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Preliminary Investigations on Therapy Thresholds for Laser Dosimetry, Cryogen Spray Cooling Duration, and Treatment Cycles for Laser Cartilage Reshaping in the New Zealand White Rabbit Auricle

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Abstract

Previous studies have demonstrated the feasibility of laser irradiation ($\lambda=1.45\ \mu\text{m}$) in tandem with cryogen spray cooling (CSC) to reshape rabbit auricular cartilage using total energy density of $14\ \text{J}/\text{cm}^2$. The aim of this study was to further explore and identify the dosimetry parameter space for laser output energy, CSC duration, and treatment cycles required to achieve shape change while limiting skin and cartilage injury. Ten New Zealand white rabbits were treated with the $1.45\ \mu\text{m}$ diode laser combined with cryogen spray cooling (Candela Smoothbeam™, Candela Co., Wayland, MA). The ear's central portion was bent around a cylindrical jig and irradiated in consecutive spots of 6 mm diameter ($13\ \text{J}/\text{cm}^2$ or $14\ \text{J}/\text{cm}^2$ per spot) along 3 rows encompassing the bend. CSC was delivered during irradiation in cycles consisting of 25–35 ms. At thin and thick portions of the ear, 4–7 and 6–10 treatment cycles were delivered, respectively. After surgery, ears were examined and splinted for 6 weeks. Treatment parameters resulting in acceptable (Grades 1 & 2) and unacceptable (Grade 3) skin injuries for thick and thin regions were identified and shape change was observed. Confocal and histological analysis of cartilage tissue revealed several outcomes correlating to laser dosimetry, CSC duration, and treatment cycles. These outcomes included expansion of cartilage layers (thickening), partial cartilage injuries, and full thickness cartilage injuries. We determined therapy thresholds for laser output energy, cryogen spray cooling duration, and treatment cycles in the rabbit auricular model. These parameters are a starting point for future clinical procedures aimed at correcting external ear deformities.

Keywords

diode laser with cryogen spray cooling (CSC); otoplasty; ear; laser; cartilage

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Introduction

Significantly protruding or prominent ears affect 5% of the Caucasian population and are congenital defects resulting from the absence of the antihelical fold, overdevelopment of concha, cartilage, and/or increased scapha-conchal angle [1-4]. Since auricular malformations are easily visualized and poorly concealed, these malformations may often lead to psychological stress, emotional trauma, low self-esteem, and behavioral problems when children reach school age [5, 6]. Otoplasty is the current gold standard of treatment for correcting ear deformities. For children this procedure is usually performed before the child enters school, typically around five years of age [1, 2]. Otoplasty is technically demanding, and often results in poor aesthetic outcomes [2]. Typically, this procedure requires general anesthesia (especially in the young children) and carries the risk of hematoma, infection, necrosis, inadequate correction, and scarring [2]. The ideal otoplasty treatment would be minimally invasive, simple to perform, incisionless, require only local anesthesia, and lead to permanent shape change of the ear. Although, some incisionless methods currently exist, they still require sutures and needles to recreate the natural anatomy of the ear.

First conceived by Helidonis et al., laser cartilage reshaping (LCR) is an emerging surgical treatment modality aimed at altering the shape of living cartilage tissues [7-12]. During LCR, tissue is held in mechanical deformation where the tissue is then heated by laser radiation, which accelerates the process of mechanical stress relaxation [13]. After the tissue is splinted in the desired shape for a period of time, a new stable shape is achieved. Since cartilage is embedded beneath skin or mucosa, protecting this tissue from thermal injury is an important element of clinical LCR. Cooling techniques have been widely used by dermatologists to reduce thermal injury in the superficial skin layers while heating subsurface tissue layers using lasers. Combining a contact cooling mechanism with laser irradiation, Mordon et al. [14] were the first to successfully demonstrate safe shape change in rabbit auricles using an Er: Glass laser (1.54 μm). Shortly following this animal study, two separate clinical investigations were conducted with the latter involving 24 patients [15, 16].

Cryogen spray cooling (CSC) may be considered the most robust and aggressive technique to cool the skin prior to laser irradiation while minimizing damage to epidermal tissue. Furthermore, this cooling technology has been used with a variety of different laser wavelengths and for a wide range of clinical applications from hair removal to pigmented lesion treatment [17-19]. As the cryogen evaporates on the skin, cryogen draws heat from the tissue's surface thus protecting the superficial layers of the skin from reaching elevated temperatures while allowing selective generation of heat in the deeper layers of the skin or subcutaneous tissues [20, 21]. Recently in 2009, Holden et al. demonstrated the feasibility of CSC in tandem with LCR to reshape the ear in adult rabbits using the Smoothbeam™ diode laser ($\lambda=1.45 \mu\text{m}$) without causing skin injury [22].

The purpose of this study was to better identify therapy thresholds for laser output energy (E), duration of cryogen spray cooling pulse (T_c), and the number of treatment cycles (N_t) that can be used to reshape the ear in-vivo with acceptable skin injury using Holden's parameters as a baseline [22]. The extent of tissue injury was determined using visual

assessment, live-dead assay, and conventional histology. Given the difficulty of modeling numerically the effect of multiple laser and CSC burst on tissue, in-vivo studies are a necessary step toward optimizing this process for clinical LCR.

Materials and Methods

Ten New Zealand White Rabbits (Western Oregon Rabbit Company, Philomath, OR) weighing approximately 5 kg were used in this study. The rabbit ear model is an established model for laser cartilage reshaping and has been used for analysis over the past decade [10, 14, 20, 22]. The protocol was approved by the UC Irvine Institutional Animal Care and Use Committee (IACUC). IACUC rules and regulations were strictly followed in this study.

Animal Surgery

Animals were anesthetized using ketamine HCl (4-5 mg/Kg) and xylazine (0.2 mg/Kg) given intramuscularly. Once anesthetized, both ears were shaved and prepped for treatment. Each ear on a given animal was treated separately using different laser and cooling settings. Our experimental setup and approach was based on Holden's [22] adaptation and modifications of Mordon's study [14]. The central portion of the ear was wrapped around a cylindrical reshaping jig (diameter 21 mm) constructed out of PVC piping. This hollow jig contained 24 holes (diameter 6 mm, 9 mm separation between spot centers) arranged in 3 rows with 8 holes in each row, which served as the irradiation template. An illuminated fiber optic endoscope light cable was inserted into the reshaping jig to transilluminate the template through the skin and provided a guide for laser irradiation [Figure 1A]. The Smoothbeam™ diode laser ($\lambda = 1.45 \mu\text{m}$, 6 mm spot diameter, Candela Corp, Wyland, MA) delivered a sequence of cooling and heating cycles repeated at a rate of 1 Hz. Each treatment cycle included five alternating cryogen spray cooling (R134a Cryogen Spray Cooling) pulses with variable duration, T_c , from 25 to 35 ms and four laser pulses over 210 ms. Following laser treatment, the ears were splinted around gauze roll (diameter 9 mm) for 6 weeks. Retention sutures (Ethibond Excel, white braided polyester suture) with silicone bolsters were placed to maintain the ears in deformation. The entire procedure, including laser treatment to both ears and splinting, required approximately 20-30 minutes. Animals were closely monitored following treatment and allowed to fully recover from anesthesia before returning to the vivarium.

Evaluating Different Laser and CSC Parameters

Our investigation focused on treating the central region of the rabbit pinna, which is approximately midway between the tip of the ear and the base of the bony external auditory canal junction. The thinner cartilage regions measuring roughly 0.10-0.50 mm are located more laterally; whereas, thicker regions measuring 0.50-1.5 mm are located more medially. Cartilage progressively increases in thickness closer to the crania. In previous work by Holden et al., a baseline set of laser, CSC, and treatment cycle parameters to effectively and safely reshape the central region of the ear without causing skin injury were identified: $E = 14 \text{ J/cm}^2$, $T_c = 33 \text{ ms}$ and $N_t = 6$ and 4 for the thick and thin regions, respectively [22]. Using these parameters as a starting point, we systematically varied the energy density, E , decreased cryogen spray cooling pulse duration, T_c , and increased the number of cycles, N_t ,

to identify potential therapeutic thresholds. This step-by-step serial iterative approach required changing one parameter at a time and observing the consequences of this therapy on the skin of the rabbit ear before moving forward to evaluate the next set of parameters. As laser and cooling parameters act synergistically, the clinical outcomes could not be estimated *a priori*. The objective was to vary laser power, decrease cryogen cooling duration, and increase the number of treatment cycles until significant skin injury was observed. The range of parameters were: 13 and 14 J/cm² (E), 25 to 35 ms of CSC pulse duration (T_c), and number of irradiation/cooling cycles (N_i) ranging from 5 to 10 on thick and from 4 to 7 on thin regions of the ear.

Post-operatively

Animals were observed and digitally photographed (Nikon D40 SLR) daily to ensure splints were in place as well as to monitor the skin. No animals developed any evidence of cutaneous infection. All animals exhibited normal behavior without signs of pain or distress. After 6 weeks, the splints were removed and ears were photographed. The ears were qualitatively evaluated for mechanical stability: each ear was re-positioned to its anatomical position and released, to confirm the retention and stabilization of the newly acquired shape. After splint removal, animals were euthanized via an intravenous pentobarbital overdose (100mg/Kg) followed by dissection of both ears and tissue harvesting for further analysis.

Grading System

Although animals were observed daily, detailed evaluations of the ear skin were conducted at 3 and 6 weeks. A grading system was designed to quantify skin injury. The grading systems was as follows: *Grade 0* - no skin injury, *Grade 1* - minimal skin injury (erythema, mild alopecia), *Grade 2* - moderate skin injury (erythema, minimal blistering, sanguineous crusting, moderate alopecia), and *Grade 3* - severe skin injury (skin changes categorized by a second degree burn including erythema, significant blistering, significant alopecia, and scarring with pigment change). Laser and CSC dosimetry parameters resulting in Grades 0-2 were determined to be acceptable skin injuries; Grade 3 was determined to be an unacceptable skin injury. Evaluation was done by one reviewer (C.C.).

Shape Change and Bend Angle

After the splints were removed, the ears were placed in front of a blue surgical cloth and digitally photographed. No modifications of bend angles were conducted after splint removal. The bend angle was measured from the digital images using Adobe Photoshop (Photoshop CS4 version 11.0, Adobe Systems, Inc). Under normal anatomic conditions, rabbit ears are straight and measure roughly at 180° degrees. After laser treatment and splinting, the ear was expected to undergo shape change whereby the bend angle is less than 180°.

Microscopy

Confocal microscopy (Meta 510, Carl Zeiss LSM, Peabody, MA) in combination with LIVE/DEAD assay were used to image chondrocyte viability [23-26]. LIVE/DEAD (Molecular Probes Inc, Eugene, OR) contains Calcein-AM (494-nm excitation, 517-nm

emission) which easily diffuses into living cells where it accumulates and fluoresces green. The second component of the dye system is ethidium homodimer-1 (528-nm excitation, 617-nm emission) which enters a cell once the plasma membrane has been ruptured where it binds to nuclear DNA. During imaging the nuclei of the “dead” cells fluoresce red. [27] The dye solution was prepped using 0.6 µl calcein-AM, 7µl ethidium homodimer-1, and 1 ml of Hanks' balanced salt solution. Once the irradiation sites were identified and harvested for imaging, the skin and perichondrium were carefully removed. Serial cross-sections 1 mm wide were cut along the irradiation sites and placed in a micro-vial containing dye solution for 30 minutes. The tissue was immediately rinsed with PBS and imaged as previously described.

Histology

After auricular tissue harvest, the laser irradiated regions were identified, carefully removed and prepped for histology sectioning. Tissue samples were fixed in 10% formalin solution for twenty-four hours, and then rinsed and placed in a phosphate buffer (pH 7.4) solution for storage at 4° C. Black tissue dye (Cancer Diagnostics, Inc., Birmingham, MI) was placed on the irradiated site to assist in identifying treated regions following histological processing. Once the dye was allowed to dry, the tissue was dehydrated in increasing concentrations of ethanol, rinsed with HistoClear (National Diagnostics, Manville, NJ), and saturated with paraffin in ATPi tissue processor (Triangle Biomedical Sciences, Inc., Durham, NC). After dehydration the tissue samples were embedded in paraffin and allowed to set. Six µm sections were obtained. Once the sections were placed on 50% albumin fixative coated glass slides, the tissue sections were deparaffinized with HistoClear and finally stained with hematoxylin and eosin.

Results

Clinical Observations

The ear splints did not remain secure in the first 6 animals (12 ears); these 6 animals did not have silicone bolsters, only sutures held the ears in flexure. In these 6 animals, the sutures “cheese wired” through the skin between 2 and 3 weeks allowing the ears to spring back to their natural anatomical position. Though ear reshaping was not achieved in these 6 animals, data on skin injury and its evolution over time was still obtained.

In the remaining 4 animals (8 ears) we improved the suture technique by using simple silicon bolsters to prevent the “cheese wiring” effect. Such bolsters are commonly used in humans for similar procedures to distribute the stress created by retention sutures across a larger surface area. The bolsters successfully helped secure the sutures for the 6 week splint duration. In two cases, after the ear splints and bolsters were removed, evidence of pressure necrosis was observed in the skin at the bolster site. In a third case, the sutures had to be removed during the first week following treatment due to frank ischemia which resolved without incident upon immediate removal of the sutures. This was due to arterial insufficiency resulting from compression of the main auricular arteries as they traversed the flexure region. As a result, we were only able to observe reshaping in 7 ears.

Fortunately, observations on auricular skin changes were obtained in 20 ears. Immediately following laser treatment, some ears exhibited erythema, yellow discoloration, and blistering on the irradiated surface. On the interior, non-irradiated surface, erythema and blistering were observed in some specimens. Skin injury was more commonly seen on thinner regions of the ear. As expected, animals who received more aggressive laser treatment (higher laser energy, shorter duration of CSC pulses, or more cycles) developed greater skin injury. Further details on skin injury relative to laser dosimetry will be discussed later.

Skin Grading & Evaluations

At 3 weeks, all injury grades were observed in the irradiated spots, though Grade 1 (n=17) and Grade 2 (n=17) were most prevalent [Figure 1B-1D]. Since this study attempted to identify therapy thresholds, aggressive parameters were used. As a result some degree of skin injury was observed in all treated ears; no Grade 0's were seen. Grade 3 injuries were less common and were observed in only 6 ears. At 6 weeks, significant wound healing occurred and there was an overall reduction in injury grade (Grade 1, n=24, Grade 2, n=10, and Grade 3, n=6). Erythema and sanguineous crusting were much less prevalent. In contrast, scarring with hypopigmentation and alopecia were in general more commonly observed. Overall, thicker cartilage regions sustained slightly milder skin injuries compared to thinner regions. A general trend of increased skin injury grade with increased total energy delivered to the skin was observed in all treatment groups.

Laser Dosimetry

Using the data obtained from the skin evaluations, we determined several dosimetry thresholds resulting in both acceptable (Grades 1 & 2) and unacceptable skin injury (Grade 3). As previously stated, these thresholds were obtained by varying laser power density, E , the cryogen cooling spurt duration, T_c , and the number of treatment cycles, N_t , delivered to the skin. For this study, 20 different combinations of laser parameters were tested to estimate dosimetry thresholds, again using an iterative technique.

Laser parameters resulting in Grades 1 or 2 to thick regions included $E=14 \text{ J/cm}^2$, $T_c=30\text{-}35 \text{ ms}$, delivered in $N_t=5\text{-}8$ cycles. Grade 3 injuries resulted from using $E=14 \text{ J/cm}^2$ at $T_c=35 \text{ ms}$ delivered in $N_t=10$ cycles. When the CSC duration was reduced to $T_c=25 \text{ ms}$ (at $E=14 \text{ J/cm}^2$) an additional Grade 3 was obtained at $N_t=6$ cycles. All three injury grades were observed at $E=14 \text{ J/cm}^2$ at $T_c=35 \text{ ms}$ delivered in $N_t=9$ cycles. [Table I]

For thin regions, Grades 1 or 2 resulted from $E=13\text{-}14 \text{ J/cm}^2$, $T_c=30\text{-}35 \text{ ms}$ delivered in $N_t=4\text{-}5$ cycles. Grade 3 injuries were obtained by using $E=13 \text{ J/cm}^2$ at $T_c=35 \text{ ms}$ delivered in $N_t=6\text{-}7$ cycles. Similar to thick regions, when the CSC duration was reduced to $T_c=25 \text{ ms}$ using $E=14 \text{ J/cm}^2$ at $N_t=4$ cycles, a Grade 3 injury resulted. [Table II]

Shape Change

Based on previous study by Holden et al. [22], we hoped to achieve a near 90° bend for all treatment dosimetries. As mentioned above, we were only able to observe reshaping over the entire survival interval in 7 ears, due to failure of our initial splinting and suturing method (without silicone bolsters). Calculated at a 95% confidence interval, the average bend angle

was 48.97° with a standard deviation of 21.40. Reshaped ear angles ranged from 21.85°-81.35°. [Figure 1E]

Confocal Microscopy and Viability—Confocal microscopy performed on tissue specimens (n=17) revealed four different outcomes: 1) no change in either the chondrocytes or cartilage matrix, 2) uniform and non-uniform thickening of the cartilage with an increase in local chondrocyte populations, 3) partial thickness injury resulting in thinning of the cartilage layer, and 4) full thickness cartilage defects. [Figure 2]

In regions which underwent less aggressive laser treatments, the chondrocytes and cartilage matrix appeared unharmed; no changes in the structure of the chondrocytes or the cartilages were observed [Figure 2A]. Cartilage layer thickening included two variations: uniform and non-uniform chondrocyte population growth under the footprint of the laser spot. In specimens with uniform distribution, viable chondrocytes were visible on the irradiated side [Figure 2B]. These chondrocytes appeared smaller and more densely packed in comparison to adjacent non-irradiated regions. In addition to this pattern of uniform growth, non-uniform chondrocyte expansion was also observed in few specimens. These discrete regions of new cartilage growth contained clusters of small and irregularly shape chondrocytes were correlated with conventional light microscopy.

Partial thickness cartilage injuries resulted in a reduction in the cartilage layer thickness, and a concave shape [Figure 2C]. Finally, the fourth outcome, full thickness injuries were observed in two patterns: 1) complete loss of the cartilage matrix and chondrocytes and; 2) preservation of an intact matrix though devoid of viable chondrocytes. Loss of the cartilage matrix resulted in scar tissue. The second variation, full thickness injury with preservation of the matrix, revealed regions devoid of living chondrocytes with only few “dead” chondrocytes (red fluorescence). For the most part, these regions primarily lacked any fluorescent signal (red or green), suggesting an empty matrix [Figure 2D].

As a general trend, thickened cartilage layers with Grade 1 skin injuries resulted in two outcomes: no abnormalities to chondrocytes/matrix or uniform cartilage layer thickening. Grades 2 & 3 skin injuries in thick regions of the ear were noted to have full thickness injuries with matrix preservation. Thin regions sustaining Grades 1 & 2 skin injuries primarily resulted in partial thickness cartilage injuries; however, full thickness injuries were occasionally observed. Finally, thin regions with Grades 3 skin injuries were devoid of viable chondrocytes and had either scar tissue or empty matrix.

Histology—Histological analysis was performed on 20 tissue specimens. Conventional light microscopy revealed four different outcomes from laser irradiation: 1) proliferation of cartilage matrix in the irradiated region e.g., thickening; 2) partial thickness injury resulting in reduction of the cartilage layer thickness and loss of chondrocytes and matrix; 3) full thickness injury with complete absence of any cartilage tissue; and 4) preservation of matrix with distinct lacunae and abnormal chondrocyte appearance.

Regions with thickened cartilage layers suggest proliferation of chondrocytes has occurred [Figure 3A]. Confocal imaging of these regions showed the new cell populations were

viable. In addition to the uniform expansion of cartilage layers at the irradiated site, two other patterns of chondrocyte proliferation were observed including an undulating proliferation of chondrocytes along the surface of the original cartilage tissue and ectopic growth of chondrocytes isolated from the main body of the specimen below the original cartilage at the irradiated site [Figure 4].

In specimens with partial thickness injury (loss of cartilage), the cartilage appeared concave with scar tissue occupying the space between the skin and remaining cartilage [Figure 3B]. With more aggressive treatments, full thickness cartilage injuries were common with tissue regions devoid of cartilage with connective tissue entirely replacing the chondrocytes [Figure 3C]. To determine if proliferating chondrocytes existed at the edges of the full-thickness defect, additional tissue analysis such as proliferating cell nuclear antigen immunolabeling (PCNA) needs to be conducted [14].

Finally, the fourth outcome revealed maintenance of the cartilage matrix with distinct lacuna and chondrocyte abnormalities. This type of injury was observed in regions which underwent several continuous laser pulse cycles (i.e. 9-10 pulse on thick regions, and 5-7 on thin). Overall, lacunae located in this region appeared more defined and thicker in comparison to non-irradiated regions. In non-treated tissue, chondrocytes, with distinct nuclei, were relatively centered inside lacunae with moderate spacing between the lacuna and chondrocyte. However, in treated regions the “chondrocytes”, lacking a defined nucleus, appeared to have been drawn out against the lacuna thus minimizing spacing between one another. [Figure 5] Confocal imaging of this region revealed neither red nor green fluorescence, signifying lack of viable chondrocytes or apoptotic chondrocytes.

In general, thick regions sustaining Grades 1 & 2 skin injuries primarily resulted in uniform cartilage “thickening”. Occasionally, partial thickness injury and matrix preservation with chondrocytes irregularities were observed. With Grade 3 skin injuries of the thick region outcomes were less predictable and included: non-uniform cartilage “thickening”, partial thickness injury, and regions of abnormal lacunae and chondrocytes. No full thickness cartilage injuries with the destruction of the matrix were observed during histological analysis of thickened cartilage regions.

For thin regions, Grade 1 skin injury resulted in both partial thickness and occasional full thickness injury with the loss of matrix. Grades 2 & 3 for thin regions presented both full thickness injury with destruction of cartilage matrix and preservation of matrix with chondrocyte abnormalities (likely non-viable).

Discussion

This study aimed to identify therapy thresholds for specific laser dosimetry and CSC parameters for auricular reshaping in the in-vivo rabbit model using a commercially available laser and CSC device. The approach was to systematically vary laser output energy, duration of cryogen spray cooling pulse, and the number of lasing/cooling cycles to identify therapy thresholds. This problem proved to be much more complex than initially thought. In addition to the thermal injury expected in this type of study, a significant

frostbite skin injury was observed from the application of CSC pulses over multiple cycles. Depending on treatment parameters, a frostbite skin injury may be present alone or in a combination with thermal (overheating) injury in cartilage.

Previous work by Holden et al. [22] identified a set of laser and CSC parameters that produced reshaping in the ex-vivo rabbit auricular model and demonstrated its potential clinical efficacy in a limited in-vivo rabbit study. Holden's in-vivo work was derived from numerous ex-vivo investigations involving the use of the Smoothbeam™ diode laser. However, the limitation of the ex-vivo approach is that both frostbite and conventional laser burn injuries cannot be evaluated independently, since in the ex-vivo tissue most of the injury-induced changes do not show immediately. Both frostbite and thermal injuries evolve over time, within the hours and days following application of therapy [28]. The 6 week post-treatment evaluation period in this study allowed sufficient time for the injury stabilization and healing [29].

Building upon previous work performed in our laboratory [22], we obtained significant information on the laser heating and cryogen spray cooling parameters required to reshape rabbit auricle tissue. That information was a starting point for the current study where we have modified treatment parameters to increase tissue injury and this resulted in both acceptable (Grades 1 & 2) and unacceptable (Grade 3) skin injuries for thick and thin regions of the rabbit ear. Acceptable injuries were defined as those producing changes to the irradiated area including erythema, minimal blistering, sanguineous crust, and moderate alopecia. On the posterior surface of the human ear, these types of skin changes would be tolerable and on par with skin incisions that accompany conventional otoplasty. In contrast, unacceptable skin injuries included burns, blistering resulting in scarring with hypopigmentation, and significant alopecia.

In this study three dosimetry parameters were varied and their impact on skin injury were examined. The three dosimetry parameters were: 1) laser output energy density (E); 2) cryogen spray cooling pulse duration (T_c); and 3) the number of laser and CSC treatment cycles (N_t). In the presence of only laser energy, it was expected that an increase in E or/and N_t would increase total energy deposited in the tissue and the subsequent temperature rise would increase tissue injury grade. However, the addition of CSC pulses interwoven with laser pulses resulted in removal of thermal energy from tissue leading to a decrease in tissue surface temperatures. The increase in this energy removal was produced by increasing T_c or/and N_t . Through our investigation, we found this energy removal can lower surface temperatures below freezing resulting in a frostbite injury. Thus, increasing N_t can produce both heat or frostbite injuries. Moreover, while laser energy is deposited volumetrically, CSC creates an intense heat flux directed from tissue depth towards the surface. CSC can remove almost all deposited laser energy from relatively thin tissue specimens. However, the same treatment parameters applied to thicker specimens can produce heating in the deep tissue layers. Overall, we have found that due to the interaction between laser heating, cryogen cooling, and varying tissue geometries, a complex structure of treatment parameter thresholds emerges.

For example, a Grade 1 skin injury in the thick cartilage region may result from $E=14 \text{ J/cm}^2$, $T_c=35 \text{ ms}$, and $N_t=6$. By increasing the number of treatment cycles to $N_t=10$ and using the same energy and CSC duration, we observed a Grade 3 injury. Frostbite resulting from repetitive exposure of the skin to cryogen or, possibly, a combination of surface frostbite with a thermal injury to the cartilage resulted in this Grade 3 injury. In contrast, another Grade 3 injury in the thick region resulted from lowering the CSC duration to $T_c=25 \text{ ms}$ at $E=14 \text{ J/cm}^2$ delivered in $N_t=6$ treatment cycles. In this specimen, we determined the thermal injury was due to insufficient cooling. In thinner ear regions, less aggressive laser and CSC parameters were necessary to acquire comparable grade scale injuries. For example a Grade 3 injury in the thin cartilage was achieved using only $E=13 \text{ J/cm}^2$, $T_c=35 \text{ ms}$, and $N_t=6$. A Grade 2 injury was obtained with $E=14 \text{ J/cm}^2$, $T_c=35 \text{ ms}$, and $N_t=5$. Frostbite was avoided in a Grade 1 injury by decreasing the number of treatment cycles using: $E=14 \text{ J/cm}^2$, $T_c=35 \text{ ms}$, and $N_t=4$. Similar to the thicker cartilage regions, decreasing the duration of the CSC pulse duration produced significant Grade 3 thermal injury with $E=14 \text{ J/cm}^2$, $T_c=25 \text{ ms}$, and $N_t=4$. The trends for laser treatment using the Smoothbeam™ diode laser were: 1) increasing laser energy density and treatment cycles resulted in frostbite skin injury or a combination of frostbite and thermal injury, 2) decreasing CSC pulse duration resulted in thermal injury in skin and cartilage tissue.

The present laser system (Smoothbeam™ diode laser, $\lambda=1450 \text{ nm}$) is currently used to treat skin conditions such as fine rhytids and acne [18, 30]. The device design allows photothermal heating and cryogen spray cooling to work synergistically to minimizing harmful superficial skin injury. CSC was designed to selectively cool superficial skin layers while spatially heating deep tissue layers, essential for adapting this technology to clinical LCR. When the pulse duration is too short ($T_c<25\text{ms}$) less protection is provided to the skin, resulting in significant thermal injury to both skin and cartilage. Confocal and histology both confirm full thickness injury with the destruction of the cartilage matrix. Similarly, a full thickness cartilage injury can result from excessive cryogen and laser application if the laser treatment is delivered in several consecutive cycles (i.e. $N_t=9-10$ pulses on thick regions, and $N_t=5-7$ pulses on thin). With multiple cycles and maximum pulse duration ($T_c=35 \text{ ms}$) freeze burns are likely contributing to Grade 3 skin injuries, while damage to the cartilage is likely due to thermal treatment. Confocal imaging of these injuries revealed full thickness cartilage injury with matrix preservation; no green fluorescence and limited red fluorescence were observed. This is expected since 6 weeks had passed following treatment allowing sufficient time for non-viable cells to undergo necrosis, apoptosis, or reabsorption. Light microscopy of these regions revealed structural integrity of the matrix, albeit distinct chondrocytes abnormalities were observed. Light microscopy must be combined with imaging methods such as confocal microscopy to gain information on the functional status of the tissue. Similar to the present findings, others have observed a wide range of outcomes in terms of cartilage tissue repair and remodeling following laser treatment which include chondrocytes proliferation, chondrocytes regeneration, as well as the absence of viable chondrocytes at the irradiation site [31-33].

Holden et al. [22] achieved an average bend angle of 89° using the Smoothbeam™ diode laser. In the current study more aggressive treatment was aimed to increase laser energy delivered to the tissue and to determine at what point injury to auricular tissue occurs. In

addition, longer splinting durations resulted in an average reshaping angle of 48.97°. This substantial improvement demonstrates this approach may be used to create the complex curvatures of the human ear, such as the antihelix. The antihelical fold is missing or effaced in most otoplasty candidates.

For the first groups of animals, the splints “cheese wired” through the skin between 2 and 3 weeks. No shape change was observed in these animals. As a result we corrected our technique by using silicon rubber bolsters, commonly used to prevent this effect in humans. Although Mordon et al. [14] and Holden et al. [22] achieved shape change in their respected studies by 1 week, it was hypothesized that more aggressive laser parameters resulted in greater injury and extended splinting duration was required to enhance shape change stabilization since tissue wound healing, remodeling, and regeneration clearly occurred. As a result we opted to splint for a duration of 6 weeks which would be compatible with the duration many surgeons ask their patients to wear head band compression dressings after otoplasty surgery.

The number of animals used in this study is a limitation (i.e. N=1 in some parameter sets); however, this is a practical evaluation using an iterative approach towards parameter selection. In future studies, we will expand the number of animals to further refine the space. We approached this study from the standpoint of parametric analysis, looking at the impact of specific changes in a stepwise fashion from what we believed were a safe set of parameters determined from previous work. Together, we have collectively looked at 20 different dosimetry combinations in samples of two different thicknesses and have scaled the laser energy, pulse number, and cryogen spray cooling duration sequentially. There are a large number of parameters that could be investigated, but to do this exhaustively would require over a 100 animals; hence the present study provides guidance for future work with reduced animal subject numbers.

In retrospect, a short coming of this study may be a bias introduced by the fact that there was not a blinded assessment of the degree of thermal and frostbite injury. However, the grading system used a very coarse Likert scale, fractionating the possible outcomes into just four levels with the extremes of Grades 0 and 3 being normal and profoundly injured. The criteria for Grades 1 and 2 were distinct and refined. Certainly there is a biased introduced into the study, but we believe this had only a minimal effect given the clarity between each of the 4 levels.

Understanding the complex interplay between laser dosimetry, cryogen spray cooling duration, and number of treatment cycles is challenging. There are competing effects of heat generation and removal that result in thermal injury produced by excessive heating combined with freeze injury. This study is a first step towards the longer goal of identifying optimal laser parameters. In fact, we have come to understand this is an exceedingly complex problem to model using the Smoothbeam™ technology and is something that has not been encountered in previous cryogen spray cooling models that were performed for dermatological applications. Future work will focus on developing a numerical model to estimate tissue injury based upon the empirical results presented herein, and also analyze the mechanical stability of reshaped auricular cartilage grafts. In addition, before clinical studies

with human subjects can be considered, dosimetry must be scaled and refined to match the axial dimensions of the human ear which are not well-described in the literature. The present study examined tissue injury and shape change in a skin-cartilage model with form-factor and dimensions thinner than that of its human counterpart. However, safe and efficient dosimetry limits for the clinical study may be estimated using numerical modeling. A similar approach has been used for auricular laser reshaping with contact cooling where outcomes obtained using a rabbit model were adapted for later use in human studies [14-16]. Clinical studies to examine the feasibility of laser combined with CSC to treat protuberant ears are forthcoming.

Conclusion

This investigation determined several therapy thresholds using the Smoothbeam™ diode laser, and provided the required information to move forward with planned clinical studies and investigations. Since no system or software modifications were required to perform the reshaping, this device may eventually have value in treating protruding or prominent ears in outpatient settings.

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Figure 1.

The ear to be treated is placed on the reshaping jig (J) which is transilluminated to provide a template for laser treatment. The “X” indicates potential irradiation sites (A). Skin injury of the thin cartilage region at 3 weeks: Grade 1 (B), Grade 2 (C), and Grade 3 (D). Digital image of a reshaped ear following the 6 week splint duration (E).

Figure 2.

Confocal images of treated cartilage tissue. Living cells emit green fluorescence and dead cells fluoresce red. Outcomes resulting from treatment include: no change to chondrocytes or cartilage matrix (A), uniform cartilage expansion (B), partial thickness injury (C), and full thickness injury with the preservation of the cartilage matrix (D). Arrows signify irradiated sites.

Figure 3. Histological images of uniform cartilage expansion (A), partial thickness injury (B), and full thickness injury with absence of cartilage tissue (C). Arrows signify irradiated sites. Note: skin tissue was removed prior to histological processing in image B. (15.75×)

Figure 4. Isolated chondrocyte proliferation below original cartilage tissue (A) and non-uniform proliferation along the irradiated surface (B). Arrows signify irradiated sites. (25×)

Figure 5.

Histological images of the cartilage matrix. For non-treated cartilage regions (A), the chondrocytes were moderately spaced inside the lacunae and contained distinct nuclei. “Chondrocytes” in treated regions (B) lacked both spacing and distinct nuclei. Confocal imaging of this region revealed neither red nor green fluorescence. (50×)

Table I

The numbers inside each cell indicate skin injury Grades (1-3) of the **thick** cartilage regions at 3 weeks. Skin injuries resulted from the combination of laser energy output (E), CSC duration (T_c), and the number of treatment cycles (N_t). All thick regions were treated with $E=14 \text{ J/cm}^2$. The arrow illustrates the trend of increased injury grade severity resulting from an increase in laser output energy, the number of treatment cycles, and the decrease in the duration of cryogen spray cooling.

	Duration of Cryogen Spray Cooling, T_c (ms)				
	25	30	33	35	
Number of Treatment Cycles, N_t	4				
	5		2,2		
	6	3	2,2	1,1,1,1	
	7				
	8		2	1,1	1,1,1,1
	9				3, 2, 1
	10				3

Table II

The numbers inside each cell indicate skin injury Grades (1-3) of the **thin** cartilage regions at 3 weeks. Skin injuries resulted from the combination of laser energy output (E), CSC duration (T_c), and the number of treatment cycles (N_t). Thin regions were treated with $E=13 \text{ J/cm}^2$ or $E=14 \text{ J/cm}^2$. Injury grades resulting from 13 J/cm^2 are distinguished from 14 J/cm^2 by an underscore. The arrow illustrates the trend of increased injury grade severity resulting from an increase in laser output energy, the number of treatment cycles, and the decrease in the duration of cryogen spray cooling.

	Duration of Cryogen Spray Cooling, T_c (ms)				
Number of Treatment Cycles, N_t		25	30	33	35
	4	3	2,2		1,1,1,1
	5		<u>2,2,2</u>	<u>1,2</u>	<u>1,2,2,2,2,2</u>
	6				<u>3</u>
	7				<u>3</u>
	8				
	9				
	10				