Inhibitory effects of low-level laser therapy on skin-flap survival in a rat model

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BACKGROUND: Although several studies have demonstrated the effects of low-level laser therapy (LLLT) on skin flap viability, the role of higher doses has been poorly investigated.

OBJECTIVE: To investigate the inhibitory effect of the LLLT ($\lambda=670$ nm) on the viability of random skin flaps in a rat model using an irradiation energy of 2.79 J at each point.

METHODS: Sixteen Wistar rats were randomly assigned into two groups: sham laser irradiation ($n=8$); and active laser irradiation ($n=8$). Animals in the active laser irradiation group were irradiated with a 670 nm diode laser with an energy of 2.79 J/point, a power output 30 mW, a beam area of 0.028 cm², an energy density of 100 J/cm², an irradiance of 1.07 W/cm² for 93 s/point. Irradiation was performed in 12 points in the cranial skin flap portion. The total energy irradiated on the tissue was 33.48 J. The necrotic area was evaluated on postoperative day 7.

RESULTS: The sham laser irradiation group presented a mean ($\pm$ SD) necrotic area of 47.96 $\pm$ 3.81%, whereas the active laser irradiation group presented 62.24 $\pm$ 7.28%. There was a significant difference in skin-flap necrosis areas between groups ($P=0.0002$).

CONCLUSION: LLLT ($\lambda=670$ nm) increased the necrotic area of random skin flaps in rats when irradiated with an energy of 2.79 J (100 J/cm²).

Key Words: Low-level laser therapy; Necrosis; Rats; Surgical flaps

Low-level laser therapy (LLLT) has been investigated as a noninvasive and sterile alternative for improvement of microcirculation and, consequently, increasing the viability of ischemic skin flaps (1-13). It is known as an athermal treatment modality due to the relatively low doses and low powers that are unable to promote any change in tissue temperature.

Several studies have demonstrated the efficacy of LLLT in accelerating the healing process, the promotion of analgesia, and an increase in microcirculation and capillarity (5,6,11). Skin flaps may progress to necrosis due to intrinsic and extrinsic factors that compromise local microcirculation (14); LLLT has been used to increase skin flap viability.

Several parameters must be considered when applying LLLT. These include wavelength, output power, emission mode, spot size and shape of the beam, irradiance, energy density, irradiation time, application technique, energy per point and total energy delivered to the tissue (9). Although all of these parameters appear to be important in modulating physiological and therapeutic responses, wavelength, energy and application time have been suggested by the World Association for Laser Therapy (WALT) as key factors (15). Both infrared (1,2,4,6,11) and red (5,8-10) lasers have been used to increase skin flap viability. Despite the positive results demonstrated with both types of lasers, some studies suggest that lasers with wavelengths in the red spectrum are more effective at increasing skin flap viability (16). The energy used in previous studies assessing the effects of red lasers in dorsal random skin flaps ranged from 0.06 J to 1.44 J per point (3,9,5,10,12). Energies <0.3 J

Les effets inhibiteurs de la thérapie laser de faible intensité sur la survie des lambeaux cutanés d'un modèle murin

HISTORIQUE : Plusieurs études ont démontré les effets de la thérapie laser de faible intensité (TLFI) sur la viabilité des lambeaux cutanés, mais le rôle de plus fortes doses a été peu évalué.

OBJECTIF : Examiner l'effet inhibiteur de la TLFI ($\lambda=670$ nm) sur la viabilité de lambeaux cutanés aléatoires d'un modèle murin au moyen d'une énergie d'irradiation de 2,79 J à chaque point.

MÉTHODOLOGIE : Les chercheurs ont réparti 16 rats Wistar en deux groupes aléatoires : irradiation laser factice ($n=8$) et irradiation laser active ($n=8$). Les animaux du groupe d'irradiation laser active ont été irradiés à l'aide d'une diode laser de 670 nm à une énergie de 2,79 J/point, une puissance de 30 mW, un faisceau de 0,028 cm², une densité énergétique de 100 J/cm² et une radiation de 1,07 W/cm² pendant 93 s/point. Douze points ont été irradiés sur la portion du lambeau cutané crânien. L'énergie totale irradiée sur les tissus s'élevait à 33,48 J. Les chercheurs ont évalué la zone nécrosée le septième jour après l'opération.

RÉSULTATS : Le groupe d'irradiation laser factice présentait une zone nécrosée moyenne de ($\pm$ ÉT) 47,96 $\pm$ 3,81 %, tandis que le groupe d'irradiation laser active présentait une zone de 62,24 $\pm$ 7,28 %. On constatait une différence importante entre les zones nécrosées des lambeaux cutanés des deux groupes ($P=0,0002$).

CONCLUSION : La TLFI ($\lambda=670$ nm) accroissait la zone nécrosée des lambeaux cutanés aléatoires lorsqu'ils étaient irradiés par une énergie de 2,79 J (100 J/cm²).

were ineffective (3,9), whereas energies from 0.3 J to 1.44 J (5,10,12) increased skin flap survival. According to Arndt-Schulz' law, very low doses are ineffective, and intense stimuli may result in negative responses in tissue physiology. It appears that energies from 0.3 J to 1.44 J are in the therapeutic window for improving skin flap survival. Nevertheless, to the best of our knowledge, no studies have demonstrated an inhibitory effect on skin flap survival using higher energies of LLLT. Thus, the present study aimed to test an energy greater than those proven to be effective. Accordingly, the objective of the present study was to investigate the inhibitory effect of LLLT ($\lambda=670$ nm) on the viability of random skin flaps in rats when irradiated with 2.79 J of energy at each point.

METHODS

Study design

The present study was approved by the Ethics and Research Committee of Paulista University (São Paulo, Brazil) under protocol number CEP 008/11.

The present analysis was an experimental, interventional, randomized study with a blinded assessor. All animals received humane care in strict compliance with the Ethical Guidelines for Animal Experiments, Council for International Organizations of Medical Sciences, Standards of Brazilian Science Society for Laboratory Animals, and current national legislation on Procedures for the Scientific Use of Animals (Federal Law 11794 [October 9, 2008]).

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Figure 1) Dorsum of the animal after trichotomy and delimitation of the area of the skin-flap elevation

Sample

In the present study, 16 adult male Wistar EPM-1 rats (*Rattus norvegicus*: var, *Albinus*, *Rodentia*, *Mammalia*) weighing between 292.4 g and 381.2 g (mean 337.3 g) were used. The sample size was calculated considering a difference of 20% in skin-flap necrosis area between the active laser irradiation and sham laser irradiation groups, and an estimated SD of 9.6% based on data from a previous study (9). For a significance level of 0.05 and 80% power, it was estimated that five animals would be required in each group (Minitab version 15, State College, USA). Allowing for attrition, eight rats were used in each group, yielding a total of 16 animals.

Equipment

A low-power laser device was used in the present experiment (Physiolux Dual, BIOSET Indústria de Tecnologia Eletrônica Ltd, Brazil) with a red diode laser emission $\lambda=670$ nm, output power of 30 mW, continuous-emission mode and round-shaped beam of 0.028 cm². The power of this equipment was measured before and after all irradiations using a power meter (LaserCheck, Coherent, USA).

Groups

The 16 rats were kept in individual cages, receiving food and water ad libitum in a controlled environment in which the light/dark cycle was 12 h/12 h at 21°C.

The animals were randomly allocated into two groups: sham laser irradiation group – random skin flap and simulation of laser irradiation (n=8); and active laser irradiation group – random skin flaps subjected to laser irradiation ($\lambda=670$ nm), energy density of 100 J/cm² with irradiance of 1.07 W/cm² for 93 s, providing 2.79 J of energy per point (n=8).

Surgical technique

Under intraperitoneal general anesthesia (tiletamine hydrochloride and zolazepam hydrochloride 25 mg/kg) and analgesic control (butorphanol 1.0 mg/kg), each animal was placed on a flat surface with their limbs in the extended position and had their dorsal area trichotomized before delimitation of the random skin flap area (Figure 1). A cranially based random skin flap was then created (10 cm long $\times$ 4 cm wide) and elevated from the dorsum of the animals in both groups following an experimental model described by McFarlane et al (17). This experimental model was developed to study skin flap necrosis area (25% to 50%) and its prevention. The flap consisted of the superficial fascia, panniculus carnosus, subcutaneous tissue and skin. After elevation, the flap was repositioned on its original location after placement of a plastic film with the same dimensions of the flap between the superficial fascia and superficial muscle layer (Figure 2). The placement of a plastic film was to prevent revascularization from vessels of the underlying tissues, thus ensuring homogeneous conditions of ischemia and necrosis in the animals, as proposed by Korlof and Ugland (18) and revised by Kaufman et al (19). This experimental

Figure 2) Elevation of the skin flap and positioning of the plastic barrier

model has been widely used in previous studies assessing the effects of different treatments to decrease skin-flap necrosis (9,10,12,21-25). A suture was performed with simple stitches using 4-0 nylon monofilament, 1 cm from one another (Figure 3).

A mold using black cardboard was fabricated with the same dimensions as the flap area. The mold had fenestrations to allow for attaching the tip of the probe and emitting laser beams to the skin of the animal (on the cranial-flap portion) without any interference in the transmission of the beam. Thus, laser irradiation was always performed on the same regions of the flaps. These points of irradiation were placed 1 cm from the edge of the flap and 1 cm from one another (assuming the centre of the fenestrations). Thus, 12 points were made in the cranial half of the mold, which were the points that received laser irradiation (Figure 4). After this procedure, the animals in the sham laser irradiated group were anesthetized for 20 min so that the probe of equipment for the emission of laser irradiation was positioned for 93 s at each point, simulating an emission (sham). The animals from the active laser irradiation group also underwent anesthesia for 20 min, then received laser irradiation with $\lambda=670$ nm, energy density 100 J/cm², 2.79 J per point (on the 12 points distributed in the cranial portion of the skin flap) for 93 s at each point, which supplied 33.48 J of energy to the animal on each day of irradiation. The irradiation technique (active or sham) had the laser probe positioned to promote a perpendicular incidence of LLLT.

All animals subjected to laser irradiation were treated immediately postoperatively, postoperative day 1 and on postoperative day 2 (9). The contact technique was used in all irradiations. The animals were euthanized on postoperative day 7 by anesthetic saturation.

Assessment of necrotic area

The percentage of the necrosis area of skin flap was assessed on postoperative day 7 using the paper-template method (26). The limit of viable tissue was characterized by soft, pink, hot skin with hair. Necrotic tissue was characterized by rigid, dark, cold and hairless skin.

Statistical analysis

The data were subjected to a Kolmogorov-Smirnov test to determine distribution. To evaluate possible differences in the percentage of necrosis of the groups, an unpaired *t* test was used. The level of rejection for the null hypothesis was set at $P \leq 0.05$ (5%). All tests were performed using GraphPad InStat 3 (GraphPad, USA).

RESULTS

The distribution of the percentage of the necrotic area of random skin flaps is shown in Table 1.

A Student's *t* test showed a statistically significant difference for the percentages of necrosis area between groups ($P=0.0002$).

Figure 3) Animal after the suture of the flap using 4-0 nylon monofilament

DISCUSSION

The present study investigated the inhibitory effects of LLLT on the viability of skin flaps when irradiated with 2.79 J of energy at each point. Results showed that LLLT led to an increase in the necrotic area of skin flaps.

Recent studies have shown that LLLT promotes increased viability of random skin flaps in rats (1,2,4,5,8-12). There have been several explanations for the findings. Some authors have suggested that the decrease in necrotic area of skin flaps occurs as a result of improved blood flow and promotion of neoangiogenesis (5,6,11,12). Amir et al (5) found significant capillary proliferation in animals subjected to LLLT. Prado et al (27) found a decrease of malondialdehyde concentration in skin flaps, which may be one explanation for the improvement in local blood flow. Some studies suggest involvement of autonomic nervous system changes related to LLLT in the improvement of blood perfusion in irradiated areas (6).

Some authors suggest that LLLT modulates the recruitment and action of fibroblasts, increases the concentration of collagen in healing processes (28), controls the action of some inflammatory agents (29), decreases the number of inflammatory cells (30) and reduces the effects of oxidative agents (27,31). All of these factors may be associated with increased viability of skin flaps. However, some studies have shown that LLLT was not effective at improving the viability of random skin flaps in rats (3,13).

In the present study, we obtained results that could be considered paradoxical compared with the other studies because the irradiated group showed an increase in necrotic area. Some authors have cited possible inhibitory effects caused by LLLT (32-34), and it is not uncommon to find authors who correlated the Arndt-Schulz law with inhibitory or paradoxical responses (35-39). This law was introduced in the late 19th century by H Schulz, who used potentially harmful chemicals in small doses and observed that they promoted biostimulatory effects (39,40). Later, together with Arndt, Schulz presented the theory that weak stimuli promoted slight acceleration in vital activities and that intense stimuli may lead to negative responses (34,41).

When comparing the doses used by authors who reported effects of increased skin flap viability with the doses used by Smith et al (3), there is great variation in the wavelengths used, average power, mode of laser emission (continuous or pulsed), duration and application technique, areas of light beam, energy density and irradiance. However, it is possible to establish a qualitative correlation with the energy administered to each irradiated point. The present study was based on parameters already established by other researchers regarding wavelength, average power, emission mode and application technique, but irradiated with a dose of 2.79 J per point in 12 equidistant points 1 cm from one another, totaling 33.12 J of energy administered to the animal. It is possible that this energetic magnitude reached energy indexes above those favourable to biological tissue in accordance with the Arndt-Schulz law.

Figure 4) Demonstration of laser-irradiated points in the cranial portion of the random skin flap

TABLE 1

Percentage of necrosis area in both groups

Animal	Group	
	Sham-irradiated	Active laser
1	47.42	66.54
2	46.51	66.66
3	54.07	54.06
4	46.72	60.50
5	50.87	66.44
6	45.16	56.77
7	42.31	73.75
8	50.00	52.67
Mean $\pm$ SD	47.96 $\pm$ 3.81	62.24 $\pm$ 7.28

Data presented as % unless otherwise indicated

Considering the results reported by Cury et al (13), it may be suggested that the lack of influence of laser irradiation on the viability of skin flaps occurred due to the correlation of the distance between the irradiated points and the energetic magnitude irradiated in each point. Additionally, Cury et al (13) took advantage of a significantly higher number of suture points than those used in the surgical technique performed in other studies, which could have led to more tension on the flap and possible complications to the local microcirculation. This may clarify the cause of the approximately 20% difference between the absolute values referring to the average percentage of necrosis found in control groups of the present and other investigations (10,12,21).

The energy used per point for a red laser to increase the viability of dorsal skin flaps in rats appears to be between 0.3 J (10) and 1.44 J (12,42), with output power ranging from 6 mW to 60 mW. Several authors who used values in this 'therapeutic window' obtained significant increases in tissue viability (5,9,10,12). Doses <0.3 J, as used by Smith et al (3), resulted in no significant alterations in tissue. However, as verified in the present study, 2.79 J per point caused more necrosis in the skin flap. It is possible that the increase in necrosis resulted from tissue bioinhibition caused by high doses of energy, leading to photodestruction of cytochromes in the respiratory chain because these mitochondrial components are important receptors of the energy emitted by the laser. This destruction would lead to decreased formation of ATP in oxidative phosphorylation, thus decreasing cellular metabolism and preventing cellular actions to increase the viability of the skin flap (43). Structural alterations of mitochondria can be observed when increasing the magnitude of energy supplied to the tissue (25,44). A surprising finding was observed in a recently published study (45). The authors reported a decrease in skin flap necrosis using a red laser (660 nm), applying 7.3 J per point. Nevertheless, the output power was 100 mW. It is possible that when using output powers >60 mW, more energy is required by the tissue. Possibly, there is a minimal length of time necessary to trigger a biostimulatory effect. This hypothesis is supported by dosage recommendations proposed by

WALT, in which higher-energy doses and higher application times are suggested for lasers with higher output powers (15). Thus, the results observed in the present study may be limited to the combination of parameters used including wavelength, output power, energy per point, total energy applied to the tissue and application time.

Because thermal changes in the flap have not been measured, it would be important to design a study evaluating possible thermal changes on tissue and its eventual influence on the viability of the flap, given that the dose used in the present study would not be sufficient to raise tissue temperature.

Over the past years, WALT has stressed the need to standardize laser-related parameters of irradiation to facilitate comparison of data among studies.

Thus, further studies regarding this subject should be conducted to understand the possible mechanisms that explain the observed effects

of different doses of laser irradiation on skin flaps, as well as agreement among researchers to report standardized irradiation parameters and equipment used. Most studies in the literature do not present data regarding laser wavelength, radiant exposure, irradiance, emission frequency, or size and shape of the beam, making the comparison of results exceedingly difficult.

CONCLUSION

LLLT ($\lambda=670$ nm) increases the necrotic area of random skin flaps in rats irradiated with 2.79 J (100 J/cm²) of energy at 12 points distributed equidistantly in the cranial half of the flap.

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