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Defocused high-power diode laser accelerates skin repair in a murine model through REDOX state modulation and reepithelization and collagen deposition stimulation

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ABSTRACT

Skin wounds represent a burden in healthcare. Our aim was to investigate for the first time the effects of defocused high-power diode laser (DHPL) on skin healing in an animal experimental model and compare it with gold standard low-level laser therapy. Male Wistar rats were divided into 5 groups: Negative control; Sham; 0.1 W laser (L0.1 W); DHPL Dual 1 W (DHPLD1 W); and DHPL Dual 2 W (DHPLD2 W). Rats were euthanized on days 3, 5, 10, 14 and 21. Clinical, morphological, PicroSirus, oxidative stress (MDA, SOD and GSH) and cytokines (IL-1β, IL-10 and TNF-α) analyses were performed. A faster clinical repair was observed in all laser groups at D10 and D14. DHPLD1 W exhibited lower inflammation and better reepithelization compared to other groups at D10. DHPL protocols modulated oxidative stress by decreasing MDA and increasing SOD and GSH. Collagen maturation was triggered by all protocols tested and L0.1 W modulated cytokines release (IL-1β and TNF-α) at D3. In conclusion, DHPL, especially DHPL1 W protocol, accelerated skin healing by triggering reepithelization and collagen maturation and modulating inflammation and oxidative stress.

1. Introduction

Skin wounds are characterized by cutaneous tissue disruptions that promote important changes in their anatomical structure and/or function [1]. In medical clinic routine, ulcerated skin lesions of various etiologies are very frequent and challenging. These injuries may lead to pain and discomfort, being a gateway to secondary infections [2–4]. Tissue repair is a life-critical complex physiological process aimed to restoring damaged tissue integrity, which involves a series of intricate, dynamic, and well-ordered events [5–8]. Several treatment protocols have been proposed to accelerate repair, relieve symptoms and improve the quality of repaired tissue [9–15], including photobiomodulation (PBM) therapy. PBM is a low-cost, noninvasive therapy that uses non-ionizing forms of light, including lasers and light emitting diodes (LEDs), in the visible ($\lambda = 400$ to 780 nm) and infrared ($\lambda = 780$ to 1064 nm) spectrum that cause photophysical and photochemical events at

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different biological degrees [16–18]. This results in therapeutic effects such as pain control, modulation of the inflammatory process, wound healing stimulation, and tissue regeneration [9,10,19–27].

The success of PBM depends on the light parameters used. Previous studies showed that clinical outcomes and biological mechanisms are dependent on light density of energy, for example [9,10,23]. However, many other parameters can be altered and finding the most effective treatments through pre-clinical studies has important translational value. As far as we are concerned, the effect and mechanisms of action of different protocols of defocused high-power diode laser (DHPL) on skin repair has never been evaluated in an animal model study until present time. Our group has recently demonstrated promising results from this therapy in oral wound healing [22]. DHPL might have advantages such as deeper tissue penetration. Moreover, the literature suggests that PBM using dual wavelength promotes better results than a single-wavelength laser [23]. Thus, our aim was to compare different PBM protocols during skin repair, using low-level laser diode and DHPL with dual wavelength. We evaluated the effect of these different protocols on important basic mechanisms such as reepithelization, inflammatory response, collagen deposition/maturation, modulation of redox state and cytokines release.

2. Materials and Methods

2.1. Study Design

This consisted in a controlled experimental study in animal model.

2.2. Animal Model and Experimental Procedure

The present study was developed following the Guide for the Care and Use of Laboratory Animals and was approved by the Ethics Committee on Animal Use of the Porto Alegre University Hospital (HCPA, Brazil) under protocol n. 2018–0624.

The sample size calculation, considering wound closure as primary outcome, was based on studies using a similar methodology [9,28], with a number of 5 animals per group per day of euthanasia. One hundred and five, 8-weeks old, male rats (*Rattus norvegicus albinus, Rodentia, Mammalia*, Wistar lineage) weighing between 250 and 300 g were used. The rats were housed in boxes with a minimum of 2 and a maximum of 4 animals and maintained in standard temperatures between 20 and 24 °C and 12-h light/dark cycles. The animals received free access to solid chow and water. To induce the skin wound, animals were anesthetized by the isoflurane inhalable technique and full-thickness circular wounds (10 mm in diameter) were created on the backs by a standard punch biopsy technique after shaving the area. Animals received two daily intraperitoneal doses of tramadol (20 mg/kg) for analgesic purposes. Animals were randomly allocated, based on body weight, into five experimental groups:

Control Group: uninjured skin ($n = 5$).

Sham Group: wound induction without treatment, only daily handling ($n = 25$).

Laser 0.1 W (L0.1 W) Group: 660 nm, 01 W and 6 J/cm^2 laser wound treatment ($n = 25$).

DHPL Dual 1 W (DHPLD1 W) Group: DHPL 810 + 980 nm, 1 W, 6 J/cm^2 wound treatment (n = 25).

DHPL Dual 2 W (DHPLD2 W) Group: DHPL 810 + 980 nm, 2 W, 6 J/cm^2 wound treatment (n = 25).

PBM treatment was performed immediately after the surgical procedure and daily until the euthanasia period of each experimental group. Treatment protocol lasted 21 consecutive days. Euthanasia was performed by means of isoflurane inhalant overdose on days 3, 5, 10, 14 and 21. At each experimental point, 5 animals in each group were killed. The back injury was photographed and removed and the specimens were divided into two fragments, where one was fixed in 10% buffered formalin solution for histopathological and picrosirius study and the other one was packaged in liquid nitrogen for cytokine analysis and antioxidant activity.

Table 1
PBM parameters.

	Experimental Groups		
	L0.1 W	DHPLD1 W	DHPLD2 W
Center wavelength (nm)	660 nm ±10 nm	810 nm + 980 nm (50%/50%)	810 nm + 980 nm (50%/50%)
Operating mode	Continuous	Pulsed	Pulsed
Frequency (Hz)	~ 50/60 Hz	50 Hz	50 Hz
Pulse duration (ms)	Continuous	2 ms	2 ms
Duty cycle (%)	–	10%	10%
Peak power (W)	0.1 W	1 W	2 W
Average power (mW)	100 mW	1000 mW	2000 mW
Polarization	Yes	No	No
Spot size (cm^2)	0.03	4.91	4.91
Beam shape	Round	Round	Round
Beam profile	Gaussian	Gaussian	Gaussian
Irradiance at target (W/cm^2)	3,33 W/cm^2	0.20 W/cm^2	0.40 W/cm^2
Exposure time (s/point)	3	30	15
Radiant exposure (J/cm^2)	10	6.11	6.11
Total radiant energy (J/point)	0.3	20	20
Number of points	6	1	1
Method of application	Light in contact	Device in contact	Device in contact
Number and frequency of treatment sessions	1× day/21 days	1× day/21 days	1× day/21 days

2.3. Parameters of PBM

Laser parameters are fully described in Table 1. L0.1 W group received irradiation with a continuous indium–gallium– aluminum–phosphide (InGaAlP) diode laser (MMOptics Ltda, São Carlos, Brazil). The irradiation was performed perpendicularly, and the laser device was put in contact to the skin, in the incision region and around the lesion area (6 points in the ulcer area). The PBM of the experimental DHPLD1 W and DHPLD2 W were performed with a pulse diode laser (Gemini®, Azena Medical, LLC, distributed by Ultradent Products, Inc.) with dual wavelength 810 + 980 nm and two distinct doses protocols (Table 1). The laser device has an adapter that is placed perpendicularly and in contact with the skin at the wound central point. This ensures a standard defocus laser light application without any ablative tissue risk. For all PBM therapies (Laser and DHPL Dual), the power output was checked as instructed by the manufacturer. The laser irradiations were done following biosafety rules.

2.4. Clinical Analysis

The animals' weight was recorded at day 0 and then each 2 days until the end of the experiment. At the euthanasia day, photographs of the wounds were taken and a reference object with previously known measures was included in the photo area to allow standard measurement. The wound area was calculated by a blinded examiner using ImageJ 1.48v software (National Institutes of Health, USA). The wound area in pixels was then converted to mm^2 using the photographed object as a reference.

2.5. Histopathological Analysis

Paraffin-embedded-formalin-fixed sections were used for histopathological analysis. The slides were stained with hematoxylin-eosin for the evaluation of reepithelization and inflammation degree. Initially, a descriptive analysis was performed followed by a semi-quantitative analysis based on reepithelization and inflammation scores. Two experienced and blinded pathologists performed the analysis based in a

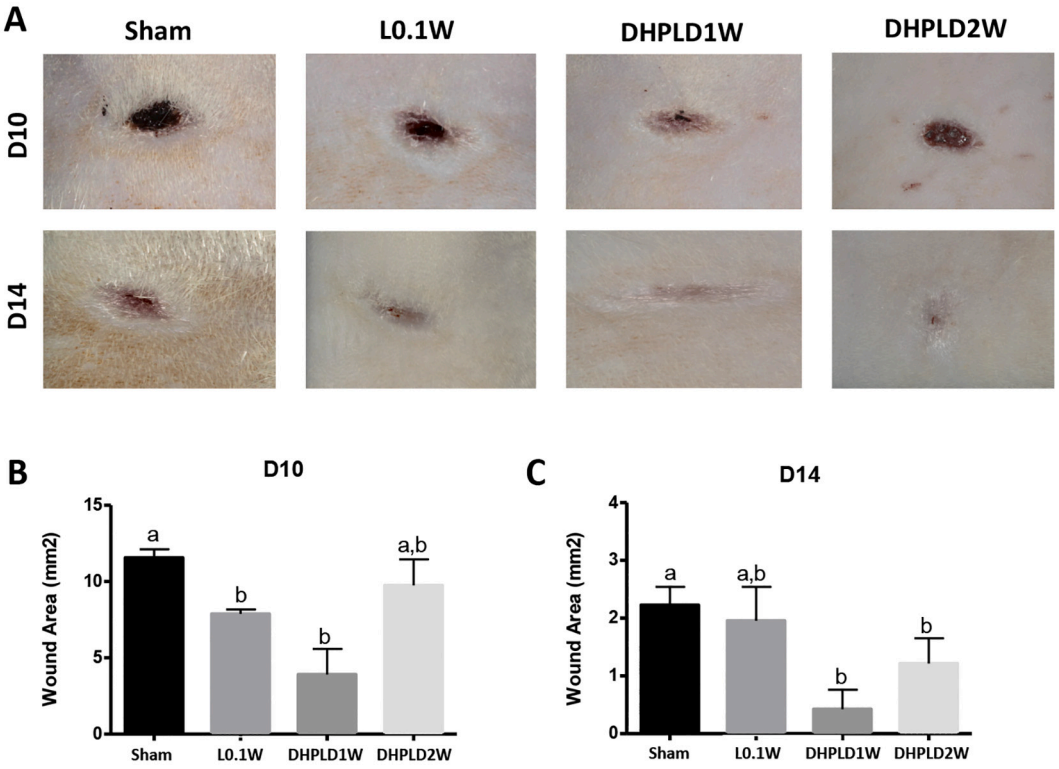

Fig. 1. (A) Representative images of clinical aspects at D10 and D14. (B) At D10, L0.1 W and DHPLD1 W showed enhanced clinical resolution of the wound compared to Sham group. (C) At D14, both DHPL, D1 W and D2 W showed advanced wound healing. Different lowercase letters ("a" and "b") in columns (intergroup analysis) denote significant difference ($p < 0.05$).

consensual final score.

Reepithelization scores have been previously described and consisted of: "Grade 0 - reepithelization at the end of the wound; Grade 1 - reepithelization covering less than half the wound; Grade 2 - reepithelization covering more than half of the wound; Grade 3 - reepithelization covering the entire wound with irregular thickness; Grade 4 - reepithelization covering the entire wound and of normal thickness" [29].

The inflammatory process scores consisted of: "Grade 1 - acute inflammation (pyogenic membrane at the most superficial wound area with no vascular events in the underlying connective tissue); Grade 2 - predominance of diffuse acute inflammation (vascular phenomena such as edema and hyperemia predominate); Grade 3 - predominance of chronic inflammatory process (presence of inflammatory cells, angiogenesis and fibroplasia); Grade 4 - resolution and healing (reduction or disappearance of chronic inflammation)" [30].

2.6. PicroSirius Red Staining

Paraffin-embedded-formalin-fixed sections of 3-μm of all groups at D10 were deparaffinized, re-hydrated and next stained with PicroSirius Red according to manufacturer's protocol (Vetec Química Fina LTDA). The images were acquired using a polarized microscope (Olympus BX51) at 40× magnification coupled with a camera device (Olympus Q-color 5 RTV) and capture software (Q-capture, version 2.0.11). The evaluation consisted in staining intensity, pattern of the collagenization and disposition of collagen fibers deposited in the wound area. An initial descriptive analysis of groups was performed. Then, two experienced and blinded pathologists graded the collagen fibers in the polarized images on the basis of a consensus. Each case was classified as previously described in "+1 (thin = green, delicate loosely arranged collagen fibers seen throughout the wound area), +2 (thin, delicate loosely arranged collagen fibers are seen in the surface and center of the wound area, but thicker and gross in the deep and margins) and +3 (thick = orange/red, gross densely arranged collagen fibers seen throughout the wound area)" [31].

2.7. REDOX Assay

2.7.1. Malonaldehyde Dosage (MDA)

The content of MDA (nanomoles of MDA per gram of tissue), a product of lipid peroxidation, was measured by methods previously described [32]. Briefly, samples were suspended in Trisma 1:5 (*w/v*) buffer. The material was incubated for 40 min at 45 °C in a water bath, centrifuged at 2500 G for 5 min at 4 °C; 300 μL was then removed, read at 586 nm, and interpolated in a standard curve. Supernatants were tested for MDA content and placed in microplates. Absorbance was measured at 586 nm.

2.7.2. Glutathione Dosage (GSH)

GSH levels (GSH units (U) per mg of tissue) were measured to verify antioxidant activity [33]. Briefly, 0.02 M EDTA were added to the prepared skin tissue and stored at −80 °C until use. The tissue was thawed and automatically homogenized for 2 min, followed by centrifugation at 3000 ×*g* for 15 min at 4 °C. Then the supernatant was removed and mixed with 0.4 M Tris buffer (pH 8.9) and 5,5′-dithiobis-(2-nitrobenzoic acid). Absorbance was measured at 420 nm.

2.7.3. Estimation of Superoxide Dismutase (SOD)

SOD levels (U/g) were also assed to measure antioxidant activity [34]. The skin sample was mixed in 1 mL of 0.4 M phosphate buffer, pH 7.0, and then centrifuged at 10,000 rpm for 15 min. Next, 0,25 mL Methionine, 0.03 mL Riboflavin, and 0.01 mL NBT were added to 0.01 mL of skin homogenate, along appropriate standard, and control samples. The samples were subjected to 10 min exposure in an illumination chamber lined with aluminum foil and fitted with a 15 W fluorescent lamp. The optical density was immediately read at 560 nm.

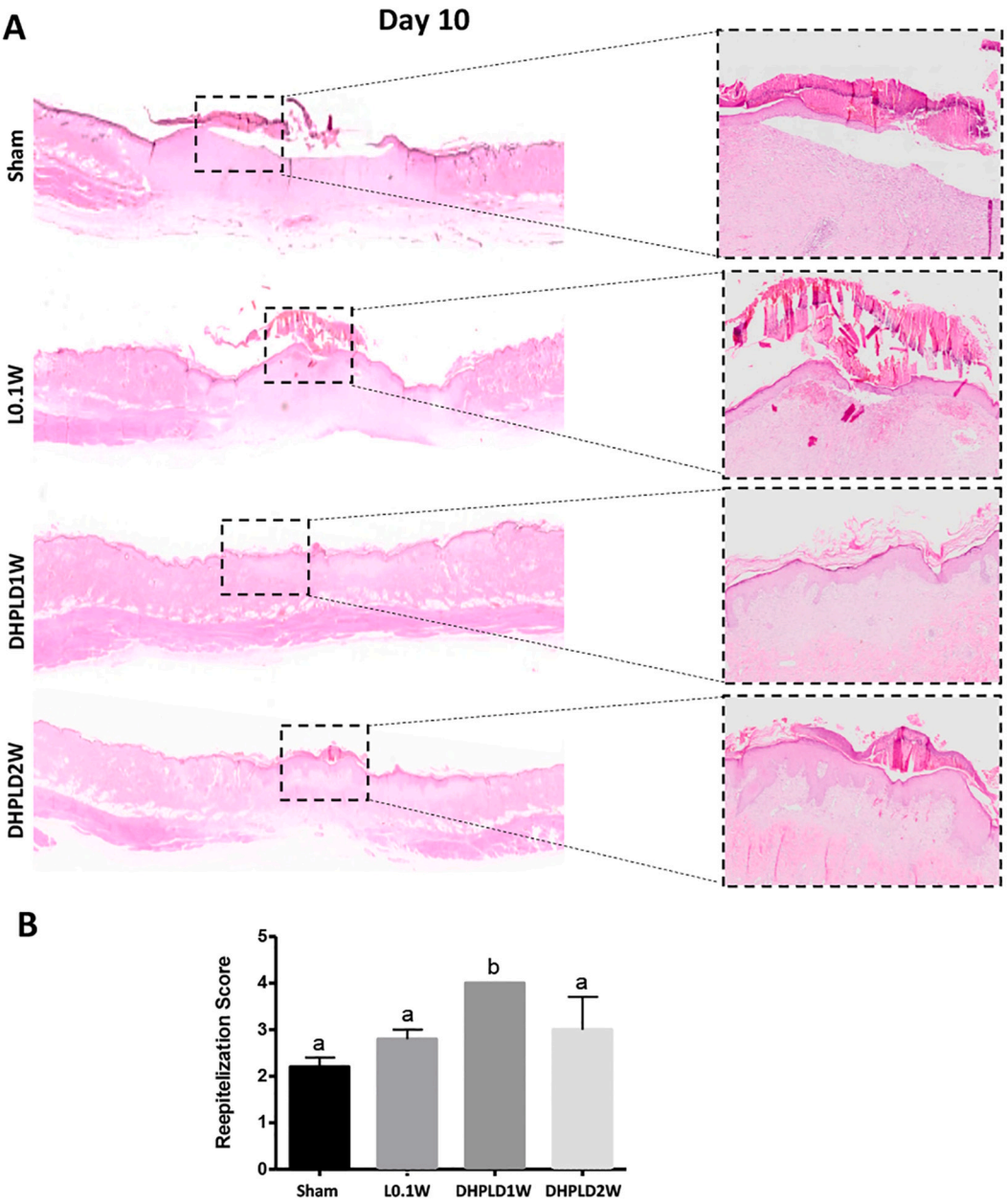

Fig. 2. Histopathological evaluation reepithelization at D10. (A) Photomicrographs of experimental groups at day 10. Sham group exhibited exposed areas of connective tissue and the L0.1 W and DHPLD2 W presented complete reepithelization with irregular thickness. Only the DHPLD1 W had epithelial tissue covering the entire wound in a regular thickness (haemotoxylin and eosin, ×40 and ×100). (B) On day 10, reepithelization degree of DHPLD1 W group was significantly higher compared to the other groups. Different lowercase letters ("a" and "b") in columns (intergroup analysis) denote significant difference ($p < 0.05$).

2.8. Cytokine Immunoassays

Interleukin (IL)-1β, IL-10 and tumour necrosis factor (TNF)-α levels (pg/mL) were assessed by commercial enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, MN), and the protocol followed the manufacturer's instructions [35]. The detection range for all cytokines was 62.5–4000 pg/mL and the minimum detection limit for IL-1β and IL-10 was 12.5 ng/mL and for TNF-α was 50 ng/ml.

2.9. Statistical Analysis

The software GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analysis. Differences between groups within each evaluation time were assessed through multiple *t*-tests. Different lowercase letters in graphs and tables denote significant difference ($p < 0.05$).

3. Results

3.1. Weight Analysis

During the experimental period, animals from all groups progressively gained weight. No significant difference was observed between groups within each experimental day ($p > 0.05$).

3.2. DHPLD1 W Has Major Effects in Stimulating Clinical Skin Repair

In the initial evaluation times (D3 and D5) all groups presented a similar wound area. At D10, L0.1 W and DHPLD1 W presented significantly smaller wounds compared to the Sham group ($p = 0.004$ and

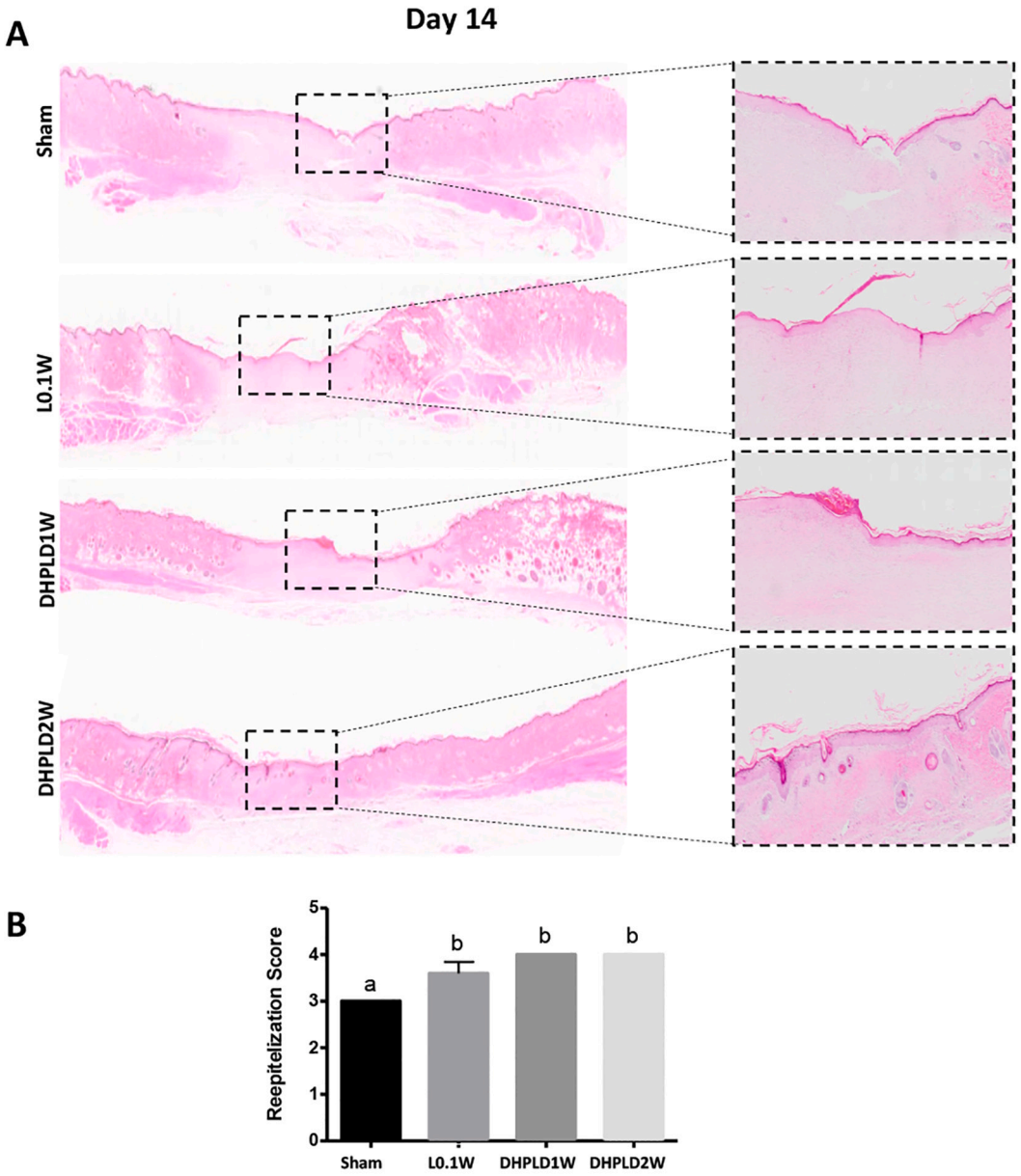

Fig. 3. Histopathological evaluation of reepithelization at D14. (A) Photomicrographs of experimental groups at day 14. All irradiated groups showed newly formed epithelial tissue covering all the extension of the wound area with a normal thickness, while Sham group still presented irregular thickness (haemotoxylin and eosin, ×40 and ×100). (B) All irradiated groups showed higher reepithelization degree compared to Sham group. Different lowercase letters ("a" and "b") in columns (intergroup analysis) denote significant difference ($p < 0.05$).

0.01, respectively) (Fig. 1A and B). At D14, the wound area of DHPLD1 W and DHPLD2 W groups were smaller compared to Sham group ($p = 0.01$ and 0.03, respectively) (Fig. 1A and C). At D21, all groups had complete wound repair with no sign of reminiscent crust.

3.3. PBM Modulates the Inflammatory Process and Accelerates the Formation of a New Epithelial Barrier

At D3 and D5, all groups presented similar amount of connective tissue covered by epithelium. Significant results were detected at days D10 and D14 (Figs. 2 and 3, respectively). At D10, only DHPLD1 W had reepithelization covering the entire wound and of normal thickness, while other groups still presenting some exposed areas (Sham) ($p < 0.001$) or irregular thickness such as L0.1 W ($p < 0.01$) and DHPLD2 W ($p < 0.05$). At D14, all irradiated groups had achieved grade 4 of reepithelization, while Sham group still presented some areas of uneven thickens. At D21, all groups presented complete formation and maturation of epithelial tissue.

Modulation of inflammatory response by PBM occurred in an early time-point (Fig. 4). While Sham group presented a pyogenic membrane with acute inflammatory infiltrate at D3, all irradiated groups had a more chronic process with more lymphocytes infiltration in the connective tissue translated as a more advanced phase of inflammatory response. At D5, no significant difference was noted between groups, which were mostly trapped in the chronic inflammatory process with collagen fibers deposition. At D10, however, DHPL irradiation induced more collagen fiber deposition and angiogenesis accompanied with a decrease in chronic inflammatory cells, which resulted in a significantly resolution of inflammatory phase (higher grade in score scale) in DHPLD1 W and DHPLD2 W groups compared to Sham group. At D14 and D21, all groups presented resolution and healing of chronic inflammation.

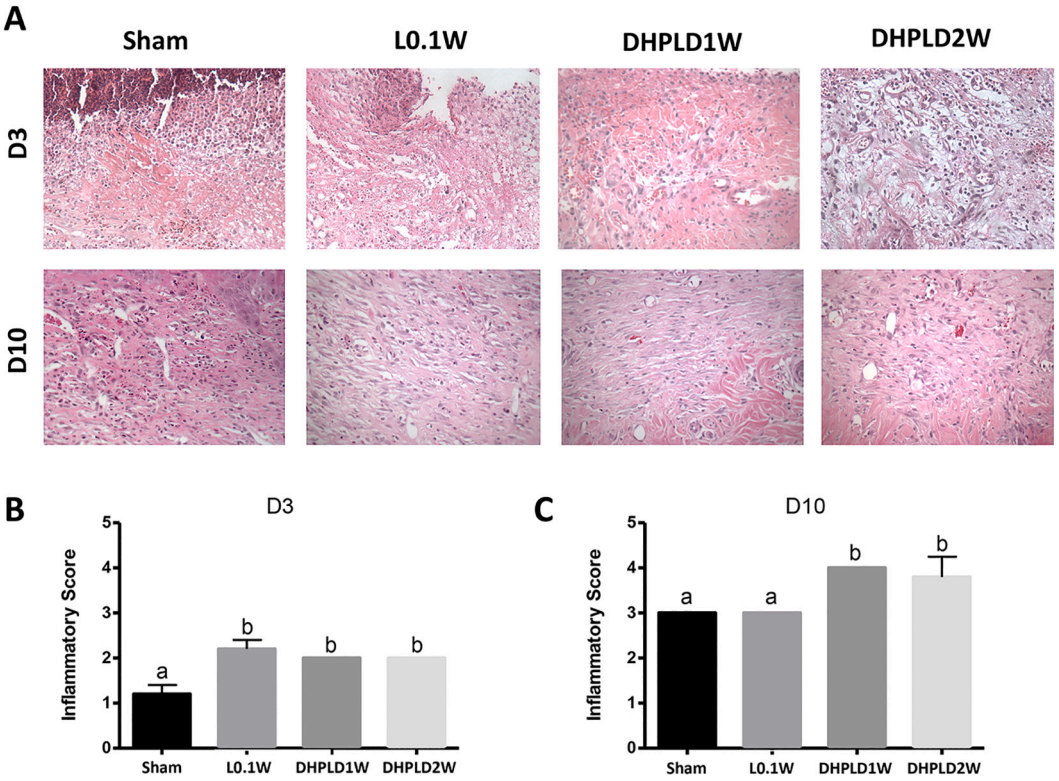

Fig. 4. Histopathological evaluation of inflammatory process. (A) Photomicrographs of experimental groups at D3 and D10. All the PBM groups showed more advanced healing demonstrated by less and more chronic inflammatory infiltrate associated to more collagen deposition in wounds (haemotoxylin and eosin, ×400). (B) At D3, all PBM presented higher inflammatory grades, which represent a more advanced healing. (c) At D10, the DHPLD1 W and DHPLD2 W presented a more advanced resolution of inflammatory phase compared to Sham and L0.1 W groups.

3.4. FBM Modulate Collagen Deposition and Maturation

PicroSirius analysis at D10 revealed that the Sham group remained with thin and loosely arranged green fibers while the irradiated groups presented denser collagen fibers, which appeared reddish in color, throughout the wound area, with a more structured pattern (Fig. 5A). Most of the fibers in irradiated groups were arranged parallel to the epithelium, however some collagen bundles among the new muscle fibers were also observed, a pattern closer to what was observed in the normal skin. Semi-quantitative analysis revealed that all irradiated groups presented significantly higher scores compared to Sham group ($p < 0.05$), which implies a more advanced stage of collagen deposition and maturation (Fig. 5B).

3.5. PBM Modulates Redox State during Skin Repair

We analyzed the impact of PBM in redox state through malondialdehyde (MDA), deduced glutathione (GSH) and superoxide dismutases (SOD) levels (Fig. 6). While MDA is a marker of oxidative damage, both GSH and SOD have antioxidant proprieties and a protective role against reactive oxygen species (ROS). At early healing phase (D3 and D5), only the DHPL irradiation significantly reduced MDA levels ($p < 0.001$), and DHPLD1 W was also associated with a significant increase in both GSH and SOD levels at D5 and D3, respectively ($p < 0.05$). At later stages, all irradiated groups presented significantly lower MDA levels compared to Sham group ($p < 0.05$). Interestingly, at D10, L0.1 W exhibited the highest SOD levels among all irradiated groups, which were also significantly different from the Sham group ($p < 0.05$).

3.6. Cytokine Released Is Not Significantly Impacted by PBM during Skin Repair

The effects of PBM on inflammatory cytokines release were evaluated through IL-1β, IL-10 and TNF-α levels (Fig. 7). At D3, an early stage of wound healing process, L0.1 W had the most meaningful results showing a significant increase in IL-1β and TNF-α levels ($p < 0.05$). DHPL irradiation had no impact on any type of cytokine release in all evaluation times.

4. Discussion

The global cost for wound care is projected to reach up to $3.5 billion in 2021 [36], a result of its high prevalence in the population: in the US, it is estimated that 3% of the population over 65 years old have open wounds [36]. PBM has been used with success in the treatment of skin and oral wounds [37–39]. To date the vast majority of clinical and experimental studies have focused on the use of low power lasers or LEDs [10,37,38]. The use of high-power lasers in a defocused way represents a possible approach to achieve the same outcome on the tissue. Our pioneer study compared the effect of two protocols of DHPL with the "gold-standard" of PBM - low power diode laser - in an animal skin-healing model. Our results showed that the low power laser culminated in a faster healing process, as expected. Remarkably, the DHPL, in particular the 1 W peak power protocol (DHPLD1 W group), was able to further accelerate this process by modulating events such as the formation of a new epithelial barrier, modulating the inflammatory process, collagen production, and oxidative stress protection by reducing the damage (MDA) and stimulating antioxidant enzymes such as GSH and SOD.

The use of DHPL has emerged as a therapeutic option that can bring some advantages such as a larger spot area, which reduces the total

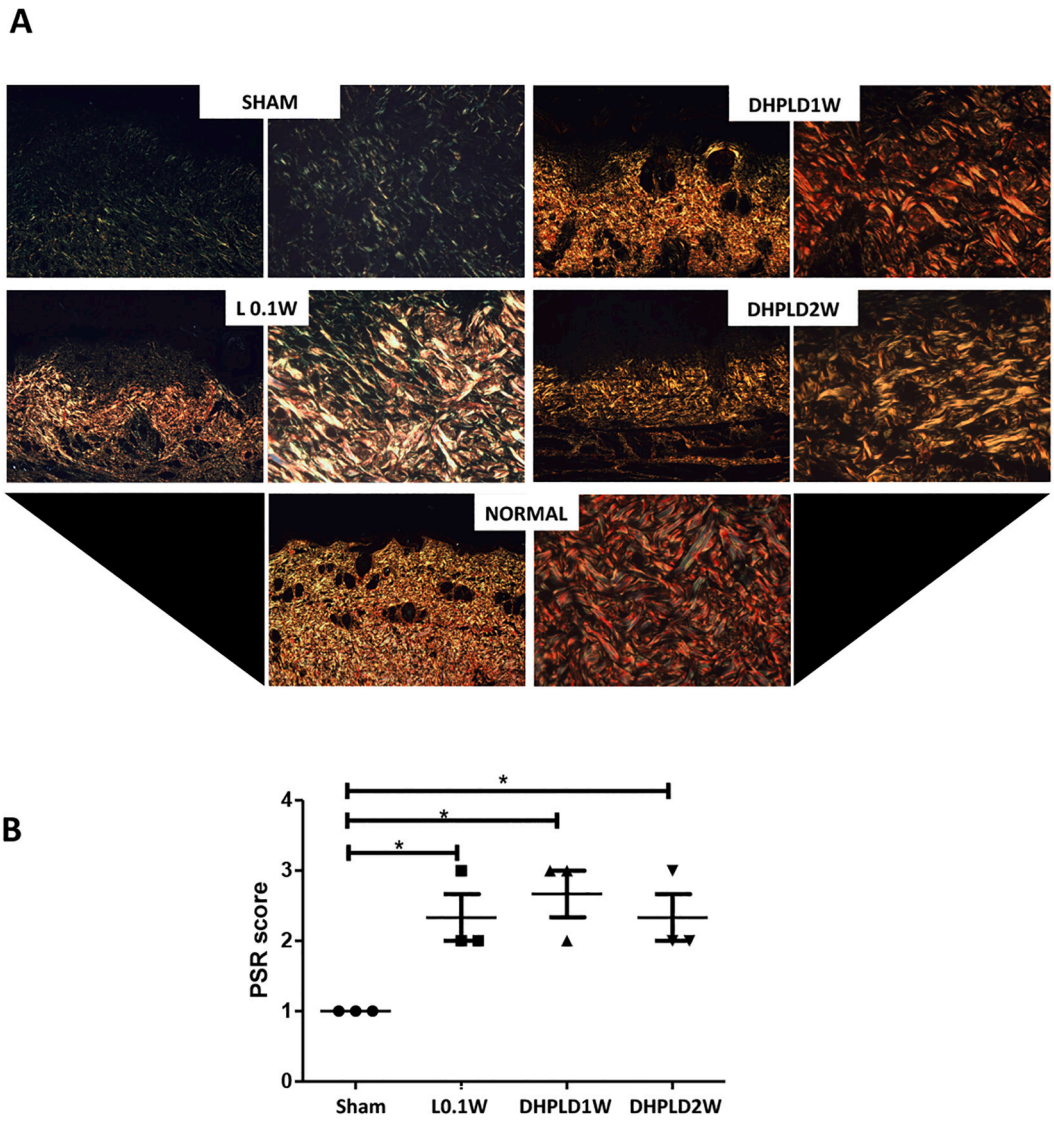

Fig. 5. Collagen evaluation at D10. (A) The Sham group exhibited thin and loosely arranged fibers (green fibers) while the irradiated group presented denser collagen fibers (reddish) throughout the wound area, with a more structured pattern, resembling the normal skin area (PicroSirius Red/ Polarization light, x40 and x400). (B) All the irradiated groups showed higher degree of collagen fibers deposition with better organization compared to the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

irradiation time in large wounds that are common on the skin and a huge challenge to treat. Also, many dermatology centers have high-powered lasers available for other clinical conditions such as hair removal, or management of vascular and pigmented lesions [40–42]. These facts support the clinical advantages and applicability of DHPL in wound healing. Yet, for this therapy to be included among clinical guidelines, studies supporting its clinical effectiveness and mechanism of action, such as ours, are needed. Concerning the protocols chosen, our study aimed to answer two main questions: 1 - Is DHPL as effective as low power diode laser in promoting skin wound healing? and 2 – Which DHPL protocol is more effective, 1 W or 2 W?

Dosimetry is a key aspect of PBM. The low-level laser therapy protocol used in the present study is within the therapeutic window described for wound healing [10,13,24,27,43], and our groups has already validated its positive effect on oral healing [9,22,27,44,45]. Our results demonstrated that from the clinical perspective DHPL protocols achieved results comparable or better to the gold-standard group. In all the further tissue, cellular and molecular analysis this pattern was sustained. Thus, the answer to our first question was: yes, DHPL appears to be as effective as low-power diode laser in promoting cutaneous wound healing in an animal experimental model. It is known that PBM, as well as other therapies, has a therapeutic window and that the increase in dose or changes in other parameters such as potency can influence the outcomes [9,39,46]. Herein, we compared 1 W to 2 W while maintaining the same optimal dose of 6 J/cm^2. Interestingly, the group treated with 1 W achieved more promising results, which answers our second question. Our comprehensive tissue, cellular and molecular analysis gave us grounds to understand the enhanced clinical effect of DHPLD1 W group.

There are still few studies, and with conflicting results, about the effect of the DHPL on wound healing and interestingly most of them are restricted to oral healing. Campos et al. (2016) found that low-power laser and LED (both with 6 and 1.2 J/cm^2 dose) were more effective than DHPL with a wavelength of 808 nm (10 J/cm^2 dose) for the healing of oral mucositis induced by chemotherapy in hamsters [47]. It's not clear if the negative results of DHPL were due to the different wavelength intrinsic to the high-power laser or due to the significant changes in dose between protocols tested. Zand et al. (2012), on the other hand, found a positive effect of CO_2 laser device operated using 1 W power in defocused continuous mode in accelerating oral healing in patients with minor recurrent aphthous stomatitis [48]. Our group has also found a

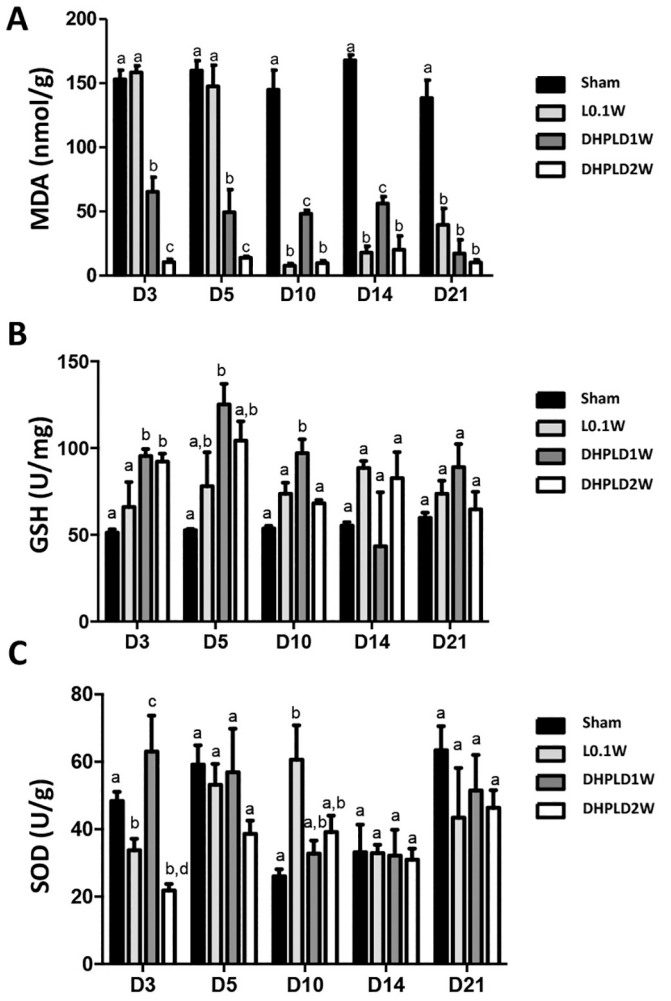

Fig. 6. Redox state analysis. (A) MDA levels (nmol/g of tissue), at D3 and D5, both groups irradiated with DHPL showed lower MDA levels compared to Sham group ($p < 0.001$). At later period of analysis, all irradiated groups presented significantly lower MDA levels ($p < 0.05$). (B) Overall, GSH levels (U/mg of tissue) were increased in irradiated groups. At day 10 DHPLD1 W group showed significantly higher levels compared to all other groups ($p < 0.05$). (c) SOD levels (U/g of protein) were significantly increased at D5 only on DHPLD1 W group ($p < 0.05$). At D10, L0.1 W presented the highest SOD values, which were significantly higher to Sham group levels ($p < 0.05$).

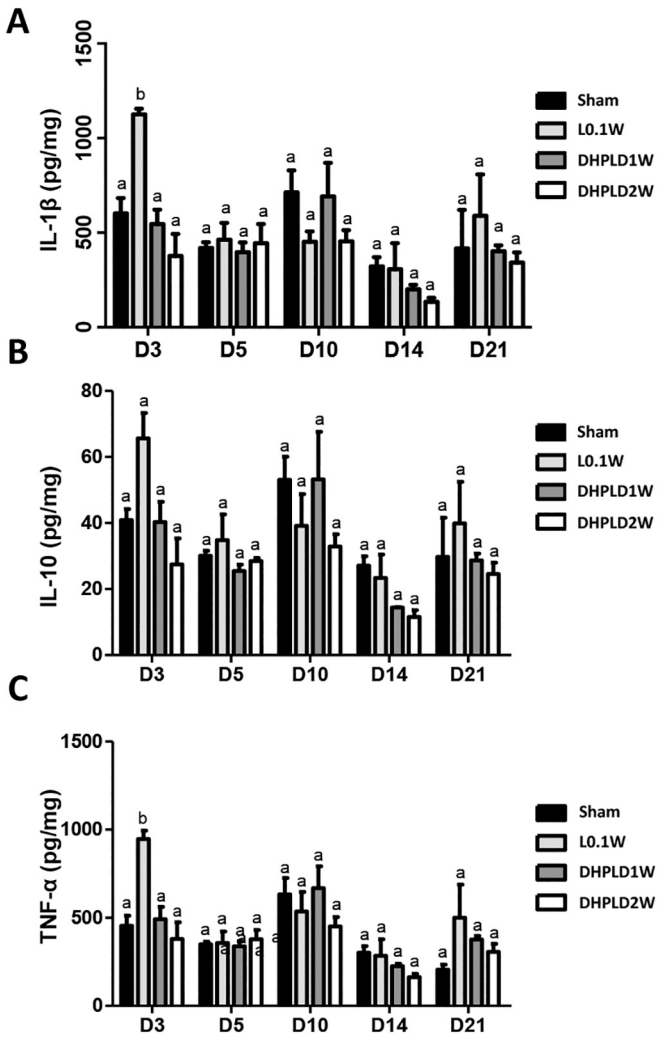

Fig. 7. Inflammatory cytokine release analysis. DHPL had no impact on any type of cytokine release in all evaluation time. (A) At D3, IL-1β levels were increased in L0.1 W group compared to all other groups ($p < 0.05$). (b) IL-10 tissue levels showed a tendency of increase in L0.1 W group at D3, but with no statistical difference. (c) TNF-α levels were increased in L0.1 W group at day 3 compared to other groups ($p < 0.05$).

positive effect of DHPL (using the same device tested herein) in promoting oral healing in OM induced in rats [22]. As far as we are concerned, this is the first experimental study assessing the effect of DHPL in animal skin healing.

Our histopathological analysis showed that PBM, in all experimental groups, reduced the inflammatory process, with the best results obtained in the DHPLD1 W group. Likewise, this group obtained the best results in relation to reepithelialization. These findings are closely related since the early formation of an epithelial barrier protects the connective tissue from possible infections, consequently corroborating for a more agile chronification or resolution of the inflammatory process [20,23,49,50]. These tissue and cellular events provide a rationale for the enhanced clinical outcome of DHPLD1 W group. Collagen deposition was also enhanced by all PBM protocols. Such results corroborate with other studies where the increase in collagen deposition with the use of the laser was observed using doses that varied from 3 and 4 J / cm^2 [19,27] to 16 J / cm^2 [10].

Another important aspect during the healing process is the release of inflammatory cytokines, that further modulate cellular and tissue response. Cytokines can contribute to stimulate cell proliferation and migration by the synthesis of growth factors [53], however in excess they can also lead to an unwanted exacerbation of th2 inflammatory process and maintenance of the wound [52,53]. Herein, only the 0.1 W laser group had a significant effect on the levels of cytokines evaluated. Both IL-1β and TNF-α levels were increased by this protocol in the initial stage of skin healing. These findings corroborate with a previous study of our group in oral mucosal healing, where we also observed increased levels of IL-1β in oral mucosa wounds treated with PBM at an early stage [27]. Our hypothesis is that this peak of IL-1β at an early stage helps to accelerate the progression of the healing process, allowing the chronic phase to start earlier.

Our results on REDOX demonstrated that the control group maintained the levels of MDA, a marker of cell damage, throughout the experimental period, while the groups DHPLD1 W and DHPLD2 W showed a significant drop on its levels, representing a decrease in oxidative damage. Concomitantly we observed an increase in the levels of GSH and SOD in DHPLD1 W and DHPLD2 W groups, which are antioxidant enzymes involved in cell protection. Previous studies have suggested that ROS are produced during laser irradiation, with the ability to activate transduction pathways for redox sensitive signals, such as: NF-kB, NRF-2 and ERK [26,54]. Tatmatsu-Rocha et al. [20]

showed that super pulsed 904 nm laser, with an average power of 40 mW, reduced markers of oxidative stress in skin healing of diabetic mice [20]. Rupel et al., demonstrated that PBM with 970 nm and 800 nm lasers devices significantly reduced ROS levels of keratinocytes exposed to H_20_2 [55]. Additionally, the authors showed that a simultaneous exposure of three different wavelengths (660 nm, 970 nm and 800 nm) was also effective in reducing the oxidative status. These results are in accordance with ours, as our DHPL protocol was performed with a mixture of wavelengths (810 nm and 980 nm), suggesting a promising outcome of combination strategy in modulating redox state.

Although our results seam promising and clinical relevant, some limitations of the study must be taken into account. It is known that there is heterogeneity of wounds in experimental animal models, which are also restricted to a more limited sample size due to ethics issues. In addition, several parameters of the PBM can be modified and this can influence the clinical outcomes. Likewise, each dose of PBM therapy can have different effects if used in other tissues, such as mucosa, bone or muscle. Thus, the beneficial results found here are related to skin repair. It is also important to note that, despite the large translational potential, studies in humans are necessary to confirm our findings.

In conclusion, the therapy using a diode DHPL, particularly with 1 W potency, accelerated cutaneous healing achieving some better results compared to the gold-standard diode low-level laser. The clinical effects were associated with faster reepithelization, modulation of inflammatory processes, increased collagen deposition, and protection of oxidative damage.

Declaration of Competing Interest

The authors declare no competing interests.

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