Lasers Med Sci (2011) 26:257–265
DOI 10.1007/s10103-010-0849-y

ORIGINAL ARTICLE

Different air-water spray regulations affect the healing of Er,Cr:YSGG laser incisions

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Received: 3 August 2010 / Accepted: 4 October 2010 / Published online: 3 November 2010
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Abstract Surgeries performed with high-intensity laser devices may be improved with accurate protocols, including the air-water spray regulation. Thus, this study sought to investigate the healing process of wounds made on the dorsum of rat tongues using an Er,Cr:YSGG laser device with different air-water spray regulations. The incisions were made on the dorsum of Wistar rat tongues using an Er,Cr:YSGG laser with three different air-water spray regulations (100/0%, 50/50%, 11/7%). Scalpel incisions functioned as controls. The sacrifices occurred between 0 and 14 days after surgery. Morphological, histological, and immunohistochemical (fibronectin and type III collagen) analysis of the wounds were performed. The air-water spray regulation influenced wound healing and the inflammatory response, especially in the earlier stages. Incisions performed using the 100/0% air/water spray regulation had the worst results, expressing a greater amount of fibronectin and type III collagen. The 50/50% air/water spray regulation brought in a non-clear surgical field and poor laser interaction with the tissue. The 11/7% air/water spray regulation showed the best clinical results and less pronounced histological events. According to the results encountered, the air-water spray should be regulated to improve surgery.

Keywords Er,Cr:YSGG laser · Wound healing · Air-water spray · Immunohistochemistry

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Introduction

Laser devices may show significant advantages when used in oral surgeries. Their interaction with tissues may vary according to the inherent capability of the single wavelength employed to be absorbed by water or chromophores inside the tissue. Due to the high level of water and intense vasculature present in the soft oral tissues, erbium-based solid-state infrared lasers, operating at approximately 3 μm, have been demonstrated to interact with these tissues [1]. Earlier investigations have shown that the Er,Cr:YSGG laser can provide efficient and precise ablation of both hard and soft tissues with minimal thermal damage to adjacent tissues in vivo [2, 3]. The Er,Cr:YSGG laser works at a wavelength of 2.78 μm, which is absorbed well by water [4]. Also, the benefit of minimal intra-operative hemorrhage when using this laser device instead of a conventional scalpel has been mentioned [5, 6].

Nevertheless, correct protocols should be determined to precisely ablate soft tissue and coagulate vessels while minimizing thermal damage [7]. Thus, the investigation of a tool incorporated in the Er,Cr:YSGG laser device, the air-water spray, is important and, up to the moment, has not been performed.

Therefore, the present study aimed to evaluate different air-water spray regulations and their relevance on wound healing in rat tongue incisions. Scalpel incisions worked as controls and represented the well-characterized archetype of a conventional incision. The evaluation of wound healing was done through morphological analysis and immunohistochemical staining.

Materials and methods

The current study was approved by the Committee of Animal Experimentation of the University of São Paulo.

Laser device

The Er,Cr:YSGG laser (Millennium, Biolase, San Clemente, CA, USA), emitting at 2.78 μm and pulsing for a duration of 140–200 μs and a repetition rate of 20 Hz, was employed in the present study. The mean power output of the laser was 2.0 W, and the energy per pulse was 100 mJ. The delivery system consisted of a fiber-optic tube that terminates in a handpiece with a 600-μm sapphire crystal tip bathed in an adjustable air-water spray. The beam spot size at the tip was 0.0028 cm^2, and the exposure time was 3 s, providing an energy density of 35.7 J/cm^2.

Animals for the experiment

Twenty-four 2-month-old female Wistar albino rats weighing between 200 and 250 g were kept in individual cages at room temperature with 12 h of light per day and 50% relative humidity. The rats received a standard laboratory diet and water ad libitum.

Surgical procedure

Animals were anesthetized by intramuscular injection with a mixture of ketamine and xylazine at 50% that was administered at 0.1 ml/100 g animal weight. Four 5-mm-long and 2-mm-deep incisions were performed on the dorsum of the tongue of each animal. One incision was made with a scalpel and the other three incisions were made with the Er,Cr:YSGG laser, varying the air-water spray regulation: 11/7%, 50/50%, 100/0% (Fig. 1a). The laser Er,Cr:YSGG was employed at 2.0 W and 100 mJ as measured by a power meter (Field Master GS, Coherent, Dieburg, Germany). The incisions were performed with the sapphire tip perpendicular to the tissue and slightly touching the mucosa. Each incision was achieved by two irradiations of 1.5 s, and the energy density was 35.7 J/cm^2. All incisions were completed by a single surgeon, and the depth was measured using a probe. After the surgical procedure, the rats were returned to the cages.

Morphological studies

The post-operative period was uneventful. Groups of four animals were killed at intervals of 0, 1, 2, 3, 7, and 14 days after surgery (groups G0, G1, G2, G3, G4, and G5, respectively). The animals were killed by CO_2 inhalation. After killing, the tongues were sectioned from the base with surgical scissors. The material was placed in individual containers with 10% formaldehyde. Samples were histologically processed, and after embedding in paraffin, 5-μm sections were obtained and stained with hematoxylin and eosin (H&E) stain. A blind qualitative histological analysis was performed by three independent operators on each set of slides using a light microscope.

Immunohistochemistry

Sections of 3 μm were obtained from the paraffin-embedded material, mounted on slides, treated with 3-aminopropyltriethoxy-silane (Sigma Chemical Co., St.

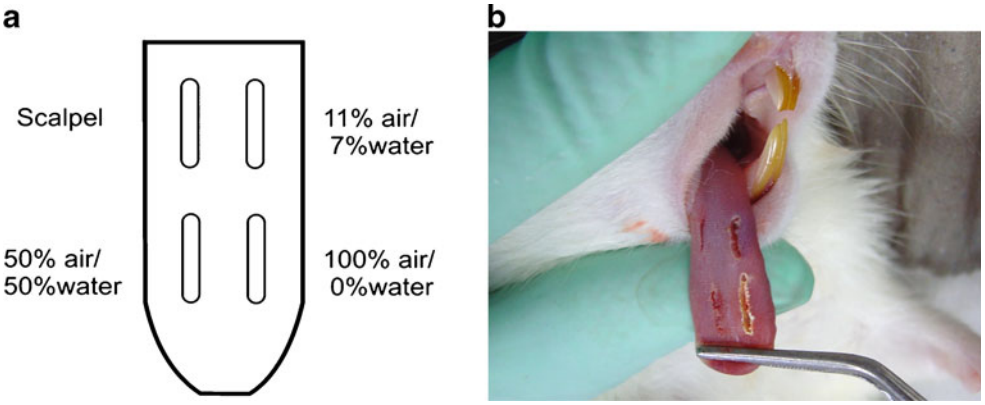


Fig. 1 **a** Position of each type of wound performed on the dorsal side of the rat tongue. **b** Final aspect of the rat tongue after surgical procedure

Louis, MO, USA), deparaffinized and hydrated. Endogenous peroxidase was quenched by incubation in 3% hydrogen peroxide in methanol (1:1) for 30 min at room temperature. Then, sections were treated for antigen retrieval according to the antibody to be used and then incubated with the antibodies (Table 1). Immunostaining was performed using the streptavidin-biotin-peroxidase method. Three pre-calibrated blind examiners analyzed the sections semi-quantitatively.

Results

Surgical procedure

All incisions performed with the scalpel appeared as a straight line of tissue disruption whereas the laser wounds looked more ulcerative (Fig. 1b). The scalpel incisions presented with a smaller width than the laser incisions. However, bleeding was more pronounced in the scalpel group and minimal in the laser groups. Laser wounds were observed as pale whitish foci of tissue ablation, as also shown by Rizoiu and coworkers [6].

Different effects could be seen with the variation of the laser air-water spray regulation. When too much water was employed, as in the 50/50% air/water condition, a clear surgical field was not achieved. Still, when using 100/0% air/water, the excessive quantity of air displaced the tissue, making it hard to reach the exact target, so the wound looked unaligned. Additionally, with the 100/0% air/water regulation, the clinical appearance was the most aggressive among all the laser groups (Fig. 1b).

Immediately after laser irradiation (G0)

Histologically, all the incisions presented with a cleft from the surface to the muscle tissue in the sub-mucosa. For the incisions performed with the laser, the cleft was deeper in the sub-mucosa, dissociating muscle fibers (Fig. 2a, b, c, and d). At this evaluation period, the incisions performed with the 50/50% air/water spray were more similar in depth to the incisions performed with the scalpel (Fig. 2a and c). For the 11/7% and 100/0% air/water regulations, tissue loss was deeper (Fig. 2b and d). The tissue loss was also wider for the 100/0% air/water regulation (Fig. 2d). For all laser samples, a fibrinous exudate lined the ulcerated area, and a coagulation necrosis was present around the incisions (Fig. 2b, c, and d). FN (fibronectin) was expressed in all incision types; however, in the scalpel incisions, this expression was a thin line around the wound area while in all of the laser incisions, FN was more scattered and less intense (Fig. 3a). COLIII (type III collagen) was not observed around the wounds at this time, only subjacent to the epithelium. In the laser specimens, some disturbance of the subepithelial collagen was noted (Fig. 4a).

One day after surgery (G1)

None of the animals presented with post-operative problems. Histologically, in the scalpel wound, a mixed inflammatory infiltrate extending laterally and downward to the sub-mucosa was seen (Fig. 2e). This infiltrate was similar to that presented by the wounds performed with the 11/7% air/water spray regulation (Fig. 2f) and more intense and scattered in the wounds performed with the 50/50% and 100/0% air/water spray regulations (Fig. 2g and h). Muscle fibers were dissociated due to edema. In the wounds performed with the 50/50% and 100/0% air/water spray, the ulcerated area was wider and sometimes lined by a fibrinous exudate (Fig. 2g and h). FN expression was more intense at this time than at the beginning, particularly at the edges of the incisions performed with the laser, and was still more scattered in the 100/0% air/water spray incisions (Fig. 3c) when compared to the 11/7% and 50/50% (Fig. 3b) air/water incisions. COLIII was expressed only in the subepithelial region; it was more intense and organized in the scalpel incisions and more diffuse in the laser incisions, especially in those performed with the 100/0% air/water spray regulation (Fig. 4c) when compared to the incisions performed with the 11/7% (Fig. 4b) and 50/50% air/water spray regulations.

Two days after surgery (G2)

A keratinized epithelium completely lined the scalpel incision area (Fig. 2i). However, a mixed inflammatory infiltrate and granulation tissue were present (Fig. 2i). In the incisions performed with the various air/water spray regulations, the wounded area was filled with a fibrinous exudate and an

Table 1 Antibodies used for the study

Antibodies	Clone	Pre-treatment	Concentration	Incubation time (min)	Incubation temperature (°C)
Fibronectin (FN)	FR1	Pepsin	1:600	120	Room temperature
Type III collagen (COLIII)	HWD1.1	Pepsin	1:300	120	Room temperature

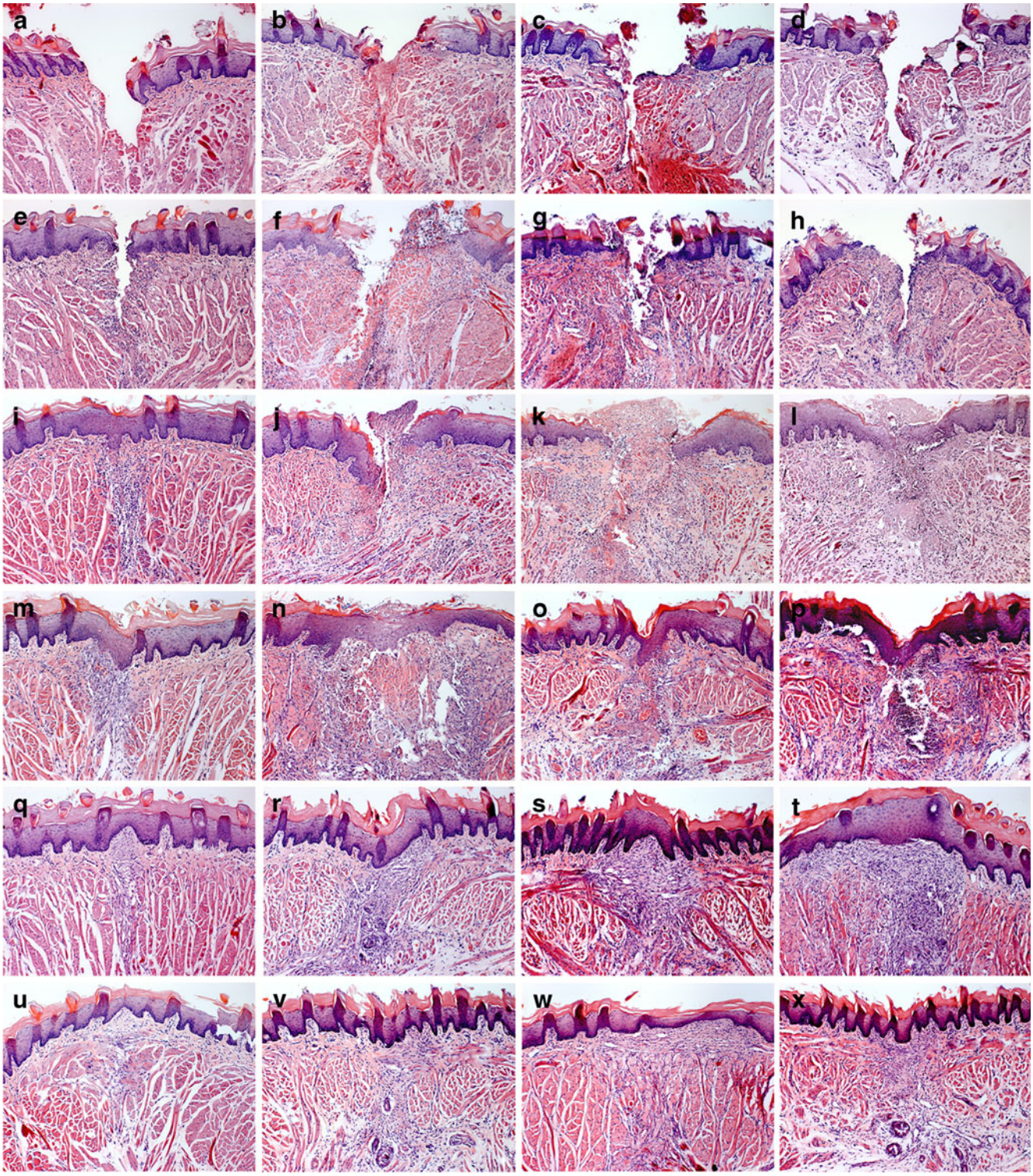


Fig. 2 **a**–**x** Morphological appearance of wounds from 0 to 14 days after surgery (H&E stain). **a** G0 scalpel. **b** G0 11% air/7% water. **c** G0 50% air/50% water. **d** G0 100% air/0% water. **e** G1 scalpel. **f** G1 11% air/7% water. **g** G1 50% air/50% water. **h** G1 100% air/0% water. **i** G2 scalpel. **j** G2 11% air/7% water. **k** G2 50% air/50% water. **l** G2 100% air/0% water. **m** G3 scalpel. **n** G3 11% air/7% water. **o** G3 50% air/50% water. **p** G3 100% air/0% water. **q** G4 scalpel. **r** G4 11% air/7% water. **s** G4 50% air/50% water. **t** G4 100% air/0% water. **u** G5 scalpel. **v** G5 11% air/7%water. **w** G5 50% air/50% water. **x** G5 100% air/0% water

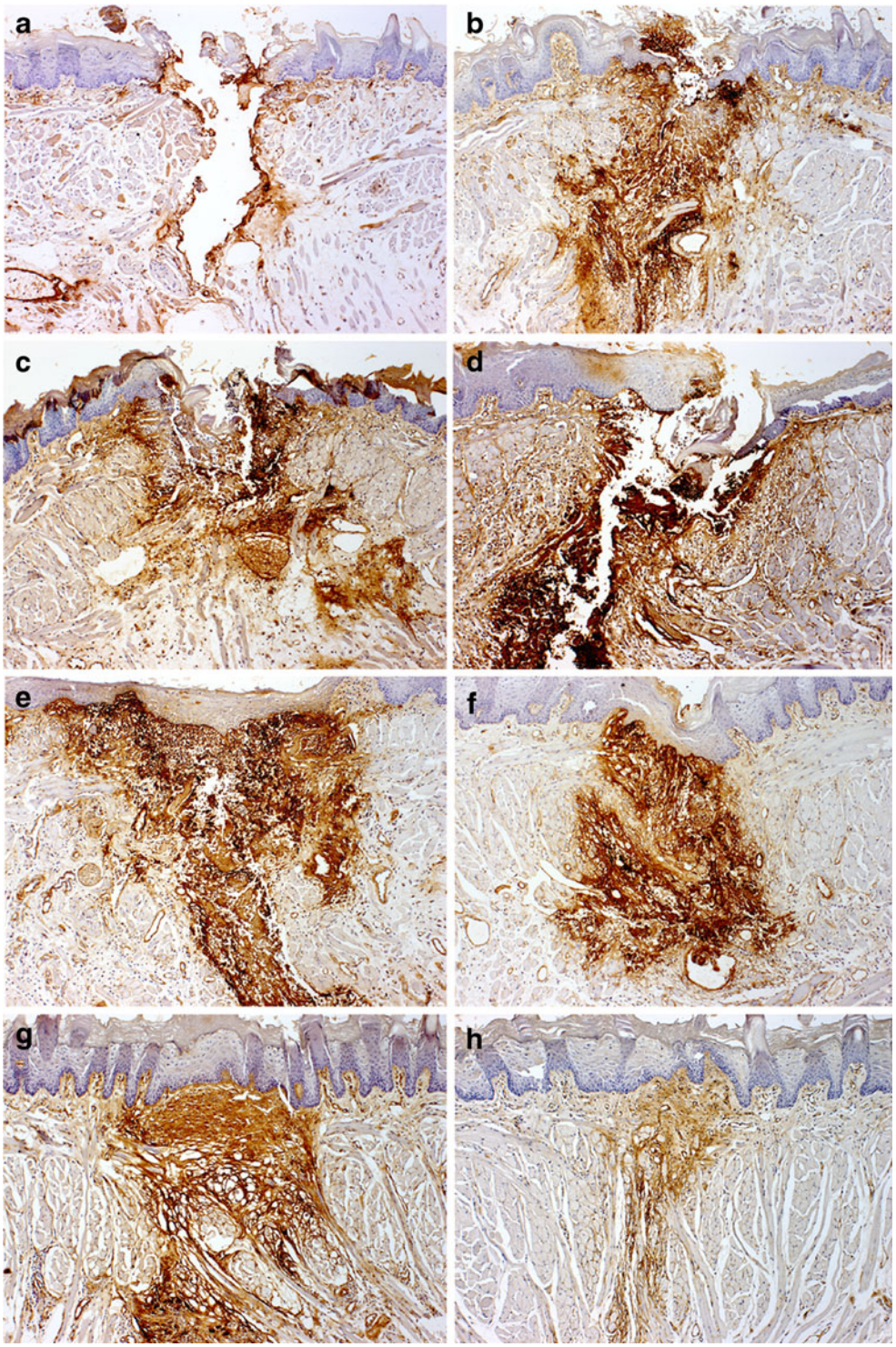


Fig. 3 **a**–**h** FN staining of wounds from 0 to 14 days after surgery. **a** G0 100% air/0% water. **b** G1 50% air/50% water. **c** G1 100% air/0% water. **d** G2 11% air/7% water. **e** G2 100% air/0% water. **f** G3 50% air/50% water. **g** G4 50% air/50% water. **h** G5 11% air/7% water

intense mixed inflammatory infiltrate. A few dissociated muscle fibers were scattered among the inflammatory infiltrate and fibrinous exudate (Fig. 2j, k and l). There was a marked difference between the scalpel and laser incisions. FN expression was similar to that observed in G1. In all the incisions, FN was more concentrated at the edges of the wound. In laser incisions, it was more expressive, as it was seen in the 11/7% air/water spray regulation (Fig. 3d) and more diffuse in the 100/0% air/water regulation (Fig. 3e). The deposition of COLIII was more intense in the 11/7% (Fig. 4d) and 50/50% laser incisions than in the scalpel incisions, and still more pronounced when the 100/0% air/water spray was employed (Fig. 4e).

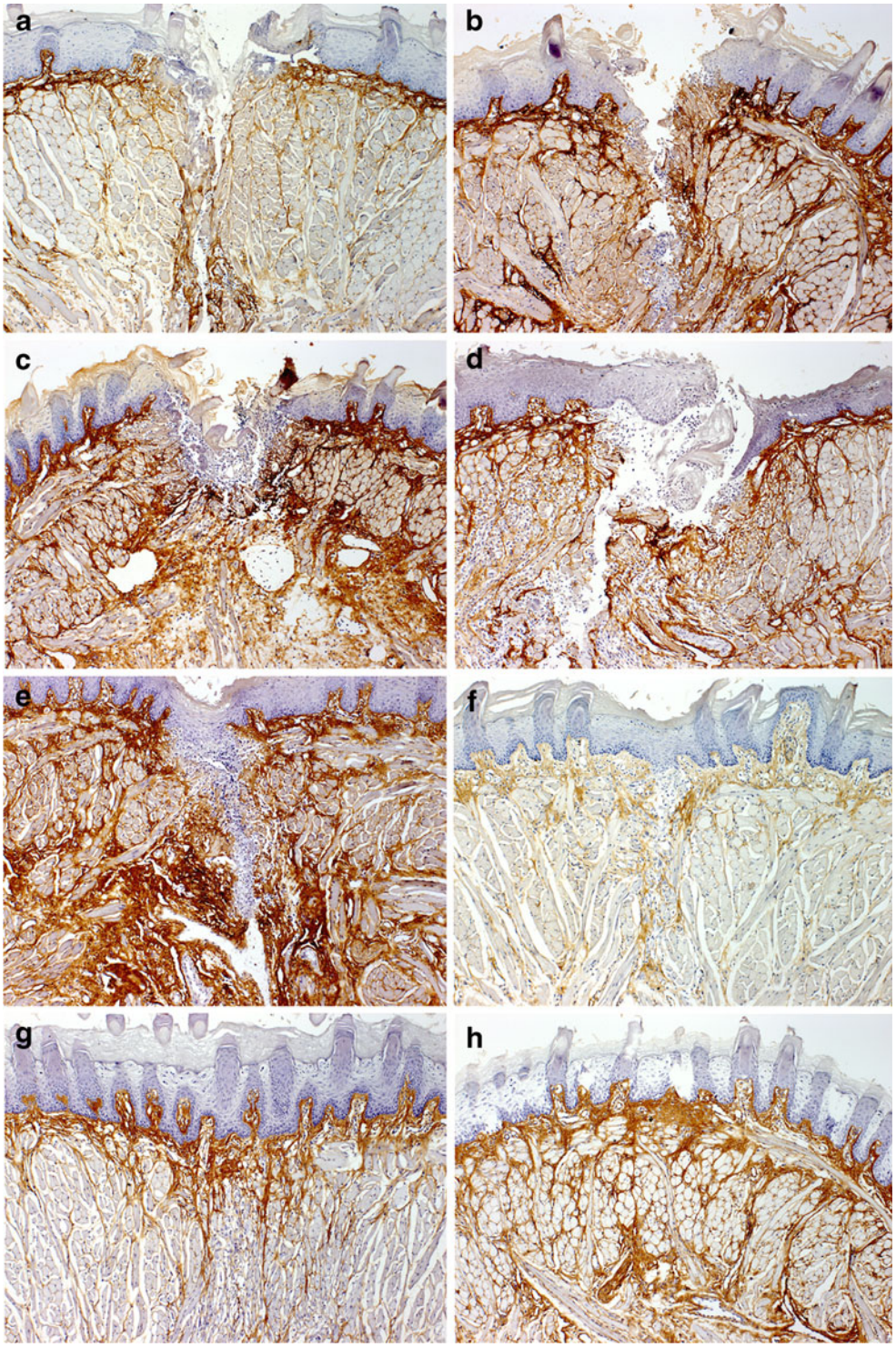


Fig. 4 **a**–**h** COLIII staining of wounds performed from 0 to 14 days after surgery. **a** G0 11% air/7% water. **b** G1 11% air/7% water. **c** G1 100% air/0% water. **d** G2 11% air/7% water **e** G2 100% air/0% water. **f** G3 scalpel. **g** G4 11% air/7% water. **h** G5 50% air/50% water

Three days after surgery (G3)

For all the incisions, granulation tissue was still present (Fig. 2m, n, o and p). However, it was more conspicuous in the laser incisions (Fig. 2n, o and p). All the incisions showed complete epithelialization, but the laser incisions did not show complete keratinization (Fig. 2n, o and p). FN was much less expressed in the scalpel incisions than in the laser incisions; the healing area of the laser incisions was clearly discernible by the concentration of FN (Fig. 3f). COLIII was present in all incisions, however, more organized and less intense in the scalpel incisions (Fig. 4f) than in the laser incisions, with no differences among the water/spray regulations.

Seven days after surgery (G4)

In addition to being epithelialized, the scalpel wound also showed lingual papillae (Fig. 2q). A small area of the sub-mucosa was still devoid of muscle fibers (Fig. 2q). A keratinized epithelium lined the laser incisions, but lingual papillae were hardly seen (Fig. 2r, s and t). Granulation tissue was still present in some samples, and muscle fibers were absent (Fig. 2r, s, and t).

A low concentration of FN was present in the scalpel incisions, and a more intense expression was seen in the laser incisions in the different air-water spray regulations though no significant differences were noted (Fig. 3g).

COLIII was evenly expressed in the scalpel incisions and scattered among the inflammatory infiltrate in the laser wounds (Fig. 4g). There were no differences among the air/water spray regulations.

Fourteen days after surgery (G5)

In the scalpel incision, only a small region of fibrous connective tissue replacing the muscle fibers was still present (Fig. 2u). In the laser incisions, the areas of connective tissue were larger (Figs. 2v, 1w, and x). FN was slightly expressed in all incisions, and COLIII was more diffusely expressed in all incisions (Figs. 3h and 4h).

Discussion

Previous investigations have shown that the Er,Cr:YSGG laser can provide efficient and precise ablation in both hard and soft tissues with minimal thermal damage to adjacent tissues in vivo [2, 3]. The benefit of minimal intra-operative hemorrhage when using this laser device instead of a conventional scalpel has been mentioned [5, 6]. Also, the Er,Cr:YSGG laser is related to a decrease in post-operative symptoms [6] and can be effectively used in oral surgery [8, 9] and endodontic treatments while increasing dentinal permeability, generating significantly lower heat than conventional tools and providing a bactericidal effect [10–13]. Despite its advantages, accurate protocols should be determined to precisely ablate soft tissue and coagulate vessels while minimizing thermal damage [7]. Therefore, the investigation of air-water spray regulations is important.

Due to the lack of published studies that focus on the air-water spray and on obtaining its best guideline for oral surgeries, three basic regulations were selected: 11/7% air/water (as recommended by the manufacturer); 50/50% air/water in order to provide results on the interaction of the laser with greater amounts of water, once Er,Cr:YSGG is considered to be the water-assisted laser; and 100/0% air/water as a way to support the importance of water irrigation along with the laser irradiation.

During the later evaluation periods, all incisions performed with either the laser or scalpel were completely repaired. Previous studies have shown that mucosal and cutaneous wounds made with the Er,Cr:YSGG laser system provide a healing process comparable to that of scalpel and punch biopsies [5, 6]. Nevertheless, our results showed that the air-water spray regulation influenced wound healing and the inflammatory response, especially in the earlier stages, being the inflammatory infiltrate more intense and scattered in the wounds performed with the 100/0% and 50/50% air/water when compared to the 11/7% air/water spray regulation.

A recent study showed that Er,Cr:YSGG laser incisions performed with 11/7% air/water spray produced significantly smaller areas of damaged tissue when compared to wounds prepared without the air/water spray and to diode laser incisions. This difference was attributed to the refrigeration capacity of the water/air spray, once Er,Cr:YSGG laser irradiation without air/water spray produced a wider extension of the thermal effect, similar to the CO_2 laser irradiation [14].

Although all incisions were done using a 2-mm depth pattern, histologically, the laser wounds extended deeper into the sub-mucosa, dissociating muscle fibers. This is likely to be due to the thermal action of the laser on the tissue; because the laser wounds have a more clinically aggressive effect on the tissue, more histological changes were expected compared to conventional scalpel incisions.

Healing was delayed in all the laser incisions, and they were completely lined by keratinized epithelium only 7 days after surgery. This delay in healing was seen when all histological parameters were analyzed. Some studies have also shown the delayed proliferation of capillaries, the slower appearance of inflammatory cell infiltration and a more prominent inflammatory reaction in laser wounds [15, 16]. Also, tissue thermal damage can be important, as was shown in a diode laser surgical procedure that produced a great inflammatory reaction [17].

Immediately after the incisions, the scalped cleft was filled with blood and tissue fluids while all the laser wounds appeared macroscopically dry. As shown by the pertinent literature [18], some amount of FN originates from the plasma, and some are synthesized in situ by fibroblasts. In that way, as mentioned in previous studies [19, 20], the FN deposits that present immediately after surgery should be more intense in the scalpel incisions than the laser wounds because the scalpel incisions produced more blood. However, the present study demonstrated more pronounced FN staining in the laser wounds when G0 was studied. This could be explained by the tissue loss being

wider and deeper when compared to the scalpel incisions despite the dried appearance of the laser wounds.

The return of the FN distribution to normal was observed 3 days after the scalpel incisions were made and only 14 days after the laser surgeries. The slower normalization of FN has already been demonstrated in a study with CO_2 lasers [20]. Also, regarding FN, a more scattered expression was noted in the 100/0% air/water spray regulation at the earlier times, as the result of a more aggressive incision than the incisions performed with 11/7% and 50/50% air/water spray. However, the normalization of FN at the end of the healing period shows that laser treatment does not alter the ability of fibroblasts to synthesize or retain FN. In addition, the accumulation of FN in laser wounds, regardless of the air/water spray regulation, was reversible in contrast to the disturbed healing in keloids [21]. Therefore, laser wounds have a lower risk of scarring as was the case for the Er:YAG laser [22].

COLIII is the predominant collagen in the oral mucosa and is also the greatest component of scars [23, 24]. As expected [25], COLIII presented a more pronounced distribution in all wound sites at the earlier times. In laser incisions, the 100/0% air/water group showed a more pronounced staining at the earlier evaluation times than the other studied air/water spray regulations, which can be related to thermal damage from non-irrigated irradiation. However, the 11/7% air/water laser group showed similar staining to the scalpel incisions, favoring the use of this parameter for clinical wounds. Luomanen and coworkers [20] have shown a slower return of collagen to normal in laser wounds, and Fisher and coworkers [26] reported less collagen formation during the healing of a CO_2-laser excision compared with conventional surgical excision in the buccal mucosa of beagles.

The Er,Cr:YSGG laser is a suitable tool for soft-tissue surgery [27–29]. Although laser wounds showed delayed healing compared to scalpel incisions, the benefits, such as the achievement of a clear surgical field with less bleeding and the decontamination provided by the laser, are relevant. In that way, the air/water spray should be regulated to improve the surgery. Fourteen days after surgical procedures, all wounds were equally healed. Nevertheless, for the earlier evaluation periods, the 100/0% air/water condition represented the most aggressive wound pattern. This regulation also implied in clinical displacement of the soft tissue; however, it must be taken into account that the rat tongue utilized in this study is a very delicate tissue. Finally, when too much water was applied (the 50/50% air/water group), the surgical field was not clear, and the laser-tissue interaction was poor.

Conclusions

According to the findings of this study, we conclude that the air-water spray should be regulated to achieve its greatest action when performing oral surgery with the Er,Cr:YSGG laser device. Excessive amounts of air and water may imply difficulties to perform the surgery, as well as in a poorer wound healing, especially at the earlier stages.

Acknowledgments The authors thank FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for its grants (numbers 05/60243-8 and 04/06794-0).

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