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ORIGINAL ARTICLE

Effect of low-level laser therapy on types I and III collagen and inflammatory cells in rats with induced third-degree burns

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Abstract Low-level laser therapy (LLLT) has been increasingly used to accelerate wound healing in third-degree burns. This study investigated the effects of lasers on the tissue repair process of third-degree burns. Burns were produced on the backs of male Wistar rats. The animals were divided into four groups (n=12): control, injury, LLLT 3 J/cm^2, and LLLT 4 J/cm^2. Each group was further divided into two subgroups; the rats in one subgroup were killed on day 8 and those in the other, on day 16 after injury. The animals in LLLT 3 J/cm^2 and LLLT 4 J/cm^2 were irradiated 1 h after injury, and irradiation was repeated every 48 h. Laser (660 nm, 35 mW) treatment at fluences of 3 and 4 J/cm^2 were used. After killing the rats, tissue fragments from the burnt area were removed for histological analysis. The LLLT-treated groups showed a significant decrease (p <0.05) in the number of inflammatory cells and increased collagen deposition compared to the injury group. Laser irradiation (both 3 and 4 J/cm^2) resulted in reduction in the inflammatory process and improved collagen deposition, thereby ameliorating the healing of third-degree burns.

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Introduction

According to the Brazilian Society of Burns, 1 million burn cases are reported every year in Brazil, of which 200,000 cases are treated in emergency rooms and 40,000 require hospitalization [1]. Although third-degree burns are less common than other forms of wounds, they result in high physiological stress. The initial injury involves heat damage of the dermis and epidermis, which is followed by a coordinated pathway of initial inflammation with subsequent repair in cases of partial thickness injuries [2].

Healing is a complex process that involves a series of events, including clotting, inflammation, granulation tissue formation, epithelialization, collagen synthesis, and tissue remodeling. Thus, it has been researched extensively, particularly, regarding factors that could delay or hinder the healing process [3].

The use of low-power lasers is becoming increasingly popular in the treatment of a variety of medical conditions, including wound healing [4], because they induce the reduction of tissue edema and inflammation, increase in phagocytosis, collagen deposition and protein synthesis [5], epithelialization [6], and improves the tensile strength of tissues [7].

Considering that burns lead to metabolic, hormonal, and immunological disturbances [8], and that laser induce the acceleration of the healing process due to their effects on biostimulants, in this study, we tested the hypothesis that lasers contribute to the tissue repair process. The specific aim of the study was to investigate the effect of laser therapy at two fluences (3 and 4 J/cm^2) on tissue regeneration in third-degree burns in rats through the quantitative evaluation

of the number of inflammatory cells and percentage of collagen.

Materials and methods

Animals

All experiments were carried out in accordance with the guidelines of the Vale do Paraíba University for animal care (protocol number: A107/CEP/2007). The experiments were performed using male Wistar rats (weight, 260±20 g) that were supplied with food and water ad libitum. The rats were placed in appropriate cages and randomly divided into experimental groups with six animals per group.

Experimental groups

Forty-eight rats were used in the experiment; the rats were divided into four groups: G1—control (non-injured, non-treated), G2—injury (injured, non-treated), G3—3 J/cm^2 (injured, treated with 3 J/cm^2 LLLT) and G4—4 J/cm^2 (injured, treated with 4 J/cm^2 LLLT). Each group was further divided into two subsets, and the rats in one subgroup were killed 8 days after the injury, and those in the other subgroup, at 16 days after injury. Animals from G3 and G4 were irradiated 1 h after inducing the burn, and the irradiation was repeated every 48 h thereafter. All animals were treated in the same way. During the procedure, animals were placed on a table in the prone position and then manually immobilized.

Induction of burns

Before inducing thermal injury, the rats were deeply anesthetized by injecting 60 mg/kg of ketamine plus 5 mg/kg of xylazine intraperitoneally (i.p.). Third-degree burns were induced using a 5.3-mm thick circumferential layer of metal (aluminum) measuring 2.5 cm in diameter, which was heated to 120 °C on a heating plate with an adjustable thermostat; the temperature of the slide was confirmed using a thermometer. The blade was held on the animals' backs for 5 s. After the burning procedure, each animal received a single dose of tetracycline i.p. (Oxytetracycline, Pfizer 20 mg/kg) as infection prophylaxis. The drug fentanyl (i.p.—0.032 mg/kg) was administered at 12-h intervals for two consecutive days for analgesic prophylaxis.

Laser irradiation

The KLD Endophoton® aluminum gallium indium phosphide (InGaAlP) laser (wavelength, 660 nm; power, 35 mW) was used in this study. Animals in all treatment groups were irradiated with an energy density of either 3 or 4 J/cm^2 punctually at the edge of the lesion, at three different points (Table 1).

Morphological analyses

The animals were killed at 8–16 days after inducing burns, and the wound area was dissected for histological analysis. Skin samples were removed with a skin punch at the edge of the lesion, thus extracting both injured and healthy skin. Samples were washed with saline and fixed with 10 % buffered (0.9 %) formalin solution. After setting, the skin samples were dehydrated, cleared, and covered with paraffin. The blocks were then cut into 4-μm thick sections by using a semiautomatic microtome. The sections were stained using hematoxylin and eosin to count the inflammatory cells and using picrosirius red, an anionic composite that distinguishes the thickness and density of collagen fibers through coloration emitted under polarized light, to estimate the percentage and type of collagen fibers. While the thin dissociated fibers typical of type III collagen appear greenish, the thickest and strong fibers of type I collagen emit colors with long wavelengths, such as red and yellow. Morphometric analysis was performed on the samples by means of image digitization and computational analysis, by using an image processing and analysis program (Image-Pro plus 4.5). To quantify the areas comprising collagen, five fields observed on a Nikon Eclipse E200 (×40 lens) microscope were digitized. The microscope was coupled to an image-capturing Sanyo digital active BLC camera, which was in turn, connected to a microcomputer equipped with a video board. All images were digitized before the quantification process, thereby standardizing the microscope light intensity and condenser height. The collagen areas were identified in the image based on their color distribution.

Table 1 Low level laser therapy—LLLT parameters

	LLLT 3 J/cm^2 (G3)	LLLT 4 J/cm^2 (G4)
Laser	InGaAlP	InGaAlP
Equipment	KLD endophoton	KLD endophoton
Frequency	Continuous	Continuous
Power	35 mW	35 mW
Power density	0.05W/cm^2	0.05W/cm^2
Spot size (cm^2)	0.63 cm^2	0.63 cm^2
Energy density	3 J/cm^2	4 J/cm^2
Energy	2.1 J	2.8 J
Time per point	60 s	80 s
Number of points	3	3
Method	Transcutaneous	Transcutaneous
Total energy	6.6 J	8.4 J

The images of histological (hematoxylin and eosin) sections were obtained using a Leica® DMLB2 microscope coupled to a digital camera with ×17.5 zoom, which was connected to a computer via a card scanner having a ×10 and ×40 objectives. To optimize the recognition and counting of inflammatory cells, the ×40 objective was used and the obtained images were processed using ImageG program. To evaluate the wound contraction, the animals were photographed on the day of the burning procedure and when they were killed, using a digital camera (Cyber-Shot Sony DSC-T10) [7.2 MP, ×3 zoom]; (Fig. 1.)

Statistical analysis

The data were tabulated using Microsoft Excel 2007 software and initially assessed for normality using the Shapiro–Wilk test. As the data was normally distributed, ANOVA with Tukey's post-hoc test was used for within-group comparisons between 8 and 16 days after the injury, and for between-group comparisons among G1 (control), G2 (injury), G3 (LLLT 3 J/cm^2), G4 (LLLT 4 J/cm^2), and samples. All data were expressed as means ± standard deviation. The GraphPad Prism 5 software program was used for all analyses, with the null hypothesis rejected at p <0.05.

Results

Counts of inflammatory cells

Eight days after injury, differences were evident with respect to the mean (± standard deviation) number of inflammatory cells present in the four groups (control: 69.33±5.7; injury: 204±59.2; LLLT 3 J/cm^2: 118±11.87; LLLT 4 J/cm^2: 108.4±38.67). ANOVA and Tukey's post-hoc test comparisons revealed statistical differences between the injury group and both the LLLT-treated groups (p <0.05). Mean (± standard deviation) inflammatory cell numbers varied among samples obtained at 16 days after injury, but the variations were less (control: 73±10.3; injury: 168±36.2; LLLT 3 J/cm^2: 110±27.5; LLLT 4 J/cm^2: 123±53.6). ANOVA and Tukey's post-hoc test results showed that only the LLLT 3 J/cm^2 and control groups had statistically significant decreases in inflammatory cell counts (p <0.05) compared to the injury group (Figs. 2 and 3).

Quantification of types I and III collagen by using polarized light

As shown in Fig. 4a, at 8 days after the injury, the percentage of type I collagen differed significantly between the injury group and other groups (p <0.05). Furthermore, the percentage of type I collagen was significantly lower in the control group than that in the groups receiving laser treatment (p <0.05). Type III collagen showed an increased in both the LLLT-treated groups (3 and 4 J/cm^2) compared to the injury group, but the increase was significantly different only from the LLLT 3 J/cm^2 group. Furthermore, as shown in Fig. 4b, no significant differences were observed in the collagen percentage between the two LLLT-treated groups (p >0.05).

Sixteen days after injury, an overall decrease in the percentage of type I collagen was observed in the LLLT groups. The percentage of type I collagen was significantly lower than that in the injury group (p <0.05) and did not differ from that in the control group (Fig. 5a). As shown in Fig. 5b, green fibers (type III collagen)

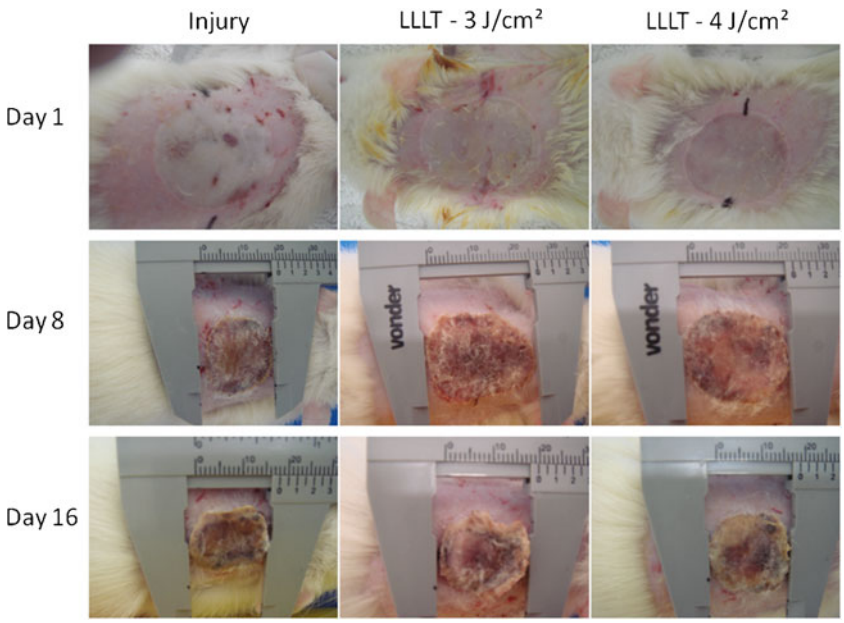

Fig. 1 Figure demonstrative clinical evolution of Burns referring to the day of confection (1 day) until the end of period (16 days)

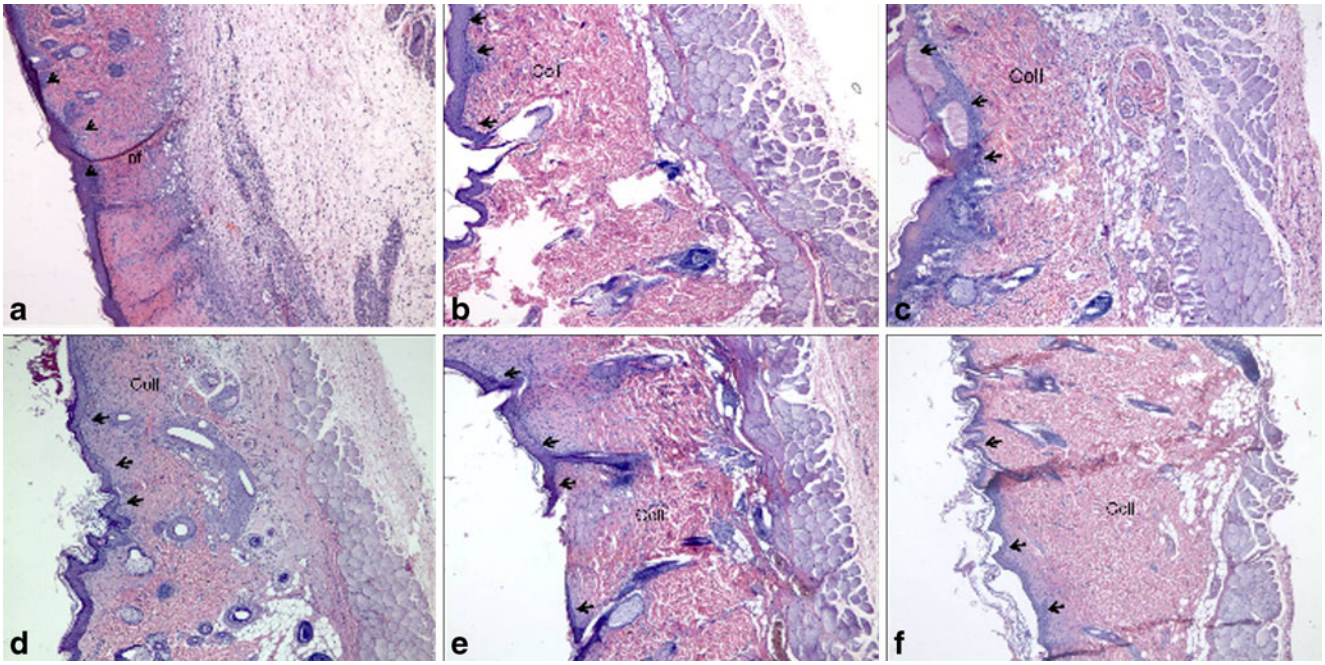

Fig. 2 Montage photomicrographs related to histological sections of the skin of animals subjected to the experimental burn. **a** Histological section refers to the group subjected injury without treatment for 8 days after the injury: observe the great loss of skin tissue and the presence of material fibrinoid-necrosis (*arrows*), the presence of intense inflammatory infiltrate (I). **b** Histological group subjected for the treatment of the lesion with laser 3 J/cm^2 at 8 days after injury: there is a smaller number of inflammatory cells at the tissue surface in (*coll*) proliferation of collagen thin. **c** Histological section for the group undergoing laser injury treatment with 4 J/cm^2 at 8 days after injury: small amount of inflammatory cells in the tissue surface rather the inflammatory infiltrate, you can see the area of skin ulceration (*arrows*) and proliferation of collagen fibers (*coll*). **d** Histological section refers to the group subjected injury without treatment at 16 days after injury: note the presence of fibrinonecrotic material (*arrows*) and presence of chronic inflammatory infiltrate. **e** Histological section refers to the group with injury-subjected laser treatment joules of 3 to 16 days after injury: proliferation of collagen (*coll*), the area of epidermis integrates with most presence of hyperplasia (*arrow*). **f** For the histological group subjected to the treatment of the lesion with laser 4 J/cm^2 16 days after injury: decrease in observing the area of skin ulceration showing the most part epidermis (*arrows*) in addition to collagen fibers in larger quantities (*coll*). Hematoxylin-eosin × 40

were rarely observed in the injury group and were significantly reduced (p <0.05) compared to the other groups. The percentage of type III collagen was similar in both the LLLT-treated groups and significantly lower than that in the control group (Fig. 6).

Discussion

In this study, we examined the hypothesis that laser therapy with a wavelength of 660 nm and fluencies of 3 and 4 J/cm^2 can facilitate wound healing of third-degree burns in an

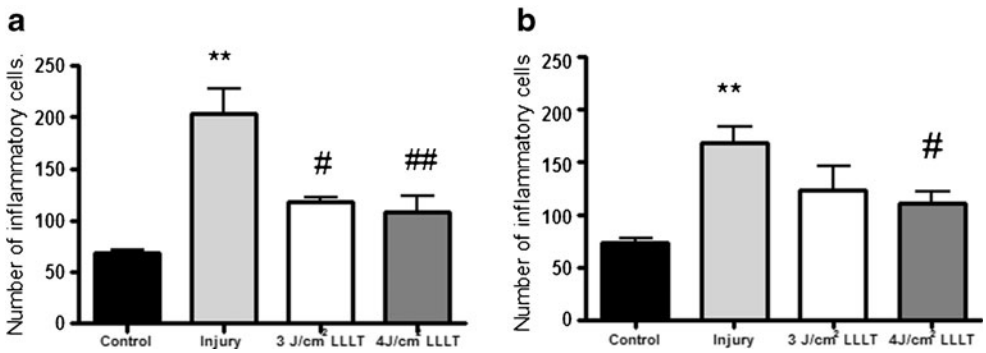

Fig. 3 **a** Means and standard deviations of the number of inflammatory cells at 8 days after the injury. Means of the groups treated using low-level laser therapy (LLLT) are significantly different (p <0.05) from that of the injury group. Furthermore, note that no difference is observed between the means of the two LLLT-treated groups (3 and 4 J/cm^2). **b** Means and standard deviations of the number of inflammatory cells at 16 days after injury. Note that the injury group is significantly different (p <0.05) from only the LLLT 3 J/cm^2 and control groups. Tukey's multiple comparison test, *p <0.05, **p <0.001

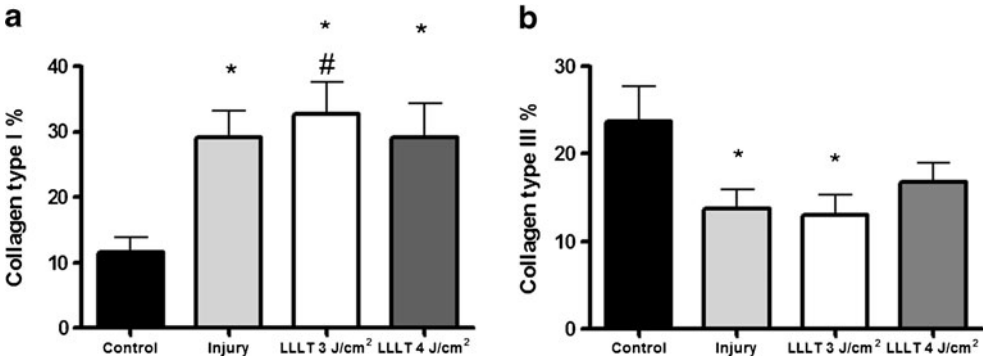

Fig. 4 **a** Means and standard deviations of the percentage of type I collagen fibers at 8 days after the injury. The injury group mean is significantly different from those of the low-level laser therapy (LLLT) groups (3 and 4 J/cm^2) and control group (p <0.05). Note that no difference is seen between the two LLLT-treated groups (p <0.05). **b** Means and standard deviations of the percentage of type III collagen fibers at 8 days after the injury. Note that the injury group differs significantly (p <0.05) from the control group and the LLLT 3 J/cm^2 group based on Tukey's multiple comparison test

animal model. The rationale for choosing two closer energy densities is the current discussion of scientific community about what is the best dose and optimal parameters for LLLT, even when doses inside "therapeutic window" are employed. Interestingly, our results showed that exposure to laser therapy can indeed accelerate the wound healing process after burning.

According to Medrado et al. [9], several experimental studies have attempted to describe the effects of lasers under various parameters with the goal of developing better outcomes in the process of tissue repair. The use of low-power lasers has increased recently in clinical applications to reduce the time of repair in skin lesions and disabling pathological conditions, such as ulcers and open lesions. The positive effects of laser therapy have led researchers to determine the optimal conditions, favorable parameters, and most appropriate wavelengths for use in these applications. Therefore, in this study, we examined the effects of lasers at two different doses (3 and 4 J/cm^2) on the healing of third-degree burns.

In the present study, a higher number of inflammatory cells were observed in the injury group samples than in the laser-irradiated group samples, which showed significant reductions of infiltrated inflammatory cells at both the periods analyzed. This finding suggests that LLLT therapy results in a higher rate of tissue repair. This result supports the anti-inflammatory effects observed in a similar study conducted by Rochkind et al. [10]. Furthermore, in an examination of the effects of laser therapy at three wavelengths on the healing process of third-degree burns, Rabelo et al. [11] also found that lasers could reduce inflammation and consequently promote tissue repair.

In the present study, we observed a significant reduction of the inflammatory response on the eighth day after inducing the burns in the LLLT-treated groups relative to the injury group. However, on the 16th day, the number of inflammatory cells was only significantly reduced in the group treated with a laser dose of 3 J/cm^2 when compared to the injury group, suggesting that the effects of laser therapy are most pronounced in the early stages of tissue

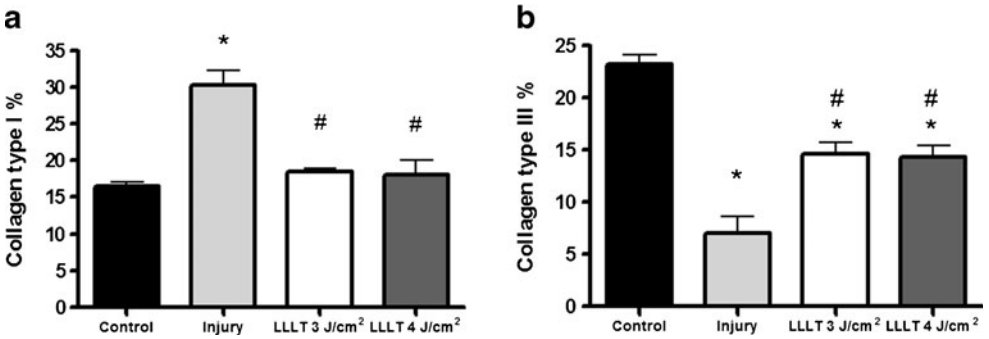

Fig. 5 **a** Means and standard deviations of the percentage of type I collagen fibers at 16 days after the injury. Note the significant difference (p <0.05) between the injury group and control group, as well as between the injury group and both the low-level laser therapy (LLLT)-treated (3 and 4 J/cm^2) groups (p <0.05). Note that no difference is seen between the LLLT-treatment groups (p <0.05). **b** Means and standard deviations of the percentage of type III collagen fibers at 16 days after the injury. Note that the injury group differs significantly (*p <0.05) from the control group and from both the LLLT-treated groups based on Tukey's multiple comparison test

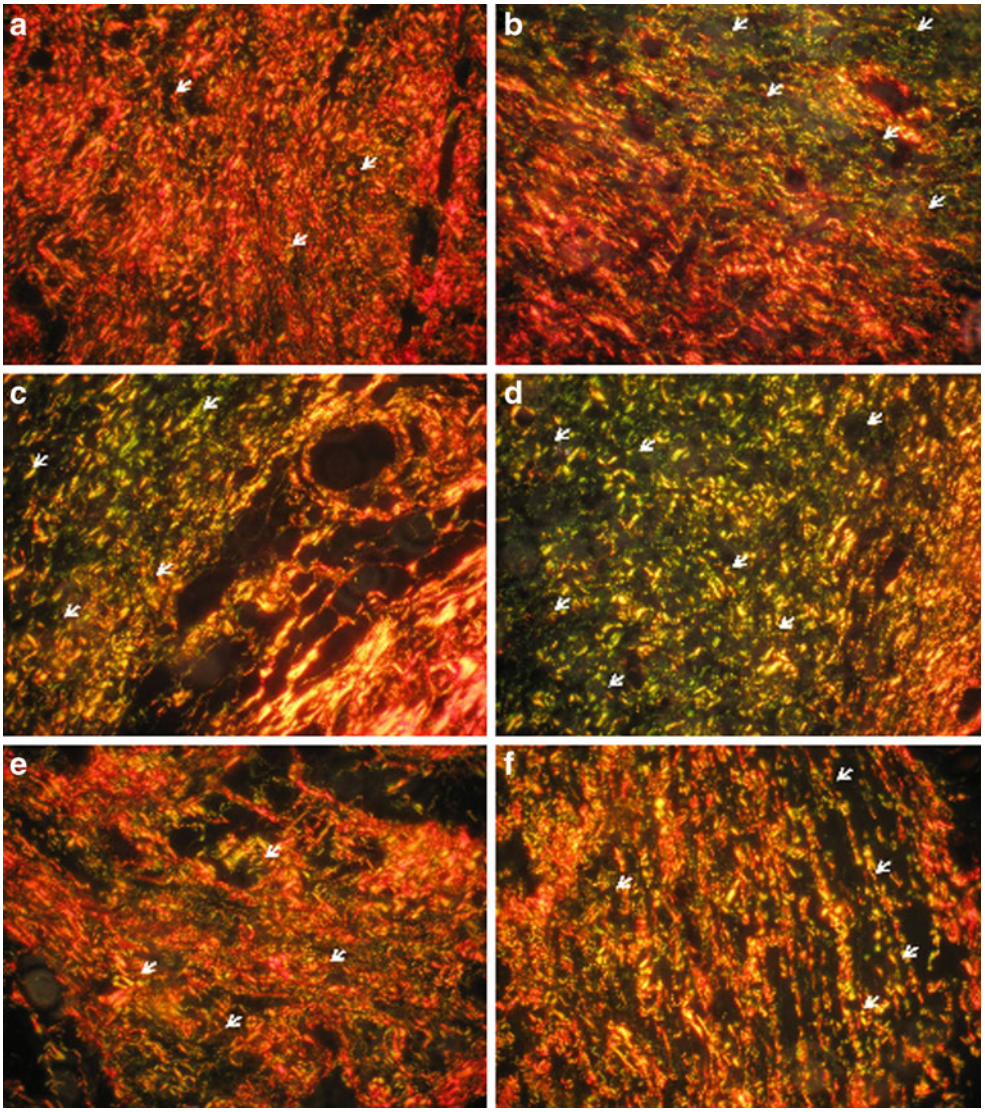

Fig. 6 Representative photomicrographs using polarized light illustrating the collagen Quantification. **a** and **b** Injury (Injured, non-treated), note the large amount of fibers are shown in red that typified the color type I collagen and the lack of greenish staining (collagen type III, marked by *arrows*). **c** and **d** The image obtained from the blade Relating to 3 J/cm^2 (injured, treated with 3 J/cm^2 LLLT), observe the areas of collagen marked by *green color*. Note the presence of two types of collagen fibers as well as their interlacing (*arrows*). **e** and **f** 4 J/cm^2 (Injured, treated with 4 J/cm^2 LLLT) note that there is a power increase of type III collagen fibers (*arrows*)

repair [12]. These results are in agreement with those of Rabelo et al. [11], who showed a significant reduction in the density of inflammatory cells (of the superficial dermis) in the early stages of tissue repair in nondiabetic and diabetic rats that received laser treatments at 632.8 nm wavelength and 10 J/cm^2. Rocha Junior et al. [13] investigated the behavior of skin wounds induced in rats after laser irradiation by using parameters similar to those used in our study. Furthermore, similar to the results in the present study, they observed that laser treatment increased neovascularization and fibroblast proliferation and decreased inflammatory infiltration, indicating that lasers were effective modulators in the tissue response process and significantly contributed to faster and more organized tissue healing.

According to several authors [14], laser therapy may facilitate tissue healing due to its inhibitory effects on the emergence of chemotactic factors in the early stages of inflammation, thereby interfering with the effects of chemical mediators that are induced by inflammation. In the present study, the effects of both laser biomodulators were effective in reducing inflammation and improved collagen deposition after third-degree burns.

On the eighth day after injury, a predominance of type I collagen was observed in the injury group, and in the laser-treated groups. Compared to the control group, both LLLT groups (3 and 4 J/cm^2) and injury group showed higher percentages of type I collagen; however, no difference in the percentage of collagen was observed between the two LLLT-treated groups, suggesting that both the doses were effective for the deposition of type I collagen. With respect to the deposition of type III collagen, it can be observed that the injury group and also group treated with 3 J/cm^2 LLLT had values of collagen type III statistically lower when compared with control group. Therefore, LLLT was most effective in the early stages of the healing process and acted as a biostimulating coadjuvant by counteracting the harmful effects of the burn to reduce inflammation and increase collagen deposition [15].

On the 16th day after the injury, we observed a predominance of type I collagen in the injury group, whereas type III collagen was predominant in the laser-treated and control groups. This suggests that the laser stimulates the production of type III collagen, thereby producing highly structured tissue.

In this study, laser therapy showed improved rates of collagen deposition at both 8 and 16 days after the injury, confirming a positive role of laser treatment in collagen synthesis and tissue remodeling, which in turn exerts stimulatory effects on connective tissue during wound healing [16, 17]. Several previous studies have shown similar results, that lasers regulate the release of cytokines responsible for fibroblast proliferation and collagen synthesis [18, 19], thus, improving collagen deposition [17], and further resulting in the improved organization of architectural collagen fibers [16].

In conclusion, this study showed that laser irradiation at doses of 3 and 4 J/cm^2 promoted a reduction of the inflammatory process and improved collagen deposition, thereby ameliorating the healing of third-degree burns.

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