of native tissue.[39] Stiffness tests in ovine models with a MACI type II collagen membrane for 1 year resulted in values 37–50% of native tissue.[40] The same implant grown in an equine model reported compression values ranging between 40 and 70% of native tissue at 8 months and 1 year, respectively.[27,41] Variability between in vivo animal models occur due to the type of animal and the repair technique. In contrast, after only 7 weeks of in vitro culture, our human constructs provide comparable or better results than multiple animal models grown for longer durations, thus providing similar or better load support.

Many studies have shown a strong correlation between the sGAG content and the compressive properties of native cartilage, but the influence of sGAG on the compressive properties of tissue engineered cartilage is less understood.[42,43] A few previous studies have identified a correlation between the sGAG content and the aggregate modulus of tissue engineered cartilage.[44,45] This study also revealed a correlation between the sGAG content and the aggregate modulus ($R^2 = 0.67$, $p = 0.046$, Fig. 6A) in addition to the sGAG content and hydraulic permeability ($R^2 = 0.64$, $p = 0.05$ Fig. 6B). Therefore, changes in the sGAG content of these tissue engineered constructs greatly influences the compressive properties.

Although sGAG content and compressive properties are correlated, the non-heterogeneous deposition of sGAG in the construct may be preventing the compressive properties from reaching similar values to native tissue. Chondrocytes on the outer surface of the scaffold tended to produce large amounts of sGAG while deposition within the scaffold was sparse.

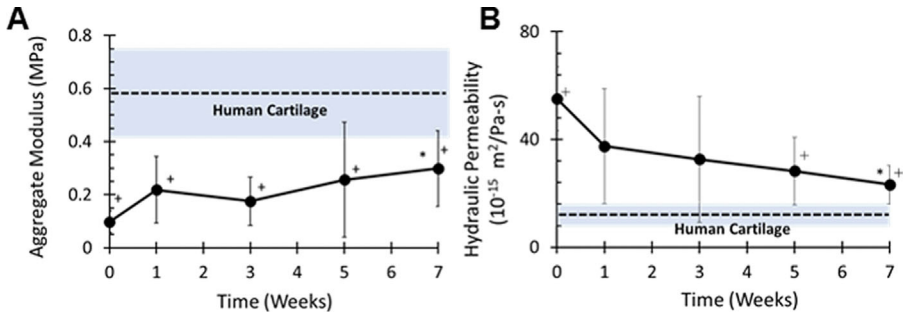

Figure 3. Changes in the confined compression tests. (A) The aggregate modulus increased from 0 weeks to 7 weeks in culture and reached values not statistically different than human cartilage at 7 weeks in culture. (B) The hydraulic permeability of constructs decreased from 0 to 7 weeks approaching human cartilage values ($^{*}p < 0.05$ vs. 0 week constructs, $+p < 0.05$ vs. human cartilage, $N = 8$ for all construct time points, $N = 3$ for human cartilage).

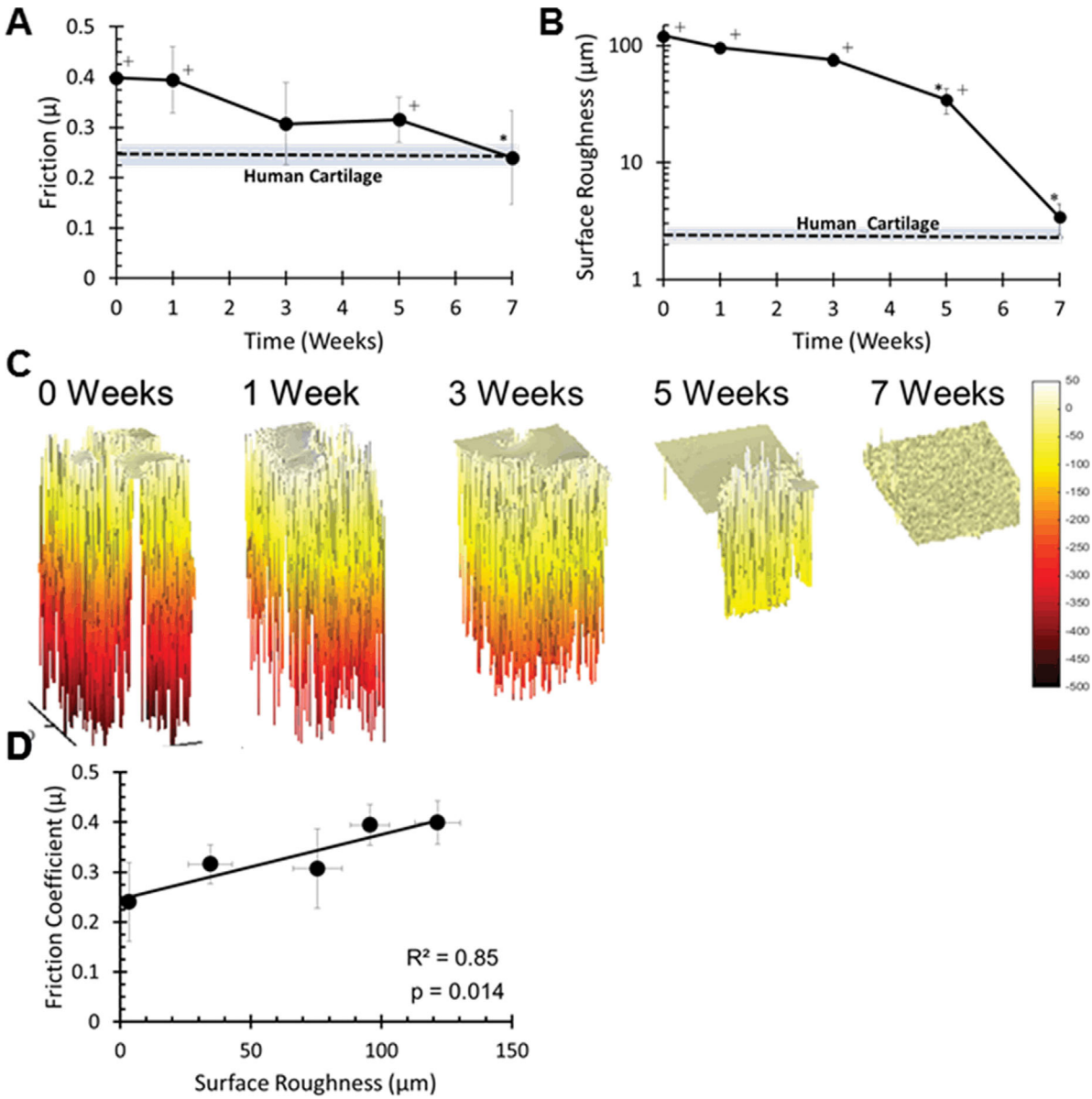

Figure 4. Friction and surface roughness of constructs over time. (A) The boundary mode friction coefficient of constructs decreased with culture time. (B) The surface roughness decreased continuously from 0 to 7 weeks. After 7 weeks in culture construct surface roughness reached values not statistically different than human cartilage. ($^{*}p < 0.05$ vs. 0 week, $+p < 0.05$ vs. human cartilage, $N = 7$–8 for all construct time points, $N = 3$ for human cartilage). (C) Representative images of the surface roughness at each time point. (D) The coefficient of friction correlates with the surface roughness of constructs ($R^2 = 0.85$, $p = 0.014$).

Lubricin staining revealed cells scattered throughout the scaffold at all time points. As such, the sGAG production and proliferation on the construct surface may be caused by the greater exposure of nutrients from the culture medium. This result has been seen in a previous porcine chondrocyte seeded collagen scaffold grown with growth factors.[36] In this porcine study histology images revealed early sGAG production was concentrated on the scaffold surface. More uniform scaffold filling occurred after 42 days of growth. In fact, more uniform defect filling has been seen after 3 months in in vivo human studies using the same scaffold and cell source as this study.[17] Since the cells are dispensed with type I collagen into the scaffold, it is possible that the sGAG and type II collagen are being produced as the type I collagen within the scaffold is slowly being degraded. The progressive ECM fill within the scaffold over time suggests that as the collagen type I is degraded access to nutrients inside the scaffold increases.

Unlike the compressive properties, which correlate with sGAG content, the friction coefficient of tissue engineered constructs is influenced by the lubricin concentration on the cartilage surface. Increased lubricin concentration on the cartilage surface is known to reduce the friction coefficient of native tissue and engineered constructs.[20,46,47] The decreased surface roughness of 7 week constructs created a smooth

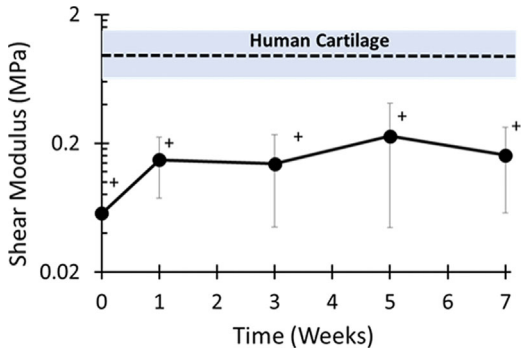

Figure 5. Shear testing of constructs reveal no significant increase in the shear modulus of constructs between 0 and 7 weeks in culture. Construct shear modulus stayed 4–16 times lower than human cartilage at all culture time points ($+p < 0.05$ vs. human cartilage, $N = 8$ for all construct time points, $N = 3$ for human cartilage).

construct surface allowing lubricin to localize to the surface. Lubricin is a boundary mode lubricant that has been shown to reduce the coefficient of friction of articular cartilage.[20] After 7 weeks in culture, these constructs provided frictional properties not statistically different than native tissue. Such data is consistent with previous tissue engineered cartilage studies, indicating frictional properties can improve to values similar to native tissue when lubricin is localized on the construct surface.[8,28]

In contrast to the compressive properties, which improve with increased sGAG content, and frictional properties, which improve with increased lubricin localization, the shear modulus did not significantly change and remained 4–16 times less than native cartilage. Similarly, previous in vitro and in vivo studies using bovine and equine chondrocytes have reported shear modulus values that remain 10 times lower than native tissue.[9,10,27] These studies attributed the improved shear modulus values to both the collagen content and organization of the engineered tissue. Additionally, the collagen content has been shown to be linearly correlated with the shear properties.[14] We observed no significant change in the shear properties of our constructs. Therefore, the total collagen content is assumed to be similar to the initial collagen content. The collagen type I matrix was expected to degrade at the same time as chondrocytes are depositing type II collagen, thus the change in collagen content is likely low for this short 7 week culture time. Unlike compressive and frictional properties, obtaining shear modulus values similar to native tissue remains a persistent challenge for the field.

There are several limitations that must be considered when interpreting these data. The collagen content of constructs was not measured because the collagen scaffold contributes a majority of the construct architecture. Any changes in the collagen content with increased growth of the constructs would be minimal in comparison to the overall amount of collagen in the scaffold. Similarly, our constructs did not undergo mechanical stimulation, which has been shown to improve collagen fiber size and organization. This collagen growth and organization leads to improved shear tissue properties.[13,48] Finally, we know these constructs undergo heterogeneous changes throughout culture due to cell matrix deposition. These changes occur on the microscale level and our bulk strain measurements might not be sensitive enough to detect these changes.

In this study, multiple mechanical properties of human chondrocyte seeded collagen constructs were extensively characterized. The mechanical properties chosen (compressive, friction, and shear) are in accordance with FDA guidance. In addition, temporal changes in the structure-function relationship of these constructs were examined. It was observed that the compressive and frictional properties of these constructs improved with increased maturation time, while the shear properties did not change significantly. Increased sGAG content was found to correlate with increased compressive properties, but no correlation between sGAG and shear properties was found. The mechanical results from this study give us insight into

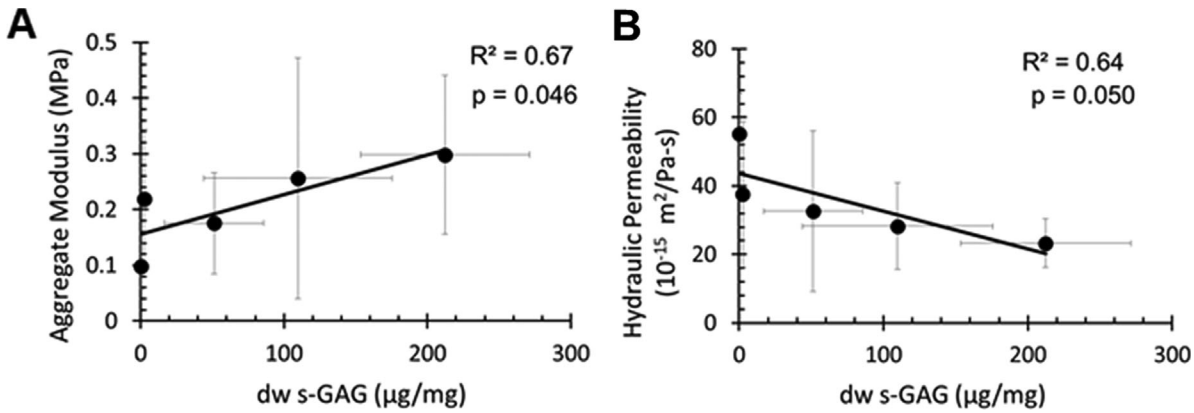

Figure 6. Structure function relationship between the mechanical properties and the sGAG content of constructs. (A) A positive correlation between the sGAG content and the aggregate modulus occurs ($R^2 = 0.67$, $p = 0.092$) (B) A negative correlation between the sGAG content and the hydraulic permeability occurs ($R^2 = 0.64$, $p = 0.10$).

the clinical success of similar tissue engineered products such as NeoCart.[16] As such, thorough mechanical evaluation of tissue engineered cartilage is critical to understanding the performance of repaired cartilage.

AUTHORS' CONTRIBUTIONS

All Authors Contributed extensively to the work presented in this paper. J.M.—friction, shear, immunohistochemistry, data analysis, and wrote manuscript. D.J.—Confined compression and data analysis. S.K., S.S., C.D., and J.S.—histology, construct development, and results discussion. I.C. discussed the results and implications and commented on the manuscript. L.B. supervised all studies of mechanical evaluation, participated in planning of all experiments, interpretation of data, and writing and editing of the manuscript.

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REFERENCES

1. Hunziker EB, Lippuner K, Keel MJB, et al. 2015. An educational review of cartilage repair: precepts & practice—myths & misconceptions—progress & prospects. Osteoarthr Cartil 23: 334–350.
2. Tuan RS. 2007. A second-generation autologous chondrocyte implantation approach to the treatment of focal articular cartilage defects. Arthritis Res Ther 9:109.
3. Brittberg M. 2010. Cell carriers as the next generation of cell therapy for cartilage repair: a review of the matrix-induced autologous chondrocyte implantation procedure. Am J Sports Med 38:1259–1271.
4. Willers C, Chen J, Wood D, et al. 2005. Autologous chondrocyte implantation with collagen bioscaffold for the treatment of osteochondral defects in rabbits. Tissue Eng 11: 1065–1076.
5. U.S. Food and Drug Administration. 2011. Guidance for Industry: Preparation of IDEs and INDs for Products Intented to Repair or Replace Knee Cartilage [Internet]. 45 p. Available from: http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.
6. Waller KA, Zhang LX, Elsaid KA, et al. 2013. Role of lubricin and boundary lubrication in the prevention of chondrocyte apoptosis. Proc Natl Acad Sci USA 110: 5852–5857.
7. Jay GD, Torres JR, Rhee DK, et al. 2007. Association between friction and wear in diarthrodial joints lacking lubricin. Arthritis Rheum 56:3662–3669.
8. Peng G, McNary SM, Athanasiou KA, et al. 2014. Surface zone articular chondrocytes modulate the bulk and surface mechanical properties of the tissue engineered cartilage. Tissue Eng Part A 20:3332–3341.
9. Ng KW, Kugler LE, Doty SB, et al. 2009. Scaffold degradation elevates the collagen content and dynamic compressive modulus in engineered articular cartilage. Osteoarthr Cartil 17:220–227.
10. Wan LQ, Jiang J, Miller DE, et al. 2011. Matrix deposition modulates the viscoelastic shear properties of hydrogel-based cartilage grafts. Tissue Eng Part A 17:1111–1122.
11. Erickson IE, Huang AH, Sengupta S, et al. 2009. Macromer density influences mesenchymal stem cell chondrogenesis and maturation in photocrosslinked hyaluronic acid hydrogels. Osteoarthr Cartil 17:1639–1648.
12. Lima EG, Bian L, Ng KW, et al. 2007. The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-β3. Osteoarthr Cartil 15: 1025–1033.
13. Stading M, Langer R. 1999. Mechanical shear properties of cell-polymer cartilage constructs. Tisssue Eng 5:241–250.
14. Byers BA, Mauck RL, Change IE, et al. 2008. Transient exposure to TGF-B3 under serum-free conditions enhances the biomechanical and biochemical maturation of tissue engineered cartilage. Tissue Eng Part A 14:1821–1834.
15. Bhumiratana S, Eton RE, Oungoulian SR, et al. 2014. Large, stratified, and mechanically functional human cartilage grown in vitro by mesenchymal condensation. Proc Natl Acad Sci USA 111:6940–6945.
16. Crawford DC, DeBerardino TM, Williams RJ. 2012. NeoCart, an autologous cartilage tissue implant, compared with microfracture for treatment of distal femoral cartilage lesions: an FDA phase-II prospective, randomized clinical trial after two years. J Bone Joint Surg Am 94:979–989.
17. Crawford DC, Heveran CM, Cannon WD, et al. 2009. An autologous cartilage tissue implant NeoCart for treatment of grade III chondral injury to the distal femur: prospective clinical safety trial at 2 years. Am J Sports Med 37: 1334–1343.
18. Puetzer JL, Brown B, Ballyns J, et al. 2013. The effect of IGF-I on anatomically shaped tissue-engineered menisci. Tissue Eng Part A 19:1443–1450.
19. Enobakhare BO, Bader DL, Lee DA. 1996. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. Anal Biochem 243:189–191.
20. Gleghorn JP, Jones ARC, Flannery CR, et al. 2007. Boundary mode frictional properties of engineered cartilaginous tissues. Eur Cells Mater 14:20–28.
21. Chang SCN, Rowley JA, Tobias G, et al. 2001. Injection molding of chondrocyte/alginate constructs in the shape of facial implants. J Biomed Mater Res 55:503–511.
22. Gleghorn JP, Bonassar LJ. 2008. Lubrication mode analysis of articular cartilage using Stribeck surfaces. J Biomech 41:1910–1918.
23. Krishnan R, Kopacz M, Ateshian GA. 2004. Experimental verification of the role of interstitial fluid pressurization in cartilage lubrication. J Orthop Res 22:565–570.
24. Buckley MR, Gleghorn JP, Bonassar LJ, et al. 2008. Mapping the depth dependence of shear properties in articular cartilage. J Biomech 41:2430–2437.
25. Griffin DJ, Vicari J, Buckley MR, et al. 2014. Effects of enzymatic treatments on the depth-dependent viscoelastic shear properties of articular cartilage. J Orthop Res 32: 1652–1657.
26. Buckley MR, Bergou AJ, Fouchard J, et al. 2010. High-resolution spatial mapping of shear properties in cartilage. J Biomech 43:796–800.
27. Griffin DJ, Bonnevie ED, Lachowsky DJ, et al. 2015. Mechanical characterization of matrix-induced autologous chondrocyte implantation (MACI®) grafts in an equine model at 53 weeks. J Biomech 48:1944–1949.
28. Bonnevie ED, Puetzer JL, Bonassar LJ. 2014. Enhanced boundary lubrication properties of engineered menisci by

lubricin localization with insulin-like growth factor I treatment. J Biomech 47:2183–2188.
29. Pleumeekers MM, Nimeskern L, Koevoet WLM, et al. 2014. The in vitro and in vivo capacity of culture-expanded human cells from several sources encapsulated in alginate to form cartilage. Eur Cells Mater 27:264–280.
30. Nebelung S, Gavenis K, Lüring C, et al. 2012. Simultaneous anabolic and catabolic responses of human chondrocytes seeded in collagen hydrogels to long-term continuous dynamic compression. Ann Anat 194:351–358.
31. Cui X, Breitenkamp K, Finn MG, et al. 2012. Direct human cartilage repair using 3D bioprinting technology. Tissue Eng 18:1304–1312.
32. Cigan AD, Roach BL, Nims RJ, et al. 2016. High seeding density of human chondrocytes in agarose produces tissue-engineered cartilage approaching native mechanical and biochemical properties. J Biomech 49:1909–1917.
33. Chahine NO, Collette NM, Thomas CB, et al. 2014. Nanocomposite scaffold for chondrocyte growth and cartilage tissue engineering: effects of carbon nanotube surface functionalization. Tissue Eng Part A 20:2305–2315.
34. Kelly TAN, Roach BL, Weidner ZD, et al. 2013. Tissue-engineered articular cartilage exhibits tension-compression nonlinearity reminiscent of the native cartilage. J Biomech 46:1784–1791.
35. Rosenzweig DH, Chicatun F, Nazhat SN, et al. 2013. Cartilaginous constructs using primary chondrocytes from continuous expansion culture seeded in dense collagen gels. Acta Biomater 9:9360–9369.
36. Krase A, Abedian R, Steck E, et al. 2014. BMP activation and Wnt-signalling affect biochemistry and functional biomechanical properties of cartilage tissue engineering constructs. Osteoarthr Cartil 22:284–292.
37. Chung C, Burdick JA. 2008. Engineering cartilage tissue. Adv Drug Deliv Rev 60:243–262.
38. Mauck RL, Nicoll SB, Seyhan SL, et al. 2003. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng 9:597–611.
39. Strauss EJ, Goodrich LR, Chen C-T, et al. 2005. Biochemical and biomechanical properties of lesion and adjacent articular cartilage after chondral defect repair in an equine model. Am J Sports Med 33:1647–1653.
40. Russlies M, Ruther P, Koller W, et al. 2003. Biomechanical properties of cartilage repair tissue after different cartilage repair procedures in sheep. Z Orthop IhreGrenzgeb 141: 465–471.
41. Nixon AJ, Rickey E, Butler TJ, et al. 2015. A chondrocyte infiltrated collagen type I/III membrane (MACI® implant) improves cartilage healing in the equine patellofemoral joint model. Osteoarthr Cartil 23:648–660.
42. Williamson AK, Chen AC, Sah RL. 2001. Compressive properties and function—composition relationships of developing bovine articular cartilage. J Orthop Res 19: 1113–1121.
43. Gannon AR, Nagel T, Bell AP, et al. 2015. The changing role of the superficial region in determining the dynamic compressive properties of articular cartilage during postnatal development. Osteoarthr Cartil 23:975–984.
44. Griffin DJ, Ortved KF, Nixon AJ, et al. 2016. Mechanical properties and structure-function relationships in articular cartilage repaired using IGF-I gene-enhanced chondrocytes. J Orthop Res 34:149–153.
45. Mauck RL, Seyhan SL, Ateshian GA, et al. 2002. Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. Ann Biomed Eng 30:1046–1056.
46. Swan D, Radin E. 1972. The molecular basis of articular lubrication. J Biol Chem 247:8069–8073.
47. Jay GD, Lane BP, Sokoloff L. 1992. Characterization of a bovine synovial fluid lubricating factor. III. The interaction with hyaluronic acid. Connect Tissue Res 28:245–255.
48. Vunjak-Novakovic G, Martin I, Obradovic B, et al. 1999. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. J Orthop Res 17:130–138.