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ORIGINAL ARTICLE

Comparison of two different laser photobiomodulation protocols on the viability of random skin flap in rats

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Abstract

To identify the best low level laser photobiomodulation application site at the same irradiation time to increase the viability of the skin flap in rats. Eighteen male rats (*Rattus norvegicus*: var. Albinus, Rodentia Mammalia) were randomly distributed into three groups ($n = 6$). Group I (GI) was submitted to simulated laser photobiomodulation; group II (GII) was submitted to laser photobiomodulation at three points in the flap cranial base, and group III (GIII) was submitted to laser photobiomodulation at 12 points distributed along the flap. All groups were irradiated with an Indium, Galium, Aluminum, and Phosphorus diode laser (InGaAlP), 660 nm, with 50 mW power, irradiated for a total time of 240 s in continuous emission mode. The treatment started immediately after performing the cranial base random skin flap (10×4 cm^2 dimension) and reapplied every 24 h, with a total of five applications. The animals were euthanized after the evaluation of the percentage of necrosis area, and the material was collected for histological analysis on the seventh postoperative day. GII animals presented a statistically significant decrease for the necrosis area when compared to the other groups, and a statistically significant increase in the quantification of collagen when compared to the control. We did not observe a statistical difference between the TGFβ and FGF expression in the different groups evaluated. The application of laser photobiomodulation at three points of the flap cranial base was more effective than at 12 points regarding the reduction of necrosis area.

Keywords Skin flap · Tissue viability · Laser photobiomodulation · Collagen · Transforming growth factor beta and fibroblast growth factor

Introduction

The skin flap is a technique widely used in plastic surgery to reconstruct skin and soft tissue defects [1]. However, it presents some complications that include tissue ischemia [2], which can vary between 2 and 20% of cases [3] depending on the area in which the flap is performed. This fact is of great concern because it may require secondary surgical interventions, lead to infections, and delay treatment. Considering the complexity of this complication and the changes that it can cause in the patients' quality of life, treatments that favor tissue vascularization have been increasingly researched. In this perspective, studies focused on low-level laser therapy or photobiomodulation (PBM) [4–7], which acts in several phases of the wound healing process including cell differentiation and proliferation, neovascularization, stimulation of collagen synthesis, and reepithelialization [8].

The increase in the number of blood vessels and release of nitric oxide are believed to be responsible for reducing the amount of necrotic tissue [9–11]. In addition, it is also known that tissues with higher concentration of collagen have greater tensile strength; characteristics are important for the quality of the tissue [12].

Transforming growth factor beta (TGFβ) and fibroblast growth factor (FGF) are families of proteins closely linked to both vascular neoformation and modulation of collagen synthesis [13]. TGFβ participates as an extracellular matrix

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modulator that helps in the synthesis of collagen and regulates the expression of several genes that encode the metalloproteinases [14]. Studies have demonstrated that both abovementioned proteins can undergo modulation effects when stimulated with PBM, thus, interfering with the skin remodeling process [15–17].

In the literature review performed by Hersant. et al. [18] on the use of PBM in plastic surgery, the authors report that the variety of parameters and their influence on cellular responses impairs the understanding of PBM effects in plastic surgery [18]. Therefore, studies have been carried out with the aim of finding the best parameters for increasing the viability of the skin flap. In this perspective, some studies compare the best wavelength [6, 19], best power [9, 20], better fluence [5, 10], and the best site of application [21–23].

Research approaching the best places for PBM application [23, 24] standardized the fluence in the different points. Our research shows a fixed value of the irradiation time delivered to the tissues in the different protocols. Therefore, the present research aimed to identify if there is a difference between the number of points in which the PBM is applied in the tissue at the same time that the increase of the skin flap viability is delivered.

Materials and method

Experimental design

The present research involved 18 male Wistar rats (*Rattus norvegicus*: var. Albinus, Rodentia Mammalia), approximately 3 months in age and body mass of 296.39 ± 26.86 g. The animals were randomly distributed by sortition in a sealed envelope in three groups with six animals per group:

- GI: control group, submitted to simulated PBM with the equipment off;
- GII: the animals received the PBM at three points (Fig. 1a);
- GIII: the animals received the PBM at 12 points (Fig. 1b).

The present research was initially approved by the Ethics Committee on the use of animals under number 6224290515 and conducted according to the international norms of ethics in animal experimentation (National Research Council, 1996).

Surgical procedure

The animals were anesthetized proportionally to their body mass, intraperitoneally, with the combination of cetamine (95 mg/kg) and xylazine (12 mg/kg) (Syntec®). After anesthetic induction, the animals were placed on a flat surface where the extension of the four limbs and their backs were digitally tricotomized. The cranial base random skin flap being 10 cm long and 4 cm wide [25] was then marked on the animals' back. The flaps were then raised from the dorsum of the animals from the deep fascia of the muscles, including the superficial fascia, fleshy panniculus and skin. A plastic barrier with the same dimensions of the flap was interposed between the flap and the donor site. The flap was then returned to its original position and sutured with simple nylon 4/0 stitches.

Protocol photobiomodulation

The animals were irradiated with a laser of Indium, Gallium, Aluminum, and Phosphorus (InGaAlP), 660 nm (Photon Laser III, DMC®, São Carlos, SP, Brazil) with a beam area of 0.028 cm^2 and 50 mW power. The GII animals were irradiated at a fluence of 150 J/cm^2 for 80 s at each point; 4 J of energy were delivered per point (three points), while the GIII animals were irradiated with a fluence of 40 J/cm^2 for 20 s at each point (twelve points), and an energy per point of 1 J was delivered, a time total irradiation of 240 s was delivered in both irradiated groups. The treatment started immediately after the operative procedure and reapplied every 24 h until the fourth postoperative day, thus totaling five applications (Table 1).

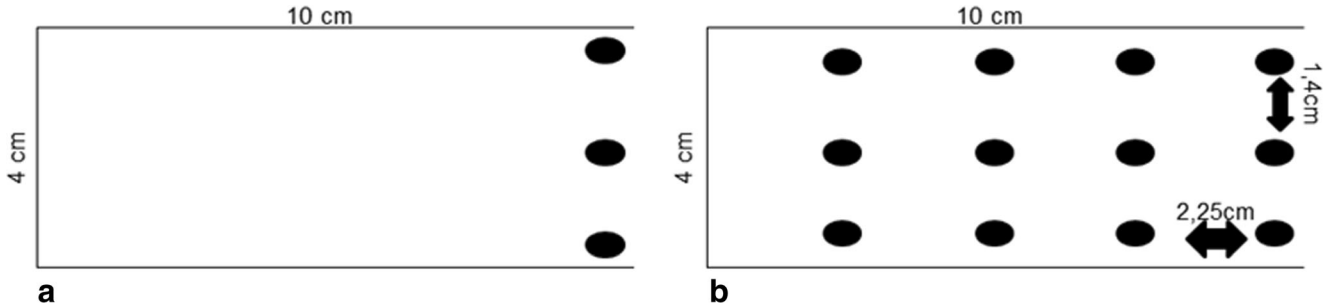

Fig. 1 GII animals received the PBM at three points (**a**). GIII animals received the PBM at 12 points (**b**)

Table 1 Treatment parameters

Parameter [uni]	GII	GII
Beam spot size at target [cm^2]	0.028	0.028
Irradiance at target [W/cm^2]	1.78	1.78
Exposure duration [sec]	264	264
Radiant exposure [J/cm^2]	150	40
Radiant energy [J]	4	1
Number of point irradiated	3	12
Area irradiated [cm^2]	0.084	3.36
Application technique	Skin contact	Skin contact
Number and frequency of treatment sessions	Five consecutive days	Five consecutive days
Total radiant energy [J}	12	12

Analysis

Method of determining the percentage of the flaps necrosis area

The determination of the percentage of necrosis area was performed on the seventh postoperative day, with the paper template method [26]. Templates of all flaps were drawn on transparent paper, within the limits of the necrotic area (dark, cold, and hairless) of the viable area (soft, pink, warm, and haired) of the flap. The templates were then trimmed and weighed on a precision balance (error of ± 0.0001 g). The necrosis area was separated from the viable area of the drawing and was also measured. The percentage of necrosis area was calculated with the following formula:

$$\%\text{of the necrosis area} = \frac{\text{Weight of paper template of flap necrosis}}{\text{weight of paper template of total area od flap}} \times 100$$

Euthanasia of animals

The animals were euthanized by anesthetic overdose with cetamine (285 mg/kg) and xilasin (36 mg/kg) on the seventh postoperative day after tissue collection.

Collection of skin sample and sample preparation for histological analysis

Skin samples were collected just above the highest point of separation between the viable and necrotic tissue, perpendicularly to the 1-cm wide lesion [6].

After the collection, the pieces remained in 10% formaldehyde solution for 24 h for fixation. They were then washed with water for 24 h. Afterwards, they were dehydrated in a solution of 70%, 90%, and 100% ethyl alcohol, and immersed for 1 h in both the first and second solution; the latest step was repeated six times for 1 h each. After the dehydration baths, the samples were diaphanized in alcohol/xylol solution (1:1) for 1 h followed by two baths of pure xylol, also for 1 h each bath. Finally, the pieces were embedded in paraffin and cut to a thickness of 5 μm on a Spencer-820 rotary microtome.

Descriptive histologic and morphometric analysis

The slides were stained with hematoxylin and eosin (H&E), (Merck, Darmstadt, Germany) and microscopically analyzed by a pathologist in an optical microscope (OLYMPUS BX53) with × 20 magnification, assessing the integrity of the skin layers, the formation of granulation tissue, and the presence of inflammatory infiltrates.

Quantification of collagen fibers

The slides stained with Picrosirus Red were analyzed for the purpose of quantifying collagen types I and III without differentiating them. The images were captured in a polarized optical microscope (OLYMPUS BX53) with × 400 magnification. These images were analyzed using the ImageJ software as proposed by Manni et al. [27] . For each slide, the reading was performed in five distinct fields and the quantification mean was used for statistical analysis.

Immunohistochemistry analysis

After deparafinization and rehydration in ethanol, the slices were treated in a Steamer (850 W potency) with 0.01 M citric acid buffer (pH 6) for 5 min for antigenic recovery.

The material was then pre-incubated in phosphate buffered saline (PBS) with 30% hydrogen peroxide for 5 min for inactivation of the endogenous peroxidase, shortly followed by the incubation of the samples with primary TGFβ antibodies (sc398, Santa Cruz Biotechnology, California, USA at 1: 1000 titer) and FGF (sc1884, Santa Cruz Biotechnology, California, USA at 1: 500 titration) for 2 h, then the slides were washed with PBS and the secondary antibody (ABC kit, PK-6200, Vector Laboratories, Burlingame, CA, USA) at a 1: 5 dilution for 30 min. Colorimetric detection with a diaminobenzidine substrate (DAB, SK-4100, Vector Laboratories, Burlingame, CA, USA) was carried out for 3 min and finally, the material was stained with hematoxylin and the blades were assembled. Digital images were captured at a magnification of × 100.

The results were evaluated both qualitatively (presence of immunomarkers) and semi-quantitatively (percentage of labeled cells). The slides were scored on a scale of 1 to 4 (1 = absent, 2 = weak, 3 = moderate, and 4 = severe) for

immunohistochemical analysis [28]. The blinded evaluation was performed by two independent experienced observers.

Statistical analysis

Data were presented through descriptive techniques, in graphs, in the form of mean and standard deviation, and median and interquartile range. Data normality was tested with the Shapiro Wilk's test and homogeneity with the Levene's test. For the comparison between groups, the one-way ANOVA variance analysis was performed in cases where the data were normal and homogeneous (necrosis area and quantification of collagen fiber); in the presence of a significant difference ($p < 0.05$), the Tukey post-hoc test was applied. The Kruskal-Wallis' test was used for non-normal or non-homogeneous data, (TGF-β and FGF expression); in the presence of a significant difference ($p < 0.05$), Mann-Whitney post-hoc test was applied.

Sample size was calculated considering a difference of 26.2% in the necrosis area (primary outcome) between the groups and an estimated standard deviation of ± 8.32% [20]. For a level of significance of 0.05 and power of 80%, the estimated sample size was four animals in each group (Minitab, v.17, State College, PA).

Results

Necrosis area

Figure 2a demonstrates the analysis of the necrosis area. We observed a statistically significant difference between GII (38.91% ± 10.75) ($p = 0.004$) and GI (63.84% ± 9.33) and with GIII (57.82% ± 13, 21) ($p = 0.027$); the GII animals presented better results.

Morphological and morphometric description

Qualitative evaluation of the tegument layers revealed that they were well organized with preserved morphology in all groups. We also observed formation of granulation tissue and inflammatory infiltrate predominantly from the hypodermis towards the fleshy paniculus in most of the lamina analyzed, independent of the group.

Quantification of collagen fibers

Figure 2b shows the comparison between groups of values obtained for collagen quantification. A statistical difference was found between the groups evaluated (0.049), and that difference was between GI (33.98% ± 3.93) and GII (44.39% ± 6.81) (0.043). The GII animals presented better results. No other statistical differences were found.

Expression of TGF-β

The immunohistochemical evaluation showed that TGF-β expression was observed mainly in the fibroblast nucleus for all groups (Fig. 3a). There was no difference between the experimental groups in the semi-quantitative analyses (Fig. 3c).

FGF expression

FGF expression was detected in the fibroblast nucleus in all groups (Fig. 3d). No difference was observed between the experimental groups in the semi-quantitative analyses (Fig. 3b).

Discussion

Some studies have already been carried out with the aim of finding the best site of irradiation with PBM for the increase of cutaneous viability [21–24]. Some of these studies standardized the fluence, while others differentiated the numbers of the points and still others the place of the application, making the time of irradiation of the tissues different. In our research, we standardized the time of irradiation of the tissue and differentiated the place of application of the PBM laser.

In the study by Pinfildi et al. [23], the authors proposed three different irradiation sites, two with the same number of points but different application sites and another with a greater number of points, being the same fluency in points. It was observed that the group that received the highest number of application points had a better result. In this context, it is important to consider that the effect of PBM is dose dependent [5, 29, 30], Thus, the result found by Pinfildi et al. [23] may have been due to either the irradiation site or by the total energy difference delivered to the tissues.

Although much has been studied about laser PBM, there is still no consensus among researchers on what would be the best metric to describe the dose of treatment used [30], so some authors [31, 32] suggest that experimental and clinical studies present in detail the most relevant parameters used, allowing their reproducibility. In the beginning, it was common to describe the dose taking into account the fluency used for irradiation, but considering that this parameter is inversely proportional to the beam diameter [31, 32], any alteration in this parameter makes the reproducibility of the protocol difficult. Another option is the standardization of irradiation exposure time values [29], which is directly proportional to energy, a parameter also widely used that influence the treatment results [32].

The results found in our research corroborate with that found by Prado et al. [24], and the authors compared the effect of the PBM applied to the laser at different numbers of points and the same fluency was standardized at all points, causing

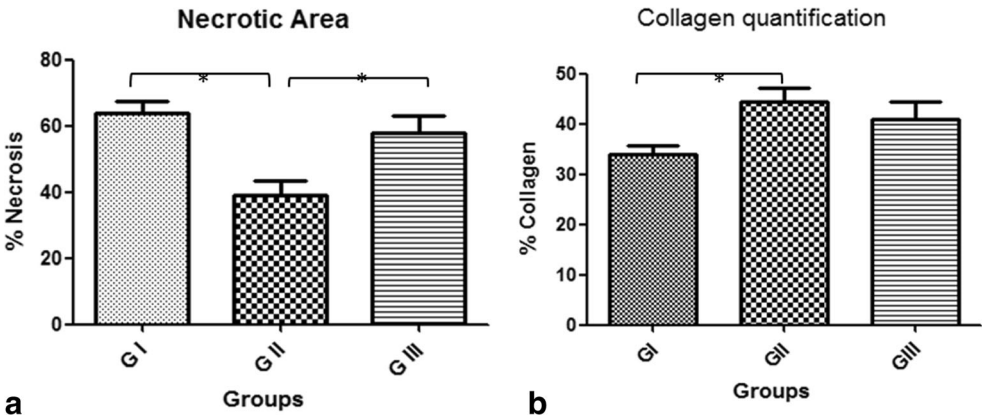

Fig. 2 **a** The analysis of the necrosis area. **b** The comparison between groups of values obtained for collagen quantification

the tissue to be exposed to different times of irradiation. The group that was irradiated for a shorter time and closer to the cranial base showed the best result, demonstrating that the place where this energy is deposited in the tissue is important, and the closer area that remains communicated with the better tissue.

Our results differ from those found by Kubota [22], in which the author did not obtain a statistical difference between irradiated animals at different points, using the same irradiation time. This disagreement in the results may have been due to the irradiation time division into dose points, thus, inferring that the manner in which the irradiation time is distributed in the tissue is related to the effectiveness of the treatment.

With regard to the results of the quantification of collagen fibers of the present study, it was observed that the groups' three points were more efficient in the increase of collagen synthesis when compared to the control group. Several studies have been carried out seeking to elucidate the acting of PBM laser in the synthesis of collagen [33–35], and has demonstrated its effect for increased collagen synthesis.

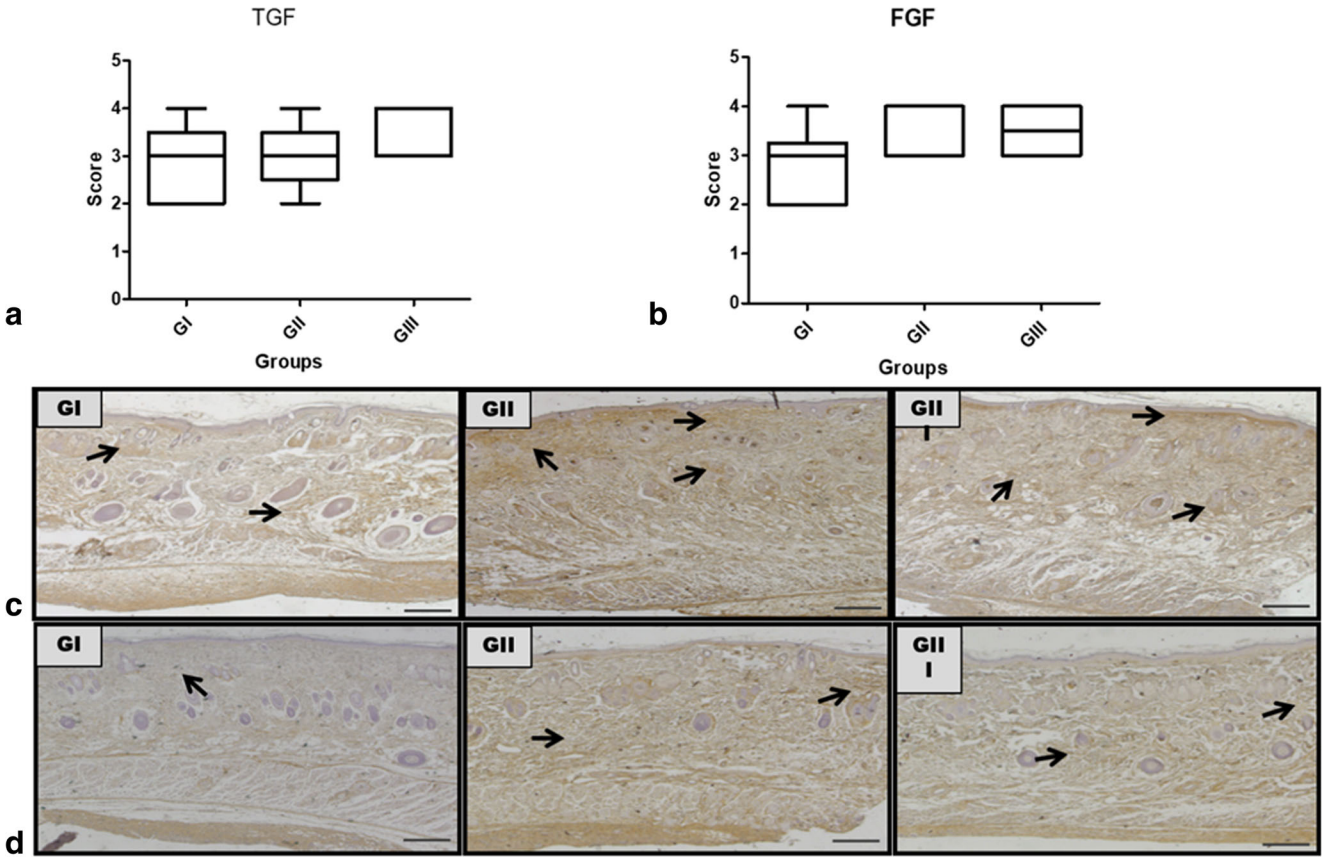

Fig. 3 **a** TGF-β expression observed mainly in the fibroblast nucleus for all groups. **b** No difference between the experimental groups in the semi-quantitative analyses. **c** No difference between the experimental groups in the semi-quantitative analyses. **d** FGF expression detected in the fibroblast nucleus in all groups

It is known that FGF is released by inflammatory cells during tissue repair and plays an important role in the stimulation and proliferation of fibroblasts to form new collagen and other extra cellular matrix components, thus favoring wound contraction. The present study did not reveal significant differences between the experimental groups, being in agreement with the results of the research by Neves et al. [16], where the comparison of 660- and 830-nm lasers effected the increased viability of the transverse abdomens muscle flap in induced and non-nicotine induced animals, and showed no significant difference in the FGF labeling between normal animals.

The limitations of the immunomaters studied in the present research may have been the number of animals. The sample calculation was performed based on our primary outcome (viability of skin flap), and we believed that studies with a larger number of animals would facilitate the understanding of the histological and immunohistochemistry results.

Conclusion

The present work concludes that the irradiation in three points of the cranial flap base was more effective than in 12 points for reducing the area of necrosis, as well as in the increase of the collagen synthesis when compared to the control group.

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