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ORIGINAL ARTICLE

Photobiomodulation regulates cytokine release and new blood vessel formation during oral wound healing in rats

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Abstract The aim of the present study was to evaluate the effects of photobiomodulation (PBM) on cytokine levels and angiogenesis during oral wound healing. Ulcers were made on the dorsum of the tongue in 48 Wistar rats. Irradiation with an indium-gallium-aluminum-phosphide (InGaAlP) laser (660 nm; output power, 40 mW; spot size, 0.04 cm^2) was performed once a day on two points of the ulcer for 14 days. Two different energy densities were used: 4 J/cm^2 (energy per point 0.16 J, total energy 0.32 J) and 20 J/cm^2 (energy per point 0.8 J, total energy 1.6 J). Tissue levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α were investigated by enzyme-linked immunosorbent assay (ELISA). Image analysis of CD31-immunostained sections was used to investigate microvessel density (MVD). PBM increased the tissue levels of IL-1β at the early stage of oral wound healing ($p < 0.01$) and increased the tissue levels of TNF-α during all stages of oral wound healing ($p < 0.05$). PBM at a dose of 4 J/cm^2 produced more significant results regarding cytokine modulation and was associated with higher MVD at day 5. Collectively, these findings indicate that cytokine modulation and increased angiogenesis are among the basic mechanisms whereby PBM improves oral wound repair.

Keywords Diode laser · Wound healing · Neovascularization · Low-level laser · Cytokines

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Introduction

Wound healing is characterized by a dynamic process regulated by a complex signaling network of growth factors, cytokines, and chemokines responsible for altering the growth, differentiation, and metabolism of target cells [1, 2]. Neutrophils are recruited to the wound area a few minutes after the injury and are responsible for intensifying the inflammatory reaction by releasing cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [3]. Despite a beneficial effect on host defense against pathogens, these cytokines can have damaging effects; TNF-α in excess, for example, is associated with chronic and slow-healing acute wounds [4]. Excess inflammation may delay the healing process, preventing progression to the next stage. Moreover, free radicals produced in this process are toxic and harmful to tissue constituents [5]. Therapeutic strategies used to accelerate wound healing are expected to have the capacity to modulate the release of pro-inflammatory cytokines and thus reduce such adverse effects.

Another important event in the wound healing process is angiogenesis. The formation of new blood vessels is a key phenomenon for granulation tissue formation, as it is responsible for supplying the oxygen and nutrients necessary to sustain cell metabolism at the wound area [6]. Recent experimental research has showed that the stimulation of blood vessel formation is associated with better clinical outcomes [7–9].

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Thus, angiogenic potential appears to be a highly desirable feature for new strategies used to accelerate wound healing.

Oral ulcers are common complaints in clinical practice that can cause mild to severe pain. Strategies that aim to accelerate the wound healing process and reduce pain are an important target of researches once there is no gold standard in treatment for this condition [10]. Several protocols using topical or systemic drugs have been described [10–12], and photobiomodulation (PBM) is an additional therapeutic option proven to promote analgesia and accelerate healing in oral lesions [13–17]. PBM has advantages over current corticosteroid-based therapies, such as noninvasiveness and absence of side effects. Our group recently demonstrated that PBM accelerates epithelial healing by activation of the mTOR signaling pathway [18] and reactive oxygen species (ROS) without inducing DNA damage [19]. In addition, PBM has been shown to increase respiratory metabolism, which results in cellular proliferation and prevention of cell death [20].

The relevance of modulating cytokine levels and promoting angiogenesis during wound healing is well established [4, 6]. However, the effect of PBM on these phenomena during oral wound healing is not entirely clear, particularly concerning which combination of parameters is able to promote them. The effect of PBM on cytokine modulation is also poorly established in the literature [21–24], especially regarding the phase of wound healing at which this effect is achieved [21, 24]. Furthermore, studies focusing on oral wound healing are extremely scarce [25]. Previous studies that analyzed the effect of PBM on stimulation of neovascularization reported controversial results; [26–29]; however, this might be explained by the different laser parameters used in each study. In addition, the use of an appropriate methodology for blood vessel counting is paramount to achieving a reliable result.

The literature is still lacking in evidence of the basic biologic process involved in the putative effects of PBM on oral ulcer wound healing. Experimental cellular and molecular biology research should expand our comprehension of the mechanisms through which PBM enhances oral wound repair. Within this context, the aim of the present study was to evaluate the effects of different PBM energy densities on tissue cytokine levels and angiogenesis during oral wound healing.

Methods

Animal model and induction of injury

The sample of the present study was obtained from a clinical and histopathological study previously published [15]. Briefly, 48 adult male Wistar rats weighing 150 to 200 g were maintained under standard conditions of temperature (20–24 °C) and light/dark cycle. Food and water were provided ad libitum throughout the experiment. Under aseptic conditions, the animals were anesthetized via intraperitoneal administration of ketamine (0.1 ml/100 g) and xylazine (0.05 ml/100 g), and a traumatic ulcer 3 mm in diameter was made on the dorsum of the tongue of each rat using a standard punch biopsy technique. The animals were then randomly divided into three cohorts of 16 each: 0-J/cm^2 control group, 4-J/cm^2 PBM group, and 20-J/cm^2 PBM group. Four rats in each group were euthanized using a CO_2 chamber on days 1, 5, 10, and 14 after the surgical procedure. The tongue was bisected, one half frozen at −80 °C, and the other half processed and paraffin-embedded.

All experiments were carried out in accordance with the US National Academy of Sciences Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Hospital de Clínicas de Porto Alegre, Brazil, under process number 12-0338.

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 cm^2, operating at a wavelength of 660 nm and an output power of 40 mW in spot and contact modes. Output power was confirmed daily using a power meter. Irradiation was performed perpendicularly to the mucosa on two points 2 mm apart at the two opposite borders of the ulcer. The energy densities used were 4 J/cm^2 (energy per point 0.16 J, total energy 0.32 J) and 20 J/cm^2 (energy per point 0.8 J, total energy 1.6 J), with respective exposure times of 4 and 20 s on each point of the ulcer. Irradiation procedures were started immediately after surgery and always performed by the same investigator, once a day for 14 consecutive days. Animals were kept under isoflurane inhalant anesthesia during laser irradiation. The control group was treated under identical conditions, but with the laser device switched off.

Enzyme-linked immunosorbent assay

Total tissue protein extraction was performed using RIPA buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA) through tissue maceration performed on ice for 30 min. Samples were centrifuged for 30 min at 4 °C, 12,000 rpm. Supernatants were collected and frozen at −80 °C. Levels of IL-1β and TNF-α were quantified by enzyme-linked immunosorbent assay (ELISA) using commercial kits (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's instructions. Briefly, polystyrene 96-well plates were pre-coated with specific capture antibody overnight at 4 °C. Nonspecific binding was avoided by blocking plates for 1 h at room temperature (RT) prior to incubating samples and standards overnight at 4 °C.

Peroxidase-conjugated secondary antibody and a biotin-avidin system were used for detection. Plates were read at 450 and 570 nm, and values obtained at 570 nm were subtracted from those obtained at 450 nm for analysis. The detection ranges of IL-1β and TNF-α ELISA were 39.0625–5000 pg/ml. A five-parameter curve was constructed for quantitation of IL-1β, whereas a four-parameter curve was used for TNF-α.

Immunohistochemistry

For immunohistochemical staining, specimens were cut into 3-μm-thick sections, deparaffinized in xylene, and hydrated in descending grades of ethanol. Endogenous peroxidase activity was blocked using 5 % hydrogen peroxide in two 15-min baths. Antigen retrieval was performed for 18 h in a citrate buffer solution heated to 90 °C in a water bath. Slides were incubated at RT for 2 h with rabbit anti-CD31 (polyclonal, 1:50, Abcam, Boston, MA, USA). The detection system used was the polyvalent HRP plus kit (Spring Bioscience, Pleasanton, CA, USA). Sections were then incubated with 3, 3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Corp., St. Louis, MO, USA) and counterstained with Mayer's hematoxylin. Cases of mouse intestine served as appropriate positive controls, and negative control was performed by suppressing the primary antibody.

Microvessel density

Vessel counts at the wound area were determined by image analysis of CD31-immunostained sections. One investigator performed microvessel density (MVD) assessment. Four high-power fields (×400 magnification) of the lesion were captured: two at the wound edges and two at the deepest part of the lesion. Vessel counts were performed using the "manual tagging" feature in the ImageProPlus software package (NIH, Bethesda, MD, USA). Vessels in each section were defined by the circular or ovoid image of the brown endothelial walls and luminal space. Capillaries, arterioles, and venules were counted. Sections from different treatment groups were indistinguishable from one another, which allowed counting to be performed in blinded fashion.

Statistical analysis

All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical analyses of the ELISA and MVD were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Asterisks denote statistical significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$).

Results

PBM increases IL-1β tissue levels at the early stage of oral wound healing

On day 1, which corresponded to the inflammatory phase of wound healing, groups that received laser irradiation with both doses (4 and 20 J/cm^2) exhibited significantly higher tissue levels of IL-1β compared to the control group ($p<0.01$) (Fig. 1a). At days 5, 10, and 14, no difference was found between any irradiated group and controls. Nevertheless, the 4-J/cm^2 group exhibited the highest tissue levels of IL-1β throughout the experimental period, while the control and 20-J/cm^2 groups exhibited similar values (Fig. 1a).

Low-level PBM decreases TNF-α tissue levels at all stages of oral wound healing

On day 1, only the 4-J/cm^2 animals exhibited lower TNF-α tissue levels compared to controls ($p<0.05$) (Fig. 1b). At days 5, 10, and 14, both irradiated groups exhibited lower TNF-α tissue levels compared to the control group. Interestingly, the 4-J/cm^2 group exhibited a greater magnitude of TNF-α reduction throughout the experimental period compared to the 20-J/cm^2 group (Fig. 1b).

New blood vessel formation appears to be stimulated by low-level PBM

On day 1, all groups exhibited a very similar number of blood vessels, which increased over time in all groups. At day 5, the 4-J/cm^2 group presented a substantially higher MVD compared to the control and 20-J/cm^2 groups (Fig. 2a). At days 10 and 14, the number of vessels in the irradiated and control groups was quite similar; only at day 5 a clear trend toward PBM-induced angiogenesis could be noted (Fig. 2b).

Discussion

Medical interest in PBM has increased progressively in the last decade. In oral surgery in particular, PBM has shown promising results in accelerating the healing of oral ulcers, chemotherapy- and radiotherapy-induced mucositis, bisphosphonate-related osteonecrosis of the jaw, and oral lichen planus, as well as shortening the clinical course of herpes simplex [16, 17, 30–32]. Furthermore, PBM is already used in clinical practice in dentistry, to reduce symptoms after a variety of procedures [33–35]. The clinical efficacy of PBM can no longer be questioned and is supported by well-controlled evidence; however, the basic mechanisms by which PBM produces the desired clinical effects in the oral mucosa remain unclear. In the present investigation, an animal model was

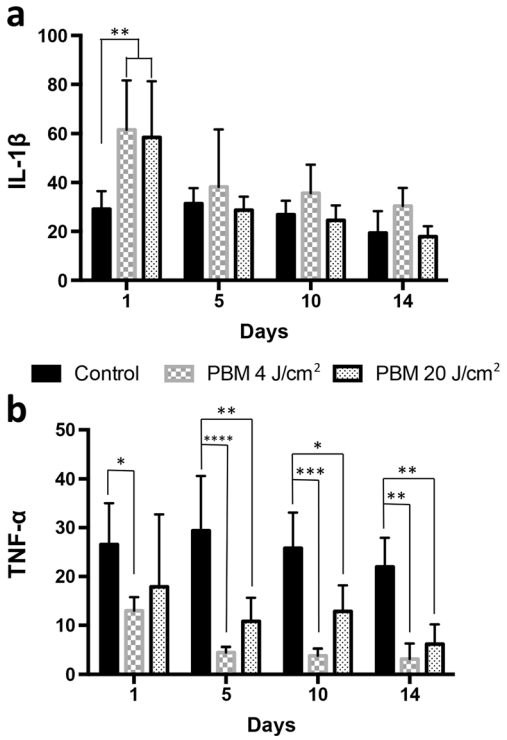

Fig. 1 PBM modulates cytokine release during oral wound healing. **a** Graphic representation of IL-1β tissue levels assessed by ELISA. PBM at both doses was able to increase IL-1β tissue levels at day 1 ($p < 0.01$ vs. control). **b** Graphic representation of TNF-α tissue levels assessed by ELISA. At day 1, only the 4-J/cm^2 group exhibited a significant decrease in TNF-α tissue levels compared to the control group ($p < 0.05$). At days 5, 10, and 14, both PBM groups exhibited significantly lower values compared to the control group. The magnitude of the decrease in TNF-α tissue levels over time was greater in the 4-J/cm^2 group than in the 20-J/cm^2 group

used to broaden comprehension of the mechanisms involved in oral wound repair mediated by PBM at two different energy densities. Two major events in the healing process were assessed: release of inflammatory cytokines and formation of new blood vessels. Our results demonstrate that PBM, especially at the low energy densities assessed, is able to modulate cytokine release by increasing IL-1β tissue levels at the early stage of oral wound healing and decreasing TNF-α tissue levels during all stages of the healing process. Moreover, higher MVD values were observed at day 5, suggesting that PBM is able to induce angiogenesis during oral healing.

Wound healing is a well-orchestrated process, which can be divided into three main, overlapping phases: inflammation, proliferation, and remodeling [5]. In the initial stage, several pro-inflammatory cytokines and chemokines are produced in the wound tissue. These cytokines play an important role in inducing infiltration of the wound area by inflammatory cells, which is necessary to remove debris and kill bacteria [1]. Previous studies with PBM demonstrated that this modality is capable of reducing gene transcription of IL-1β after 7 days in skeletal muscle repair [23] and after 24 h in joint inflammation [22]. Nevertheless, these inflammatory models do not correspond to oral wound healing. In the present study, PBM was associated with an increase in IL-1β levels as compared to a sham control group. Oton-Leite et al. (2015) evaluated salivary IL-1β levels in patients with head and neck cancer who received PBM to treat radiotherapy-induced oral mucositis. An increase in IL-1β levels was observed at day 7 after the initiation of radiotherapy [25], which is consistent with our results. Interestingly, at days 21 and, especially, 35, this result was reversed, and the PBM-treated group exhibited lower salivary IL-1β levels [25]. PBM is probably able to induce IL-1β release in the inflammatory site during the initial phases of oral wound healing.

Among its several roles during wound healing, IL-1β is capable of acting directly and indirectly on endothelial cells, as well as regulating inflammation-induced angiogenesis [1, 36]. Carmi et al. (2009) demonstrated that angiogenesis in Matrigel was abrogated when IL-1β was neutralized. This effect occurs because IL-1β is capable of activating infiltrating cells to produce endothelial cell-activating factors, such as vascular endothelial growth factor [37]. The increase in IL-1β tissue levels induced by PBM at the early (inflammatory) phase of oral wound healing could be associated with this pro-angiogenic effect, as new blood vessels are essential for progression to the proliferative phase of healing [1]. As damaged capillaries are replaced, the supply of oxygen, blood constituents, and nutrients to the wound site is restored. Failure in this process results in delayed wound healing [38]. In the present study, we observed increased angiogenesis in the 4-J/cm^2 group at day 5, which corresponds temporally to the proliferative phase of healing. Our results corroborate those of Corazza et al. (2007), who demonstrated that lower energy densities are more efficient in inducing new blood vessel formation compared to higher doses of laser energy [39]. Using the same parameters of the present study, our group previously demonstrated in vitro and in vivo that PBM at an energy density of 4 J/cm^2 was capable of inducing re-epithelialization, another major event of the proliferative phase that is extremely important to restoration of cellular homeostasis [15, 18].

TNF-α is an acute-phase protein responsible for triggering a cascade of other cytokines. This pro-inflammatory cytokine increases vascular permeability and restrains the inflammatory process by recruiting inflammatory cells to the site of infection [40]. Nevertheless, overexpression of TNF-α can give rise to chronic and slow-healing acute wounds, and is associated with host-damaging effects [4]. In the present study, PBM decreased TNF-α tissue levels throughout the experimental period. While low levels of TNF-α can promote wound healing by stimulating growth factor release, higher levels, especially for prolonged periods, hamper the wound healing process. Usually, TNF-α and IL-1β have the ability to stimulate each other, perpetuating their expression during wound

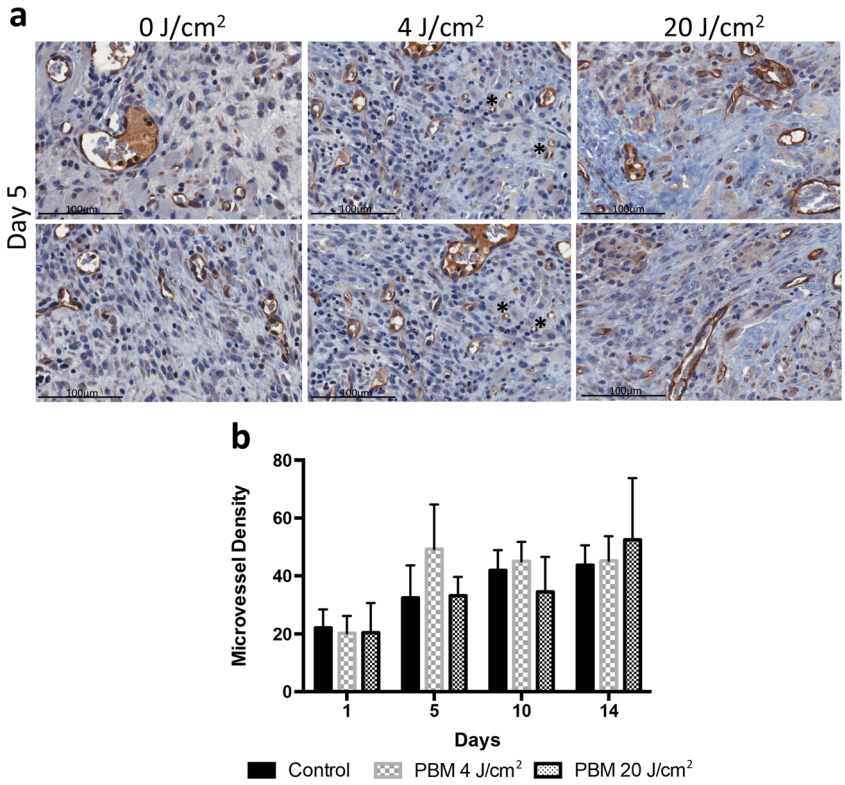

Fig. 2 Effect of PBM on angiogenesis during oral wound healing. **a** Representative examples of CD31 immunostaining at day 5. A greater degree of small vessel neoformation (*asterisk*) is visible in the 4-J/cm^2 group compared to the other groups. **b** Statistical analysis revealed no significant difference among protocols over time. However, there is a clear trend toward increased vessel numbers in the 4-J/cm^2 group on day 5

healing [41]. In the present study, IL-1β tissue levels in the irradiated groups were elevated at day 1 compared to those of controls. On the subsequent days of oral wound healing, all groups exhibited similar IL-1β tissue levels; thus, in the irradiated groups, this decrease was more pronounced. The significantly lower expression of TNF-α during all stages of oral wound healing may have contributed to this modulation of IL-1β.

An important point evaluated in the present study was the combination of PBM parameters needed to achieve the desired effects during oral wound healing. In all outcomes evaluated (IL-1β, TNF-α, and MVD), the 4-J/cm^2 group achieved better results compared to the 20-J/cm^2 group. The lower energy density was associated with a greater increase in IL-1β tissue levels (Fig. 1a) and MVD (Fig. 2b), as well as a greater decrease in TNF-α tissue levels (Fig. 1b). Several studies evaluating the role of PBM in wound healing lack standardization of parameters, thus hindering comparison of their results. Our group previously evaluated the differences between low (4 J/cm^2) and high (20 J/cm^2) PBM energy densities in oral wound healing in vivo and in vitro with all other parameters remaining equal (660 nm; output power 40 mW; spot size 0.04 cm^2) [15, 18, 19]. In vitro, PBM administered at a low energy density (4 J/cm^2) resulted in the accumulation of ROS without inducing DNA damage [17] and accelerated cellular migration of oral keratinocytes through the activation of the mTOR signaling pathway [18]. In vivo, the same parameters were associated with faster and more organized re-epithelialization and increased collagen deposition [15]. These previous results, combined with the findings of the present study, provide basic mechanistic evidence to support the hypothesis that low energy densities of irradiation lead to better clinical effects compared with higher energy densities, at least in wound healing [15, 42]. These findings are consistent with the Arndt–Schultz law, which states that while a small stimulus can stimulate physiological activities, a higher stimulus is capable of inhibiting such activity [43].

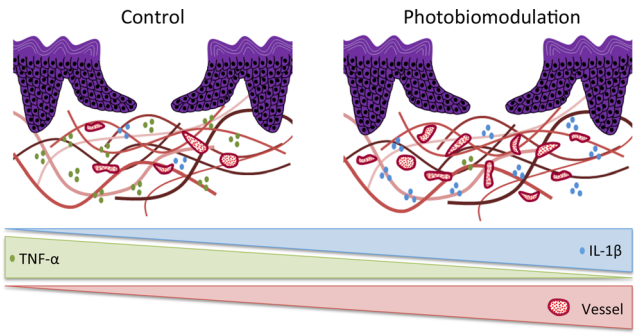

Fig. 3 Schematic illustration of the mechanisms involved in PBM-mediated oral wound healing. PBM was able to modulate cytokine release by decreasing the tissue levels of TNF-α and increasing the levels of IL-1β. A clear trend toward increased angiogenesis was noted in the 4-J/cm^2 group at day 5

Overall, PBM is able to modulate cytokine release during oral wound healing, decreasing tissue levels of TNF-α and increasing levels of IL-1β (Fig. 3). Interestingly, the lower energy density (4 J/cm^2) assessed in this study was associated

with more significant results in terms of cytokine release, as well as with a greater number of new blood vessels, especially at day 5.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval All experiments were carried out in accordance with the US National Academy of Sciences Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Hospital de Clínicas de Porto Alegre, Brazil, under process number 12-0338.

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