Effect of Low-Level Laser Therapy on Mast Cells in Viability of the Transverse Rectus Abdominis Musculocutaneous Flap

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Abstract

Objective: To assess the effect of low-level laser therapy (LLLT) on viability of mast cells of the transverse rectus abdominis musculocutaneous (TRAM) flap. Background Data: LLLT has been recently used on the TRAM flap to stimulate mast cells. Materials and Methods: Eighty-four Wistar rats were randomly divided into seven groups of 12 rats in each: group 1 (sham laser therapy); group 2 received 3 J/cm² at one point; group 3 received 3 J/cm² at 24 points; group 4 received 72 J/cm² at 1 point; group 5 received 6 J/cm² at 1 point; group 6 received 6 J/cm² at 24 points; and group 7 received 144 J/cm² at 1 point. All experimental groups underwent LLLT immediately after TRAM surgery and on the next two following days, for three sessions in total. The percentage of the area of skin flap necrosis was calculated on the fourth postoperative day and two samples of skin were collected from each rat with a 1-cm² punch to perform mast cell evaluations with toluidine blue dye. Results: Statistically significant differences were found in the percentage of necrosis, and higher values were seen in group 1 than in all other groups. Among groups 3–7 no statistically significant differences were found (p < 0.292). For mast cells, when group 1 was compared to groups 5 (6 J/cm² at 1 point) and 7 (144 J/cm² at 1 point), it had fewer mast cells. Conclusion: LLLT at a wavelength of 670 nm was effective at reducing the necrotic area, and we found that it can stimulate mast cells growth to increase vascular perfusion.

Introduction

Since the classical study of Hartrampf et al. in 1982,1 the transverse rectus abdominis musculocutaneous (TRAM) flap has become the preferred type of autogenous tissue to use for breast reconstruction.2–4 However, the TRAM flap is not a perfect solution due to complications related to abdominal wall integrity, as well as insufficient flap perfusion, which may cause partial loss of the flap.5–7 These complications may lead to a need for further surgical intervention, thus increasing hospital stay and delaying the return to activities of daily life.8–10

In an attempt to find alternatives that reduce or prevent tissue necrosis, some studies using experimental models have tried different kinds of drugs, such as vasodilators, anticoagulants, antioxidants, prostaglandin inhibitors, calcium channel blockers, and anti-adrenergic agents.9,10 The use of some of these drugs, however, has undesired systemic effects, increasing the need for non-pharmacologic means, such as acupuncture and electroacupuncture,11 low-level laser therapy (LLLT),12–15 and polarized low-frequency electric current.16–18 To improve the viability of flaps, the use of LLLT is being increasingly highlighted because of its ease of application and its good results at both the macroscopic and cellular levels.

In the literature, there are no studies that evaluate the number of mast cells found in cutaneous and TRAM flaps, but there are studies proving the importance of mast cell degranulation in stimulating blood flow.19,20 Mast cells are IgE-sensitive, and when they come into contact, they stimulate the delivery of chemical mediators found within mast cell vesicles (degranulation) such as serotonin, heparin, and histamine. Both serotonin and histamine have vasoactive properties that are important in improving vascular perfusion.21

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Pinfildi et al.\textsuperscript{15} used a helium-neon laser with a 632.8-nm wavelength, and they had statistically significant results in improving viability of skin flaps in rats. Currently, there is no consensus about the most efficient wavelength to use to increase ischemic flap viability.

Therefore, this study was designed to assess the effect of LLLT on mast cells and TRAM flap viability.

Materials and Methods

To perform this study, 84 adult 3-mo-old male rats (\textit{Rattus norvegicus}) of the Wistar EPM-1 (UNIFESP) strain, weighing 260–320 grams each, were used. The animals were kept in individual cages, with a normal day-night light cycle under controlled temperature, and were fed a standard diet and had access to water \textit{ad libitum}. This study was approved by the UNIFESP Ethics Committee in Research.

Groups and experimental protocol

Before the experiment, all 84 rats were weighted and randomly assigned using Urn Randomization Software from the University of Connecticut Health Center to seven groups of 12 animals each.

In group 1 (sham LLLT), the animals received simulated laser irradiation with the device turned off. Group 2 (3 J/cm\textsuperscript{2} at 1 point) received laser irradiation with only one irradiated spot on the TRAM pedicle, at a total energy dose of 0.18 J (6 sec of irradiation). Group 3 (3 J/cm\textsuperscript{2} at 24 points) received laser irradiation at 24 spots, in and around the TRAM flap, for a total energy dose of 4.32 J (2 min 24 sec of irradiation). Group 4 (72 J/cm\textsuperscript{2} at 1 point) received just one laser irradiation spot on the TRAM pedicle, with a total energy dose of 4.32 J, also for 2 min 24 sec. Group 5 (6 J/cm\textsuperscript{2} at 1 point) received just one laser irradiation spot on the TRAM pedicle, with a total energy dose of 0.36 J (12 sec of irradiation). Group 6 (6 J/cm\textsuperscript{2} at 24 points) received 24 laser irradiation spots, in and around the TRAM flap, for a total energy dose of 8.64 J (4 min 48 sec of irradiation). Group 7 (144 J/cm\textsuperscript{2} at 1 point) received just one laser irradiation spot on the TRAM pedicle, for a total energy dose of 8.64 J, also for 4 min 48 sec (Fig. 1).

The technique used for the laser irradiation was contact mode, in which the laser probe remains in contact with the animal during the entire irradiation period of each spot. The laser probe was kept at a 90° angle, perpendicular to the flap. A plastic template was used to mark the sites to be irradiated. All applications were performed in the same areas on every animal. The distance between the spots and the flap border was 1 cm in all cases.

Laser irradiation was performed immediately after surgery, and also on the two following days, so all animals underwent three applications.

Equipment

We used a diode low-level InGaAlP laser in the visible red range (wavelength 670 nm), with a power output of 30 mW. The operator wore protective glasses, and the spot size was 0.06 cm\textsuperscript{2} and the laser was used in continuous mode.

Surgical technique

To perform the TRAM procedure, the animals were anesthetized by intraperitoneal administration of 50 mg/kg of tiletamine hydrochloride and zolazepam hydrochloride. The researcher who performed the TRAM procedure did not know to which group each animal belonged. The flap was 3 × 5 cm in size, and was positioned 1 cm caudal to the xiphoid appendix. The skin was cut around the flap perim-

![Fig. 1](image-url)

\textbf{FIG. 1.} (A) Site of laser irradiation with a single spot on the TRAM pedicle. (B) Site of laser irradiation with 24 spots, on and around the TRAM pedicle.
eter. The flap dissection began on the left side of the pedicle, with dissection on the supra-aponeurotic plane from lateral to medial, until the midline was reached.

On the right side of the pedicle, the flap was dissected until the lateral border of the right rectus abdominis muscle was reached. The flap was then elevated by incision along the midline (linea alba). Elevation of the flap was completed by incision of the cranial portion and the lateral border of the rectus abdominis muscle, so the flap remained attached only by the right rectus abdominis muscle, near the right caudal epigastric artery.

Then the area from which the muscle was removed was closed with a running suture of 5-0 nylon. The skin was then sutured with 5-0 nylon, first with separate sutures for fixation of the flap to the midline, then on the four edges of the flap. The closure of the skin was done with a running suture over the perimeter of the flap.

**Macroscopic evaluation of the necrotic area**

The percentage of the area of skin flap necrosis was calculated on the fourth postoperative day using a paper template. The line between the viable tissue, characterized by soft, warm skin and a reddish color, and the necrotic tissue, which was stiff, dark, and cool, was demarcated on each animal. A sketch of the entire flap and necrotic area was drawn and cut out of transparent paper, then the weight was checked with a precision balance accurate to ± 0.001 gram. Then the following equation was used to calculate the percentage of necrosis present:

$$\text{Percentage of necrotic area of the TRAM flap} = \frac{\text{weight of paper template of TRAM flap necrosis}}{\text{weight of paper template of total area of TRAM flap}} \times 100$$

**Mast cell histology and morphometry**

Immediately after measurement, two samples of skin were collected using an 1-cm² punch to perform mast cell evaluation with toluidine blue dye to count mast cells. The first sample (sample A) was collected ipsilateral to the pedicle (right side) of the flap, from the portion over the pedicle 1 cm above the flap’s caudal border. The second sample (sample B) was taken from outside the flap, 2 cm from the flap’s left caudal border (Fig. 2).

The samples were examined with an optical microscope (Olympus BH-2; Olympus, São Paulo, Brazil) at 400× magnification. The quantitative study was performed by two different investigators, who using an ocular lens with 10× magnification, placed a checkered grid over the area to be assessed. The grid had 100 squares 0.25 mm in size and was coupled to the microscope, so only cells within the squares were counted. For the morphometric assessment, five areas

![FIG. 2. Sites where skin samples A and B were taken for mast-cell assessment.](image_url)

| Table 1. Average of the Percentage of Necrotic Area and Mast Cell Value in the Study Groups |
|---------------------------------------------|----------------|------------------|-----------------|----------------|----------------|
| Animal | Control | 3 J/cm² 1 pt. | 3 J/cm² 24 pt. | 72 J/cm² 1 pt. | 6 J/cm² 1 pt. | 6 J/cm² 24 pt. | 144 J/cm² 1 pt. |
| 01 | 50 | 20.98 | 12.12 | 9.52 | 1.07 | 9.41 | 5.55 |
| 02 | 31.57 | 13.63 | 31.88 | 9.87 | 14 | 4.44 | 4.91 |
| 03 | 28.57 | 19.11 | 11.62 | 2.32 | 13 | 0 | 0 |
| 04 | 37.77 | 23.61 | 4.30 | 6.89 | 0 | 4.44 | 4.25 |
| 05 | 43.75 | 16.43 | 5.12 | 7.93 | 24.19 | 13.63 | 11.11 |
| 06 | 25.56 | 29.03 | 0 | 5.88 | 0 | 14.28 | 10.86 |
| 07 | 40 | 35.89 | 0 | 18.36 | 21.34 | 10 | 6.38 |
| 08 | 23.58 | 17.33 | 11.62 | 6.66 | 0 | 8.51 | 8.16 |
| 09 | 39.24 | 37.73 | 19.60 | 6.665.49 | 6.81 | 5.55 |
| 10 | 44.82 | 13.08 | 0 | 9.90 | 21.12 | 6.66 | 11.53 |
| 11 | 29.33 | 26.08 | 3.03 | 6.97 | 10.44 | 7.69 | 19.14 |
| 12 | 56.06 | 22.5 | 15.38 | 9.30 | 5.94 | 10.41 | 13.63 |
| Average | 37.49% | 22.95% | 9.55% | 8.28% | 9.71% | 8.57% | 8.42% |

Mast cells (A) | 1.0 | 0.9 | 1.1 | 1.1 | 1.7 | 1.4 | 1.7 |
| Mast cells (B) | 1.1 | 0.8 | 1.3 | 1.0 | 1.4 | 1.0 | 1.0 |

Necrosis in group 1 > groups 2–7 ($p < 0.000$).
of each sample were examined. The investigators did not know to which group each sample belonged.

Statistical analysis

To determine if statistically significant differences existed among the seven study groups in terms of percentage of necrosis, ANOVA was performed.

To ascertain whether statistically significant differences existed among the numbers of mast cells present, the Mann-Whitney U-test was applied to the relative values of mast cells in samples A and B. Then the Kruskal-Wallis test was performed to check whether statistically significant differences existed between the group averages for mast cells in samples A and B.

To determine whether a statistically significant relationship existed between the percentage of necrotic area and the numbers of mast cells, Spearman’s correlation coefficient was applied to the evaluations of the first investigator. Statistical significance for all values was set at $p < 0.05$.

Results

As shown in Table 1, statistically significant differences were found among percentage of necrosis, with the highest values of all groups found in group 1. Among groups 3–7, no statistically significant differences were found ($p < 0.292$).

Group 1 (control) had fewer mast cells than groups 5 (6 J/cm$^2$ at 1 point) and 7 (144 J/cm$^2$ at 1 point). Group 2 (3 J/cm$^2$ at 1 point) had fewer mast cells than groups 5 (6 J/cm$^2$ at 1 point), 6 (6 J/cm$^2$ at 24 points), and 7 (144 J/cm$^2$ at 1 point). Group 4 (72 J/cm$^2$ at 1 point) had fewer mast cells than group 7 (144 J/cm$^2$ at 1 point). Group 2 (3 J/cm$^2$ at 1 point) had fewer mast cells than groups 3 (3 J/cm$^2$ at 24 points) and 5 (6 J/cm$^2$ at 1 point). Group 3 (3 J/cm$^2$ at 24 points) had fewer mast cells than groups 4 (72 J/cm$^2$ at 1 point) and 7 (144 J/cm$^2$ at 1 point). Group 5 (6 J/cm$^2$ at 1 Point) had more mast cells than the other groups.

According to results seen in Table 2 a negative correlation was found in the mast cell evaluation, meaning that as the value of a variable increases, the values of the others decrease, and vice versa. However, this was not a statistically significant difference.

Discussion

The present study showed that low-level laser (InGaAlP) therapy at 670 nm improved TRAM flap viability and enhanced mast cell numbers. One advantage of this is that most TRAM flap complications are due to poor perfusion.$^{7,24}$ Because it is a pedicled flap on the right rectus abdominis muscle, its perfusion depends on the right caudal epigastric artery, which makes it susceptible to necrosis of the pedicle’s contralateral region.$^{25}$

As flap viability is dependent on the quality of perfusion, anything that can be done to improve perfusion is desirable, and to this end LLLT was first studied in cutaneous flaps by Kami et al.$^{26}$ who found significant results in terms of increasing flap viability.

Soon afterward, other studies were carried out, but there was standardization of techniques, and studies such as those done by Smith et al.$^{27}$ Kubota and Oshiro,$^{12}$ and Amir et al.$^{13}$ all found good results, but comparisons between their results are impossible due to the range of techniques and parameters they used. In another study of this technique by Pinfieldi et al.$^{15}$ they found that application both inside and around a cutaneous flap with laser energy at 632.8 nm (HeNe) resulted in 21% of necrotic area, compared to 48% in controls, which led to an increase of flap viability. Prado et al.$^{14}$ also studied this technique, with application to only one point on the cranial aspect of a cutaneous flap, but they used a wavelength of 830 nm (AsGaAl), but they also found good results in terms of reduction of tissue necrosis.

Aside from the study by Kubota,$^{28}$ in which a blood flow increase was found after laser irradiation at 830 nm (AsGaAl) of a single site on the pedicle of an axial flap, there is a lack of studies of axial flaps and LLLT.

Thus, the utilization of LLLT in TRAM flaps is a new contribution to the literature. In the present study we found an average necrotic area of 37.49%, which is in accordance with studies by Sano et al.$^{23}$ who found an average necrotic area of 36% in animals undergoing TRAM flap procedures.

These techniques were applied with the purpose of evaluating whether the stimulation of only a single spot on the TRAM flap right caudal epigastric artery (as in groups 2, 4, and 5 here) would be sufficient to improve viability, and whether the application of LLLT to 24 sites distributed over and around the flap (as in groups 3, 6, and 7 here) would be enough to stimulate angiogenesis of the flap and its surrounding area, thus leading to an increase in TRAM flap viability.

Another determining factor for the application was the energy doses used. Reports in the literature disagree about this parameter, with doses ranging from 0.082 J/cm$^2$ (in Smith et al.$^{27}$) to 185 J/cm$^2$ (in Kubota.$^{28}$). In the present study we used doses of 3, 6, 72, and 144 J/cm$^2$ to ascertain the effect of total dose, as well as the effect of a single versus many points.

### Table 2. Spearman Correlation Coefficient Related to Percentages of Necrosis and Values of Mast Cells

<table>
<thead>
<tr>
<th>Analyzed variable</th>
<th>$r$ Value</th>
<th>Probability</th>
</tr>
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<tbody>
<tr>
<td>Necrosis $\times$ mast cells A</td>
<td>-0.166</td>
<td>0.193</td>
</tr>
<tr>
<td>Necrosis $\times$ mast cells B</td>
<td>-0.013</td>
<td>0.920</td>
</tr>
</tbody>
</table>

Significance is set at $p < 0.05$. 

![Figure 3](image-url)  
**FIG. 3.** Averages of tissue necrosis percentages in the seven study groups.
In the present study, the energy levels used were 0.18, 4.32, 0.36, and 8.64 J, which are smaller values than those used by others. This is a relevant factor to consider, as in the study by Kubota, who used a total dose of 185 J/cm², but at an energy level of 1 J, which was less than the levels we used, and those researchers used a dose of 144 J/cm² and total energy of 8.64 J, which was concentrated on just one application point. Other studies such as the one by Amir et al., who used energy rates of 1.8 J and 3.6 J and found significant results, but in a different kind of cutaneous flap.

In the present study, the same energy rates were delivered in different ways to the tissue, namely on just 1 spot in some cases, and in other distributed over 24 spots in and around the TRAM flap, to assess whether distributing the energy more widely would be better than its application to just one site.

For analyzing mast cells, toluidine blue dye was used because it is specific for marking mast cells, which become dark blue and are easily seen for morphometry.

Another mechanism evaluated in this study aside from blood flow increase, is mast cell exocytosis, which favors histamine delivery, improving vasodilation.

We found increased amounts of mast cells in the groups irradiated with energy levels of 6 and 144 J/cm²; however, the techniques used to apply the energy were different between the groups. Group 5 was irradiated on only one site over the pedicle, with total energy of 0.36 J (6 J/cm²), which showed an average increase of 1.7. Group 6 was irradiated on 24 sites inside and outside the TRAM flap, with a total energy of 8.64 J (6 J/cm²) which showed an average increase of 1.4, which differed from the findings in group 7 which was irradiated similarly to group 5, with just a single site, and it showed an average increase of 1.7, but with a total energy dose of 8.64 J (144 J/cm²).

The increase in the amount of mast cells in the present study is in line with the findings of El Sayed and Dyson, who studied mast cell increase and degranulation with laser irradiation of rat skin wounds (ulcers). In spite of using an infrared laser at 820 nm, the authors found a significant increase in mast cells compared to the control group.

Vasheghani et al. also studied the effect of LLLT with a visible red laser on the increase and degranulation of mast cells. The laser used had a wavelength of 632.8 nm (He-Ne), and in the present study we used a diode laser of 670 nm (InGaAlP).

The results found by Vasheghani et al. were obtained in rats that had second-degree burns, and the authors saw a significant increase in mast cells, mainly in their degranulation, in animals undergoing laser therapy at a level of 2.4 J/cm². When comparing energy levels, we can see that the levels we used in the present study, 3 J/cm² with just one application site or with 24 sites, did not show any significant increase compared to higher flows of 6 J/cm².

According to the results we found, we can see that the effects on mast cells depend on the energy level used, and thus are dose-dependent. Higher flows with only a single site of application to the pedicle of 6 and 144 J/cm² showed better results than those seen in the other experimental groups.

An increase in the amount of mast cells with laser irradiation with the 670-nm wavelength has also been confirmed the findings by Sawasaki et al., who studied LLLT with the same type of laser and a flow of 8 J/cm² and total energy

**FIG. 4.** Averages of mast cell values of skin samples A in the seven study groups. *, group with the most mast cell average.

**FIG. 5.** (A) Skin sample stained with toluidine blue viewed at 200× magnification. Mast cells after mast cell irradiation at a dose of 144 J/cm². (B) Similar image at 400× magnification. Shows degranulated mast cells.
dose of 12 J on oral mucosa in humans. According to the results of both the present study and those of Sawasaki et al., mast cells appear to be sensitive to higher energy levels, as seen with the level of 8.64 J in group 7.

Samples B (viable tissue) which did not receive laser therapy, had an increased amount of mast cells, with averages of 1.3 and 1.4 for groups 3 and 5 respectively, significant results when compared to those of control samples B. Group 2 showed a small number of mast cells (0.9 and 0.8) for both samples A and B when compared to all other groups, but without significant differences compared to the control group, although they showed statistically significant differences compared to groups 3, 5, and 7.

No physiologic answers were found for the finding of mast cell reductions in group 2, which was irradiated with a flow of 3 J/cm² (0.18J) on only a single site. In terms of tissue necrosis, group 2 was the only one showing a higher percentage of necrosis, with 22.95%. One possible explanation may be related to the low energy delivered to the tissue, since the groups showing a significant increase in mast cells were irradiated with energy doses of up to 144 J/cm² (8.64 J).

These findings show that mast cells are sensitive to different levels of LLLT, and future studies can be designed to find the ideal dose to stimulate mast cell degranulation. Another important perspective would be to study the amount of VEGF and blood vessel formation, to better clarify both angiogenesis and blood flow increases associated with mast cell increase.

Conclusion

Low-level laser therapy with at 670 nm was effective at reducing the necrotic area in TRAM flaps. In terms of mast cell changes, group 5 (6 J/cm² at 1 point) and group 7 (144 J/cm² at 1 point) had increases that were greater than those seen in the other groups. The technique of applying energy to a single spot on the TRAM pedicle was shown to be better than the distribution of sites around the flap perimeter, showing that the stimulation of vessel growth responsible for flap perfusion is important in increasing the amount of mast cells, and to reduce flap necrosis.

Acknowledgments

The authors gratefully acknowledge funding from the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

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