In Vivo Laser Cartilage Reshaping With Carbon Dioxide Spray Cooling in a Rabbit Ear Model: A Pilot Study

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Background/Objectives: Similar to conventional cryogen spray cooling, carbon dioxide (CO_2) spray may be used in combination with laser cartilage reshaping (LCR) to produce cartilage shape change while minimizing cutaneous thermal injury. Recent *ex vivo* evaluation of LCR with CO_2 cooling in a rabbit model has identified a promising initial parameter space for in vivo safety and efficacy evaluation. This pilot study aimed to evaluate shape change and cutaneous injury following LCR with CO_2 cooling in 5 live rabbits.

Study Design/Materials and Methods: The midportion of live rabbit ears were irradiated with a 1.45 μm wavelength diode laser (12 J/cm^2) with simultaneous CO_2 spray cooling (85 millisecond duration, 4 alternating heating/cooling cycles per site, 5 to 6 irradiation sites per row for 3 rows per ear). Experimental and control ears (no LCR) were splinted in the flexed position for 30 days following exposure. A total of 5 ears each were allocated to the experimental and control groups.

Results: Shape change was observed in all irradiated ears (mean $70 \pm 3^{\circ}$), which was statistically different from control (mean $37 \pm 11^{\circ}$, $P = 0.009$). No significant thermal cutaneous injury was observed, with preservation of the full thickness of skin, microvasculature, and adnexal structures. Confocal microscopy and histology demonstrated an intact and viable chondrocyte population surrounding irradiated sites.

Conclusions: LCR with CO_2 spray cooling can produce clinically significant shape change in the rabbit auricle while minimizing thermal cutaneous and cartilaginous injury and frostbite. This pilot study lends support for the potential use of CO_2 spray as an adjunct to existing thermal-based cartilage reshaping modalities. An *in vivo* systematic evaluation of optimal laser dosimetry and cooling parameters is required. Lasers Surg. Med. 46:791–795, 2014. © 2014 Wiley Periodicals, Inc.

Key words: facial plastic surgery; macrotia; otolaryngology; laser

INTRODUCTION

Cartilage plays an important structural and functional role within the head and neck, forming the architectural basis of the ear, nose, and airway. As such, means of precisely reshaping live auricular, nasal, and laryngotracheal cartilage have become an active topic of research in otolaryngology and plastic surgery. One clinical application for cartilage reshaping involves treating congenital malformations of the external ear. The incidence of auricular deformities is estimated at 5–15% of all newborns in the United States [1]. Children born with the most common deformity, macrotia (protuberant ear), frequently experience teasing from their peers, often leading to social and emotional trauma [2,3]. In the majority of cases, macrotia is corrected by performing an otoplasty. Traditional otoplasty techniques are based on cutting, carving, and suturing cartilage into a desired shape to balance the intrinsic elastic and structural forces, which are largely determined by the skills and preferences of each surgeon. All classic otoplasty operations require incisions of the skin to allow the surgeon access to the conchal cartilage.

More recently, several minimally invasive techniques for reshaping cartilage have been developed, of which laser cartilage reshaping (LCR) has been the most extensively studied (4–11). In LCR, thermal energy in the form of a laser beam is delivered to the cartilage tissue, which causes stress relaxation leading to shape change at temperatures between $50 - 70^{\circ}$C [12,13]. For clinical applications, LCR may permit shape change through an incision-free, potentially outpatient-based approach, which limits tissue injury, reduces costs associated with surgery and anesthesia, and shortens post-procedure recovery time. The main limitation of LCR, however, is its potential for diffusion of thermal energy to non-targeted tissues, most notably the

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superficial skin layer overlying cartilage, resulting in a burn.

To circumvent cutaneous thermal injury, LCR has been combined with simultaneous cutaneous cooling techniques. The best established modality for cutaneous cooling involves cryogen spray, which employs tetrafluoroethane (R134a) as the coolant, with demonstrated success in dermatologic applications [14–18]. However, LCR applied to tissues with skin thicker than that of the human face, such as that of the auricle, would require higher laser power and, in turn, longer cooling times, increasing the risk for frostbite when R134a is used [18].

To reduce the risk of tissue freezing, liquid carbon dioxide spray (CO_2) has been proposed as an alternative cooling modality. Unlike R134a, CO_2 does not deposit residues on the skin (thereby decreasing the risk of frostbite) and is relatively inexpensive [19]. We have recently completed an *ex vivo* study which identified several promising laser and cooling parameters that may be translated for *in vivo* evaluation [20]. These parameters have been shown to be safe (i.e., minimizing thermal cutaneous injury) and efficacious (i.e., producing clinically significant shape change) in the *ex vivo* rabbit ear model. The goal of this pilot study is to evaluate shape change and cutaneous injury following LCR with CO_2 cooling *in vivo* in five rabbits.

MATERIALS AND METHODS

LCR and Cooling Procedure

We performed our experiments based on an established live rabbit ear protocol previously employed in our lab in a manner nearly identical to that used for the *ex vivo* study [20,21]. The University of California, Irvine Institutional Animal Care and Use Committee approved this protocol.

Five New Zealand white rabbits, each weighing between 3.5–4 kg, were anesthetized using intramuscular injections of ketamine hydrochloride (4–5 mg/kg) and xylazine (0.2 mg/kg). The ears were thoroughly shaven using an electronic hair clipper. The midportions of the right ears were mechanically deformed around a curved cylindrical jig and, while under deformation, irradiated using a 1.45 μm wavelength diode laser with a 6 mm diameter spot size (Smoothbeam™ Syneron/Candela, Wayland, MA) with concurrent application of CO_2 spray to cool the surface. Exact irradiation sites were approximated using built-in perforations (6 mm diameter) 2 mm apart from one another on the curved jig; a light source placed into the hollow jig clearly outlines the perforations [20]. A modified laser hand piece was used to incorporate the CO_2 spray, which was delivered from a heated aluminum paintball tank (Pursuit Marketing Inc, Santa Fe Springs, CA) through a solenoid valve and a straight-tube nozzle. The tank was heated to a temperature that was higher than the operating temperature of the solenoid valve so that no vaporization occurred therein. The selected parameter set was 12 J/cm^2 (fluence) and 85 millisecond (cooling duration), with 4 alternating heating/cooling cycles (laser pulse duration 52.5 millisecond, pre-laser cooling spray duration 10 millisecond, intermediate cooling spray duration 15 millisecond, post-laser cooling spray duration 30 millisecond) per site and 5–6 irradiation sites per row for 3 rows per ear. Following irradiation, the ears were splinted over a gauze roll (1 cm diameter) and maintained in flexion through two 3–0 nylon horizontal mattress sutures. The left ears were designated as controls (i.e., prepped, shaved, and splinted in same manner but not irradiated/cooled).

Photographs of the ear were taken on post-irradiation days 0, 3, and 7. The rabbits were sacrificed on day 30 using a lethal dose of intravenous pentobarbital and phenytoin (Euthasol, Virbac Animal Health, Fort Worth, TX), and the ears were unsplinted, removed, and photographed for analysis of overall bend angle and evidence of gross injury (NIH Image J, Bethesda, MD). Bend angles were measured while the ears were inverted to account for the ability of treated ears to bend against gravity, which is suggestive of tissue remodeling. Differences in bend angle were compared using a two-tailed Student's *t*-test using a significance level of 0.05.

Histologic Analysis

Irradiated/cooled sections of the ear were then harvested for histologic and confocal microscopic analysis. These segments were fixed in formalin for 24 hours, dehydrated with ethanol, embedded in paraffin, and then sectioned to 6 μm thickness using a microtome through the irradiated site. Hematoxylin and eosin staining was performed and the prepared sample viewed with a light microscope at 1× and 10× magnifications to examine the overall tissue architecture.

Viability Analysis

In addition, confocal microscopy in conjunction with a LIVE/DEAD assay (Molecular Probes Inc, Eugene, OR) was performed to evaluate overall tissue viability [21]. In this assay, live chondrocytes appear fluorescent green while dead chondrocytes appear fluorescent red. Specimens were stained under low light with calcein acetomethoxy and ethidium homodimer-1 and viewed using confocal microscopy within hours of euthanasia to prevent premature fluorescence of the dyes. Imaging was performed using a 480 nm argon laser confocal microscope at 10× magnification and areas of live and dead chondrocytes were differentiated.

RESULTS

All five rabbits tolerated the procedure without complications. There was minimal to no gross thermal injury on any of the ears immediately following irradiation/cooling (Fig. 1). Subsequent inspection of the ears on days 3, 7, and 30 also did not reveal signs of obvious gross cutaneous injury (e.g., blistering, crusting, excoriation, slough), suggesting the cooling efficacy of the CO_2 spray. At sacrifice, clinically significant shape change was observed in all irradiated ears (mean 70 ± 3 degrees), which was statistically different from control (mean 37 ± 11 degrees, $P = 0.009$) (Fig. 2).

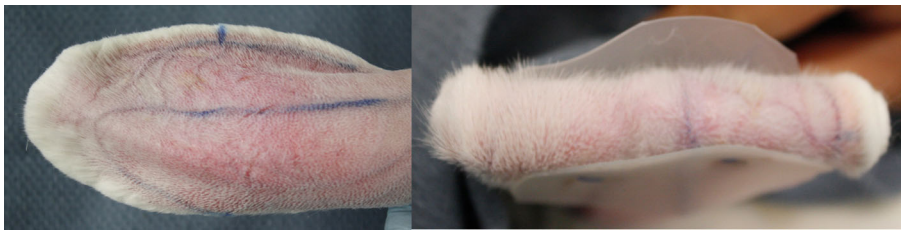

Fig. 1. Gross appearance of treated rabbit ears immediately after (left) and 3 days after (right) irradiation/cooling. No gross thermal injury was observed.

On histologic analysis, no significant thermal cutaneous injury was observed in the irradiated ears, with preservation of the full skin thickness, microvasculature, and adnexal structures (Fig. 3). Confocal microscopy at 30 days post-treatment demonstrated an intact and viable chondrocyte population surrounding the irradiated sites (Fig. 4). Irradiated samples demonstrated similar histologic and confocal microscopic findings as controls.

DISCUSSION

Due to its minimal risk of frostbite during prolonged application to tissues and ability to effectively cool skin surfaces, CO_2 spray cooling is a potential substitute to R134a spray cooling in LCR, and may be the preferred cooling medium in thicker tissues such as the ear. In previous *ex vivo* evaluations [20] and in the current pilot live study, LCR with CO_2 spray cooling appears to produce clinically significant, persistent shape change in the rabbit auricle while minimizing thermal cutaneous and cartilaginous injury, though a systematic *in vivo* evaluation of viable heating and cooling parameters is required to determine the true clinical value of this technology.

The *in vivo* rabbit ear model is a suitable tissue model for clinical applications for several reasons. First, the thickness and tissue characteristics of the rabbit ear are comparable to that of the human pediatric ear [22,23]. Second, cartilage tissue among rabbits is quite uniform and thus lends itself to the possibility of performing large scale, systematic evaluations of the effect of laser and cooling parameters on shape change and tissue injury. Porcine ear is also an appropriate model for cartilage reshaping, though planning for a larger scale, preclinical, systematic evaluation of this technology would be more feasible in a rabbit ear model [24]. Third, the pliability of the rabbit ear allows for easy deformation of the cartilage tissue around a bend, which is a prerequisite for stress relaxation following LCR. To this end, we purposely selected the midportion of the auricle for irradiation to note structural changes which are independent of muscular control (e.g., the auricular muscles are located at the base of the auricle which was not irradiated).

The current study supports the feasibility of performing LCR in a minimally invasive, outpatient-based manner. Once under anesthesia, equipment setup, reinforcing the

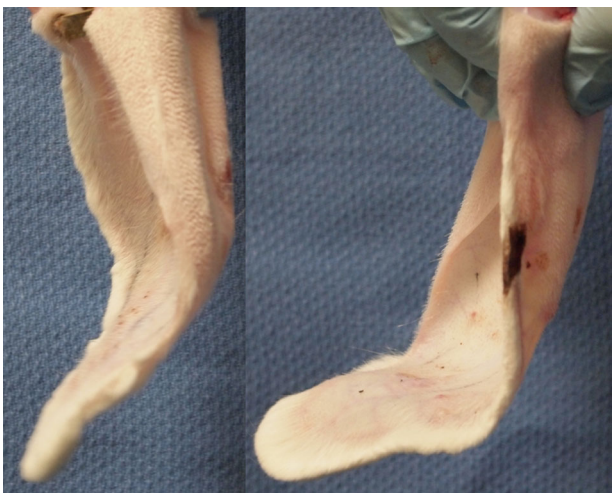

Fig. 2. Inversion of ears (from same rabbit) for bend angle measurement. The left ear is an example of control. The right ear has been treated with laser and CO_2 cooling.

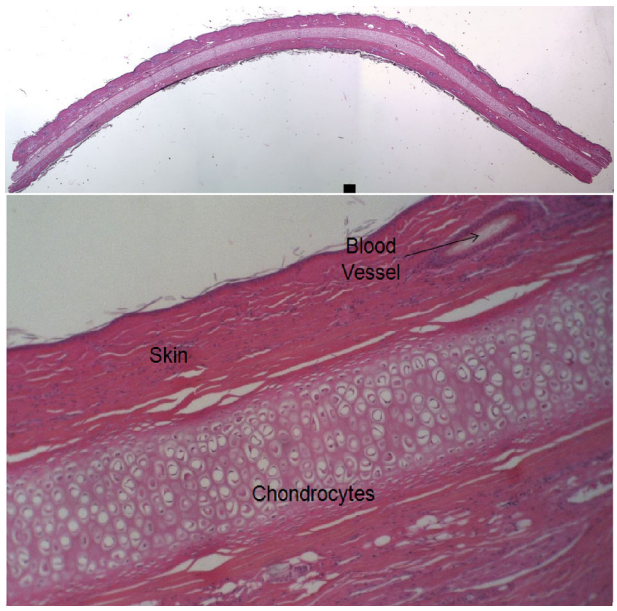

Fig. 3. Histological analysis (top, 1×; bottom, 10×) revealed preservation of the full thickness of skin without cutaneous injury, as well as intact microvasculature and adnexal structures. There was an intact chondrocyte population with no neochondrogenesis.

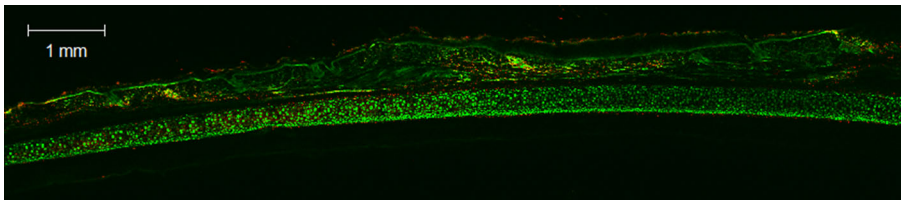

Fig. 4. Confocal analysis demonstrates a population of exclusively live chondrocytes (green signal) at 30 days post-treatment, which is consistent with previous experiments involving cryogen spray cooling.

laser safety checking system, implementing the irradiation/cooling treatment, and splinting the ear can be performed very quickly, within approximately 10 minutes. In clinical applications, patients may be pre-treated with topical, local, or regional anesthetics and undergo the treatment in the clinic. Although the treatment is minimally invasive, multiple treatments may be necessary to obtain the desired outcome. Thus, an improved understanding of the relationship between laser dosimetry and resultant shape change would optimize results while minimizing morbidity and recovery from multiple treatments.

No gross or histologic injury was observed in any of the ears under the current parameter set, which employs a rather high cooling duration (85 millisecond). As both processes depend on rapid shifts in local tissue temperature, frostbite and thermal injury can be difficult to distinguish grossly and microscopically in the absence of specialized techniques [25]. With a live animal model, thermal or frostbite injury may be evaluated in the setting of an intact healing response under normal physiologic conditions. In this study, neither frostbite nor classic burn injury was seen either on gross examination or using microscopy, which is consistent with the results from previous *ex vivo* investigations, once again suggesting that CO_2 cooling circumvents a problem inherent to R134a cryogen spray cooling.

In the current study, viability analysis demonstrated an entirely live chondrocyte population in the treatment zone 30 days post-treatment. These results are partially consistent with previous experiments conducted in our lab on LCR and cryogen spray cooling, in which the entire chondrocyte population was viable at 30 days post-treatment, but that there was neochondrogenesis (not observed in this current study) [21]. The significance of neochondrogenesis is unknown and appears to be a phenomenon unique to facial cartilage. Regardless, the persistence of chondrocyte viability over a month-long period, in combination with preservation of cutaneous and adnexal structures on histology, suggests that LCR with CO_2 cooling may potentially be used as a tissue-sparing, nonablative procedure. Of course, other facial applications exist in terms of adapting the technology for use in septoplasty and rhinoplasty.

CONCLUSION

In this pilot study, LCR with CO_2 cooling appears to produce effective auricular shape change while preventing frostbite or thermal injury to the overlying skin in an *in vivo* setting. Though the results from the current study are promising, the limited sample size will require validation through a large scale, systematic analysis of the complex relationship among laser dosimetry, cooling duration, thermal injury, and shape change. Future studies reflecting this goal are underway.

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