Published in final edited form as:

Otolaryngol Head Neck Surg. 2013 April ; 148(4): 576–581. doi:10.1177/0194599812474228.

Flexural Properties of Native and Tissue Engineered Human Septal Cartilage

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Abstract

Objective—To determine and compare the bending moduli of native and engineered human septal cartilage.

Study Design—Prospective, basic science.

Setting—Research laboratory.

Subjects and Methods—Neocartilage constructs were fabricated from expanded human septal chondrocytes cultured in differentiation medium for 10 weeks. Constructs (n=10) and native septal cartilage (n=5) were tested in a 3-point bending apparatus, and the bending moduli were calculated using Euler–Bernoulli beam theory.

Results—All samples were tested successfully and returned to their initial shape after unloading. The bending modulus of engineered constructs (0.32 ± 0.25 MPa, mean $\pm$ SD) was 16% of that of native septal cartilage (1.97 ± 1.25 MPa).

Conclusion—Human septal constructs, fabricated from cultured human septal chondrocytes, are more compliant in bending than native human septal tissue. The bending modulus of engineered septal cartilage can be measured, and this modulus provides a useful measure of construct rigidity while undergoing maturation relative to native tissue.

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This article was presented at the 2012 AAO-HNSF Annual Meeting and OTO EXPO, Washington, DC, September 9–12, 2012.

Keywords

bending modulus; septal cartilage; tissue engineering

Introduction

Craniofacial and maxillofacial surgical reconstruction often uses implants or grafts to repair defects created by trauma, congenital deformities, or previous surgical resection. Autologous, allogeneic and synthetic implants and grafts have all been successfully used; however, each has limitations.^{1–3} Synthetic materials are associated with resorption, remodeling, infection, and extrusion, while allogeneic materials carry the risk of immune rejection and disease transmission.³ Thus, autologous sources are often preferred, and donor sites from the nasal septum, auricular conchal bowl, and costal region have all been used.⁴ Of the available autologous cartilage sources, nasal septal cartilage is favored for nasal reconstruction due to its mechanical properties, ease of harvest, and minimal donor site morbidity. However, use of nasal septal cartilage is limited by the size and shape of available material. Tissue engineering offers the potential to create an abundant quantity of septal cartilage from a small donor specimen.

The formation of clinically useful, engineered septal cartilage requires that the engineered tissue possess the necessary mechanical properties to withstand surgical manipulation and implantation as well as mechanical stresses *in situ*. The mechanical properties of tissue engineered septal cartilage created by the alginate-recovered chondrocyte (ARC) method have been previously assessed using confined compression and tension tests.^{5–7} These tests provide parameters that reflect intrinsic material properties. However, the conditions of surgical handling and the mechanical stresses present in the post-implant environment impose more complex loads onto septal cartilage. One such key mechanical challenge to these tissues is flexural loading, which occurs during bending. A common approach to assessing cartilage flexural properties is to perform a bending test.^{8,9} In such a test, cartilage is cut to a beam shape and mounted in a 3-point loading configuration, which supports the ends of the beam while applying load at the center and measuring the load-displacement relationship. From the load-displacement relationship, which reflects the dimension-dependent structural properties and is quantified as stiffness or failure load, material properties such as modulus or failure strength have been estimated assuming small or large deflection models.

Using such bending tests, the flexural moduli of native human septal, auricular, and costal cartilage have been reported.^{8–10} Cadaveric human septal tissue was tested at high deflection rate (1mm/s) to failure and the flexural modulus was reported to be 0.15 MPa.¹⁰ While the deflection model used to find the flexural modulus was not stated, this value is smaller than either the compressive modulus (0.4 – 0.71 MPa) or tensile modulus (3.0 MPa) of human septal cartilage, and thus outside the expected range.^{5,6} The flexural moduli of native human costal cartilage were found to be 8.8 MPa and 7.1 MPa, while native human auricular cartilage has a flexural modulus of 4.6 MPa.^{8,9}

Regarding tissue engineered cartilage constructs, only the flexural moduli of costal and auricular neocartilage constructs have been determined. Values of 1.21 MPa and 0.66 MPa, respectively, were determined for such constructs after 12 weeks of *in vivo* maturation in nude mice.⁸ The flexural modulus of tissue engineered human septal neocartilage has not previously been reported.

This work serves to establish benchmark values for human septal cartilage flexural properties at small strain and low strain rate, and to compare these properties with those of a current tissue engineered septal cartilage prototype that is fabricated using the ARC method. Specifically, flexural stiffness and modulus were determined for native and tissue engineered septal cartilage.

Materials and Methods

Collection of Human Septal Cartilage

Human septal cartilage specimens removed during routine septoplasty and septorhinoplasty at the University of California, San Diego Medical Center and/or San Diego Veterans Affairs Medical Center were used for the study (prior approval by the Human Subjects Committee of the Veterans Administration San Diego Healthcare System and University of California, San Diego Human Research Protection Programs). All cartilage specimens were taken from the inferior septum (just superior to the maxillary crest). Specimens were dissected free of perichondrium and the remaining full thickness septal cartilage was carefully inspected for damage or trauma that could compromise mechanical evaluation. At the time of harvest, specimens were placed in sterile normal saline and transported to the laboratory at 4°C within 24 hours.

Creation of Tissue Engineered Septal Neocartilage Constructs

Neocartilage constructs were created using the previously published ARC method.⁷ Briefly, chondrocytes were first prepared from human septal cartilage. Cartilage was diced into ~1mm³ pieces and subjected to enzymatic digestion. The isolated chondrocytes were resuspended in cell culture medium and seeded in flasks at a density of 5,000 cells per cm² surface area. Cells were then incubated at 37°C with 5% CO₂/air in cell culture medium (DMEM, 2% pooled human AB serum (HS), 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, 1ng/ml TGF-β1, 5 ng/mL FGF-2, and 10 ng/mL PDGF-ββ).

Next, the expanded cells were incubated in alginate beads. The expanded cells were released from monolayer and resuspended in alginate at a density of 4×10^6 cells/mL. Alginate-chondrocyte droplets were polymerized in 102 mM calcium chloride for five minutes. The volume of each bead was approximately 10 mm³ (40,000 cells). The beads were then incubated in cell culture medium (DMEM/F-12, 2% HS, 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, 200

ng/mL Insulin-like growth factor 1 (IGF-I), and 100 ng/mL growth differentiation factor 5 (GDF-5)) for two weeks.

Cartilaginous constructs were then formed. The alginate beads were depolymerized and chondrocytes with ECM were recovered by centrifugation. The cell pellet was resuspended in culture medium and seeded into 12mm transwells at a density of 1.33×10^6 cells/cm². After 6 weeks in culture, neocartilage constructs were transferred to 50 mL rotary cell culture vessels (Synthecon, Inc., Houston, TX) and cultured for an additional 4 weeks before mechanical testing. Culture medium changed every 2–3 days throughout the construct culture period.

Sample Preparation and Mechanical Testing

All samples were prepared for 3-point bending tests by measuring and cutting samples into strips of specific geometry. Native cartilage (from n=5 patients) and tissues engineered constructs (from the cartilage of n=10 patients) were cut into rectangular 10 mm × 3 mm strips of varying thickness. Thickness was measured at 3 sites over the length of the sample using a laser displacement sensor (± 0.024 mm resolution). Samples were kept moist with PBS containing protease inhibitors during preparation.

Samples were then tested using a 3-point bending test to determine load and displacement as a function of time. Tissue strips were placed on the supports (span, L = 8mm) of a custom 3-point bending apparatus (Figure 1). A cylindrical stainless steel pin ($\phi=4.75$ mm) was attached to a micromechanical testing system (Mach1TM V500cs, Biosyntech, Montreal, Canada) with a 1 kg load cell. The pin was lowered at a constant rate of 0.05mm/s until a tare load of 0.2g was reached to establish contact with the sample. The upper pin was then lowered at a constant rate of 0.05mm/s until a displacement of 3 mm was reached. Load and displacement signals were sampled at a rate of 10Hz.

Data Analysis

The flexural stiffness (D) and modulus (E_f) were calculated for each specimen using the sample dimensions, load and displacement data, and classical Euler–Bernoulli beam theory.¹¹ The displacement data were analyzed for a maximum of 5% percent strain, to allow application of the beam theory which is applicable in a small strain regime.

The 5% strain point was determined using the following relationship for flexural strain within a beam of rectangular cross-section, which is

$$\epsilon_f = \frac{6h\Delta d}{L^2}$$

where L is the span length of the specimen, h is the thickness of the specimen, and Δd is the sample deflection; ie, displacement applied to the specimen, which yields 5% strain.

The flexural stiffness was found using the slope of the load-displacement curve, from the relationship

$$D = \frac{\Delta F}{\Delta d}$$

where F/d is the slope of the load-displacement curve in the linear region, as determined by linear regression through the origin, performed using MATLAB R2012a (MathWorks, Natick, MA).

This flexural stiffness was then related to flexural modulus by normalizing to the dimensions of the sample and span width. In particular, the theoretical flexural modulus for a beam of rectangular cross-section is

$$E_f = \frac{DL^3}{4bh^3}$$

where b is the specimen width.^{9,11}

Statistical Analysis

Donor age, sample thickness, and the flexural stiffness and modulus of the native and tissue engineered cartilage constructs were compared using a two-tailed Mann-Whitney U test. The significance threshold was chosen to be $p < 0.05$. All statistics were performed in SYSTAT 10.2 and Microsoft Excel 2010. All values are reported as mean $\pm$ standard deviation (SD).

Results

The native and tissue engineered (Figure 2) groups were from patients of similar age ranges, had similar sample thicknesses, but were from patients of somewhat different gender distribution. There was no significant difference in age between the native (35.6 ± 10.7 (21–48) years old) and tissue engineered (42.3 ± 12.7 (27–61) years old) groups ($p=0.30$). There was also no significant difference in thickness between the native (1.66 ± 0.38 mm) and tissue engineered (1.34 ± 0.48 mm) groups ($p=0.18$). The native tissue group consisted of 5 male donors, while the tissue engineered group consisted of 4 male and 6 female donors.

In 3-point bending tests, loads (Figure 3A, C) and displacements (Figure 3B, D) increased approximately linearly with time for both the native and tissue engineered samples. There was also a linear trend in the average load vs. displacement curve for each group (Figure 4A, B). The correlation coefficients (R^2) for the linear regressions were 0.94 ± 0.06 and 0.86 ± 0.14 for the native and tissue engineered cartilage groups, respectively. No samples fractured during testing and all samples returned, grossly, to their initial shape after unloading.

At the 5% strain point, there was no statistical difference between the native (0.34 ± 0.09 mm) and tissue engineered (0.45 ± 0.16 mm) sample deflections (d) ($p=0.18$). At this strain level, the load (F) generated by the native tissue (0.06 ± 0.04 N) was almost 10-fold higher

than that of the tissue engineered constructs (0.007 ± 0.006 N, $p < 0.005$). The resultant flexural stiffness was much higher for the native tissue (0.19 ± 0.15 N/mm) compared to the tissue engineered constructs (0.014 ± 0.019 N/mm, 7% of native, $p < 0.005$).

Normalization of flexural stiffness to geometrical parameters yielded the material property of flexural modulus, which was distinct between the two groups. Flexural modulus for native tissue (1.97 ± 1.25 MPa) was substantially higher than that of the tissue engineered construct (0.32 ± 0.25 MPa, 16% of native, $p < 0.05$).

Discussion

In this study, the bending properties of native and tissue engineered septal cartilage were characterized by assessment of flexural stiffness and modulus using a 3-point bending test. Using samples of similar thickness and donor age range, bending tests showed linear load-displacement curves with a greater stiffness and bending modulus for the native septal cartilage than for the tissue engineered constructs.

There are a number of limitations to the present study. All native cartilage specimens were from male donors, while the tissue engineered cartilage specimens were gender-mixed. However, no trends with gender were found within the tissue engineered cartilage group (data not shown), and this imbalance may not have affected the results substantially, as age and gender have not been shown to significantly or markedly affect tensile or compressive properties of human nasal septal cartilage.^{5,6}

There are a number of advantages and disadvantages associated with the 3-point bending test, and the application of Euler-Bernoulli beam theory. Some advantages of this approach are the relatively simple test configuration, the ability to define deflections within a small strain ($< 5\%$) regimen, and the relative insensitivity of the results to the deformation rate (data not shown). However, this theory does not account for transverse shear strains or beam elongation, and also certain cartilage material properties. In particular, the linear material assumption is an approximation since septal cartilage exhibits mechanical non-linearity at higher tensile strains⁵, and a non-linear tension-compression relationship, evident in bending.¹² Nevertheless, in the 0–5% strain region, the linear regressions of stiffness showed high R^2 values for both groups. The return to original shape after loading also suggests that the samples remained in the elastic region. Thus, an apparent bending modulus can be readily estimated.

The flexural modulus of native human septal cartilage calculated in this study was substantially greater than that from a recent study of cadaveric human septa¹⁰, and slightly less than values reported for native human auricular cartilage.⁸ The difference in flexural modulus for native human septal cartilage reported in this study and that of cadaveric human septal cartilage¹⁰ could be due to the method of determining flexural modulus. Alkan et al used the linear region of the stress vs. strain curve, which may have extended beyond small strains, whereas the present study utilized the 0–5% strain region. Alternatively, regional differences in flexural moduli across the septa may explain differences. Alkan et al tested

strips from the central septum¹⁰, while all samples analyzed in this study came from the inferior septum. Sample thickness appeared to be very similar between the two studies.

In the normal nose, gravitational and elastic forces are present within the nasal skin, muscle, and fibrocartilaginous connections in-between the cartilage components. The flexural properties of the hyaline cartilage of the nasal framework provide spring-like resistance to loading, and the arch-like shape of the nose distributes the internal and external compressive forces. It is mainly the flexural property provided by the shaped cartilaginous framework of the nose that maintains the airway function of the nose. In cases involving structural grafting, the airway function would need to be maintained or reestablished. Therefore, it is essential that grafts used in such situations have appropriate load-bearing and shape-maintaining properties.

The results for the tissue engineered septal cartilage prototype extend characterization of such tissues. The tissue engineered septal constructs had properties similar to tissue engineered auricular cartilage, and both were substantially more compliant than native tissue.⁸ In contrast, unlike the present study, Roy et al. measured flexural properties after subcutaneous implantation in nude mice for 12 weeks, which leads to cartilage stiffening.¹³ Thus, the present study, together with two others, represent some of the first attempts to characterize one important property of engineered craniofacial cartilage.

The flexural stiffness values may prove to be useful for fabricating engineered neocartilage tissue for specific grafting purposes. In addition, the bending stresses of both native and tissue engineered septal cartilage implants can be calculated by knowing the flexural modulus and tissue deflection.

This study provides a foundation for further septal cartilage bending studies. The testing modality may be conveniently applied to future studies involving native and tissue engineered implants or grafts. It may also be used to estimate physiological stresses and strains associated with surgical implant. In addition, analysis of time-dependent properties, and effects of varying bending strain rates, may also elucidate loading behavior for improving surgical implantation methods and long term cartilage grafting outcomes. To increase the flexural modulus of tissue engineered constructs to be closer to native values, future studies may assess the effects of donor cell source and region, as well as the growth factor stimuli used during culture. In addition, static or dynamic mechanical stimulation during the culture period may be investigated in the future.

Acknowledgments

This material is based upon work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development (BL R&D Merit Award (D.W.)) and NIH R01 AR044058 (R.L.S.).

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Figure 1.
Schematic of 3-point bending apparatus loaded with sample tissue in the unloaded (A) and loaded (B) positions.

Figure 2.
Representative 12mm tissue engineered human septal neocartilage construct after 10 weeks in culture.

Figure 3.

Raw load vs. time and representative load vs. displacement data between tare and 5% strain for the median native (A, C) and tissue engineered (B, D) cartilage samples with correlation coefficients (R^2) shown.

Figure 4. Average load-displacement curves from 3-point bending tests of native (A) and tissue engineered (B) cartilage with correlation coefficients (R^2) shown. Error bars represent SD.