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ORIGINAL ARTICLE

Comparison of photobiomodulation in the treatment of skin injury with an open wound in mice

Ketlyn Germann Hendler[1,2] · Jaquelini Betta Canever[1] · Luana Gabriel de Souza[1,2] · Laís Mara Siqueira das Neves[1] · Marisa de Cássia Registro Fonseca[3] · Heloyse Uliam Kuriki[1,2] · Aderbal da Silva Aguiar Junior[1,2] · Rafael Inácio Barbosa[1,2] · Alexandre Marcio Marcolino[1,2]

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Abstract

This study aimed to investigate the effects of photobiomodulation at a wavelength of 660 and 830 nm at different numbers of application points in the healing of open wounds in mice. In total, 120 mice were divided into 10 groups. The animals were submitted to cutaneous lesion of the open wound type (1.5 × 1.5 cm). Photobiomodulation at a wavelength of 660 and 830 nm and total energy of 3.6 J were used, applied at 1, 4, 5, and 9 points, for 14 days. The animals were subjected to analysis of the lesion area, skin temperature, and histological analysis. Macroscopic analysis results showed a difference ($p < 0.05$) between the irradiated groups and the sham group at 14 days PO. There was no statistical difference in skin temperature. Histological analysis findings showed better results for the epidermis thickness. Regarding the number of blood vessels, a difference was found between the 1- and 5-point 830-nm photobiomodulation groups and between the 4-point 660-nm group and the naive group. A significant difference in the number of fibroblasts was observed between the 830- and 660-nm photobiomodulation groups and the naive and sham groups. When comparing photobiomodulation wavelength, the 830-nm groups were more effective, and we emphasize the groups irradiated at 5 points, which showed an improvement in macroscopic analysis and epidermis thickness, an increase in the number of vessels, and a lower number of fibroblasts on the 14th day after skin injury.

Keywords Low-intensity laser therapy · Mice · Healing · Histology · Injuries

Introduction

Skin wounds are characterized as an anatomical change in skin integrity caused by cell rupture and occur due to multiple factors such as hypoxia, trauma, or pressure [1, 2]. Injuries to the integumentary system can be caused acutely, such as in operative wounds, traumatic injuries, and cut injuries, or late, highlighting pressure injuries and those caused intentionally such as grafts or skin flaps that are used in surgical procedures. Regardless of how they are caused, all of these injuries require proper management to minimize the risk of infections, tissue necrosis, and hypertrophic scars [3, 4]. Tissue healing can be impaired by local factors such as ischemia, infection, and elevated tissue pressure, or systemic factors such as immunosuppression, diabetes mellitus, hypothyroidism, and smoking [1].

Lesion treatments that affect the cutaneous tissue aim to reduce healing time and improve the appearance of the healing result. Among the different forms of treatment, we can mention wound debridement, use of dressings, medications [5, 6], nutritional supplementation for malnourished individuals, pressure relief with decubitus changes [7], vacuum therapy [8], extracorporeal shock waves [9], high-voltage electrical stimulation [10–12], therapeutic ultrasound [7], radio frequency [13, 14], and photobiomodulation [15, 16].

✉ Alexandre Marcio Marcolino
alexandre.marcolino@ufsc.br

[1] Laboratory of Assessment and Rehabilitation of the Locomotor Apparatus, Department of Health Sciences, Universidade Federal de Santa Catarina, Campus Araranguá - Campus Mato Alto, Rua Pedro João Pereira, 150, Araranguá, Santa Catarina 88905-120, Brazil

[2] Postgraduate Program in Rehabilitation Sciences, Federal University of Santa Catarina (LARAL/UFSC), Rua Pedro João Pereira, 150, Araranguá, Santa Catarina 88905-120, Brazil

[3] Postgraduate Program in Rehabilitation and Functional Performance, Ribeirão Preto Medical School of the University of São Paulo (USP), Av. dos Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil

Regarding the various treatments for tissue injuries, photobiomodulation stands out as an ally in wound healing due to its photobiomodulator effect which accelerates the tissue repair process, causing a reduction of the inflammatory reaction and improved speed of the soft tissue repair process. Its irradiation of the injured tissues triggers a series of physiological effects due to the absorption of photon energy by photoreceptors. When the energy interacts with cells, it causes the activation of mitochondrial ATP due to absorption of light by cytochrome c oxidase, resulting in the photodissociation of nitric oxide and the proliferation of several cells, promoting anti-inflammatory effects and triggering increased proliferation–migration and cell differentiation, cytokine modulation, growth factor production, and deposition of extracellular matrix [17–22].

There is a variety of research on the healing of cutaneous lesions, with different parameters in the treatment for regeneration and viability, and without a consensus or therapeutic window described, in addition to a lack of studies or standardization of the parameters used in the different ways of applying stitches to injuries. Given the above, this study aimed to investigate the effect of photobiomodulation applied at different wavelengths and different numbers of points to cutaneous wounds in mice.

Materials and methods

This is an experimental study with animals, containing intervention groups and a control group (Fig. 1). It used 120 Swiss lineage male mice (40–45 g) with a mean age of 60 days, which were kept in the sectoral vivarium at the Araranguá Campus of the Federal University of Santa Catarina (UFSC).

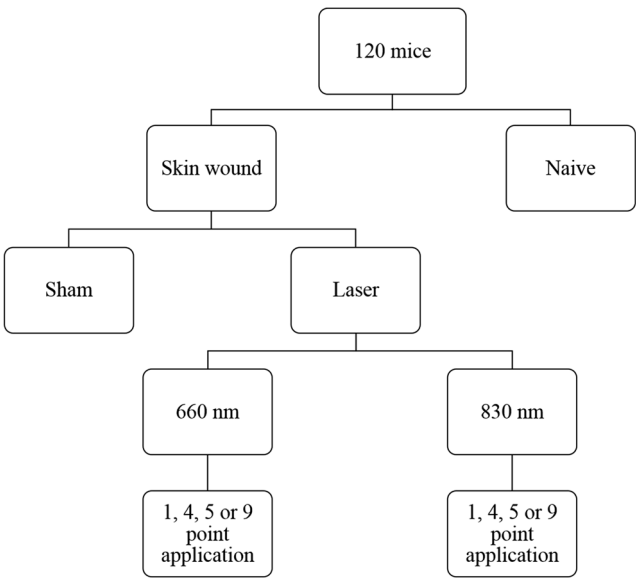

Fig. 1 Flowchart of the division of the groups evaluated in the study

The interventions were carried out in a room for animal experimentation at UFSC, following all the environmental precautions recommended by the Animal Use Ethics Commission (CEUA) and approved under number 4017201117; the ARRIVE checklist was used.

The experiments were performed during the clear cycle (from 7 am to 7 pm), and the animals were kept in the laboratory for acclimatization for at least 30 min before the evaluations were performed. All animals received tramadol analgesic every 8 h for 3 days [23, 24].

Surgical procedure

Mice were anesthetized with intraperitoneal (IP) injection of 100 mg/kg ketamine hydrochloride (Agener União®) associated with 10 mg/kg xylazine hydrochloride (Dopaser®) [25, 26]. Then, trichotomy was performed by manual traction of the hair on the back of the animals. They were then submitted to surgical incision: 1.5 × 1.5 cm of skin was surgically removed using a template developed for the experiment (Fig. 2a, b).

Intervention (photobiomodulation therapy)

The photobiomodulation therapy was performed at wavelengths of 830 nm (AsGaAl) and 660 nm (AlGaInP) using Ibramed® Medical Equipment (São Paulo, Brazil). The parameters used in this paper are shown in Table 1, and Fig. 2c demonstrates the localization of the application points.

Analysis of the samples

The animal analysis procedures were carried out in the sectoral bioterium and in the microscopy laboratory of the Center of Sciences, Technology, and Health at the Araranguá Campus of UFSC.

Skin lesions were assessed daily by macroscopic observation before the application of photobiomodulation. All animals were photographed with a Cyber-Shot DSC-P72 digital camera (5.1 megapixels, Zoom 3.2; Sony®, USA) kept at a constant distance of 20 cm; photographs were later analyzed using ImageJ® software. Analyses were performed immediately after surgery and on the 7th and 14th days after the surgical procedure.

Thermography is a technique that consists of observing temperature through high-resolution infrared technology. The evaluations were performed after irradiation of the photobiomodulation (PBM), in the following times: immediately after surgical incision, 7 and 14 days after the surgery[4]. We used a constant distance of 20 cm between the FLIR C2 camera and the animals' dorsal region to record the temperature of the lesion region, which was later analyzed using FLIR Tools™ software.

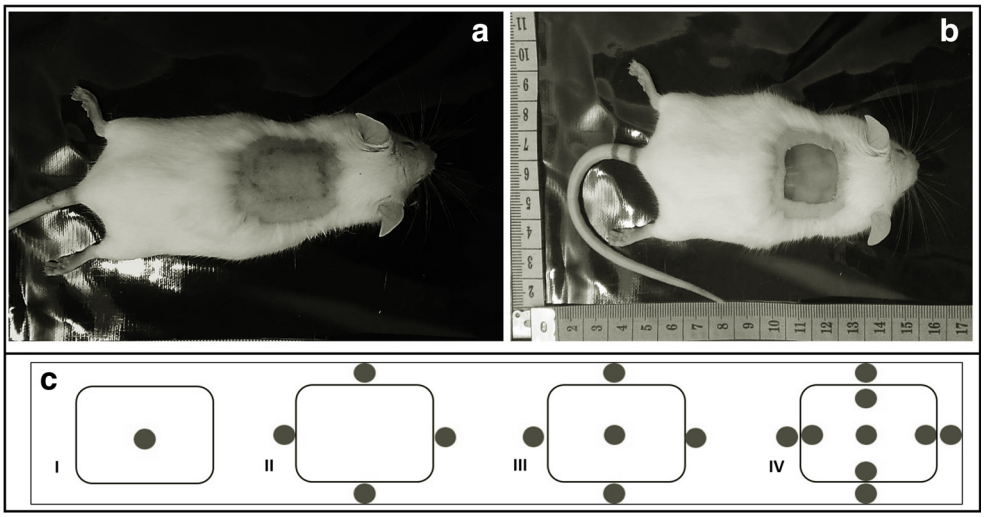

Fig. 2 Surgical procedure. **a** Manual hair trichotomy and demarcation of the back of the animals for skin removal. **b** Final model of the open wound after removal of a 1.5 × 1.5 cm piece of the skin. **c** Application of photobiomodulation points on the backs of animals

After euthanizing the animals by anesthetic overdose on the 14th day after the surgical procedure, skin samples were removed and immersed in 10% formalin for 48 h. The samples were fixed, dehydrated, diaphanized, embedded in paraffin, and then cut by a microtome to obtain 5–6-µm-thick non-serial sections. We stained the skin samples with hematoxylin and eosin (HE) for histological evaluation by light microscopy.

A trinocular microscope and 14-megapixel digital camera, both from Global Optics, were used to acquire histological images.

To determine the thickness (in micrometers) of the epidermis of each of the samples, quantitative analysis of the images of the histological sections was performed using ImageJ® software (Fig. 3a).

The number of blood vessels was determined from the images of the samples which were standardized for counting a grid with 1.5 × 1.5 cm squares, totaling 100 squares in the lower quadrants (Fig. 3b).

The number of fibroblasts was determined using the ImageJ® cell counter tool which counts cells by hand marking (Fig. 3c).

Table 1 Photobiomodulation therapy parameters with a wavelength of 660 nm and 830 nm

Laser irradiation (nm)	660			
Power (mW)	30			
Beam area (cm^2)	0.06			
Application points	1	4	5	9
Fluency per point (J/cm^2)	60	15	12	6.67
Total fluency per point (J/cm^2)	840	210	168	93.38
Time (s)	120	30	24	13
Energy per point (J)	3.6	0.9	0.7	0.4
Total energy per point (J)	50.4	12.6	10.08	5.6
Total energy (J)	50.4	50.4	50.4	50.4
Laser irradiation (nm)	830			
Power (mW)	30			
Beam area (cm^2)	0.11			
Application points	1	4	5	9
Fluency per point (J/cm^2)	32.72	8.18	6.54	3.63
Total fluency per point (J/cm^2)	458.08	114.52	91.56	50.82
Time (s)	120	30	24	13
Energy per point (J)	3.6	0.9	0.7	0.4
Total energy per point (J)	50.4	12.6	10.08	5.6
Total energy (J)	50.4	50.4	50.4	50.4

Nm, nanometers; *mW*, milliwatts; cm^2, square centimeter; J/cm^2, joule per square centimeter; *s*, seconds; *J*, joules

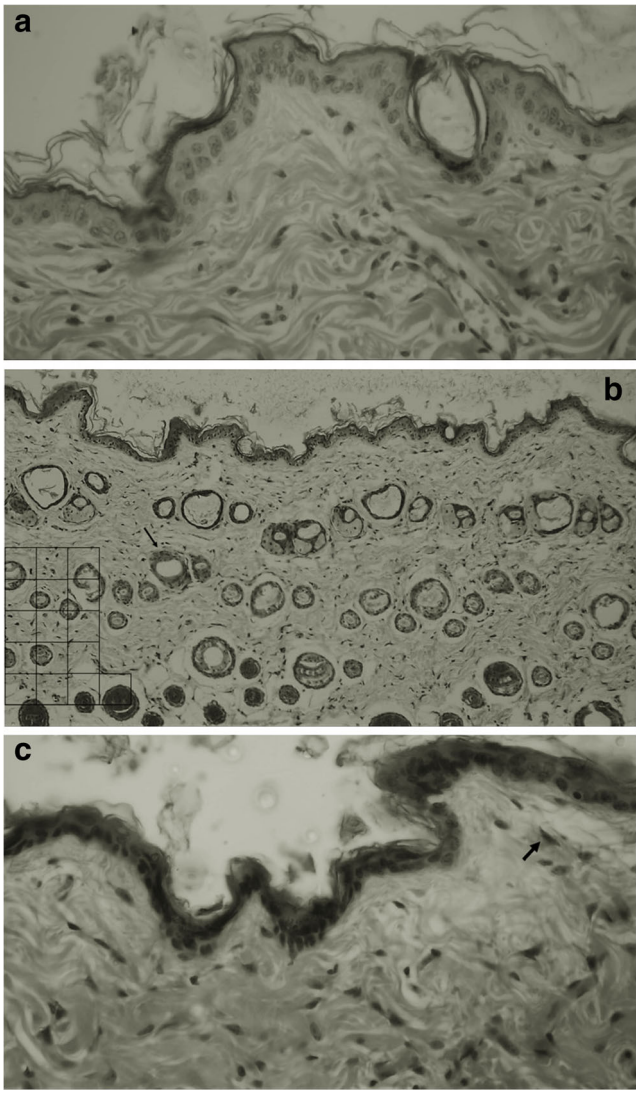

Fig. 3 Illustration of histological evaluation. **a** Analysis of epithelial thickness, objective × 40. **b** Analysis of the number of vessels; the arrow indicates a blood vessel and the grids in the left corner demonstrate the form used for counting. **c** Counting of fibroblasts; the arrow indicates the location of a cell

Statistical analysis

We verified the normality of the data by the Shapiro–Wilk test, with the variables presenting normal distribution. We evaluated the lesion area and skin temperature with one-way ANOVA with repeated measures. Statistical analysis for histology was performed using one-way ANOVA and post hoc Tukey's tests with GraphPad Prism 8.0 software.

Results

To obtain the data related to the present study, we used 125 male Swiss mice (40–45 g), mean age 60 days. During the procedure, some losses occurred due to autophagy (2 animals) and soon after anesthesia (3 animals). All 120 animals were distributed in 10 groups, 9 experimental groups and a naive group. The following results are described according to the analysis performed in this research.

Macroscopic observation of the wound area was performed; photos for evaluation were taken at three different times (immediately postoperative, and 7 and 14 days after the surgery) and area analysis was performed using ImageJ® software. Figure 4 shows the data regarding the wound area (cm^2) at the three evaluation points, for the sham group and the groups irradiated with a laser at wavelengths of 660 and 830 nm. Analyses were performed with one-way ANOVA with repeated measures, p value < 0.05; in the first (immediately postoperative) and second (7 days) evaluations, no statistically significant differences were observed between the groups. In the third evaluation (14 days), the photobiomodulation groups were statistically different ($p < 0.05$) to the sham group, except for the 830-nm photobiomodulation group irradiated at 9 points.

Histological analysis was performed using a sample of cutaneous tissue taken from the dorsal region of the animals. The procedure was performed on the 14th day after euthanasia by using excess anesthetics. Microscopic analysis of skin thickness, permeated blood vessels, and the number of fibroblasts was performed. We used the one-way ANOVA statistical method to analyze the obtained data with $p < 0.05$.

Epidermis thickness in the group irradiated at 5 points at 830 nm was significantly different ($p < 0.05$) to that in the naive, sham, 1-point 660-nm, 5-point 660-nm, and 1-point 830-nm groups; the group irradiated at 4 points at 830 nm was significantly different to the 1-point 660-nm and 830-nm groups; and the group irradiated at 9 points at 830 nm was significantly different to the group irradiated at 1 point at 660 nm. All data are shown in Fig. 5.

Analysis of the number of permeated blood vessels was performed using a grid developed with 1.5 × 1.5 cm squares, containing 100 squares distributed in the lower quadrant of the image, totaling the number of vessels. Figure 6 shows a difference between the 1- and 5-point 830-nm groups and the 4-point 660-nm group vs the naive group.

Figure 7 demonstrates the histological analysis regarding the number of fibroblasts. A significant difference is observed between all the 830-nm groups (1, 4, 5, and 9 points of application) and the naive and sham groups; between the 1-, 4-, and 5-point 660-nm groups and the naive group and between the 1-, 4-, 5-, and 9-point 660-nm groups and the sham group. There is also a significant difference between the 1-point 660-nm group and the 4- and 5-point 660-nm groups.

Skin temperature analysis is shown in Fig. 8. There was no statistical difference ($p > 0.05$) between the groups evaluated.

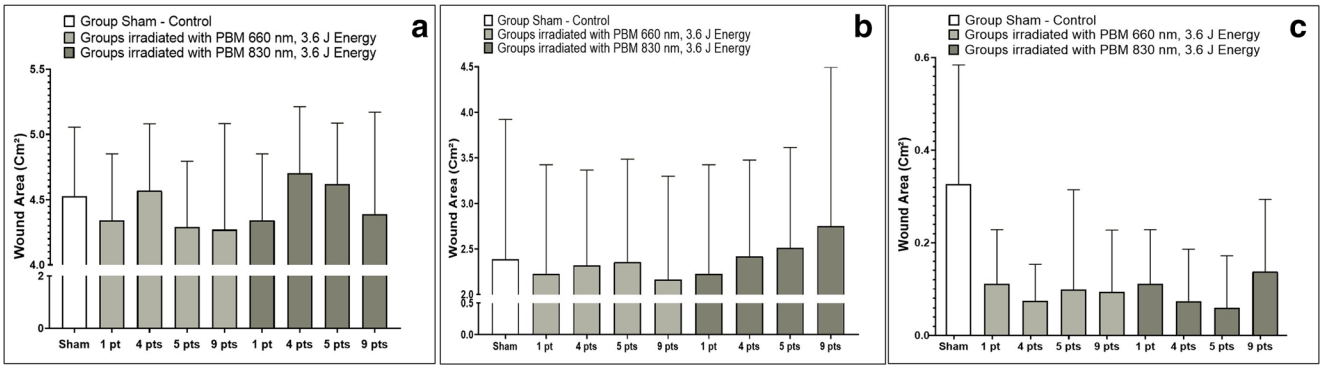

Fig. 4 Evolution of the wound area during the three analyses performed in the study. **a** Immediately postoperative. **b** 7 days after surgical procedure. **c** 14 days after surgical procedure. *$p < 0.05$, 830-nm (1, 4, and 5 points) and 660-nm (1, 4, 5, and 9 points) groups vs the sham group

Discussion

The present study aimed to investigate and compare the effect of laser PBM applied at different numbers of points and different wavelengths on healing of an open skin wound in mice. The animals described as sham are those that received the surgical intervention but were treated with placebo PBM (device off) for the time proposed for the animals that received the intervention (120 s). Naive animals, on the other hand, did not receive surgical intervention or any form of treatment, being necessary as controls for temperature and histological analysis.

Several studies on the healing of integumentary lesions performed with PBM at different wavelengths found positive effects of this treatment. Three of the studies investigated the effects of PBM in the red spectrum (635–670 nm) and found evidence of its effectiveness [27, 28] and effects similar to those of infrared wavelength (830 nm), except for an increase in the number of fibroblasts [29]. Most studies (six) found efficacy of healing for PBM at 810–870 nm, both in isolation and compared with infrared PBM [4, 30–34]. In line with most studies, we found superior PBM effects at 830-nm wavelength but, in some respects, we also observed positive results in the 660-nm groups.

Various authors have described that the dose–response of PBM is influenced by the intensity or time of exposure, and by parameters such as target tissue depth, attenuation, treatment interval, and wavelength. The present study sought to

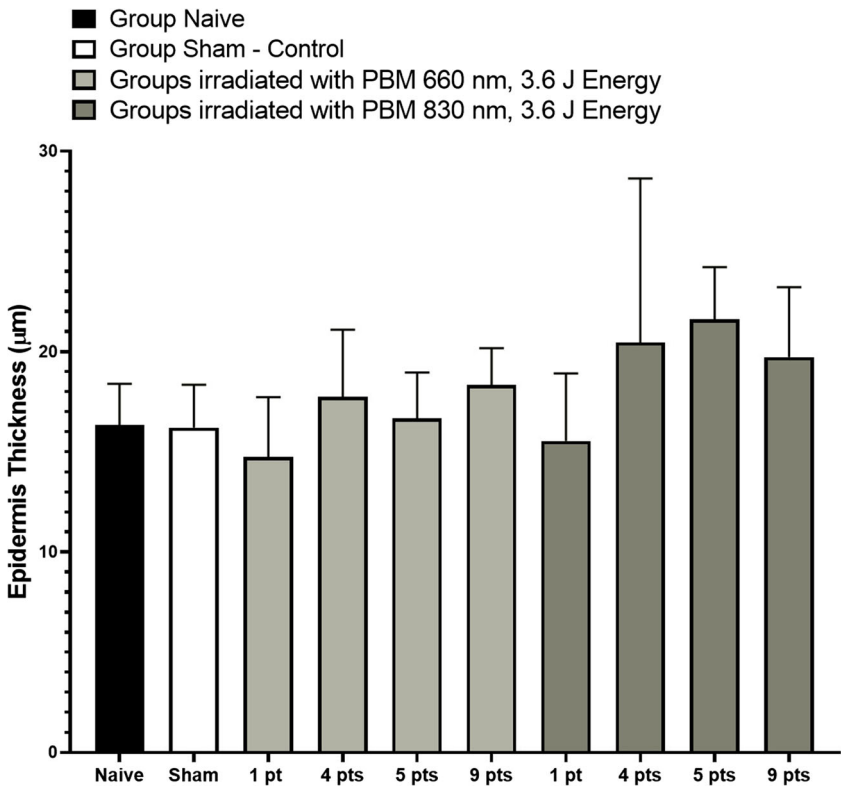

Fig. 5 Histological analysis of skin thickness in all groups. *$p < 0.05$, 4-point 830-nm group vs 1-point 660-nm and 830-nm groups. **$p < 0.05$, the 5-point 830-nm group vs the naive, sham, 1-point and 5-point 660-nm, and 1-point 830-nm groups

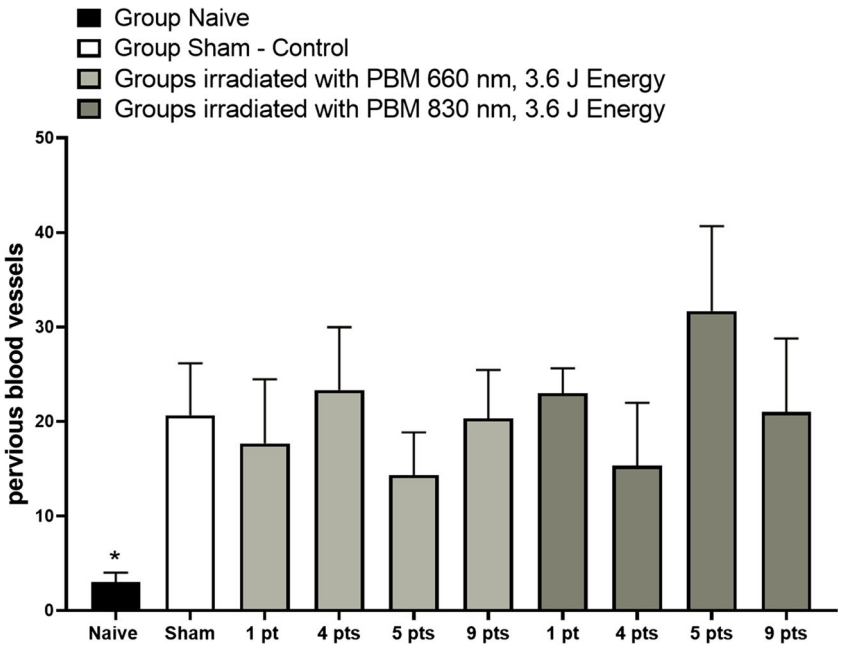

Fig. 6 Histological analysis of the number of pervious blood vessels in all groups. *$p < 0.05$, the 1- and 5-point 830-nm groups and the 4-point 660-nm group vs the naive group

compare the effects on the tissue of two wavelengths and different numbers of points of application [35–37].

Based on this, we note that the results related to application at different numbers of points of the skin wound may have been influenced by the number of points and the division of energy deposited at each point. Several studies have used PBM in skin tissue injuries; most of these used an experimental skin flap model and demonstrated positive results for improving tissue viability. However, there is no consensus when analyzing the dose used that ranged from 3 to 144 J/cm^2 or the number of points of application that varied from 1 to 54 points [38–41].

Based on that and the area of injury that we get with the experimental model, we chose to score the application at 1, 4, 5, and 9 points. Thus, we can verify that the parameters used can influence the result given that, in our study, we found that

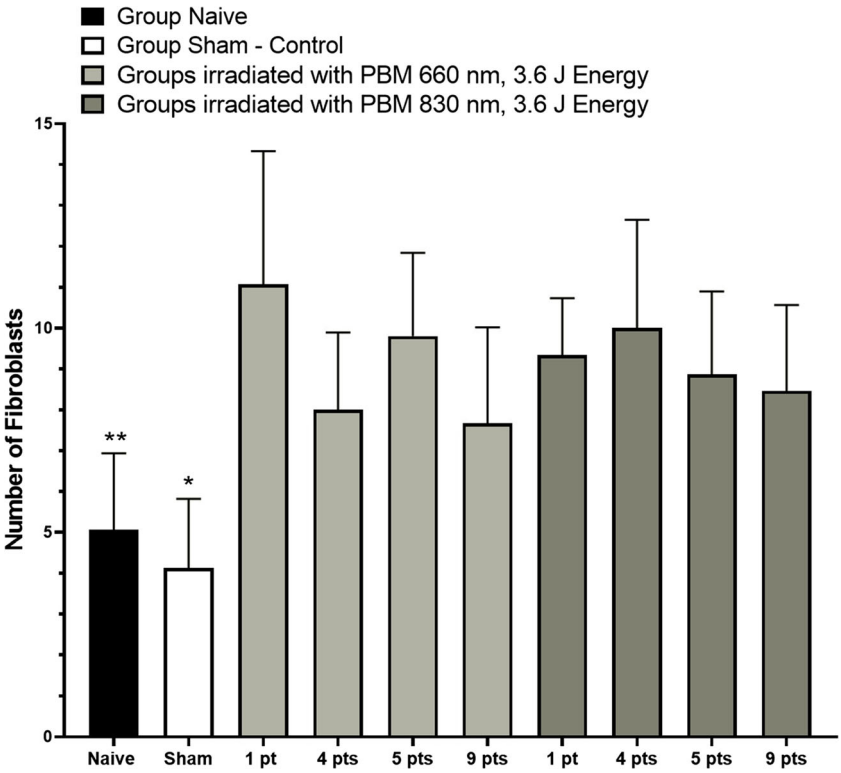

Fig. 7 Histological analysis of the number of fibroblasts in all groups. *$p < 0.05$, sham vs the 830-nm (1, 4, 5, and 9 points) and 660-nm (1, 4, 5, and 9 points) groups. **$p < 0.05$, the naive vs 830-nm (1, 4, 5, and 9 points) and 660-nm (1, 4, and 5 points) groups

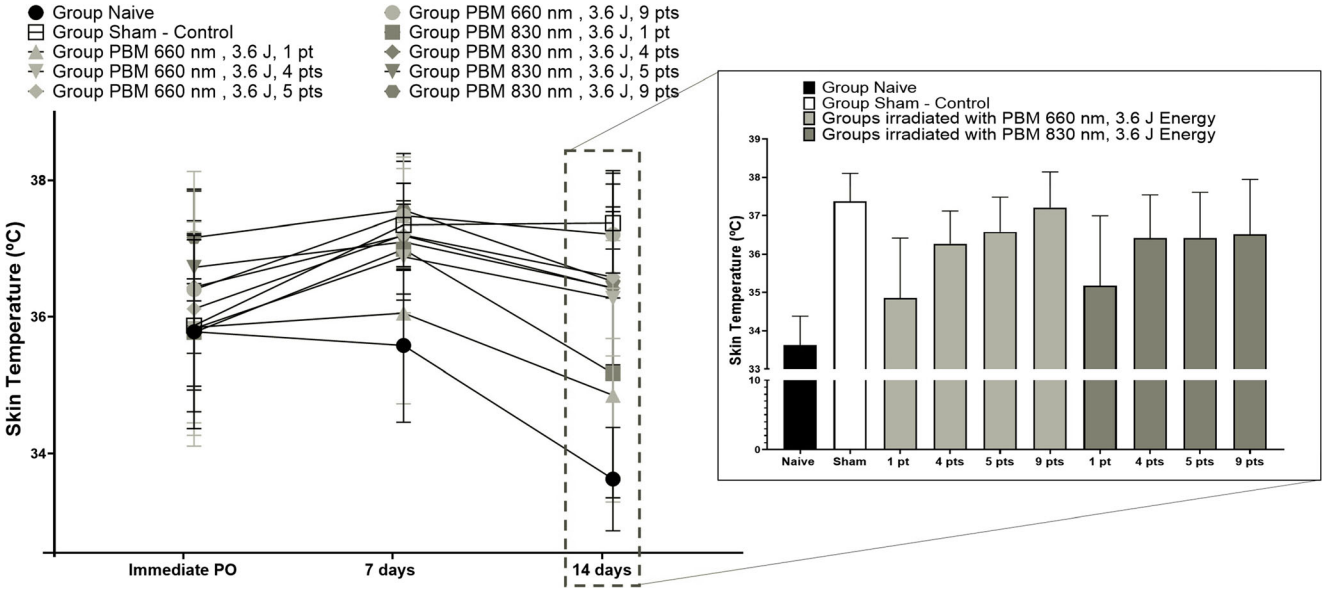

Fig. 8 Analysis of skin temperature using thermal images obtained with an FLIR C2 camera and later analyzed using FLIR Tools™ software

application at 4 and 5 points was more effective than that at 1 or 9 points. Where our study differs from those presented is that the others used several points with the sum of the parameters, at either different times or energy application, for example, and in our research, we made a control so that the final parameters were equal in all groups (total time, total energy, energy per point, fluence per point, and total fluence).

Regarding macroscopic analysis, we observed significant differences only in the groups in which 830-nm PBM was applied at 4 or 5 points, indicating that treatment in these groups was more effective. All groups presented a healing process in its natural course.

PBM therapy on the skin wounds can influence neoangiogenesis, epithelial and fibroblast proliferation, collagen synthesis and deposition, revascularization, and wound contraction, having a beneficial effect in accelerating skin wound healing. Corroborating our findings above, we obtained significant results for epithelium thickness, the 5-point 830-nm PBM group being different to the naive, sham, 1-point 830-nm, and 1-point 660-nm groups, and the 4-point 830-nm group being different to the 1-point 660-nm group [20, 42–46].

Leite et al. [47] investigated the effect of pulsed electric field and laser PBM on the viability of the TRAM flap in diabetic rats and found that PBM causes an increase in epidermis thickness; reduces necrosis area and leukocyte number; increases mast cells, vascular endothelial growth factor, and fibroblast growth factor; and enlarges the neoformed blood vessels. Their studies corroborate our findings, in which we observed an increase in epithelium thickness.

Regarding the number of vessels, there was a difference between the naive control and three of the treatment groups: the 660-nm group with 4 application points and the 830-nm groups with 1 and 5 points; the latter stands out, with a large increase in the number of vessels.

Melo et al. [48] aimed to evaluate the effect of low-power laser therapy at a wavelength of 904 nm on the healing of surgical wounds in rats. They found a reduced inflammatory response, better collagen fiber deposition, and an increase in the mean number of newly formed vessels.

Wagner et al. [49] evaluated the effects of PBM on the cytokine levels and angiogenesis during oral wound healing and concluded that cytokine modulation and increased angiogenesis are among the mechanisms of PBM that improve oral wound repair. Medeiros et al. [50] verified the effects of low-level laser therapy on matrix metalloproteinase (MMP-2) immunoexpression in wound healing and angiogenic processes and found that laser therapy improved wound healing, especially at 14 days, as evidenced by contraction of the wound, anti-inflammatory activity, neocollagenesis, and an increase in the number of vessels formed (neoangiogenesis). In our findings, we also found an increase in the number of blood vessels but in only three of the groups studied compared with the naive group.

Results regarding the number of fibroblasts show statistical differences for almost all treatment groups compared with the naive and sham groups, except when comparing the naive group with the 9-point 660-nm group. Fibroblasts are related to the production of collagen and extracellular matrix, which is an important component in wound healing.

The study by Golçalves et al. [51] aimed to compare the effects of low-level gallium–aluminum–arsenide laser therapy at 830 nm and healing ointment on cutaneous wound healing, in blood vessels and collagen maturation of skin wounds in Wistar rats. They found an increase in the number of blood vessels in the 830-nm PBM-treated group, in addition to a

higher number of mature collagen fibers, but no difference was observed between the groups concerning fibroblasts.

Corroborating the present study, Sampaio et al. [52], Chaves et al. [53], and Solmaz, Ulgen, and Gulsoy [54] described that PBM increases fibroblast proliferation and new blood vessels, reduces inflammatory cells, stimulates angiogenesis and the formation of granulation tissue, and increases collagen synthesis and, consequently, healing of the wound.

Regarding temperature, we did not observe significant differences between the groups. However, it is possible to observe a lower temperature trend in the naive group which can be explained by the absence of the inflammatory process that occurs after an injury, it being influenced only by the variation of normal body temperature, followed by the treated groups and finally the sham group. Among researches that used thermographic evaluation, Christensen et al. [55] emphasized that thermography cannot be used to assess absolute temperature changes due to normal variations in skin temperature over time and is a complimentary assessment. Neves et al. [10] evaluated temperature in the flap and found a temperature increase on the 4th postoperative day in both groups evaluated (control and treatment with high-voltage electrical stimulation).

Dostalova and collaborators [56] used thermography after third molar extraction and found no significant changes in temperature. The study by Carvalho et al. [57] aimed to evaluate the anti-inflammatory potential of gallium arsenide (904 nm) in the healing of skin wounds by measuring the surface temperature of the skin wound and by histopathological examination; they observed an increase in the temperature of the treated group without confirming an anti-inflammatory action of PBM.

As we can see, those studies used thermography for evaluation and follow-up during the inflammatory phase of some lesions/procedures, but not during longer-term follow-up, as performed in this study. This would explain the results found in the study, with no significant difference between groups based on the healing of almost closed wounds, without the absence of inflammatory infiltrate.

Some limitations that should be considered are the non-quantification of myofibroblasts and collagen fibers and the lack of analysis of cytokines and important growth factors in the wound healing process, which could add relevant information to the study.

Conclusion

Based on the sample evaluated in this study, in the comparison of wavelengths, 830 nm was more effective when compared with the naive and sham groups and those irradiated at 660 nm. Macroscopic analysis results demonstrated a positive intervention result at both wavelengths, with reduction of wound area size compared with the control group, except in the 9-point 830-nm PBM group. When observing the epidermis thickness, there was a general statistical difference in the 830-nm PBM groups compared with the 1- or 5-point 660-nm PBM groups. Analysis of the number of permeated blood vessels showed a significant difference of the groups irradiated by 830-nm PBM (1 and 5 points) and PBM 660 nm (4 points) in relation to the naive group. Regarding quantification of fibroblasts, an increase was observed in the groups treated with 830-nm PBM in relation to the control groups (sham and naive) and between groups irradiated with 660-nm PBM. Temperature analysis results showed no significant difference between groups. Comparing the number of points, we highlight application at 4 and 5 points in the open injury, with emphasis on the group with 5 points of PBM application which showed an improvement in macroscopic analysis and epithelial thickness, an increase in the number of vessels, and fewer fibroblasts on the 14th day.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethics Committee (CEUA) under number 4017201117.

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