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Volume Expansion of Tissue Engineered Human Nasal Septal Cartilage

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Abstract

Importance—Cartilaginous craniofacial defects range in size and autologous cartilaginous tissue is preferred for repair of these defects. Therefore, it is important to have the ability to produce large size cartilaginous constructs for repair of cartilaginous abnormalities.

Objectives—

1. To produce autologous human septal neocartilage constructs substantially larger in size than previously produced constructs
2. To demonstrate that volume expanded neocartilage constructs possess comparable histological and biochemical properties to standard size constructs
3. To show that volume expanded neocartilage constructs retain similar biomechanical properties to standard size constructs

Design—Prospective, basic science

Setting—Laboratory

Participants—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. Cartilage from a total of 8 donors was collected.

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Conflict of Interest

There is no conflict of interest among authors.

Main Outcomes Measured—Human septal chondrocytes from 8 donors were used to create 12mm and 24mm neocartilage constructs. These were cultured for a total of 10 weeks. Photo documentation, histological, biochemical, and biomechanical properties were measured and compared.

Results—The 24mm diameter constructs were qualitatively similar to the 12mm constructs. They possessed adequate strength and durability to be manually manipulated. Histological analysis of the constructs demonstrated similar staining patterns in standard and volume expanded constructs. Proliferation, as measured by DNA content, was similar in 24mm and 12mm constructs. Additionally, glycosaminoglycan (GAG) and total collagen content did not significantly differ between the two construct sizes. Biomechanical analysis of the 24mm and 12mm constructs demonstrated comparable compressive and tensile properties.

Conclusion and Relevance—Volume expanded human septal neocartilage constructs are qualitatively and histologically similar to standard 12mm constructs. Biochemical and biomechanical analysis of the constructs demonstrated equivalent properties. This study shows that modification of existing protocols is not required to successfully produce neocartilage constructs in larger sizes for reconstruction of more substantial craniofacial defects.

Level of Evidence—NA.

Keywords

Cartilage tissue engineering; Human septal cartilage; Cartilage construct

Introduction

Cartilaginous craniofacial defects may result from tumor resection, trauma, and congenital deformities. Analogous reconstructive material is used to repair these defects to produce optimal structural and functional results. Components used for reconstruction include autologous, allogenic, and synthetic materials. Autologous tissue is favored as the use of synthetic grafts may be complicated by infection and extrusion, while allogenic grafts carry the risk of immune rejection and disease transmission [1-4]. The nasal septum, auricle, and rib are potential donor sites for autologous cartilage. Nasal septal cartilage possesses significant advantages over auricular and costal cartilage due to its superior structural properties, ease of harvest, and minimal donor site morbidity. However, only a finite supply of nasal septal cartilage in a predefined configuration is available for grafting. Moreover, this can be further limited by trauma, congenital deformities, or iatrogenic septal defects. Previous studies have successfully produced tissue engineered autologous neocartilage that may eventually be used for reconstructive surgery [5-7].

Nasal septal cartilage engineering begins with harvest of cartilage from a donor followed by isolation of chondrocytes. Chondrocytes are then proliferated in monolayer culture, initiating a phenotypic shift to a fibroblastic structure 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 extracellular matrix (ECM) [10-12]. The redifferentiated cells are then incubated to form neocartilage constructs which can ultimately be used for clinical application.

There have been many advances in nasal septal cartilage engineering culminating in the development of tissue engineered nasal septal constructs that nearly mimic the biological and biomechanical properties of native septal cartilage. However, the production of nasal septal constructs has been limited to 12mm flat discs. While this accomplishment is extraordinary, the repair of certain head and neck defects is limited, nevertheless. Traumatic, congenital, and iatrogenic deformities come in myriad sizes and shapes. Therefore, the application of autologous tissue engineering for repair of these defects requires the formation of septal constructs that are larger in size and can acquire various configurations.

In theory, the use of autologous tissue engineered septal cartilage for a clinical case will involve several steps. During the initial procedure, a small piece of septal cartilage will be harvested from the patient and placed in a sterile vial. The tissue would be sent to a laboratory where the tissue processing can begin. The tissue will be digested and cultured as described above. A key element of this process would involve tailoring the tissue engineering process to the specific needs of the patient. The surgeon would define the quantity of tissue required as well as the particular size and shape needed to repair a patient's defect. In essence, the surgeon would dictate the exact product to be produced. The culture of larger septal constructs is the next step in bringing this plan to fruition. However, there are no existing studies demonstrating the production of clinically relevant septal neocartilage constructs of large size. The goal of this investigation is to apply previous culture methods to the production of 24mm constructs [5,6]. Structural, biochemical, and biomechanical properties of neocartilage constructs will be evaluated and compared to those of standard 12mm constructs.

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. IRB approval was obtained at both institutions. Cartilage from a total of 8 donors was collected. The cartilage from these donors was dissected free of perichondrium and diced into pieces (1 mm^3). The fragments were digested as reported previously [5]. Suspensions of digested cartilage were filtered (70 mm), 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 μg/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 μg/mL streptomycin sulfate, 0.25 μg/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 (5,000 cells per cm^2 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 1ng/mL transforming growth factor - beta-1 (TGFr-bb (PDGF-bb), and changed every two 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.5After 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 μg/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 bone morphogenic protein-14(BMP-14), and 200ng/ml insulin growth factor-1 (IGF-1) were placed in the media bottle and changed every 2-3 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 55mM sodium citrate and 0.15mM NaCl. Centrifugation at 750 g for five 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 4×10^6cells/mL. This suspension was used to seed at least one 12mm and one 24mm diameter transwell clear polyester membrane insert (Corning, Inc., Corning, NY) per donor at 1.33×10^6cells/cm^2. Culture medium was changed every other day for 6 weeks. After 6 weeks of culture, the two 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 millilitres of culture medium (DMEM/F-12, 25 μg/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 BMP-14, and 200ng/ml IGF-1were placed in the rotary culture vessel and changed every 2-3 days. The constructs were cultured for an additional 4 weeks in the rotary culture vessel.

Culture Termination

Each construct was weighed upon culture termination. A portion of each construct was set aside for biomechanical testing. The remainder of the construct was divided for structural and biochemical testing. The portions of sample used for biochemical testing were digested, one with proteinase K (PK) in phosphate-buffered EDTA and the other with pepsin, overnight. The remainder of the construct was placed in optimum cutting temperature (OCT) compound and frozen for histochemical analysis.

Biochemical Analysis

Cellularity of the constructs was tested using the PicoGreen DNA content determination assay as described in a previous report [5,13]. 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 dimethyl-methylene blue (DMMB) reaction [5]. 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].

Histology

Constructs were analyzed by histochemistry to localize GAG and by H&E staining. Samples to undergo histochemical analysis were placed in OCT compound and frozen by immersion in liquid nitrogen-cooled isopentane. They were sectioned in a cryostat at either 30μm (bioreactor constructs) or 40μm (transwell and static bottle-cultured constructs) thickness. The sections were placed on poly-L lysine coated slides (Polysciences Inc., Warrington, PA) and allowed to dry overnight. Staining with H&E was performed as previously described [17]. For histochemical localization of GAG, slides from each sample group were stained with 0.1% alcian blue in buffer (0.4 M $MgCl_2$, 0.025 M Sodium acetate, 2.5% glutaraldehyde, pH 5.6) overnight, and destained with 3% acetic acid until clear [18]. Samples were then observed and photodocumented using light microscopy.

Immunohistochemistry was performed with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), a peroxidase-based detection system. The slides were prepared as described above. After rinsing in ABC buffer, the samples were blocked with 2.5% normal horse serum for 20 minutes. The samples were then probed with one of three antibodies: anti-collagen type I (Sigma, St. Louis, Mo) at a dilution of 1:2000, anti-collagen type II (Chrondrex Inc, Redmond, WA) at a dilution of 1:10000, or a mouse nonspecific IgG used at 1 ug/mL as a negative control. Sections were counterstained with methyl green nuclear stain (Vector Laboratories, Burlingame, CA). Samples were then documented by photomicroscopy.

Biomechanical Testing

Each construct was subjected to compression testing. A 4.8 mm diameter disc was punched out from the constructs and the average thickness was determined from 3 measured locations manually using a current sensing micrometer. The discs were then transferred to a confined test chamber between porous platens. The chamber was filled with phosphate buffered saline (PBS) with proteinase inhibitors at 22°C and attached to a mechanical spectrometer (DynaStat, IMASS, Accord, MA). Automated electromechanical testing and data acquisition were implemented by interfacing a computer-controlled function generator (HP33120A, Hewlett-Packard, Palo Alto, CA) to the mechanical spectrometer, and the load and displacement signals from the spectrometer as well as the streaming potential signal from the amplifier to a multi-function 16 bit I/O board (NB-MIO-16XH-42, National Instruments, Austin, TX). A one kilogram load cell with attached plunger was used. The test sequence consisted of applying 15, 30, and 45% ramp compression over 400 seconds each to the sample and allowing the resultant load to relax to equilibrium for 1200 seconds. This was followed by application of a series of oscillatory displacements decreasing in amplitude (relative to the compressed thickness) at frequencies of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 Hz while the load was measured. The compressive properties of the constructs were estimated from the acquired data assuming tissue homogeneity.

To perform tensile testing, tensile strips (0.8 mm gauge wide × 8 mm long) were punched from remaining tissue constructs after compression samples were removed. Average thickness was determined from 3 measured locations manually using a current sensing micrometer. Tensile tests were performed on a materials testing machine according to methods used previously [19]. Briefly, samples were clamped and hydrated with PBS with proteinase inhibitors. Specimens were subjected to a tare load of 0.05 N and then elongated to 10% and 20% strain at 0.25%/s with 900 s of relaxation to equilibrium at each strain step. Specimens were then pulled to failure at 5 mm/min. An equilibrium tensile modulus was determined from the slope of a linear least-squares fit of the data at 0%, 10%, and 20% strain steps. A dynamic tensile modulus was determined as the slope of a linear least squares fit of the data between 25-75% of the maximum stress during the pull to failure. Strength and failure strain were determined as the maximum stress and corresponding strain.

Statistical Analysis

Analysis was performed using Systat 10.2 (Systat Software, Chicago, IL). Means are presented ± the standard deviation (SD). Differences in DNA content per mg wet weight, GAG per DNA and mg wet weight, and total collagen per DNA and wet weight, confined compression modulus, peak stress, and stiffness at failure strain were assessed using a two-sample t-test. A difference was considered significant when $p \leq 0.05$.

Results

Septal cartilage was obtained from a total of 8 donors with a mean age of 37.75 years; 6 of these patients were male and 2 were female. The 12mm and 24mm diameter constructs were qualitatively similar with a smooth, white, and opaque surface and comparable thickness (Figure 1). They possessed adequate strength and robustness to be manually manipulated.

Biochemical analysis did not demonstrate a significant difference in composition between the two construct sizes (Figure 2). Cellularity did not differ between the 12mm and 24mm diameter constructs (0.182 ± 0.06 and 0.180 ± 0.06 μg DNA per mg wet weight tissue, respectively; p=0.892). Additionally, the 12mm and 24mm diameter constructs possessed similar quantities of GAG (85.22 ± 60.13 and 73.36 ± 44.64 μg GAG per μg DNA, respectively; p=0.329). The total collagen content did not vary significantly between the two constructs sizes (363.37 ± 118.31 (in 12mm) and 303.98 ± 98.69 (in 24 mm) μg total collagen per μg DNA; p=0.052). However, the p-value approaches significance, implying the larger constructs tended to contain less collagen per wet weight compared to the smaller constructs.

Histological examination of the neocartilage constructs confirmed the biochemical findings above. H&E staining of the constructs demonstrated analogous proliferation and quantity of ECM surrounding each cell in both construct sizes. Alcian Blue staining demonstrated robust staining in 12mm and 24mm constructs, indicating comparable presence of sulfated GAGs. Immunohistochemical staining showed significant type II collagen content and confirmed the absence of type I collagen in the constructs. Histological data is not shown.

Compression testing indicated similar compressive properties in both constructs (Figure 3). The confined compression modulus of the 12mm and 24mm diameter constructs was 0.048 ± 0.014 and 0.024 ± 0.020 MPa, respectively (p=0.436). The tensile properties of the constructs were also analogous (Figure 3). Peak stress was measured at 0.519 ± 0.431 and 0.388 ± 0.542 MPa for the 12mm and 24mm diameter constructs, respectively (p=0.719) Stiffness at failure strain was 2.313 ± 2.593 and 2.411 ± 1.812 MPa for the 12mm and 24mm diameter constructs, respectively (p=0.952).

Discussion

Acute Volume expanded human septal neocartilage constructs exhibit equivalent cell proliferation and ECM accumulation compared with previously generated constructs of standard size (12mm). Moreover, total collagen content in the two size constructs is comparable. The analogous biochemical properties of the constructs are reflected in their gross similarity and resilience to manipulation. Additionally, histological analysis confirmed that the constructs possessed similar composition as exhibited by biochemical analysis. Biomechanical analysis of the constructs consisted of compression and tensile testing. Both analyses showed similar biomechanical properties of the two construct sizes.

When compared with native human nasal septal cartilage, the 12mm and 24mm constructs contain less DNA (1.38-fold) and GAG (2.2-fold). Total collagen content was also slightly lower in both size constructs compared with native human septal cartilage (1.41-fold and 1.7-fold in 12mm and 24mm constructs, respectively) [20]. The constructs are also softer than native human septal cartilage tissue with confined compression modulus values that are 9.2 and 18.3-folds lower (12mm and 24mm constructs, respectively) [21]. The tensile strength of the constructs is 3.66 and 4.89-folds lower than that of nasal septal cartilage (12mm and 24mm constructs, respectively). Similarly, the constructs possessed tensile stiffness at failure strain values that are 2.1-fold lower than native tissue [22]. While the biochemical and biomechanical properties of the constructs produced in this study are not equivalent to those of native human septal cartilage, they are very close to approaching similitude. Moreover, analysis of both size constructs in this study produced values that are far superior to previous studies performed by our group (pending publication). We believe this results from implementation of our new culture protocol that involves culture in a bioreactor. Overall, we have successfully created improved constructs, bringing us closer to the eventual development of clinically useful constructs that more closely resemble native septal cartilage.

This study was limited by the small sample size. Additionally, the sample group possessed more male than female donors. However, this imbalance was not believed to greatly affect results, as gender has not been shown to significantly affect biochemical properties, tensile strength, or compressive properties in human nasal septal cartilage [21,22].

Previous cartilage tissue engineering studies have successfully created articular and auricular cartilage constructs of comparable size to the standard 12mm nasal septal cartilage constructs created in this and previous studies. However, there has been limited research investigating the production of larger constructs. Whitney et al. [23] used auricular and

articular chondrocytes from mature rabbits to produce large scaffold-free cartilage constructs. Their study successfully created 4cm by 4cm cartilage sheets. Similarly, Weidenbecher et al. [19] used auricular chondrocytes from rabbits to engineer scaffold-free cartilage sheets that were then used to create a neotrachea. Both studies used rabbit chondrocytes for their investigation. The use of human nasal septal chondrocytes for the production of larger cartilage constructs has not been previously evaluated. Traumatic, congenital, and iatrogenic cartilaginous craniofacial defects can range from small to very large. Therefore, it is paramount to possess the ability to apply cartilage tissue engineering for the creation of larger pieces of autologous tissue for reconstruction of these defects. This is the first description of the creation of human nasal septal neocartilage constructs of a larger size.

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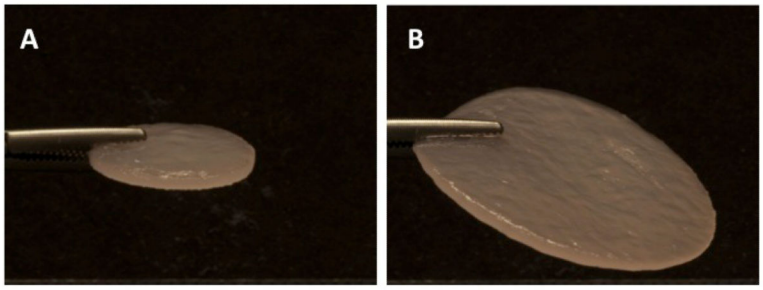

Figure 1.

Photographs of neocartilage constructs from one patient sample. (A) 12mm and (B) 24 mm constructs are shown. The constructs were similar in gross morphology and thickness.

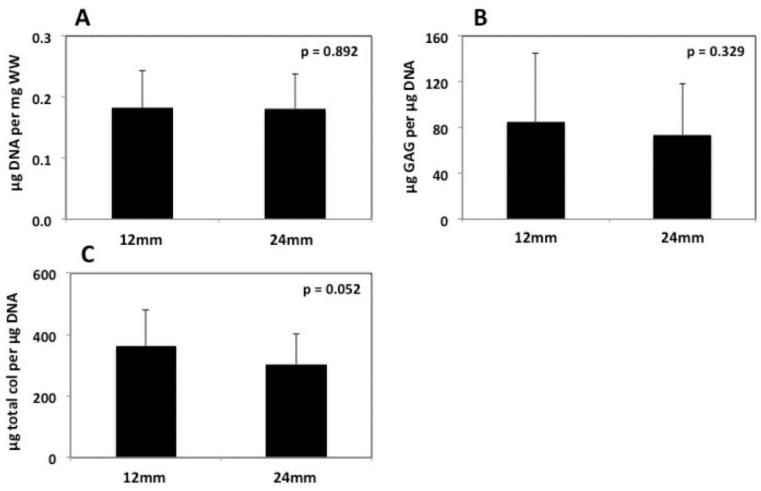

Figure 2.

Biochemical properties of 12mm and 24mm diameter human septal neocartilage constructs. (A) DNA per mg of tissue wet weight, (B) GAG per DNA, and (C) total collagen per DNA are shown. DNA content, GAG, and total collagen content did not significantly differ between the two construct sizes. Error bars depict standard deviation. GAG, glycosaminoglycan; WW, wet weight; col, collagen.

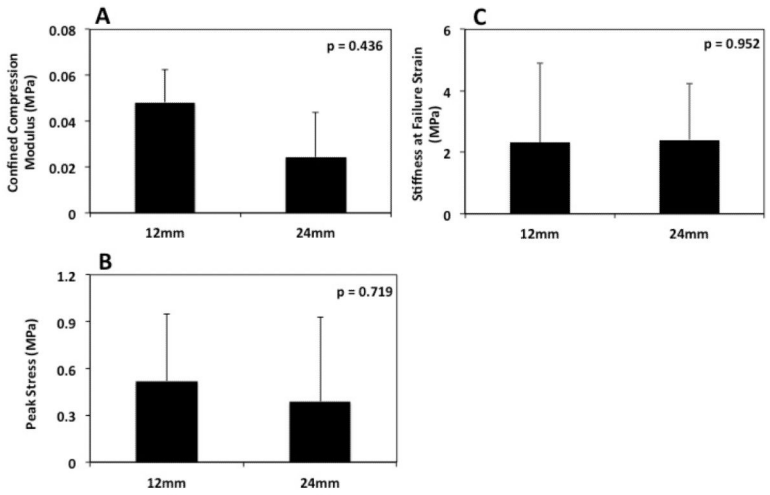

Figure 3.

(A) Compression testing showed that the larger constructs possessed similar compressive properties to the smaller constructs with no statistically significant difference between the two. (B) Tensile testing showed that the constructs exhibited similar strength and (C) stiffness as measured by peak stress and stiffness at failure strain.