



The effects of transcutaneous low-level laser therapy on the skin healing process: an experimental model

Felipe Scholz Ramos¹ · Laura Beatriz Mesiano Maifrino² · Sarah Alves³ · Beatriz da Costa Aguiar Alves¹ · Matheus Moreira Perez¹ · David Feder¹ · Ligia Ajaimé Azzalis³ · Virginia Berlanga Campos Junqueira³ · Fernando Luiz Affonso Fonseca^{1,3}

Received: 4 September 2017 / Accepted: 21 December 2017
© Springer-Verlag London Ltd., part of Springer Nature 2017

Abstract

We aim to evaluate the action of transcutaneous laser in the initial wound healing process. The use of low-level laser therapy (LLLT) has proven to be effective on inflammatory modulation and wound healing. The trial was performed on five groups of rats, through a dorsal incision. All groups received treatment on auricular artery. Groups 1 and 3 were treated with transcutaneous LLLT over a period of 15 min. Groups 2 and 4 received one and two inactive laser applications (placebo), respectively. Group 5 was the control one. Blood samples were collected 2 h after the last application of LLLT so that cytokine levels could be measured by ELISA. Tissue fragments were harvested for morphometric, histomorphometric, and RT-qPCR analyses. The morphometric analysis revealed a greater decrease in the wounded area in G1 when compared with G2, whereas in G3, the improvement in the area was greater when compared with G4. Finally, the histomorphometric analysis showed that G1 was the group closer to G5 in terms of collagen fiber count. G2 and G4 had higher amounts of collagen fibers than G5 while G3 had a lower quantity. The use of the transcutaneous LLLT in the current study influenced the wound healing process.

Keywords Low-level laser intensity · Wound · Healing · Inflammation

Introduction

The skin is an organ with numberless complex physiological processes, among which one may mention wound healing, a mechanism that is divided into tissue repair (characterized by the regeneration of the injured tissue) and tissue restoration (defined as the replacement of the injured tissue with scar tissue) [1].

In the process of tissue restoration, there will inevitably be a sequel as the end result; however, such a cicatricial sequela may be replaced by an integral skin-like tissue. In both (tissue restoration and tissue repair) processes, a healing process is

divided into four phases, namely hemostasis, inflammation, proliferation, and remodeling. Each step consists of complex biochemical processes which result in the participation and interaction of endothelial cells, platelets, neutrophils, macrophages, pro- and anti-inflammatory cytokines (TNF- α , IL-1, IL-2, IL-6, IL-8), growth factors (PDGF, EGF, FGF, VEGF), proteoglycans, and prostaglandins, among other thousands of molecules and cells [2].

In the event of abnormalities in the wound healing process, such as excessive inflammation or extensive/constant traumas, unaesthetic scars may occur. Among them, atrophic, hypertrophic, and/or keloid scars can be mentioned. Furthermore, with associated pathologies, the tissue injury may not heal, and in such cases, the use of therapeutic resources is recommended in an attempt to boost the wound healing process [3].

Some of these resources can be used for the inflammatory control, time optimization, and healing quality. The low-level laser therapy (LLLT) is one of these methods. The term LASER stands for “amplification of light by stimulated emission of radiation.” In addition, the term “low level” refers to the category with therapeutic actions while the high-intensity

✉ Fernando Luiz Affonso Fonseca
profferfonseca@gmail.com

¹ Faculdade de Medicina do ABC, Laboratório de Análises Clínicas, Av. Príncipe de Gales, 821, Santo André, SP 09060-650, Brazil

² Laboratório de Histomorfologia, Universidade São Judas Tadeu, R. Taquari, 546, São Paulo, SP 03166-000, Brazil

³ Departamento de Ciências Biológicas, Universidade Federal de São Paulo, R. Prof. Artur Riedel, 275, Diadema, SP 09972-270, Brazil

lasers have destructive actions: cutting, vaporizing, ablating, and/or coagulating a biological tissue. This resource deals with parameters like wavelength, intensity expressed in watts (W), energy expressed in joules (J), the use of different techniques (such as punctual, sweep, intravascular), and the application on acupuncture points [4].

Intravascular laser irradiation of blood (ILIB) is an invasive application technique of the LLLT developed in Russia. It consists of the insertion of an optical fiber into a vascular access catheter, which is then placed in a vein in the upper arm. However, a non-invasive ILIB technique has been created, also known as modified ILIB, which comprises the connection of the LLLT to a wristband that is placed over the radial artery, resulting in benefits that will occur in the circulatory system through the light absorption by chromophores in the blood (hemoglobin and myoglobin), directly (ILIB) or indirectly (modified ILIB) [5].

Pathologies that encompass large body areas, although they may be applied punctually, are often treated with scanning by ILIB or transcutaneous ILIB. The techniques of ILIB and transcutaneous ILIB are even more desirable in those cases where systemic effects are intended and with emphasis on the circulatory, immune, and endocrine systems [4]. The ILIB technique is applied through the use of an optical fiber inserted into a catheter with access to a blood vessel in order to irradiate the blood, distributing the energy systemically throughout the body. In addition, the transcutaneous ILIB technique is modified from the previous one by making use of a bracelet where the laser is inserted, without the need to break the skin [4].

The use of LLLT can bring many beneficial results. Some of them are volemic balance, normalization of lipoprotein blood transport, decrease in the possibility of platelet aggregation, improvement in the immune system, regulation of inflammatory mediators, mitochondrial ATP synthesis, mobility of fibroblasts, collagen synthesis, synthesis of endogenous antioxidants, neovascularization, oxygen supply, systemic and peripheral metabolism, the amount of red blood cells, and improvement in microcirculation [6–8].

When applied, these physiological effects can be used in the treatment of a great variety of diseases and soft tissue conditions like, among others, neuropathies, autoimmune diseases, cerebrovascular accidents, severe chronic injuries, or wounds and burns [9]. Due to conflicting results and the lack of standardization of the LLLT parameters used in the articles, this resource is not currently used in large scale within the health area. Parameters such as application interval, application time, intensity, power, and wavelength are some data that usually vary between studies, so there is a difficulty in accepting this feature. Both techniques, ILIB and transcutaneous modified ILIB, have not been frequently studied or published given the little credibility they have among researchers and health professionals [4]. Therefore, the aim of the current

study is to analyze the action of the transcutaneous low-level laser therapy in the initial phase of the wound healing process.

Material and methods

This study was designed as a controlled clinical trial, and it was carried out after the ethical approval of the Research Ethics Committee of the Faculdade de Medicina do ABC (FMABC)–Fundação ABC. It was performed with the participation of Dr. Giuliana Petri in the animal facility of the FMABC, and the collected samples were analyzed in the molecular biology laboratory of the same institution.

Samples

A total of 25 male Wistar rats (*Rattus norvegicus*), weighing 300–400 g, were equally divided into five groups (Table 1). For assessing the risk of bias in animal studies, the 10-item SYRCLE's tool was followed [10]. As placebo, a sham irradiation was performed. All animals were kept in polypropylene containers with stain-steel lids, and they all received filtered water and Nuvilab CR-1 chow (NuVital). The containers were stored in a temperature-controlled room (20–28 °C) under a 12:12-h light/dark (12L:12D) cycle and 60–85 humidity, with 20 air changes per h. The containers were daily cleaned with 10% sodium hypochlorite and 15% benzalkonium (Herbalvet T.A.) solutions.

Laser parameters

The LLLT equipment used in this research was Therapy XT (DMC[®] Equipment, São Carlos, Brazil) (ANVISA 80030810157) with the parameters and configurations: laser-type indium-gallium-aluminum-phosphorus (InGaAlP) diode laser, wavelength of 660 nm ± 10 nm, radiant power of 0.1 W/cm² ± 20%, power density of 3.57 W/cm², emission mode continuum, mode of treatment in contact with the skin (treatment local: on the left auricular artery), optical fiber diameter of 600 μm, beam spot size of 0.028 cm², energy density of 3.213 J/cm², and radiant energy of 90 J, with total energy of 6.426 J. Application time was of 900 s, and total treatment sessions varied according to the group treated as described above (design); therefore, the total treatment session was one session (groups 1 and 2) and two sessions (groups 3, 4, and 5) with an interval of 48 h. The technique used was the modified ILIB. The parameters are based on doses used clinically in humans, adapted by the research group for use in animals.

Surgical technique and treatment protocol

The animals were intraperitoneally anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine for the subcutaneous

Table 1 Sample distribution and group constitution

Group	Procedure
Group 1	Surgical incision followed by 1 application of transcutaneous LLLT at 600 nm 2 h after wound was induced; animals were sacrificed 2 h after the application
Group 2	Surgical incision followed by placebo transcutaneous laser application (sham irradiation); animals were sacrificed 2 h after the application
Group 3	Surgical incision followed by 2 applications of transcutaneous LLLT (2-h and 3-day intervals) at 600 nm after wound was induced; animals were sacrificed 2 h after application 2
Group 4	Surgical incision followed by 2 applications of placebo transcutaneous LLLT (sham irradiation; 2-h and 3-day intervals); animals were sacrificed 2 h after application 2
Group 5	No surgical incisions Transcutaneous LLLT applications at 660 nm on days 1 and 3; animals were sacrificed 2 h after application 2. This group represents LLLT effect control in healthy animals

surgical incision. After anesthesia, the animals were submitted to trichotomy, followed by an incision at the dorsal longitudinal subcutaneous level of 3 cm in length. After the induction of inflammation by surgical incision of 3 cm; transcutaneous LBP applications with reanesthetized animals were performed after 2 h of inflammation induction. LLLT application was performed by fixating the laser head tip directly on the skin, at the area of the left auricular artery. Each application took a total of 15 min. Aiming to reduce the animals' discomfort, IM tramadol 10 mg/kg was administered after the experiment was carried out.

At the end of the study, the animals were sacrificed in a CO₂ euthanasia chamber in compliance with the standard guidelines of the Animal Facility of the FMABC, and the carcasses were accordingly disposed of as hospital waste. The LLLT protocol fluxogram can be found in Fig. 1.

Analysis processing

With the animals still anesthetized, the collection of venous blood and the harvest of cutaneous and subcutaneous tissue fragments were performed 2 h after the treatment was done. The drawn blood was used for the analysis of inflammatory parameters by means of the enzyme-linked immunosorbent assay (ELISA) test, whereas the cutaneous fragments were used for the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) evaluation of gene expression and Masson's trichrome histological staining for further analysis, namely digital morphometry and histomorphometry.

Inflammatory parameters

The protocol for the analysis of the inflammatory parameters was followed as described below. Blood was drawn at two different times according to each group division: 2 h (G1 and G2) and 2 h and 3 days (G3, G4, G5) after the application of the transcutaneous LLLT. The inflammatory markers measured by ELISA were tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and ultrasensitive C-reactive protein (us-

CRP). Siemens reagents were used, and the material was analyzed according to the good clinical laboratory practice guidelines.

Antioxidant parameters

For qRT-PCR analysis, the gene expression of the enzymatic antioxidant markers superoxide dismutase 1 (SOD1), catalase (CAT), and glutathione 4 (GPx4) was evaluated (Table 2). The expression of the endogenous gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as the reference gene was also evaluated. The objective was the normalization of the expression of the target genes according to the expression values.

The analysis was performed by using 20 longitudinally sliced paraffin-embedded histological samples, which were placed in Eppendorf tubes and processed according to the following protocols: standard xylol deparaffinization, using the protocol from the RNeasy FFPE kit (Qiagen[®]) for RNA extraction, and the protocol from the QuantiNova Reverse Transcription kit (Qiagen[®]) for complementary DNA (cDNA) synthesis.

The initial standardization of the real-time PCR amplification occurred in a thermal cycler (Applied Biosystems 7500 Real-Time PCR Systems, Foster City, CA, USA), and the reaction volume per well was 15 μ L. The first step of the initial cyclic parameters was a holding stage at 95 °C for 10 min, followed by a cycling stage with 40 cycles of 95 °C for 15 s and subsequently 60 °C for 25 s. Finally, the melt curve stage consisted of two steps: the first at 95 °C for 15 s and the second at 60 °C for 1 min. The following was added to the qRT-PCR reaction: 1 \times SYBR Green, 0.25 μ M of each oligonucleotide, cDNA, and RNase-free deionized water. The final volume was 15 μ L.

Histological analysis

After the fragments were harvested, the samples were stored in a collection container with 10% formaldehyde solution for

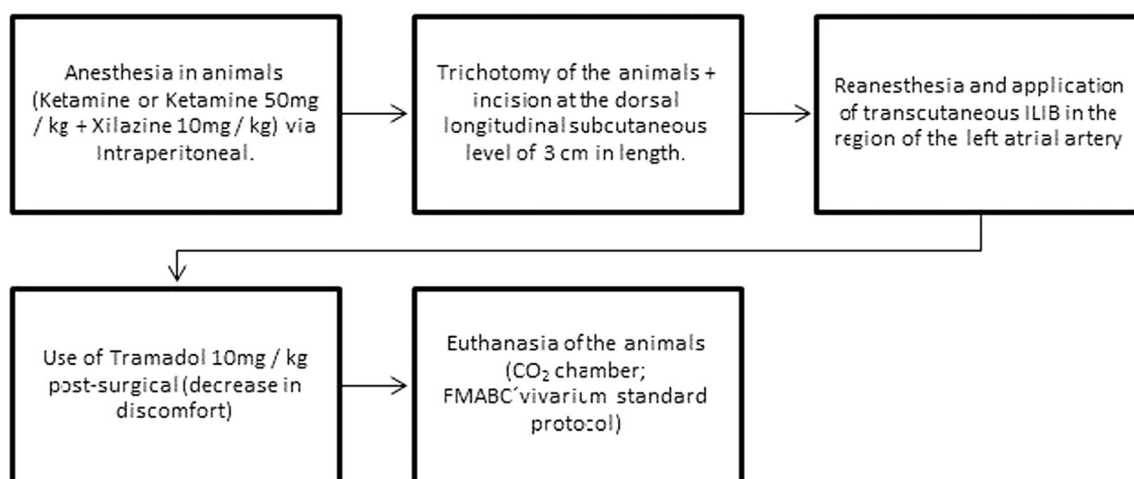


Fig. 1 Flowchart of the transcutaneous LLLT surgical and treatment protocol

24 h. Later on, dehydration, diaphonization, and paraffin embedding processes were performed in alcohol, xylol, and paraffin, respectively. The samples were cut in a microtome, fixed in slides, and then deparaffinized with xylol, alcohol, and water. They were cover slipped with xylol and Entellan and finally stained with Masson's trichrome (MT).

Morphometric analysis

For the morphometric analysis (area and perimeter), the MT-stained slides were photographed using a Sony DSC-WX50 camera. A ruler was positioned beside each animal so that the image pixels could be standardly converted to centimeters with the aid of the software ImageJ 1.49v (Fig. 2). For the morphometric analysis of the wounds, mean, standard deviation, and minimum and maximum values of the wound perimeters and areas were measured before and after the application of the transcutaneous LLLT on the auricular artery of the animals at time intervals according to the treated group.

Histomorphometric analysis

The histological slides were photographed with a Sony microcamera coupled to an optical microscope. Images were 5× zoomed and transferred to the computer using the software ZEN 2. For the histomorphometric analysis of the images, the

ImageJ 1.9v was used. For the standardization of the images, a proximal quadrant was delimited to the edges of the wound, measuring 4 × 5 ruler grids and adjusted to 50,000 pixels, totaling an area of 10,416,51 pixels (Fig. 3).

The histomorphometric analysis was performed according to the digital images of the left and right proximal segments of the edges of the wounds from the epidermal and dermal tissues. A total of eight samples were set aside for G1 and ten samples for groups 2, 3, 4, and 5 (Table 3). The analysis identified the following structures in the samples: stratum corneum disjunctum, empty spaces, follicular unit, sebaceous gland, epidermis (stratum corneum compactum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale), and collagen fiber. For the histomorphometric data collection, the comparison was established between the 1-day treatment group (G1) and the 1-day placebo group (G2), the 3-day treatment group (G3), and the 3-day placebo group (G4). Finally, all the groups were compared with the baseline provided by the control group (G5).

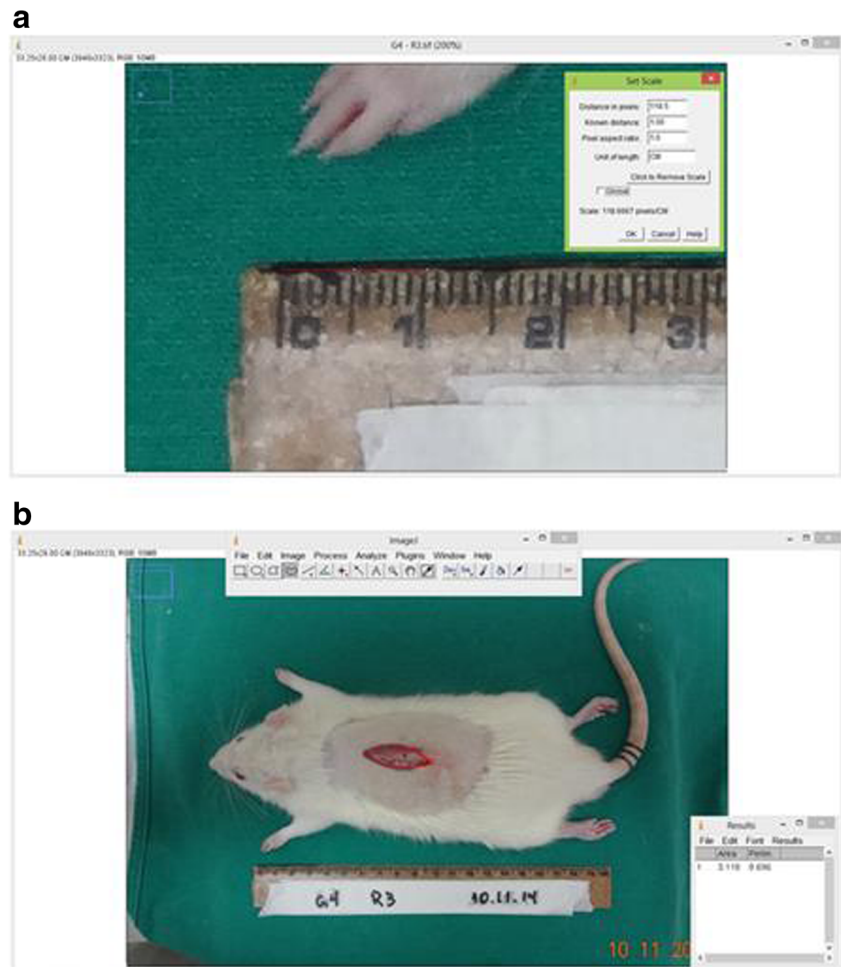
Statistical analysis

Quantitative morphometric variables with normal distribution were expressed as means, standard deviation, and minimum and maximum values (Shapiro-Wilk, $p > 0.05$). Student's *t* test was used to analyze the association between groups and the

Table 2 Primer sequences and amplicons used in molecular analysis

Gene	Sequences (5'–3')	Amplicon (bp)
Superoxide dismutase	F - GCGTCATTCACTTCGAGCAG	191
	R - CCTCTTTCATCCGCTGGAC	
Catalase	F - GCGGATTCTGAGAGAGTGG	188
	R - GAGGGTCACGAACTGTGTCA	
Glutathione peroxidase 4	F - CCTCGCAATGAGGCAAAACC	249
	R - CCCTTGGGCTGGACTTTCAAT	

Fig. 2 a Pixel conversion into centimeters: adjust to 1 cm. Standardization in centimeters and the measuring of the area and perimeter were carried out according to the following protocol: straight tool ► measuring of 1 cm on the ruler ► analyze ► set scale ► know distance ► adjust in 1 ► unit of length ► adjust in cm ► OK ► freehand selection tool ► 200% zoom ► demarcation of the wound from the outer edge with the help of the mouse ► analyze ► measure. **b** ImageJ protocol illustration (area and perimeter)



before and after measures of the diameter and perimeter of the area. The statistical package of choice was the Stata 11.0. For the qualitative histomorphometric variables, absolute and relative values were used to describe them. An ANOVA test was conducted to evaluate the association of the corneal layer, empty spaces, sebaceous glands, and epidermis according to the groups. Finally, the Kruskal-Wallis test was applied in order to analyze the relation between the follicular unit and collagen fibers according to the groups.

Results

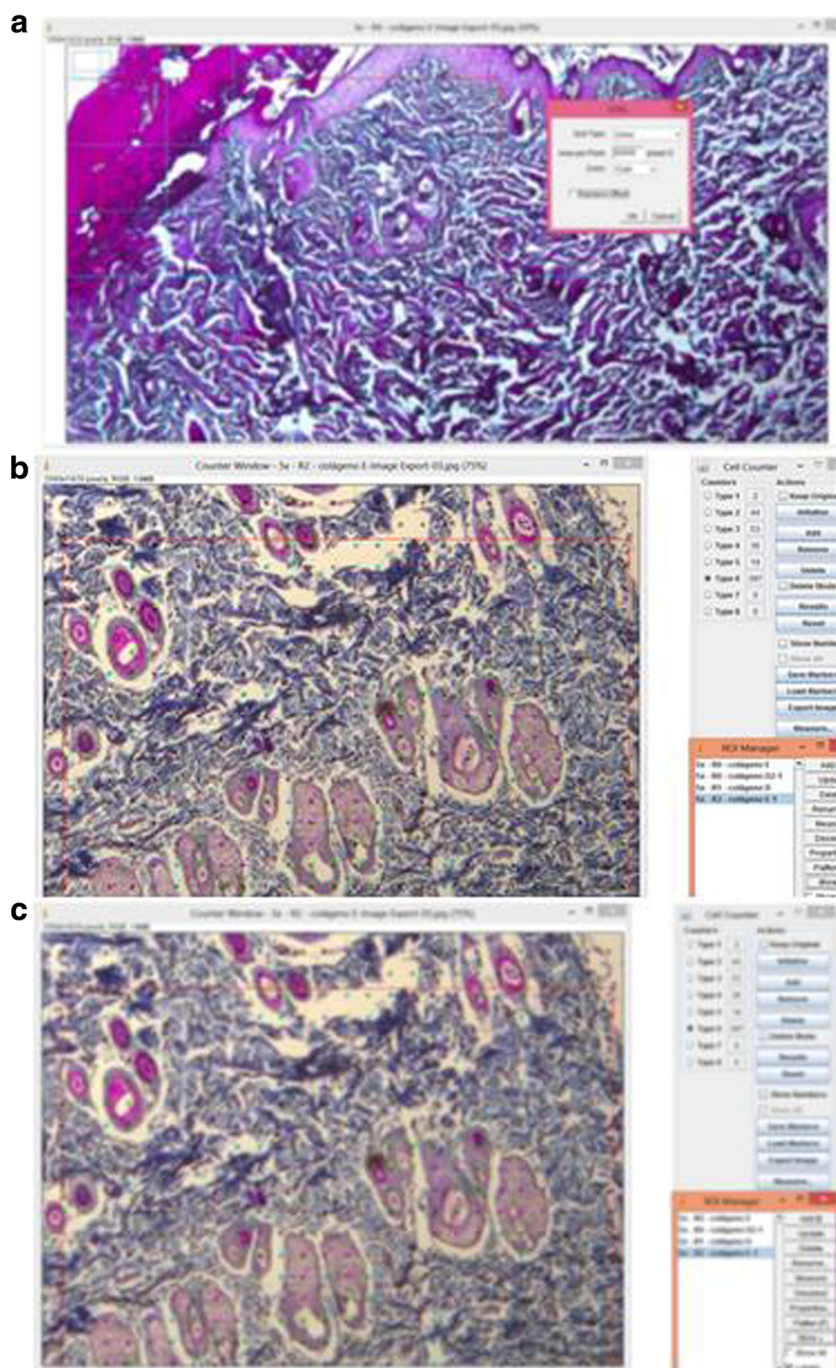
Regarding the evaluated ELISA parameters (Table 4), no statistical differences could be observed in the groups. The obtained results for the analysis of molecular parameters of the antioxidant system did not show any differences in expressions between the studied groups. Amplification of the internal gene GAPDH could be observed in all the samples. Due to the absence of target gene expression (SOD1, CAT, and GPx4), the results were not shown.

Morphometry of the wounds

The mean initial area of G1 showed a tendency to statistical significance. A reduction could be observed when compared with the mean final measurement after the treatment. On the other hand, when G2 was compared with the mean final measurement, there were no significant statistical differences. Concerning G3, when the mean initial measurement was compared with the final, a statistical significance could be found with an increase in the diameter of the area. However, G4 had a reduction in the diameter of the area when mean initial area was compared with the final (Table 5).

As to the perimeter, it can be observed that G1 showed a tendency to statistical significance once a reduction in the final mean could be seen when compared with the initial mean. Nevertheless, G2 did not present statistical differences. G3 and G4 showed statistical significance. G3 had an increase in the perimeter, despite the increase in the perimeter, revealing a smaller significance level (Table 6). All the data provided by the area-related observations indicate the overall reduction in size of the wound, whereas the perimeter data show the tensile strength of the edges required for the wound closure.

Fig. 3 a Quadrant demarcation for histomorphometric count. The following protocol was employed: plugins ► analyze ► grid type ► lines ► area per point ► 50,000 ► OK ► rectangular tool. **b** Fixation of the quadrant used for histomorphometric count. For the fixation and standardization of the delimited quadrant, the following protocol was used: image ► overlay ► to ROI manager ► place 4×5 rectangle on the area to be worked on ► click on Add (t) in the ROI Manager window. **c** Counting of the histological structures. After demarcation and fixation, the protocol for the counting of the histological structures was undertaken as follows: plugins ► analyze ► cell counter ► initialize ► straight tool ► start counting by clicking on the dots



Histomorphometry of the wounds

Upon analyzing G1, the significant statistical differences are remarkable for the stratum corneum disjunctum, sebaceous gland, and epidermis when compared with G2. In G1, a greater number of these structures could be observed (Table 7). When G3 and G4 were compared, it was observed that the stratum corneum disjunctum and empty spaces were more present in G3. However, there was a decrease in the sebaceous gland and epidermis in G3 when compared with G4. Finally,

when G1, G2, G3, and G4 were compared with G5, it could be observed that G1 was the closest when the epidermis was analyzed; G3 stood out concerning the corneal layer; G2 was closer to G5 in relation to empty spaces and to G4, in regard to sebaceous gland. In sum, there was no predominance of any specific group concerning the evaluated structures (Table 7).

When it came to the follicular unit, there was a statistical difference between G1 and G2. G1 had a higher median of this structure when compared with G2. G3 and G4 also showed

Table 3 Group constitution and skin structure characterization

Characteristics		
	<i>n</i>	%
Group		
G1	8	16.8
G2	10	20.8
G3	10	20.8
G4	10	20.8
G5	10	20.8
	Mean (SD)	Minimum–maximum
Stratum corneum disjunctum	0.05 (0.03)	0.0–0.18
Empty spaces	0.12 (0.06)	0.0–0.32
Follicular unit	0.07 (0.05)	0.0–0.19
Sebaceous gland	0.01 (0.02)	0.0–0.06
Epidermis	0.04 (0.03)	0.0–0.12
Collagen fiber	0.69 (0.03)	0.53–1.0

SD standard deviation

differences, but it was G4 that had a higher median when compared with G3. G1 was the group with the median closer to control (G5) (Table 8).

In relation to the collagen fiber, G1 had a lower median value when compared with G2; however, it was the group closest to G5. Regarding G3 and G4, it could be observed that G3 had a lower median when compared with G4 and G5. On the other hand, G4 showed a median value higher than that of G5 and closer to that of G2 (Table 8).

Discussion

The aim of the current study was to evaluate the effects of transcutaneous low-level laser therapy (modified ILIB) on the inflammatory and healing phases of skin wounds inflicted on rats. The evaluation started with the quantitative measurement of inflammatory mediators (cytokines) and enzymatic endogenous antioxidants. Then, the quantities of collagen fibers, sebaceous glands, blood vessels, empty spaces,

Table 4 Inflammatory markers quantified by ELISA

Groups	TNF- α Mean (pg/mL)	IL-6 Mean (pg/mL)	us-CRP Mean (mg/L)
G1	< 4.00	< 2.00	< 0.30
G2	< 4.00	< 2.00	< 0.30
G3	< 4.00	< 2.00	< 0.30
G4	< 4.00	< 2.00	< 0.30
G5	< 4.00	< 2.00	< 0.31

G1 = 8 animals, G2 to G5 = 10 animals each

Table 5 Comparison among groups according to the measurements of the wound diameters before and after

Variables	Diameter of the area		<i>p</i> *
	Before Mean (95% CI)	After	
G1	2500.5 (1610.5; 3390.5)	2168.2 (1373.0; 2963.5)	0.061
G2	2697.6 (2037.5; 3357.7)	2563.8 (1769.5; 3358.1)	0.085
G3	3046.0 (2410.4; 3681.6)	3179.0 (2810.0; 3548.0)	0.391
G4	2722.0 (2069.1; 3374.9)	2505.4 (2041.7; 2969.1)	0.525

G1 = 8 animals; G2 to G4 = 10 animals each

95% *CI* confidence interval of 95%

*Paired Student's *t* test

epidermal tissue, and stratum corneum disjunctum were measured, followed by the wound area and perimeter measurement.

Regarding the endogenous antioxidant response of the enzymes superoxide dismutase (SOD), CAT, and GPx, no differences could be observed when the groups were compared. According to Kurahashi and Fujii [11], antioxidant enzymes support redox homeostasis by balancing the levels of reactive oxygen species (ROS) and antioxidants. The excess of ROS may lead to damaging results, such as exacerbation in the inflammatory response or even a delay in wound healing. As a counterpoint, the excess of the antioxidants SOD, CAT, and GPx may also make the wound healing process more difficult [11].

Silveira et al. [12] conducted a research that contrasts the results found in this study. It was carried out with rats incised with 8-mm-diameter wounds, followed by two applications of helium-neon (HeNe) laser at 1 J/cm² (2 s) and 3 J/cm² (6 s) at intervals of 2, 12, 24, 48, 72, 96, and 120 h after the wound incision. An improvement in the wound healing process and a decrease in SOD and CAT activities suggested a decrease in the inflammatory process and a decrease in the demand of these enzymes.

Table 6 Comparison among groups according to the perimeter of the area measured before and after the treatment

Variables	Perimeter of the area		<i>p</i> *
	Before Mean (95% CI)	After	
G1	8327.0 (6975.3; 9678.7)	8088.2 (6466.6; 9709.9)	0.074
G2	8150.8 (7373.2; 8928.4)	7893.8 (7024.8; 8762.8)	0.133
G3	8380.4 (7694.1; 9066.7)	9815.4 (9153.2; 10,477.6)	< 0.001
G4	8089.8 (7065.8; 9113.8)	9166.2 (8251.1; 10,081.4)	0.035

G1 = 8 animals; G2 to G4 = 10 animals each

95% *CI* confidence interval of 95%

*Paired Student's *t* test

Table 7 Association of the corneal layer, empty spaces, sebaceous gland, and epidermis between groups

Groups	Corneal layer Mean (95% CI)	<i>p</i> *	Empty spaces Mean (95% CI)	<i>p</i> *	Sebaceous gland Mean (95% CI)	<i>p</i> *	Epidermis Mean (95% CI)	<i>p</i> *
G1	20.6 (11.1; 30.1)	0.041	76.1 (49.0; 103.3)	0.060	18.2 (9.42; 27.1)	0.014	18.1 (13.8; 22.4)	0.007
G2	18.0 (11.1; 24.9)		70.4 (52.4; 88.3)		7.4 (3.4; 11.6)		13.7 (10.3; 16.9)	
G3	42.4 (19.5; 65.3)		89.0 (62.5; 115.4)		7.1 (2.6; 11.6)		24.7 (12.3; 37.1)	
G4	23.7 (12.7; 34.6)		49.6 (37.9; 61.3)		12.0 (7.0; 17.0)		32.6 (21.8; 43.4)	
G5	32.7 (22.4; 43.0)		66.2 (44.1; 88.3)		10.9 (7.1; 14.7)		19.3 (16.3; 22.3)	

G1 = 8 animals; G2 to G5 = 10 animals each

95% CI confidence interval of 95%

*ANOVA

Ribeiro et al. [13] analyzed the activity of endogenous enzymatic antioxidants with the use of low-intensity laser (600 nm, beam area of 0.04 cm², output power of 40 mW, power density of 1 W/cm², and total energy of 3.2 J for 10 s). Comparing with the period prior to the wound incision, a decrease in SOD activity could be observed 1 day after the laser application. However, there was an increase in SOD activity in the protocol of application before and after the wound was inflicted on days 3 and 7. Catalase activity increased in the treated group with the application of LLLT before and after the wound 1, 3, and 7 days later. Finally, no differences in GPx activity could be observed within the compared groups.

Chen et al. [14] reveal that the synthesis of the endogenous enzymatic antioxidants may be attributed to the nuclear factor kappa B (NF-κB) action, which is activated from ROS. NF-κB is responsible for the release of different molecules such as SOD. Therefore, a pro-oxidant effect of the LLLT with ROS release in the short run and an antioxidant response in the long run may be hypothesized, thus suggesting longer treatment times with the objective of analyzing the modulation of the enzymatic antioxidant agents since in this research, the treated, placebo, and control groups did not present differences in the levels of these molecules, before and after and not even between the groups.

Table 8 Association between follicular unit and collagen fibers according to the groups

Groups	Follicular unit Median (95% CI)	<i>p</i> *	Collagen fiber Median (95% CI)	<i>p</i> *
G1	44.0 (29.0; 65.1)	0.485	373.5 (321.0; 412.6)	0.091
G2	29.0 (15.1; 81.8)		404.0 (365.5; 438.7)	
G3	29.5 (22.0; 45.4)		350.5 (310.5; 374.9)	
G4	37.0 (16.6; 65.0)		390.0 (326.6; 444.2)	
G5	47.0 (31.2; 63.2)		365.0 (337.0; 418.2)	

G1 = 8 animals; G2 to G5 = 10 animals each

95% CI confidence interval of 95%

*Kruskal-Wallis

Still on the subject of inflammatory process, the results found in the current study show similar levels of cytokines among the treated and non-treated groups. Nevertheless, Rambo et al. [15] compared the levels of TNF-α, IL-1β, and IL-10 in young rats incised with 8-mm-diameter wounds, with and without the application of LLLT, and in old rats under the same conditions. The parameters used for the laser application were the following: wavelength of 600 nm, output power of 30 mW, power density of 1.07 W/cm², beam spot area of 0.028 cm², fluence of 72 J/cm², and total energy of 2 J. It was observed that the administration of LLLT contributed to the decrease in the expression of IL-1β and TNF-α when treatment and control groups were compared in the young and old samples 3 days after wound was inflicted. On the other hand, a significant increase in the expression of IL-10 was seen in the treated groups when compared with both young and old control groups on days 3, 7, and 14 after the wound was inflicted.

Lima et al. [16] evaluated rats treated with LLLT (670 nm, 0.031 W/cm², 9 mW, beam spot area of 0.28 cm²) and non-treated rats, both groups with 6-mm-diameter wounds. Through the ELISA test, a significant decrease in levels of IL-1β and IL-6 was observed 6 and 12 h after the treatment when compared with the control group. However, TNF-α and IL-10 levels were not significantly different among the groups at different time intervals, and it is hypothesized that in this research, as in Lima et al. [16], the inflammatory level of the lesions was not enough to generate perceptible systemic alterations.

Yet, from a morphometric viewpoint, the results here described showed an improvement in the wound in the 1-day treatment group when compared with the 1-day placebo group. However, the 3-day treatment group showed inhibition and a resultant worsening in wound healing when compared with the 3-day placebo group.

Gupta et al. [17] evaluated the action of laser (635 nm, 4 J/cm², 10 mW/cm²) on partial thickness dermal abrasion of 1.2 × 1.2 cm in rats inflicted with an electric clipper. The obtained results showed contraction of the wounded area on day 1 in relation to the control group, which only showed some improvement from day 4 on.

From a histomorphometric viewpoint, the results obtained in group 1 of the current study showed a quantity of collagen fibers close to the quantity in the control group (group 5, unwounded), whereas group 2 had higher quantities when compared with group 1 and control. Gupta et al.'s results [17], such as an increase in collagen deposition, neovascularization, and the re-epithelialization stimulated by LLLT, support the results here found. Still, the work of Huang et al. [18] reports that therapies with low levels of energy and intensity of the laser are usually more effective in wound repair than those with higher levels. Actually, higher doses of laser end up bringing inhibitory responses or no responses at all, and the former case could be observed in groups 3 and 4 of this study.

Although spot-shaped LLLT is clinically used and has positive results in animals and humans, there are still variables that result in negative or no response, especially because of the configuration parameters of the equipment that still creates a lot of confusion. Density of energy, energy, total energy, and output energy are some of the nomenclatures that are used without standard definitions creating confusion in the interpretation of a study. Like these parameters, some others also generate confusion, bringing limitations as to the standardization of doses to be used to achieve positive therapeutic effects.

Further, regarding the confounding of the configuration parameters, negative effects can be observed when used, for example, at high doses, where inhibitory effects occur, retarding wound healing. Still, there is no standardization if high energy doses with larger intervals between sessions are better than low energy doses with smaller intervals. Empirically, in clinical care, we believe that lower doses with smaller intervals are better.

For ILIB and transcutaneous ILIB, the commonly used parameters are wavelengths from 365 to 635 nm, times from 7 to 20 min or greater than 20 min, and powers of 1.5 up to 150 mW [19, 20]. Therefore, it is possible to observe that the parameters are still very variable, requiring further studies aimed at standardizing the doses used, with emphasis on the differences between them in animal and human studies in order to avoid null or undesirable effects when using doses of animals in humans or from humans to animals.

In addition, the biological effects promoted by ILIB initially occur from the absorption of light by the cytochrome c oxidase (Cox) of blood cells, releasing components such as nitric oxide (NO), responsible for vasodilation and consequently increased tissue oxygenation [22, 23]. Another structure that has its activity stimulated facilitating the metabolic changes is the cell membrane. In convergence, all these effects result in increased cellular metabolism, i.e., increase in ATP, reactive oxygen species (EROS), and cellular signaling [6, 21, 22]. It is also seen that the ILIB promotes changes throughout the blood (lipids, red blood cells, platelets) and also in the structure of the vascular wall, resulting in a systemic therapeutic effects [21]. Increase in the amount of red blood cells,

granulocytes, lymphocytes, neutrophils, basophils, and eosinophils; reduction in erythrocyte sedimentation rate; and increase in cell activity and enzymes are some of the possible effects promoted by ILIB.

In summary, analysis of other cellular and molecular markers (such as morphology of red and white blood cells; nitric oxide dosage; NF- κ B; interleukins 1, 2, 8, and 10; and growth factors) to ensure an overview of the effects of transcutaneous LLLT on the organism is suggested.

Conclusion and summary

In sum, the findings of this study reveal that the levels of endogenous enzymatic antioxidants as well as the levels of inflammatory mediators did not show any differences among the analyzed groups, not even when the comparison was between the incised-wound groups and control. Moreover, these levels did not suffer any influence from the transcutaneous low-level laser therapy (modified ILIB).

On the other hand, regarding the evaluation of the histopathological and morphometric parameters, the treated groups showed the influence of the transcutaneous LLLT when compared with the placebo groups. Surprisingly, the 1-day treatment group was the one with levels of collagen fibers closer to ideal; therefore, new studies should be conducted with different laser doses, and other biochemical markers should be analyzed so that the effects of transcutaneous LLLT can be better elucidated.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Not applicable.

References

1. Reinke JM, Sorg H (2012) Wound repair and regeneration. *Eur Surg Res* 49:35–43
2. Olczyk P, Mencner L, Vasev KK (2009) The role of the extracellular matrix components in cutaneous wound healing. *BioMed Res Int* 747584
3. Wolfram D, Tzankov A, Püzl P et al (2009) Hypertrophic scars and keloids: a review of their pathophysiology, risk factors, and therapeutic management. *Dermatol Surg* 35:171–181
4. Hamblin MR, Sousa MVP, Arany PR et al (2015) Low level laser (light) therapy and photobiomodulation: the path forward. *Proc SPIE* 9309
5. Mikhaylov V (2009) Development and clinical applications of intravenous laser blood irradiation (ILBI). *Laser Ther* 18:69–83

6. Farivar S, Malekshahabi T, Shiari R (2014) Biological effects of low level laser therapy. *J Lasers Med Sci* 5:58–62
7. Mikhaylov V (2015) The use of intravenous laser blood irradiation (ILBI) at 630–640 nm to prevent vascular diseases and to increase life expectancy. *Laser Ther* 24:15–26
8. Rola P, Doroszko A, Derkacz A (2014) The use of low-level energy laser radiation in basic and clinical research. *Adv Clin Exp Med* 23: 835–842
9. Loreti EH, Pascoal VL, Nogueira BV et al (2015) Use of laser therapy in the healing process: a literature review. *Photomed Laser Surg* 33:104–116
10. Hooijmans CR, Rovers MM, de Vries RB, Leenaars M, Ritskes-Hoitinga M, Langendam MW (2014) SYRCL's risk of bias tool for animal studies. *BMC Med Res Methodol* 14:43
11. Kurahashi T, Fujii J (2015) Roles of antioxidative enzymes in wound healing. *J Dev Biol* 3(2):57–70. <https://doi.org/10.3390/jdb3020057>
12. Silveira PC, Silva LA, Freitas TP et al (2011) Effects of low power laser irradiation (LPLI) at different wavelengths and doses on oxidative stress and fibrogenesis. *Lasers Med Sci* 26:125–131
13. Ribeiro BG, Alves NA, Santos LAD et al (2016) Red and infrared low-level laser therapy prior to injury with or without administration after injury modulate oxidative stress during the muscle repair process. *PLoS One* 11:e0153618
14. Chen ACH, Huang YY, Arany PR et al (2009) Role of reactive oxygen species in low level light therapy. *Proc SPIE* 7165: 716502–716511
15. Rambo CSM, Silva JA Jr, Serra AJ et al (2014) Comparative analysis of low-level laser therapy (660 nm) on inflammatory biomarker expression during the skin wound-repair process in young and aged rats. *Lasers Med Sci* 29:1723–1733
16. Lima AA, Spínola LG, Baccan G et al (2013) Evaluation of corticosterone and IL-1 β , IL-6, IL-10 and TNF- α expression after 670-nm laser photobiomodulation in rats. *Lasers Med Sci* 29:709–715
17. Gupta A, Dai T, Hamblin MR (2014) Effect of red and near infrared wavelengths on low-level laser (light) therapy induced healing of partial-thickness dermal abrasion in mice. *Lasers Med Sci* 29:257–265
18. Huang YY, Chen ACH, Carroll JD et al (2009) Biphasic dose response in low level light therapy. *Dose Res* 7:358–383
19. Moskvina SV (2017) Low-level laser therapy in Russia: history, science and practice. *J Lasers Med Sci* 8(2):56–65
20. Sotoudeh A, Jahanshahi A, Zareiy S, Darvishi M, Roodbari N, Bazzazan A (2015) The influence of low-level laser irradiation on spinal cord injuries following ischemia–reperfusion in rats. *Acta Cir Bras* 30(9):611
21. Chung H, Dai T, Sharma S, Ying-Ying H, Carroll JD, Hamblin MR (2012) The nuts and bolts of low-level laser (light) therapy. *Ann Biomed Eng* 40(2):516–533
22. Hamblin MR, Huang YY (2014) *Handbook of photomedicine*. CRC
23. Avci P, Gupta A, Sadasivam M, Vecchio D, Pam Z, Pam N, Hamblin MR (2013) Low-level laser (light) therapy (LLLT) in skin: stimulating, healing, restoring. *Semin Cutan Med Surg* 32(1):41–52