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ORIGINAL ARTICLE

Low-level laser therapy (670 nm) on viability of random skin flap in rats

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Abstract This study investigated the effects of 670 nm laser, at different fluences, on the viability of skin flap in rats. One hundred male animals were used. The animals were divided into control group; group treated with 3 J/cm^2; group treated with 6 J/cm^2; group treated with 12 J/cm^2 and group treated with 24 J/cm^2. The skin flap was made on the backs of all animals studied, with a plastic sheet interposed between the flap and the donor site. Laser irradiation was done immediately after the surgery and on days 1, 2, 3 and 4 after surgery. The percentage of necrosis of the flap was calculated at the 7th postoperative day. Additionally, a sample of each flap was collected to enable us to count the blood vessels. Treated animals showed a statistically significant smaller area of necrosis than did the control group. The necrosis in the treated groups was 41.82% (group 2), 36.51% (group 3), 29.45% (group 4) and 20.37% (group 5). We also demonstrated that laser irradiation at 670 nm, at all doses used, had a stimulatory effect on angiogenesis. Our study showed that the 670 nm laser was efficient to increase the viability of the skin flap, at all fluences used, with a tendency of reaching better results at higher doses.

Keywords Laser therapy · Microcirculation · Necrosis · Surgical flaps

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Introduction

Skin flap is a common surgical procedure used in plastic and reconstruction surgeries. However, the failure of the flap, mainly due to inadequate vascularization, is a frequent problem found in this type of intervention [1]. Ischemia is responsible for tissue necrosis, causing an undesirable failure of the proposed treatment. In contrast, if blood flow is sufficient in the distal portion of the flap, then flap necrosis becomes much less of a problem [2].

Some authors have studied the effects of low-level laser therapy (LLLT) on the viability of skin flaps [3–5]. The action of LLLT is based on the absorption of the light by tissues, which will generate modifications in cell metabolism [6, 7]. Studies have shown that laser irradiation increased mitochondrial respiration and ATP synthesis in isolated cells in culture [8]. Other studies have shown that laser light affects calcium exchange through the cell membrane, causing transient changes in the cytoplasmic calcium levels [6, 7]. These modifications can increase the synthesis of DNA, RNA and cell-cycle regulatory proteins, stimulating cell proliferation, which could, therefore, be beneficial for the re-establishment of connective tissue

during tissue repair and wound healing, contributing to the increase of skin flap viability [7].

Pinfildi et al. [3] and Prado et al. [4] demonstrated that laser irradiation was able to decrease the area of necrosis of the skin flap. In addition, other authors suggest that the positive effects of laser irradiation on skin flap viability is due to positive effects on stimulating the formation of new blood vessels, improving blood flow in the skin flap area [8–10].

The use of LLLT on tissue regeneration has increased in recent years. However, knowledge about the mechanisms by which laser therapy acts and the lack of protocols of treatment are still limited and need further investigation [11, 12].

In order to progress our understanding of the clinical parameters involved in the field of laser therapy and to determine the responses of tissue regeneration to different fluences of LLLT, the aim of this study was to investigate the dose–response effects of 670 nm laser irradiation on the viability of skin flaps in rats.

Methods

This study was conducted in accordance with the Guide for Care and Use of Laboratory Animals and was approved by the Animal Ethics Committee of the Federal University of Sao Carlos. At the beginning of the experiment, 100 adult male rats (12 weeks, 260–320 g) were randomly divided into five groups, with 20 animals each: group 1 (control group); group 2 (treated with 3 J/cm^2); group 3 (treated with 6 J/cm^2); group 4 (treated with 12 J/cm^2) and group 5 (treated with 24 J/cm^2).

All animals were anesthetized with ketamine (95 mg/kg) and xylazine (12 mg/kg) intraperitoneally and they were also depilated. A random skin flap measuring 10 cm×4 cm was made with a cranial base on the back of each rat [3]. A plastic barrier with the same dimensions was placed between the flap and its donor site. Flaps were closed with simple nylon 4–0 stitches (Fig. 1).

Fig. 1 Random skin flap (10 cm×4 cm)

Laser irradiation was performed immediately after the surgery and on days 1, 2, 3 and 4 after surgery. A low-energy AlGaInP laser, 670 nm (Ibramed Equipamentos Médicos Ltda), continuous wave (CW), 0.6 mm beam diameter, 30 W cm^{-2} was used. Laser irradiation was at fluences of 3 J/cm^2 for 6 s (total energy 4.32 J), 6 J/cm^2 for 12 s (total energy 8.64 J), 12 J/cm^2 for 24 s (total energy 17.28 J) and 24 J/cm^2 for 48 s (total energy 34.56 J). Twenty-four points, on the skin flap surface and surrounding it, were irradiated by the punctate contact technique (Fig. 2). The irradiation was performed with a plastic template on the skin flap with demarcation points for each group [3].

Analysis

Skin flap necrosis

The percentage of skin flap necrosis was calculated on the 7th post-operative day through the paper-template method. The rats were sedated, and the limit between viable tissue characterized by soft skin, reddish, warm and haired, and necrotic tissue (stiff, dark, cool, and hairless skin) was demarcated on the animals [3]. A mold of the entire flap and the necrotic area was drawn on transparent paper and cut out, being checked with a precision balance (0.001 g error). After that, the following equation was used:

$$\text{Percentage of necrosis area of the flap:} = \frac{\text{Weight of paper template of flap necrosis}}{\text{Weight of paper template of total area of flap}} \text{ X } 100$$

After this procedure, a sample of the skin flap was taken for histological analysis. Then, the rats were humanely killed by an overdose of general anesthetic so that we could verify the necrotic areas.

Histological analysis

The specimens were retrieved en bloc. A sample of 2 cm^2 in the region of the necrosis line transition for each skin

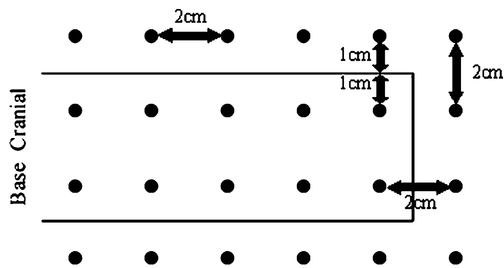

Fig. 2 Scheme of laser irradiation on 24 points, using the punctate technique

flap was taken and processed (Fig. 3). The flap samples were fixed in 10% buffered formalin (Merck, Darmstadt, Germany), embedded in paraffin blocks and cut into transverse sections (5 µm). Five laminae of each part of the sample were stained with hematoxylin and eosin (HE, Merck) and analyzed. Histologic evaluation was performed under a light microscope (Zeiss Axioshopt, Carl Zeiss, Rio de Janeiro, Brazil), with a ×40 objective. The number of blood vessels were counted for each lamina by two experienced pathologists, who were blind to the treatment. A mean of the number of blood vessels for the laminae of each skin flap was considered for statistical analysis.

Statistical analysis

The results are given as means and standard deviations. We used analysis of variance (ANOVA) to compare changes among the groups and the Tukey test to identify the differences. Correlation between the areas of necrosis and the number of blood vessels was assessed with the Pearson's correlation coefficients. A P level ≤0.05 was considered as being statically significant.

Results

Figure 4 shows the means and the standard deviations (SD) of the necrotic areas found in each group. Non-irradiated animals (control group) showed statistically significant higher values of necrotic areas (49.92%) than did the other groups. Moreover, LLLT produced a statistically significant increase in skin flap viability, mainly at higher fluences (24 J/cm^2). The animals of group 5 presented a lower mean percentage of necrotic area (20.37%) than the other groups (group 2, 41.84%; group 3, 36.51% and group 4, 29.45%). Moreover, animals irradiated with 3 J/cm^2 demonstrated no difference when compared to animals irradiated with 6 J/cm^2 and showed lower values than animals irradiated with 12 J/cm^2 and 24 J/cm^2. Mean percentage of necrosis shown in animals irradiated with 6 J/cm^2 was statistically significant different when compared with that in animals irradiated with 12 J/cm^2 (P<0.05) and was highly statistically significant different when compared with that in animals irradiated with 24 J/cm^2, P<0.01). Animals irradiated with 12 J/cm^2 and 24 J/cm^2 were highly statistically significant different (P<0.01).

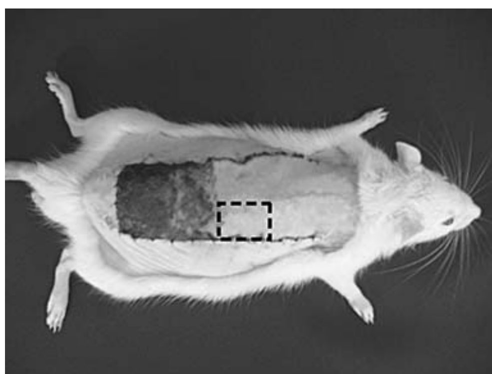

Fig. 3 Blood vessel

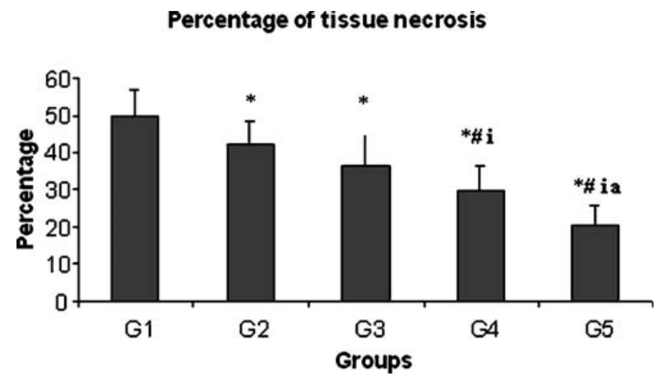

Fig. 4 Percentage of tissue necrosis. *G1* control group; *G2* treated with 3 J/cm^2); *G3* treated with 6 J/cm^2; *G4* treated with 12 J/cm^2 and *G5* treated with 24 J/cm^2. * Statistically significant when compared to control

We also demonstrated that laser irradiation at 670 nm, at all fluences used, had a stimulatory effect on the increase of the number of blood vessels at the necrosis transition line (Figs. 5 and 6). The irradiated animals showed a statistically significant higher number of blood vessels than did the non-irradiated animals, mainly at the dose of 24 J/cm^2.

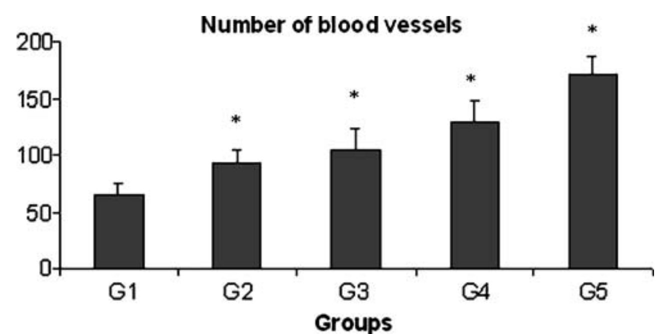

Fig. 5 Number of blood vessels. *G1* control group; *G2* treated with 3 J/cm^2); *G3* treated with 6 J/cm^2; *G4* treated with 12 J/cm^2 and *G5* treated with 24 J/cm^2. * Statistically significant when compared to control

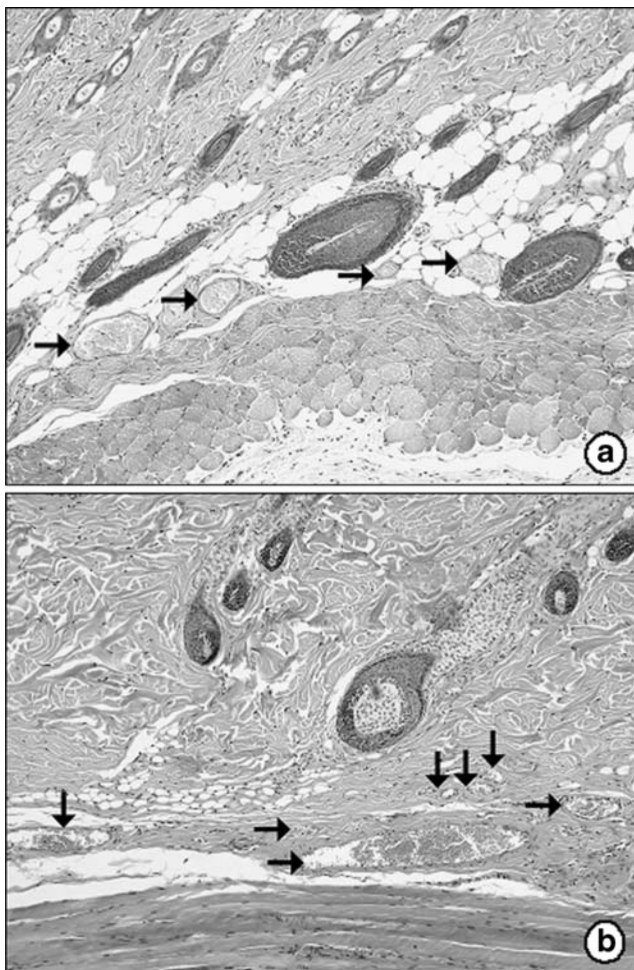

Fig. 6 Number of blood vessels. **a** control group; **b** treated with 24 J/cm^2

Figure 6 demonstrates the analysis of blood vessels in the control group compared to group 5.

Pearson's correlation coefficient showed a significant negative correlation between the number of blood vessels and the area of necrosis (P=−0.972; P=0.0001), suggesting that the higher number of blood vessels was one of the factors that could have contributed to the decrease of the necrotic areas.

Discussion

We investigated the effects of 670 nm laser irradiation at different laser fluences. Our observations indicated that laser irradiation produced an increase in skin flap viability, at all fluences used, especially at 24 J/cm^2.

Recent studies have shown that laser stimulated mitochondrial activity, which led to a stimulation of cell proliferation, promotion of fibrin absorption from injured tissue and enhancement of the conversion of myofibroblasts from fibroblasts, which could explain the enlarged surviving area of the skin flap after laser irradiation [13].

Our results corroborate those of Pinfildi et al. [3] and Amir et al. [14], who also found decreases in tissue necrosis of the skin flap after 632.8 nm laser irradiation at fluences of 3 J/cm^2 and 2.9 J/cm^2, respectively.

Furthermore, our study demonstrated a stimulatory effect of different 670 nm laser doses on blood vessel growth. Adequate blood perfusion is essential to guarantee skin flap survival and consequently the success of the repair procedure [15, 16]. Moreover, Chang et al. [17] demonstrated that both arterial and venous augmentation were effective for increasing flap survival. Vascular photomodulation can be associated with the reduction of inflammatory cells and with the stimulation of macrophages, T-lymphocytes, endothelial cells, fibroblast migration during the healing process, decreasing flap necrosis [18].

Enwemeka et al. [19] stated that the development of new blood vessels is an essential part of the healing process and that the re-establishment of the circulation at the injury site limits ischemic necrosis and permits repair. Salate et al. [20] observed that the 660 nm laser was able to stimulate the formation of new blood vessels in injured tendons of rats compared to control animals. Kubota [1] found that 830 nm laser irradiation produced higher vascular perfusion and larger flap survival areas than control flaps that were not irradiated.

Moreover, our findings further support the existence of a dose–response curve, demonstrating that higher fluences were more likely to produce a response than lower fluences were. The same results were found in other studies investigating the effects of laser on hard and soft tissues [13, 16, 21, 22]. Probably, the fluence of 24 J/cm^2 was more efficient in accelerating the inflammatory response and in stimulating the migration of cells related to tissue regeneration, as macrophages and fibroblasts. Moreover, as discussed above, the higher dose used in this study was more efficient at stimulating angiogenesis in the skin flap, which probably contributed to the increased flap viability.

Similarly, Prado et al. [4] and Kubota and Oshiro [23] found an increase in vascular perfusion and a decrease in tissue necrosis in skin flaps at high fluences (36 J/cm^2). However, the best fluence to be used in skin flap regeneration is still controversial and need further investigation.

The methodology employed in this study is aligned with previous reports found in the literature [3, 4, 14]. The plastic film used between the flap and the donor site prevents revascularization of the flap through donor site vessels, assuring homogeneous ischemia [14]. In addition, the paper-template method is a simple and quick method to measure necrotic areas and only requires transparent paper and an accurate scale [3, 4]. Moreover, the punctate contact

technique of irradiation used (on the skin flap surface and around it) was effective in stimulating skin flap viability.

We should point out some limitations of our work. Although we found an increase in the number of blood vessels at the region of the necrosis transition line in the irradiated animals, we did not measure the blood flow or the proportion of the different types of vessels (arteries or capillaries). Moreover, as the region of the transition line on the backs of the animals varied among groups, the region of the histological analysis varied as well. The inclusion of a group that had not been operated on would allow us to have a standard of the behavior of the number of blood vessels on the backs of the animal. Moreover, the analysis of blood flux in the skin flap would give more specific information about the distribution of the blood flow.

Although the effects of LLLT have been demonstrated in many studies, the regulatory mechanisms of laser on tissues are poorly understood [23, 24]. Such mechanisms probably involve increases in cell proliferation through changes in cell metabolism, affecting RNA synthesis and the expression of various cell regulatory proteins [9, 10, 25, 26]. However, the reasons for the stimulatory effects of laser remain unclear and need further investigation.

Conclusion

Our study demonstrated the positive effects of 670 nm laser on skin flap viability, mainly at the higher dose used. These data highlight the importance of using adequate laser wavelength and dose to elicit the best tissue response. Studies investigating the effects of different fluences of laser on tissue repair are important in determining the efficacy of laser therapy. Further studies are required to investigate possible mechanisms of action that may explain the effects of laser on skin flap viability.

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