Background and Objectives: To evaluate the long-term effect of laser cartilage reshaping on rabbit nasal septal cartilage viability and mechanical integrity in an in vivo model.

Study Design/Materials and Methods: In vivo animal investigation. Rabbit septal cartilage specimens were laser (Nd:YAG, λ = 1.32 μm, spot size 5.4-mm diameter, 10 W, 10 seconds, 50 Hz PPR) reshaped and subsequently reimplanted into an interscapular subcutaneous pocket. Specimens were harvested at 8 and 12 months and evaluated using photography, flow cytometry, and histology.

Results: Grossly, specimens showed alteration in the physical integrity with varying degrees of tissue resorption. The non-irradiated control specimens demonstrated significantly increased stiffness. Histologically, there was marked depletion of the extracellular matrix and an overall reduction in tissue mass in laser irradiated tissues. However, flow cytometry data identified viable chondrocytes in laser-irradiated specimens that were identical to those observed in controls.

Conclusions: Study results demonstrate that the rabbit nasal septal cartilage model can be effectively used to study laser reshaping, however alternative recipient sites with perichondrial lining, such as the pinna, may provide a more realistic physiologic environment for reshaped graft tissue. The dosimetry used in this pilot study likely led to significant thermal injury. Study results underscore the importance of elucidating the optimal laser dosimetry required to initiate permanent shape change while minimizing thermal damage. Lasers Surg. Med. 36:147–154, 2005.

Key words: laser; laser reshaping; cartilage; Nd:YAG; flow cytometry; reshaping; rabbit; septum; viability; histology

INTRODUCTION

Laser cartilage reshaping (LCR), first introduced in 1992 [1,2], is a novel technique designed to permanently alter the shape of cartilaginous structures without the use of scalpels, scoring, sutures, or morselization [3–9]. Several animal studies [10–13], and one published clinical trial [14] have focused on demonstrating the clinical efficacy of this laser application. In parallel, numerous biophysical studies have been performed which have focused on both determination of the mechanism of action and optimization of dosimetry [15–20]. Previous animal studies of LCR have used primarily porcine, rabbit, or canine auricular or tracheal cartilage which differs substantially from nasal septal cartilage in that these tissues are either highly elastic or surrounded by very thick skin [10,11,13]. These tissues are not the optimal models to simulate the morphologic cartilage of the nasal septum, which is more hyaline in nature with less elastin fibers. The need for a more representative model prompted characterization of rabbit and porcine nasal septal cartilages and the development of a surgical technique to safely remove cartilage from the rabbit nasal fossa and replant it in a subcutaneous pocket [21–24]. A further limitation of these previous studies is that cartilage specimens were not irradiated in a uniform manner with precisely controlled dosimetry. In fact the effect of dosimetry alone on the long-term viability (e.g., >6 months) of chondrocytes has not been systematically

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The objectives of this study are to begin evaluating the long-term stability of these grafts after laser reshaping in an in vivo rabbit septal cartilage model and control the laser dosimetry using a computer-controlled reshaping device [16]. The present in vivo pilot animal investigation builds upon previous ex vivo studies performed in our laboratory that focused on determining optimal laser dosimetry [25–28] using the rabbit nasal septal cartilage model [23,24]. Being a pilot investigation, a relatively high laser power is used to establish the biophysical response of rabbit septal cartilage to laser irradiation at the extrema of the dosimetry parameter space.

METHODS

Four Pasteurella-free New Zealand white rabbits (3.5–4.5 kg) ranging from 9 to 12 months in age were used. All protocols and experimental design parameters were reviewed and approved by the University of California, Irvine Institutional Animal Care and Use Committee.

Animal Surgery

Anesthesia, preoperative preparation, and the surgical technique have been described elsewhere [23,28] and only an overview will be provided here. Animals were induced by giving a 2:1 ml ratio of Ketamine (100 mg/ml) and (Xylazine 20 mg/ml), respectively, via an intramuscular injection, intubated, and then maintained on 2% isoflurane gas. A midline 3.0-cm long nasal dorsal incision from the level of the fronto-nasal suture to 1.0 cm above the nasal tip was made to provide exposure for a laterally based osteoplastic flap (1.0 mm x 2.5 mm) centered over the septum. After exposing the nasal cavity, bilateral sub-mucoperichondrial flaps were elevated, and a 1.0 x 2.5 central segment of the septal cartilage was removed. Both sides of the septal mucosa was approximated, the osteoplastic flap closed, and skin incisions sutured together.

Laser Reshaping

Figure 1 schematically illustrates the experimental protocol. The excised cartilage graft was immediately divided into two rectangular slabs (5 mm x 15 mm) with a razor blade. One served as control while the other was irradiated with the laser. The cartilage specimen was placed in a custom jig assembly designed to maintain the specimen in a curved semi-circular geometry (Fig. 2). The jig was attached to a computer controlled rotary translation stage, which positioned the specimen relative to the laser beam. Diffusely transmitted light from a diode probe laser aimed at the...
Nd:YAG laser (used for specimen heating) irradiation site ($\lambda = 635 \text{ nm}$, 5 mW, Lasermate, Walnut, CA) was collected by a fiber optic cable (Ceramoptic, East Longmeadow, MA) connected to a photoreceiver (Model 2001, New Focus, Mountain View, CA) and detected using a lock-in amplifier (Model, Stanford Research System, Sunnyvale, CA). The fiber optic cable was inserted into the center of the jig, and had prism attached to its tip to allow collection of light directly. Light from a Nd:YAG laser ($\lambda = 1.32 \text{ mm}$, spot size 5.4-mm diameter, 10 W, 10 seconds, 50 Hz PPR, New Stars Lasers, Roseville, CA) was directed perpendicularly at the specimen using a multimode optical fiber (600 microns), coupled to an infrared thermopile detector which was used to estimate temperature [17]. The laser was directed at three predetermined positions in a non-overlapping vertical arrangement along the surface of the specimen (Fig. 2C). Signals from the thermopile and lock-in amplifier were recorded using an analog to digital converter and a PC workstation. After irradiation, the cartilage specimen was immersed in an ambient temperature saline bath for 15 minutes while still maintained in deformation by the jig. Control specimens were only secured in the jig for 15 minutes (while immersed). Then, after removal from the jig both control and laser irradiated specimens were immersed three times (15 minutes each) in antibiotic solution containing phosphate buffered saline (PBS) with gentamicin (200 mg/l) and amphotericin B (22.4 mg/l) under sterile conditions.

Re-Implantation

A small vertical incision was made in the interscapular region over the rabbit’s dorsal fat pad. A subcutaneous pocket was developed and the cartilage slabs placed on opposite sides and secured with 5-0 Prolene sutures. To facilitate identification, the laser treated specimen was consistently placed on the left side of the pocket. The
incision was then sutured together. Following surgery, the animals were observed daily and examined for signs of pain, wound infection, and other operative complications.

**Specimen Harvest**

One rabbit died at 8 months due to complications associated with a massive urinary vesicular calculus. In this animal, both the control and laser irradiated specimen were removed and immediately immersed in formalin (10%) for histologic study, since flow cytometry resources at that time were unavailable. The remaining three animals were euthanized after 12 months and the cartilage specimens were removed, photographed, and prepared for histology and viability analysis.

**Viability Analysis**

In one pair of specimens, each graft was divided in half longitudinally. One half was preserved in formalin for histology while the other specimen was enzymatically digested using a three-step enzymatic digestion process [30]. After digestion, isolated chondrocytes were transferred to a centrifuge tube and prepared for viability staining as previously described [25].

Two-color viability dye system was used (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR). The pelleted cells were resuspended in the two component dye and prepared for flow cytometric analysis, as previously described [25].

Flow cytometry (FACScan, Benton-Dickinson, Franklin Lakes, NJ) was performed and an average of 10,000 cells were analyzed per tissue specimen. Light scattering and fluorescence data were analyzed using CellQuest acquisition and analysis software (BD Biosciences Immunocytometry Systems, San Jose, CA) running on a personal computer (Apple, Cupertino, CA). Scatter plots were generated for each sample by plotting all events red intensity (X-axis) and green intensity (Y-axis).

**Histology**

Following fixation, specimens were serially dehydrated using graded ethanol solutions and then embedded in paraffin. The microsections were sectioned (6 μm) and stained with hematoxylin & eosin and alcian blue (for analysis of proteoglycan content), and examined microscopically at (10–40×).

**RESULTS**

The non-irradiated control and laser-irradiated specimens were significantly stiffer than cartilage specimens immediately removed from the nasal septum, and some evidence of tissue resorption was evident (Fig. 3). All four laser irradiated specimens demonstrated varying degrees of tissue resorption varying from being intact with some loss of structural integrity to complete resorption in one case (Fig. 4). There was limited preservation of shape in all laser-irradiated specimens; the specimen, which was removed from the subject at 8 months, was the most structurally intact.

**Biophysical Properties**

Real-time measurements of cartilage surface temperature obtained for each specimen were recorded during irradiation. Each 10-second pulse of the Nd:YAG laser resulted in cartilage surface temperature increasing from room temperature to 100–120°C (Fig. 5). A corresponding maxima in the diffuse reflectance curve was seen in all irradiated samples, corresponding to the onset of accelerated stress relaxation [15–19]. The peak in the diffuse reflectance curve occurred between 60 and 80°C.

**Histology**

Histologically, there was marked depletion of the extracellular matrix and an overall reduction in tissue mass in laser irradiated tissues (Fig. 6A,C). Specimens stained with alcian blue showed a weak binding of the stain, indicating loss of the proteoglycan component within the extracellular matrix (Fig. 6B,D).

**Flow Cytometry**

Flow cytometry using a “live-dead assay” demonstrated identical scattering and emission patterns indicating the presence of live and absence of dead chondrocytes. Notably, at this power and pulse duration, low tissue viability would be expected based upon acute viability studies [25]. Unlike previous studies, cell counts did not normalize for specimen mass [25].

**DISCUSSION**

The laser heating of mechanically deformed cartilage specimens accelerates stress relaxation, leading to permanent shape change. In theory, LCR produces subtle, temperature-induced alterations in the tissue matrix properties that result in marked changes in bulk material characteristics such as the elastic modulus [29,30] and heat capacity [31]. Heating tissue with laser or any other source results in a number of effects, each of which is largely determined by the space- and time-dependence of the temperature field. In industry, deposition of thermal energy is
routinely used to induce subtle chemical transitions and modify material properties of semiconductors, alloys, ceramics, and polymers. In medicine, thermal interactions have been used primarily to coagulate, ablate, and destroy targeted tissue structures. In contrast to these traditional applications in medicine, LCR exploits the properties of cartilage as a composite polymer biomaterial. As the only viscoelastic hard tissue in the body, cartilage provides a unique opportunity to develop truly plastic surgical procedures. This pilot investigation is an important step toward the optimization of LCR, particularly since cartilage grafts were removed from the specimen, irradiated, and returned to an orthotopic recipient site devoid of a stem-cell rich perichondrium, unlike previous reported animal studies, which used the ear or trachea as the recipient site, and used laser wavelengths that often resulted in non-uniform axial temperature gradients that may better preserve viability of deeper cartilage tissue layers. Laser heating of cartilage not only affects the proteoglycan rich matrix, and permits stable reshaping of cartilage structure, but may also affects changes in cellular function and interaction, local tissue reaction, and modification of vascular flow in the in vivo environment. Although results of both in vivo animal studies and the sole human study [14] indicate that a stable shape change could be produced using a laser, whether irradiated cartilage tissue contains viable

Fig. 4. Photographic montage of laser-irradiated cartilage explants at 12 months, demonstrating the extremes of gross tissue resorption encountered from minimal to significant resorption.

Fig. 5. Biophysical response of cartilage during laser-mediated reshaping. Mechanically deformed slabs are irradiated with an Nd:YAG laser while measuring tissue surface temperature (—) and diffuse reflectance (–□–). A change in slope of the temperature curve (arrow), at 60–70 °C, coincides with the peak diffuse reflectance, which marks the beginning of tissue (stress) relaxation.
chondrocytes is unknown. In Ovchinikov’s clinical study [14], the laser dosimetry used may have resulted in chondrocyte injury, most likely in the superficial layers of the specimen in view of the spatial selectivity of the wavelengths employed. The absence of severe complications (e.g., septal perforation) may be related to this spatial selectivity of the laser device (shallow penetration depth, small spot size) combined with being surrounded by normal cartilage tissue and stem-cell rich perichondrium than by selection of a set of “optimal” dosimetry parameters that reshapes tissue and minimizes cell injury in the laser irradiated region.

This pilot study is the first in vivo viability study to use septal cartilage tissue. The majority of previous in vivo animal investigations have used auricular cartilage [10–13]. In fact, the clinical success of LCR may depend more upon the spatial selectivity of laser irradiation (i.e., laser spot ~1–2 mm with shallow penetration depth) than the preservation of viable chondrocytes within the region of optical energy deposition. In clinical nasal septal LCR, which has now been performed on over 200 human subjects (E. Sobol, 2003, personal communication), focal deposition of laser energy both laterally and axially likely produce only a partial thickness thermal insult in a discrete and limited region of the cartilage. The reshaping is performed with the laser energy delivered through the mucosa, which creates very small circular regions of devitalized mucosal tissue. This is in contrast to the model described herein, in which the entire surface of the cartilage is diffusely irradiated with a deeper penetrating Nd:YAG laser and a large laser spot. Irradiating the entire surface of an intact graft as in this study eliminates artifact that might result from chondrocytes that survive just because they were not irradiated, which underscores the aim of this study to establish information regarding dosimetry and cell viability.

Fig. 6. A, B: Photographic montage of histology taken from controls (A, B) and laser-irradiated cartilage (C, D). A, C: They represent a H&E-stained section viewed at 40×. C: Note the gross loss of tissue matrix and dysmorphic character of the chondrocytes. B, D: They illustrate a section stained with alcian blue (40×). Note in (D), the weak-staining pattern, indicating depletion of the proteoglycan substrate within the extracellular matrix.
In this study, the reshaped cartilage, devoid of the perichondrium, is then re-implanted into an orthotopic site. It is well known that the perichondrium is the source of nutrients for facial and airway cartilage. It is also an important source of mesenchymal stem cells, which are thought to play an important role in chondrocyte survival and regeneration as well as maintenance of structural integrity. Despite using high laser fluences, both laser irradiated and control specimens had identical scattering patterns using flow cytometry and live-dead assays. Nevertheless, matrix depletion and abnormal chondrocyte morphology were seen on light microscopy.

While challenges related to surgical access and technique were not encountered, the selection of a donor site was problematic. Subcutaneous pockets as recipient sites have been used primarily in tissue engineering studies focused on determining the long-term stability of artificial constructs populated by autogenous chondrocytes. Unfortunately, rabbit septal cartilage is relatively soft and pliable like its human counterpart. Following harvest 8–12 months after transplantation to a subcutaneous pocket, this physical feature was lost and both control and laser irradiated specimens became stiff and non-compliant. The lack of perichondrium or a highly vascularized tissue bed may account for part of this material change. Further, the implantation of the curved (laser reshaped) cartilage graft into a relatively flat intrascapular subcutaneous pocket produces forces that act upon the specimens over months and may counter shape change in both laser reshaped specimens and specimens that are naturally curved as well. In future work, the reshaped specimen will be implanted in a geometrically favorable location, such as within a vascularized subperichondrial pocket of the pinna where the forces are more favorable to the retention of shape.

CONCLUSION

LCR remains a promising technology with many potential clinical applications in head and neck surgery. This study was the first in vivo rabbit nasal septal cartilage study to examine the long-term stability of laser reshaped cartilage grafts and correlates these findings with cell viability; albeit using high laser fluences. This information underscores the importance of laser dosimetry and resultant thermal damage. Though the cellular viability was maintained, histologic inspection of the cartilage clearly demonstrates matrix loss in the laser-treated regions. Future investigations must assess chondrocyte viability as a function of varied laser doses in order to determine the optimal thermal range required to maximize both shape retention and graft stability.

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