Participation of the Immune System and Hedgehog Signaling in Neoangiogenesis Under Laser Photobiomodulation

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Abstract

Introduction: This study aimed to characterize immune and endothelial cells, myofibroblasts and pericytes, and positive cells for hedgehog proteins in late tissue repair of rats skin wounds treated with 670 nm photobiomodulation therapy (PBMT).

Methods: A blind experimental study was conducted, in order to assess the effect of PBMT in later stages of healing, with emphasis on neoangiogenesis, immune cells and Hedgehog signaling. Forty Wistar rats were allocated randomly in two groups; control and treated with a diode GaAlAs laser (9 mW, 670 nm, 0.031 W/cm², spot size of 0.28 cm², fluence of 4 J/cm² applied every other day, until a total dose of 16 J/cm² was achieved). Standardized skin wounds were performed and the animals were euthanized at 14, 21, 28 and 35 days. Tissue sections were subjected to hematoxylin-eosin and immunohistochemistry for CD31, NG2, smooth muscle alpha actin, CD8, CD68, Ptch, Gli-2 and Ihh. All histomorphometric data were statistically analyzed and significance level was at P<0.05.

Results: At late stages of wound healing, neoangiogenesis persisted as revealed for the number of CD31+ cells (P=0.016) and NG2+ and smooth muscle alpha actin positive pericytes (P=0.025), for both experimental groups. By day 21, laser-treated group had decreased CD68+ cells (P=0.032) and increased CD8+ (P=0.038). At remodeling stage, there were positive cells for the hedgehog signaling pathway family which seemed to be activated.

Conclusion: These data suggest that photobiomodulation therapy was able to modulate extracellular matrix remodelling even at the later stages of wound healing.

Keywords: Wound healing; Low-level light therapy; Immunohistochemistry; Neoangiogenesis.
Animals were irradiated with a 21, 28, and 35 after surgery in a CO₂ group.

Skin tissue from the damaged area was removed and Histopathology

emission, in the same pattern as described for the laser with the active tip of a low-level laser device, without light seconds per point and 124 seconds per session.

dose of 16 J/cm² was reached. The irradiation time was 31 seconds per point and 124 seconds per session.

Control Group: The animals were subjected to contact with the active tip of a low-level laser device, without light emission, in the same pattern as described for the laser group.

Animals from both groups were euthanized on days 14, 21, 28, and 35 after surgery in a CO₂ chamber.

Histopathology

Skin tissue from the damaged area was removed and fixed in 4% buffered formalin solution with pH of 7.4 for 24 hours. Tissue samples were subjected to further histological processing and embedding in paraffin blocks.

Paraffin-embedded tissue samples were cut using a microtome and 4 µm sections were obtained. They were stained with hematoxylin-eosin (HE).

Immunohistochemistry

Slides were pre-treated with organosilane-based adhesive (3-Aminopropyltriethoxysilane, Sigma, St. Louis, MO, USA). All the antibodies used in the study were standardized (Anti-CD31/1:1000, SC-1506, Santa Cruz Biotech; Anti-Pitch1/1:1000, NB200-118, Novus Biological; Anti-Gli-2/1:1000, GWB-CE7858, GenWay Biotech; Anti-Ihh/1:250, AB 39634, Abcam; Anti-NG2/1:200, MAB 5384, Millipore; Anti-Actina/1:200, M0851, DakoCytomation; Anti-CD8/1:10, AR0801, Biosource; Anti-CD68/1:200, MCA341R, AbD Serotec). The peroxidase method was used for immunoblotting.

Histological sections were subjected to deparaffinization in xylene and rehydrated by treatment with absolute ethanol and then with water at room temperature (23°C). Antigen retrieval was performed with a 10 mM citrate buffer at pH of 6.0 in a 96°C water bath and subsequent blocking of endogenous peroxidase using Dako Dual Enzyme Block (DAKO, Carpinteria, CA, USA). To eliminate non-specific binding, a serum-free protein block (DAKO, Carpinteria, CA, USA) was used and the sections were incubated at 4°C with primary antibodies in a humidified chamber overnight. The sections were further incubated with a secondary antibody, Dako envision polymer peroxidase (Dako, Carpinteria, CA, USA), for 30 minutes at room temperature. The reaction was revealed using DAB (Dako, Carpinteria, CA, USA) with a 3% hydrogen peroxide solution. The slides were counterstained with Harris hematoxylin and mounted with Canada balsam.

A qualitative and quantitative graduation was adopted to indicate the intensity of the immunohistochemical reaction in which zero was considered non-reactive. The other levels were + (discrete), ++ (moderate) and +++ (severe).

Histomorphometry

Histological and immunohistochemical analysis of all sections was performed without prior knowledge of the groups to which the animals belonged. The area containing standard positive staining cells was measured by morphometry and the results were tabulated and subjected to statistical analysis. The morphometric study was performed using images captured by Motic Images Advanced 3.0 software. A standard area was used to analyze all cases (12,288,000,000 pixels). Five areas of each section were analyzed. Adobe Photoshop CS5 Extended software version 12.0 x32 (Adobe Systems Incorporated, USA) was used to measure the percentage of stained

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Materials and Methods

Experimental Groups

Forty male Wistar rats, Rattus norvegicus, weighing between 200–300 g were kept in individual cages under controlled temperature, humidity, and lighting. They received a standard commercial diet and water ad libitum.

All the procedures were in accordance with Fortuna et al. Anesthesia was administered intraperitoneally with a combination of ketamine (50 mg/kg) and xylazine (10 mg/kg) at a dose of 0.2 mL/100 g of body weight. Then, the animals were shaved on the dorsal region and an iodophor was used as a sanitizer (Biochemistry, Rio de Janeiro, Brazil). A standardized tissue fragment was removed from the skin with a circular 8 mm punch (Stiefel Biopsy Punch, Germany). The wounds were not sutured. After the removal of surgical tissue, animals were maintained in individual cages and inspected daily for bleeding, loss of crust, and the presence of any kind of exudate.

The animals were randomly distributed into 2 experimental groups by means of their registered laboratory number.

Laser Group: Animals were irradiated with a semiconductor laser device, namely a continuous-wave (9 mW, 670 nm, 0.031 W/cm²) diode laser of gallium aluminum arsenide (AlGaAs), with a 0.28 cm² active tip area (Laser VR-KC-610, Dentoflex, Brazil). There was an everyday application of 1 J/cm² at 4 equidistant points from the circular wound border, totaling 4 J/cm² per session. This type of application proceeded until a total dose of 16 J/cm² was reached. The irradiation time was 31 seconds per point and 124 seconds per session.

Control Group: The animals were subjected to contact with the active tip of a low-level laser device, without light emission, in the same pattern as described for the laser group.

Animals from both groups were euthanized on days 14, 21, 28, and 35 after surgery in a CO₂ chamber.

Histological and histomorphometric analysis, the immune and the endothelial cells, myofibroblasts and pericytes, and the positive cells of Hedgehog proteins in the late phase of tissue repair of rat skin wounds treated with 670 nm LPBM.
tissue. Then, these data were compared to the total area selected. All examinations were performed double-blinded to avoid bias.

Statistical Analysis
A database was created in Microsoft Excel (2010) and data were analyzed using R software (version 3.1.3), where a descriptive analysis was obtained (median and quartiles) to identify the general and specific characteristics of the study sample. The Shapiro–Wilk test was used to verify the normal distribution of data. According to this analysis, the variables were analyzed using the non-parametric student’s t test and the Mann-Whitney test was used for independent samples. The significance level for this study was 5% ($P < 0.05$).

Results

14 Days
The experimental groups showed intense cellularity on the wounded area corresponding to fibroplasia. Spindle and rhomboid-shaped cells with intense basophilia were seen near the blood capillaries. The laser group exhibited neoangiogenesis with basophilic cells proliferating from the wall of vascular endothelium to the extracellular matrix. These cells were smooth muscle alpha actin and NG2 positive cells. The presence of monomorphonuclear cells, CD8 and CD68, was observed in both experimental groups ($P > 0.05$). There were more CD31+ cells in the control group than in the laser group ($P < 0.05$). Alpha smooth muscle cell actin and NG2+ cells were more numerous in the control group; however, this data was statistically significant only for smooth muscle alpha actin positive cells ($P < 0.001$) (Table 1).

Components of the hedgehog pathway showed the more expressive distribution of Ihh+ and Ptc+ cells than Gly-2+ cells in both control and laser groups, mainly in fusiform cells present in the extracellular matrix (Figure 1). However, when the two groups were compared, the differences were not significant ($P > 0.05$).

21 Days
Similar features to those described in the 14 days’ section were observed after 21 days. Rhomboid-shaped cells were more concentrated below the epidermis, near the basal membrane. In the control group, few vessels were seen. There was mild inflammatory infiltrate composed of lymphocytes and plasma cells in the reticular dermis. In the laser group, the blood vessels were congested.

During this period, the presence of monomorphonuclear cells increased. There was a higher level of CD8+ cells in the control group ($P = 0.038$). The laser group showed fewer CD68+ cells ($P = 0.032$). There were also a greater number of CD31+ cells in the laser group compared with the control group ($P = 0.016$) (Table 1; Figure 2).

The cells identified as myofibroblasts and pericytes and positive stained smooth muscle alpha actin and the NG2 cells were observed in the extracellular matrix of the control group ($P = 0.025$ and $P < 0.05$ respectively) (Table 1).

There was a higher number of Gli-2+ and Ihh+ cells in the control group. Moreover, an inverse relationship was observed regarding the Ptc+ cells, although these differences were not statistically significant ($P > 0.05$) (Table 2).

Table 1. Immunohistochemical Expression of CD8, CD31, CD68, Smooth Muscle Alpha Actin, and NG-2

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CD-8+</th>
<th>CD-31+</th>
<th>CD-68+</th>
<th>aActina+</th>
<th>NG-2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>14d Laser</td>
<td>21.1 (14.2;47.3)a</td>
<td>42.2 (15.1;55.0)b</td>
<td>71.7 (37.1;84.6)b</td>
<td>32.5 (18.4;46.5)a</td>
<td>215.0 (83.0;1077.7)a</td>
</tr>
<tr>
<td>14d Control</td>
<td>38.9 (28.1;111.5)b</td>
<td>62.9 (49.8;75.3)b</td>
<td>49.8 (44.9;73.1)b</td>
<td>206.4 (182.6;227.4)a</td>
<td>548.2 (256.5;839.8)b</td>
</tr>
<tr>
<td>21d Laser</td>
<td>110.8 (91.3;130.4)a</td>
<td>1196.0 (475.4;3379)a</td>
<td>58.3 (48.6;87.1)a</td>
<td>59.0 (21.4;80.6)a</td>
<td>288.7 (206.9;1060.1)a</td>
</tr>
<tr>
<td>21d Control</td>
<td>66.4 (43.9;92.6)a</td>
<td>55.3 (13.3;100.7)a</td>
<td>101.0 (88.7;435.6)a</td>
<td>117.4 (83.8;151.0)a</td>
<td>95.0 (487.4;2980.9)a</td>
</tr>
</tbody>
</table>

* Significant findings ($P < 0.05$); * Student’s t test; b Mann-Whitney test.
Different letters represent significant findings ($P < 0.05$) between groups in each variable studied (A, B) [among groups 21d and laser 28d CD-31 antibody ($P = 0.014$) and 14d and 28d Laser NG-2 antibody ($P = 0.014$)].
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There was a decrease in cellularity in the dermis in both groups, although a significant concentration of basophilic cells was observed, sometimes fusiform and/or rhomboid, in the subepithelial area in the laser-treated group. Few blood vessels were seen in the reticular dermis. Although monomorphonuclear inflammatory cells were more common in the laser group, the difference was not significant ($P > 0.05$).

The number of CD31$^+$ cells was similar in both experimental groups. However, in the laser group, the CD31$^+$ cells significantly decreased compared to the previous study period ($P = 0.014$) (Table 1).

The expression of the NG2$^+$ cells was higher on day 28 (Figure 3) compared to day 14 ($P = 0.014$) (Table 1). However, when the experimental groups were compared at this period, there was no significant difference in the smooth muscle alpha actin positive cells and the NG2$^+$ cells, and in the Gli-2$^+$ and Ihh$^+$ cells between both groups ($P > 0.05$) (Table 2).

### 28 Days

There was a decrease in cellularity in the dermis in both groups, although a significant concentration of basophilic cells was observed, sometimes fusiform and/or rhomboid, in the subepithelial area in the laser-treated group. Few blood vessels were seen in the reticular dermis. Although monomorphonuclear inflammatory cells were more common in the laser group, the difference was not significant ($P > 0.05$).

The number of CD31$^+$ cells was similar in both experimental groups. However, in the laser group, the CD31$^+$ cells significantly decreased compared to the previous study period ($P = 0.014$) (Table 1).

The expression of the NG2$^+$ cells was higher on day 28 (Figure 3) compared to day 14 ($P = 0.014$) (Table 1). However, when the experimental groups were compared at this period, there was no significant difference in the smooth muscle alpha actin positive cells and the NG2$^+$ cells, and in the Gli-2$^+$ and Ihh$^+$ cells between both groups ($P > 0.05$) (Table 2).

### 35 Days

The tissue sections obtained from the laser-treated and control groups showed extensive areas of fibroplasia, absence of skin appendages, and a reduced number of CD8$^+$ and CD68$^+$ cells ($P > 0.05$) (Table 1).

The CD31$^+$ cells in the laser group were lower than those in the control group, although the difference was not statistically significant ($P > 0.05$). There was also no statistically significant difference in the presence of the smooth muscle alpha actin positive cells between the groups, although the laser group showed a higher number. An inverse relationship was observed for the

| Table 2. Expression of Gli-2$^+$, Ihh$^+$ and Ptch$^+$ cells in the control and laser groups |

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Median (Q1-Q3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gli-2</td>
<td>Med</td>
</tr>
<tr>
<td>14 d Laser</td>
<td>80.7 (46.9;92.3)</td>
</tr>
<tr>
<td>14 d Control</td>
<td>89.0 (52.4;126.9)</td>
</tr>
<tr>
<td>21 d Laser</td>
<td>79.0 (53.0;117.5)</td>
</tr>
<tr>
<td>21 d Control</td>
<td>117.0 (77.5;160.3)</td>
</tr>
<tr>
<td>28 d Laser</td>
<td>152.6 (92.9;188.2)</td>
</tr>
<tr>
<td>28 d Control</td>
<td>98.5 (72.3;130.7)</td>
</tr>
<tr>
<td>35 d Laser</td>
<td>134.4 (127.3;180.9)</td>
</tr>
<tr>
<td>35 d Control</td>
<td>125.4 (86.0;159.1)</td>
</tr>
</tbody>
</table>
NG2+ cells, although it was not statistically significant ($P > 0.05$) (Table 1).

The number of Gli2+ cells was higher in the laser group but the Ihh+ and Ptch+ cells predominated in the control group ($P > 0.05$). The Gli-2+ cells were numerous only in the laser group on day 35 compared to days 14 and 21 ($P > 0.05$) (Table 2).

**Discussion**

There is increasing evidence that cellular, biochemical and molecular mechanisms that take place in wound healing often perpetuate themselves for a longer period during tissue repair. The final clinical aspect of an injury may not reflect simultaneous biological phenomena going on in the tissue. The present study aimed to characterize the populations of immunocompetent cells, myofibroblasts, pericytes, and other cell types that provide phenotypic evidence of hedgehog signaling pathway expression in later stages of tissue repair. In addition, the study investigated whether the primary effects triggered by LPBM could influence the course of biological events as the day advanced.

The experimental model of cutaneous wound healing in rats has been used to investigate the different stages of repair. In particular, the action of possible endogenous and exogenous factors as biomodulating agents has been considered. LPBM is an example of such a biomodulating factor. In a study conducted by Medrado et al., 670 nm diode lasers were used on cutaneous ulcers in rats, and a significant increase in the trans-differentiation of myofibroblasts was observed during the early phase of wound healing. However, the authors investigated only the initial days of repair. Later in 2008, the same authors evaluated cutaneous healing aggravated by the action of silica until 60 days after the induction of the same standardized skin wound. Although there were no statistically significant differences between the experimental groups in the later periods of repair, on 30th and 60th days, it was observed that neoangiogenesis persisted until the 60th day. Could LPBM modulate the course of this process and some cellular populations during the later phase of repair? To answer this question, the present study was conducted from day 14 to day 35.

On day 14, the number of CD8+ cells were smaller in the laser group. This finding confirmed that the potent anti-inflammatory action of LPBM was prolonged. There is evidence from *in vivo* and *in vitro* studies that CD8+ T lymphocytes can also exert regulatory effects. Boyce et al showed an increase in CD8 T cell suppressors in later stages of healing and suggested their involvement in the process, probably mediated by the release of Th-1 cytokines/growth factors. Although it was not the aim of the present study to evaluate the cytokine profile produced by such cells *in situ*, our results demonstrated an increase in CD8+ cell counts from day 21 ($P = 0.038$) to day 28 in the laser group. The number of CD68+ cells was smaller on day 21 in the laser group ($P < 0.005$). Probably, the regulatory action of CD8+ lymphocytes caused a decrease in CD68+ cells. Some studies have shown the role of LPBM in macrophage activity at the beginning of wound healing, but the presence of these cells have not yet been investigated in later stages of repair. We can suggest that the inflammatory response that takes place in the early phase of wound repair indeed extends into later stages of this process, even when the wound is clinically healed.

The expression of smooth muscle alpha actin, biomodulated by phototherapy, has previously been investigated. Although the presence of smooth muscle alpha actin indicates the trans-differentiation of fibroblasts to myofibroblasts, other cell types can also express such a biomarker. Medrado et al suggested that myofibroblasts present in tissue sections of laser-treated animals could be derived from perivascular cells (pericytes) or pre-existing fibroblasts in the extracellular matrix since these cells have the simultaneous expression of NG2, smooth muscle alpha actin, and desmin during healing. The authors suggested that the maximum contraction of the wound occurred on day 7 as laser-irradiated animals exhibited a significant increase in myofibroblasts and pericytes in the newly formed dermis. In the current study, there was no...
significant difference between the experimental groups. The control group had more NG2 and alpha smooth actin positive cells in all periods of the study. This fact seems to indicate that wound contraction occurs later in the control animals than in the laser-treated animals. The roles played by smooth muscle alpha actin and NG2+ cells in the wound bed cover a broad spectrum of biological processes such as collagen production and repopulation of tissues as well as their respective essential structures and functions.20

The formation of new capillaries from the pre-existing ones is an essential component of healing due to an increased demand for nutrients.7 According to Andrade,21 angiogenesis is a biological process that has a positive impact on wound remodeling. In this study, maximum neoangiogenesis occurred on day 21 and increased in the animals irradiated with lasers (p < 0.05). The remaining vessels in the micro-environment of the injury during this period may contribute to ensuring not only an increased supply of oxygen and nutrients, but also local cell repopulation.22 Besides, several studies have shown that proliferating pericytes may protrude from the wall of the capillary and accumulate in the damaged tissues where they turn into myofibroblasts and begin to synthesize the extracellular matrix.23

The Hedgehog pathway is involved in many aspects of embryonic development, including morphogenesis, angiogenesis, and repair.11 Its role in repair is still a relatively unexplored topic. There was no significant difference between the control and laser groups regarding Ihh+, Gli+, and Ptch+ cells in the present study. According to Athar et al.,24 the transducer protein, Shh, has been demonstrated to be the most active protein in cutaneous healing. It is necessary to consider the possibility that the activation of Hedgehog signaling may be an early event in wound healing. As only later phases of repair were studied in our research, our understanding of how these proteins regulate the entire process is limited. Sicklick et al.25 reported that myofibroblasts could produce Hedgehog ligand which controls tissue reconstruction and remodeling. This binder can also regulate the viability and migratory activity of various cell types stimulated by Hedgehog signaling. Thus, additional investigations to evaluate the expression of transducer signal proteins of this family upon skin repair are necessary.

The findings of this study suggest that LPBM slightly continue to modulate extracellular matrix remodeling during later experimental wound healing. While primary action of lasers on first days of repair is well known, it can persist over time in the micro-environment of the lesion, though the effects may be discrete or even imperceptible from a clinical point of view. Further studies in this area, which evaluate the expression of these and other signaling proteins through techniques that are more sensitive and other experimental models, would contribute to the elucidation of late biological events related to the action of LPBM in wound healing.

Ethical Considerations
The experimental research was conducted according to the laws of the Gonçalo Moniz Research Center, Fiocruz, with the approval of the Institutional Animal Care and Use Committee under the protocol number 010/11.

Conflict of Interests
The authors declare no conflict of interest.

References


