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Effects of diode laser setting for laryngeal surgery in a rabbit model

Helena Hotz Arroyo-Ramos[1] · Larissa Neri[1] · Marilia Wellichan Mancini[2] · Amaro Nunes Duarte Neto[3] · Thais Mauad[3] · Rui Imamura[1]

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Abstract

Purpose To study the damaging effect of different diode laser settings on vocal folds 7 days after injury in a rabbit model.
Methods Twenty-one male New Zealand white rabbits were randomized into three groups with seven animals per group. A 980-nm diode laser was used to create a single spot injury in each vocal fold. Different modulation frequencies (10 Hz versus 1000 Hz) in pulsed mode, different powers (3 W versus 5 W), and distinct wave modes of radiation (pulsed versus continuous) were compared.
Results The extent of the inflammatory infiltrate and ablation crater were greater when using 5-W optical power compared with 3 W. The extent and depth of the inflammatory infiltrate, and the width and depth of the ablation crater were greater with continuous wave mode compared with pulsed mode. The density of collagen fibers only increased when using the laser in continuous wave mode.
Conclusion The use of the 980-nm diode laser with an output power of 5 W produced an increased extent of thermal injury compared to an output power of 3 W and, more importantly, using continuous rather than pulsed wave mode significantly increased the extent and depth of thermal injury in rabbit vocal folds.

Keywords Larynx · Vocal fold · Laser injury · Wound healing · Diode laser · Endolaryngeal surgery

Introduction

The diode laser-based technique with optical emission wavelength within the range 808–980 nm has been used in the last decade for the treatment of laryngeal diseases [1–12] owing to its ablative and hemostatic properties. In addition, the diode laser is delivered by a fine glass optical fiber that allows the surgeon to hold it in a pencil-like holder for manipulation and to access areas that are difficult to reach with the CO_2 laser, the preferred laser for transoral laser microsurgery (TLM) [4, 13]. However, there is great variability among practicing otolaryngologists with regard to the use of the diode laser for TLM, especially with regard to the settings of the device [14]. To our knowledge, no descriptions of the extent of lesions or of vocal fold healing after injury using the 980-nm wavelength diode laser technology have been reported to date.

The purpose of the present study was to address this lack of information by comparing the damaging effect of different 980-nm wavelength diode laser settings 7 days post-injury in rabbit vocal folds.

Methods

The study was approved by the Ethics Committee for the Analysis of Research Projects of the University of Sao Paulo School of Medicine (research protocol No. 177/13). The research was conducted at the University of Sao Paulo School of Medicine. All experiments were performed in accordance with the ethical standards of the Brazilian College of Animal Experimentation.

✉ Helena Hotz Arroyo-Ramos
helenaharroyo@hotmail.com

[1] Department of Otolaryngology, The University of Sao Paulo School of Medicine, Av. Dr. Enéas de Carvalho Aguiar, 255-6º andar-sala 6167, São Paulo, SP CEP 05403-000, Brazil

[2] Research and Education Center for Phototherapy in Health Sciences (NUPEN), São Carlos, SP, Brazil

[3] Department of Pathology, The University of Sao Paulo School of Medicine, São Paulo, SP, Brazil

Determination of laser injury standard

All procedures were performed under direct laryngoscopy followed by endoscopic guidance using a 4.0-mm, 30° Hopkins telescope (Karl Storz, Tuttlingen, Germany) and a 980 nm diode laser (Medilaser—DMC, Sao Carlos-SP, Brazil) delivered through a 400-µm-core diameter optical fiber (Medilaser—DMC). An angled stainless-steel rigid catheter was specially developed to permit directional control of the fiber optic cable of the diode laser (ENT cannula model X2-400-10-140-30 ST-DMC).

A pilot study was carried out and two rabbits underwent direct laryngoscopy. Punctate lesions were administered over different time intervals (5 s, 10 s and 20 s) in one rabbit, and cordectomy type II in the other, in an effort to determine which laser injury could be studied more objectively 7 days after injury. After analysis, a single spot injury over a 20-s interval in each vocal fold was chosen as the standard procedure for this study and the laser parameters were adjusted according to the groups discussed below. The fiber tip was used in superficial contact with the tissue, that is, the researcher touched the fiber tip perpendicular to the vocal fold surface, but not pressing into the mucosa [15, 16].

Animals

In total, 22 male New Zealand white rabbits weighing 2.8–3.5 kg were used in this study. One of these rabbits was not subjected to any surgical procedure but to direct laryngoscopy to check the larynx, to obtain an injury-free reference. The vocal folds in this animal were subjected to all of the stains used in the experimental groups. Data from that rabbit were not included in the statistical analysis; they were used as a non-surgical control.

The 21 remaining animals were randomized into three groups with seven animals per group, with different laser parameters applied to each vocal fold. In Group I, we fixed the power at 3 W with pulsed wave and compared two frequencies: 10 Hz and 1000 Hz. In Group II, we fixed the pulsed wave at a frequency of 10 Hz and compared the power settings: 3 W and 5 W. In group III, we fixed the power setting at 3 W and compared the pulsed wave with the continuous wave. All injuries were administered with the diode laser fiber in stationary contact with the vocal fold for 20 s, and without applying pressure. Comparisons were made between vocal folds in the same animal. The side of the vocal fold that received each parameter was also randomly selected, and blinded to the surgeon. The total energy applied (E) in each procedure was expressed in Joules (J) (Fig. 1). The 400-µm fiber core optical area was 1.25×10^{-3} cm^2, hence, the power densities applied were as follows: 1.2×10^3 W/cm^2 and 2.4×10^3 W/cm^2 (3-W pulsed and continuous waves, respectively) and 2.0×10^3 W/cm^2 (5-W pulsed wave). Fluence values for the three groups are shown in Fig. 1 and are with reference to the 20 s exposure time: 24×10^3 J/cm^2 and 48×10^3 J/cm^2 (3-W pulsed and continuous waves, respectively) and 40×10^3 J/cm^2 (5-W pulsed wave). Pulsed mode was used with 10 Hz and 1000 Hz frequencies, 50% duty circle, i.e., 50 ms on/50 ms off and 0.5 ms on/0.5 ms/off cycles, respectively. It is important to note that, in pulsed mode, the mean power, irradiance (power density) and fluency are reduced to 50% of the corresponding continuous mode values, due to the pulsed mode parameters used (50% duty cycle).

Surgical procedure

Anesthesia was administered to all animals by intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) (Vetbrands, Paulınia, Brazil) and they were maintained under spontaneous ventilation. The animals underwent direct laryngoscopy, and diode laser injury was performed on the true vocal folds (Fig. 2) respecting the standard laser injury procedure as previously described and with laser parameters chosen according to the three groups (Fig. 1). The laser irradiation parameters followed instructions from the manufacturer and were based on literature values [14]. The same

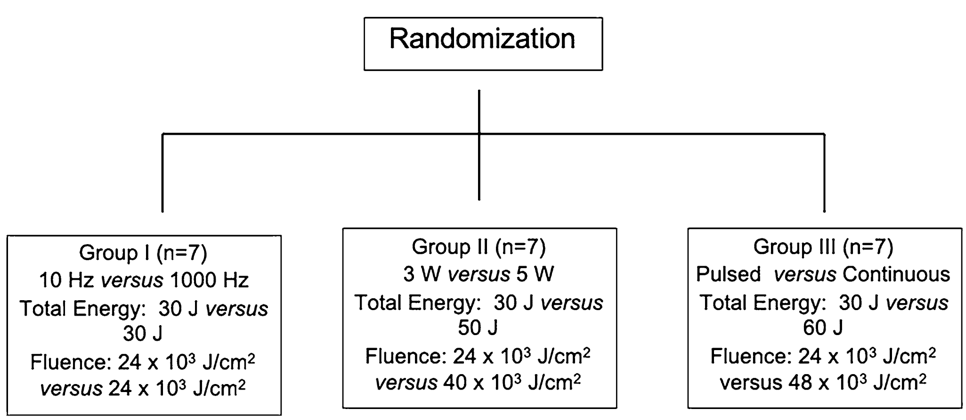

Fig. 1 Randomization of 21 animals into three groups

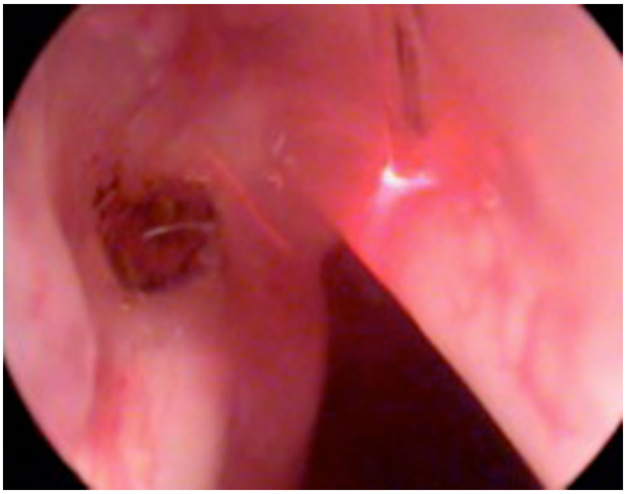

Fig. 2 Intraoperative image of the laser injury on the left vocal fold just after the procedure and the 980-nm diode laser fiber in contact with the right vocal fold mucosa about to start the procedure

researcher (HHAR) performed all surgical procedures and was blinded to the parameters set at the laser device. After surgery, the rabbits were returned to their cages, without activity limitations. The animals were killed 7 days after the procedure. Anesthesia was induced as noted above, and a lethal dose of propofol was administered intravenously.

Histology and histochemistry

After sacrifice, the larynges were harvested and placed in paraformaldehyde for 24 h for fixation. After that, they were dehydrated in an increasing alcohol gradient, and embedded in paraffin. Successive 3.0-µm-thick sections were cut in the coronal plane. The sections were made in the same systematic manner from the anterior to the posterior portion of the vocal fold. All sections were placed on a glass slide and each fifth section was stained with hematoxylin and eosin (H&E), using standard methods. Two blinded pathologists examined each H&E slide in an effort to choose the one that could best represent the center of the lesion. Additionally, histochemical stains for elastin fibers (Weigert's resorcin–fuchsin after oxidation with oxone) and collagen (PicroSirius Red) were performed.

Quantitative histological analysis

All slides selected for the study were scanned with the Pannoramic Scan digital slide scanner (3DHistech, Budapest, BP, Hungary) for digital image analysis.

The H&E-stained scanned slides were evaluated by a blinded pathologist along with the main researcher (HHAR). Using objective (continuous) and subjective (categorical) analyses, they both examined the cell nuclei of the inflammatory infiltrate surrounding the injury and the degree of injury. The objective analyses were performed using Pannoramic Viewer® 1.3 software for Windows® (3DHistech). Lateral thermal injury was determined by measuring the extent and depth of the inflammatory infiltrate (µm). In the same way, the extent and depth of the ablation crater were measured. The data obtained were expressed as mean ± standard deviation. The categorical analyses of wound healing components were performed using subjective scales (Table 1). Different magnifications were used for these analyses.

The densities of collagen stained with PicroSirius Red and elastin stained with resorcin–fuchsin were calculated as the fraction of the positively stained area to the total area of the vocal fold (percentage) using Image Pro Plus 4.5 (Media Cybernetics, Rockville, MD, USA).

Table 1 Subjective scales for categorical analyses of wound healing components

Variables	Scale			
Grade of inflammatory infiltrate	0 = absence	1 = slight	2 = moderate	3 = severe
Type of inflammatory infiltrate	1 = suppurative	2 = mixed	3 = lymphomono nuclear	4 = granulomatous
Depth of the inflammatory infiltrate	1 = superficial muscle layer	2 = deep muscle layer	3 = perichondrium	4 = cartilage
Edema of lamina propria	1 = little	2 = moderate	3 = intense	
Amount of fibroblasts	1 = little	2 = moderate	3 = intense	
Amount of eosinophils	1 = little	2 = moderate	3 = intense	
Integrity of the epithelium	1 = complete re-epithelialization	2 = incomplete re-epithelialization		
Exocytosis	0 = absence	1 = presence		
Spongiosis	0 = absence	1 = presence		
Blister	0 = absence	1 = presence		
Ulceration	0 = absence	1 = presence		
Coagulative necrosis	0 = absence	1 = presence		
Fibrosis	0 = absence	1 = presence		

Statistical analysis

Statistical analyses were performed with STATA version 11.2 (Stata Corp, College Station, TX, USA). The Wilcoxon non-parametric test was used for continuous variables and Fisher's Exact Test for categorical variables, using a threshold for statistical significance of $p < 0.05$. We did not correct type I errors due to the exploratory nature of this study.

Results

H&E analysis

An overview analysis of the H&E staining demonstrated thermal injury characterized by exocytosis of inflammatory cells; edema of mucosa and submucosa; massive cellular infiltration around the ulcer, with a combination of polymorphonuclear cells (especially eosinophils), lymphocytes and histiocytes; the presence of granulation tissue with fibroblasts and neoformed vessels and areas of coagulative necrosis. In some cases (two in group I, four in group II, and two in group III), suppurative abscess formation was observed in the lesion area. In three other animals (one in group II and two in group III), there was formation of ectopic cartilage.

In group I, although the extent and depth of the ablation crater presented relatively higher measurements at 1000 Hz compared with 10 Hz, there was no statistically significant difference in the objective (Table 2) or in the subjective analyses. The most significant difference in group II was that related to the extent of the inflammatory infiltrate and the extent of the ablation crater, which were statistically significantly greater at 5 W than at 3 W (Table 2; Figs. 3, 4). There was no statistically significant difference in any of the subjective analyses in group II. In group III, the extent of the inflammatory infiltrate, the extent of the ablation crater, the depth of the ablation crater (Table 2; Figs. 3, 4), and the subjective analysis of the depth of the inflammatory process were statistically significantly greater in continuous wave mode compared with pulsed wave mode (Fig. 5).

Analysis of collagen and elastin fibers

Elastic fibers were fragmented, disorganized (close to ulcers, they were vertically distributed), with loss of linearity in the submucosal layer and sparsely distributed in all layers of the vocal folds that received laser injury, regardless of the group analyzed. There was no statistically significant difference in the density of elastic fibers in rabbits in the same group.

The collagen fibers were thin and poorly organized in the areas of granulation tissue in all groups analyzed. The density of collagen fibers was higher when the laser was used in continuous wave mode ($p = 0.018$). There was no statistically significant difference with regard to collagen density in relation to laser frequency (group I) or laser power (group II).

Discussion

Inflammatory and fibrotic processes can result in voice limitations due to altered tissue viscoelasticity and oscillatory function of the vocal fold [17]. As a result, treatments that provide maximum preservation of structure and function are highly desirable for vocal fold pathologies [13, 18–20]. The carbon dioxide laser has been the preferred laser since the introduction of TLM [4, 13], but it has some limitations such as high cost, limited tissue penetration depth, and limited portability. The diode laser has demonstrated early promise as a treatment modality for a number of vocal fold diseases as it is portable, smaller and simpler to use. However, there

Table 2 Comparison of objective (continuous) inflammatory variables analyzed by H&E for groups I, II and III (Wilcoxon test)

	Group I			Group II			Group III		
	10 Hz	1000 Hz	*p*	3 W	5 W	*p*	Pulsed	Continuos	*p*
Extension of the inflammatory infiltrate (μm)	3730.87 (1942.71)	4157.67 (2136.65)	0.735	4628.83 (794.43)	6880.74 (1332.67)	0.018	7711.28 (4365.17)	11124.26 (4160.99)	0.018
Extension of the ablation crater (μm)	565.65 (895.79)	1657.02 (1414.80)	0.102	1547.97 (992.02)	2840.39 (1194.11)	0.018	993.39 (1078.95)	2884.04 (2375.96)	0.028
Depth of the inflammatory infiltrate (μm)	1131.37 (670.97)	1235.40 (834.57)	0.866	1753.90 (311.82)	1805.10 (387.38)	0.865	1179.23 (645.09)	1491.27 (431.90)	0.091
Depth of the ablation crater (μm)	145.68 (206.09)	415.34 (549.18)	0.102	295.61 (221.54)	199,66 (147.72)	0.398	186.67 (309.62)	255.64 (300.67)	0.028

Values refer to "mean (SD)", p = significance

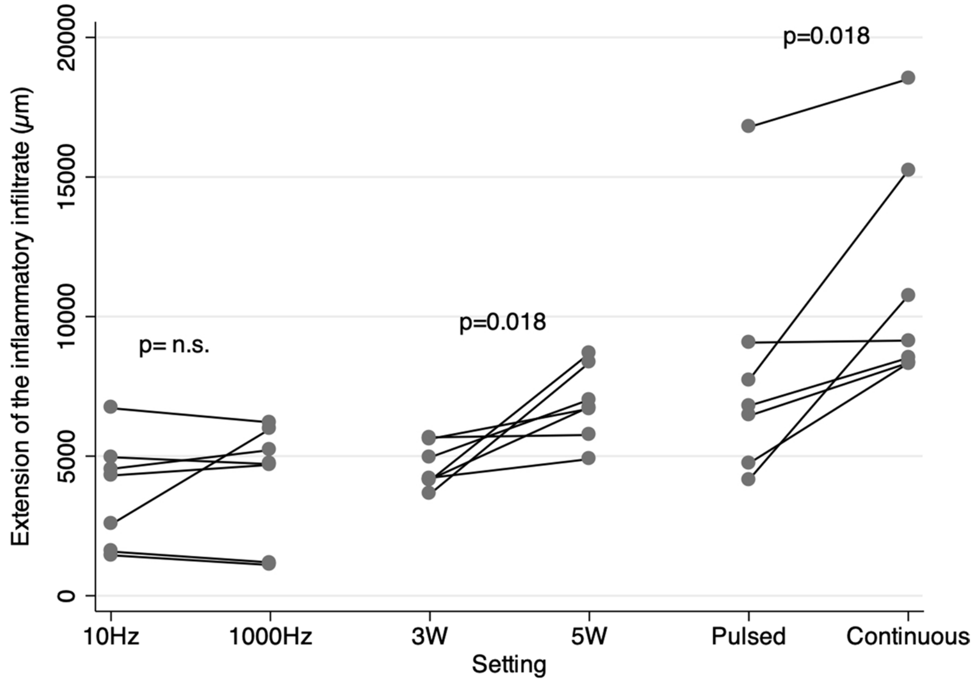

Fig. 3 Results from Wilcoxon non-parametric test applied to analyze the extension of the inflammatory infiltrate in groups I, II and III

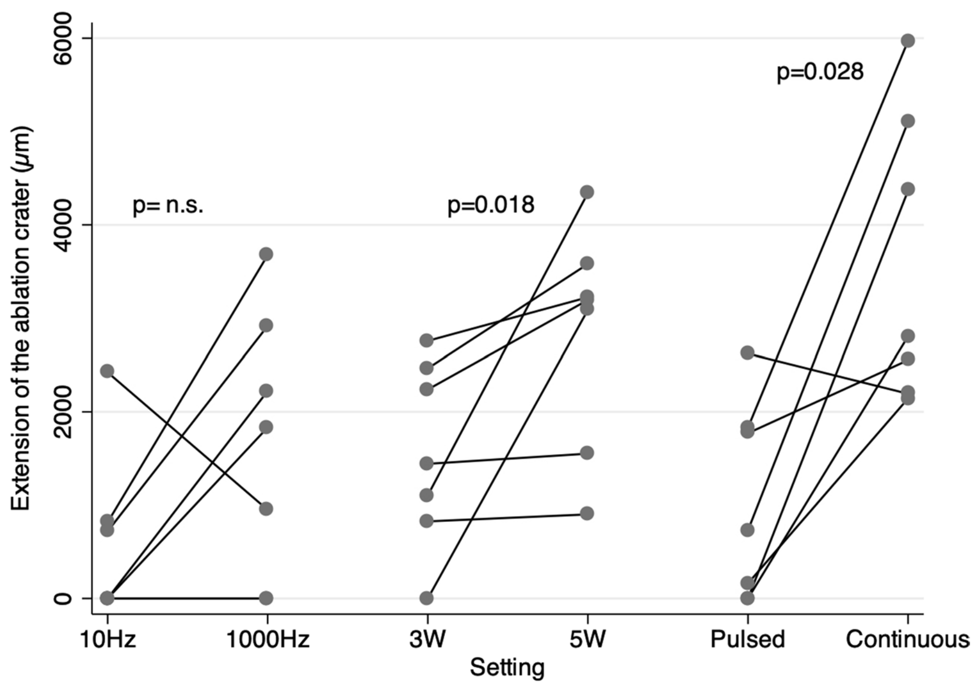

Fig. 4 Results from Wilcoxon non-parametric test applied to analyze the extension of the ablation crater in groups I, II and III

are limited data addressing the damaging effect of this laser and its influence on healing in vocal fold tissues.

Compared to the CO_2 laser ($\lambda = 10,600$ nm), the 980-nm diode laser has lower water absorption but it is also absorbed by other chromophores such oxyhemoglobin. These features confer on the diode laser a surgical precision only slightly inferior to the CO_2 laser but with superior capability of coagulation [1]. As a result, the diode laser has a greater penetration depth in biologic tissue, which makes it ideal for photocoagulation, while the CO_2 laser is best suited for tissue ablation [21].

In our study, the extent of the inflammatory infiltrate and the extent of the ablation crater were significantly smaller when the tissues received an output power of 3 W compared with 5 W (group II). This slight change could be insignificant if tested in other tissues; however, the vocal fold has a highly complex microstructure which makes it very delicate and susceptible to minimal damage. These findings are in

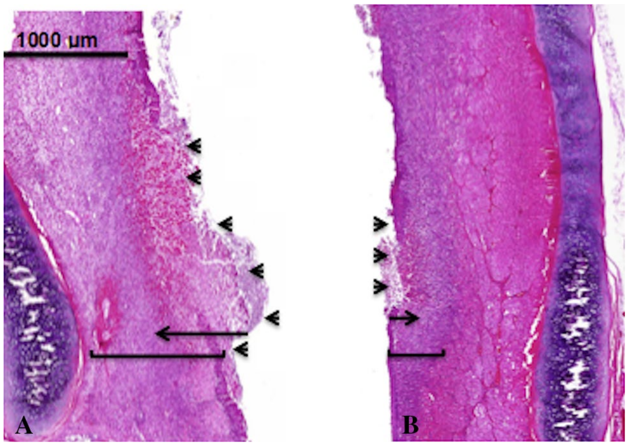

Fig. 5 Representative histological coronal sections of a rabbit larynx from group III stained with hematoxylin and eosin showing the extent of the ablation crater (arrowheads), the depth of the ablation crater (black arrow) and depth of the inflammatory process (bracket): **a** right vocal fold which received continuous wave mode with more pronounced injury, **b** left vocal fold which received pulsed wave mode (×20)

accordance with previous studies that demonstrated laser parameters interfering in the healing mechanism, especially the effects of increased output power [22–24]. The surgeon must remember that the action of the laser at lower power output (3 W) can minimize thermal damage to the tissue but can also reduce the effectiveness of the incision (ablation rate). The laser settings should be carefully chosen depending on the clinical demands and the surgeon's experience.

In continuous wave (CW) mode, the optical energy is emitted constantly as the laser is always on. In pulsed mode, the optical energy is modulated (alternately on/off), in an effort to minimize lateral thermal damage. This is possible since the thermal relaxation time (time required for the heated tissue to lose 50% of its heat through diffusion [25]) is sufficient to allow thermal recovery, with adequate time between exposures [26, 27], before the next pulse of energy. Our results showed that the CW mode diode laser produces more pronounced changes than pulsed wave mode with greater lateral and depth extent of the inflammatory infiltrate and ablation crater. This is due to the higher heat generation in situ (optical to thermal energy conversion process). This result corroborates those of other authors who compared CW versus pulsed mode of CO_2 laser in the vocal fold of dogs [28] and the oral mucosa of dogs [23, 29], and found lower lateral thermal damage with pulsed wave mode. Based on this study and on the surgeon's training (HHAR) with this laser, pulsed wave mode is sufficient for good outcomes when operating on lesions in the larynx. In any event, the correct laser settings should be carefully chosen depending on the clinical demands and the different tissue characteristics [22], choosing the least traumatizing procedure whenever possible [30]. In this sense, perhaps surgeons could consider the use of pulsed mode of the diode laser in an effort to avoid local damage and buildup of heat, minimizing damage to contiguous tissues, especially in benign lesions and early glottic cancer where preservation of the surgical margin is important [31]. Conversely, the literature reviewed shows the surgeons' preference for CW mode [1–12].

We compared two different frequencies, 10 Hz versus 1000 Hz (pulsed mode); however, there were no statistically significant differences in the data, although the extent and depth of the ablation crater were relatively greater when using a 1000 Hz repetition rate.

Elastic fibers contribute to the biomechanical properties of the vocal fold, related not only to its density but also to its disorganization in cicatricial tissue [16, 32, 33]. In normal human vocal fold, they are more abundant in the middle layer. In our study, the elastic fibers were fragmented, disorganized, with loss of linearity in the submucosal layer, and sparsely distributed in all layers of the vocal folds that received laser injury.

The density of type I and type III collagen fibers is high in acute response to injury [34]. It is known that tissues with a higher concentration of collagen are more likely to develop scarring and fibrosis. We measured the percentage of collagen to quantify the acute tissue damage produced by the diode laser and found that CW mode can increase the density of collagen, which may predict poorer wound healing.

This study has several limitations. First, we did not compare the diode laser to other lasers, or to cold instruments. We aimed to study the effect of different parameters of the 980-nm diode laser on the vocal folds, as the diode laser is currently being used in TLM in different centers around the world without a thorough scientific background [1–12]. Generalization of results of this experimental animal model to humans is inappropriate. Nevertheless, we were able to estimate the biological effect of different diode laser parameters on the vocal folds, controlling for biases that could have otherwise compromised the validity of the study results. Another limitation is that we only evaluated the healing process at 7 days post-injury. The decision to study the early phase of vocal fold wound healing was based on previous studies [16, 17, 34–38]. Branski et al. [35] and Tateya et al. [34] advocated that the acute healing process is a critical period during which therapeutic intervention can attenuate vocal fold scar formation. Ling et al. [36] argued that knowledge of acute cellular changes that follow vocal fold mucosal injury is fundamental to eventual therapeutic manipulation of the vocal fold wound healing process. We chose to evaluate the healing process on the seventh day based on the information that, when considering acute vocal fold wound healing, a more mature inflammatory reaction can be found at day 7 [16], and the process of active tissue remodeling followed this [17]. In the study by Tateya et al. [34], extracellular matrix components such as hyaluronic acid, collagen, and fibronectin were produced immediately after surgical incision in the vocal fold and were more abundant between the third and fifth days. In addition, Branski et al. [35] reported a massive proliferation of inflammatory cells and fibroblasts around the surgically induced vocal fold wound at day 3. New collagen was deposited by day 5, with fibroblasts being the predominant cells at that time, and finally, a more mature collagen was detected by day 7 [35]; however, it is not possible to predict long-term results from our study and further studies over longer periods are needed, as well as studies in humans.

The diode laser is a viable method that should be considered for TLM. It seems to be a promising laser device, so we expect that new diode laser wavelengths will continue to emerge, making it an interesting alternative to the CO_2 laser, but with lower cost.

Conclusion

In the present study, the use of the 980-nm diode laser with an output power of 5 W produced an increased extent of thermal injury compared to an output power of 3 W and, more importantly, using continuous rather than pulsed wave mode significantly increased the extent and depth of thermal injury in rabbit vocal folds.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval The study was approved by the Ethics Committee for the Analysis of Research Projects of the University of Sao Paulo School of Medicine (research protocol no. 177/13). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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