Laser mediated cartilage reshaping
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The Porcine and Lagomorph Septal Cartilages: Models for Tissue Engineering and Morphologic Cartilage Research

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ABSTRACT

Interest in reconstruction and modification of the facial cartilaginous frameworks using advanced technology and instrumentation is growing rapidly. Despite this maturing interest, no animal model has been established to provide morphologic cartilage tissue with similar characteristics to human septum in suitable quantities. The objective of this study was to characterize porcine and lagomorph (rabbit) nasal septal cartilage tissue. Both models share great similarity with their human counterpart and provide a low-cost, high volume, and easily obtained source of bulk cartilage tissue. We present a technique for harvesting intact septal cartilages from these species, and characterize select cellular, metabolic, and physical properties using pulse-chase radiolabeling, flow cytometry, and mechanical analysis. Our selective evaluation of key tissue properties establishes these species as appropriate animal models for nasal septal cartilaginous surgery. (American Journal of Rhinology, 2001, in press)

Introduction

Human septal cartilage tissue is difficult to obtain in sufficient quantities for many physical and biological experiments. The small amounts of excess cartilage available for use following nasal surgery are often inadequate for studies requiring repeated or multiple physical and biologic analysis. Cadaveric sources are also scarce and associated with variable procurement schedules, elderly donors (with ossified and chondrocyte sparse tissue), and prolonged delays between death and tissue harvest. Human tissue also has the additional risk of infection due to viral agents such as hepatitis and HIV. The need for an appropriate non-human septal cartilage tissue model has been amplified by growing research and development interest in tissue engineering, laser reshaping and powered surgical instrumentation. The septum (a morphologic cartilage) consists of hyaline cartilage with little elastic fiber components (1). Traditionally, biologists and biomechanical engineers have used non-nasal cartilage tissues obtained from either small rodents or articular joints of large mammals. However these conventional sources are of limited use with respect to the human septum in size, shape, or tissue microarchitecture. This prompted our search for a low-cost readily obtained animal tissue model with similarities to human septal cartilage that could also be used to simulate cartilage surgery, train surgical residents, and evaluate new instrumentation.

Bulk quantities of porcine septal cartilage are easily obtained from local packing houses at low-cost, often within minutes of sacrifice. As domestic pigs are highly inbred strains, anatomic variation among individual animals is limited. Although much longer and of different shape rectangular), porcine septal cartilage (Figure 1a) grossly approximates human septum in terms of thickness and physical behavior on manual inspection. Porcine septum has been studied extensively for laser-tissue interactions for several years (2-8), but little is known about its physical or biologic properties (9, 10). This study characterized several cellular (cell density and size), metabolic (proteoglycan synthesis rates), and physical (elastic modulus, mass density) properties of porcine septum with respect to caudal and cranial locations. Characterization of the spatial differences in biological and physical properties is important as these are known to vary with location in the septum (10). As a practical matter, we also evaluated these properties in rabbit (lagomorph)
septi, which is also a morphologic cartilage. While the rabbit septum (Figure 1 b) is different in thickness and shape to human tissue, rabbit surgical studies are relatively low cost and useful for in vivo investigations.

Materials and Methods

Specimen Harvest

Fresh porcine heads from 100 kg domestic pigs were obtained immediately following slaughter from a local abattoir (Clougherty Packing Company, Vernon, CA). Soft tissue was removed from the nasal, maxillary, and frontal bones down to the periosteum. Osteotomies along the junction between the nasal bone and maxilla were extended superiorly to just over the frontal sinus with a carpenter’s chisel.

Figure 2 Extraction of porcine septal cartilage. Removal of nasal bones en bloc (a), septum (b), and separation of perichondrium and mucosa from the cartilage (c).

The nasal bone complex was removed exposing the septal cartilage (Figure 2 a). The chisel was then used to disarticulate the cranial septum from its attachments to the osseous septum. The cartilaginous septum was then extracted from the nasal floor using the chisel directed along the maxillary crest (Figure 2 b). Soft tissue including the perichondrium was dissected free from the specimen with the chisel or elevator leaving only the cartilage material (Figure 2 c). The pig septum is approximately 15 x 5 cm in dimension with thickness varying from 6 mm anteriorly to 3-4 mm posteriorly. The septum was arbitrarily divided into six regions in order to determine spatial variation in the physical and biologic properties (Figure 3). Cartilage specimens were cut into slabs of precise user-specified thickness using a custom guillotine microtome. The microtome removed at least 0.5-mm of cartilage tissue thus ensuring no perichondrial fibers remained in the specimen. For biochemical and cellular analyses a 6 mm biopsy punch was used to obtain disc shaped specimens.

The heads of freshly sacrificed New Zealand white rabbits (2 kg) were obtained from a local hutchery (B&B Rabbits, Fontana, CA). The septum was harvested using surgical instruments as described above for pigs with slight modification. The perichondrium of the rabbit septum is not rigidly adherent to the cartilage as in pigs (or humans) and, therefore can be completely removed using an elevator. The septum measures approximately 3.75-4 x 1-1.5 cm with thickness varying from 0.25 mm anteriorly to 0.75 mm posteriorly and was also divided into six equally spaced regions from cranial to caudal (Figure 3). For mechanical measurements, the specimens were cut into rectangular slabs (10 x 3 mm) using a razor blade. Biochemical and cellular specimens were prepared as above.

Figure 3 Schematic of septal cartilage divided into six regions.

Mass Density Measurements

Mass density (referred to here as density) was calculated by measuring the mass of a disc shaped specimen (with a digital analytic balance) and dividing this value by the tissue volume (product of disc diameter and thickness measured with a precision micrometer). Four specimens were evaluated from each of the six regions from three different pigs. Density was measured from one specimen in each region from eight rabbits. The thickness of rabbit specimens measured using a micrometer was the actual thickness of the septal cartilage free of perichondrial tissue. The same number of samples and specimens were used for measurements of cell density and cell size (see below).

Cell Density and Size

Cell density (number of cells/gram of tissue) was calculated following enzymatic isolation of individual chondrocytes from cultured septum specimens. A flow cytometer (fluorescence activated cell sorter- FACS) was used to count individual cells within a sample of known tissue mass. Flow cytometry measures the scattering of coherent light from individual cells in suspension as they pass through an optical cuvette. The intensity of both forward and side scattered light allows discrimination of individual cells from matrix debris and also the estimation of
approximate cell size. As the technique is automated, 10,000 individual cells can be analyzed in seconds. Porcine and rabbit tissue was analyzed in identical fashion.

**Cell Culture and Isolation:** The cartilage discs were washed three times in antibiotic solution containing phosphate buffered saline (PBS) with gentamicin (200 mg/L) and amphotericin B (22.4 mg/L) for 15 minutes under sterile conditions. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) with/without 10% fetal calf serum (FCS), gentamicin (10 mg/mL), penicillin (100 μg/mL), streptomycin (100 μg/mL), and L-glutamine (29.2 g/mL) was used as the digestion media. Chondrocytes were isolated under sterile conditions using a three-step enzymatic digestion protocol: 1) hyaluronidase (1 mg/mL in culture medium without FCS) (Sigma Chemical Co., St. Louis, Mo.) for 15 minutes; 2) pronase (0.01 mg/mL in culture medium without FCS) (Sigma Chemical Co.) for one hour; and 3) collagenase II (1 mg/ml in culture medium including FCS) (Worthington Biochemical Corporation, Lakewood, NJ) for 15 hours (11). All digestion steps were performed at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air. After digestion, isolated chondrocytes were transferred to 15-mL centrifuge tubes and pelleted by spinning at 1300 rpm for 5 minutes. The supernatant was then aspirated, leaving only the cells. The isolates were then resuspended in a known volume of Hank's solution (Gibco).

**Flow Cytometry:** Flow cytometry analysis was performed on isolated cell suspensions using a system (FACScan, Becton Dickinson, San Jose, CA) equipped with a MAC PowerPC 7600/132 computer (Apple Computer Co., Cupertino, CA) and CellQuest acquisition and analysis software (Becton Dickinson). The instrument is equipped with a 35 mW argon ion laser (\(\lambda = 488 \text{nm}\)) and several photomultiplier tubes and photodiodes which record forward and side scattered light. The cytometer analyzed 60 μL of resuspended chondrocyte solution per minute. A population gate was established using forward and side scatter profiles to include only intact cells, and exclude cellular debris. All events (including debris) were recorded, but analysis was terminated when 10,000 population-gated events were detected. The acquisition time required to count 10,000 gated events was recorded. Cells per gram of tissue was calculated knowing the time required to count 10,000 cells, cytometer flow rate, and original specimen mass in the isolated cell suspension. Forward scattered light intensity was recorded for each population gated event. Cell size was estimated by comparing these measurements with a calibration curve established from the flow cytometry of polystyrene spheres of known diameter (10, 16, 18, and 29 μm diameter, Coulter, Hialeah, FL). Statistics for cell size in each specimen were calculated.

**Modulus of Elasticity**

The mechanical properties of a tissue are best represented by the elastic modulus, which describes the intrinsic stress-strain relationships in a material independent of geometry. Static stress and strain measurements were recorded in fresh cartilage slabs (25 x 5 x 2 mm) selected from the caudal (anterior), cephalic (posterior), and central regions of the pig septal cartilage. Measurements were made only in these three central regions rather than the six areas defined above because only limited tissue with appropriate uniformity is available after processing. Similar limitations were encountered with rabbit septal cartilage. Anteriorly toward the nasal tip, the cartilage is extremely thin (~0.25 mm) and reproducible measurements are difficult to obtain. Posteriorly, the septum is irregularly shaped, and often this region of the tissue is damaged during the extraction process. Because of these practical concerns, the elastic modulus was determined only in the central two regions (III and IV) of the rabbit septum. The rectangular slabs (10 x 3 mm) were cut from the septum with a razor, with the long axis of the slabs oriented transversely with respect to the long axis of the intact rabbit septum. Septum width was determined by actual septum thickness, free of perichondrial tissue.

**Figure 4** Schematic of apparatus used to measure elastic modulus.

A custom mechanical testing device was used to generate force (stress) versus displacement curves in cantilevered specimens secured between two sandpaper-lined aluminum blocks under gentle pressure (Figure 4). The distal end of the specimen was deflected upwards by movement of a
motorized micropositioner (model 860A, Newport Electronics Corporation, Irvine, CA) while force was measured continuously with a load cell (227 g rating, OMEGA Engineering, Stamford, CT). A square wave (0.1 Hz, 0 V DC bias) produced by a function generator (DS 345, Stanford Research Systems, Sunnyvale, CA) supplied the input signal to a linear servo amplifier circuit which in turn provided the drive voltage for the micropositioner. Specimen translation velocities were 0.76 and 0.130 mm/sec for porcine and rabbit tissue, respectively. The excitation voltage for the load cell was provided by a DC power supply (PPS-2322, Amrel, Arcadia, CA). Output voltage from the load cell was amplified and low-pass filtered (3Hz, 3dB cutoff, SR560, Stanford Research Systems). Data were acquired using a 16-bit AD converter (AT-MIO-16XE-50, National Instruments, Austin, TX) and a personal computer (Equicomp Solutions, Westminster, CA) using software written in LabView (National Instruments). Maximum displacement during each cycle was measured using a micrometer. Data from each trial was used to generate force vs. displacement curves. Elastic modulus was determined assuming linear viscoelastic behavior using a model for flexure of a cantilevered beam with the load concentrated at the free end, and expressed in terms of Mega-Pascals (MPa) (12).

Proteoglycan Synthesis Rates

Measurement of cartilage tissue metabolism can be assessed using biochemical, molecular, or histologic techniques. The most widely used technique measures the incorporation of Na$_2^{35}$SO$_4$ into new proteoglycan (PTG) molecules. PTGs are complex macromolecules (100 - 200 MD) primarily responsible for the mechanical properties of cartilage, and are rapidly synthesized by chondrocytes as their half-lives are relatively short. Scintillation counting permits measurement of relative biochemical activity within a set of specimens.

Normalized scintillation counts were measured using a modification of the protocol by Wong et al (6). Cartilage discs were washed in antibiotic solution three times and then placed in tissue culture and incubated overnight (see above). After 24 hours, 1 ml of Na$_2^{35}$SO$_4$ (activity 10 uCi/ml) containing growth medium was added. After an additional 72 hours, isotope containing medium was removed and the cartilage discs washed with PBS until all radioisotope activity in the eluent was at trace levels. Fresh growth medium was again placed into each well and allowed to incubate for 4-6 hours. Discs were subsequently washed three times in PBS and then enzymatically digested overnight using the three-step protocol outlined above with serum free media. Radioactivity from a 100 µl aliquot of the digested cell suspension added to 10 ml of Ecoscint (National Diagnostics, Atlanta, GA) was measured using a liquid scintillation counter (LS5801, Beckman Instruments, Fullerton, CA). Total protein content in each specimen was measured using a spectrophotometer (λ=562 nm, model DU 650, Beckman Instruments, Fullerton, CA). Bovine Serum Albumin (BSA) in concentrations varying from 1 to 100 µg/ml was used as the calibration standard for the assay. Protein content was derived, and used to normalize scintillation counts for cell and tissue mass in porcine specimens. Scintillation counts were normalized by mass in rabbit specimens. One specimen from each region in six pigs and four rabbits were used.

Results

Figures 5-8 (a and b) depict the regional variation in mass density, cell density, cell size, and proteoglycan synthesis rates for the six regions of the pig and rabbit septi, respectively. The error bars denote standard error (s.e.). Regional variation was observed for each of the latter three biological properties. There was little spatial variation in mass density, hence average values for all specimens for pig and rabbit were calculated (Table I). Proteoglycan synthesis rates were normalized (a.u.) and expressed in terms of scintillation count rate (s$^{-1}$). Elastic moduli values are summarized in Table I.

Discussion

Porcine and rabbit septal cartilage provides a convenient source of morphologic tissue that can be used for basic investigations, surgical device evaluation and design, and teaching. In fact, porcine septal cartilage warps dramatically when cut or scraped with a scalpel thus providing a graphic means to demonstrate Gilles and Gibson's principles of balanced cross sections (13, 14). Our initial motivation for selecting porcine septi was entirely practical: 1) easily obtained in large quantities at low cost; 2) grossly approximates human septum in texture and thickness allowing for the simulation of surgical procedures; and 3) specimens are sufficiently large to allow for repeat or multiple experiments from the same animal. However, in investigations focused on measuring tissue optical, mechanical, and biologic properties, it became readily apparent that there was spatial variation in tissue properties within the septum of

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Figure 5  Mass density as a function of spatial location on the pig (a) and rabbit (b) septi.

Figure 6  Cell density as a function of spatial location on the pig (a) and rabbit (b) septi.

Figure 7  Cell size as a function of spatial location on the pig (a) and rabbit (b) septi.

Figure 8  Normalized PTG synthesis rates as a function of spatial location on the pig (a) and rabbit (b) septi. Note the scale change for region I.
an individual animal in addition to inter-animal differences within the same species (6). This prompted the systematic evaluation of selected tissue properties that we summarized in this study.

**Mass Density**

Specific cartilage tissues types (articular, morphologic, fibrocartilage, elastic) differ in the architectural arrangement of water (80%), extracellular components (Type II collagen 13% and PTG 7%), and cell density. For example, articular cartilage is a highly ordered laminar structure while morphologic cartilage consists primarily of amorphous hyaline tissue. Hence the physical and biological parameters that are known for one tissue type are of limited relevance to another. In morphologic cartilage such as the septum, the lack of any higher order structure within the tissue suggests that basic physical properties would not demonstrate any spatial variation. The most basic tissue properties such as density were remarkably similar from region to region in both the pig and rabbit (Figure 5 a and b). This is not surprising, as the ratio of water to formed or cellular elements is generally very constant. Further, values for cartilage density (averaged over all measurements) for each species were not significant different (Table 1).

<table>
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<th>Animal</th>
<th>Property</th>
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**Elastic Modulus**

The elastic modulus characterizes material deformation in response to an applied force and is independent of specimen geometry. The modulus is required for any rigorous mechanical analysis and simulation, and reliable measurements of septal cartilage elastic moduli are unavailable. This provided motivation for the present study. Specimen deformation in response to an applied force was measured using a single-ended cantilevered beam geometry, which is relevant to our current research focus on cartilage warping and reshaping. Slight differences in the elastic modulus were observed between cephalic, central, and caudal regions of the pig septum, which is consistent with gross observations of specimen “stiffness”. The values obtained in this study [5.5-4.8 (pig) and 7.92 (rabbit) Mpa] are in good agreement with previous reported measurements of nasal and costal cartilages, but differ substantially from softer articular tissues (15). The rabbit septal cartilage (evaluated only in the central III and IV regions of the specimen) was more rigid than pig tissue.

Tissue modulus is a function of the arrangement of constitutive elements within the tissue matrix. Calculation of the modulus used here assumed linear viscoelastic behavior, which is a valid approximation for the small forces and displacements generated using the current apparatus and measurement technique. Large deformations result in deviation from ideal behavior; time-dependent tissue creep and stress relaxation are observed along with mechanical hysteresis. In molecular terms, the mechanical properties during compression are largely due to proteoglycan interactions while the collagen framework governs tension (16). During flexure, both mechanisms are in action.

**Cell Density, Cell Size and Proteoglycan Synthesis**

In previous studies, differences in PTG synthesis rates were observed along the caudal to cephalic axis of the septum (6). This observation emphasized the importance of quantifying these spatial differences as synthesis rates in native tissue may vary over an order of magnitude as observed in the pig (Figure 8 a). PTG synthesis rates were by far greatest in the most cephalic region of the specimen (region I). Such massive differences in native biological behavior would obscure differences resulting from experimental manipulation with growth factors, physical alteration (e.g. radio-frequency or laser heating), or pharmacologic agents. Hence it is imperative that the appropriate control be used in any investigations focused on measuring biochemical or cellular activity in porcine septal cartilage. This native spatial variation in metabolic activity is greater than what might be expected given the relatively uniform mass density and elastic modulus measurements. Similar variations (albeit in the same order of magnitude) were observed in PTG synthesis rates in rabbits (Figure 8 b), albeit peak activity was observed in the central regions.

Determining the nature of this difference in synthetic activity prompted characterization of
the cellular structure and organization of the septum on a regional basis. Cell density (cells/g) was measured in each of the six regions in order to determine if the observed variations in synthetic activity corresponded to higher cell density (Figure 6a and b). Cell density can be measured using several standard methods. Light microscopy is simplest, but is time consuming, subject to sampling errors, and involves fixation. Hemocytometry following chondrocyte isolation allows slightly more accurate estimation of cell concentration, but still requires a manual count. Automated cell counting devices can also be used on cell suspensions. A Coulter counter device was initially used to measure cell density and estimate size, but abandoned due to the inability to discriminate accurately cells from debris. These counters also require relatively large volumes of cell suspension. FACS was used because the method counts large numbers of cells rapidly with precise exclusion of debris and non-cellular material based on optical scattering properties. As this technology relies upon measurement of coherent light scattering from individual particles, cell size can also be estimated at the same time. It must be emphasized that FACS techniques only estimates cell size, and that cells in suspension differ in geometry from cells in situ. FACS can be combined with selective staining of specific cell populations with fluorescent dyes sensitive to specific enzymatic function or structural features.

In porcine septum, both cell density (cells/g) and cell size (μm) increased along the cephalic to caudal axis (Figure 6a) which is surprising as PTG synthesis decreased moving in the same direction (Figure 8a). As chondrocytes are solely responsible for the biosynthesis of proteins within the matrix, one would expect PTG synthesis to reflect changes in cell composition. No specific correlation between PTG synthesis and cell density and size was observed in rabbit tissue. Cell density increased along the cephalic to caudal direction (Figure 6b) as similarly observed in the pig. Cell size was relatively constant from region to region (Figure 7b). The basis for these regional differences in metabolic activity is unclear, but because of these findings, we use exclusively the cephalic component in our current investigations that focus on measuring cell synthetic activity. The pig and rabbit cartilages differed significantly in cell density and cell size. Pig chondrocytes varied from 14-20 μm in diameter, whereas the rabbit chondrocytes were much larger (50-60 μm), and these measurements are in good agreement with histologic observations (unpublished results). Cell density in the pig (200,000 to 350,000 cells/g) was much lower than that observed in the rabbit (2-8 million cells/g). The relative age of the specimens analyzed may account for this large difference. The rabbits used in this study were at the peak of their growth phase (2 kg). Cell density measurements in mature New Zealand white rabbits (5-7 kg) were an order of magnitude lower (unpublished results). We opted to use younger animals for entirely practical reasons; at this weight rabbits are slaughtered for commercial consumption.

Conclusion

Porcine sepal cartilage is very similar to its human counterpart and provides a low-cost, high volume, and easily obtained source of cartilage tissue. Our selective measurement of tissues biological and physical characteristics forms the basis for using this species as an animal sepal cartilage model. While articular cartilage has been characterized extensively, particularly with respect to its biomechanical properties, few investigations have focused on morphologic cartilages such as the septum. The properties determined in this study are primarily relevant to investigations that focus on tissue metabolism, cell density, and basic biomechanical behavior. Inasmuch as the rabbit is a practical animal model for many head and neck surgical investigations, we also characterized the similar tissue properties for the rabbit septum. The data collected here are by no means exhaustive. We plan to expand the list of properties as our interest in cartilage tissue biophysics and tissue engineering grows.

Acknowledgment

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References


