Lasers Med Sci (2013) 28:633–641
DOI 10.1007/s10103-012-1105-4

ORIGINAL ARTICLE

The influence of red laser irradiation timeline on burn healing in rats

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Received: 17 January 2012 / Accepted: 16 April 2012 / Published online: 23 May 2012

Abstract Low-level laser therapy (LLLT) promotes biomodulation of wound healing and literature reports that light delivery during the inflammation could play a different role compared with latter phases of the healing process. The objective of this study was to investigate whether single dose of a red laser (λ=660 nm) is different from fractionated delivery protocol in full thickness burns. Two lesions were inflicted on the back of 36 rats. In the fractionated dose group (FG), the lesions were irradiated with 1 J/cm² on days 1, 3, 8, and 10 post-wounding. In the single dose group (SG), the lesions were irradiated with 4 J/cm² on day 1, immediately after injury. Control lesions (CG) received no light and were left to heal spontaneously. Blood flow was measured on days 1, 3, 8, 10, 15, and 21 using laser Doppler flowmetry. Animals were killed on days 3, 8, 10, 15, and 21. Skin specimens were obtained and routinely processed for hematoxylin and eosin. The specimens were evaluated according to differential leukocyte counting and angiogenesis. Statistical analysis was performed, and significance was accepted at $p<0.05$. Irradiated groups showed a peak of new vessels on day 15 while, for CG, the peak was on day 21. On day 21, FG exhibited a significantly greater number of cumulative neutrophils while SG showed a higher number of mononuclear cells. Our results confirm that both protocols used accelerate angiogenesis and stimulate leukocyte chemotaxis on burn treatment. In addition, this work suggests that a single-dose LLLT accelerates the inflammatory phase of skin repair.

Keywords Angiogenesis · Blood flow · Laser Doppler · Leukocytes · Skin repair

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Introduction

One of the most common causes of human lesions is burn injuries. The burn wound faces the double challenge of repair and regeneration from the periphery along with the clearance of necrotic tissue from more central areas. The margins of the burn wound are not clearly demarcated as thermal injury extends beyond the tissue that is immediately in contact with the heated element that produces the burn [1]. Moreover, the structural damage may not necessarily correlate with functional loss, definitely not for early biopsies when considering the progressive nature of a burn wound [2].

With current therapy, both cosmetic and functional outcomes are often unsatisfactory. Novel strategies designed to heal burn wounds with tissue regeneration rather than

fibrous scarring would have tremendous potential for individuals suffering from burn injuries. Restoring the vascular bed of the dermis after a burn is an important potential component of tissue regeneration [1].

In wound healing, the sequence of events awaiting a complete wound closure and repair can be divided into three overlapping phases: inflammation, proliferation, and matrix formation and remodeling. Literature reports show that phototherapy accelerates wound healing through the stimulation of reepithelization, proliferation and differentiation of fibroblasts, increased protein synthesis, reduction of inflammatory process, augmented production of extracellular matrix, and higher organization of collagen bundles [3–5].

Defining protocols for photobiomodulation is not an easy task, since this therapeutic modality involves several dosimetric parameters such as wavelength, output power, exposure time, energy, dose, number of treatments, and treatment intervals, among others [6]. Additionally to the light source parameters, tissue optical characteristics should be considered [7]. Obviously, tissue optical coefficients change depending on tissue condition (e.g., healthy and injured tissue) in such way that different regimen treatments should be proposed [8, 9].

Although studies have demonstrated acceleration on wound repair, in reality, there is a scarcity of works about different dose regimens. It is still unknown the effects of a single irradiation compared with multiple irradiations resulting in the same total energy but applied in different moments during the healing process. However, the different tissue responses according to the phases of wound healing to phototherapy were reported in some papers. Laser treatment during inflammatory phase of tendon repair produced much greater stimulatory effects than irradiation on proliferative phase, indicating that the moment of intervention may be critical [10].

One hypothesis for a different effect of fractionated light application as compared with a single irradiation would be the different tissue optics as a consequence of the different cellular targets found in the wound during the healing course. On the inflammatory phase, targets would be the pro-inflammatory mediators, as, for instance, the ones released by mast cells, promoting initial vascular reactions [11]. In contrast, on the proliferative phase, pro-angiogenic factors as well as fibroblast stimulation mediators would be more profuse targets [12]. Therefore, multiple irradiations may stimulate different events. On the other hand, a single irradiation on the beginning of the process may produce enough consequences on the signal cell transduction mechanism that its effect may be observed in the whole course of the healing process.

The present study was designed to investigate the effects promoted by laser irradiation when it is applied at specific moments of the healing process. We also studied if there is any difference in tissue response when single or fractionated dosage is applied to treat full thickness burns. We evaluated morphological and functional changes counting leukocytes and blood vessels, and by using laser Doppler flowmetry.

Methods

Animals

Thirty-six healthy male adult Wistar rats with approximately 300 g of body mass were used in the trial. During the experimental period, all animals were individually housed in acrylic plastic isolators in a 12-h light/dark cycle and fed with granulated food and water ad libitum. The animals were anesthetized by intraperitoneal injecting 80 mg/kg ketamine and 10 mg/kg xilazine before thermal injury and during experimental procedures. The animals had their back fur removed by a shaver, and the skin was cleaned with povidone-iodine solution. All procedures, care, and handling of the animals were carried out according to the Ethical Principles of Animal Experimentation formulated by the Brazilian College for Animal Experimentation and were approved by Ethics Committee on Research Animal Care of IPEN/CNEN-SP.

Injury model

For burn induction, the dorsum of each rat was exposed for 5 s to the tip of a flexible rubber tube, 5 mm in diameter, connected to a source of boiling water conducting the steam. Two burns were produced resulting in reproducible and non-lethal wounds. The burns presented clear limits (around 6 mm^2), and previous histological analysis revealed that epidermis and dermis were completely injured.

Laser irradiation

The animals (n=36) were randomly divided into two groups. A GaAlAs diode laser (Kondortech, São Carlos, Brazil) emitting red light (λ=660 nm) was used in the experiment. The laser output power (30 mW) was measured by a laser power meter (LM-01, Coherent, USA) ensuring a density power of 30 mW/cm^2. The irradiations were performed in the same manner with the laser probe in contact with the wound. The laser beam covered the entire burn including the boundaries illuminating an area of 1 cm^2.

In the fractionated dose group (FG), the lesions (n=18) were irradiated with 1 J/cm^2 corresponding to an exposure time of 33 s on day 1 immediately after wounding and on days 3, 8, and 10 post-wounding. In the single dose group (SG), the lesions (n=18) were irradiated with 4 J/cm^2 (Δt= 133 s) on day 1, immediately after injury. Control lesions

(CG, n=36) received no light and were left to heal spontaneously. The control and irradiated lesions were always more than 1 cm apart to avoid light scattering and therefore any interference on the healing process. Each experimental animal acted as its own control due to the individual variability on the duration and quality of repair [12].

Histomorphometrical analysis

Three animals per group (FG and SG) were euthanized in a CO_2 chamber on moments 3, 8, 10, and 15 post-treatment for histological analyses. Six animals/group remained alive for flow measurements, and three were killed on day 21 (n= 3 lesions per group per time point). The skin samples were carefully collected to include the adjacent healthy tissue and all the healed tissue in depth.

All specimens were fixed in 10% buffered formalin (pH 7.4) and paraffin embedded. The microscopic features were evaluated from the analysis of one 5-μm section of each sample, stained routinely with hematoxylin and eosin. All burns were classified as full thickness because there was collagen denaturation and microvascular damage with deep thrombosed vessels. Stained sections were observed and photographed using a calibrated ocular on a light microscope (Leica DMLP, Wetzlar, Germany).

Quantitative assessment of new vessels

For a quantitative assessment of new vessels, the vessels localized in the dermis were counted inside three square areas from the deep of the lesion measuring 10,000 μm^2 each using the Image J (free software, NIH, Bethesda, Maryland, USA) in such way that the total number of vessels was divided per the number of squares in each slide. At least three slides from each animal were observed. Healthy skin was also evaluated.

Leukocyte differential counting

The cell count was performed by an experienced pathologist with no previous knowledge of the samples. The absolute counts of leukocytes and relative proportions of polymorphonuclear neutrophils and mononuclear cells (lymphocytes and macrophages) were determined separately in the deep of the burn wound. All counts were performed in ten alternate microscopic high-power fields (×1,000) using an integration graticule (Leica Microsystems, Wetzlar, Germany).

Laser doppler flowmetry

Before injury, the blood flow register was performed at three selected sites over the vertebral column always in head–tail direction. The blood flow was measured using the method previously described [13]. Briefly, a Flolab® flowmeter (Moor Instruments Ltd., Denvo, UK) was used equipped with a 1 mW laser, emitting at λ= 780 nm. The probe was a MP13 (Moor Instruments, 1.5 mm external diameter, two 0.25 mm optical fibers, 0.5 mm spaced apart). MP13 is a non-contact probe that prevents flow alterations due to mechanical contact with the skin. The Doppler filter of the instrument was fixed at 15 kHz. The flowmeter probe was fixed by a metallic arm, avoiding involuntary movements due to its manipulation, which could affect the recorded signals. The data were analyzed according to the proposed hemodynamic parameter—$F(\%)=100\ (F_{LS}/F_{CS})/F_0$, where F_{LS} and F_{CS} are the mean blood flow from lesion site (LS) and control normal site (CS), respectively. F_0 is F_{LS}/F_{CS}, both measured prior to injury, i.e., the baseline. $F(\%)$ means the percentage of perfusion variation of the LS, referred to the CS, and referred to the measurements on the first day. Thus, calculated values of $F(\%)$ from the day 1 prior to lesion are always 100%. Detailed mathematical modeling of the normalized parameter $F(\%)$ showed its lower sensitivity to systemic and environmental flow variations to instrument calibration errors, as well as to the dispersion of values among samples.

After registering the blood flow in the three different areas, two sites were randomly selected to receive the burn, and the remaining one served as the healthy skin control, to provide basis of comparison regarding normal skin blood flow during experimental period. Lesion sites were located at the middle of the back at 3 and 6 cm from the base of the tail and received a burn. CS was located at 1 cm from the base of the tail. In this site, the control measurements of healthy skin blood flow were performed. Lesion and control sites were standardized due to the great spatial variation of blood flow even in close anatomic areas [13]. Thus, randomized sites could make the analysis of the results unfeasible, because of the dispersion of flow values. The probe distance from the skin was adjusted using a 1 mm spacer. For both sites (LS and CS), a 6 mm diameter area was selected, in which three 30-s distinct measurements were carried out, to compute a mean blood flow of each site.

The blood flow was measured on days 1, 3, 8, 10, 15, and 21 post-wounding. The perfusion registers were stored in a personal computer and were analyzed using software supplied by the manufacturer of flowmeter (MoorSoft Windows®/moorLAB, v1.2). Laser Doppler values are presented as percentage changes from initial blood flow obtained on day 1, before carrying out any procedures. Mathematical modeling of the hemodynamic parameter $F(\%)$ used in this work was described previously [13].

Statistical analysis

It is well known that physiological parameters, as discussed in this work, do not follow a Gaussian (normal) distribution but a log-normal one [14]. In this manner, the number of new vessels, values of leukocyte differential counting, and percentage of blood flow were normalized replacing them with the log (ten base) of their values. To show the actual distribution of the measured data, we used a graph based on mean value and standard deviation. Parametric methods were used to detect differences between all possible pairs in the within-group and within-day analysis. Parametric two-sample *t* test was used as an inference test. Null hypotheses were rejected at the 0.05 risk level. For data analysis, Origin Pro 8® software (OriginLab, Northampton, USA) was used. The number of new vessels, values of differential counting, and cumulative blood flow were also analyzed by integrating the data of each group from day 3 until day 21 of the experiment. Once obtained behavior of the cumulative data of each group, a linear fit was performed to obtain information about the speed in which the increased data, using the slope of the fit. For implementation of this mathematical analysis, the software Origin 8® was also used.

Results

Morphological analysis

Morphological analysis of the burns showed that the structural characteristics of the repaired skin after LLLT are not dependent on the moment of irradiation during the healing process (Fig. 1). We also observed that, during the first 10 days post-wounding, no significant differences were observed when irradiated, and control lesions were compared. All groups showed thermally injured hyaline collagen fibers, chronic inflammatory cells, and cell debris. On the tenth day post-wounding, some new vessels could be observed in the dermis, and most of them were seen in close proximity to the necrotic area (Fig. 1a, c and e). Cutaneous ulcers were still present in all groups, but irradiated groups showed more advancing epithelial sheets (compare Fig. 1a and c, e).

After 15 days, morphological differences were also observed among irradiated and control groups, and these differences increased until day 21. Control and laser groups showed complete epithelization of the wound, however, control group was in proliferative phase, with granulation tissue subjacent to the lesion still presenting many inflammatory cells, blood vessels, and immature collagen beginning to be oriented (Fig. 1b). SG and FG groups were in the remodeling phase, with devascularization, scanty inflammatory cells, and enlarged fibroblasts distinguished among the few acidophilic collagen fibers (Fig. 1d and f). Besides, the number of new vessels for CG was higher than irradiated groups (compare Fig. 1b and d, f). No appendages of the skin or sebaceous glands were observed in the regions of repaired dermis.

Quantitative assessment of new vessels

The effects of single and fractionated doses on vessels count are plotted in Fig. 2. An increase of the number of new vessels was observed during the experimental procedure (Fig. 2a). On days 8 and 15, no statistically significant differences were observed among groups. On day 10, a significantly higher quantity of vessels was observed on FG when compared with the CG ($p=0.00027$), while SG and CG revealed no significant differences. On day 21, the number of vessels was significantly higher on CG than on irradiated groups ($p=0.01933$ and 0.03439 regarding FG and SG, respectively). A noteworthy remark is that the greatest amount of vessels for SG and FG was observed on day 15 whereas for CG the max out was detected on day 21.

Figure 2b shows cumulative new vessels number from day 3 until day 21. According to linear fit performed, no statistically significant differences were observed among groups.

Leukocyte differential counting

On day 3, counting of neutrophils for SG and FG groups was significantly higher than control ($p=0.03143$ and $p=0.02165$, respectively), but it was similar to each other. On day 10, FG showed a significant increase in neutrophils when compared with SG ($p=0.03239$) and CG ($p=0.03814$). On days 8, 15, and 21, no statistically significant differences were detected (Fig. 3a).

Figure 3b exhibits the cumulative neutrophil counting during the whole experimental period. The highest slope value was observed for FG (2.12±0.19) indicating that neutrophils in this group appear prior than SG (1.77±0.15) and CG (1.67±0.21). On days 8, 10, and 15 post-injury, neutrophil counting was significantly high for laser groups compared with CG. On day 21, only FG showed statistically significant differences compared with CG ($p<0.05$).

Lymphocytes and macrophages, on the other hand, presented a different behavior (Fig. 4). Only on day 10 was a significant increase of these cells detected for FG and SG compared with CG ($p=0.03681$ and $p=0.01856$, respectively). Once again, no statistically significant differences were observed between irradiated groups at this moment (Fig. 4a).

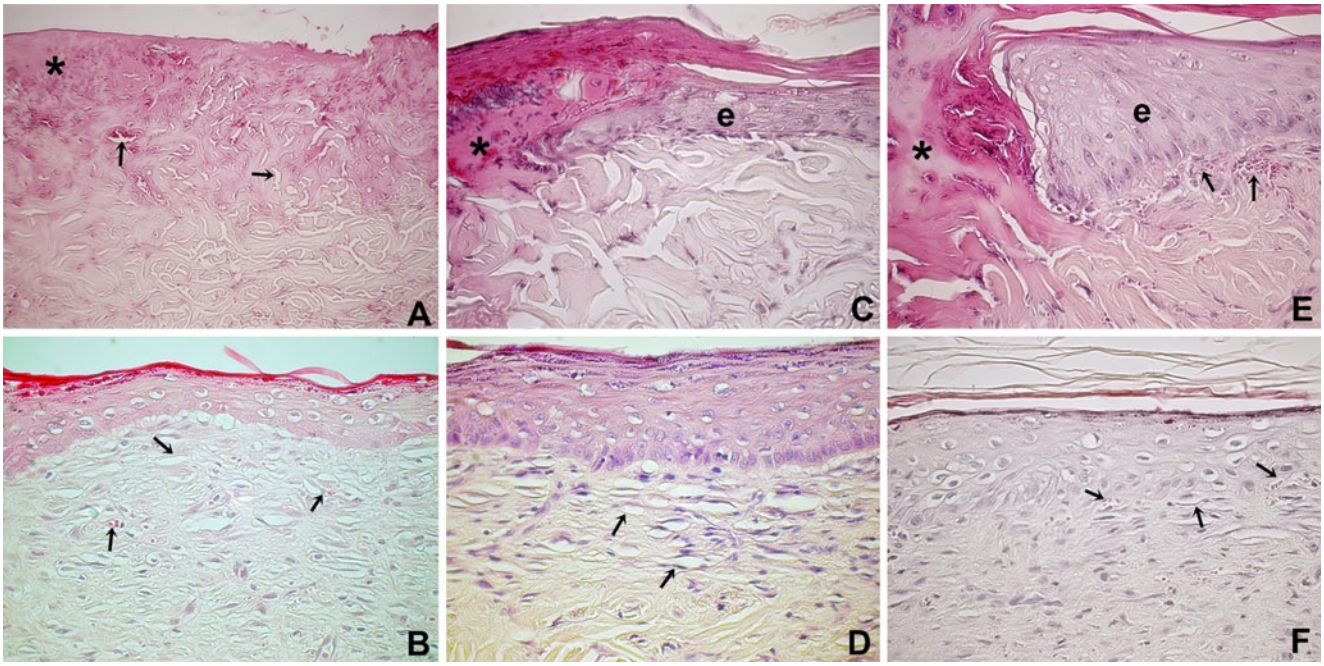

Fig. 1 Photomicrographs of control (**a** and **b**), fractionated dose (**c** and **d**), and single dose (**e** and **f**) groups' injured skin. **a** CG 10 days post-wounding (p.w.) showing denatured collagen (*), *arrows* show blood vessels in the dermis; **b** 21 days p.w. showing complete epithelization, granulation tissue in the remodeling phase with oriented collagen fibers, and angiogenesis (*arrows*); **c** SG 10 days p.w. showing denatured collagen fibers (*) and incomplete epithelial layer (*e*); **d** on day 21 p.w. showing a complete thick epithelial layer and granulation tissue with newly formed blood vessel subjacent to the epithelium (*arrows*); **e** FG on day 10 p.w. with denatured collagen (*) and incomplete epithelial layer (*e*) but with more blood vessels in the dermis (*arrows*); **f** FG on day 21 p.w. with intact epithelial layer and granulation tissue in the dermis (*arrows* point to angiogenesis) (HE, original magnification ×160)

Regarding the appearance of these cells in tissue, SG showed a greater slope value (2.36±0.07) than FG (2.18±0.13) or CG (2.02±0.06) (Fig. 4b). For both 15 and 21 days post-burning, SG exhibited a statistically significant higher accumulation of mononuclear cells than CG. On day 21, SG was also significantly different from FG ($p<0.05$).

Laser doppler flowmetry

We then investigated the blood supply on tissue. In Fig. 5a, it can be seen that no statistically significant differences are observed regarding FG, SG, and CG on days 3, 8, 10, and 15 post-injury. On the 21st day, blood flow is similar for SG and FG, but both are significantly different from CG ($p=0.02356$ and $p=0.00692$, respectively).

When the cumulative flow was measured for the whole experiment, significant differences were noted (Fig. 5b). It was found that CG had a slower increase of blood flow (slope value=2.11±0.06) compared with other groups. SG and FG had similar boost of flow (2.23±0.05 and 2.22±0.05, respectively). On day 21, both FG and SG presented a significantly higher accumulation of blood flow compared with CG ($p<0.05$).

Discussion

Our results corroborate that both regimens used to treat full-thickness burns increase leukocyte influx and accelerate angiogenesis compared with untreated control. Some punctual significant differences depending on the analyzed moment were detected, indicating that single dosage accelerates tissue response to repair. This finding may be extremely interesting to critical burn patients as a single irradiation could hasten the clinical response.

There have been numerous reports in the literature over the last 40 years that low-level laser therapy can stimulate wound healing [15–17] in both animals and humans including burns [18, 19]. Healing of soft tissues is a complex three-phase process involving inflammatory phase, formation of granulation tissue, and remodeling [20]. In our work, particularly, on day 10 post-burning, we observed a significant difference between SG and FG regarding quantity of neutrophils and mononuclear cells. In fact, in the inflammatory stage, there is a neutrophil exudation peak followed by a peak of mononuclear cells. Thus, our results suggest that single irradiation accelerated neutrophilic leakage since FG showed the highest neutrophil counting.

An interesting remark that deserves further investigation was the pronounced highest slope value for SG (see

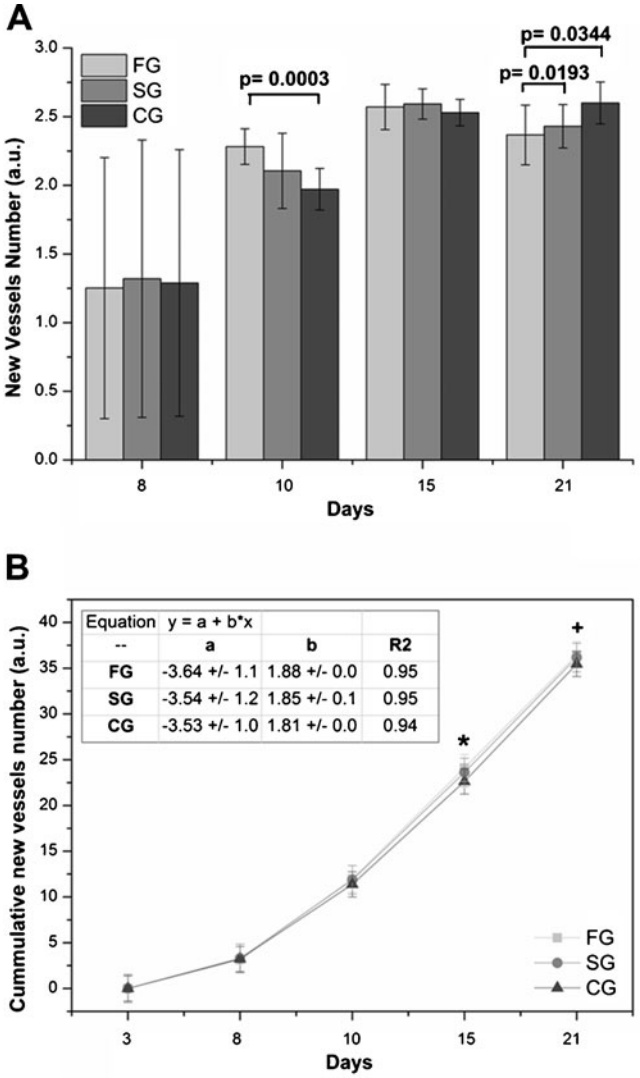

Fig. 2 New vessels number (**a**) and cumulative new vessels during the whole experimental period (**b**). Fractionated dose group (*FG*), single dose group (*SG*), and control group (*CG*). The *bars* represent standard deviation of the mean

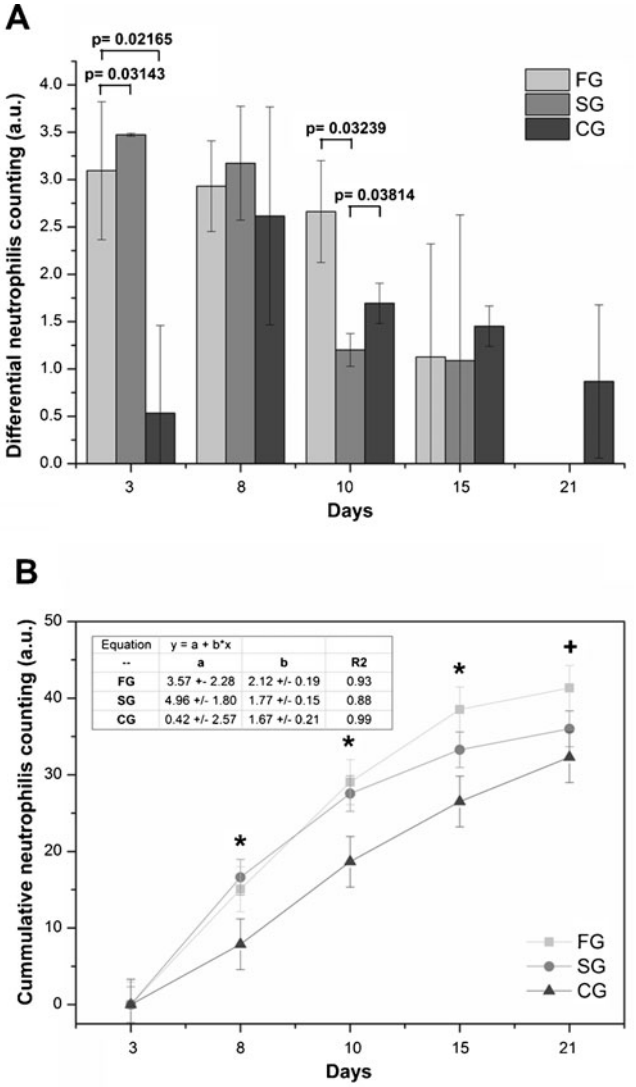

Fig. 3 Neutrophil counting (**a**) and cumulative neutrophils during the whole experimental period (**b**). Fractionated dose group (*FG*), single dose group (*SG*), and control group (*CG*). The *bars* represent standard deviation of the mean. *Asterisk* indicates SG and FG significantly different of CG; *plus sign* indicates FG significantly different of CG ($p<0.05$)

Fig. 5b). This finding indicates that lymphocytes and macrophages appeared prior in this group. We can hypothesize that single dosage may be able to anticipate the cellular signalization, which triggers inflammation in a more effective way compared with slit dosages.

On the other hand, evidence suggests that fluence rate and power density are key biological parameters of LLLT, and they may both work with lower and upper thresholds. Therefore, between these values, LLLT is effective, and outside of them, LLLT is too weak to have any effect (values below the threshold) or so intense that the effects may be inhibited (values above the threshold) [21].

The work of Haxsen and co-authors describes the role of power density reporting that the osteoblastic response is altered by the power density rather than by fluence. They describe a logarithmic association with a threshold and not a linear dose-dependency response [22]. In our work, the power density was kept during the experiments. Therefore, we may infer that the 30 mW/cm^2 was inside the power density threshold.

In contrast, Boschi et al. also report a threshold but for energy instead of power density [23]. They report that the LLLT anti-inflammatory effect response is energy-dependent. According to their results, laser treatment with 2.1 J was more effective than 0.9 and 4.2 J. Thus, the hypothesis that in our study fractionated dose 1 J/cm^2 had not reached threshold energy for some of the considered parameters (neutrophils and mononuclear cells) could be further investigated.

The stimulatory effects of LLLT seem to be related to specific events during the first two phases of wound healing:

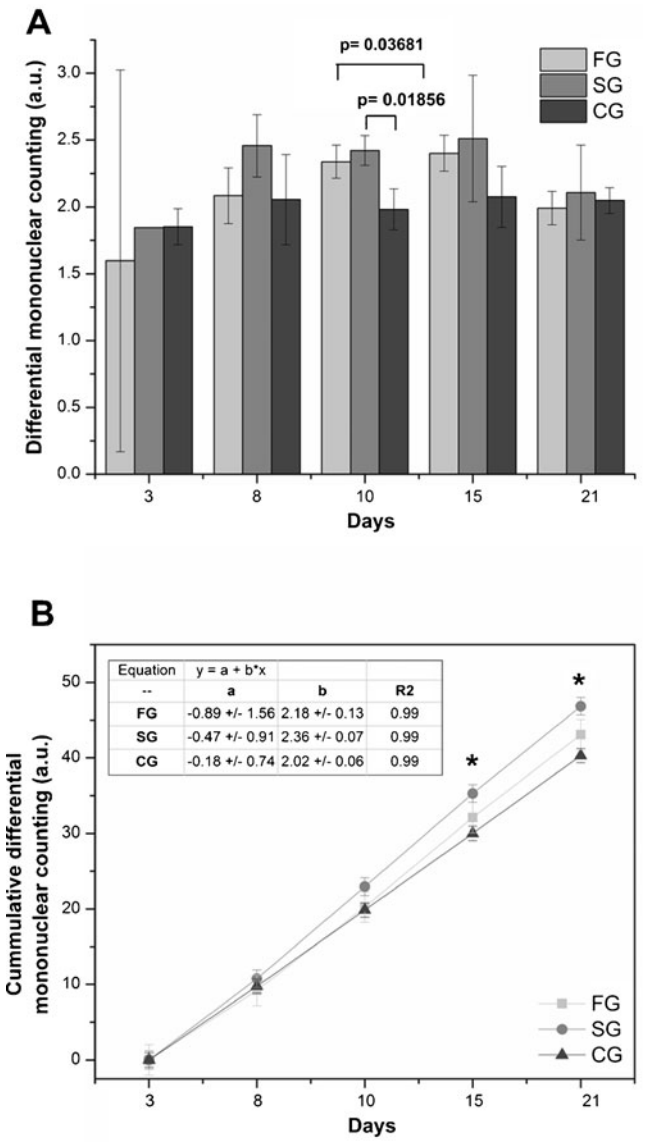

Fig. 4 Lymphocyte and macrophage counting (**a**) and cumulative lymphocytes and macrophages during the whole experimental period (**b**). Fractionated dose group (*FG*), single dose group (*SG*), and control group (*CG*). The *bars* represent standard deviation of the mean. *Asterisk* indicates SG significantly different of CG; *plus sign* indicates SG significantly different of FG and CG ($p<0.05$)

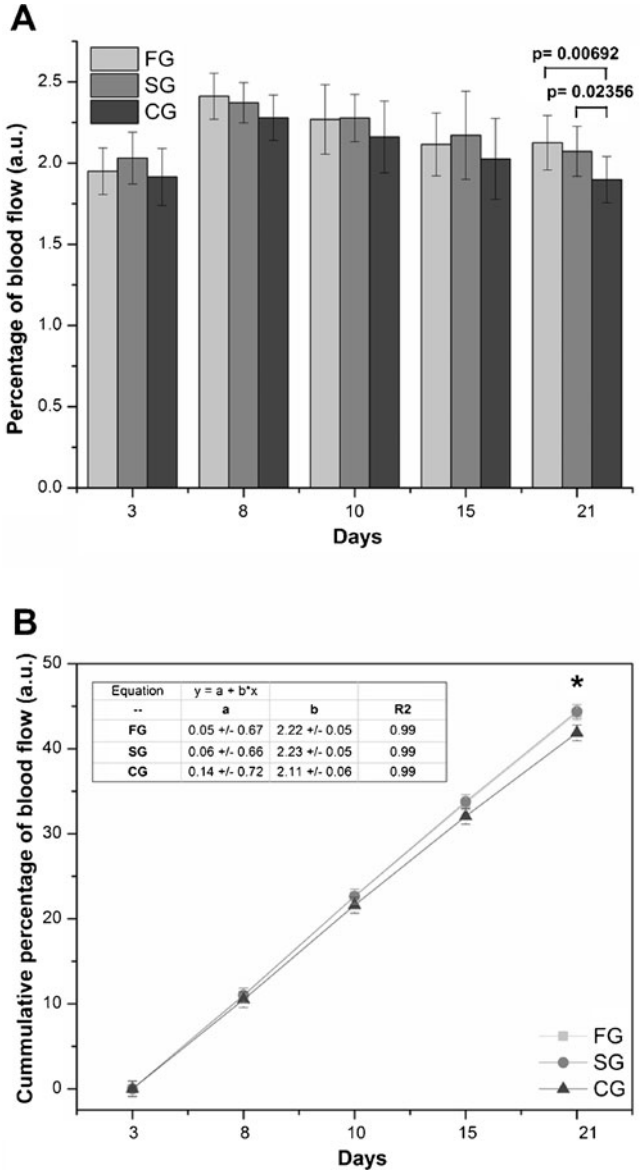

Fig. 5 Percentage of blood flow (**a**) and cumulative blood flow during the whole experimental period (**b**). Fractionated dose group (*FG*), single dose group (*SG*), and control group (*CG*). The *bars* represent standard deviation of the mean. *Asterisk* indicates SG and FG significantly different of CG ($p<0.05$)

the inflammatory and the proliferative phase, indicating that the moment of irradiation may be important [10]. For this reason, for FG, the treatment was performed at days 1 and 3, aiming the inflammatory phase, and at days 8 and 11, aiming the proliferative phase. For SG, the treatment was performed on the first day with a higher dosage, therefore acting over the initial inflammatory phase. In fact, laser treatment would reduce the intensity and duration of the inflammatory phase [11, 24].

The relationship of burn depth and microvascular blood flow is well established. Laser Doppler technique has been used since 1975 for monitoring the cutaneous circulation. Doppler flowmetry is based on the Doppler principles stating that, when monofrequency light waves are reflected off moving objects, they undergo a change in frequency. Laser Doppler imaging (LDI) is the only technique that has been approved by the American Federal Drug Administration specifically for the assessment of burns. This follows a long and consistent body of works demonstrating the efficiency of LDI in clinical studies. The technique was first used by Niazi et al. who demonstrated that the accuracy of LDI assessment was 100% compared with biopsy–histology and only 65% for clinical assessment [25].

When comparing the cumulative flow among the groups, SG shows more rapid flow increase compared to other groups, indicating better vascularization. This difference is not noticeable when comparing the flow in every moment of the experiment.

Interestingly, the vessel count for FG on day 10 was considered significantly different from CG, but it was not different from SG, while SG was not different from CG. These data may require that a single greater energy density delivery (4 J/cm^2), which is more cost effective and also present a good compliance, would be the best to be performed since no differences were observed between FG and SG.

The earliest increase on the amount of vessels observed on irradiated groups (on day 15 for irradiated groups and on day 21 for CG) may contribute to the healing events triggered by LLLT. More vessels may indicate a better distribution of the irrigation on the tissue without necessarily meaning that the flux (measured by *F*%) is different. Briefly, the laser Doppler signal is related to the flow of the blood cells (mostly red blood cells), which is defined as the product of the number of blood cells and their velocities within the measured skin volume. It is generated by the movement of blood cells in both the sub-capillary thermoregulatory vascular bed and the nutritional capillaries [14]. Therefore, if we have more vessels, it does not necessarily mean that we would have more or faster red blood cells at the moment when the measurement is being performed. In summary, in any specified moment, if the pre-capillaries sphincter on a given area is closed, the blood flow will be low, although the number of blood vessels present in this area may be elevated.

It is important to note that blood flow and the number of blood vessels on microcirculation (mostly capillaries) do not present a linear relationship since capillary microcirculation is controlled by a complex pattern of opening and closing of precapillary sphincters, a phenomenon known as vasomotion.

The quantity of new vessels present on irradiated groups showed a similar pattern with that observed in a healthy skin sample on day 21. These results suggest that SG and FG are already in the remodeling phase, which is the last healing stage. At the same time-point, CG showed a higher number of vessels than healthy skin and also than irradiated groups suggesting that the healing is delayed compared with the two experimental groups.

The importance of dose versus irradiation moment is still a matter of investigation. If a single laser exposure would be enough to produce the same effect as three or four exposures, regarding the compliance of the therapy, and also the costs involved, a single application would be better. A single near-infrared laser treatment showed a positive effect on a full-thickness wound healing model [15]. If the dose or effects are cumulative, the best way of treatment has to be found regarding to the physiological process involved on the healing as well as patient's safety and comfort.

Acknowledgments The authors kindly thank Stella T. Sugayama for technical support. Martha S. Ribeiro thanks CNPq for financial support.

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