Angiogenesis induced by low-intensity laser therapy: comparative study between single and fractioned dose on burn healing

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ABSTRACT:

Severe burns cause extensive damage and are complicated by loss of body fluids, injury in the cutaneous vasculature and delayed wound healing. Low-intensity laser therapy (LILT) has been studied as an alternative method to accelerate wound healing. This study was carried out to evaluate LILT effects (λ= 660 nm) in rat burned skin with two different dose regimens. Thirty-six male adult Wistar rats with two burns created on their back using steam water were divided into 3 groups. In the fractioned dose laser group (FG), the lesions were irradiated with 1J/cm² on days 1, 3, 8 and 10; in the single dose laser group (SG), the lesions were irradiated with 4J/cm² on day 1. On control group (CG), lesions were not irradiated. Three animals per group were sacrificed on days 1, 3, 8, 10, 15 and 21 post-wounding and skin specimens were collected and processed to histomorphometry. At days 1, 3 and 8, statistical significant differences were not observed among groups. On the 10th day, mean values of the number of blood vessels for FG was significantly higher than CG. Irradiated groups showed a peak of new blood vessels formation at day 15 while for CG the peak was at day 21. The number of vessels in CG was significantly higher than FG and SG at day 21. These findings suggest that LILT may accelerate angiogenesis compared to control group, however, no significant differences were observed between laser groups with fractioned or single dose during all experiment.

Keywords: Blood vessels, burns, low-intensity laser, photobiomodulation, red laser, skin repair, wound healing

1- INTRODUCTION

It is estimated that two million people sustain different degrees of burns annually in the US[1,2]. Burns often happen unexpectedly and have the potential to cause dysfunction, lifelong disfigurement and death. The repair of extensive burn wounds has long been a problem[3]. This has come more into focus in recent times because the emphasis from more conservative treatment, with wound closure being undertaken as the most important advent preventing life-threatening infections, decreasing morbidity and mortality with also a better post healing prognostic[4].

There has been recently an increased interest in the use of low-intensity laser radiation to accelerate wound healing including burn wounds[5,6]. In laboratory animals, as reported by several investigators, the biostimulation of wound healing process as reported by several investigators results stimulation of fibroblasts proliferation; significant increases in reepithelialization, collagen synthesis, granulation tissue formation; acceleration of wound closure, macrophage stimulation, and extracellular matrix production[7,8]. Nowadays, this therapy is successfully applied in clinical practice although the mechanisms underlying laser effects are still poorly understood[9].

The sequence of events that culminate in a complete wound closure and repair can be divided into three overlapping phases: inflammation, reepithelization and remodeling, which begin almost simultaneously with reepithelization. Most reports of laser biostimulation suggest that its greatest effect appear to be related to specific events during the first two phases of wound healing, i.e., the inflammatory phase and the proliferative phase indicating that the period of intervention may be critical[8].

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Although studies have demonstrated acceleration on wound healing process, in reality, no data about different dose regimens have been reported. It is still unknown the effects of a single dose compared to fractionated doses applied in different moments during the healing process.

The purpose of the present study was to evaluate the effects of LILT during skin repair in rats using two different irradiation protocols by histomorphometry analyses.

2- MATERIAL AND METHODS

Thirty-six healthy male adult Wistar rats weighing approximately 300g were used in the experiment. During the experimental period, all animals were kept in individual cages in a 12 h-light/12 h-dark cycle, and fed with granulated ration and water *ad libitum*. The animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (0.32 mL/Kg) and xilazina (0.2 mL/Kg), and had their dorsal area shaved and cleaned with povidone-iodine.

After anesthesia, the back of each rat was exposed for 5s to the external tip of a cylinder, 5mm in diameter connected to a source of boiling water. Two round burns were created. National and international principles of laboratory animal care were followed. Histological examinations in a previous pilot study revealed that epidermis and the whole dermis were injured.

The lesions (n=72) were randomly divided into three groups. The laser used was a GaAlAs diode laser (Kondortech, São Carlos, Brazil) emitting red light (λ= 660nm), with output power of P=30 mW. Power output was calibrated by a laser power meter (LM-01, Coherent, USA). The laser probe was applied in contact with the wound. In the fractioned dose laser group (FG), the lesions (n=24) were irradiated with 1 J/cm² on days 1, 3, 8 and 10; in the single dose laser group (SG) the lesions (n= 24) were irradiated with 4 J/cm² on day 1, immediately following injury. On control group (CG) the lesions (n= 24) were not irradiated.

The animals were sacrificed in a CO_2 chamber on days 1, 3, 8, 10, 15 and 21 (n=4 lesions per time point). The skin samples were carefully collected to include the adjacent healthy tissue and all the healed tissue in depth. All samples 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 technique. Stained sections were observed and photographed using a calibrated ocular on a Laica light microscope.

For a quantitative assessment of new blood vessels, blood vessels localized in the dermis were counted inside 3 selected square areas measuring 10,000 μm^2 each using the Image J software in such way that the total number of blood 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. Statistical analysis of the number of blood vessels was performed. Values are given as means and bars are standard deviations. Statistical comparisons between groups were carried out with the Student t-test, which retains the overall significance level at 5% ($p<0.05$).

3-RESULTS

Histological findings showed differences between irradiated and control groups; however no noteworthy differences were observed between irradiated groups (table 1).

On day 3, all groups showed moderate quantity of polimorfonuclear infiltration and intense presence of necrotic tissue. On day 8, both irradiated and control lesions showed strong signals of inflammatory activity, but the number of fibroblasts and neovascularization activity were higher than at day 3.

After 10 days, however, morphological differences were observed between irradiated and control groups. A variety of inflammatory cells and cell debris was present in the dermis subjacent to the damaged epidermis. Part of the injured skin of the control lesions was still devoid of epidermis. Inflammatory cells were observed in a higher amount on control group than in the irradiated groups. On the other hand, the proliferation of fibroblasts was higher on the irradiated groups than in control group. The most obvious difference was the more advanced epithelization of the skin in irradiated wounds when compared to the controls. Another morphological difference was related to the necrosis, which was much higher in the control lesions than in the irradiated animals. At this moment, the number of new blood vessels in lesions of FG was significantly higher than control group but no statistically significant differences were observed between SG and CG neither between SG and FG.

At 15 days post-wounding, the number of fibroblasts and new vessels was higher in SG and FG compared to CG. Epithelization for all groups was more accelerated than at day 10. Irradiated groups showed complete epithelization while CG showed parts devoid of epidermis and moderate necrosis (figures 1A and 1B). Angiogenesis was more pronounced than on day 10 but no significant differences were observed among groups. Irradiated groups showed a peak of new blood vessels formation at this moment (figure 2).

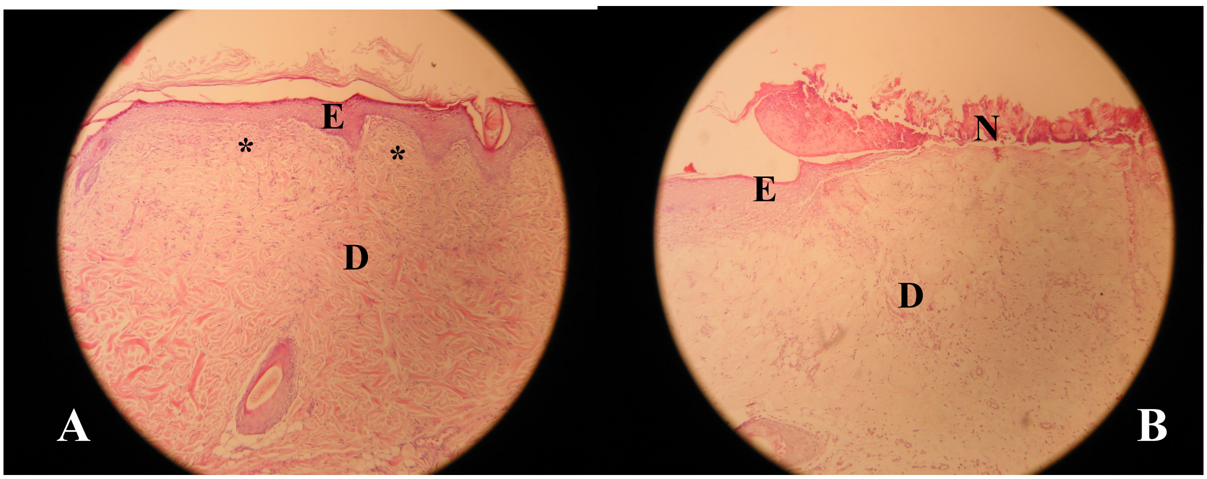

Figure 1- Photomicrograph of rat skin 15 days post-wounding. A- FG: Observe intense presence of new vessels in the superficial dermis (*) and epithelial tissue recovering all injured area. E- epidermis; D- dermis. HEx100. B- CG: Note the epidermis (E) recovering part of the burned area with moderate presence of necrotic tissue (N). D- dermis. HEx100.

On day 21, irradiated and control groups were totally recovered by a new epidermis. It is possible to observe from figure 2 that irradiated groups at this moment presented a mean value of new vessels significantly lower than CG and more similar to healthy skin, which contained an average of 17 vessels/30,000 μm^2 area.

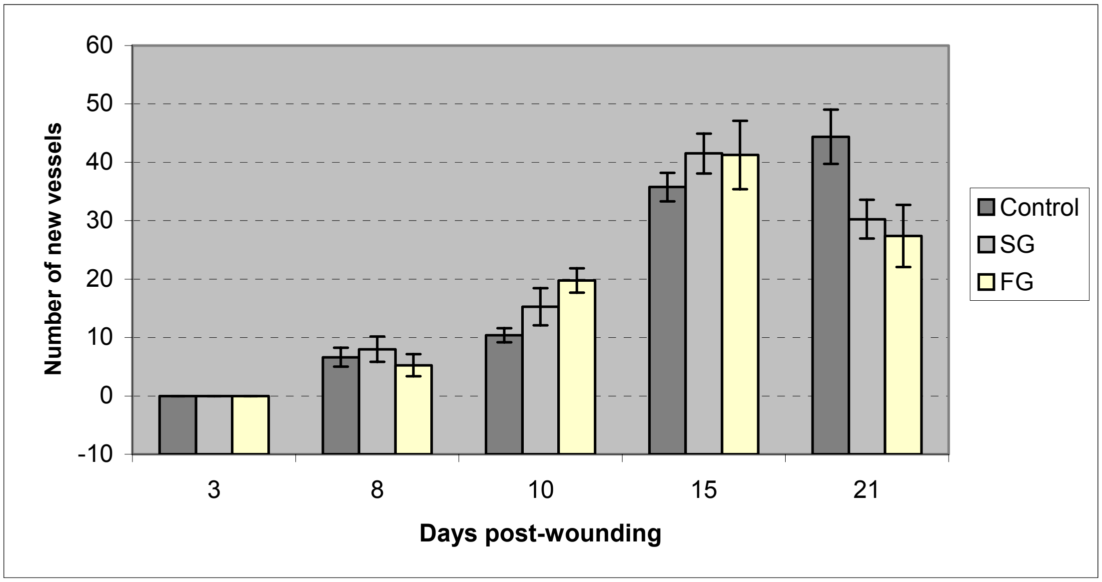

Figure 2: Mean values ± SD of the number of new blood vessels during the experimental period. See details in the text.

4-DISCUSSION

The biostimulatory effects of LILT have been reported since the initial years of laser development. Many studies focused on the effects of laser photostimulation on a variety of pathological conditions, including burns[2,6,10-15]. The repair of extensive burn has long been a problem. This has come more into focus in recent times because the emphasis has shifted to more conservative treatments.

This study aimed to understand the importance of laser radiation on specific time-points during wound healing. For this purpose the rats were separated into 3 groups, one control group without treatment, one group that received laser radiation immediately after injury with $4J/cm^2$, and another group that received $1J/cm^2$ in four different periods (days 1,3,8 and 10)

The important point is that the stimulatory effects of laser appear to be related to specific events during the first two phases of wound healing: the inflammatory phase and the proliferative phase, indicating that the period of intervention may be critical[8]. For this reason, in this study, in one of the laser groups the treatment was performed at days 1 and 3 aiming the inflammatory phase and at days 8 and 10 aiming the proliferative phase. In the second laser experimental group the treatment was performed on the 1st day, therefore acting over the inflammatory phase. According to Medrado and collaborators laser treatment would reduce the intensity and duration of the inflammatory phase[16].

Although a vast literature is currently available about laser biostimulation, the information is quite diverse and conflicting, especially because of the differences in experimental procedures. Another important parameter is the specific analyzed event and its magnitude. According to Karu[17] even in cell culture, the specific event that would be triggered by the radiation can not be predicted with precision, since a fibroblast cell culture when irradiated may start an accelerated division phase or it may start to produce more collagen fibers. The mechanism behind this manifestation is not currently understood. In previous work of our group[11] that aimed to analyze blood flow in irradiated groups, there were not significant differences between irradiated and control groups. Thus, according to the results of the present research although an increased blood flow may not be significant the number of blood vessels, on the other hand may contribute to the healing events triggered by red radiation.

In another perspective, several authors suggest that the laser dose is cumulative while others suggest that the effects of laser dose are cumulative not the dose itself. In this study the differences between the two dose regimes were not detected, even though on day 10 a significant difference on the blood vessels number was observed in FG compared to control, but it was not significant when compare to SG, and the former was considered equal to CG.

The differences among groups were observed after 10 days, when the wounds were at the proliferative phase. This data agree with some authors, since in this day the irradiated groups showed epithelization surrounding the wounds, while in control group there was not a signal of initial epithelization. Regarding the importance of this early epithelium formation, it would be quite obvious that as soon as possible the protective tissue should be back, reestablishing the protective barrier against microorganisms and also diminishing the patient's discomfort. Also on day 10, the results showed higher fibroblasts proliferation in irradiated groups than in CG, and this may be the factor that leads to an earlier epithelization.

Ribeiro et al.[12] investigated the influence of a low-intensity polarized visible laser with a radiant exposure of 1 J/cm^2, applied on specific moments of skin healing after a cold burn inflicted in rats. Two groups of burns were irradiated by He-Ne laser with two different linear polarization alignment and the histological analysis showed that the healing of irradiated wounds was faster than the non-irradiated wounds, therefore even with a low dose (1J/cm^2), the beneficial effects of the laser radiation can be detected.

On day 15, the process continued and the faster epithelization on irradiated lesions continued when compared to control group showing even more fibroblast proliferation matched up to day 10 and also with control group.

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 showed that the SG and FG are already in the remodeling phase, which is the last healing stage. At the same time point the CG showed a higher number of vessels than healthy skin and than irradiated groups suggesting that the healing is not complete and granulation tissue may still be present.

The importance of radiant exposure versus irradiation moment is still a matter of investigation. Even though FG at days 1, 3 and 8 had received 1J/cm^2 at each time, therefore less than the 4J/cm^2 received by SG, there was not a significant difference compared to the 4J/cm^2 received by SG at day 1. If a single laser exposure would be enough to produce the same effect as 3 or 4 exposures, regarding the compliance of the therapy and also the costs involved, a single application would be better. In a recent study Rezende et al [18] using a single near-infrared laser treatment observed a positive effect on wound healing. If the dose or effects are cumulative, the best way of treatment has to be found regarding the physiological process involved on the healing as well as patients safety and comfort.

5- CONCLUSION

The results obtained in this work indicate that low-intensity red laser therapy accelerates angiogenesis during burn healing process compared to a non-irradiated control. However, no statistically significant differences were observed between fractionated and single doses under the tested parameters.

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