Graded Carbon Dioxide Laser–Induced Subglottic Injury in the Rabbit Model

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Objective: To conduct an endoscopic and histologic analysis of the subglottic effects of various carbon dioxide laser–induced injuries in the rabbit model.

Design: Animals were assigned to either a control (cricothyroidotomy only) group or 4 (cricothyroidotomy and posterior subglottic laser) groups that were injured using varying systematically controlled carbon dioxide laser power exposures (5 W, 8 W, and 12 W), with durations of 2 or 4 seconds, and surface area exposures (25% or 40%).

Subjects: Twenty-seven New Zealand white rabbits.

Interventions: The subglottis was approached via cricothyroidotomy. Control airways were immediately closed, while injured airways were subjected to graded carbon dioxide laser exposures prior to closure. Airways were endoscopically monitored preoperatively, immediately postoperatively, and on postoperative days 1, 7, 14, and 21, after which the animals were humanely killed and subglottic tissue harvested for histological evaluation.

Results: Clinical observation revealed no significantly obstructive (acute) stenosis during the duration of the study. Endoscopic visualization revealed the formation of posterior subglottic scarring. Histological analysis of the mucosa revealed that use of carbon dioxide laser resulted in a statistically significant (unpaired, 2-tailed t test, P<.05) proportional thickening of the lamina propria layer, without significant changes in the epithelial and cartilaginous layers. In addition, mucosal blood vessel size increased proportional to the power of the laser delivered to the area (P<.05).

Conclusions: Carbon dioxide laser–induced injury to the subglottis caused localized scarring, lamina propria thickening, and increased vascularity, which resolved with time and was not associated with significant airway obstruction. This model describes a systematic, controlled, and reproducible method of investigating subglottic injury.

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Although the incidence is diminishing, the development of airway stenosis remains a challenging problem to manage and correct. This decline is largely attributed to improved management of the infant requiring prolonged intubation for assisted ventilation. However, other factors are well known to create, perpetuate, and exacerbate this condition. These include gastroesophageal reflux disease, infection, traumatic injury to the airway, surgical trauma, and the use of lasers in the management of airway lesions. Various lasers have been implemented in the treatment of different pathogenic airway processes, including recurrent respiratory papillomas, cysts, vascular malformations and hemangiomas, webs, and strictures. Ironically, lasers have been associated with creating the very condition they have been used to correct, with several reports indicating posttreatment stenosis in laser-treated patients.

It is now widely held that one must address circumferential lesions in a periodic segmental approach to avoid creating a cicatricial stenotic lesion. What are not clearly defined are the limits of exposure the tissue can tolerate without irreversible damage and stenosis. The effect of exposure in terms of duration and intensity and that of repetitive exposure to a particular area are not clearly understood. Our goal was to determine the response of the posterior subglottic area to varying laser intensities and degrees of area exposure. To accomplish this, we chose the rabbit model because it is a proven acceptable model of airway injury and healing and has produced reliable and reproducible results. We should note that this represents one of only a handful of published studies that address, in a prospective, controlled manner, the impact of lasers on the subglottic mucosa. As such, we believe that our findings help elucidate not only the mucosal response to laser thermal injury but also improve our understanding of the subglottic epithelium's response to laser therapy.
standing of basic aspects of mucosal repair such as extracellular matrix deposition and revascularization.

METHODS

ANIMALS

For this study, the adult New Zealand white rabbit was selected. This animal model has been shown to be applicable for the study of subglottic stenosis and mucosal airway wound healing.29-32 Three experiments were compiled for this study, for a total of 27 animals.

AIRWAY WOUNDING AND VISUALIZATION

All animal experiments were conducted under approved protocols compliant with Institutional Animal Care and Use Committee regulations. Under general anesthesia (ketamine hydrochloride, 35 mg/kg, and xylazine hydrochloride, 5 mg/kg), spontaneous ventilation was maintained, and each animal underwent a preoperative direct laryngoscopy and video endoscopy of the subglottis and upper trachea. A Hopkins (Karl Storz Inc, Tuttingen, Germany) 0º, 3-mm rigid telescope and Storz camera and imaging system were used for evaluation and video imaging. Patency of the airway and absence of subglottic stenosis or other abnormalities were ensured prior to proceeding. Figure 1 reviews the key aspects of the surgical approach. With the animal in the supine position, cervical fur was clipped, and the skin was prepared with a povidone-iodine (Betadine; Purdue Pharma LP, Stamford, Conn) and 70% alcohol scrub. Repeated doses of ketamine and xylazine were administered to maintain adequate anesthesia during the procedure as needed. With a shoulder roll placed, a vertical midline neck incision was made, and with proper dissection, the cervical strap muscles were identified and retracted laterally to expose the laryngotraheal complex. The overlying pretracheal fascia was incised and a cricothyroidotomy was performed, including the lower third of the thyroid cartilage, the cricothyroid membrane, and the first 2 tracheal rings. With the posterior subglottic mucosa widely exposed, further intervention was based on group assignment. Sham control airways were closed, while the posterior subglottic mucosa of the other groups was exposed to the carbon dioxide laser, attached to a 3-mm spot-size manipulator, using different power settings and number of exposures. All exposures involved a 1-second continuous pulse beam held at standard distance from the mucosa (3 cm), with power settings of 5 W, 8 W, and 12 W, each with 2 pulses covering approximately 25% of the airway circumference. A final group of exposures at 8 W with 4 pulses, effecting approximately 40% to 50% of the airways, was studied as well. The depth of injury was through the level of the perichondrium and into the cartilage, as characterized by the “welder’s flash.”

The cricoid and tracheal rings were placed back into anatomic position, and the subcutaneous tissue and skin layer were closed with 5.0-nylon sutures in a loose interrupted fashion. The animals were once again examined and photographed endoscopically and then allowed to recover in the surgical suite. When alert and demonstrating purposeful movement, they were transferred back to the cages, where they were allowed to eat and drink without restriction. They were monitored daily for their general health and signs of stridor, airway restriction, and increased work of breathing. The animals were then endoscopically examined on postoperative days 1, 7, 14, and 21. Under general anesthesia, the rabbit airways were visualized using a direct laryngoscopic approach. Airways were video captured and converted into digital movie files and images (Metamorph; Molecule Devices Corporation, Downingtown, Pa).

A parallel animal study was conducted using an identical experimental protocol but with earlier kill times. Data from this study are currently being analyzed (V.C.S., unpublished data, December 2005–February 2006), though 1 image from an acutely injured mucosa 48 hours after injury is included (Figure 2B) to provide gross evidence of the acute extent of laser-induced injury.

Figure 1. Airway wounding. A, The soft tissues of the neck were dissected in the midline, after which the strap muscles were separated. B, The overlying pretracheal fascia was incised and the subglottis entered via a midline cricothyroidotomy, and the posterior subglottic mucosa was exposed. C, The posterior subglottis was injured via application of a carbon dioxide laser (1-second continuous pulse, 3-mm beam). Each pulse was designed to injure one eighth of the subglottic mucosa.

Figure 2. Gross imaging. Sham surgery did not induce scarring of the posterior subglottic mucosa at 21 days after injury (A). At 48 hours after injury, the damaged posterior mucosa can be observed macroscopically (the arrowhead indicates the 2 laser injuries) (B). At 21 days after injury, laser mucosal damage results in the development of a chronic scar localized to the posterior subglottic mucosa in the 12-W (2 pulses) (C) and 8-W (4 pulses) (D) groups (the arrowheads indicate the presence of posterior subglottic web in the carbon dioxide laser–injured airways)
TISSUE ANALYSIS

Animals were humanely killed by initiating heavy sedation with ketamine hydrochloride and xylazine hydrochloride mixture (35 mg/kg and 5 mg/kg, respectively), followed by intracardiac administration of pentobarbital sodium (50 mg/kg). The previous operative incision was opened, and the larynx and proximal trachea were harvested. The specimen was immediately dissected vertically in the anterior midline and photodocumented using a dissecting microscope (Olympus America Inc, Melville, NY) connected to a charged-coupled device (CCD) camera (Diagnostic Instruments Inc, Sterling Heights, Mich) coupled to dedicated imaging software (Metamorph). The airway was bisected vertically and processed for histological analysis.

MICROSCOPIC ANALYSIS

Tissue specimens were fixed (10% buffered formalin), paraffin embedded, and sectioned into 10-μm thick sections. Sections were stained with hematoxylin-eosin to ascertain the following histological features: cellularity, vascularity, and overall morphologic features of the epithelial, lamina propria, and cartilage layers. For histological analysis, 17 rabbits were used: sham (n=5), 5 W with 2 pulses (n=2), 8 W with 2 pulses (n=3), 12 W with 2 pulses (n=4), and 8 W with 4 pulses (n=3). The animals were chosen based on visual inspection of the sectioned airways, which determined that all 3 layers were relatively intact and appropriate for subsequent measurements. While there exists the possibility that this resulted in some selection bias, it was deemed necessary for a thorough and appropriate histological analysis. The thickness of the lamina propria and cartilage layers was calculated using the aforementioned imaging software averaged across the respective experimental groups and compared using statistical analysis (unpaired, 2-tailed t test). The size and number of blood vessels (as identified histologically by the presence of lumen and red blood cells within lumen) was recorded and compared between the respective experimental groups (unpaired, 2-tailed t test). P<.05 was considered statistically significant.

RESULTS

CLINICAL OBSERVATIONS

A total of 4 animals died prior to the termination of the study. Three died immediately following administration of anesthesia during endoscopic visualization. The remaining animal died on postoperative night 2, having had no prior observable symptoms. Postmortem dissection revealed a sizeable mucous plug in the proximal tracheal region. One additional rabbit (exposure, 5 W) developed stridor and subcutaneous emphysema, both relieved by opening a small portion of the skin incision. No other unexpected events were encountered throughout the duration of the study.

ENDOSCOPIC VISUALIZATION AND GROSS IMAGING

Immediately following surgery and on postoperative days 1, 7, 14, and 21, an endoscopic examination was performed on each animal, allowing serial documentation of wound maturation and scar development. Figure 3 provides representative examples from a member of 3 of the groups, documenting wound progression over time. The sham group showed no induced scarring or other changes of the posterior glottic mucosa throughout the period of observation. In contrast, the carbon dioxide laser induced initial damage to the posterior mucosa, which, at least partially, resolved over time, resulting in the formation of a chronic scar localized to the posterior subglottic mucosa. POD indicates postoperative day.
ing webs being localized and defined. Persistent contracture, cartilage deformity, complete obstruction, and extension of the stenotic region were not noted in any of the groups 3 weeks after surgery.

These findings correlate with those observed on gross imaging, following postoperative day 21, the time the animals were killed and subglottic tissue harvested. Having been removed postmortem, the airways were comprehensively imaged prior to additional processing (Figure 2). Again, no abnormalities were noted in the posterior subglottis in the sham group. However, as indicated by the arrowheads in Figure 2, the specimens in the laser groups contained narrowband scar formation in the surgical region. This did not extend into adjacent mucosa, since the web remains localized to the site of injury. An example of an acutely injured airway is given in Figure 2 as a demonstration of the extent of injury 48 hours after administration of the minimal laser power used in the study. The posterior mucosa can be seen to be discolored, surrounded by an erythematous ring and containing eschar indicative of each of the 2 laser pulses.

HISTOLOGICAL ANALYSIS

Following gross imaging, airways were sectioned and stained with hematoxylin-eosin to highlight connective tissue. The 3 areas of particular attention were the posterior subglottic mucosa, lamina propria, and cartilaginous layers. In Figure 4, representative examples of 3 different groups are represented. Compared with the sham group with normal appropriately configured layers, the laser specimens revealed several changes 3 weeks following injury. In the epithelial layer, complete regeneration of the pseudostratified ciliated columnar epithelium was observed. No abnormalities with regards to width or composition were encountered. However, at the level of the lamina propria, notable differences were observed. The healing process at this level appeared to be reparative, with increased thickness when compared with controls. This enlargement was primarily due to increased connective tissue deposition, primary collagen, which was determined with Masson trichrome stain (data not shown). In addition, this layer contains lymphatic, glandular, and vascular structures. The latter demonstrated clear differences compared with the sham controls. Both the number of vessels and their size were increased. Finally, despite our witnessed penetration of the perichondrium and exposed cartilage, the luminal border appeared intact, smooth, and without scar formation. Overall thickness at this level was similar among all groups, and fibrosis was not noted.

MORPHOMETRIC MEASUREMENTS

At the time of sectioning for histological analysis, the thickness of the subglottis in each animal was measured circumferentially. Figure 5A demonstrates the values for lamina propria thickness for each group. Compared with the sham control group, all laser-injured groups demonstrated substantial increases in thickness of the lamina propria layer, which translates into greater mucosal thickness. This trend reached statistical significance in the 12-W pulse group (P = .02) and in the 8-W (4 pulses) group (P = .01). No substantial or significant changes were noted in the thickness of the cartilaginous layer (Figure 5B). With regard to vascular changes in the lamina propria, Figure 5D reveals that the vessel numbers are greater in most of the laser-injured groups, with statistical significance in the 5-W group (P = .03). In addition, vessel size was measured and averaged for each group, with significantly larger values noted in each laser-injured group compared with the sham controls (Figure 5C).

COMMENT

Many airway conditions have been successfully treated with the carbon dioxide laser. Two of the more frequent and well-described applications are in the treatment of subglottic hemangioma and juvenile recurrent respiratory papillomas. Unfortunately, laser use in the treatment of subglottic hemangiomas has been associated with an incidence of stenosis of up to 20%, with 10% requiring tracheotomy for airway control, warning that the extent into the trachea and rapid recurrence produce elevated risks for stenosis. Studies have noted that repeated exposure without circumferential involvement can induce significant stenosis, likely owing to the increased depth of injury in tissue already altered from previous exposure. Similarly, while it is regarded as the primary mode of therapy for juvenile recurrent respiratory papillomas, increased laser use has been associated with an increased incidence of airway stenosis and tracheotomy. Meanwhile, other studies have concluded that laser use has reduced the need for tracheotomy by over 30% in this population with no reported cases of subglottic stenosis.
With the multiple applications for the use of lasers in the airway, their safety and efficacy are important considerations and require intense scrutiny. While the risk of airway fire has been well described and laser precautions have become commonplace, the risk of permanent injury to the mucosa has not been clearly ascertained. Our goal was to develop a model for investigating the effects of altered laser power intensity and area of exposure on the airway (posterior subglottic) mucosa.12,29

The animal model has several advantages, which includes allowing a prospective study with varying laser settings, the results of which could be repetitively studied endoscopically, followed by histological analysis. The rabbit model, in many ways, is ideal, since it is accepted as a standard small animal airway model. It is an inexpensive, reliable, convenient, and readily available model with reproducible results.12,29-32 We chose to use adult specimens to obviate the need to compensate for luminal expansion with animal growth. Animal size also allowed a larger, less vulnerable airway for study.

There have been multiple methods of inducing airway stenosis described in animal models, including knife incision,33 wire brushings,34 electrocautery,35,36 pneumatic drill,37 hydrochloric acid,29 or silver nitrate to the mucosa.38 Many of these methods do not mimic the actual clinical scenario and yield more significant acute and chronic stenosis, resulting in the early death or need for premature kill times in 22% to 88% of animals. However, the use of the carbon dioxide laser has many clinical applications in the airway, with experimental studies revealing rates of premature death due to obstruction at up to 80%38 and 100% of specimens having greater than 50% stenosis.20

Wound healing after airway intervention/injury is a process of intense restoration and reorganization with, in some cases, irreversible scarring replacing previously normal tissue when the injury reaches or exceeds the lamina propria. The subglottis is particularly vulnerable because in children it is the narrowest part of the larynx and is the only level completely surrounded by cartilage. Out model verifies both qualitatively (histologically) and quantitatively (image analysis) that minimal chronic scarring develops after isolated laser exposure, with the mild changes occurring in the lamina propria layer. Many studies note that in this layer and at the level of perichondrium and cartilage, a reparative rather than regenerative processes occurs.20,22,29,39,40 These result in an increased thickness and associated luminal compression, with often much more extensive results reported than those in our study. These changes at the level of the lamina propria are associated with increased scar tissue, which has been frequently observed in previous studies; however, to our knowledge, the vascular changes we encountered have not previously been described. In fact, the only previous study mentioning vascular composition that we found reported no difference before and after injury in this respect.14,20,22,36,60,91 Appropriate tissue repair has long been thought to be highly dependent on appropriate tissue vascularity. When normal vascular structures are damaged, revascularization is crucial to a timely reparative process. As is the case with other aspects of connective tissue healing, new vessel formation

Figure 5. Morphometric assessment. Compared with the sham surgery mucosa, the thickness of the laser-injured lamina propria (A) was greater, while cartilage thickness (B) remained unchanged. The vascularity of the mucosal layer was analyzed with respect to blood vessel size (C) and number (D). Compared with the sham surgery mucosa, the average vessel size was greater in each of the laser treatment groups, while vessel number was higher only in the 5-W group. Data are presented as averages of the airway dimensions, with error bars representing standard errors of the mean.
is often poorly organized and excessive. Our data suggest that revascularization of the subglottic mucosa following laser injury results in abnormally large vessels and a slight increase in total vessel number. This might represent an increased metabolic demand during mucosal repair, necessitating increased oxygen and nutrient supply. Subsequent studies will need to elucidate outstanding issues. First, how does this revascularization eventually resolve later into the chronic phases of tissue repair and begin to approximate the initial vessel density and size? Second, how does this phenomenon contribute to the overall course of mucosal wound healing?

Several aspects of our findings were somewhat surprising. We found it interesting that the laser did not induce a more severe, lasting gross or histological injury. Although it induced a local response (scar), it did not induce significant circumferential stenosis. Thus, we must consider that either we did not damage the cartilage or the cartilage healed after damage. The former is difficult to accept, since we witnessed mucosal vaporization, as well as perichondrial and cartilaginous exposure and injury with each procedure. Based on previous studies, our assumption was that a full-thickness injury would induce a more severe cicatricial stenosis or at least local cartilage framework deformation.32,33 Other studies have also noted severe responses to similar injuries with electrocautery, in which severe stenosis was incurred in 80% of canines that had to be killed early.37 Correa et al48 described a similar approach in the canine model with carbon dioxide laser application at 6 W, again, resulting in 80% requiring early kill times. The duration of exposure and beam spot size were not mentioned. In contrast, our study would indicate that the lamina propria and especially the cartilaginous layer may be, under certain conditions, capable of a certain degree of regeneration. The technique used resulted in considerably less stenosis than the literature has previously noted.

One might argue that the settings were not sufficient to cause damage. However, the settings were chosen to reflect those used clinically and were able to induce a “welder’s flash” and visual evidence characteristic of cartilaginous penetration. Despite visible tissue destruction, it is encouraging from a clinical standpoint that perichondrial and cartilaginous exposure and injury with each procedure. Based on previous studies, our assumption was that a full-thickness injury would induce a more severe cicatricial stenosis or at least local cartilage framework deformation.32,33 Other studies have also noted severe responses to similar injuries with electrocautery, in which severe stenosis was incurred in 80% of canines that had to be killed early.37 Correa et al48 described a similar approach in the canine model with carbon dioxide laser application at 6 W, again, resulting in 80% requiring early kill times. The duration of exposure and beam spot size were not mentioned. In contrast, our study would indicate that the lamina propria and especially the cartilaginous layer may be, under certain conditions, capable of a certain degree of regeneration. The technique used resulted in considerably less stenosis than the literature has previously noted.

Perhaps our mechanism of injury was crucial to the observed wound healing response. One of the advantages of using the laser is that it provides a consistent, controlled, well-visualized injury, compared with many other methods that are associated with poor visualization while inducing injury, cumbersome techniques with limited clinical relevance, and relatively poor ability in terms of providing a consistent and reproducible injury.

Our study’s weakness is that the model does not exactly replicate the human clinical scenario, since these animal airways are manipulated without an original underlying pathologic process. Despite this, our pilot study revealed findings that are important and serve as a basis for further investigation. Our method of injury could be criticized for using a laryngofissure, which is a significant added variable;36; however, this does not cause airway narrowing, confirmed histologically.43 In addition, by using an open technique, the vector of the carbon dioxide laser toward the posterior subglottis is changed compared with that in traditional endoscopic techniques. While this represents a departure from established clinical approaches, it actually enhances the utility of the carbon dioxide laser as an experimental tool. The direct vector allows us to more precisely standardize both the distance between the probe and the tissue as well as the boundaries of the thermal pulse. It is possible that this may result in increased energy delivered to the subglottic tissue compared with more clinically appropriate vectors. As such, the degree and extent of tissue injury may be somewhat greater than would be encountered in a clinical scenario. Subsequent, larger studies will address this issue by comparing the direct approach with endoscopic approaches to the rabbit subglottis. Nevertheless, in the present study, this method has the benefit of standardization of wound depth and dimension not afforded by an endoscopic approach.

In conclusion, to our knowledge, this study is the first to demonstrate, on both gross and histological examination, the effects of laser injury of varying intensities and extent of mucosal exposure in the animal airway. Our results indicate a marked initial and limited chronic degree of scar formation, with significant changes in thickness at the level of the lamina propria. Changes at the level of the perichondrium and cartilage were not observed. We also noted increased blood vessel size and, to a lesser degree, blood vessel number, findings not commonly noted in previous studies. Based on these results, the initial use of the carbon dioxide laser in the airway using acceptable clinical settings induces a very limited chronic response (scar). Future areas of investigation include histochemical analysis of the secretions during the short-term healing process in comparison with other mechanisms of injury.

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Author Contributions: Drs Chafin, Sandulache, Otteson, Hebda, and Dohar had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Sandulache, Hebda, and Dohar. Acquisition of data: Chafin, Otteson, Hoffman, and Hebda. Analysis and interpretation of data: Chafin, Dunklebarger, Hoffman, Hebda, and Dohar. Drafting of the manuscript: Chafin, Sandulache, Hoffman, and Hebda. Critical revision of the manuscript for important intellectual content: Chafin, Dunklebarger, Otteson, Hebda, and Dohar. Statistical analysis: Sandulache, Dunklebarger, and Hebda. Ob-
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REFERENCES


Correction

Error in Byline. In the Original Article titled “Graded Carbon Dioxide Laser–Induced Subglottic Injury in the Rabbit Model,” published in the April issue of the Archives (2007;133[4]:358-364), an error occurred in the presentation of the fifth author’s name in the byline on page 358. The byline should have appeared as follows: “J. Brett Chafin, MD; Vlad C. Sandulache, PhD; Joshua L. Dunklebarger, MD; Todd D. Otteson, MD; Paul J. Hoffmann, BS; Patricia A. Heba, PhD; Joseph E. Dohar, MD, MS.”