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Effect of Hyaluronidase on Tissue Engineered Human Septal Cartilage

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Abstract

Objectives—Structural properties of tissue-engineered cartilage can be optimized by altering its *collagen to sulfated glycosaminoglycan (sGAG)* ratio with hyaluronidase. The objective was to determine if treatment of neocartilage constructs with hyaluronidase leads to increased *collagen:sGAG* ratios, as seen in native tissue, and improved tensile properties.

Study Design—Prospective, basic science

Methods—Engineered human septal cartilage from 12 patients were treated with hyaluronidase prior to culture. Control and treated constructs were analyzed at 3, 6, or 9 weeks for their biochemical, biomechanical, and histological properties.

Results—Levels of sGAG were significantly reduced in treated compared to control constructs at 3, 6 and 9 weeks. Treated constructs had higher *collagen:sGAG* ratios when compared with control constructs at 3, 6, and 9 weeks. Treated constructs had greater tensile strength, strain at failure as well as increased stiffness as measured by the equilibrium and ramp tensile moduli when compared with the untreated control constructs. Continued time in culture improved tensile strength in both treated and control constructs.

Conclusions—Hyaluronidase treatment of engineered septal cartilage decreased total sGAG content without inhibiting expansive growth of the constructs. Decreased sGAG in treated constructs resulted in increased *collagen to sGAG* ratios and was associated with an increase in tensile strength and stiffness. With additional culture time, sGAG increased modestly in depleted constructs, and some initial gains in tensile properties were dampened. Alterations in the dosage of

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Conflict of Interest: None

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hyalurondiase during neocartilage fabrication can create constructs that have improved biomechanical properties for eventual surgical implantation.

Level of Evidence—NA

Introduction

The repair of craniofacial cartilaginous defects created by trauma, previous surgical resection, and congenital deformities requires analogous reconstructive material to obtain optimal results. Unfortunately, the use of synthetic grafts may be complicated by infection and extrusion, while allogenic grafts carry the risk of immune rejection and disease transmission (1,2). Autologous cartilage grafts as an alternative can be harvested from the nasal septum, auricle, and rib. Tissue engineering of autologous neocartilage offers the potential to produce ample quantities of tissue from a small donor specimen and the creation of grafts in defined shapes and sizes.

Cartilage tissue engineering is possible with chondrocytes obtained from the septum, auricle, rib, and articular cartilage (3–5). Multiple studies suggest that human nasal septal chondrocytes may be optimal for the production of neocartilage constructs for use in head and neck reconstructive surgery (6,7).

Nasal septal cartilage engineering involves several key steps. Cartilage is harvested from a donor and chondrocytes are isolated by digesting the extracellular matrix (ECM). Chondrocyte cell numbers are dramatically increased in monolayer culture which causes the chondrocytes to undergo a shift toward a fibroblastic phenotype in a process called dedifferentiation (8,9). The cells are then cultured in a three-dimensional (3D) configuration which induces redifferentiation to the chondrocyte phenotype with production of functional cartilaginous ECM (10–12). The alginate-recovered-chondrocyte (ARC) method is a successful 3D scaffold-free culture system (13) that has been used previously to create cartilage constructs from human nasal septal chondrocytes (14).

Numerous studies have examined the ability of growth factors and serum to promote redifferentiation and matrix production in nasal septal chondrocytes (7,15,16). Culture of human nasal septal chondrocytes in medium supplemented with 10% pooled human serum (HS) was found to improve cell proliferation and biochemical properties of neocartilage (15) and key growth factor combinations for culture make a significant difference (16). Nasal septal cartilage constructs were cultured in media supplemented with HS, insulin-like growth factor-1 (IGF-1), and growth differentiation factor 5 (GDF-5). Culture with this combination of growth factors produced 12-fold thicker constructs with more sGAG and type II collagen compared with HS alone. In addition, these constructs showed the greatest confined compression modulus in biomechanical testing.

Additional factors that influence the mechanical strength and stability of cartilage are the relative abundance of certain structural molecules in the ECM of the chondrocytes (17). In human articular cartilage, the tensile modulus and strength have been attributed primarily to the collagen network (17,18–21). Within this tissue type, the collagen content is approximately 2–10 times higher than GAG content (22), whereas in the native human nasal

septum, collagen content has been found to be approximately 2.7 times higher than GAG content (11,18,23).

Tissue engineered cartilage constructs grown in static conditions lack maturational growth and overproduce GAGs, resulting in an imbalance between the collagen and GAG ratio seen in native tissue. This reduces the tissue tensile properties (24,25). The typical ratio of collagen:GAG in engineered cartilage is 1:1 or less (26–28).

Tensile mechanical properties of articular cartilage can be modulated through depletion of the proteoglycan components of cartilage ECM using chemical extractions or enzymatic treatments such as hyaluronidase. A number of studies have demonstrated improved tensile properties of articular cartilage tissue after GAG depletion (29–32). Treatment of articular cartilage explants (32) and tissue engineered articular cartilage (33,34) with the GAG-depleting enzyme chondroitinase-ABC (C-ABC) resulted in increased collagen content and improved tensile properties (32).

One explanation for the increase in articular cartilage tensile stiffness and strength is that proteoglycan depletion allows the collagen fibrils to associate linearly (end-to-end) or laterally (side-to-side) to increase the fibril diameter (35–37). Hyaluronidase selectively cleaves hyaluronic acid (HA) resulting in release of proteoglycan aggregates from cartilage tissue explants (38). In a recent study of immature articular cartilage explants, quantitative polarized light microscopic (qPLM) analysis of the orientation and parallelism index as an measure of collagen network microstructure, revealed that treatment with hyaluronidase induced collagen network organization (increased parallelism index). When dynamic compressive compaction was added to the hyaluronidase treatment, a relatively high parallelism index was induced near the articular surface, resembling that of mature cartilage (39).

Materials and Methods

Collection of Cartilage and Chondrocyte Monolayer Expansion

Remnant human septal specimens from 12 patients (9 male, 3 female; age 39.5 ± 13.5 years) removed during routine surgery from the sponsoring institution were used (prior approval by the Human Subjects Committee of our institution was granted). At the time of harvest, specimens were placed in sterile normal saline and transported to the laboratory at 4°C within 24 hours.

Cartilage specimens were dissected free of perichondrium and diced into ~1mm^3 pieces. The tissue was then digested by incubation at 37°C in 0.2% Pronase type XIV (Sigma, St. Louis, MO) in medium (Dulbecco modified Eagle medium [DMEM], 0.4M L-proline, 2mM L-glutamine, 0.1mM nonessential amino acids, 10mM HEPES (hydroxyethyl-piperazineethanesulfonic acid) buffer, 100U/mL penicillin G, 100μg/mL streptomycin sulfate, 0.25μg/mL amphotericin B, 2% pooled human AB serum (HS) (Gemini Bioproducts, Woodland, CA)) for 60 minutes followed by incubation with 0.025% collagenase P (Roche Diagnostics, Indianapolis, IN) in medium for 16 hours.

After filtration through a 70μm mesh to remove undigested material, the cells were recovered by centrifugation. The cell pellet was resuspended in culture medium and counted with a hemacytometer using Trypan blue to determine the number of chondrocytes isolated from each specimen. Cells from each donor were placed in monolayer culture in T-175 culture flasks at a seeding density of 5,000 cells per cm^2 surface area. Monolayer cultures were incubated at 37°C with 5% carbon dioxide/air. Culture medium was supplemented with 2% HS, 1ng/ml Transforming Growth Factor (TGF)-β1, 5ng/mL Fibroblast Growth Factor (FGF)-2, and 10ng/mL Platelet-derived Growth Factor (PDGF)-BB, and changed every two to three days. Chondrocytes were grown until confluency, which typically occurs after seven to ten days under these conditions. Confluency was evaluated with transmitted light microscopy.

Creation of Cartilage Constructs

The expanded cells were released from monolayer, resuspended in alginate at a density of 4×10^6cells/mL in a solution of 1.2% low-viscosity alginate (Kelco LV, San Diego, CA). Droplets of the alginate-chondrocyte suspension were polymerized in 102mM calcium chloride for five minutes. Uniform bead size was ensured by carefully controlling the rate of flow through the syringe used to create the droplets. After washing with 0.9% saline, the beads were transferred to a 250mL Nalgene PETG square media bottle filled with 50 mL of alginate culture medium (DMEM/F-12, 25μg/mL ascorbate, 0.4mM L-proline, 2mM L-glutamine, 0.1mM nonessential amino acids, 10mM L HEPES buffer, 100U/mL penicillin G, 100μg/mL streptomycin sulfate, 0.25μg/mL amphotericin B) supplemented with 2%HS, 100ng/mL GDF-5 and 200ng/mL IGF-1 and cultured for two weeks. Media was changed every 2–3 days.

The alginate beads were terminated on day 14 and depolymerized using a solution of 55mM sodium citrate and 0.15mM NaCl. The chondrocytes and their ECM were recovered by centrifugation. The cell pellet was resuspended in culture medium supplemented with 2%HS, 100ng/ml GDF-5, and 200ng/ml IGF-1 and used to seed multiple transwell clear polyester membrane cell inserts (Corning, Inc., Corning, NY) at a density of 1.33×10^6cells/cm^2. Culture medium was changed every 2–3 days.

Hyaluronidase Treatment

After a culture period of 7–11 weeks (8.8 ± 1.6weeks), three constructs per patient were treated with either culture medium containing 600U/mL bovine hyaluronidase (Sigma Aldrich, St. Louis, Mo) or a mock treatment of culture medium without the enzyme for 24 hours at 37°C with 5% carbon dioxide. After treatment, the constructs were rinsed in PBS and transferred into 6-well culture plate (Corning, Inc., Corning, NY) containing culture medium and cultured for another 3, 6, or 9 weeks. At the end of the culture period, constructs were harvested, weighed, and evaluated.

Biochemical Testing

Cellularity of the constructs were tested using the PicoGreen DNA content determination assay (Invitrogen, Carlsbad, CA) after digestion with Proteinase K (PK). Portions of each sample digest were mixed with PicoGreen reagent. Fluorescence was measured with an

excitation light wavelength of 480nm and emission wavelength of 520nm in a spectrofluorimetric plate reader. Fluorescence values were converted to DNA quantity using standards of human DNA in the appropriate buffer solution. DNA content was normalized to determine cell number and also to milligram of wet tissue weight (40).

The sGAG content was determined using portions of the PK digests and the dimethyl-methylene blue (DMMB) reaction. Portions of each sample digest were mixed with DMMB dye. Absorbance at 525nm was measured in a spectrophotometric plate reader and compared to a plot of standards made from shark chondroitin sulfate type C (Sigma, St. Louis, MO). GAG content was normalized to DNA content.

The hydroxyproline assay was used to determine the amount of total collagen in the PK digests. In a fume hood, a volume of 12.1N hydrochloric acid (HCl) (Sigma) equal to the sample volume was added to the sample and incubated at 110°C for 16–18 hours. The samples were removed from heat, allowed to equilibrate to room temperature, centrifuged briefly, and placed at 110°C with the caps open in order to allow the HCl to evaporate (16–24 hours). The dried sample was dissolved in assay buffer (238mM citric acid monohydrate, 882mM sodium acetate trihydrate). A volume of 150μl of the reconstituted samples and Hydroxyproline standards were transferred into a 96-well plate. A volume of 75μl of Chloramine-T-reagent (50mM Chloramine-T, 26% n-Propanol, 238mM Citric Acid monohydrate, 882mM sodium acetate trihydrate) was then added to each sample in the plate. The plates were incubated at room temperature for 20 minutes. Finally, a volume of 75μl DMBA (1M p-Diaminobenzaldehyde, 60% n-Propanol, 16% Perchloric Acid) was added to each sample in the plate. The plate was incubated at 60°C for 15 minutes, cooled to room temperature for 5 minutes, and the absorbance was read at 560nm using an EMAX precision microplate reader (Molecular Devices, Sunnyvale, CA). The sample absorbance was compared to a plot of the Hydroxyproline standards. Hydroxyproline content was normalized to DNA content. The amount of collagen in the sample was estimated using the formula: collagen(g)/(tissue(g)-7.25=hydroxyproline(g).

Histology and Immunohistochemistry

Histochemistry was used to localize GAG. Samples were placed in Optimal Cutting Temperature Compound and frozen prior to sectioning in a cryostat at 50μm thickness. For histochemical localization of GAG, slides from each sample group were stained with 0.1% Alcian blue in buffer (0.4M magnesium chloride, 0.025M sodium acetate, 2.5% glutaraldehyde, (pH 5.6) overnight, and destained with 3% acetic acid until clear. Samples were then observed using light microscopy. Samples were be documented by photomicroscopy.

Mechanical Testing

Portions of the constructs (N=5) were analyzed by tensile testing. Specimens for tensile testing were kept hydrated with PBS supplemented with calcium and magnesium during testing. The thickness of each construct was measured at 5 points distributed around the circumference and center of each sample, using a contact-sensing micrometer.

For tensile testing, a tapered specimen with a central gauge region measuring 10mm in length by 0.8mm in width was isolated using a custom-made punch. Tapered specimens were held in clamps that provide a secure grip on the ends of the specimens, just outside the gauge region, and subjected to ramp-relaxation extension to determine tensile equilibrium modulus, ramp modulus, strength, and strain at failure (22,41). A computer-controlled mechanical test that controls displacement while measuring load was used. The test sequence consisted of applying a positive displacement of 0.5mm/min until a tare load of 0.05N (equivalent to a stress of 0.2MPa) or 5% extension was obtained (whichever comes first, with 5% being sufficient to remove slack in the sample), then elongating the specimen to 10% strain (using the grip-to-grip distance, containing the gauge region, the tare state) at a constant strain, allowing stress relaxation to equilibrium, and then elongating the specimen at a constant rate of 5mm/min until failure. From the displacement and load data, tensile mechanical properties were determined.

Statistical Analysis

Analysis was performed using Systat10 (Systat Software, Chicago, IL). Means were presented ± the standard deviation. The effects of hyaluronidase treatment on each of the biochemical and tensile parameters were analyzed by one-way ANOVA. If the ANOVA identified an overall significant effect, post-hoc Tukey's Honestly Significant Differences tests were used to identify significant differences. A difference was considered significant when $p<0.05$.

Results

Tissue engineered human nasal septal neocartilage constructs from 12 patients (mean age 39.5 years--9 males, 3 females) were either treated with culture medium containing hyaluronidase or culture medium alone for 24 hours. Control and treated constructs were harvested at 3, 6, or 9 weeks after additional culture time and analyzed for their biochemical, biomechanical, and histological properties. Both treated and untreated constructs were opaque with smooth surfaces and strong enough to manipulate with a forceps (figure 1).

As expected, treated constructs had a marked reduction in sGAG when compared to control constructs at 3 (22.6±6.19 and 121±54.0μg sGAG per μg DNA, respectively; p=0.00), 6 (60.1±15.1 and 170±91.2μg sGAG per μg DNA, respectively; p=0.001) and 9 (112±37.9 and 205±104μg sGAG per μg DNA, respectively; p=0.001) weeks post-treatment (Figure 2 and Table 1).

Collagen levels were similar in treated and control constructs at 3 (204 ± 50.1 and 202 ± 49.5μg collagen per μg DNA, respectively; p=0.73) and 6 (288±54.5 and 273±58.8μg collagen per μg DNA, respectively; p=0.18) weeks post-treatment (Table 1) with more deposition of collagen occurring in the 3 to 6 weeks culture interval (p=0.001 and p=0.01, respectively).

The ratio of collagen to sGAG remained higher in the treated constructs when compared with control constructs at 3 (9.29±1.95 and 2.01±0.98μg collagen per μg sGAG, respectively; p=0.00), 6 (5.04±1.43 and 2.01±1.04μg collagen per μg sGAG, respectively;

p=0.00), and 9 (2.60±0.72 and 2.06±1.50μg collagen per μg sGAG, respectively; p=0.06) weeks post-treatment (Figure 2 and Table 1). By 9 weeks of culture time, the ratio of collagen to sGAG in treated constructs steadily decreased as sGAG levels began to rise (p=0.001). In contrast, the ratio of collagen to sGAG remained stable in control constructs over the 9 week post-treatment culture period (p=0.99).

After 3 weeks of post-treatment culture, treated constructs had greater tensile strength (σ_{ult}, 0.27±0.18 and 0.13± 0.10MPa, respectively; p=0.19), strain at failure (ε_{ult}, 0.49±0.35 and 0.0.31±0.15MPa, respectively; p=0.24) as well as increased equilibrium ($E_{t,eq}$, 0.19±0.14 and 0.07±0.11N/mm, respectively; p=0.19) and ramp tensile moduli ($T_{t,ramp}$, 0.37±0.20 and 0.23±0.13N/mm, respectively; p=0.09) when compared with the untreated control constructs (Figure 2 and Table 1). Continued time in culture improved the σ_{ult} (0.42±0.29 and 0.31±0.27MPa, respectively; p=0.42 and p=0.37) and ε_{ult} (1.42±0.93 and 1.39±0.27MPa, respectively; p=0.07 and p=0.09) of both treated and control constructs, while $E_{t,eq}$ (0.09±0.05 and 0.06±0.07N/mm, respectively; p=0.08 and p=0.99) and $T_{t,ramp}$ (0.18±0.10 and 0.15±0.06N/mm, respectively; p=0.09 and p=0.40) were not improved.

The histochemical analyses (H&E, Alcian Blue and collagen immunohistochemistry-type I +II) corroborated the biochemical results. The level of sGAG in treated constructs is markedly reduced at 6 weeks of culture time (Figure 3).

Discussion

The abundance of certain structural molecules in the ECM plays an important role in the mechanical stability of cartilage. However, the ratios of ECM components from tissue-engineered neocartilage are rarely characterized.

In the current study, hyaluronidase treatment of neocartilage constructs was effective in decreasing the total sGAG content without eliminating subsequent deposition of collagen. The values reflected in Table 1 demonstrate a steady increase in both GAG and collagen levels during additional culture time.

Biochemically, treated constructs had a marked decrease in sGAG (<20% of control after 3 weeks). This was associated with an initial stiffening of the tissue and a greater than 2-fold increase in tensile strength and stiffness (equilibrium tensile modulus). This impact is notable in Figure 2 and Table 1. As the tensile strength of the cartilage increases, the tissue can withstand a higher stress before failure, which is shown as Strain at Failure in Figure 2. Concurrently, increased equilibrium and ramp tensile moduli reflect a reduction in the degree of cartilage stretching. Increases in these tensile parameters may indicate increased biomechanical maturity of the engineered cartilage tissue.

Depletion of sGAG resulted in a striking increase in the ratio of collagen to sGAG compared to the untreated controls (Figure 2 and Table 1). By allowing the incubation time to exceed 3 weeks, sGAG levels began to rise in depleted constructs, and continued to increase in untreated control constructs. As a result, the tensile improvement which was gained initially with hyaluronidase treatment becomes dampened with additional culture time (Table 1). In previous studies of human nasal septal cartilage, it has been found that the biomechanical

shortcomings of tissue engineered human septal neocartilage are likely due to relatively low quantities of type II collagen and the mature collagen crosslink, hydroxylysylpyridinoline, relative to sGAG. Native human septal cartilage has a collagen:sGAG ratio of approximately 2.7 (42). Efforts to optimize chondrocyte differentiation and neocartilage construct culture conditions have improved the collagen:sGAG ratio to approximately 1.8.

The mechanical properties of the constructs that were improved from the single treatment with hyaluronidase include: tensile strength, strain at failure, and equilibrium and ramp tensile moduli. With additional culture time to 9 weeks, GAG content increased steadily in the depleted constructs, causing a dampening effect on the elevated collagen:GAG ratio. This may correspond to the observed reduction in the equilibrium and ramp tensile moduli seen later. These findings represent an opportunity to enhance the fabrication of neocartilage with hyaluronidase. Varying the dose of the enzyme or using multiple treatments over strategic time points during the culture period are approaches for future investigation.

Conclusions

The biosynthesis of extracellular matrix components in cartilage engineering can dictate the biomechanical properties of the construct. This study was able to modify the levels of two key ECM components: collagen and GAG. The addition of hyaluronidase depletes the proteoglycan in the ECM and allows collagen levels to rise relative to GAG levels. As the collagen:GAG ratio increased, so did the biomechanical maturity of the engineered cartilage. The engineered product achieved tensile properties that more closely resemble those of native tissue, compared to untreated neocartilage constructs.

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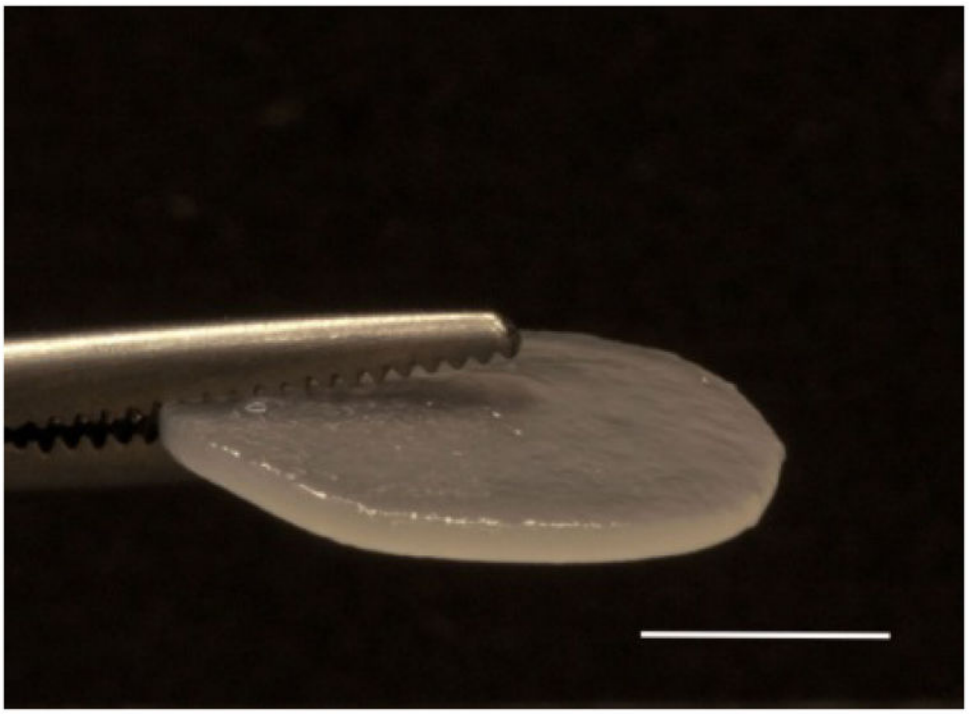

Figure 1.
Hyaluronidase-treated and control tissue engineered human septal cartilage constructs were firm, white, and opaque with smooth surfaces. Constructs treated with hyaluronidase were more pliable than untreated control constructs. Scale bar is 5mm.

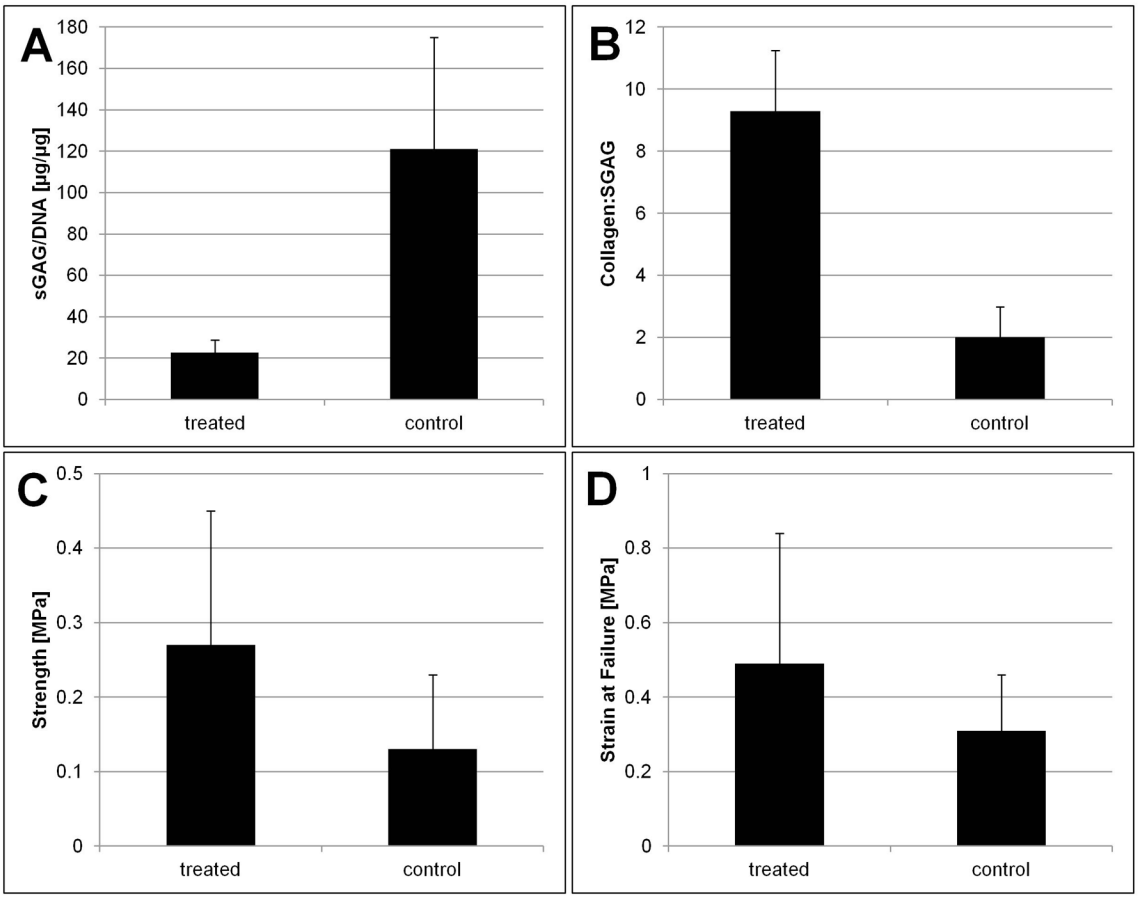

Figure 2.

Constructs treated with hyaluronidase had marked reduction in levels of sGAG compared to controls (A), and higher ratios of collagen to GAG (B). Treated constructs had elevated tensile strength (C) as well as strain at failure (D). n=12

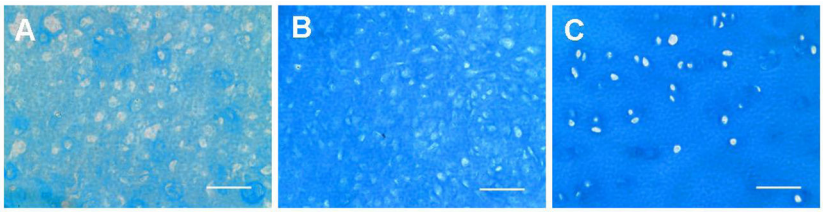

Figure 3.

Alcian Blue stained tissue engineered human septal neocartilage 6 weeks after treatment with hyaluronidase (A) or untreated (B) compared with native human septal cartilage (C). After 6 weeks post-treatment culture, the level of sGAG in treated constructs is still markedly reduced when compared with untreated control constructs. Scale bar is 100 μm.

Table 1

Biochemical and tensile biomechanical properties of hyaluronidase-treated and untreated control tissue engineered neocartilage constructs after 3, 6, or 9 weeks post-treatment culture. Hyaluronidase treatment decreased construct wet weights and sGAG content. The initial collagen:sGAG ratio increased as did all of the tensile mechanical properties when compared with untreated control constructs (see underlined properties). N=12 for biochemical analyses (wet weight, DNA, sGAG/DNA, collagen/DNA and collagen:sGAG). N=5 for tensile biomechanical testing (strength, strain at failure, Eq and ramp moduli). sGAG, sulfated glycosaminoglycan; Eq Modulus, equilibrium tensile modulus.

	3 weeks		6 weeks		9 weeks	
Properties	**Treated**	**Control**	**Treated**	**Control**	**Treated**	**Control**
Wet Weight (mg)	84.2 ± 12.3	120 ± 27.1	90.6 ± 16.2	130 ± 32.0	101 ± 19.8	135 ± 34.3
DNA (μg)	12.9 ± 3.38	9.80 ± 1.90	10.6 ± 2.14	10.0 ± 1.71	8.34 ± 1.08	8.94 ± 1.77
sGAG/DNA (μg/μg)	22.6 ± 6.19	121 ± 54.0	60.1 ± 15.1	170 ± 91.2	112 ± 37.9	205 ± 104
Collagen/DNA (μg/μg)	204 ± 50.1	202 ± 49.5	288 ± 54.5	273 ± 58.8	270 ± 42.6	311 ± 54.0
Collagen:sGAG	9.29 ± 1.95	2.01 ± 0.98	5.04 ± 1.43	2.01 ± 1.04	2.60 ± 0.72	2.06 ± 1.50
Strength (MPa)	0.27 ± 0.18	0.13 ± 0.10	0.27 ± 0.07	0.29 ± 0.16	0.42 ± 0.29	0.31 ± 0.27
Strain at Failure (MPa)	0.49 ± 0.35	0.31 ± 0.15	0.96 ± 0.33	0.78 ± 0.56	1.42 ± 0.93	1.39 ± 0.89
Eq Modulus (N/mm)	0.19 ± 0.14	0.07 ± 0.11	0.06 ± 0.02	0.07 ± 0.06	0.09 ± 0.05	0.06 ± 0.07
Ramp modulus (N/mm)	0.37 ± 0.20	0.23 ± 0.13	0.11 ± 0.04	0.17 ± 0.07	0.18 ± 0.10	0.15 ± 0.06