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ORIGINAL ARTICLE

Effect of photodynamic therapy on the healing of cutaneous third-degree-burn: histological study in rats

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Abstract The aim of this study was to conduct a histological assessment of the effect of photodynamic therapy (PDT) on the repairing of third-degree-burn wounds made on the backs of rats with a heated scalpel. Ninety-six rats were divided into groups: G1, control (n=24), cold scalpel; G2, burned, heated scalpel (n=24); G3, low-level laser therapy (LLLT) (n=24), on burns; and G4, photodynamic therapy (PDT) (n=24), toluidine-O blue (100 μg/ml) and LLLT treatment on burns. The laser (685 nm) was applied in continuous mode, 50 mW, 4.5 J/cm^2, contact mode at nine points (9 s/point). Eight animals in each group were killed at 3 days, 7 days or 14 days after surgery, and tissue specimens containing the whole wounded area were removed and processed for histological analysis; the results were statistically analyzed with Kruskal–Wallis and Dunn's tests (P<0.05). The results demonstrated significant differences between G2 and G3, and between G2 and G4, at both 3 days and 7 days, with regard to acute inflammation scores; G1 and G2 showed significant differences when compared with G4 at 3 days, with regard to neo-angiogenesis scores; G1 and G2 were statistically different from G3 and G4 at both 3 days and 7 days, with regard to re-epithelization scores; G2 showed statistically significant differences when compared with G3 and G4 with regard to collagen fiber scores at 7 days. LLLT and PDT acted as a biostimulating coadjuvant agent, balancing the undesirable effect of the burn on the wound healing process, acting mainly in the early healing stages, hastening inflammation and increasing collagen deposition.

Keywords Wound healing · Burns · Laser biomodulation · Photodynamic therapy

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Introduction

Burns are severe injuries that may result in loss of tissue fluids and are associated with tissue destruction and infection, as well as pain; all these factors may result in death. Great social and psychological impairment is commonly seen in most burn victims [1]. Healing is a complex process that involves a dynamic series of events, including clotting, inflammation, granulation tissue formation, epithelialization, collagen synthesis and tissue remodeling. This is still the subject of a large number of studies conducted by several researchers, especially regarding factors that delay and hinder the healing process [2]. In addition, burns represent a significant socioeconomic burden, due to loss of income and increasing health care costs. The development of an effective, fast acting, and low-cost treatment for these kinds of wounds would represent enormous benefits for both patient's life quality and economic status [3].

Several therapeutic methods have been used to minimize the damaging effect of burns [4]. The use of low-level laser therapy (LLLT) has been shown to result in increasing

amounts of both acute and chronic inflammatory cells [5]. This advance of inflammatory response helps to excite the maturation of granulation tissue and increases the number of fibroblasts, consequently increasing the deposition of collagen fibers and improving neovascularization [5]. These aspects are seen at the early stages of the healing process and they probably occur due to the effects of laser energy at the cellular level [6, 7]. Recent findings suggest that LLLT could be useful to treat severe burns, especially if it is initiated at the early healing stage [8, 9]. It appears that laser at 660 nm showed better results during the early wound healing stages, and the use of 780 nm laser showed beneficial effects throughout the experimental period [9].

Photodynamic therapy (PDT) is the combination of a photosensitizer that binds to a target cell and light of a suitable wavelength, activated in the presence of oxygen [10]. PDT has been used in the treatment of several conditions, including periodontal diseases [11], peri-implantitis [12], and cutaneous wound healing [13], and it seems to be promising for other conditions, such as tumors, arthritis, skin disorders and many other non-oncologic diseases [14, 15]. PDT may favor the repair process when associated with LLLT, which shows the advantage of promoting biomodulation in the tissue to be repaired and reducing inflammation. LLLT increases mitochondrial respiratory chain and adenosine triphosphate (ATP) synthesis, favors the repair process, induces cell proliferation, promotes production of nucleic acid, and increases both cell division and collagen synthesis [16].

Bacterial attachment to an open wound during the healing process is the prominent etiological factor in the pathogenesis of infections. PDT has shown that photosensitizers are effective in the photo-inactivation of bacteria, viruses, fungi and parasites [14]. During photodynamic therapy, reactive oxygen is produced to kill target cells when the photosensitizer is excited by light in the presence of oxygen. This provides photodynamic therapy with many attractive advantages, such as a non-invasive nature, selective targeting, easy repeatability and great safety [15]. Recently, a study has suggested that photodynamic therapy is also effective in suppressing the community of microorganisms in the biofilm [17].

There are few published reports on the effect of PDT on burn healing [17]. The aim of our study was to evaluate histologically the wound healing process in open burns made on the backs of rats submitted to laser and photodynamic therapy.

Material and methods

Animals

This study protocol was approved by the Committee of Animal Institutional Care and Usage. Ninety-six male adult Wistar rats (*Rattus norvegicus* var. *albinus*), weighing between 180 g and 220 g, were used. Before the surgical procedures, all the animals were acclimatized to the laboratory environment for 5 days.

Surgical procedures

The animals were anesthetized with an intramuscular injection of ketamine (Francotar, São Paulo, SP, Brazil; 0.04 ml/100 g body weight) and xylazine (Coopers, São Paulo, SP, Brazil; 0.02 ml/100 g body weight). Immediately after anesthesia, the dorsal region of all animals was trichotomized and disinfected with povidone–iodine (Riodeine 1%, Rioquímica, São José Rio Preto, SP, Brazil). A circular area of skin (approximately 8 mm in diameter) was removed from the medial dorsal region with a punch, which was placed on the medium portion of the median sagittal plane.

The 96 rats were randomly allocated by a computer-generated table to the treatments: control, burns, LLLT and PDT. In order to achieve better standardization, we chose animal 1 first, followed by 2 and 3, respectively. The animals were randomly divided into four groups of 24 rats each: G1 (control group), where the lesions were made with a cold punch and no treatment was given; G2 (burn group), where the lesions were made with a heated punch and no treatment was given; G3 (LLLT), where the lesions were made with a heated punch and treated with LLLT; and G4 (PDT), where the lesions were made with a heated punch and treated with locally applied photosensitizer and low-level laser therapy (PDT). In the wounds of groups 2, 3 and 4, the ¨punch¨ was kept heated with the blue flame of a Bunsen burner, which was regulated at 80°C, and then pressed on the rats' skin for 30 s to make a third-degree burn.

Photosensitizer and LLLT

The photosensitizer used in this study was toluidine-O blue (TBO) (Vigor-farma, São Paulo, Brazil) at a dosage of 100 μg, applied to the lesions drop by drop around all the wound margins (1 ml). The low-intensity laser used in this study was a gallium aluminum arsenide (GaAlAs) one (IR 500, Laser Beam Industry and Technology Ltd, Rio de Janeiro, Brazil) with a wavelength of 685 nm and spot area of 0.01 cm^2. The treatment was conducted in contact with the tissue, at a power of 0.05 W, power density of 0.5 W/cm^2, fluency of 4.5 J/cm^2/point (nine s/point), with a full exposure time of 81 s (nine points). Irradiation was performed punctually on eight distinct regions around all wound margins and also in a central point, scanning the wound all over in accordance with Pessoa et al. [5], in the LLLT Group. In the PDT Group the same parameters of

laser irradiation were used, 1 min after photosensitizer had been poured into the wound.

Experimental periods

After 3 days, 7 days, and 14 days of surgery and treatment, eight animals in each group were killed with thiopental at lethal doses (150 mg/kg; Cristalia, Chemical Pharmacy Ltd, Itapira, SP, Brazil). Although the control group animals had not been irradiated, they were handled the same way as the animals in the experiment, so that the same stress levels were produced.

Laboratory procedures

The pieces obtained were fixed in 10% formalin for 24 h. After this, they went through routine laboratory procedures until they were set in paraffin, directed in a way to allow 6-μm-thick semi-serial traverse sections to be obtained. The sections were stained with hematoxylin and eosin (HE) and Masson's trichrome (MT) for histological analysis by light microscopy (×40).

Intra-examiner reproducibility

Before the histological analysis was performed, the examiner was trained through double measurements of 12 specimens with a 1-week interval. Paired *t*-tests was run, and no differences were observed in the mean values for comparison ($P>0.05$). Additionally, Pearson's correlation coefficient was obtained for the two measurements and revealed a very high correlation (0.99; $P=0.000$).

Statistical analysis

The criteria used for this histological analysis are shown in Table 1. The analysis was based on the studies by Meireles et al. [18] and Iordanou et al. [19]. Statistical analysis was carried out with Kruskal–Wallis and Dunn's tests. Statistical significance was set at $P<0.05$.

Results

G1, control

After 3 days, the control specimens showed moderate acute inflammatory reactions (score 2) with discrete collagen deposition (score 1). Re-epithelization was absent (score 0) and neo-angiogenesis was slight in this group (score 1). After 7 days, acute inflammation was found to be slight to moderate, although small numbers of chronic inflammatory cells were present (score 1). Collagen deposition and neo-angiogenesis were slight in this period (score 1). Re-epithelization was not seen at this point (score 0). After 14 days, chronic inflammation was moderate (score 2), with small numbers of acute inflammatory cells (score 1). Moderate collagen deposition (score 2) and intense newly formed blood vessels (score 3) were seen in all cases. The epithelium covered 100% of the wound, with irregular thickness (score 3).

G2, burned

At 3 days postoperatively, thermal necrosis extending inward to the hypodermis and signs of intense acute

Table 1 Criteria used for light microscopy analysis

Criterion	Score		
Re- epithelization	Absent (0)	Present, covering < 50% of the wound (1) Present, covering > 50% of the wound (2) Present, covering 100%, with irregular thickness (3) Present, covering 100%, with regular thickness (4)	
Acute inflammation	Slight, presence of <25% of neutrophils among all cells in the field (1)	Moderate, presence of <25–50% of neutrophils among all cells in the field (2)	Intense, presence of > 50% neutrophils among all cells in the field (3)
Chronic inflammation	Slight, presence of <25% of chronic inflammatory cells in the field (1)	Moderate, presence of <25–50% of chronic inflammatory cells in the field (2)	Intense, presence of > 50% chronic inflammatory cells in the field (3)
Number of collagen fibers	Slight, Masson's trichrome stain less intense than that seen in the healthy adjacent tissue (1)	Moderate, Masson's trichrome stain similarly intense to that seen in the healthy adjacent tissue (2)	Intense, Masson's trichrome stain more intense than that seen in the healthy adjacent tissue (3)
Neo-angiogenesis	Slight, a smaller amount than that seen in healthy adjacent tissue (1)	Moderate, an amount similar to that seen in healthy adjacent tissue (2)	Intense, an amount greater than that seen in healthy adjacent tissue (3)

inflammation could be seen at this stage (score 3). Collagen deposition was slight in all specimens (score 1). Neo-angiogenesis was mostly slight (score 1). Re-epithelization was absent in all specimens (score 0).

After 7 days, acute inflammation varied from intense to moderate, with chronic inflammation varying from discrete to moderate, at this time (Fig. 1). Collagen deposition was uneven, and, when present, occurred in small amounts (Fig. 2). Angiogenesis was discrete in all animals. Re-epithelization was absent in all specimens. Fourteen days after the burn, acute inflammatory reaction was discrete (score 1), with moderate chronic inflammation (score 2). The numbers of collagen fibers varied from discrete to moderate. Connective tissue comprised an intense number of congested blood vessels. Re-epithelization covered <50% of the wound in all specimens analyzed (score 0).

G3, LLLT

Three days after treatment, slight acute and chronic inflammation reactions were observed (score 1). The use of laser energy resulted in the presence of a small number of fibroblasts that were migrating toward the area to be repaired, following moderate collagen deposition (score 2). Neo-angiogenesis was slight, and re-epithelization was present, covering <50% of the wound margins (score 1). After 7 days, the acute inflammatory reaction was discrete, and chronic inflammation was moderate in most specimens (Fig. 3). In all of them, collagen deposition was intense at this stage (score 3). Re-epithelization was present, covering <50% of wound (score 1). Fourteen days after laser treatment, epithelium was present, covering <50% of the wound (score 1). There were neutrophils and macrophages underlying the epithelium, showing a moderate chronic (score 2) and discrete acute (score 1) inflammatory reaction. Some tortuous newly formed vessels showed moderate neo-angiogenesis (score 2). Collagen fibers were immature, delicate and sometimes fragmented. An intense number of collagen fibers (score 3) was observed under light microscopy, and the fibers were parallel to the wound surface when stained with Masson's dye.

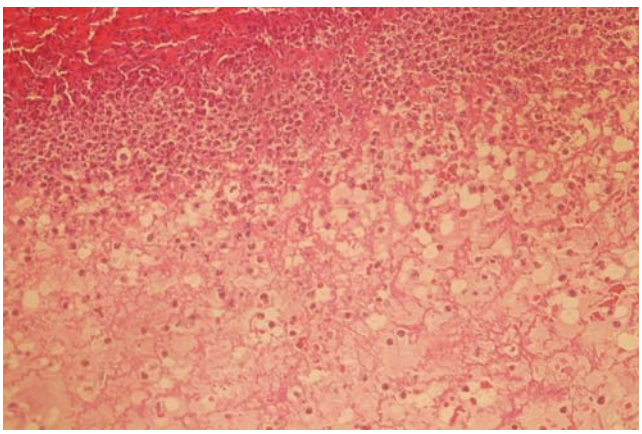

Fig. 1 Photomicrograph of a burn specimen 7 days after the burn, showing an intense acute inflammatory reaction (HE, ×40)

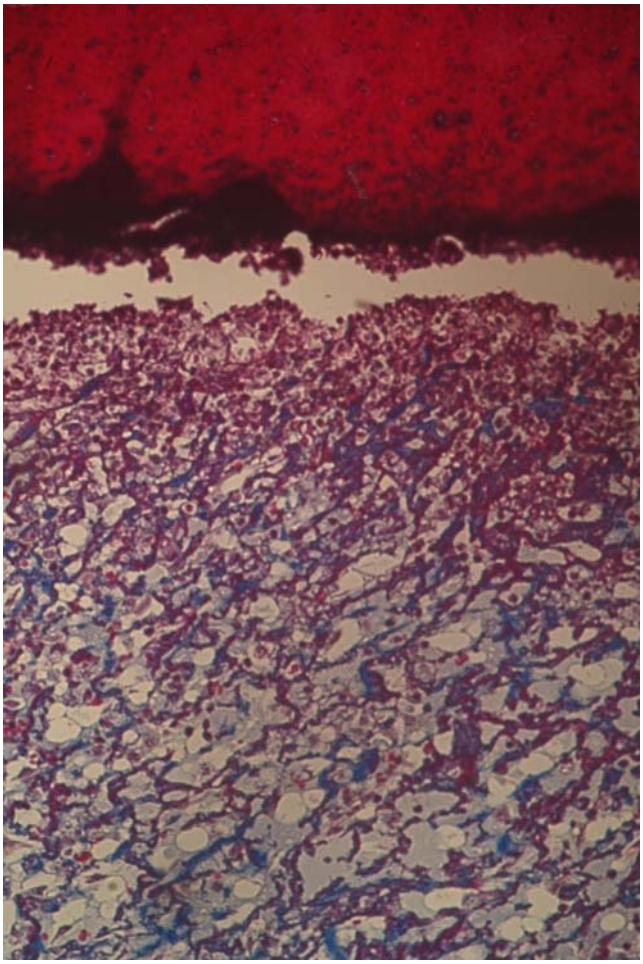

Fig. 2 Photomicrograph of a burn specimen 7 days after the burn, showing slight collagen deposition and absence of re-epithelization (MT, ×40)

G4, PDT

After 3 days, the inflammation was discrete (score 1) in most specimens, with a moderate number of collagen fibers (score 2) and intense neo-angiogenesis (score 3). Re-epithelization was present, covering <50% of the wound margins (score 1). Seven days after treatment, acute inflammation was mostly slight (score 1) and chronic inflammation was moderate (score 2). There was intense deposition of collagen fibers (score 3), which were organized and parallel, within the wound (Fig. 4), with moderate neo-angiogenesis. The epithelium was present, covering <50% of the wound margins (score 1). A discrete amount of mixed inflammatory exudate was seen after 14 days, with slight numbers of acute and chronic

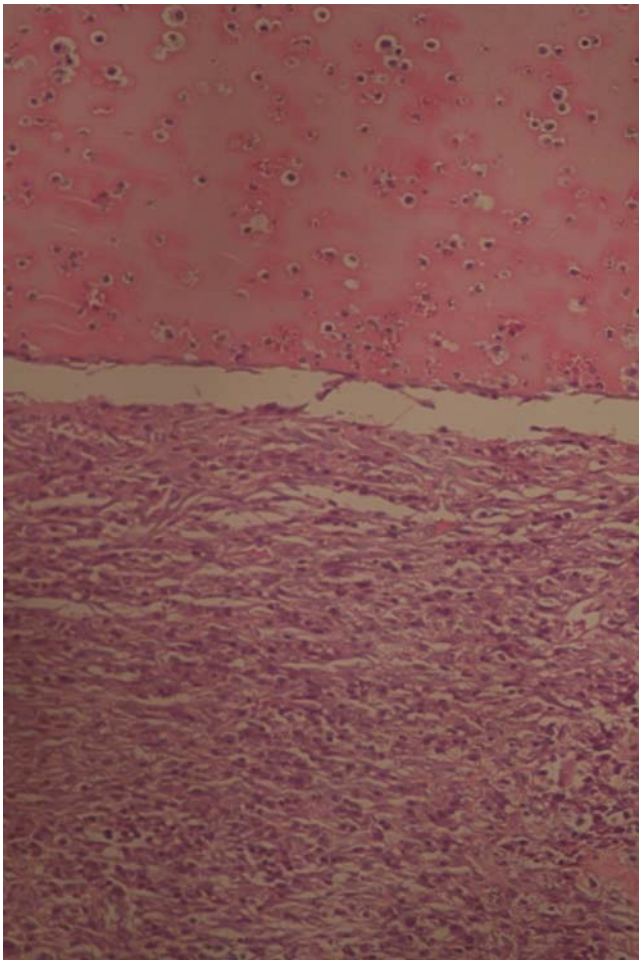

Fig. 3 Photomicrograph of an LLLT specimen 7 days after the burn, showing slight acute inflammation (HE, ×40)

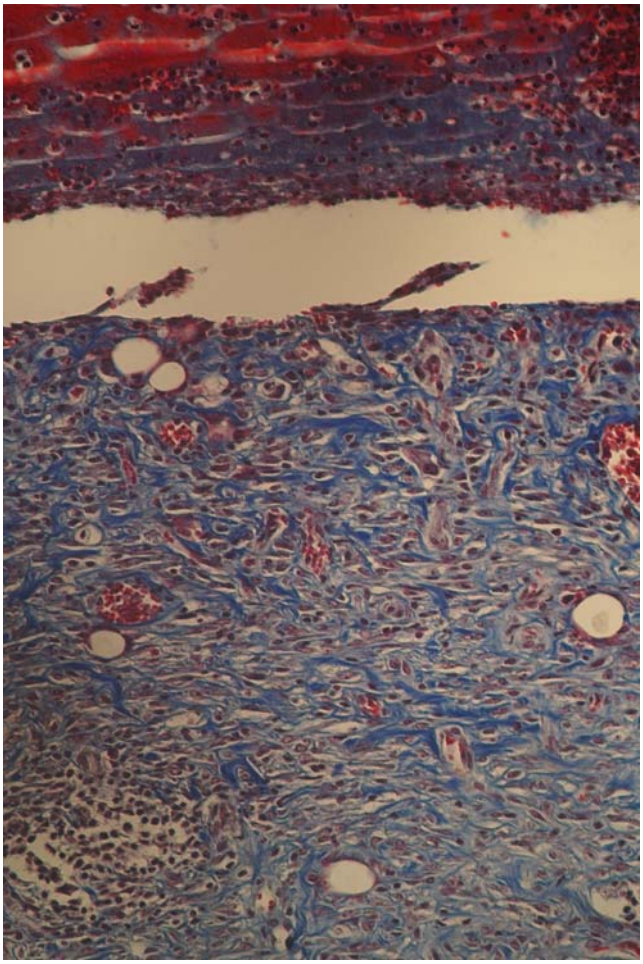

Fig. 4 Photomicrograph of a PDT specimen 7 days after the burn, showing intense collagen deposition with fibers parallel to the wound surface (MT, ×40)

inflammatory cells (score 1). Re-epithelization was present, covering 100% of the wound, with an irregular thickness (score 3; Fig. 5). The dermis showed a small number of newly formed congested blood vessels and a large number of collagen fibers in all specimens (score 3).

Data analysis

Considering each group as an independent variable, we performed the non-parametric Kruskal–Wallis test for each histological criterion. The results showed a significant difference among the groups evaluated in each criterion ($P<0.05$). Dunn's test demonstrated a significant difference between groups G2 and G3, and between G2 and G4, after both 3 days and 7 days, with regard to acute inflammation scores. For the neo-angiogenesis scores, groups G1 and G2 showed significant differences when compared with group G4 at 3 days. With regard to re-epithelization scores, groups G1 and G2 were statistically different from G3 and G4 after both 3 days and 7 days. After 7 days, group G2

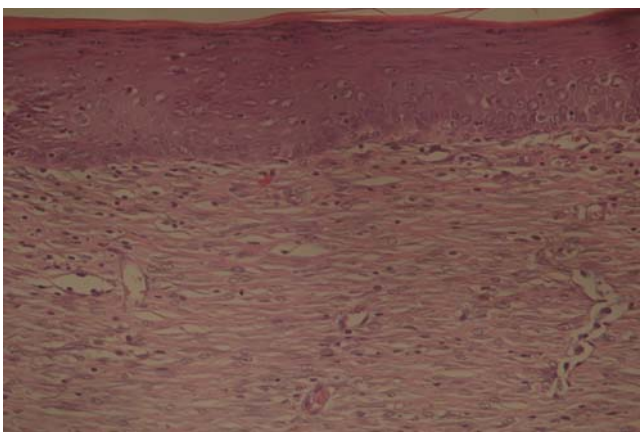

Fig. 5 Photomicrograph of a PDT specimen 14 days after the burn, showing intense collagen deposition, with fibers parallel to the wound surface, and re-epithelization covering 100% of wound, with irregular thickness (HE, ×40)

showed statistically significant differences when compared with groups G3 and G4 with regard to collagen fiber scores.

Discussion

Wound healing is a complex process that involves the response of several local and systemic tissues, which is regulated by many different cellular and humoral factors [20]. It normally proceeds in four overlapping phases: inflammation, granulation, matrix formation and remodeling [21].

In vitro [22] and in vivo [3, 5, 6, 9, 23] studies have demonstrated that phototherapy acts on the events of healing. The meta-analysis of LLLT efficacy indicated that laser therapy is highly effective in promoting tissue repair [24]. There are several reports in the literature on the beneficial effect of laser on local vascularization, edema, pain, and inflammation, as well as the deposition and organization of both extracellular matrix and collagen [25, 26], but there are few published reports [17] on the effect of PDT on the healing of a burn. In our study, both LLLT and PDT were used, due to the severity of burns that deeply affected the skin and made the treatment difficult.

In our study, at the early stages of the repair process, it was observed that wounds treated with LLLT and PDT showed early re-epithelization after 3 days but only on the border of the wound. Acute inflammation was also mostly slight in the specimens treated with LLLT and PDT. Control specimens showed moderate acute inflammation, while burn specimens showed intense acute inflammation. Chronic inflammation varied from slight to moderate in the wounds treated with laser. In the PDT groups, chronic inflammation was slight in a great number of specimens. Some wounds showed a chronic inflammation reaction in both control and burn groups. In this investigation, the effects of LLLT and PDT on third-degree burns were found to be considerable at the early stages. During this healing process, laser energy and PDT hastened the change from exudative to proliferative phase, mainly at the third and seventh days. This effect on the inflammation process was maintained throughout the experimental period. This may have been related to the repair of third-degree burns that initially require the removal of the necrotic tissue by phagocytosis [2]. This takes a long time in third-degree burns, but the period of this study was short so that we could detect more suitable differences among the groups. In fact, it is necessary to provide a longer time so that real differences can be found between the groups. Studies have suggested that LLLT hastens inflammation [5], modulates prostaglandin levels [8], enhances the action of macrophages [8], promotes fibroblast proliferation [22], facilitates collagen synthesis, and fosters immunity [27].

Neo-angiogenesis, as seen in most of the animals treated with PDT, varied from moderate to intense. In the LLLT groups, it varied from slight to moderate; in the control and burn groups it was mostly intense after 14 days. Considering this parameter, we found that LLLT and PDT were most effective; however, PDT showed an early more satisfactory repair. This was in accordance with the findings by Meireles et al. [2], who found that third-degree burns treated with 660 nm laser showed satisfactory repair in the early experimental period (at days 3 and 5). One important aspect of the findings of our study was the wide difference seen in the amounts of neo-angiogenesis in the PDT and LLLT groups. The hastening effects of LLLT and PDT on the wound healing process is related to low intensity laser irradiation, showing its capacity for modifying capillary density [28], stimulating local microcirculation [29] and mainly increasing not only collagen synthesis but also cell proliferation [30]. Furthermore, it favors cellular chemotaxis and promotes local vasodilatation and angiogenesis [31]. Thus, there must be an increase in oxygen diffusion through the tissue during PDT and LLLT, favoring the repair process, because collagen secretion by fibroblasts in extracellular spaces occurs only in the presence of high oxygen pressure [31]. Susceptibility of cells to irradiation, and activation ability, are linked to their physiologic states. Cells with decreased redox potential (some pathologic states) are more sensitive to irradiation [16]. Low levels of laser energy have been shown to increase blood flow rate and volume, as well as accelerate the wound healing process [32].

The beneficial effect of LLLT and PDT was evident in re-epithelization and collagen matrix deposition. Re-epithelization is important, as it restores skin integrity, making it less vulnerable to infection [33]. Our results demonstrated that the LLLT and PDT used in this study enhanced wound healing, as evidenced by the statistical significance of epithelization after the 3rd, 7th and 14th days, when compared with that in the control and burn groups. In the PDT specimens, there was a tendency to higher quality and better organization of the epithelium after the 14th day. The epithelium of the PDT specimens was covering 100% of the wound, with irregular thickness, while, in the LLLT group, the epithelium covered <50% of the wound. A possible explanation for the faster and better epithelization could be the increase in keratinocyte proliferation and migration, from local or systemic effects of photomodulation [19]. Photons enter the tissue and are absorbed in the mitochondria and at the cell membrane [34]. The photonic energy is converted into chemical energy within the cell, in the form of ATP, which leads to normalization of cell function, pain relief and healing [34]. Cell membrane permeability is altered, and then physiological changes occur. These physiological changes affect

macrophages, fibroblasts, endothelial cells, mast cells, bradykinin, and nerve conduction rates [34]. Low doses of light intensify the formation of the transmembrane electrochemical proton gradient in the mitochondria, which is followed by calcium release from the mitochondria into the cytoplasm by an antiport process, which, in turn, triggers or stimulates various biological processes such as the synthesis of RNA and DNA, cell mitosis, protein secretion and cell proliferation [34, 35].

The deposition of collagen was slight in the burn wounds and varied from slight to moderate in the control wounds. In the LLLT group collagen deposition varied from moderate to intense, while, in the group irradiated by PDT, most specimens showed an intense deposition of collagen. Collagen, however, cannot be synthesized in the absence of an adequate oxygen supply, and this was clearly the direct result of the blood supply provided for the increase in angiogenesis [36]. Positive responses were seen with LLLT and PDT in fibroblast proliferation. Both collagen deposition and organization of the collagen matrix are important in animals with weak mechanical resistance against infections and damage [37]. Photons produced by lasers can stimulate healing in acute and chronic wounds that are healing slowly [38]. This inference creates gradient forces in the cell, inducing the change in the distribution of organelles [39]. Different substances absorb light of different wavelengths, and the cells of injured skin are more sensitive to light than those of intact tissue [16]. Once target cells have absorbed photons, the cascade of biochemical events occurs, resulting in a hastening of the wound healing [40]. In human fibroblasts there are several molecules that serve as photoreceptors [39].

Low-level laser irradiation stimulates macrophages to release chemical mediators, cytokines and growth factors, which, in turn, activate the latter stages of wound repair [41]. A recent study has demonstrated that irradiation with an argon laser (630 nm and 660 nm) caused the release of transforming growth factor and platelet-derived growth factor from fibroblasts [41]. Growth factors include cytokines, which are essential to wound healing and attract useful cells and proteins to the wound, including immunity cells to oppose infection and other cells to form connective tissue. They stimulate and increase production of connective tissue and create the new supply of blood vessels to nourish the site and promote remodeling [42]. An increase in cytokine expression is a way of hastening the reparative process [43]. A proposed mechanism by which LLLT stimulates wound healing is the absorption of light energy, which stimulates the release of chemical mediators. [38].

PDT is defined as an oxygen-dependent photochemical reaction that occurs upon the light-mediated activation of the photosensitizing compound that leads to the generation of cytotoxic reactive oxygen species, predominantly singlet oxygen, which are toxic to microorganisms [10]. The major advantages of PDT are its specific therapy on target cells, no collateral effects, activity initiation only when exposed to light, and the lack of development of resistant bacterial species [44].

In our study the use of 685 nm laser and PDT had positive effects on collagen matrix deposition, proliferation and neo-angiogenesis after days 3, 7 and 14. Positive effects on inflammation could be observed after the third and seventh days. At the end of the experimental period, the effects of LLLT and PDT on re-epithelization and collagen deposition were more evident than in the inflammation process. Although no statistically significant difference was found between the two groups tested (PDT and LLLT), the results in the LLLT and PDT groups were consistently better than those of the control and burn animals. These results are in accordance with those of several other authors, who found that laser energy hastens the wound healing process mainly at the early stages [2, 5, 8, 9, 18, 45].

Conclusion

Within the limits of this study, the results suggested that LLLT (685 nm) and PDT (TBO plus irradiation at 685 nm) could be useful to treat cutaneous burns, especially if they are initiated at an early stage of healing, but there was no advantage in the use of PDT (LLLT+ TBO) to heal burns when compared with LLLT alone. Although there was no statistical difference between LLLT and PDT, there was a histological tendency for better cicatrization after the use of PDT in burn healing.

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