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Vivian Petersen Wagner
Luise Meurer
Marco Antonio Trevizani Martins
Chris Krebs Danilevicz
Alessandra Selinger Magnusson
Márcia Martins Marques
Manoel Sant’Ana Filho
Cristiane Helena Squarize
Manoela Domingues Martins
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Vivian Petersen Wagner,a Luise Meurer,b Marco Antonio Trevizani Martins,a Chris Krebs Danilevicz,a Alessandra Selinger Magnusson,a Márícia Martins Marques,a Manoel Sant'Ana Filho,a Cristiane Helena Squarize,d and Manoela Domingues Martinsa

aUniversidade Federal do Rio Grande do Sul, School of Dentistry, Department of Oral Pathology, Porto Alegre, Rio Grande do Sul, 90035-003, Brazil
bUniversidade Federal do Rio Grande do Sul, Hospital de Clínicas de Porto Alegre, Department of Pathology, Porto Alegre, Rio Grande do Sul, 90035-903, Brazil
cUniversidade de São Paulo, School of Dentistry, Porto Alegre, Rio Grande do Sul, 90035-000 Brazil
dUniversity of Michigan School of Dentistry, Laboratory of Epithelial Biology, Department of Periodontics and Oral Medicine, Ann Arbor, Michigan 48109-1078

Abstract. The aim of the present prospective study was to evaluate the impact of laser phototherapy (LPT) on the healing of oral ulcers. Different power densities were used on oral wounds in Wistar rats (n = 72) randomly divided into three groups: control (0 J/cm²), 4 J/cm² laser, and 20 J/cm² laser. Ulcers (3 mm in diameter) were made on the dorsum of the tongue with a punch. Irradiation with an indium-gallium-aluminum-phosphide laser (660 nm; output power: 40 mW; spot size: 0.04 cm²) was performed once a day in close contact with the ulcer for 14 consecutive days. A statistically significant acceleration in healing time was found with wounds treated with 4 J/cm² LPT. Moreover, striking differences were found in the ulcer area, healing percentage, degree of reepithelialization, and collagen deposition. The most significant changes occurred after 5 days of irradiation. Based on the conditions employed in the present study, LPT is capable of accelerating the oral mucosa wound-healing process. Moreover, faster and more organized reepithelialization and tissue healing of the oral mucosa were achieved with an energy density of 4 J/cm² in comparison to 20 J/cm². © 2013 Society of Photo-Optical Instrumentation Engineers (SPIE)

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1 Introduction

Oral ulcers, whether originating from traumatic, immunological, or other pathological processes, are one of the most common complaints involving the oral mucosa and can cause mild to severe pain. These lesions are characterized by damage to both the epithelium and connective tissue (lamina propria), which are usually repaired within weeks if the etiological factor is removed. The aim of treatment is to relieve the symptoms and accelerate the repair process, as patients with oral ulcers, such as recurrent aphthous stomatitis and mucositis, report a reduction in oral health-related quality of life.

Wound healing is a dynamic and complex process that involves biochemical and physiological phenomena, and is made up of three main overlapping phases: inflammation, proliferation, and remodeling. Different therapeutic protocols, such as analgesics, corticosteroids, anti-inflammatory agents, and phototherapy, have been tested to accelerate the wound healing process and reduce pain.

The laser phototherapy (LPT) can be employed to modulate a number of biological processes in a phenomenon known as photobiomodulation. The LPT increases cell metabolism and is reported to induce analgesia, anti-inflammatory action, and tissue repair. Meste and collaborators were one of the first groups that describe enhanced healing and pain relief achieved with LPT. Studies involving this method of therapy report its effects on myofibroblasts, lymphoid cell proliferation and collagen synthesis, as well as its anti-inflammatory properties, neo-vascularization potential, and the release of growth factors. The effects of LPT on the healing process of skin wounds have been studied, particularly using the dorsum skin model to investigate the effect of different irradiation parameters. A growing body of evidence suggests that LPT has anti-inflammatory action and accelerates tissue repair. However, these effects are dependent on irradiation parameters such as wavelength, output power, and energy density. Moreover, the same parameters can have different effects on different cell types.

The impact of LPT on wound healing of the oral mucosa is not completely understood. It is therefore of paramount importance to determine the correct combination of parameters to achieve desirable effects regarding the healing of oral ulcers. The aim of the present study was to evaluate the effects of different LPT energy densities on clinicopathological aspects of oral ulcers. Herein, the present study showed the acceleration in the healing process, as demonstrated by clinical and histological findings, in function of the laser energy density protocol, with 4 J/cm² achieving better results.

2 Methods

2.1 Animal Model

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and received

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approval from the ethics committee of the Porto Alegre University Hospital (Brazil) under the process number 12-0338. Seventy-two male Wistar rats weighing 150 to 200 g were kept under standard conditions of temperature (20 to 24°C) and light/dark cycle with solid chow and water ad libitum. The animals were randomly divided into three cohorts of 24 animals each: control group (0 J/cm²), 4 J/cm² laser group, and 20 J/cm² laser group.

2.2 Wound Procedure
Under aseptic conditions, the three groups were anesthetized with an intraperitoneal administration of ketamine (0.1 ml/100 g) and xylazine (0.05 ml/100 g). Traumatic ulcers measuring 3 mm in diameter were made on the dorsum of the tongue using a standard punch biopsy technique.

2.3 Laser Irradiation
Laser irradiation was delivered with a continuous-wave indium-gallium-aluminum-phosphide (InGaAlP) diode laser (MM Optics Ltd., São Carlos, SP, Brazil) with a spot size of 0.04 cm², operating at a wavelength of 660 nm and an output power of 40 mW in punctual and contact modes. Irradiation was performed perpendicularly to the mucosa in two points with a 3-mm distance to each other in the two opposite borders of the ulcer. The energy densities were 4 J/cm² (energy per point of 0.16 J, total energy of 0.32 J) and 20 J/cm² (energy per point of 0.8 J, total energy of 1.6 J) with respective exposure times of 4 and 20 s on each point of the ulcer. The LPT was applied after the immediately the wound procedure. The same investigator applied the LPT once a day on the wounds under isoflurane inhalant anesthesia during 14 consecutive days. The control group was treated under identical conditions but with the laser equipment switched off.

2.4 Clinical Analysis
Six rats in each group were euthanized using a CO₂ chamber on days 1, 5, 10, and 14 after the surgical procedure. The wounds were measured and photographed. Ulcer area was calculated based on the length and width using a digital caliper. These measurements were multiplied for the determination of area in square centimeters. The percentage of wound healing and healing time were recorded, as described elsewhere. Briefly, percentage of healing was calculated as [(initial area – area at respective evaluation time)/initial area] ×100.

2.5 Histopathological Analysis
After euthanasia, the tongues were removed and fixed in a 10% buffered formalin solution for 48 h. After washing with water, the specimens were dehydrated and embedded in paraffin. Slices measuring 5 μm in thickness were obtained and stained with hematoxylin-eosin.

Descriptive analyses of each group/evaluation time were performed, followed by a semi-quantitative analysis. The degree of reepithelialization was determined by a grading system (0 to 4), as described elsewhere. Grade 0 = reepithelialization on the margins of the wound; Grade 1 = reepithelialization covering less than half of the wound; Grade 2 = reepithelialization covering more than half of the wound; Grade 3 = reepithelialization covering the entire wound with irregular thickness; and Grade 4 = reepithelialization covering the entire wound with normal thickness. Inflammation was also evaluated using a grading system as described elsewhere. Grade 1 = acute inflammation (pyogenic membrane); Grade 2 = prevalence of acute diffuse inflammation; Grade 3 = prevalence of chronic inflammation (fibroblast proliferation); and Grade 4 = resolution and healing (reduction in or disappearance of chronic inflammation, despite the persistence of some inflammatory cells).

2.6 Picrosirius Red Staining for Collagen
Picrosirius red staining was used to analyze the collagen fibers. When examined under polarized light, larger collagen fibers appear strongly birefringent and appear greenish in color. This birefringence is highly specific to collagen.

2.7 Statistical Analysis
The data were expressed as mean and standard deviation values. The SPSS version 18.0 was employed for the statistical analyses. Groups, evaluation times, and the interaction between group and evaluation time were compared using the generalized estimating equation, followed by a post-hoc Bonferroni correction when necessary. The significance level was set at 5% (p < 0.05). The Kappa coefficient was calculated to determine inter-examiner agreement regarding the scores of reepithelialization and inflammation.

3 Results
3.1 Clinical Analysis
No differences were found in wound area, and no detectable signs of repair were evident on day 1. A decrease in mean wound area was found in all groups on day 5, with the smallest area in the 4 J/cm² laser group, followed by the 20 and 0 J/cm² laser groups [Fig. 1(a)]. On day 10, all animals in the 4 J/cm² laser group exhibited repaired lesions, whereas this clinical situation was only observed at the end of the experiment (day 14) in the other groups. No statistically significant differences were found among groups on days 10 and 14 [Figs. 1(b) and 1(c)].

Figure 1(d) displays the percentages of wound healing. No significant differences among groups were found on day 1. The 4 J/cm² laser group exhibited a significant increase in the percentage of healing on day 5. On days 10 and 14, most of the wounds were healed and no significant differences were found among the groups at these evaluation times.

3.2 Histopathological Analysis
3.2.1 Descriptive analysis
Exposure of connective tissue was observed on day 1. Discrete or no migration of epithelial cells in the center of the wound was found in all groups. Intense diffuse acute inflammation (polymorphonuclear infiltrate) and red blood cells were noticed in the wound area.

On day 5, reepithelialization covering more than half of the wound was found in the 0 J/cm² group. Some focal points of acute inflammation were still present in most of the animals, but the number of mononuclear cells was higher than that on day 1. Notably, all animals in the 4 J/cm² laser group exhibited full reepithelialization at this time. Granulation tissue was evident, and no focal points of acute inflammation were found.
Mononuclear infiltrate, neovascularization, and fibroblast proliferation were evident. Collagen fibers were predominantly organized and paralleled to the basal layer of the epithelium. The 20 J/cm² laser group exhibited reepithelialization covering more than half of the wound in some animals and covering the entire wound with irregular thickness in other animals. Inflammatory infiltrate revealed the formation of granulation tissue in most of the wound beds, and focal points of acute inflammation still occurred in some cases.

On day 10, all groups exhibited reepithelialization covering the entire wound. The control group (0 J/cm²) exhibited epithelium with irregular thickness, whereas the 4 and 20 J/cm² laser groups exhibited new epithelium with normal thickness. Inflammatory infiltrate revealed the formation of granulation tissue in most of the wound beds, and focal points of acute inflammation still occurred in some cases.

3.2.2 Degree of reepithelialization

The Kappa coefficient for inter-examiner agreement regarding the degree of reepithelialization was 0.91, indicating a high level of agreement. On day 5, the 4 J/cm² group exhibited a significantly greater degree of reepithelialization, whereas the 0 and 20 J/cm² laser groups exhibited similar degrees of connective tissue exposure. On day 10, the 20 J/cm² laser group exhibited a significantly greater degree of reepithelialization [Figs. 2(a)–2(c)].

3.2.3 Degree of inflammation

The Kappa coefficient for inter-examiner agreement regarding the degree of inflammation was 0.96, indicating a high level of agreement. Although variations were found in the pattern of inflammatory infiltrate, no statistically significant differences were found among the groups [Fig. 2(d)].

3.2.4 Organization and distribution of collagen fibers

On day 1, Picrosirius red staining revealed no red or green birefringence, indicating the absence of collagen. The wounds were filled with blood clots. On day 5, all groups displayed thin collagen fibers (characterized by green birefringence), which were poorly organized. In the groups treated with laser, some denser collagen fibers, which appeared reddish in color, were noted in the wound. These fibers were parallel to the epithelium and

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**Fig. 1** (a) Clinical aspect of oral ulcers in different groups on day 5, smaller ulcer area in 4 J/cm² group; (b and c) Clinical evaluation of mean and standard error of area (in mm²); and (d) Clinical evaluation of percentage of wound healing (%).
demonstrated a more organized pattern in comparison to the control group. On day 10, collagen fibers had begun to rearrange into bundles among the new muscle fibers in all three groups. On day 14, all groups exhibited organized collagen bundles mainly with red birefringence around the perimysium. The groups treated with laser exhibited an organization pattern similar to normal tongue mucosa, with a predominance of reddish fibers organized parallel to the epithelium in the adjacent lamina propria and bundled in the submucosa area around the muscle fibers (Fig. 3).

4 Discussion

The effects of LPT on the healing process of skin wounds have been studied with different irradiation parameters. A growing body of evidence suggests that LPT has anti-inflammatory action and accelerates tissue repair. However, these effects are dependent on wavelength, output power, and energy density. Moreover, the same parameters can have different effects on different cell types, and few studies have been conducted to analyze the influence of LPT on the wound-healing process involving the oral mucosa. It is therefore of paramount importance to determine the correct combination of parameters to achieve desirable effects on oral ulcers.

In the present investigation, two different energy densities were tested (4 and 20 J/cm²) with the same wavelength, spot size, power output, irradiation frequency, and interval between irradiations. Moreover, both methods were performed in contact with the ulcer and using the punctual irradiation mode. The choice of testing two very different energy densities was based on the previous studies, where better results regarding wound healing with smaller energy densities were observed in comparison to larger energy densities. Indeed, such findings followed the Arndt–Schultz law, which states that mild stimulus excites physiological activity, whereas strong stimulus can inhibit such activity. Therefore, 4 J/cm² was used as the low-energy density, and 20 J/cm² was used as the high-energy density in the present study. The 4 J/cm² dose has been widely used in clinical practice and studied in clinical trials. However, few clinical studies have been conducted analyzing higher doses of LPT. Other important aspects regarding the laser parameter are the energy per point that seems to be relevant because it considers the output power and the time of tissue exposure to laser, improving the study reproducibility.

![Photomicrographs of experimental groups on day 5, reepithelialization covering entire wound and more chronic inflammatory infiltrate in the 4 J/cm² group (hematoxylin-eosin; magnification: ×100 (a, c, and e) and ×200 (b, d, and f)); (b and c) Histopathological evaluation of degree of reepitelialization (mean and standard error); and (d) Histopathological evaluation of degree of inflammation (mean and standard error).](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics)
study, the 4 J/cm² laser group with 0.16 J of energy per point showed better results in oral wound healing than the 20 J/cm² laser group with 0.8 J of the total energy.

The in vivo results demonstrated that LPT at the same power density induced the acceleration of the healing process as a function of energy density. Irradiation with 660 nm, 40 mW, and 4 J/cm² accelerated the healing of oral ulcers in comparison to 0 and 20 J/cm². On day 5, the 4 J/cm² laser group exhibited positive clinical behavior with a greater decrease in the mean area of the oral ulcers and higher percentages of healing in comparison to the control and higher energy density laser groups. Moreover, the histopathological analysis revealed a more advanced stage of repair in the 4 J/cm² laser group on day 5. This finding is supported by the degree of reepithelialization, with the epithelium covering the entire wound in all animals, and by the histopathological aspect of the connective tissue. Chronicity of the inflammatory infiltrate was also noticed in this group on day 5, whereas the 0 and 20 J/cm² laser groups still exhibited focal areas of acute inflammation. These histopathological aspects may be associated with the clinical finding of a reduction in ulcer area and greater percentage of healing on day 5, demonstrating that the irradiation with 4 J/cm² led to the faster and more organized healing pattern. Similar results have been obtained for excisional wound healing in mice, in which a biphasic relationship has been found with positive effects at 2 J/cm² and an inhibitory effect at 50 J/cm².

However, studies have predominately attributed the inhibitory effect to higher power levels rather than energy density per se.

It is difficult to compare the present findings with observations of wound healing in the literature, as most studies were performed using different tissue models, such as skin, which has a different repair process than the oral mucosa healing. Furthermore, many studies only report the power and wavelength of the laser irradiation, while energy density is not mentioned and cannot be calculated due to the absence of other important parameters such as spot size and irradiation time. Nonetheless, an increase in energy density has been shown to impair wound healing in vivo.

The LPT with 4 J/cm² for skin wound repair has demonstrated a decrease in polymorphonuclear infiltrate and neovascularization as well as greater deposition of collagen and elastic fibers. The irradiation with 4 J/cm² can promote a greater number of newly formed epithelial layers and the irradiation with 3 and 5 J/cm² is capable of increasing the growth rate of cultured epithelial cells. Moreover, mononuclear infiltrate, neovascularization, and fibroblast proliferation were evident in 5 days when the tissue was irradiated with the 4 J/cm². This was expected since other authors have shown increased production of growth factors, such as bFGF, as a result of red laser irradiation with small energy densities. Additionally, the irradiation with 4 J/cm² for wound healing induces the formation of more fusiform cells expressing desmin and

![Fig. 3 Photomicrographs of Picrosirius red staining in experimental groups on days 5, 10, and 14 and in normal mucosa (original magnification ×100).](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics)
alpha-smooth muscle actin decreases the levels of IL-1β mRNA increases populations of intact and degranulated mast cells, type I collagen, and fibronectin deposition and enhances synthesis activity.

Low-intensity laser irradiation is absorbed by cellular photosensitizers, such as cytochromes and flavins, which promote a cascade event that results in Ca2+ influx, affecting the levels of cyclic nucleotides, interfering with DNA and RNA syntheses, and modulating cell proliferation. A further increase in dose induces cellular antioxidant activity and can cause the destruction of photoreceptors, which is accompanied by growth inhibition and cell lethality, as expected from the Arndt–Schulz law.

Overall, LPT has been found to accelerate wound healing as well as to reduce pain and the inflammatory response. In the present study, LPT with the energy density of 4 J/cm² and the other parameters applied caused important oral mucosa wound-healing effects, accelerating two of the most substantial healing phases: inflammation and proliferation. This protocol decreases acute inflammation and induces reepithelialization, fibroplasia, and granulation tissue formation. However, the correlation between the results obtained with this animal model and clinical outcomes remains to be established. Therefore, care should be taken before extrapolating these results to clinical practice without additional testing.

5 Conclusion

Based on the conditions employed in the present study, LPT using red laser (660 nm) and an output power of 40 mW is capable of accelerating the oral mucosa wound-healing process. Moreover, faster and more organized reepithelialization and tissue healing of the oral mucosa were achieved with an energy density of 4 J/cm² in comparison to 20 J/cm².

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