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ORIGINAL ARTICLE

Comparative analysis of low-level laser therapy (660 nm) on inflammatory biomarker expression during the skin wound-repair process in young and aged rats

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Abstract The wound-healing process plays an essential role in the protective response to epidermal injury by tissue regeneration. In the elderly, skin functions deteriorate as a consequence of morphological and structural changes. This study aimed to evaluate and compare the effect of low-level laser therapy (LLLT) in cutaneous wound healing in young and aged rats. A total of 60 male rats comprising 30 young (±30 days) and 30 aged (±500 days) was used. The animals were divided into four experimental groups and underwent skin wound and/or treatment with LLLT (660 nm, 30 mW, 1.07 W/cm^2, 0.028 cm^2, 72 J/cm^2, and 2 J). Analyses were conducted to verify the effects of LLLT in the tissue repair process, in the gene expression, and protein expression of TNF-α, IL-1β, and IL-10, obtained in skin wound model. Results showed that there were significant differences between the young control group and the aged control group and their respective treated groups (LLLT young and LLLT aged). We conclude that LLLT has shown to be effective in the treatment of skin wounds in young and aged animals at different stages of the tissue repair process, which suggests that different LLLT dosimetry should be considered in treatment of subjects of different ages. Further clinical trials are needed to confirm these findings in clinical settings.

Keywords Wound healing · Low-level laser · Inflammatory mediators · Aged

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Introduction

Wound healing is a complex process that has attracted the attention of researchers for many years; research has focused particularly on factors that delay or hinder the wound-healing process. The most important failures in wound healing are those that occur during the initial stages, which can accentuate edema, reduce vascular proliferation, and decrease levels of particular cells involved in the wound-healing process, including leukocytes, macrophages, and fibroblasts [1].

Cutaneous wound healing involves numerous types of cells, tissues, cytokines, chemokines, growth factors, and proteolytic enzymes. The activities of the cells that include migration, proliferation, phagocytosis, and the synthesis of proteins that form the extracellular matrix (ECM), are tightly regulated, and it is critical that the chronological phases of wound healing that encompass hemostasis, inflammation, proliferation, and tissue remodeling, are precisely executed to achieve the best possible repair [2].

Studies dating back almost one century describe an age-related decline in the rate of repair. Many more recent studies have investigated age-related alterations in the proliferative aspects of wound repair, including keratinocyte proliferation, ECM synthesis, and angiogenesis. By contrast, age-related changes associated with the function of inflammatory cells within wounds have been less well studied [3].

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Recent evidence strongly supports the notion that molecular inflammatory processes play a pivotal role in the aging process and in age-related diseases. During aging, the generation of cyclooxygenase (COX)-derived reactive species (RS) increases and the expression of particular genes is upregulated, including interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), COX-2, and inducible nitric oxide synthase. COX activity and the production of thromboxane A2 and prostaglandin 12 also increase during aging. Other pro-inflammatory proteins, such as adhesion molecules (e.g., vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and P- and E-selectin), are all upregulated during aging [4].

Over the last 10 years, several animal and human studies have shown that low-level laser therapy (LLLT) has modulatory effects on the inflammatory markers prostaglandin E2, TNF-α, IL-1β, and plasminogen activator, that it mitigates the processes associated with inflammation, including edema, hemorrhage formation, necrosis, and neutrophil influx, and modulates the activity of leucocytes, including macrophages, lymphocytes, and neutrophils [5]. Other studies have shown that LLLT modulates and accelerates wound healing by increasing mitotic activity, activity in fibroblasts, collagen synthesis, and neovascularization, as well as reducing inflammation and oxidative stress [1, 5–9].

However, whether the healing process in aged animals is similar to that in young animals, whether aged animals respond differently to laser treatment compared with young animals, and at what age any change in response occurs, remains debatable. This study aimed to evaluate and compare the effect of LLLT in wound healing by using an experimental model developed in young and aged rats.

Materials and methods

Animals

The animals used for this study comprised 60 male Wistar rats (*Rattus norvegicus albinus*), which included 30 young rats that were aged 30 days and weighed 130–150 g, and 30 aged rats that were aged 500 days and weighed 400–450 g. The animals were obtained from the animal facility of the Universidade Nove de Julho, São Paulo, SP, Brazil, and were kept under controlled conditions of light and temperature, with free access to water and food. All experimental procedures were approved by the Institutional Research Ethics Committee (AN 0016/2011), and they were carried out in accordance with the guidelines stipulated by the Brazilian College for Animal Experimentation and the International Council for Laboratory Animal Science Standards.

The animals (30 young and 30 aged rats) were assigned to four groups, with five animals allocated per subgroup based on the sampling times, which were 3, 7, and 14 days. The four groups were as follows: G1, control aged rats, comprising older animals that only underwent skin injury; G2, treated aged rats, comprising older animals that underwent skin injury and were administered LLLT; G3, control young rats, comprising animals that only underwent skin injury; and G4, treated young rats, comprising young animals underwent skin injury and were administered LLLT (Fig. 1).

Skin-wounding operation

The animals were anesthetized with an intramuscular injection of a 7 % ketamine solution (Cetamin, Syntec, Cotia, SP, Brazil) and a 0.3 % xylene solution (Xilazin, Syntec) at a ratio of 2:1 (0.2 mL/100 g). All care was taken to avoid any discomfort to the animals. Once anesthetized, the animals were placed in the prone position, the wound site was sterilized with an alcohol-iodine solution, and they were held while the dorsum was shaved. To wound the skin, an 8-mm skin punch was performed and a circular area of skin was removed. Each animal was wounded four times with the wounds located in the middle portion of the median sagittal plane. After the wounds were dressed, the animals were placed in clean cages, with five rats in each cage, and they had free access to water and food. The analgesic, dipyrone, was administered for 2 days after the operations at a dose of 0.1 mL/animal, every 4 h.

Laser application

A DMC Laser Photon Laser III® (DMC, São Carlos, SP, Brazil) model was used, as well as radiation (Table 1). The application used a single-point transcutaneous method, with a total energy of 2 J/wound, an energy density of 72 J/cm^2, and a time of 67 s. The laser was applied immediately after wounding the skin and on alternate days after injury until the rats in each group were euthanized. The control group underwent the same wounding operation and experienced the same experimental conditions, but did not receive laser treatment. A single operator performed all of the procedures relating to the execution of the experiments.

Euthanasia

The animals were identified, weighed, and then euthanized via intracardiac administration of thiopental sodium (Cristália Itapira, SP, Brazil) at a dose of 0.05 mL/100 g body weight, 3, 7, and 14 days after injury. After euthanasia, a 1-cm margin of skin around the wound was surgically incised to the depth of the fascia and removed. The skin samples (two animals for each sampling point) were split into two, then frozen in liquid nitrogen and stored at −80 °C. One sample was used for histology, and the other sample was used to investigate protein

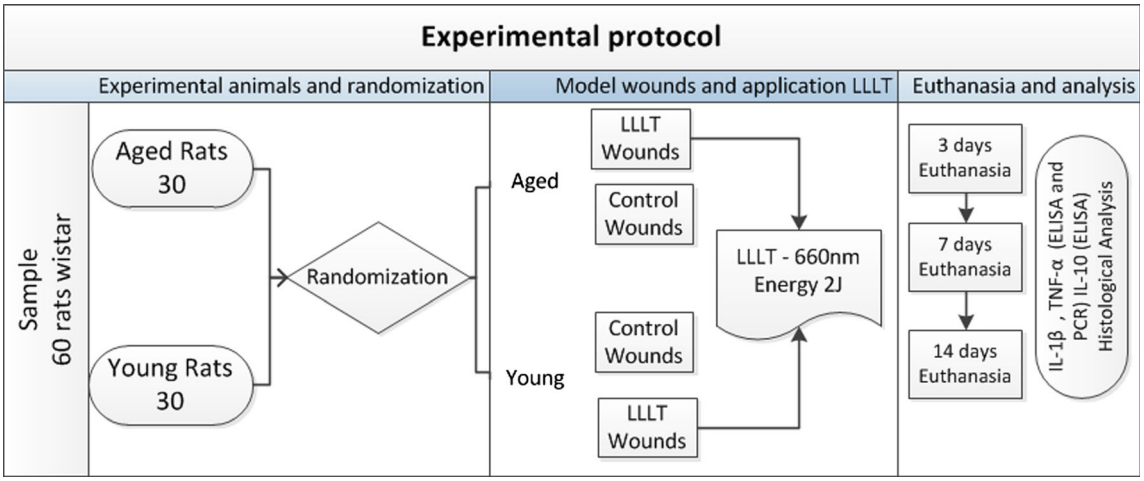

Fig. 1 Flow chart representing the experimental design

expression using the enzyme-linked immunosorbent assay (ELISA) and to examine gene expression by using the real-time reverse-transcription polymerase chain reaction (RT-PCR).

Histological procedure

Samples that had been frozen in liquid nitrogen were embedded in Tissue-Tek® OCT™ Compound (Tissue-Tek; Sakura Finetek USA), and 4-μm sections were prepared using a cryostat (Leica CM 1850, Wetzlar, Germany). The tissue sections were stained with hematoxylin and eosin.

Quantification of gene expression

Total RNA was extracted from skin wound samples and RT-PCR assays were performed to quantify mRNA expression, as described next. Thawed tissues were homogenized in TRIzol (1 mL; Gibco BRL, Gaithersburg, MD, USA) and total RNA was isolated according to the manufacturer´s instructions. Total RNA (1 μg) was used for complementary DNA synthesis and RT-PCR. Contaminating DNA was initially removed by incubating the sample for 15 min at 37 °C with DNase I (Invitrogen Life Technologies; Carlsbad, CA, USA) at 1 U/μg RNA in 20 mM Tris–HCl containing 2 mM $MgCl_2$ at pH 8.4, followed by an incubation at 95 °C for 5 min to inactivate the enzyme. Then, the reverse transcription (RT) was carried out in a 200-μL reaction volume in the presence of 50 mM Tris–HCl (pH 8.3) containing 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, and 50 ng of random primers with 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reaction conditions were 20 °C for 10 min, 42 °C for 45 min, and 95 °C for 5 min.

The reaction product was amplified with RT-PCR by using the ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the SYBR Green Core Reaction Kit (Applied Biosystems). The thermal cycling conditions were 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Experiments were performed in triplicate for each time point. Target gene mRNA was quantified as a relative value compared with an internal reference, glyceraldehyde 3-phosphate dehydrogenase, the abundance of which was considered not to change with the varying experimental conditions. The primers used for RT-PCR were IL-1β, GenBank® accession number M98820 (forward primer, 5′-CACCTCTCAAGCAGAGCACAG-3′; reverse primer, 5′-GGGTTCCATGGTGAAGTCAAC-3′) and TNF-α (forward primer, 5′-CAGAGGGAAGAGTTCCCCAG-3′; reverse primer, 5′-CCTTGGTCTGGTAGGAGACG-3′). Beta-actin was used as an internal control (forward primer, 5′-AAGATTTGGCACCACACTTTCTACA-3′; reverse primer, 5′-CGGTGAGCAGCACAGGGT-3′). For RT-PCR, 1 μL of the RT reaction volume was used [10].

Evaluation of IL-1β, TNF-α and IL-10 protein expression in wound healing

IL-1β, TNF-α, and IL-10 protein expression in the skin wounds was quantified by using ELISA according to the

Table 1 Low-level laser therapy parameters

Wavelength (nm)	Frequency	Power density (W/cm^2)	Power output (mW)	Spot size (cm^2)	Energy density (J/cm^2)	Total energy delivered (J)	Irradiation time per treatment (s)
660	Continuous	1.07	30	0.028	72	2	67

manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 96-well plates were coated with 100 μL of the monoclonal antibodies specific for the cytokines, anti-IL-1β, and TNF-α, which were diluted in sodium carbonate buffer (0.1 M, pH 9.6), and the plates were incubated at 4 °C for 18 h. To block the plates, they were washed four times with phosphate-buffered saline containing 0.05 % Tween 20 (PBST), then filled with blocking solution comprising 3 % gelatin in PBST (300 μL/well; Sigma, St Louis, MO, USA) and incubated at 37 °C for 3 h before being subjected to a new washing cycle. Next, 100 μL of the appropriately diluted samples or recombinant cytokine standard were added to the plates, and they were incubated at 4 °C for 18 h. After washing, 100 μL of the biotinylated antibodies specific for the detection of each cytokine were added to the wells, and they were incubated at room temperature (22 °C) for 1 h. The plates were washed, and then streptavidin peroxidase (100 μL) was added to the wells, and they were incubated at room temperature (22 °C) for 1 h, followed by further washes. The reaction was visualized by adding 3,3′,5,5′-tetramethylbenzidine solution (100 μL/well), which was followed by the addition of 2 N sulfuric acid (50 μL/well) to stop the reaction. The plates were read with a Spectrum Max Plus 384 spectrophotometer (Sunnyvale, CA, USA) at a wavelength of 450 nm with correction at 570 nm. The concentrations of the cytokines in the samples were calculated from the standard curves obtained from the recombinant cytokines [11, 12].

Statistical analysis

The data were tabulated using Microsoft Excel 2007 software and initially assessed for normality using the Shapiro–Wilk test. As a normal distribution was observed, ANOVA with Tukey's post hoc test was used for comparisons between experimental groups. All of the data are expressed as mean and standard deviation values. GraphPad Prism 5 software program was used. Significant differences from the null hypothesis were considered to exist when $p<0.05$.

Results

Histological analysis of samples taken 3, 7, and 14 days after injury

Photomicrographs of the sections of tissues removed from the animals in the experimental groups for histological analysis was mounted to facilitate comparisons of the tissues' responses to the different treatments received. A blinded assessor performed the descriptive analysis involving the main events of the repair process such as presence of crust, polymorph nuclear, macrophages, lymphocytes, re-epithelialization, angiogenesis, and fibrogenesis (Figs. 2, 3, and 4).

Effect of LLLT on IL-1β and TNF-α protein expression in wound healing

Expression of IL-1β and TNF-α in the healing wounds of young and aged rats treated with LLLT and euthanized 3, 7, and 14 days after injury is presented here.

LLLT administration led to a statistically significant decrease in the expression of IL-1β protein when comparing the young and aged rat control groups, 3 days after injury ($p<0.001$). IL-1β protein expression showed a significant decline in the aged rats that received LLLT when compared with the aged rat control group ($p<0.05$). Three days after injury, IL-1β protein expression in groups of young rats was similar irrespective of whether or not the animals had received LLLT. The LLLT administration led to a statistically significant decrease in the expression of IL-1β protein compared with the young and aged rat control groups, 3 days after injury ($p<0.05$). In addition, decrease in IL-1β protein expression was observed when aged rats were treated with LLLT when compared with the control group aged rat and when the young rats were treated with LLLT in comparison with the young rats control group ($p<0.05$) 7 days after injury (Fig. 5a, b). IL-1β expression 14 days after injury and the statistical analysis was similar to the analysis obtained at 7 days after injury, with apparent statistical differences between the groups of young and old animals and between both young and aged rat control groups and LLLT-treated animals ($p<0.05$). By also checking the date relating to TNF-α protein expression, both groups of rats administered with LLLT tended to show a decline in TNF-α protein expression 3, 7, and 14 days after injury (Fig. 5c). Statistically significant differences in the TNF-α expression can also be observed between the young and aged rat control groups ($p<0.05$) and between the young and aged rat groups administered LLLT ($p<0.05$), 3 days after injury. TNF-α protein expression 7 days after injury shows apparent statistical differences between the young and aged rat control groups, aged rats administered with LLLT and the aged rat control group, young rats administered with LLLT and the young rat control group, and between the two LLLT administered groups ($p<0.05$). Healing is observed 14 days after injury with an elevation of TNF-α levels in both groups administered with LLLT; However, the statistical analysis only detected differences between the control rat groups ($p<0.05$) and between the groups that received LLLT ($p<0.05$) (Fig. 5d–f).

Effect of LLLT on IL-1β and TNF-α mRNA expression in wound healing

Here, we present the results of action of LLLT on IL-1β and TNF-α mRNA expression in young and aged rats, 3, 7, and 14 days after injury. We observed a statistically significant increase in IL-1β mRNA expression, which occurred 3 days

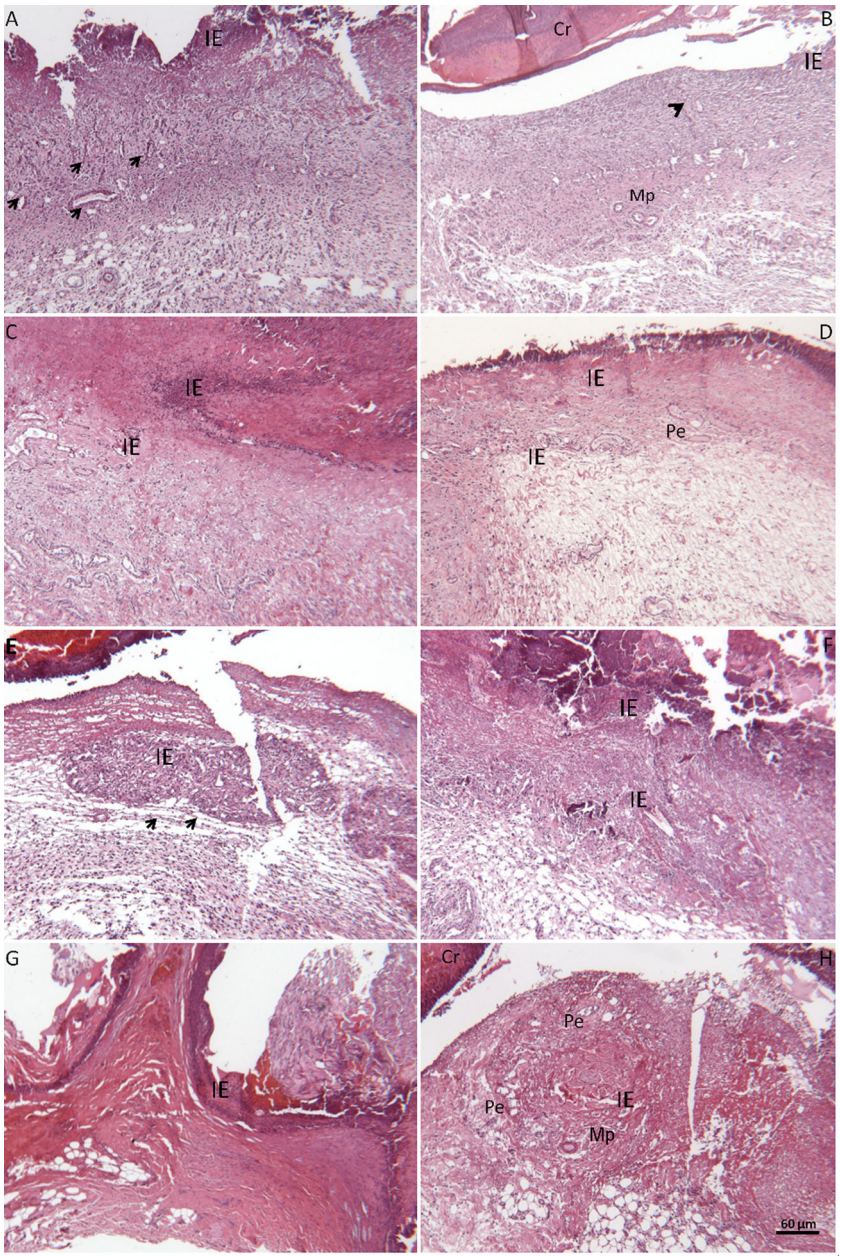

Fig. 2 Light-field photomicrographs of rat skin subjected to surgical skin wounding, 3 days after injury. **a**, **b** Skin sections from the young rat control group; **c**, **d** skin sections from the aged rat control group; **e**, **f** skin sections from the young rats subjected to LLLT; and **g**, **h** skin sections from the aged rats subjected to LLLT. Note the formation of the crust comprising fibrin and leukocytes (*Cr*) and the areas of acute inflammation accompanied by plasma exudate (*IE*). Areas of angiogenesis are apparent, indicating the process of repair (*arrowheads*). Dilated blood vessels can be seen showing intense immune cell migration, or diapedesis, and plasma extravasation (*Pe*). Chronic inflammation consisting mainly of macrophages (*M*) and plasma (*P*) cells is present. The sections were stained with hematoxylin and eosin and viewed with a×10 objective. *Scale bar*, 60 μm

after injury among the aged control group as compared with the young rat control group ($p<0.001$). A statistically significant reduction in IL-1β mRNA expression occurred 3 days after injury in the young rat group receiving LLLT compared with the young rat control group ($p<0.05$). When aged groups are compared, we observed a reduction in IL-1β mRNA expression in aged rats administered with LLLT compared with the control group aged rat and a statistically significant difference in IL-1β mRNA expression between the young and aged rat groups administered with LLLT ($p<0.05$) (Fig. 6a).

At 7 days after injury, difference between IL-1β mRNA expression was observed in the aged rat control group compared with young rats in the control group, which was statistically significant ($p<0.001$) 7 days after injury. Furthermore, 7 days after injury, the difference in IL-1β mRNA expression between aged control rats and the aged rats administered with LLLT was statistically significant ($p<0.001$). IL-1β mRNA expression 7 days after injury showed a statistically significant difference between the young and old rats administered with LLLT ($p<0.001$) (Fig. 6b). IL-1β mRNA expression 14 days after injury showed significant differences between the young and aged rat control groups ($p<0.05$) and between the aged rats and young rats receiving LLLT ($p<0.05$) (Fig. 6c).

With respect to TNF-α, mRNA expression can be seen to decrease in both groups administered with LLLT, 3, 7, and 14 days after injury. The expression of TNF-α mRNA was increased at all times in the aged rat control group with statistically significant differences apparent between the

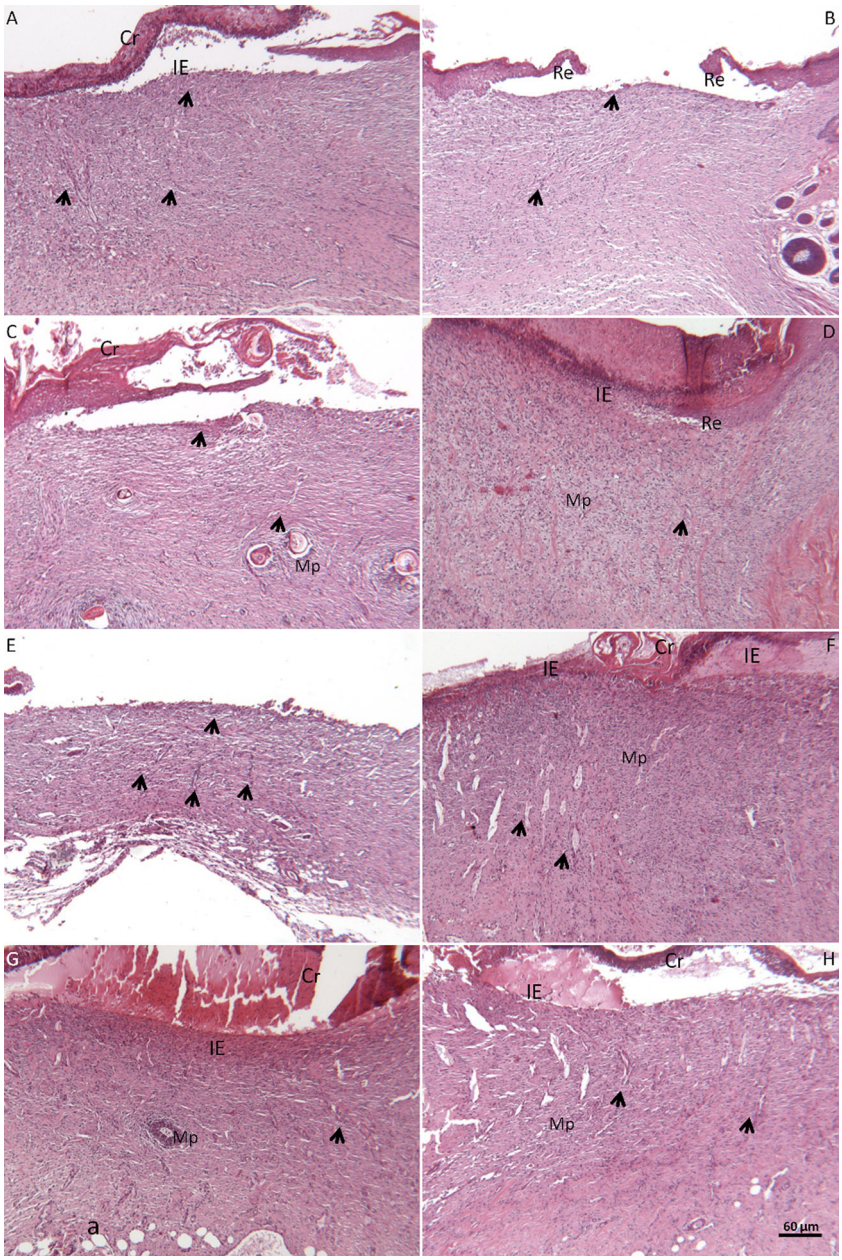

Fig. 3 Light-field photomicrographs of rat skin subjected to surgical skin wounding, 7 days after injury. **a**, **b** Skin sections from the young rat control group; **c**, **d** skin sections from the aged rat control group; **e**, **f** skin sections from young rats subjected to LLLT; and **g**, **h** skin sections from the aged rats subjected to LLLT. Note the formation of the crust comprising fibrin and leukocytes (*Cr*) and areas of acute inflammation accompanied by plasma exudate (*IE*). Areas of angiogenesis are apparent, indicating the process of repair (*arrowheads*). Dilated blood vessels can be seen showing intense immune cell migration, or diapedesis, and plasma extravasation (*Pe*). Chronic inflammation consisting mainly of macrophages (*M*) and plasma (*P*) cells is present. The sections were stained with hematoxylin and eosin and viewed with a×10 objective. *Scale bar*, 60 μm

young and the aged rat control groups. Furthermore, the differences in the expression of TNF-α mRNA between the aged rats in the control group and the aged rats administered with LLLT were observed at 3, 7, and 14 days after injury, which were statistically significant ($p<0.05$). A statistically significant difference in TNF-α mRNA expression between the young rat control group and the young LLLT administered rats, 7 days after injury (Fig. 6d–f) was observed.

Effect of LLLT on IL-10 protein expression

Here, we analyze the effect of LLLT on IL-10 protein expression. IL-10 protein expression significantly increased in the groups administered with LLLT when comparing both the young and the aged control animals ($p<0.05$). We also noted that with the exception of day 7 after injury, the aged rat control group tended to express higher levels of IL-10 than the young rat control group, but these differences were not statistically significant. Furthermore, at 3, 7, and 14 days after injury, the groups administered with LLLT statistically showed significant differences in their expression of IL-10 ($p<0.05$) (Fig. 7a–c).

Discussion

The study was designed to seek possible explanations whether the behavior of the healing process in an elderly individual

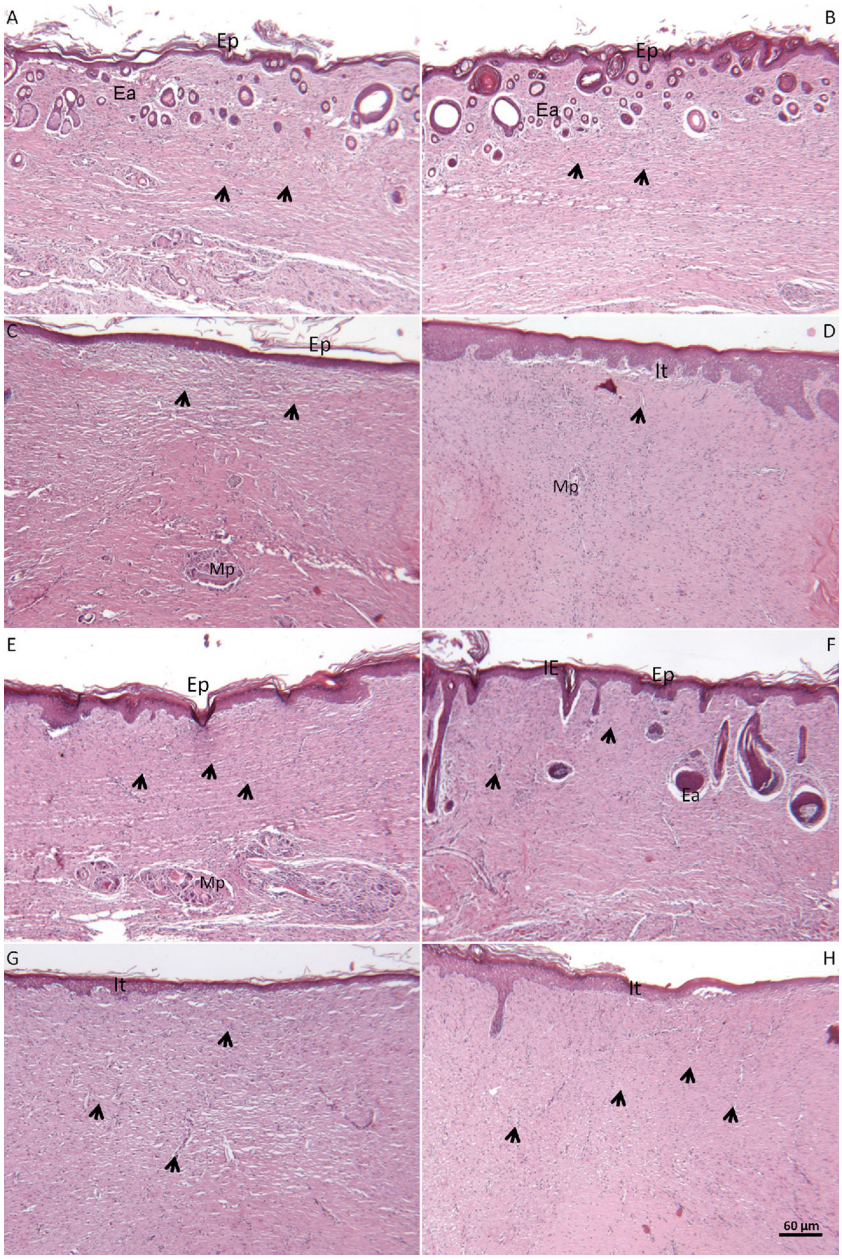

Fig. 4 Light-field photomicrographs of rat skin subjected to surgical skin wounding, 14 days after injury, showing the same visual fields. **a**, **b** Skin sections from the young rat control group; **c**, **d** skin sections from the aged rat control group; **e**, **f** skin sections from the young rats subjected to LLLT; and **g**, **h** skin sections from the aged rats subjected to LLLT. Note the complete re-epithelialization of the wound (*Ep*) showing epithelium that is typical of the skin. In some instances, the epithelium shows clear signs of increasing thickness (*It*) because of the immaturity of the skin cells in the group of aged rats. The presence of epithelial appendages, including the hair follicles, sebaceous glands, and sweat glands (*Ea*), is evident in the skin from young animals. Some isolated areas of chronic inflammation are present that comprise mainly macrophages (*M*) and plasma (*P*) cells. The tissue is undergoing processes associated with early tissue repair (*arrowhead*). The sections were stained with hematoxylin and eosin and viewed with a×10 objective. *Scale bar*, 60 μm

may respond in the same way as that of a young individual and the response of the LLLT is done differently at this stage. The study is conducted to analyze the gene and protein expressions of two pro-inflammatory cytokines (TNF-α and IL-1β) and an anti-inflammatory cytokine IL-10 and include a histological analysis that addressed three different experimental team points (3, 7, and 14 days) used as an experimental model of cutaneous wound in young and aged rats.

Limitations of the current study mainly stem from the restrictions regarding dosimetry. The absence of a detailed study with a dose–response curve could help decisively in the investigations. Although the results suggest that there is an advantage of 2-J dose-treated group for the young, we must stress that the elderly group also benefited from LLLT, i.e., a study with various doses could confirm if this trend continues. We believe that future investigations on the analysis of the expression of the transcription factor, NF-kB and IL-receptors r 1 (IL-1RA) and tumor necrosis factor receptor (TNFR) could provide a more detailed view of the action of LLLT on the modulation of pro-inflammatory cytokines IL-1 and TNF.

The main findings of the present include the following: The behavior of the repair process occurred differently between experimental groups young and aged, with obvious delay in the aged group as can be verified by histology analysis, which can descriptively also check that both groups treated with LLLT showed an increase of the repair process when

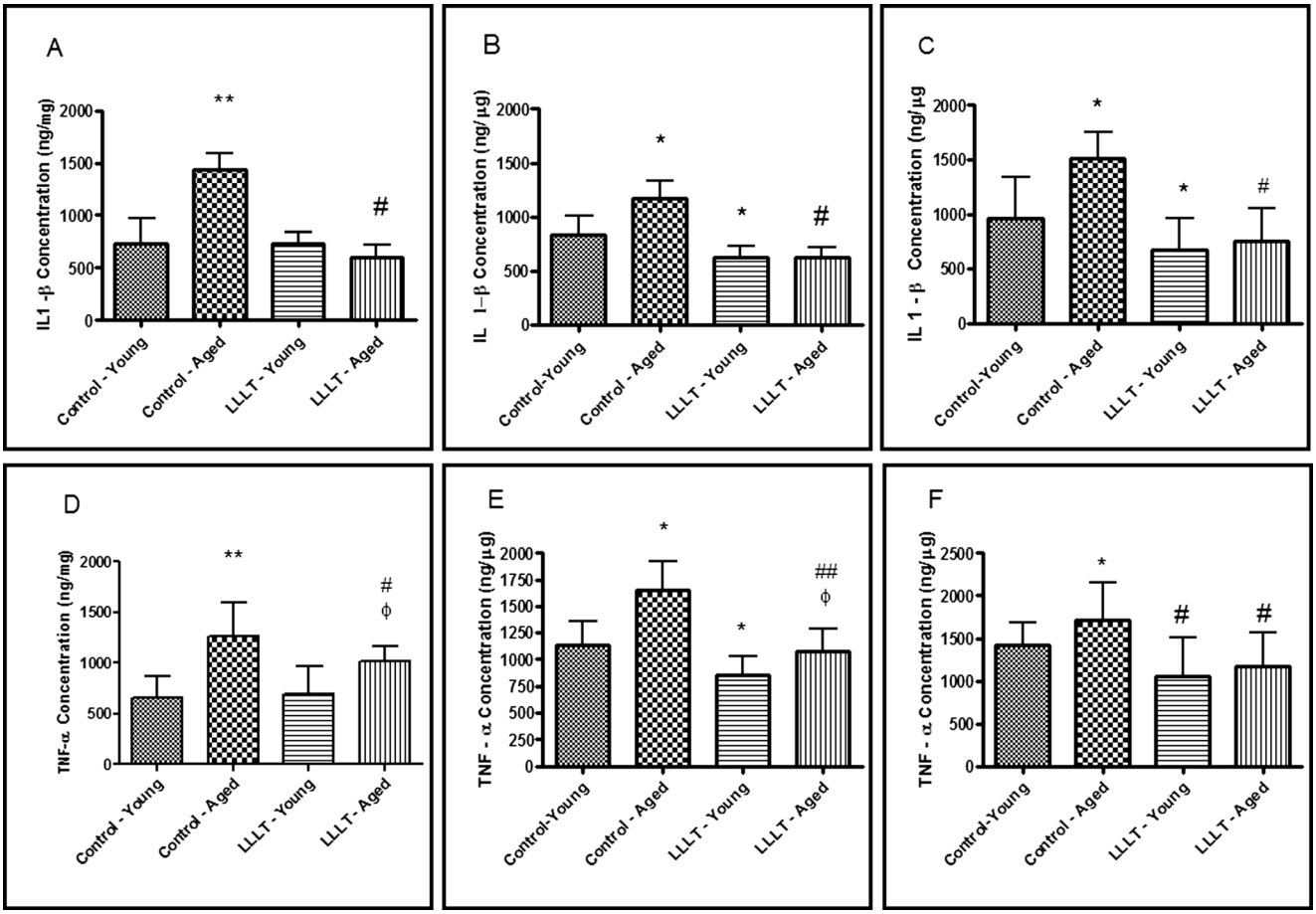

Fig. 5 Comparisons of the mean and standard deviation concentrations of interleukin-1β and tumor necrosis factor alpha obtained using the enzyme-linked immunosorbent assay. **a**, **d** Concentrations in wounds 3 days after injury; **b**, **e** concentrations in wounds 7 days after injury; and **c**, **f** concentrations in wounds 14 days after injury. $*p<0.05$; $**p<0.001$—using Tukey's test with comparisons against the young rat control group; $^{\#}p<0.05$; $^{\#\#}p<0.001$—using Tukey's test with comparisons against the aged rat control group; $p<0.05$—using Tukey's test comparing the young rat group administered LLLT with the aged rat group administered LLLT

compared with their control groups; however, the group treated with LLLT aged still showed lag mainly as regards the inflammatory phase (polymorph nuclear, macrophages, and lymphocytes) and the proliferation phase—re-epithelialization, angiogenesis, and fibrogenesis [13].

We observed that LLLT was effective in decreasing the expression (gene and protein) on the pro-inflammatory mediators (IL-1 and TNF) throughout the experimental period although we can see the increased expression of the protein of the anti-inflammatory cytokine IL-10. However, we observed a higher level of this anti-inflammatory cytokine in the young LLLT group compared with the aged LLLT group. This finding again suggests a new proposal for LLLT dosimetry, which may be used in aged animals to achieve better results.

These results confirm the findings reported by Sarkar and Fisher [14], showing increased serological levels of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and IL-8 in elderly individuals compared with younger ones.

According to Harris et al. [15], circulating levels of TNF-α are the best indicator of mortality in frail elderly people.

Histological analysis was also performed during this study, at all of the experimental time points. At day 3 and starting from the wound's edges, a noticeable epithelial thickening with visible proliferation of thin epithelium, fibroblast and macrophage-derived cells, leukocyte infiltration, and pronounced angiogenesis were observed in the young control and young LLLT groups. By contrast, in both the aged control and aged LLLT groups, the epithelium was noticeable only near the edges of the lesion, which presented intense acute inflammatory exudate and dilated blood vessels but no signs of angiogenesis.

These results confirm that the early infiltration of inflammatory cells in the young control and young LLLT groups demonstrate decelerated wound healing in both the aged control and aged LLLT groups. Moreover, these findings are in agreement with the study by Ashcroft et al. [16], which reports that decreased proliferation and migration of inflammatory cells and a consequent delay in angiogenesis contribute to slowing re-epithelialization and neovascularization during the wound-healing process in the elderly.

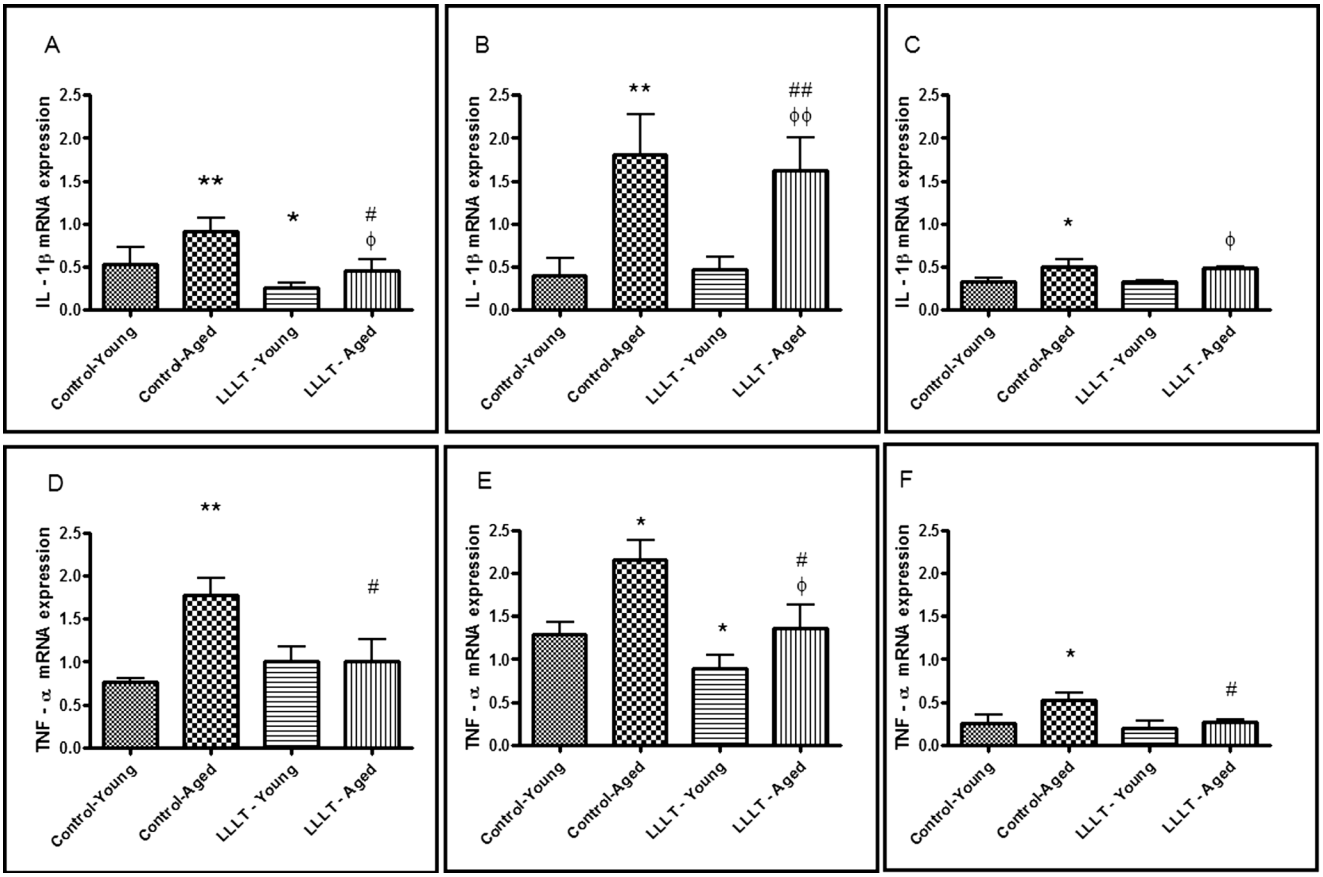

Fig. 6 Comparison of the mean and standard deviation levels of mRNA expression for interleukin-1β and tumor necrosis factor alpha, extracted from skin wounds and quantified using the real-time polymerase chain reaction. **a**, **d** Concentrations in wounds 3 days after injury; **b**, **e** concentrations in wounds 7 days after injury; and **c**, **f** concentrations in wounds 14 days after injury. *$p<0.05$; **$p<0.001$—using Tukey's test with comparisons against the young rat control group; $^{\#}p<0.05$; $^{\#\#}p<0.001$—using Tukey's test with comparisons against the aged rat control group; $p<0.05$—using Tukey's test comparing the young rat group administered LLLT with the aged rat group administered LLLT

The effectiveness of LLLT at day 3 was clear, especially in the LLLT young group compared with the control young group, thus emphasizing acceleration of the wound-healing process when higher numbers of inflammatory cells migrate early to the site of an injury, a phenomenon that occurs concomitantly with an intense presence of plasma fibrinoid

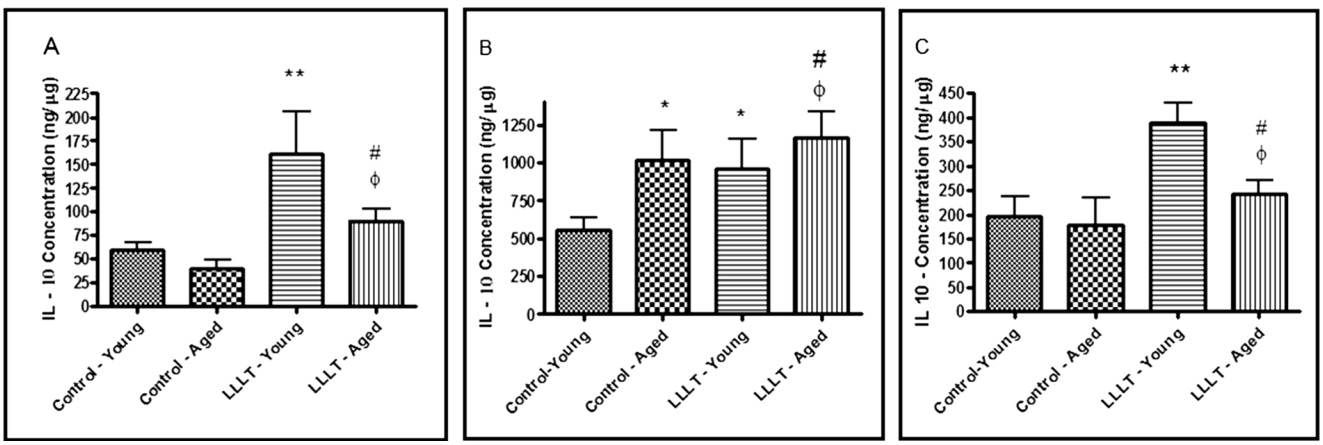

Fig. 7 Comparison of the mean and standard deviation concentrations of interleukin-10 obtained using the enzyme-linked immunosorbent assay. **a** Concentrations in wounds 3 days after injury; **b** concentrations in wounds 7 days after injury; and **c** concentrations in wounds 14 days after injury. *$p<0.05$; **$p<0.001$—using Tukey's test with comparisons against the young rat control group; $^{\#}p<0.05$; $^{\#\#}p<0.001$—using Tukey's test with comparisons against the aged rat control group; $p<0.05$—using Tukey's test comparing the young rat group administered LLLT with the aged rat group administered LLLT

exudate and areas of collagen hyalinization. The effectiveness of LLLT in our study corroborates the results obtained by Medrado et al. [17] who assessed the effects of LLLT on wound healing in rats at the 24-, 48- and 72-h time periods and concluded that animals treated with LLLT showed decreased inflammatory responses, increased collagen deposition, and enhanced proliferation of myofibroblasts in experimentally induced skin wounds.

In our study, intense angiogenesis was observed on day 7, suggesting that the healing process occurs in all groups, although it was more evident in the young control, young LLLT, and aged LLLT groups. Especially in the aged LLLT group, an intense dermal tissue repair process characterized by a marked increase in angiogenesis and re-epithelialization of the wound's edges was observed. These results are consistent with the findings published by Tacon et al. [18], who, after histological analysis, reported healing activity on day 5 as evidenced by increased angiogenesis and decreased inflammatory infiltrates in the wounds of mice treated with a 660-nm AlGaInP laser with energy densities of 3 and 6 J/cm^2.

Changes in cell infiltration in elderly individuals may affect the early inflammatory response that occurs during wound healing. In a comparative study between wounds in older and younger individuals, Ashcroft et al. [19] showed an increase in early neutrophil response, slowed monocyte influx, and increased numbers of mature macrophages.

Our results showed areas of chronic inflammation that were mainly characterized by macrophage infiltration in the aged control group on days 7 and 14. Interestingly, these events were described in a study published by Sarkar and Fisher [14], in which several indications for chronic inflammation, such as macrophage infiltration and levels of circulating pro-inflammatory chemical mediators, were reported. According to these investigators, the presence of such activated macrophages may be both beneficial and harmful in the sense that activated macrophages may also induce the release of toxic components that could damage healthy host tissues.

IL-1, IL-6, IL-8, and TNF-α, among other inflammatory mediators, belong to the chemokine family, which influences macrophage migration [14]. IL-10 is an anti-inflammatory cytokine capable of inhibiting the inflammatory response via decreasing pro-inflammatory cytokines and suppressing monocyte activation [20]. With regard to neutrophils, IL-10 inhibits macrophage chemotaxis [21].

Therefore, our results confirm the effectiveness of LLLT in promoting a decrease in inflammatory cytokines and are in agreement with studies using LLLT in various inflammatory conditions in which satisfactory chemokine mediation results were achieved [22–25].

During the wound-healing process, around day 10, the wound bed is completely filled with granulation tissue crossed by a capillary network and fully regenerated lymphatic vessels. At the end of this phase, skin appendages such as hair follicles and glands undergo controlled regeneration, and the scars become hypovascularized as a consequence of neocapillary loss [13].

In this study, on day 14, the young control and LLLT groups showed dense connective tissue with cell density (mainly fibroblasts) as well as vascular density typical of the healing process. Complete re-epithelialization of the wounds in which the epithelium returns to normal with the presence of epithelial appendages such as hair follicles and sebaceous and sweat glands was also observed. Unlike the aged control and LLLT groups, the fibroblast-derived cells resembling fibrocytes, indicative of low metabolic activity as well as the absence of epithelial appendages, suggest a decelerated re-epithelialization process. This result on day 14 demonstrated that although LLLT was effective in the young LLLT group, it was critical in the aged LLLT group.

Therefore, after analyzing all the study results, we conclude that LLLT is effective in the treatment of skin wounds in young and aged animals during different phases of the tissue regeneration process. However, the effects of LLLT obtained in the aged animals (aged LLLT group) suggest that new dosimetries should be tested to achieve better results.

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