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Shape Fidelity of Native and Engineered Human Nasal Septal Cartilage

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Abstract

Objective—To test engineered and native septal cartilage for resistance to deformation and remodeling under sustained bending loads and to determine the effect of bending loads on the biochemical properties of constructs.

Study Design—Prospective, basic science.

Setting—Laboratory.

Subjects and Methods—Human septal chondrocytes from 6 donors were used to create 12-mm constructs. These were cultured for 10 weeks and subjected to bending for 6 days. Free-swelling controls and native tissue from 6 donors were used for comparison. Shape retention, photo documentation, live-dead staining, and biochemical properties were measured.

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Author Contributions

Marsha S. Reuther, conception, design, conduct of research, acquisition of data, analysis and interpretation of data, drafting and revising the article, final approval; **Kristen K. Briggs**, conception, design, conduct of research, acquisition of data, analysis and interpretation of data, drafting and revising the article; **Monica K. Neuman**, conduct of research, acquisition of data, analysis and interpretation of data; **Koichi Masuda**, conception, design, data interpretation; **Robert L. Sah**, conception, design, data interpretation; **Deborah Watson**, conception, design, provision of patients, data interpretation, manuscript writing, revision, and final approval.

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Results—Live-dead staining showed no difference in cell survival between loaded constructs and free-swelling controls. The immediate shape retention of the constructs was 39.0% versus 24.4% for native tissue ($P = .13$). After 2 and 24 hours of relaxation, the constructs possessed similar shape retention to native tissue (26.9% and 16.4%; $P = .126$; 21.7% and 14.4%; $P = .153$). There was no significant change in construct shape retention from immediately after release to 2 hours of relaxation (39.0% and 26.9%, respectively; $P = .238$). In addition, the retention did not change significantly between 2 and 24 hours of relaxation (26.9% and 21.7%; $P = .48$). There was no significant difference in biochemical properties between loaded constructs and controls.

Conclusion—The shape retention properties of human septal neocartilage constructs are comparable to human native septal cartilage. In addition, mechanical loading of neocartilage constructs does not adversely affect cell viability or biochemical properties. This study demonstrates that neocartilage constructs possess adequate shape fidelity for use as septal cartilage graft material.

Keywords

cartilage tissue engineering; human septal cartilage; shape fidelity

Autologous cartilage grafts are favored for the repair of craniofacial defects created by trauma, tumor resection, and congenital deformities. Cartilage may be obtained from the nasal septum, auricle, and rib for reconstructive purposes. However, nasal septal cartilage is preferred because of its superior structural properties, ease of harvest, and minimal donor site morbidity. Regrettably, only a finite amount of nasal septal tissue in a predefined configuration is available for use as grafting material. This can be further limited by trauma and congenital or iatrogenic defects of the nose. Previous studies have successfully produced tissue-engineered autologous neocartilage that eventually may be used for reconstructive surgery.^{1–3} This technology offers the potential to produce large quantities of autologous cartilage in defined shapes and sizes. Clinically useful septal neocartilage constructs must possess sufficient mechanical strength to withstand manipulation during surgery and the in situ mechanical stresses associated with the contractile healing forces from the nasal skin–soft-tissue envelope.

Cartilage is a mechanosensitive tissue, and its responses to physical forces have been studied both in vivo and in vitro. Mechanical stimuli play an important role in the development and growth of cartilage in vivo and the functional adaptation of tissue nearing maturation.^{4,5} The metabolic responses of chondrocytes and cartilage explants to controlled mechanical stimuli in vitro have also been extensively studied.^{6,7} The mechanical properties of tissue-engineered septal neocartilage have been studied using confined compression and tensile testing.^{1–3,8,9} While the results of these tests reflect intrinsic mechanical properties of the neocartilage, the mechanical stresses associated with the postculture and implantation environment are not being tested. One such mechanical stress is sustained bending. The mechanical behavior and shape fidelity of native septal cartilage and tissue-engineered neocartilage constructs under a physiologically relevant sustained bending load have not been analyzed.

A common method used to assess cartilage response to a sustained bending load is to perform a bending test. Previous articular cartilage-bending studies have validated the use of a custom-made bending bioreactor to produce a controlled bending deformation.^{10–12} The loading apparatus is housed in a standard incubator and produces a controlled bending deformation on cartilage strips during in vitro culture. The central cylindrical loading post distributes stress across the specimen surface, yielding a smooth curvature. In these previous studies, cartilage explants were subjected to bending deformations using the bending bioreactor. Changes in sample shape were quantified after in vitro growth at various time points, thereby providing a measure of tissue shape fidelity. Similar measurements have not been performed using native septal cartilage or tissue-engineered septal neocartilage constructs.

This work serves to establish a benchmark measure of shape fidelity of native human septal cartilage and to compare this with our current tissue-engineered septal neocartilage constructs. In addition, the effect of bending loads on the biochemical properties of neocartilage constructs is evaluated.

Methods

Human Septal Cartilage Collection and Chondrocyte Expansion

The study used remnant human septal specimens removed during routine surgery at the University of California, San Diego Medical Center or San Diego Veterans Affairs Medical Center. Institutional Review Board approval was obtained at both institutions. Cartilage from a total of 12 donors was collected. Six specimens were used for testing of native tissue. The cartilage from 6 donors was dissected free of perichondrium and diced into pieces (1 mm³). The fragments were digested as reported previously.¹ Suspensions of digested cartilage were filtered (70 μ m), then washed and centrifuged. Cells were resuspended in cell culture medium (DMEM [low glucose], 2% pooled human AB serum [HS; Gemini Bioproducts, Woodland, CA), 25 mg/mL ascorbate, 0.4 mmol/L L-proline, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 10 mmol/L HEPES buffer, 100 U/mL penicillin G, 100 mg/mL streptomycin sulfate, 0.25 mg/mL amphotericin B). The number of chondrocytes was determined by hemacytometer counting after trypan blue exclusion.

For each patient, isolated chondrocytes were seeded at low density (5000 cells per cm² surface area) into T-175 flasks. Monolayer cultures were incubated in a humidified atmosphere at 37°C with 5% carbon dioxide/air. Culture medium was supplemented with 1 ng/mL transforming growth factor- β -1, 5 ng/mL fibroblast growth factor-2, and 10 ng/mL platelet-derived growth factor- β and changed every 2 days. Chondrocytes were grown until confluency (6–8 days).

Culture in Alginate

The expanded cells were released from monolayer and resuspended in alginate as described previously.¹ After washing with 0.9% saline, the alginate beads were transferred to a 250-mL Nalgene PETG square media bottle. Fifty milliliters of alginate culture medium (DMEM/F-12, 25 μ g/mL ascorbate, 0.4 mM L-proline, 2 mM L-glutamine, 0.1 mM

nonessential amino acids, 10 mM L HEPES buffer, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B) supplemented with 2% HS, 100 ng/mL bone morphogenic protein-14 (BMP-14), and 200 ng/mL insulin growth factor-1 (IGF-1) were placed in the media bottle and changed every 2 to 3 days.

Release from Alginate Culture and Formation of Constructs

Culture of alginate beads was terminated after 14 days. The alginate beads were depolymerized using a solution of 55 mM sodium citrate and 0.15 mM NaCl. Centrifugation at 750g for 5 minutes was then undertaken to separate the supernatant from the pellet consisting of recovered chondrocytes with associated ECM. The recovered cells and ECM were resuspended in chondrocyte culture medium at a cell density of 4×10^6 cells/mL. This suspension was used to seed at least two 12-mm-diameter transwell polyester membrane inserts (Corning Inc, Corning, NY) per donor at 1.33×10^6 cells/cm². Culture medium was changed every other day for 6 weeks. After 6 weeks of culture, 2 constructs from each sample were released from the transwell insert. Both constructs were placed in a 50-mL disposable rotary cell culture vessel (Synthecon Inc, Houston, TX). Fifty milliliters of culture medium (DMEM/F-12, 25 µg/mL ascorbate, 0.4 mM L-proline, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM L HEPES buffer, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B) supplemented with 2% HS, 100 ng/mL BMP-14, and 200 ng/mL IGF-1 was placed in the rotary culture vessel and changed every 2 to 3 days. The constructs were cultured for an additional 4 weeks in the rotary culture vessel.

Bending Bioreactor Culture

Upon culture termination, 2 flat strips of similar size ($\sim 10 \times 2 \times 1$ mm³) were prepared from the neocartilage constructs and the 6 native cartilage specimens. The cartilage and construct strips were loaded either into the bending bioreactor or cultured in a 6-well plate as free-swelling controls (Figure 1A and B). The bending chambers were housed in a standard incubator to provide environmental control (temperature, pH, humidity) and had accessible ports for medium changes. The central cylindrical loading post distributed stress across the specimen surface, yielding a smooth curvature. A 5-mm post diameter was used, which was shown to impose a noninjurious deformation to the samples as revealed by cell viability assays in a pilot study. Digital images of specimens were acquired before culture in the initial configuration and after culture, both while loaded (imposed shape), directly after unloading (0 hour), after a 2-hour period of free-swelling following unloading (2 hour), and after 24 hours of free-swelling. The sample shape in these 4 configurations was quantified as an opening angle, defined by the 2 inner corners with a vertex at the midpoint (Figure 1C). The angle was measured using ImageJ software (National Institutes of Health, Bethesda, MD). The percentage of the imposed shape that was retained following each relaxation period was calculated as the shape change between the initial and relaxed configurations divided by the shape change between initial and imposed configurations and expressed as a percentage. According to this measure, a sample that fully returns to the initial flat configuration after unloading would have 0% shape retention, whereas a sample that remained in the imposed curved shape after unloading would have 100% shape retention.

Biochemical Analysis and Cell Viability

After shape analysis was completed, a central portion of each neocartilage construct was weighed and digested with proteinase K (Roche Diagnostics, Indianapolis, IN) for biochemical analysis. To ascertain whether chondrocyte viability was maintained during sample preparation and culture under a bending load, pieces of tissue adjacent to the removed biochemical portions were assessed using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes Inc, Eugene, OR). Calcein-AM and ethidium homodimer-1 dyes were prepared according to package instructions to 100 × concentration and then further diluted by combining 5 µL of each stain with 500 µL of phosphate-buffered saline (PBS). A slice of each construct was added to the dye solution at room temperature and protected from light. After 20 minutes, each slice was rinsed twice in 1 mL of PBS (10 minutes per rinse) and again protected from light. After staining, the construct was analyzed for cellular viability using fluorescence microscopy.

Cellularity of the constructs was tested using the PicoGreen DNA content determination assay as described in a previous report.^{1,13} DNA content was normalized per milligram wet weight. The glycosaminoglycan (GAG) content was determined, as reported previously, using portions of the proteinase K digests and the dimethylmethylene blue reaction.¹ GAG content was then normalized per milligram wet weight and by DNA content.¹⁴ The quantity of hydroxyproline in the constructs was determined as described previously.¹⁵ Hydroxyproline content was converted to collagen content using a mass ratio of collagen to hydroxyproline of 7.1.¹⁶

Statistical Analysis

Analysis was performed using Systat 10.2 (Systat Software, Chicago, IL). Means are presented ± the standard deviation (SD). Differences in shape retention, DNA content per milligram wet weight, GAG per DNA and milligram wet weight, and total collagen per DNA and wet weight were assessed using a 2-sample *t* test. A difference was considered significant when $P < .05$.

Results

The native and tissue-engineered groups had similar donor age ranges but different gender composition. There was no significant difference in age between the native (37.2 ± 13.2 [21–52] years old) and tissue-engineered (45.2 ± 13.4 [31–61] years old) groups ($P = .16$). The native tissue group consisted of 5 male and 1 female donors, while the tissue-engineered group consisted of 3 male and 3 female donors.

Live-dead staining demonstrated no difference in cell viability between the free-swelling and loaded neocartilage constructs. No samples fractured during loading. The immediate shape retention of the constructs was $39.0\% \pm 19.4\%$ versus $24.4\% \pm 9.9\%$ for native tissue, which was not significantly different ($P = .13$). After 2 hours of relaxation, the constructs possessed similar shape retention to native tissue ($26.9\% \pm 13.8\%$ and $16.4\% \pm 4.2\%$, respectively; $P = .13$). Similarly, the shape retention after 24 hours of relaxation did not differ significantly between constructs and native tissue ($21.7\% \pm 10.4\%$ and $14.4\% \pm 4.9\%$,

respectively; $P = .15$). The shape retention of both native tissue and constructs tended to decrease with increased relaxation time (Figure 2). However, there was no significant change in construct shape retention from immediately after release to 2 hours of relaxation ($39.0\% \pm 19.4\%$ and $26.9\% \pm 13.8\%$, respectively; $P = .238$). In addition, the retention did not change significantly between 2 and 24 hours of relaxation ($26.9\% \pm 13.8\%$ and $21.7\% \pm 10.4\%$; $P = .48$; Figure 3).

The biochemical properties of free-swelling and loaded neocartilage constructs were not significantly different. Cellularity did not differ between loaded and free-swelling constructs (0.21 ± 0.08 and 0.21 ± 0.04 μg DNA per milligram wet weight sample, respectively; $P = .85$). The loaded and free-swelling constructs also possessed similar quantities of GAG (15.19 ± 6.7 and 14.6 ± 6.2 μg GAG per milligram wet weight sample, respectively; $P = .78$). In addition, the collagen content did not vary significantly between loaded and free-swelling constructs (19.30 ± 3.02 and 18.40 ± 5.34 μg total collagen per milligram wet weight sample, respectively; $P = .78$).

Discussion

This study characterizes the resistance of native septal cartilage to deformation and remodeling under a sustained bending load during *in vitro* culture, thereby representing the first description of shape fidelity of the native human nasal septum. Additionally, the shape fidelity of tissue engineered septal neocartilage constructs is compared with native tissue and the biochemical properties of loaded constructs and free-swelling controls are evaluated. Native septal cartilage and neocartilage constructs exhibited a high level of shape fidelity. These tissues possessed a marked resilience to an applied bending deformation and almost fully recovered to their initial shape when removed from the bending bioreactor. Such a high degree of shape fidelity is desirable for engineered cartilage tissue. Moreover, bending the neocartilage constructs did not negatively impact the biochemical properties of the constructs. This is extremely important as it supports future studies in which more immature constructs may be shaped in defined configurations for use in reconstructing specific defects.

To test the shape fidelity of native tissue and neocartilage constructs, we successfully used a technique that was previously developed to reshape immature cartilage through application of bending mechanical loads during *in vitro* culture.^{10–12} In these previous studies, it was demonstrated that *in vitro* culture of immature bovine cartilage in the bending bioreactor imposed a significant shape change on the tissue. Therefore, we chose this technique to test the shape fidelity of neocartilage constructs and native cartilage.

This study was limited by the small sample size. A post hoc calculation of sample size using a power level of 0.8 demonstrated that the sample size of 6 was not large enough to detect a significant difference between the sample groups. However, the data from this pilot study suggest that the shape fidelity properties of native and engineered septal tissue are comparable. In addition, the native tissue group possessed more male donors than the construct group. However, this imbalance was not believed to affect the results greatly, as

age and gender have not been shown to affect significantly tensile strength or compressive properties in human nasal septal cartilage.^{8,9}

The formation of clinically useful, engineered septal cartilage grafts requires that the engineered cartilage possess the necessary mechanical properties to withstand surgical manipulation and implantation and to support the structures of the nose. Engineered human nasal septal tissue intended for surgical grafting must not only resist deformation under acute mechanical loads but also demonstrate the ability to withstand plastic deformations or permanent shape changes when subjected to repeated or sustained loading. As a structural member of the nose, cartilage grafts are subjected to the ever-present forces produced by supporting nasal tissues and those generated during wound healing following surgery. Under the influence of such sustained loads, a cartilage graft may remodel and become permanently altered in shape, potentially compromising its functional or aesthetic roles. Therefore, to produce clinically useful tissue-engineered septal neocartilage, the construct resistance to bending deformation must be examined. This is the first description of the shape fidelity of native septal cartilage. This study successfully demonstrates that the resistance to deformation of engineered septal cartilage constructs is comparable to native septal tissue and, therefore, that neo-cartilage constructs have adequate shape fidelity for use as septal cartilage graft material.

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References

1. Alexander TH, Sage AB, Chen AC, et al. IGF-1 and GDF-5-promote the formation of tissue-engineered human nasal septal cartilage. *Tissue Eng Part C Methods*. 2010; 16:1213–1221. [PubMed: 20178406]
2. Alexander TH, Sage AB, Schumacher BL, Sah RL, Watson D. Human serum for tissue engineering of human nasal septal cartilage. *Otolaryngol Head Neck Surg*. 2006; 135:397–403. [PubMed: 16949971]
3. Chang AA, Reuther MS, Briggs KK, et al. In vivo implantation of tissue-engineered human nasal septal neocartilage constructs: a pilot study. *Otolaryngol Head Neck Surg*. 2012; 146:46–52. [PubMed: 22031592]
4. Kiviranta I, Tammi M, Jurvelin J, Saamanen AM, Helminen HJ. Moderate running exercise augments glycosaminoglycans and thickness of articular cartilage in the knee joint of young beagle dogs. *J Orthop Res*. 1988; 6:188–195. [PubMed: 3278079]
5. Plochocki JH, Riscigno CJ, Garcia M. Functional adaptation of the femoral head to voluntary exercise. *Anat Rec A Discov Mol Cell Evol Biol*. 2006; 288:776–781. [PubMed: 16761292]
6. Guilak, F.; Sah, RL.; Setton, LA. Physical regulation of cartilage metabolism. In: Mow, VC.; Hayes, WC., editors. *Basic Orthopaedic Biomechanics*. 2. New York: Raven Press; 1997. p. 179-207.
7. Grodzinsky AJ, Levenston ME, Jin M, Frank EH. Cartilage tissue remodeling in response to mechanical forces. *Annu Rev Biomed Eng*. 2000; 2:691–713. [PubMed: 11701528]
8. Richmon JD, Sage A, Chen A, Schumacher BL, Sah RL, Watson D. Tensile biomechanical properties of human nasal septal cartilage. *Am J Rhinol*. 2005; 19:617–622. [PubMed: 16402652]
9. Richmon JD, Sage A, Chen A, Sah RL, Watson D. Compressive biomechanical properties of human nasal septal cartilage. *Am J Rhinol*. 2006; 20:496–501. [PubMed: 17063745]

10. Williams GM, Lin JW, Sah RL. Cartilage reshaping via in vitro mechanical loading. *Tissue Eng.* 2007; 13:2903–2911. [PubMed: 17716002]
11. Williams GM, Gratz KR, Sah RL. Asymmetrical strain distributions and neutral axis location of cartilage in flexure. *J Biomech.* 2009; 42:325–330. [PubMed: 19117571]
12. Williams GM, Sah RL. *In vitro* modulation of cartilage shape plasticity by biochemical regulation of matrix remodeling. *Tissue Eng Part A.* 2011; 17:17–23. [PubMed: 20649477]
13. McGowan KB, Kurtis MS, Lottman LM, Watson D, Sah RL. Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen and Hoechst 33258. *Osteoarthritis Cartilage.* 2002; 10:580. [PubMed: 12127839]
14. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta.* 1986; 883:173–177. [PubMed: 3091074]
15. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys.* 1961; 93:440–447. [PubMed: 13786180]
16. Jackson DS, Cleary EG. The determination of collagen and elastin. *Methods Biochem Anal.* 1967; 15:25–76. [PubMed: 4899619]

Figure 1. Cartilage strip positioned horizontally in loading apparatus before (A) and after (B) application of bending load. (C) The shape was quantified as an opening angle.

Figure 2.

The immediate shape retention of the constructs was 39.0% versus 24.4% for native tissue ($P = .13$). After 2 hours and 24 hours of relaxation, the constructs possessed similar shape retention to native tissue.

Figure 3.

There was no significant change in construct shape retention from immediately after release to 2 hours of relaxation (39.0% and 26.9%, respectively; $P = .238$). In addition, the retention did not change significantly between 2 and 24 hours of relaxation (26.9% and 21.7%; $P = .48$).