Tissue Engineering of Human Septal Cartilage Using a Rotary Bioreactor

Marsha S. Reuther[1,2], Van W. Wong[3,4], Kristen K. Briggs[3,4,*], Barbara L. Schumacher[3,4], Robert L. Sah[3], Koichi Masuda[5] and Deborah Watson[1,2]

[1]*Division of Otolaryngology-Head and Neck Surgery, University of California, San Diego, La Jolla, CA, USA*

[2]*Head and Neck Surgery Section, VA San Diego Healthcare System, San Diego, CA, USA*

[3]*Department of Bioengineering, University of California, San Diego, La Jolla, CA, USA*

[4]*Research Service, VA San Diego Healthcare System, San Diego, CA, USA*

[5]*Department of Orthopedic Surgery, University of California, San Diego, La Jolla, CA, USA*

Abstract: Tissue engineering of human septal neocartilage offers the potential to produce large quantities of autologous material for use in the repair of cartilaginous craniofacial defects. Culture of septal neocartilage constructs in a rotary cell culture system bioreactor may improve the biochemical and biomechanical properties of engineered tissue. The objective of this study was to characterize the morphological, compositional and mechanical properties of engineered human septal cartilage constructs when cultured in three different environments. Human septal cartilage constructs were cultured in transwell plates for 6 weeks and subsequently cultured for an additional 4 weeks in transwell plates, free-floating in a sterile media bottle, or in a rotary cell culture system bioreactor. The histologic, biochemical, and biomechanical properties of the constructs were examined. All constructs maintained their radial dimensions throughout the culture period. Qualitatively, the constructs cultured in the rotary cell culture system bioreactor were firmer and more resilient compared with the constructs cultured under free-floating static and transwell plate conditions. Culture of human septal neocartilage constructs in a rotary cell culture system bioreactor augmented cell proliferation and extracellular matrix production when compared with constructs cultured in transwell plates and free-floating static conditions. Additionally, rotary cell culture system bioreactor culture enhanced total and type II collagen production. The rotary cell culture system bioreactor constructs possessed improved biomechanical properties compared with the other conditions and this is reflected in their superior resistance to manipulation. These findings suggest that culture in a rotary cell culture bioreactor can modulate the composition and mechanical function of engineered scaffold-free human septal cartilage constructs to produce constructs better suited for reconstructive surgery.

Keywords: Cartilage tissue engineering, human nasal septal tissue, bioreactor, rotary vessel, cartilage constructs, cartilage reconstruction.

INTRODUCTION

The repair of cartilaginous craniofacial defects created by trauma, tumor resection, or congenital deformities requires analogous reconstructive material to obtain optimal results. Autologous, allogenic, and synthetic structures are used for grafting. Allogenic grafts pose the risk of immune rejection and disease transmission, while the use of synthetic grafts is associated with infection and extrusion [1-4]. Due to these limitations, autologous grafts are favored. The nasal septum, auricle, and rib are potential autologous cartilage donor sites. Nasal septal cartilage offers significant advantages over these other cartilage donor sites mentioned due to its superior structural properties, ease of harvest, and minimal donor site morbidity. However, the use of nasal septal cartilage is limited by the finite amount of tissue available and potentially suboptimal geometric structure for repair of some defects. Tissue engineering of autologous neocartilage, therefore, offers the potential to produce large quantities of autologous cartilage from a small donor specimen and affords the ability to create grafts in defined shapes and sizes.

Nasal septal cartilage engineering involves several key steps. After cartilage is harvested from a donor, the extracellular matrix (ECM) is digested, thereby isolating the chondrocytes. Chondrocytes are then proliferated in monolayer culture which causes them to undergo a shift toward a fibroblastic phenotype in a process called dedifferentiation. This step is critical for expansion of cell number. Monolayer culture is accompanied by a change from the production of type II collagen (characteristic of native chondrocytes) to type I collagen (characteristic of fibroblasts) [5, 6]. The dedifferentiated chondrocyte may then be induced to re-differentiate into its native phenotype, thereby redifferentiating. To promote redifferentiation, chondrocytes are cultured in a three-dimensional (3D) configuration which induces redifferentiation and restores the chondrocyte phenotype, enabling

*Address correspondence to this author at the Cartilage Tissue Engineering Lab, University of California, San Diego, PFBH, Room 314, 9500 Gilman Drive, MC 0412, La Jolla, CA 92093-0412, USA; Tel: (858) 248-2866; Fax: (858) 552-7466; E-mail: briggskk@gmail.com

E-ISSN: 2311-1755/14

production of functional cartilaginous ECM and the subsequent formation of neocartilage constructs [7-9]. Chondrocyte redifferentiation from the fibroblastic phenotype is influenced by multiple additional factors, including cell seeding density, media composition, growth factors, 3D scaffold properties, diffusion, and mechanical stimulation.

Optimization of the media formulation used for chondrocyte redifferentiation improved the histological, biochemical and biomechanical properties of engineered septal neocartilage constructs [10, 11]. However, the biochemical and biomechanical properties of these constructs were still significantly lower than values reported for native human septal cartilage [12-15]. These properties were further improved when engineered septal cartilage constructs were subjected to a period of *in vivo* maturation in athymic nude mice [16]. In an effort to develop an *in vitro* process for promoting maturation of engineered septal cartilage, bioreactor systems that have been demonstrated suitable for the three-dimensional culture of scaffold-free tissues and impart a mechanical stimulus were considered.

Studies have shown that cartilage formation is favorably influenced by mechanical stimulation [17]. Consequently, this is an important aspect of the culture environment that can be manipulated to potentially produce superior tissue engineered cartilage. In addition, it has been reported that the supply of oxygen and soluble nutrients is limited during static *in vitro* culture of 3D tissue with studies demonstrating that cellular spheroids larger than 1mm in diameter are composed of necrotic hypoxic centers rimmed by viable cells [18]. Bioreactors have been developed to control mechanical stimuli and fluid flow in order to address this issue.

The rotary cell culture bioreactor (RCCS bioreactor), developed at NASA's Johnson Space Center, provides an environment in which the cells or tissues are in perpetual free fall countered by the upward force provided by rotation of the culture medium simulating a microgravity environment [19, 20]. The lack of impellers, airlifts, bubbles or agitators provides a low shear stress environment. Cells within the rotating bioreactor maintain a uniform orbit within the fluid suspension. A coaxial silicone membrane delivers oxygen to the unit without generating bubbles that can cause cell-damaging turbulence. Rotation of the culture medium transmits mechanical stimulus to the cells, and increases the exchange of oxygen, nutrients, and metabolic wastes. These features provide an improved culture environment when compared with conventional culture systems such as flasks and dishes [21].

The application of RCCS bioreactors to septal cartilage tissue engineering has been limited. Moreover, the development of tissue engineered nasal septal constructs that possess the biomechanical and biological properties of native tissue has not yet been achieved. The objective of the present study was to compare the effect of three different culture conditions--RCCS bioreactor, free-floating in a sterile media bottle or in a transwell plate--on the morphological, histological, biochemical and mechanical properties of engineered human septal cartilage constructs.

MATERIALS AND METHODS

Ethics Statement

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. Written informed consent was obtained from patients prior to surgery. Human protocols and consent forms were reviewed and approved by the Human Subjects Committee of the Veterans Administration San Diego Healthcare System and University of California, San Diego Human Research Protection Programs.

Chondrocyte Isolation and Expansion

The cartilage specimens were dissected free of perichondrium and diced into pieces (1 mm^3). The fragments were digested as reported previously [11]. Suspensions of digested cartilage were filtered (70 μm), 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 non-essential 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

(TGFβ-1), 5ng/mL fibroblast growth factor-2 (FGF-2), and 10ng/mL platelet-derived growth factor-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 [11]. 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 µg/mL ascorbate, 0.4mM L-proline, 2mM L-glutamine, 0.1mM non-essential 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 x 10^6 cells/mL. This suspension was used to seed at least three 12mm diameter transwell polyester membrane inserts (Corning, Inc., Corning, NY) per donor at 1.33 x 10^6 cells/cm^2. Culture medium was changed every other day for 6 weeks. After 6 weeks of culture, two constructs from each sample were released from the transwell insert, while the third construct remained in transwell culture. One construct from each sample was placed in a 250 mL Nalgene PETG square media bottle and a second construct was 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, 0.4mM L-proline, 2mM L-glutamine, 0.1mM non-essential 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 rotary culture vessel and changed every 2-3 days. Two milliliters of the same medium were used for each construct in the transwell inserts and this was changed every 2-3 days. The rotation speed of the rotary cell culture vessel was adjusted throughout the culture period to maintain each construct at a relatively steady position within the vessel. The constructs were cultured for an additional 4 weeks under these three conditions.

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.

Quantitative Assay for Cellularity

Cellularity of the constructs was tested using the PicoGreen DNA content determination assay as described in a previous report [11, 22]. DNA content was normalized per milligram wet weight.

Quantitative Assay for Glycosaminoglycan (GAG)

The GAG content was determined, as reported previously, using portions of the PK digests and the dimethyl-methylene blue (DMMB) reaction [23]. GAG content was then normalized per milligram wet weight [13].

Quantitative Assays for Total Collagen and Types I and II Collagen

The amount of solubilized type I and type II collagen in the constructs was quantified by enzyme-linked immunosorbent assay (ELISA) using human type I and native type II collagen kits (Chondrex Inc., Redmond, WA) [16]. Collagen was solubilized by sequential incubation with 5000 U/ml bovine hyaluronidase (Sigma-Aldrich, St. Louis, MO) at 4ºC overnight, followed by two incubations with 10mg/mL pepsin (Sigma-Aldrich, St. Louis, MO) dissolved in 0.05M acetic acid at 4ºC overnight. Finally, the samples were incubated with 1mg/mL pancreatic elastase (Sigma-Aldrich, St. Louis, MO) at 4ºC for 16 hours. Separate type I and type II collagen ELISAs were performed following the manufacturer's instructions. The optical density was read at 490nm using a spectrophotometric

plate reader. Collagen values were then normalized per milligram wet weight. The quantity of hydroxyproline in the constructs was determined as described previously [24]. Hydroxyproline content was converted to collagen content using a mass ratio of collagen to hydroxyproline of 7.1 [25].

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 [13]. 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 NaAcetate, 2.5% glutaraldehyde, pH 5.6) overnight, and destained with 3% acetic acid until clear [26]. Samples were then observed and photodocumented using light microscopy.

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.

Statistical Analysis

Analysis was performed using Systat 10.2 (Systat Software, Chicago, IL). Means are presented ± the standard error (SE). Differences in DNA content per mg wet weight, GAG per mg wet weight, type II collagen per wet weight, total collagen per wet weight, and confined compression modulus were assessed using a one-way analysis of variance (ANOVA). If the ANOVA identified an overall significant effect, post-hoc Tukey's HSD tests were used to identify significant differences between culture conditions. A difference was considered significant when $p \leq 0.05$.

RESULTS

The neocartilage constructs cultured in the media bottle (free floating static) and RCCS bioreactor (Figure **1**) resembled native septal cartilage with a smooth, firm, and opaque surface of solid, white tissue.

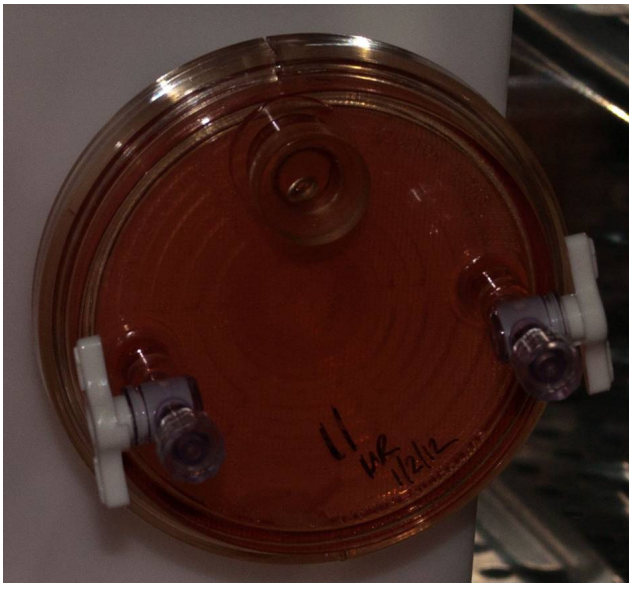

Figure 1: Photograph of a disposable rotary cell culture bioreactor vessel. The disc-shaped high aspect ratio vessel provides gas exchange through a membrane that forms the inside wall of the vessel. Ports allow for media exchange as well as sample addition and extraction. Engineered neocartilage constructs maintain a uniform orbit in the rotating culture medium.

Conversely, the constructs cultured in the transwell plate were softer and failed to maintain their shape

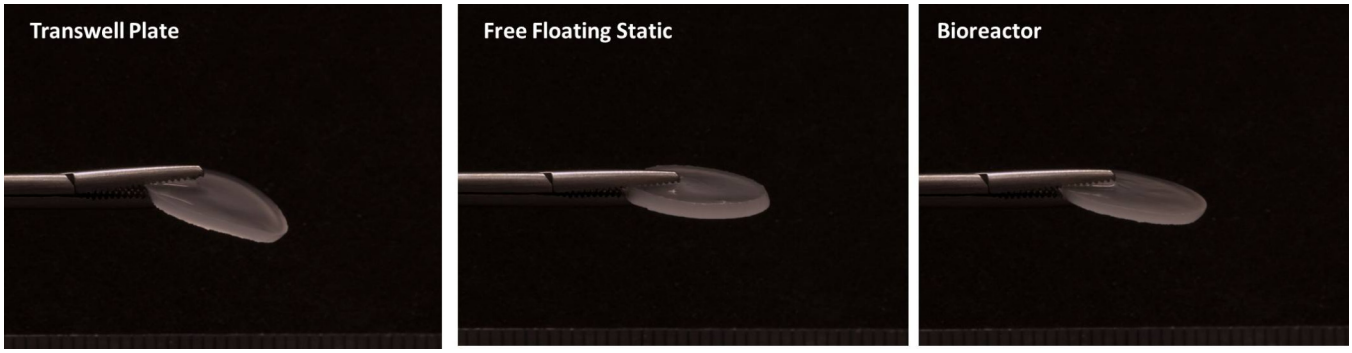

Figure 2: Photographs of representative constructs. The constructs cultured in the RCCS bioreactor and free floating static conditions were more robust and possessed sufficient strength to be manipulated and maintain their shape, while the construct cultured in the plate was more pliable and failed to maintain its shape during photography.

during manipulation (Figure **2**). Constructs cultured in all three conditions maintained their radial dimensions (Figure **2**).

The construct wet weight tended to decrease and the thickness tended to increase from the transwell condition to the static free floating condition, as well as in the RCCS bioreactor; however, the weights were not significantly different (Table **1**).

Biochemical testing demonstrated a significant difference in proliferation between the three culture conditions. The static free floating constructs possessed significantly more DNA per mg wet weight compared with the transwell plate constructs ($p < 0.05$; Table **1**). Additionally, the bioreactor constructs contained significantly more DNA than both the transwell and static constructs ($p < 0.0001$; Table **1**). GAG accumulation was significantly greater in the static and bioreactor constructs compared with the transwell constructs and did not differ significantly between the static and bioreactor conditions ($p < 0.05$; Table **1**). The total collagen content was greatest in the bioreactor constructs, followed by the static constructs ($p < 0.01$; Table **1**). The transwell constructs contained significantly less total collagen than constructs cultured under the other two conditions ($p < 0.05$; Table **1**). The content of solubilized type I collagen was below the limit of detection for the assay (0.08 µg/mL) for all conditions (data not shown). The bioreactor constructs showed a robust accumulation of solubilized type II collagen that did not differ significantly between the static and bioreactor culture conditions ($p > 0.05$; Table **1**). There was significantly less solubilized type II collagen in the transwell constructs compared with the static ($p < 0.05$) and bioreactor ($p < 0.01$) constructs (Table **1**).

Histologic examination of the neocartilage constructs supported the biochemical findings above.

Table 1: Composition and Mechanical Properties of Tissue-Engineered and Native Septal Cartilage

Parameter	Tissue-Engineered Septal Cartilage Constructs			Native Septal Cartilage
	Transwell	Free-Floating Static	RCCS Bioreactor	
Construct Composition				
Weight (mg)	259 ± 51.5 (9)	202 ± 41.2 (9)	172 ± 31.4 (9)	—
Thickness (mm)	1.77 ± 0.22 (9)	2.21 ± 0.73 (9)	2.43 ± 0.54 (9)	1.32 ± 0.20 (21)
DNA (µg/mg wet weight)	0.09 ± 0.01 (9)	0.15 ± 0.02 (9)	0.28 ± 0.03 (9)	0.19 ± 0.07 (33)
sGAG (µg/mg wet weight)	3.82 ± 0.15 (9)	8.34 ± 1.13 (9)	8.24 ± 1.28 (9)	28.7 ± 9.2 (33)
Total Collagen (µg/mg wet weight)	7.91 ± 1.07 (9)	13.1 ± 1.32 (9)	19.4 ± 1.54 (9)	87.1 ± 20.1 (33)
Soluble Type II Collagen (µg/mg wet weight)	1.19 ± 0.31 (9)	1.77 ± 0.43 (9)	2.01 ± 0.55 (9)	—
Mechanical Behavior				
Aggregate Compressive Modulus (KPa)	1.46 ± 1.55 (6)	4.74 ± 5.53 (4)	8.53 ± 14.4 (6)	440 ± 40 (21)
Hydraulic permeability (30%, $x10^{-15}$ m^2/Pa.s)	299 ± 851 (6)	18.6 ± 40.0 (6)	4.05 ± 6.51 (6)	0.45 ± 0.48 (21)

Comparison of the construct composition and mechanical properties of native septal cartilage tissue and septal neocartilage constructs for each of the three culture conditions. Data represent average ± standard deviation. Parentheses indicate the number of samples analyzed per group. Values for native septal cartilage are from previously published studies [12-14].

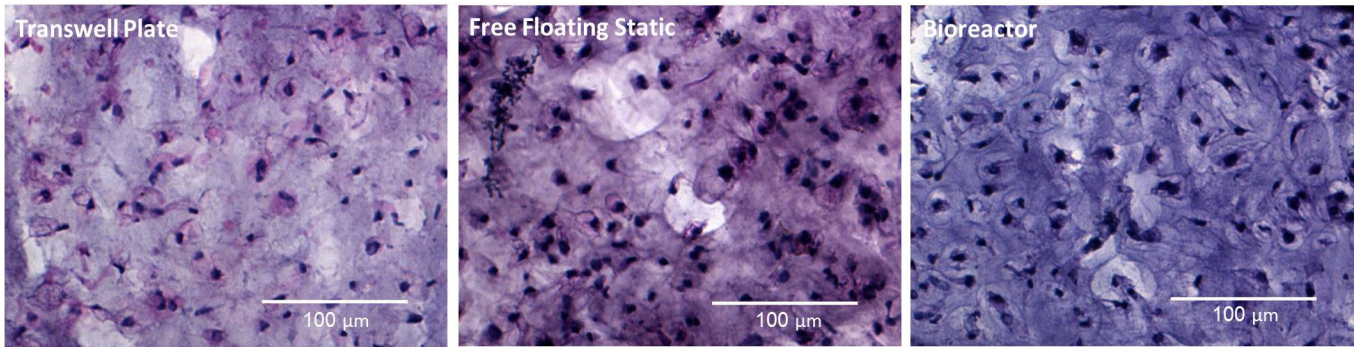

Figure 3: H&E staining of construct sections. Culture of constructs in the free floating static or RCCS bioreactor conditions resulted in constructs with stronger tissue with increased cell numbers and greater ECM deposition per cell compared with constructs cultured in transwell plates (20X magnification).

H&E staining of the constructs demonstrated increased robustness of the tissue structure as well as increased proliferation and amount of ECM surrounding each cell in the bioreactor and static constructs compared with constructs cultured in transwell plates (Figure **3**).

Alcian Blue staining demonstrated robust uniform staining in bioreactor and static constructs, indicating the presence of GAGs (Figure **4**).

The confined compression aggregate modulus (H_{A0}) tended to increase and the hydraulic permeability at 30% offset strain (k_p, ε=0.3) tended to decrease, from the transwell constructs to the static free floating and the bioreactor constructs, with the highest modulus and lowest hydraulic permeability observed in the bioreactor constructs. However, these differences were not significant ($p > 0.05$, Table **1**).

DISCUSSION

Human septal neocartilage constructs cultured in a RCCS bioreactor exhibited enhanced cell proliferation and ECM accumulation compared with constructs cultured in transwells and static free floating conditions. Culture in a RCCS bioreactor led to significantly greater total collagen and improved solubilized type II collagen deposition over culture in transwells. The improved biochemical properties of the RCCS bioreactor constructs were reflected in their firmness and gross similarity to native septal cartilage. The RCCS bioreactor constructs possessed improved biochemical properties compared with the other conditions and this is reflected in their superior resistance to manipulation. Construct mechanical properties improved with RCCS bioreactor culture resulting in an increase in the aggregate confined compression modulus, H_{A0}, and a decrease in hydraulic permeability at 30% offset strain (k_p, ε=0.3). Constructs cultured under all three conditions maintained their radial dimensions and shape, although the RCCS bioreactor constructs tended to be thicker than those cultured in either the static or transwell conditions. When compared with native human nasal septal cartilage, RCCS bioreactor constructs contain slightly more DNA (1.4-fold) and less GAG (3.5-fold) and total collagen (4.5-fold) (Table **1**) [12, 13]. It is thus not surprising that, although superior to constructs cultured in static free-floating and transwell conditions, the mechanical properties of RCCS bioreactor constructs were substantially less than reported values for native human septal cartilage (Table **1**) [14].

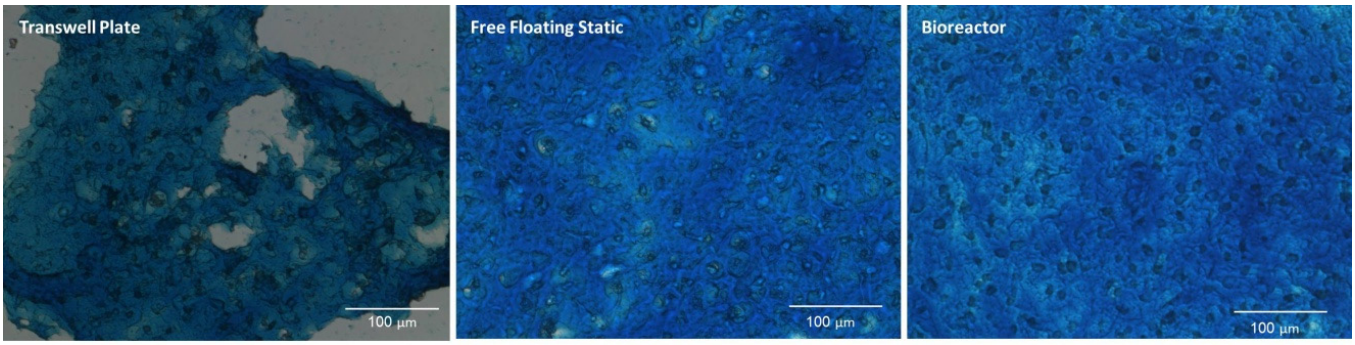

Figure 4: Alcian Blue staining of construct sections. Constructs cultured under static free floating and RCCS bioreactor conditions exhibit robust staining with Alcian Blue, indicating abundant accumulation of GAG (20X magnification).

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 9 was not large enough to detect a significant difference between the sample groups for all parameters tested. However, the data from this pilot study suggest that RCCS bioreactor culture of scaffold-free human septal chondrocyte constructs results in improved biochemical composition and mechanical behavior.

Culture in a bioreactor provides control of fluid flow in order to address limitations in diffusion of nutrients and waste [27]. Bioreactors have also been used in cell culture to provide mechanical stimulation during growth. It has been demonstrated that mechanical stimulation favorably influences cartilage formation *in vivo* [17]. The chondrogenic effect of rotary cell culture vessel bioreactors on cultured articular chondrocytes has been well established. In a study by Li and colleagues [28], bovine articular chondrocytes were seeded onto nanofiber scaffolds and cultured in a RCCS bioreactor for 45 days. These constructs exhibited enhanced accumulation of GAG and total collagen, increased expression of cartilage-associated genes, and enhanced mechanical properties when compared with constructs produced under static conditions. Similarly, Sheehy and colleagues [29] subjected agarose-embedded porcine chondrocyte constructs to rotational culture and found that, compared with static culture, the rotation caused a significant increase in GAG and collagen production. Vunjak-Novakovic and colleagues [30] compared culture of bovine articular chondrocytes seeded into fibrous polyglycolic acid scaffolds in static flasks, spinner flasks, and rotating vessels. After 6 weeks of culture, the static culture and spinner flasks produced constructs with poor mechanical properties while the constructs from the RCCS bioreactors possessed superior mechanical properties, robust ECM, and the highest percentage of GAG and collagen content. In a study by Pound and colleagues [31], human articular chondrocytes were encapsulated in alginate/chitosan microcapsules and cultures in a rotary RCCS bioreactor, perfused bioreactor, or static conditions for 28 days. They found that chondrocytes cultured in the rotating bioreactor showed similar histologic properties to native cartilage, while those cultured under the other conditions were less organized. The RCCS bioreactor constructs showed enhanced proliferation and total protein content compared with the other conditions.

The RCCS bioreactor system has also been shown to promote chondrogenesis of chondrocytes cultured free from scaffold materials. Aged human articular chondrocytes cultured in a RCCS bioreactor produced tissue that was histologically positive for type II collagen and proteoglycan deposition [32]. Ohyabu and colleagues [33] reported that culture of rabbit bone marrow cells in a rotary cell culture system enhanced expression of aggrecan and type II collagen mRNA and resulted in significantly more GAG/DNA and histological staining for proteoglycan deposition. In a study by Sakai and colleagues [34], scaffold-free culture of adult human bone marrow-derived cells in a RCCS bioreactor resulted in enhanced production of GAG and deposition of type II collagen. These studies suggest that the application of RCCS bioreactors in the culture of scaffold-free cartilage constructs may promote chondrogenesis and enhance tissue maturation.

There has been limited study of RCCS bioreactor culture of human nasal septal cartilage constructs. Gorti and colleagues [35] reported positive histological staining for GAG when human septal chondrocytes seeded into polygalactin scaffolds were cultured in a RCCS bioreactor for 6 weeks. In a recent study, RCCS bioreactor and static culture of human septal chondrocytes embedded in alginate were compared. In this case, chondrocytes cultured using these two conditions exhibited no significant difference in cell proliferation, deposition of GAG, deposition of type II collagen and compressive properties [36].

Overall, the use of RCCS bioreactors in tissue engineering of articular and septal cartilage has been promising. However, prior to this study, quantification of tissue properties of scaffold-free human septal constructs cultured in a RCCS bioreactor system had not been reported.

In this study, we successfully demonstrated that scaffold-free human septal neocartilage constructs cultured in a RCCS bioreactor maintained their radial dimensions and exhibited enhanced cell proliferation and total collagen production compared with transwell and static culture conditions. These improved biochemical properties are reflected in the constructs' resilience with manipulation and superior biomechanical properties. The application of RCCS bioreactors to the culture of human nasal septal neocartilage may ultimately lead to the formation of neocartilage that is comparable to native tissue.

ACKNOWLEDGEMENTS

This project was supported by NIH T32 DC 28-20, NIH R01 AR044058, and the Department of Veterans

Affairs, Veterans Health Administration, Office of Research and Development (BL R&D Merit Review Award).

This work was performed in the Cartilage Tissue Engineering laboratory at the University of California, San Diego.

REFERENCES

[1] Tardy ME Jr., Denneny J 3rd, Fritsch MH. The versatile cartilage autograft in reconstruction of the nose and face. Laryngoscope 1985; 95: 523-33. http://dx.doi.org/10.1288/00005537-198505000-00003

[2] Komender J, Marczynski W, Tylman D, Malczewska H, Komender A, Sladowski D. Preserved tissue allografts in reconstructive surgery. Cell Tissue Bank 2001; 2: 103-12. http://dx.doi.org/10.1023/A:1014333022132

[3] Collawn SS, Fix RJ, Moore JR, Vasconez LO. Nasal cartilage grafts: more than a decade of experience. Plast Reconstr Surg 1997; 100: 1547-52. http://dx.doi.org/10.1097/00006534-199711000-00027

[4] Romo T 3rd, McLaughlin LA, Levine JM, Sclafani AP. Nasal implants: autogenous, semisynthetic, and synthetic. Facial Plast Surg Clin North Am 2002; 10: 155-66. http://dx.doi.org/10.1016/S1064-7406(02)00006-8

[5] von der Mark K, Gauss V, von der Mark H, Muller P. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature 1977; 267: 531-2. http://dx.doi.org/10.1038/267531a0

[6] Homicz MR, Schumacher BL, Sah RL, Watson D. Effects of serial expansion of septal chondrocytes on tissue-engineered neocartilage composition. Otolaryngol Head Neck Surg 2002; 127: 398-408. http://dx.doi.org/10.1067/mhn.2002.129730

[7] Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982; 30: 215-24. http://dx.doi.org/10.1016/0092-8674(82)90027-7

[8] Homicz MR, Chia SH, Schumacher BL, *et al*. Human septal chondrocyte redifferentiation in alginate, polyglycolic acid scaffold, and monolayer culture. Laryngoscope 2003; 113: 25-32. http://dx.doi.org/10.1097/00005537-200301000-00005

[9] Chia SH, Homicz MR, Schumacher BL, *et al*. Characterization of human nasal septal chondrocytes cultured in alginate. Characterization of human nasal septal chondrocytes cultured in alginate. J Am Coll Surg 2005; 200: 691-704. http://dx.doi.org/10.1016/j.jamcollsurg.2005.01.006

[10] 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. http://dx.doi.org/10.1016/j.otohns.2006.05.029

[11] 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: 1213-21. http://dx.doi.org/10.1089/ten.tec.2009.0396

[12] Homicz MR, McGowan KB, Lottman LM, Beh G, Sah RL, Watson D. A compositional analysis of human nasal septal cartilage. Arch Facial Plast Surg 2003; 5: 53-8. http://dx.doi.org/10.1001/archfaci.5.1.53

[13] Neuman MK, Briggs KK, Masuda K, Sah RL, Watson D. A compositional analysis of cadaveric human nasal septal cartilage. Laryngosope 2013; 123: 2120-4. http://dx.doi.org/10.1002/lary.23727

[14] Richmon JD, Sage A, Wong VW, Chen AC, Sah RL, Watson D. Compressive biomechanical properties of human nasal septal cartilage. Am J Rhinol 2006; 20: 496-501. http://dx.doi.org/10.2500/ajr.2006.20.2932

[15] Richmon JD, Sage AB, Wong VW, *et al*. Tensile biomechanical properties of human nasal septal cartilage. Am J Rhinol 2005; 19: 617-22.

[16] 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. http://dx.doi.org/10.1177/0194599811425141

[17] Vinatier C, Mrugala D, Jorgensen C, Guicheux J, Noel D. Cartilage engineering: a crucial combination of cells, biomaterials and biofactors. Trends Biotechnol 2009; 27: 307-14. http://dx.doi.org/10.1016/j.tibtech.2009.02.005

[18] Sutherland SM, Sordat B, Bamat J, Gabbert H, Bourrat B, Mueller-Klieser W. Oxygenation and differentiation in multicellular spheroids of human colon carcinoma. Cancer Res 1986; 46: 5320-9.

[19] Freed LE, Langer R, Martin I, Pellis NR, Vunjak-Nokakovic G. Tissue engineering of cartilage in space. Proc Natl Acad Sci USA 1997; 94: 13885-90. http://dx.doi.org/10.1073/pnas.94.25.13885

[20] Lappa M. Organic tissues in rotating bioreactors: fluid-mechanical aspects, dynamic growth models, and morphological evolution. Biotechnol Bioeng 2003; 84: 518-32. http://dx.doi.org/10.1002/bit.10821

[21] Martin I, Obradovic B, Treppo S, *et al*. Modulation of the mechanical properties of tissue engineered cartilage. Biorheology 2000; 37: 141-7.

[22] McGowan KB, Kurtis MS, Lottman L, Watson D, Sah RL. Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen and Hoechst 33258. Osteoarthritis Cartilage 2002; 10: 580-7. http://dx.doi.org/10.1053/joca.2002.0794

[23] Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986; 883: 173-7. http://dx.doi.org/10.1016/0304-4165(86)90306-5

[24] Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961; 93: 440-7. http://dx.doi.org/10.1016/0003-9861(61)90291-0

[25] Jackson DS, Cleary EG. The determination of collagen and elastin. Methods Biochem Anal 1967; 15: 25-76. http://dx.doi.org/10.1002/9780470110331.ch2

[26] Scott JE, Dorling J. Differential staining of acid glycosaminoglycans (mucopolysaccharides) by Alcian blue in salt solutions. Histochemie 1965; 5: 221-33. http://dx.doi.org/10.1007/BF00306130

[27] Martin I, Wendt D, Heberer M. The role of bioreactors in tissue engineering. Trends Biotechnol 2004; 22: 80-6. http://dx.doi.org/10.1016/j.tibtech.2003.12.001

[28] Li WJ, Jiang YJ, Tuan RS. Cell-Nanofiber-based cartilage tissue engineering using improved cell seeding, growth factor, and bioreactor technologies. Tissue Eng Part A 2008; 14: 639-48. http://dx.doi.org/10.1089/tea.2007.0136

[29] Sheehy, EJ, Buckley CT, Kelly DJ. Chondrocytes and bone marrow-derived mesenchymal stem cells undergoing chondrogenesis in agarose hydrogels of solid and channelled

architectures respond differentially to dynamic culture conditions. J Tissue Eng Regen Med 2011; 5: 747-58. http://dx.doi.org/10.1002/term.385

[30] Vunjak-Novakovic G, Martin I, Obradovic B, *et al*. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. J Orthop Res 1999; 17: 130-8. http://dx.doi.org/10.1002/jor.1100170119

[31] Pound JC, Green DW, Chaudhuri JB, Mann S, Roach HI, Oreffo RO. Strategies to promote chondrogenesis and osteogenesis from human bone marrow cells and articular chondrocytes encapsulated in polysaccharide templates. Tissue Eng 2006; 12: 2789-99. http://dx.doi.org/10.1089/ten.2006.12.2789

[32] Marlovits S, Tichy B, Truppe M, Gruber D, Vécsei V. Chondrogenesis of aged human articular cartilage in a scaffold-free bioreactor. Tissue Eng 2003; 9: 1215-26. http://dx.doi.org/10.1089/10763270360728125

[33] Ohyabu Y, Kida N, Kojima H, Taguchi T, Tanaka J, Uemura T. Cartilaginous tissue formation from bone marrow cells using rotating wall vessel (RWV) bioreactor. Biotechnol Bioeng 2006; 95: 1003-8. http://dx.doi.org/10.1002/bit.20892

[34] Sakai S, Mishima H, Ishii T, *et al*. Rotating three-dimensional dynamic culture of adult human bone marrow-derived cells for tissue engineering of hyaline cartilage. J Orthop Res 2009; 27: 517-21. http://dx.doi.org/10.1002/jor.20566

[35] Gorti GK, Lo J, Falsafi S, *et al*. Cartilage tissue engineering using cyrogenic chondrocytes. Arch Otolaryngol Head Neck Surg 2003; 129: 889-93. http://dx.doi.org/10.1001/archotol.129.8.889

[36] Reuther MS, Wong VW, Briggs KK, *et al*. Culture of human septal chondrocytes in a rotary bioreactor. Orolaryngol Head Neck Surg 2012; 147: 661-7. http://dx.doi.org/10.1177/0194599812448049

Received on 14-02-2014 Accepted on 19-03-2014 Published on 11-04-2014

DOI: http://dx.doi.org/10.12970/2311-1755.2014.02.01.3