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Effects of a single near-infrared laser treatment on cutaneous wound healing: Biometrical and histological study in rats

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Abstract

Background: Low intensity laser therapy has been recommended to support the cutaneous repair; however, so far studies do not have evaluated the tissue response following a single laser treatment. This study investigated the effect of a single laser irradiation on the healing of full-thickness skin lesions in rats.
Methods: Forty-eight male rats were randomly divided into three groups. One surgical lesion was created on the back of rats using a punch of 8 mm in diameter. One group was not submitted to any treatment after surgery and it was used as control. Two energy doses from an 830-nm near-infrared diode laser were used immediately post-wounding: 1.3 J cm^{-2} and 3 J cm^{-2}. The laser intensity 53 mW cm^{-2} was kept for both groups. Biometrical and histological analyses were accomplished at days 3, 7 and 14 post-wounding.
Results: Irradiated lesions presented a more advanced healing process than control group. The dose of 1.3 J cm^{-2} leaded to better results. Lesions of the group irradiated with 1.3 J cm^{-2} presented faster lesion contraction showing quicker re-epithelization and reformed connective tissue with more organized collagen fibers.
Conclusions: Low-intensity laser therapy may accelerate cutaneous wound healing in a rat model even if a single laser treatment is performed. This finding might broaden current treatment regimens.

Keywords: GaAlAs diode laser; Full-thickness cutaneous defects; Laser biomodulation; Low intensity laser therapy; Skin repair

1. Introduction

The biomodulatory effects of low intensity laser therapy (LILT) have been reported since the beginning of laser development for biological applications. Many studies have focused on the effects of laser photomodulation on a broad range of pathological conditions such as wound healing, reduction of edema, and the relief of pain from various etiologies [1–5].

Wound healing is a complex biologic and biochemical process that starts right after an injury. Several studies had been performed aiming to clarify the different mechanisms that interfere with it. Recently, LILT effects have been investigated not only at experimental basis, but also in clinical trials [6–8]. This has been motivated by the necessity of enhancements on the healing process of post-surgical wounds, burns, implants and flap surgeries, leading to a shorter length of stay in a health care facility, which results in a lower risk of nosocomial infections.

It could be shown that LILT at adequate parameters may promote alterations in cellular metabolism leading, for instance, to an increased proliferation of fibroblasts,

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faster formation of granulation tissue, promotion of collagen synthesis, angiogenesis and earlier epithelization [9–13].

Although studies have demonstrated acceleration on wound healing process, in reality, no data about LILT effects following a single treatment have been reported. During a flap surgery, the irradiation of the open wound could provide an interesting approach, leading to a better clinical result. As this therapy can be easily performed with a few minutes of laser application, improved results would be achieved if a single treatment would be able to generate a better clinical outcome.

Biometry and light microscopy are two powerful tools to study wound healing, allowing a clinical and morphological evaluation of the results. Nevertheless, these tools are rarely used to investigate the effect of LILT on cutaneous wound healing.

Thus, the purpose of this study was to investigate two energy doses using a near-infrared 830 nm diode laser to compare the healing of full-thickness cutaneous defects after a single laser treatment in a rat model.

2. Materials and methods

For the present study, forty-eight adult male rats (Rattus norvegicus, albinus, Wistar) with body mass between 200 g and 220 g were used. During the experimental period, all animals were kept in individual cages in a 12 h light/12 h dark schedule at 22 °C and feed with granulated ration and water ad libitum. The rats were anaesthetized with chloral hydrate at 10% (0.4 mL per 100 g of body mass) and shaved at the dorsal region. After anesthesia, a circular fragment of skin was removed from the median region of the back by a punch with 8 mm in diameter. National and international principles of laboratory animal care were followed.

The animals were divided into three groups of 16 rats according to the following description:

- Group 1 (G1) – Control group. No light treatment was performed.
- Group 2 (G2) – The lesions were submitted to a single laser irradiation. The energy dose was approximately 1.3 J cm^{-2} using 60 mW during 25 s [3].
- Group 3 (G3) – The lesions were submitted to a single laser irradiation with an energy dose of approximately 3 J cm^{-2} using 60 mW during 56 s [14].

For irradiation procedures, a low power diode laser integrated with a proper driver, beam-delivery fiber and handpiece was used (Kondortech, BioWave LLLT, Brazil). This laser emits at 830 nm, with total output power of 60 mW checked at the end of the delivery system using a power meter (LM-01, Coherent, USA).

The laser beam diameter was measured and its area at the handpiece was 0.07 cm^2, with a divergence of 20°. A spacer was constructed with a 52 mm-long hollow white PVC cylinder, which had an adaptation fixed in the handpiece tip at one end and was open at the other extremity. The laser beam propagated freely inside the spacer and reflected in the internal walls so the laser output power was measured at the end of the delivery system. The internal spacer had 1.2 cm in diameter. In this way, the beam was expanded to ensure a homogeneous light intensity on the entire lesion at 1.13 cm^2. The device was built up to ensure that both laser groups would receive exactly the same irradiance with a homogeneous distribution of the energy into the entire wound, therefore the only difference between the two irradiated groups would be the fluence. The irradiation was performed without contact with the wound at a constant distance of 52 mm provided by the beam expander cylinder. Hence, with this device, the entire lesion was irradiated at once, since the wound area was 0.5 cm^2 and the laser beam covered an area of 1.13 cm^2. The beam was large enough to assure that the wound and the health skin in the vicinities would be irradiated. Without the expansion, the original beam area (0.07 cm^2) would not be enough to produce a uniform irradiation of the whole wound.

Table 1 summarizes the treatment parameters.

Twenty-one of the 48 animals were evaluated regarding wound contraction. Measurements of the internal wound diameter were performed at days 1, i.e., immediately after irradiation 3, 7 and 14 post-wounding (p.w.) by using a caliper rule. The diameter was measured in the midsagittal plane from left to right. The percentage changes in wound size relative to the initial dimension (day 1) were calculated. The means and standard errors were computed and differences among groups during experimental period were analyzed by using ANOVA-test. To identify the differences regarding the variables time and group, multiple comparisons for the means by LSD (least square difference) method were performed. Significance was accepted at $p < 0.05$.

The remaining 27 animals were sacrificed for histological analysis at the same periods (3, 7 and 14 days p.w.). Three animals for each group per time point were sacrificed. The skin biopsies were carefully collected to include the adjacent healthy skin and all the healed tissue in depth. All biopsies were fixed in formalin 10% for 24 h. Thereafter, biopsies were included in paraffin and 6 μm thickness transversal sections were obtained. The sections were stained by using the hematoxylin–eosin (HE) and Masson-Trichrome techniques. Stained sections were observed and photographed with an Olympus – BHT (BH2, Japan) light microscope.

Histological analyses of the specimens were performed in a blind method. Three pathologists were not informed, prior to the examination, about the experimental conditions of the samples. At least four slides from each animal, containing three sections each, were analyzed. In addition with the histological evaluations, the pathologists also performed a semiquantitative evaluation considering the following morphological parameters: continuity of the epithelium surface, polimorphonuclear infiltration, presence

Table 1
Treatment parameters applied in this research

Groups ($n = 16$)	Exposure time (s)	Power (mW)	Dose (J cm^{-2})	Intensity (W cm^{-2})
G1 (control)	No treatment was performed			
G2	25	60	1.3	0.053
G3	56	60	3.0	0.053

Rats were irradiated immediately after lesion creation and received a single laser exposure. No antibiotics were used and uncovered wounds were allowed to heal by secondary intention.

and morphological features of the blood vessels (neovascularization), presence and morphological features of the fibroblasts. Semiquantitative results were organized according to the following scores: 0 – absent; 1 – slight; 2 – moderate; 3 – intense. Collagen arrangement and density was also evaluated.

3. Results

3.1. Biometrical analysis

Fig. 1 shows mean values and standard error for the percentage of wound diameter variation relative to the initial size at day 1 p.w. (100%) during all experimental period. Figs. 2 and 3 illustrate the aspect of the lesions during the 14 days of experiment for G1 (control) and G2 ($D = 1.3$ J cm^{-2}), respectively. Irradiated groups (G2 and G3) presented similar cutaneous repair. As it was expected, the wound size decreases for all groups during the first 14 days p.w. At day 3 p.w., there is not a statistically significant difference among the groups regarding wound contraction. Significant differences were observed between G2 and control group (G1) at day 7 p.w. ($p = 0.017$) (compare Figs. 2C and 3C). On the 14th day p.w., G2 and G3 presented similar values for wound contraction. At this moment, both groups showed significant differences compared to the G1 values ($p = 0.021$ and $p = 0.038$ for G2 and G3, respectively) (compare Figs. 2D and 3D).

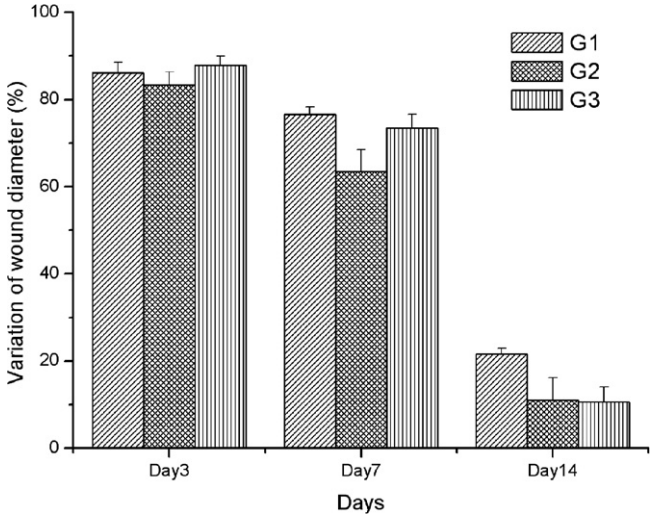


Fig. 1. Mean values and standard errors for the percentage of wound diameter variation related to initial size during experimental period. Seven rats were used for each group.

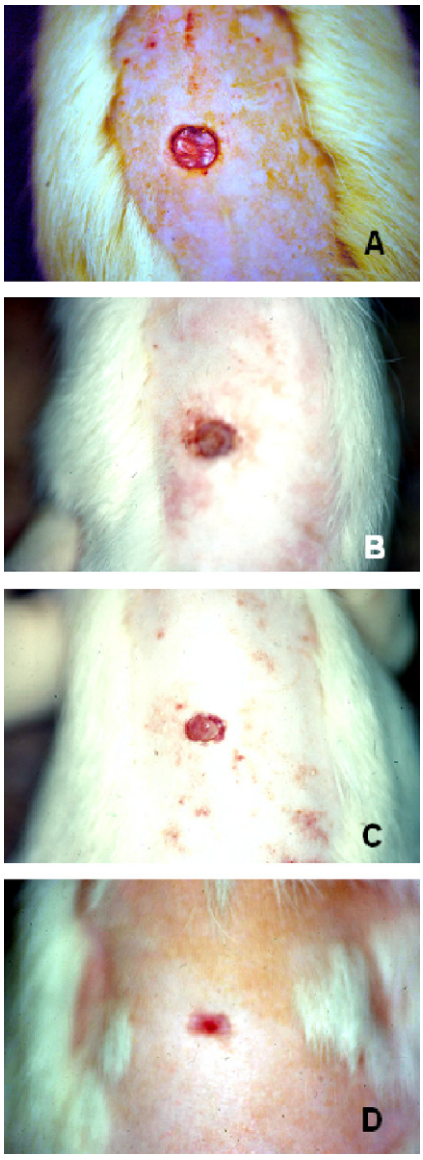


Fig. 2. Lesion aspect of the control group (G1) during experimental period. (A) day 1 p.w.; (B) day 3 p.w.; (C) day 7 p.w.; (D) day 14 p.w.

3.2. Histological analysis

At day 3 p.w., G1 showed a discrete proliferation of epithelium near to the wound edges. Above the surface, a thick layer of crust with cell debris was noted. In the subjacent dermis, it was observed a high amount of neutrophils, some of them in degeneration, and absence of connective tissue proliferation was also observed. The injured deep dermis showed inflammatory exudate, some lymphocytes and macrophages (Fig. 4A).

For irradiated groups (G2 and G3), the epithelial proliferation was similar to control group. Neutrophils were observed in the subjacent dermis, which also presented inflammatory exudate, lymphocytes and macrophages

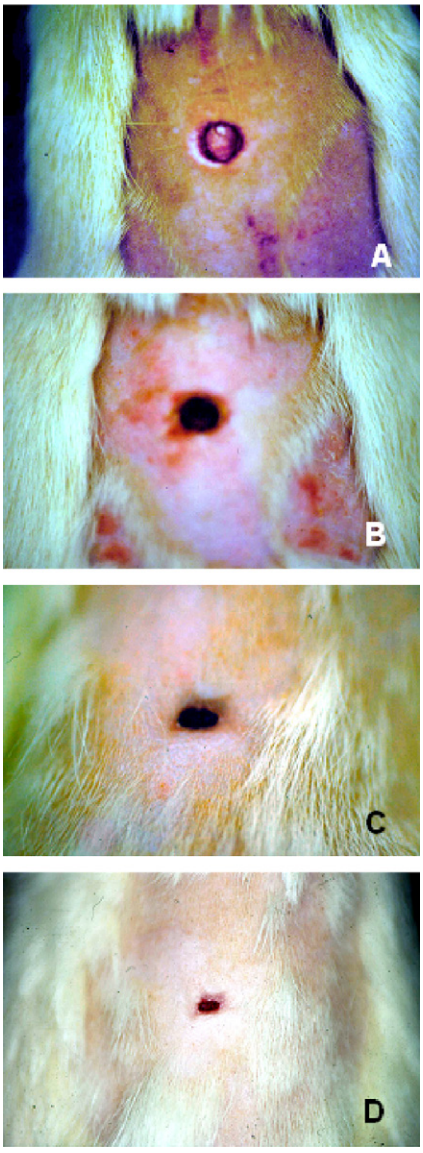


Fig. 3. Lesion aspect of the irradiated group (G2) during experimental period. (A) day 1 p.w.; (B) day 3 p.w.; (C) day 7 p.w.; (D) day 14 p.w.

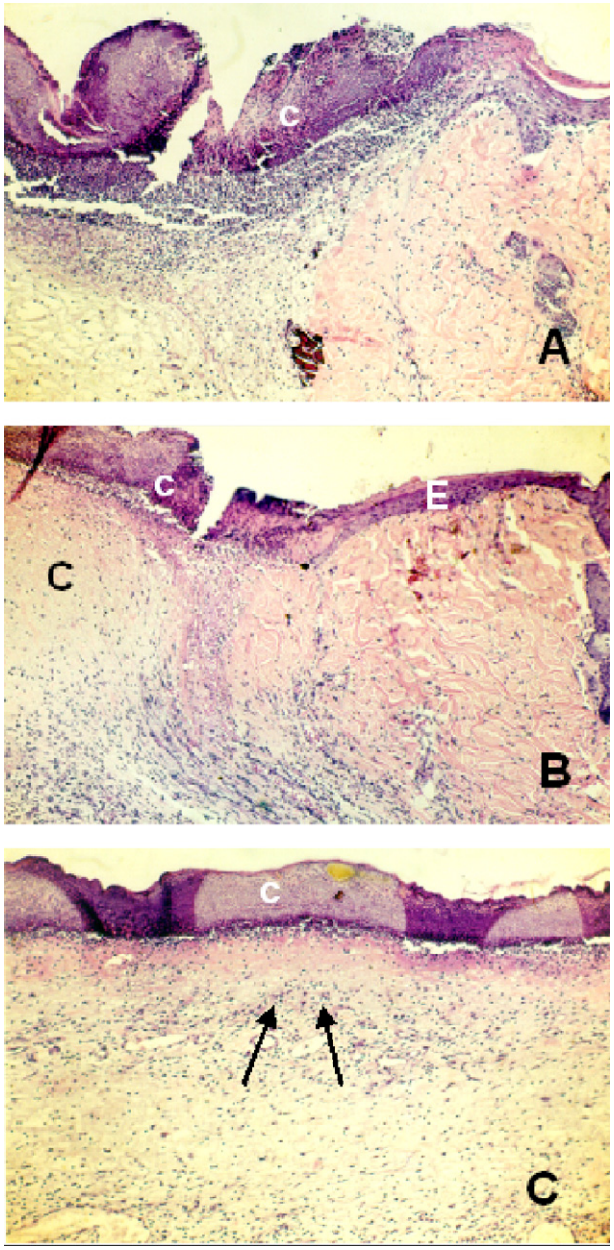


Fig. 4. (A–C) Photomicrograph of rat skin on day 3 p.w. (A) Control dermis; (B) Irradiated dermis ($D = 1.3$ J cm^{-2}); (C) Irradiated dermis ($D = 3$ J cm^{-2}). The wound edge is partially recovered by a thick epithelial layer (E) growing under the crust (c) through the other side of the wound; Observe re-formed connective tissue (C) in B. Lymphoplasmocitary infiltrate (arrow) is observed particularly in C. Three rats per group were analyzed. HE × 80.

(Fig. 4B). However, some differences were detected between irradiated and control groups regarding the characteristics of the wounded deep dermis. Re-formed capillaries, fibroblasts and small collagen fibers were observed in the laser treated lesions (Fig. 4C).

Seven days after injury, G1 analyses showed that part of the injured skin was still devoid of epidermis. Cell debris was observed near to the edges of the epidermis. The inflammatory process was still present (Fig. 5A) and connective tissue was unorganized, exhibiting a slight number of capillaries, fibroblasts and small collagen fibers.

For groups G2 and G3, epidermis was completely recovered at this time. At this moment a difference was noted between irradiated groups. The samples from G3 presented a connective tissue less organized than G2. A variety of inflammatory cells such as lymphocytes and macrophages was observed in G3, but not found in G2. The samples from G2 also exhibited a higher content of fibroblast cells and more new formed capillaries than G3 (Fig. 5B and C).

On the 14th day p.w., the epidermis was totally restored in all groups. However, for control group the epithelium was less differentiated than in irradiated groups (compare Fig. 6A, B and C). The superficial and deep dermis presented a moderate number of fibroblasts, with parallel orientation with the wound surface, and blood vessels. Some lymphocytes and macrophages were still observed (Figs. 6A and 7A). Irradiated groups

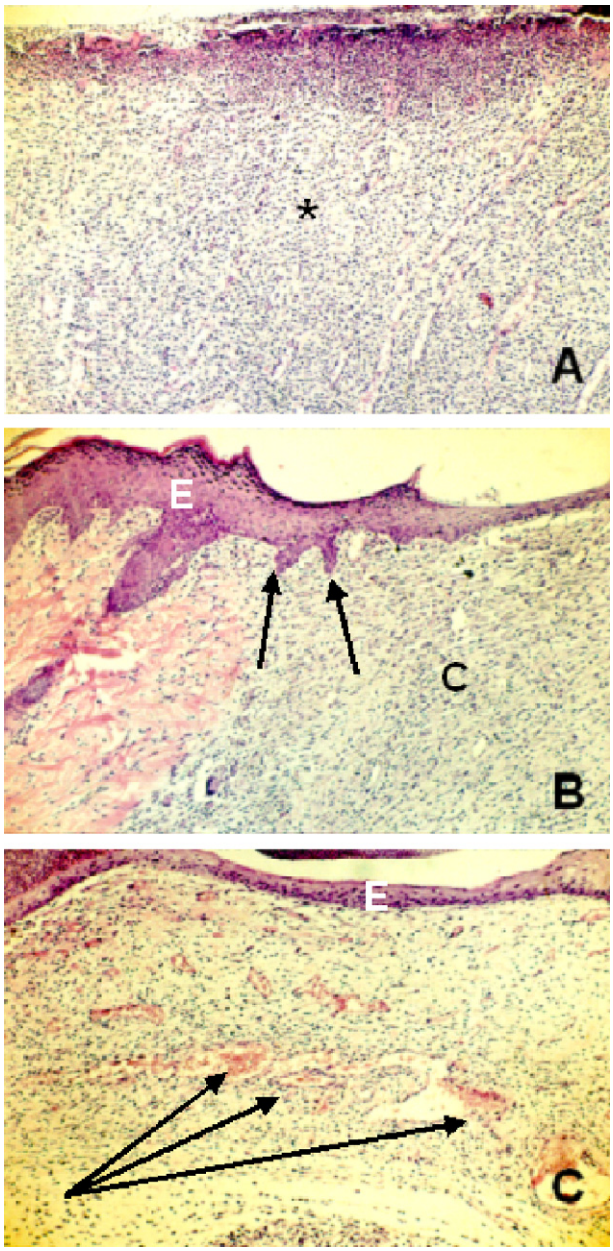


Fig. 5. (A–C) Photomicrograph of rat skin on day 7 p.w. (A) Control dermis shows an intense inflammatory infiltrate (*); (B) Irradiated dermis ($D = 1.3\ J\ cm^{-2}$); Observe the new epithelial layer covering almost completely the injured dermis (E). A re-formed connective tissue can also be observed (C). The arrow shows invagination of the epithelium. (C) Irradiated dermis ($D = 3\ J\ cm^{-2}$). A new epidermis covers the lesion area (E). A moderate neovascularization is observed in the dermis. Three rats per group were analyzed. HE × 80.

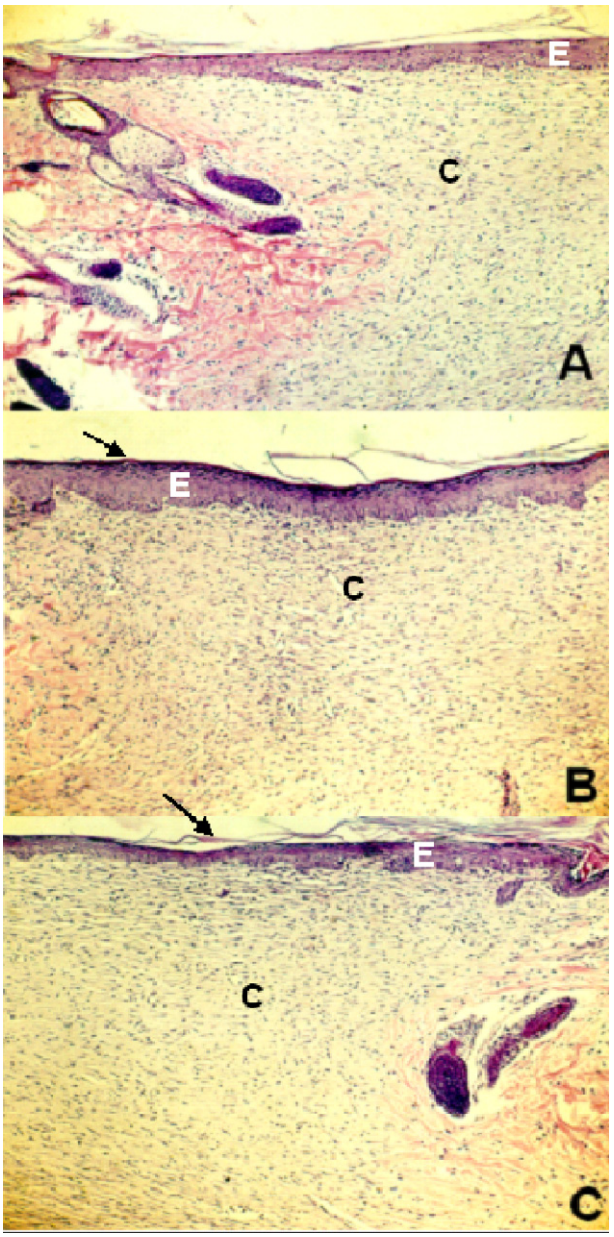


Fig. 6. (A–C) Photomicrograph of rat skin on day 14 p.w. The repaired dermis is easily distinguished from the original ones through the observation of the lack of glands and hair follicles, by the great number of large fibroblasts and by the organization of the extracellular matrix. Lesions are completely re-epithelized. (A) Control dermis. The arrow points to re-formed epithelium (E) and connective tissue (C). (B) Irradiated dermis ($D = 1.3\ J\ cm^{-2}$) and (C) Irradiated dermis ($D = 3\ J\ cm^{-2}$). Inflammatory cells were rare. Note a rose connective tissue (C) indicating collagen fibers presence. The arrow indicates keratin presence in the epidermis (E). Note a higher differentiated epithelium in (B). Three rats per group were analyzed. HE × 80.

showed a larger amount of fibroblasts, which presented conspicuous nuclei, indicating the intense activity of these cells (Fig. 6B and C). Thin collagen fibers were in a parallel alignment with the epithelium surface. Particularly, G2 samples showed better fibers arrangement than G3 (compare Fig. 7B and C).

Table 2 is a semiquantitative summary that shows the main histological findings determined from the analysis of laser treated and untreated wounds obtained from the pathologists' examination.

4. Discussion

The aim of the present study was to verify the effect of a single irradiation from an 830-nm GaAlAs laser on the rate

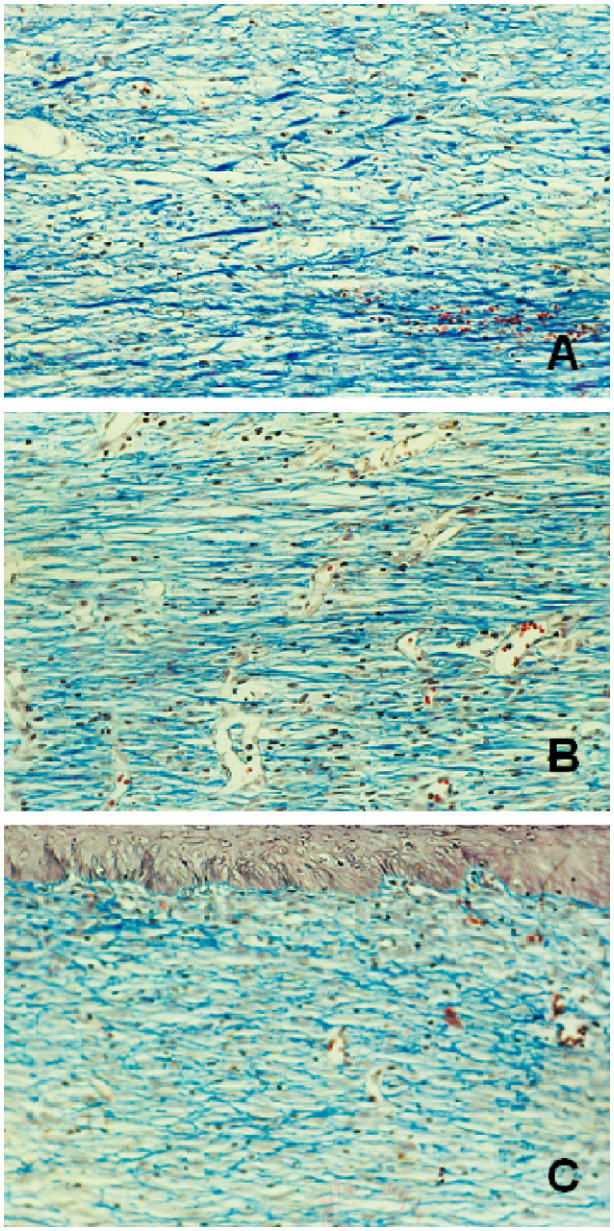
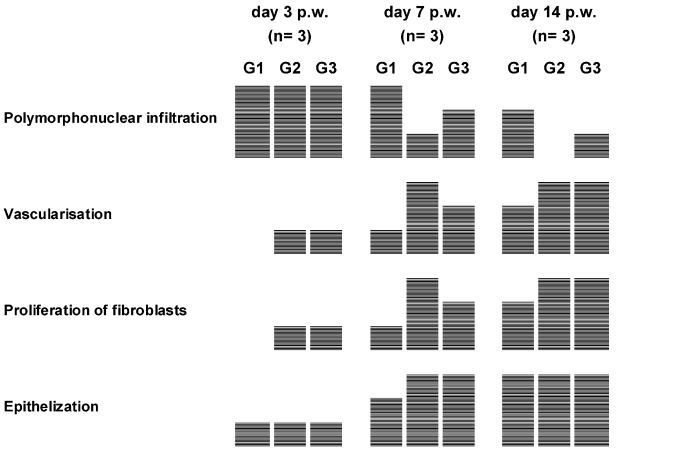


Fig. 7. (A–C) Photomicrograph of rat skin on day 14 p.w. Enlighten the collagen fibers organization. Comparing figures A, B, and C, a more organized connective tissue can be observed in B. Masson-Thricrome × 160.

Table 2
Schematic representation of the cutaneous wound healing process during experimental period



The size of each bar represents a semiquantitative evaluation of several morphological parameters. Semiquantitative results were organized according to the following scores: Absent, no box; slight, 1 box; moderate, 2 boxes; intense, 3 boxes. Three animals per group were examined on each day. The results reflect an overview of each group of animals.

of wound contraction and to determine whether any effect was dose-dependent. Treatment dosage is one of the most important clinical parameters. Specifying the right dose for a particular condition is not an easy task. Besides dose, other parameters must be taken into account, such as wavelength, irradiance, contact or non-contact application, exposure time, type of tissue, physiological conditions, as well as optical properties of the tissue [15]. In this study, the wavelength was unchanged, the irradiance was kept the same, the laser application was always non-contact, the target tissue was the same for all groups and the animal's physiological conditions were controlled. Thus, the studied variable, beside the single treatment proposition, was the dose, which means the amount of energy per area delivered to the tissue.

Differences observed between control and irradiated wounds indicate that laser irradiation affects various aspects of the healing process, including the degree of inflammation, formation and organization of the collagen, neovascularization and epithelization after a single light treatment.

The results obtained through the histological analysis also showed the importance of the treatment dose. The improvement on skin repair as well as the structural characteristics of the healing tissue represented by the amount of inflammatory cells observed, and the quality of the repaired connective tissue were associated with the energy density. The dose of 1.3 J cm^{-2} leaded to a better biological response in the earlier period of this study according to the biometrical evaluation and to the semi-quantitative histological study. This finding is in agreement with other studies, where low doses and near-infrared wavelengths were used [6,8,16–18]. Grossman et al. [18], for instance, showed in vitro that to obtain fibroblast proliferation with a 780 nm laser, 0.5 J cm^{-2} is needed.

The work of Hawkins and Abrahamse performed in cell cultures aimed to establish cellular responses to He–Ne laser irradiation using different laser fluences (0.5, 2.5, 5, 10, and 16 J cm^{-2}) on normal and wounded human skin fibroblasts [19]. The results showed that morphologically, wounded cells exposed to 5 J cm^{-2} migrate rapidly across the wound margin indicating a modulator or positive influence of phototherapy. Higher doses 10 and 16 J cm^{-2} were characterized by a decrease in cell viability and cell proliferation. The authors concluded that laser irradiation can modify cellular processes in a dose or flu-

ence dependent manner. The results of their research corroborate with our data. In fact, according to Vladimirov et al. [20] a beneficial action of the low-intensity laser light, as well as other light sources, as LED (light-emitting diode) is based on three different photochemical reactions: the photoreactivation of Cu–Zn–superoxide dismutase, which can be inactivated at low pH in hypoxic sites; the photodynamic action of endogenous sensitizers, mainly hematoporphyrin, that can be present in higher content in pathological conditions; and the photolysis of complexes of metal-containing proteins with nitric oxide, which leads to the release of free NO and in turn, this free compound may promote alterations on mitochondrial respiratory chain. In accordance with the authors, since laser radiation at low doses promotes a stimulatory effect on cells, and at higher doses no effect or even inhibitory effects may be observed, the amount of endogenous photosensitizer, as for instance, the porphyrin content into the tissue, may play an important role on dosimetry in light therapy.

According to Kujawa et al., LILT affects red blood cells not only in a fluence dependent manner but also in a radiant exposure (irradiance) dependent manner [21]. According to our results, even with the same irradiance the alterations were fluence dependents. In addition, Sommer and collaborators [22] have reported that the intensity necessary for biomodulation has to surpass a threshold value. Light intensities lower than this threshold would not produce biomodulatory effects. In our study, an intensity of 53 mW cm^{-2} was used. Our results show that this value was adequate to observe a faster healing process in laser treated wounds compared to control wounds. Moreover, in this study we irradiated the lesions in a non-contact, uniform way. The laser beam created a homogeneous light intensity on the tissue surface. Despite literature suggests the use of direct contact to reduce the energy loss due to reflection [23], non-contact treatment has the advantage of producing a more homogeneous irradiation in extended surfaces; besides, this method avoid the contamination of the laser tip with blood and debris during treatment. Furthermore, in this work it was used a non-polarized diode laser beam with perpendicular incidence, which results in a minimum energy loss due to reflection. This method of irradiation leads to a 4–7% of radiation trouncing due to reflection [26].

Usually the fluence has to be evaluated regarding the target tissue. In uncovered tissues, as open wounds, low fluences (1–5 J cm^{-2}) may present good results [23]; conversely, if bone tissue is the target, higher fluences may present better results. In fact, Silva and Camilli showed that the highest fluence (10.2 J cm^{-2}) was better than the lowest (5.1 J cm^{-2}) to repair bone defects [24]. Therefore, to produce a dose–response type curve not only the fluence and the irradiance must be taken into account but also the target tissue and its optical properties on the moment that the irradiation is performed. Actually, the physical properties of the near-infrared radiation permit a higher penetration in skin since its absorption by water and blood is weak [23,25,26].

The single irradiation method proposed in this study could be particularly interesting to medical practice. A variety of techniques has been developed aiming to restore the function and configuration of the damaged tissues. However, flap necrosis due to a lack of vascularization still remains as a problem in this type of surgery. The study of Kubota showed increased perfusion of axial pattern flaps and a better flap survival after irradiation with an 830 nm diode laser. Due to the simplicity of this therapy, it could be performed immediately after surgery or even during surgery to improve the clinical outcome [27].

The severity of the injury and the response of the individual are the main factors that control the entire outcome of the repair process. It is possible that laser radiation affects the individual response to an injury. Several chemical mediators are responsible for the host reaction to an injury and they can be detected as soon as the injury is inflicted. According to the work of Albertini et al., the low power laser irradiation possibly exerts anti-inflammatory effects by modulating the release of adrenal corticosteroid hormones [28]. The work of Kandolf-Sekulovic et al. demonstrated that LILT shows a systemic immunomodulatory effect on contact hypersensitivity (CHS) reaction in rats [29]. Decreased ear swelling observed in the elicitation phase was associated with diminished proliferative responses of the draining lymph node cells in the induction phase of CHS reaction. The effects were observed after a single treatment with a near-infrared laser with a fluence of 3.6 J cm^{-2}. In our study, a significant difference on wound closure was observed between G2 (dose = 1.3 J cm^{-2}) and control group (G1) at day 7 p.w. This finding suggests that laser radiation can act on the cellular events that happen during inflammatory stage. The reduction on the duration of the inflammatory phase may result in a faster entry into the proliferative stage of healing, when granulation tissue is produced. This mechanism of action fits with our histopathological analysis of the healing process on the 7th day p.w. Lesions of G2 presented a higher fibroblast proliferation, a lower polimorphonuclear infiltration and neovascularization more pronounced than control lesions. However, to explain the exact modulator mechanism of laser radiation on inflammation or even during the repair process does not seem to be a simple task. Nevertheless, as this study shows, it is important to emphasize that even after a single exposure, not only early effects can be observed but, during the whole repair process, the effect of a single exposure can be detected.

The findings of this study also suggest a better arrangement of the collagen fibers in the irradiated tissues. Interestingly, wounds irradiated with the dose of 1.3 J cm^{-2} showed collagen fibers more organized and a ticker epithelial layer than those irradiated with 3 J cm^{-2} (compare Figs. 6B and C and 7B and C). This finding is extremely relevant on the beginning of the healing process since, the

resistance of the skin is impaired, and thus increased tensile strength in the wound would be important to avoid postoperative complications. Stadler et al. [30] using an 830 nm diode laser showed an increased tensile strength during skin repair in the irradiated groups in a diabetic murine model.

Some studies report no beneficial effects of laser light on wound healing processes [31,32]. Schlager et al. [31], for example, used a 670-nm laser light with 2 J cm^{-2} and 250 mW of output power during 10 consecutive days in rats burned skin. According to the authors, no beneficial effects of the laser treatment were observed. Probably, 10 consecutive exposures brought inhibition of the laser effects since a therapeutic window had been previously reported [23,33].

Our results also demonstrate that there are determined dose values, which conduce to good biological response, with viable times for clinical practice. Significant results on wound healing improvement were obtained by a methodology that required only a single laser treatment. This is an important issue regarding the clinical practice. Monstrey and collaborators [34] reported that treatment of deep dermal wounds with polarized-light irradiation resulted in a significantly shorter healing time, with almost no hypertrophic scarring, and optimal aesthetic and functional results at a long-term follow-up, however, despite the conservative treatment and no extension of the hospital stay, the patients were treated until complete closure of the wound and in some cases the treatment was performed for as long as a month.

In conclusion, low-intensity laser therapy may provide acceleration of cutaneous wound healing in rats. A uniform exposure from a single irradiation with a laser emitting at 830 nm could be used to hasten the biological response in a conservative way. The effects were to some extend dependent upon the dose. The dose of 1.3 J cm^{-2} produced a better result when compared to the dose of 3 J cm^{-2}, according to the histological analyses, although the biometrical analysis did not show differences between these two doses.

5. Abbreviations

CHS	contact hypersensitivity
D	energy dose
GaAlAs	gallium aluminium arsenide
HE	hematoxylin–eosin
He–Ne	helium–neon
LED	light-emitting diode
LILT	low intensity laser therapy
LSD	least square difference
G1	Group 1
G2	Group 2
G3	Group 3
PVC	polyvinyl chloride
p.w.	post-wounding

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