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Low-Level Laser Therapy (LLLT) Reduces Inflammatory Infiltrate and Enhances Skeletal Muscle Repair: Histomorphometric Parameters[1]

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Abstract—Low level laser therapy (LLLT) has been suggested as an effective therapeutics in inflammatory processes modulation and tissue repairing. However, there is a lack of studies that analyze the anti-inflammatory effects of the infrared lasers in muscular skeletal injury. The aim of this study was to investigate the effects of low-level laser therapy 904 nm in the repair process of skeletal muscle tissue. Swiss mice were submitted to cryoinjury and divided in test (LLLT-treated) and control groups. Histological sections were stained with hematoxylin-eosin to assess general morphology and inflammatory influx, and Picrossirus to quantify collagen fibers deposition. Our results showed significant reduction in inflammatory infiltrated in irradiated mice after 4 days of treatment compared to control ($p = 0.01$). After 8 days, the irradiated group showed high levels at regenerating myofibers with significant statistically differences in relation at control group ($p < 0.01$). Collagen deposition was significantly increased in the final stages of regeneration at test group, when compared with control group ($p = 0.05$). Our data suggests that LLLT reduces the inflammatory response in the initial stages of injury and accelerates the process of muscular tissue repair.

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1. INTRODUCTION

Muscle injuries are frequent when it comes to physical rehabilitation. 30% of pathologies treated in clinics and specialized centers involve this kind of injury. They might occur due to several reasons, like direct trauma, ischemia, unnerving, poisoning, among many others. However, generally speaking and regardless of etiological factors, muscular repairing is considered similar [1]. Muscular injuries are characterized by structural damages, such as membrane disorders and sarcomerae damages which result in inflammatory process with cytokines liberation and fagocitic cells infiltration [2].

Inflammation is a process characterized by organism response to different traumas and infections, like a defense mechanism that also starts the tissue repairing process. After 1–4 h that this injury process was started, inflammatory cells (neutrophils and macrophages) invade in the injured or infected area. Repair process after muscle damage involves a synchronized activation of numerous cellular and molecular responses, being the balance between inflammation and regeneration a key role for a beneficial result. That process can be seen as two interdependent stages: degeneration and regeneration, and the success in the repairing process might be directly related to the muscle interaction with the inflammatory process, where muscular regeneration relies on the balance between the pro and anti-inflammatory factor [3–5].

In the last decades, low level laser therapy (LLLT) has been a therapeutical technique quite used in rehabilitation sciences due to its anti-inflammatory and healing effect [6–9]. These actions are discussed in literature, especially when it concerns the modulation of tissue repair inducing the increasing in the fibroblasts amount, collagen synthesis, mitotic activities and neovascularization [10]. Researchers reported that low level laser therapy reduces the inflammatory process and speeds up the repair of muscular injuries and tendons [11–14]. Other authors talk about activation and proliferation of satellite cells, migration and differentiation into myoblasts, as well as fusion of these cells forming new muscular fibers replacing degenerated ones after the use of LLLT [15–17].

[1] The article is published in the original.

Though low LLLT could be considered useful therapeutics in the treatment of inflammatory and regenerative processes, clinical and experimental evidences are not conclusive, showing considerable controversy that comes from contradicting results informed by several authors, in part, due to methodological problems and also by parameters used by some researchers which are not sufficiently clear resulting in negative and precipitated conclusions over the effectiveness of this technique [18].

Some studies have shown anti-inflammatory effects from visible red laser therapy. However, little was investigated the reduction of the inflammatory process when it comes to in vivo muscular injuries, influencing skeletal muscle repair, using lasers with infrared wavelength, such as Gallium Arsenet 904 nanometers (GaAs 904 nm) [19]. Leal Junior et al., reported that the penetration in human tissue is slightly better and also that clinical studies have shown significantly better results using 904 nm in relation to other wavelengths [2]. In spite of this benefit, most studies have used small doses and there almost is an absence of experiments using bigger doses at this kind of muscular injuries shown in this study. Besides, we propose a treatment protocol with irradiation in alternate days, similar to the clinical treatment.

Therefore, due to the extensive controversy observed in the literature the aim of this study was to investigate the effects of specific protocol of GaAs LLLT (904 nm) during different phases of skeletal muscle repair through histomorphometric parameters.

2. MATERIAL AND METHODS

2.1. Animals

Male swiss mice (22–25 g) were maintained at the animal housing at Faculty of Minas—FAMINAS, under a controlled temperature (around 22°C), relative humidity (40–60%), light/dark cycle (12 h), with access food and water ad libitum. All procedures were approved by the Institutional Animal Care (protocol number: 010/2008) and were conducted according to Brazilian Ethics Guidelines for Animal Studies (COBEA).

2.2. Experimental Muscular Injury

The animals were anesthetized with an intraperitoneal injection of a premixed solution containing ketamine (90 mg/kg; Ketalar; Parke-Davis, SP, Brazil) and xylazine (10 mg/kg; Rompun; Bayer, SP, Brazil) and after tricotomy and assepsy a longitudinal incision was done in each animal's right posterior paw in order to have their gastrocnemius muscle exposed. Muscular injury was performed as previously described [20], through a stainless steel haste (4 mm of diameter), dove into liquid nitrogen for 30 s and then kept in touch with their tissue for 10 s. Control groups were kept under the same experimental conditions. Mice were sacrificed 1, 4, 8, and 12 days post injury.

2.3. Therapeutical Protocol and Experimental Groups

Both test and control groups were divided into subgroups according at kinetic from analysis to monitor the influx of inflammatory cells and tissue regeneration process as well as deposition of collagen. After 30 min of injury induction, biostimulation was carried out using a GaAs laser device (LASERPULSE, IBRAMED, Amparo, SP, Brazil) with the following parameters: pulsate emission, wavelength: 904 nm (infrared), power: 70 Wpico, pulse duration: 60 ns, frequency: 9.5 kHz, energy density: 9 J/cm^2, for 27 s.

Irradiation was applied in alternate days (48 h interval), through punctual technique in touch with the tissue at the injury location. Irradiation incidence angle was perpendicular (90°) in relation to the irradiated surface. The device was calibrated by the maintenance department from the institution, keeping periodical calibrations.

2.4. Histological Staining and Morphometric Analysis

Gastrocnemius muscles from test and control mice were carefully removed, fixed in formalin-buffered Millonig fixative (pH 7.2) for 24 h. The 5 μm-thick sections of paraplast-embedded tissue (Sigma) were stained with hematoxilin–eosin and sirius red to analyze histological alterations and collagen deposition, respectively. Images of all cross-sections from three test and control mice at each time point were acquired with a microdigital camera mounted on a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Degenerating and necrotic fibers were identified by homogeneous pale eosinophilic sarcoplasm, whereas regenerating fibers by strong basophilia and centrally located nuclei [21]. To identify the presence of collagen, each image area was automatically measured by colorimetric differential and the results were expressed in percentage in relation to the total image area. Areas occupied by inflammatory infiltrates, degeneration, regeneration and collagen deposition were determined with Image-pro Plus 4.5 software (Media Cybernetics Inc., Silver Spring, MD).

2.5. Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) *one-way* to determined the differences of conditions (1, 4, 8, and 12 days post injury) and Student *t* test was applied to obtain statistically significant differences between the process of regeneration/degeneration, inflammatory response and collagen deposition in both groups analyzed, considering as significant when p value is ≤ 0.05.

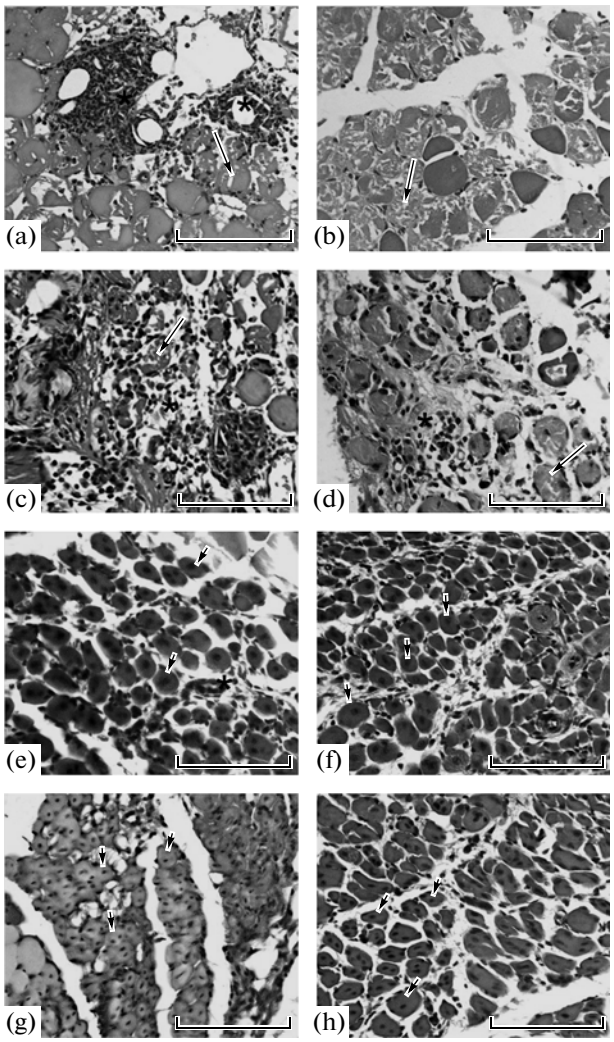

Fig. 1. Histological cross-sections of gastrocnemium muscle stained by Hematoxilin eosin (HE). Photomicrography of control group (a, c, e, g) and test group (b, d, f, h) after 1 (a, b), 4 (c, d), 8 (e, f) and 12 (g, h) days induction injury. Asterisks (*) show inflammatory infiltrate; arrows indicate myonecrosis and arrow heads indicate regenerating myofibers. Scale bars = 100 μm.

3. RESULTS

3.1. LLLT Reduces the Inflammatory Infiltrate in the Initials Stages of Injury

One day after injury, we observed that the citoarchitecture of the fiber was gone in both groups and an large areas of myonecrosis with predominance of inflammatory infiltrates in the control group in relation to the group treated with GaAs (904 nm) laser (Figs. 1a and 1b). Morphometrical analysis showed that after the first day of treatment there were no significant statistically differences at inflammatory infiltrates between control and test groups (1004 ± 427.7 vs. 635.5 ± 325.5; $p = 0.58$; respectively). After 4 days, the inflammatory response kept the same histomorphometrical profile, with significant statistically

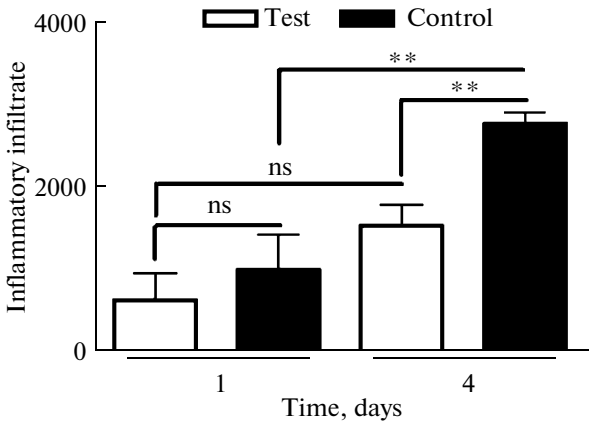

Fig. 2. Histomorphometry of the inflammatory infiltrate presence. Bars represent the mean values and the respective standard deviation (sd) from the results obtained from 3 animals per group. Statistical analysis was based on unpaired t-student test. ** $p = 0.01$ and ns = not significant.

difference (2774 ± 148.3 vs. 1540 ± 252.8; $p = 0.01$) between both control and test groups, respectively (Figs. 1c, 1d and 2).

3.2. LLLT Increases the Number of Regenerating Cells

Figure 3a showed that there wasn't any significant difference at presence of myofibers degenerating in both control and test groups at the first and fourth day of treatment, with $p = 0.15$ and 0.34, respectively. Even though results showed myonecrosis decrease in test group from the first to the fourth day, there was no significant statistical difference ($p = 0.12$). In 8 days (Figs. 1e and 1f), we could observe a predominant increase in regenerating myofibers, stressed by central nucleation and few areas of inflammatory infiltrates. In 12 days, both groups presented regenerating myofibers predominance with central nucleation. Analyzing the presence of myofibers with central nucleation between control and test groups (374.7 ± 24.8 vs. 128.7 ± 31.05, respectively), we could see significant statistical differences on the eighth day of treatment ($p = 0.003$). With twelve days of treatment there was no significant change between control group and the one treated with lasers (367 ± 63.66 vs. 217.3 ± 129.8; $p = 0.35$) (Fig. 3b).

3.3. LLLT Increase Levels of Collagen Deposition in the Final Stage of Regeneration

After identifying anti-inflammatory results in the early stages of treatment, collagen deposition was evaluated. After 4 days of treatment, collagen deposition in the control group was statistically larger than in the test one ($p = 0.01$). However, from the fourth to the twelfth day of treatment, we observed a large increase in collagen deposition in the test group (0.89 ± 0.48 vs.

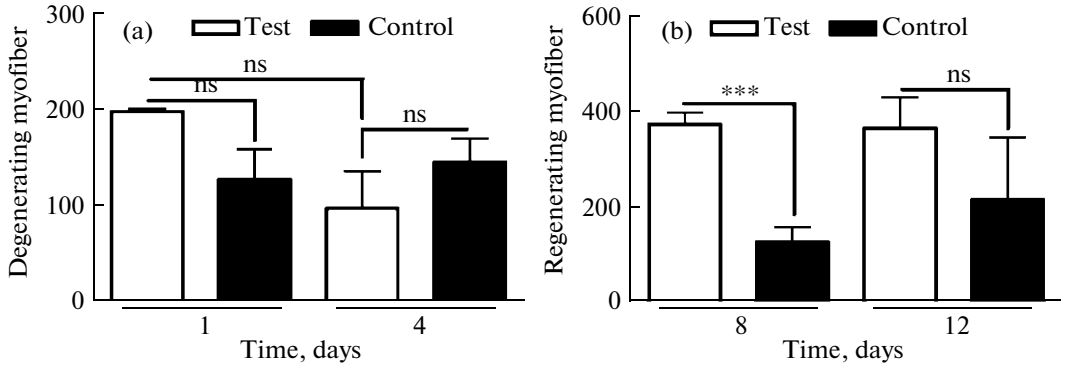

Fig. 3. Histomorphometry of degenerating (a) and regenerating myofibers (b). Bars represent the mean values and the respective standard deviation (sd) from the results obtained from 3 animals per group. Statistical analysis was based on unpaired *t*-Student test. *** $p = 0.003$ and ns = not significant.

10.96 ± 0.96; $p = 0.004$; respectively). High levels of collagen deposition seen in the test group was also statistically significant when compared with the control group after 12 days of treatment (10.96 ± 0.96 vs. 7.06 ± 0.52; $p = 0.05$; respectively) (Fig. 4).

4. DISCUSSION

The inflammatory process represents the first event following tissue injury, whose main function is to eliminate cellular debris and activate the repair process. This reaction is absolutely required to provide repair, although its long-term persistence is considered one of the most important reasons of delay in the regeneration process [22]. Low level laser therapy has been widely used on research biological [23–28] and clinical [29–32]. Several studies have described the anti-inflammatory effects of LLLT in numerous models of tissue injury. Correa et al. demonstrated a reduction in the neutrophils' levels in peritonitis induced by lipopolissacarydes (LPS) after treatment with GaAs 904 nm laser [19]. Barbosa et al. and Dourado et al. used wavelengths of 685 and 904 nm have shown a decrease of inflammatory process of lesions caused by toxins after laser therapy treatment [6, 7, 33]. Rizzi et al. have pointed out a reduction in the liberation of reactive oxygen species (ROS) and in the NFkB activation induced by trauma after treatment with 904 nm laser suggesting that this therapeutics reduces the inflammatory response induced by muscle injury [1]. Others authors also showed a decrease in the expression of proinflammatory mediators, including IL-1, tumor necrosis factor (TNFα), and prostaglandins [34].

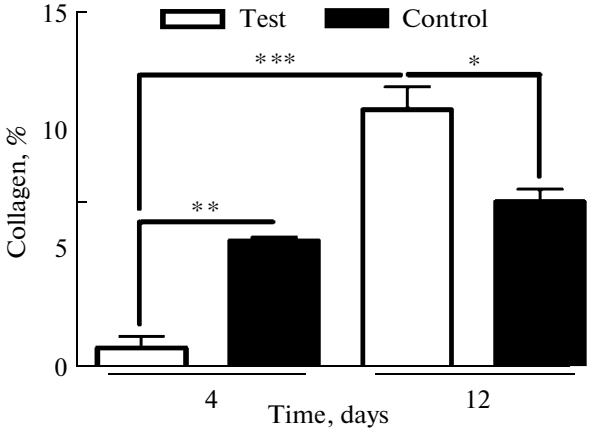

Fig. 4. Histomorphometry of collagen deposition at the site of injury. Bars represent the mean values and the respective standard deviation (sd) from the results obtained from 3 animals per group. Statistical analysis was based on unpaired t-student test. *** $p = 0.004$, ** $p = 0.01$, and * $p = 0.05$.

The results obtained in our work showed a significant decrease at the intensity of the inflammatory process in 4 days after performing tissue injury. These findings suggest that this protocol of Laser irradiation is able to downregulate the inflammatory response and improve the acceleration of biological events responsible for the regeneration process. Similar results previously described corroborate the anti-inflammatory effects shown in our results [1, 19]. This modulatory effect of LLLT on the inflammatory response can also be result of the inhibitory effect this therapy in synthesis of prostaglandins, a chemical mediator widely supposed to provide chemotactic signals for myeloid cells [35]. Therefore, LLLT might promote a fast acute inflammatory response in earlier stages of regeneration, accelerating phagocytic inflammatory phase of the tissue repair.

During muscular degeneration, the repairing process is activated and occurs a cellular proliferation to the tissue's regeneration. Satellite cells, stem cells, trophic factors and extracellular matrix have a significant role in this regeneration and myofiber reconstruction. After myofiber lesion, satellite cells are activated to start the cellular cycle and proliferate, allowing the expansion of myogenic cells to happen. After

this proliferation, satellite cells differentiate and contribute to the formation of new myofibers as well as they help in the repairing of the damaged fiber, with centrally located nuclei. Therefore, satellite cells have a major role during muscular skeletal repair after injury [36, 37]. Shefer's group showed beneficial effects of LLLT at the satellite cells survival and proliferation, suggesting this therapy as an effective means to mitigate the consequences long-term from muscle injury [16, 17, 38]. A recent study showed that LLLI can activate the myoblasts proliferation and increase the expression of cell cycle related proteins. These findings suggest that stimulating the quiecentes myoblasts to enter into proliferative stage may be an important cellular mechanism involved in the healing of skeletal muscle [39].

Our results showed a significant increase in the number of regenerating myofibers, characterized by the presence of central nucleation, after 8 days of treatment with LLLT, suggesting an improvement in tissue repair process. Evidence showed that LLLT in the infrared spectrum was more effective in treatment of lesions of oral mucositis, and on repair of bone defects, in relation to the red visible spectrum, which can be explained by the greater power of penetration of infrared rays [40–42]. Study showed in models osteopenic fractures, 904 nm laser accelerated the repair process, especially in the initial phase of bone regeneration [43]. Other findings showed that 904 nm laser (energy density 4 J/cm^2) reduces myonecrosis after snake envenoming [33]. Despite different therapeutic protocols, all these data suggest a beneficial effect of 904 nm laser in tissue repair process and corroborate the findings of this study.

In a natural process the inflammatory phase is gradually replaced by proliferative phase, characterized by the migration of fibroblasts, responsible for synthesizing, depositing and remodeling the collagen fibers required to repair after tissue injury. In physiological situations, collagen provide strength, integrity and structure, but, after tissue injury, collagen deposition is essential for replacing the tissue injured and restoring anatomic structure and function [22, 37]. Biological events, such as formation of new capillaries associated at progressive deposition of collagen can result in complete tissue regeneration [44]. Bayat et al. suggest that LLLT seems to accelerate some scarring processes and might improve the roles of the fibroblasts, including a permanent production of extracellular matrix (including collagen, glycosaminoglycans and proteoglycans) and granulation tissue [45].

This study, LLLT significantly increased collagen deposition in the final stages of tissue repair, suggesting a beneficial effect of this therapy in the modulation of colagenogenesis. Studies in vitro using the same wavelength (904 nm) have suggested that LLLT increases collagen production [46], corroborating our findings, however, other authors have shown decrease in collagen synthesis and production using 904 nm laser [47]. Both authors have used low energy density compared with our protocol.

Study in vivo with different wavelength showed that LLLT stimulates collagen fibers deposition at the final stages of repair, confirming our results, but other researchers haven't found beneficial effects at collagen deposition [48, 49]. Using the same wavelength, but with low energy density authors also reported that LLLT stimulates the deposition of collagen [22]. Another recent study, using the same model of muscle injury, showed that LLLT promotes an increase in collagen fibers [50].

5. CONCLUSIONS

Our data suggest that this protocol was successful in improving skeletal muscle repair, modulating the inflammatory response, promoting deposition of collagen fibers. The exact explanation for these beneficial results is not completely understood yet, and investigations new are still required to elucidate the mechanisms involved at the effects observed in this study, evaluating its potential use in the treatment of myopathy and other pathologies.

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