

## Effects of 660 nm low-level laser therapy on muscle healing process after cryolesion

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**Abstract**—The aim of this study was to evaluate the effects of 660 nm low-level laser therapy (LLLT) on muscle regeneration after cryolesion in rat tibialis anterior muscle. Sixty-three Wistar rats were divided into a control group, 10 J/cm<sup>2</sup> laser-treated group, and 50 J/cm<sup>2</sup> laser-treated group. Each group formed three subgroups ( $n = 7$  per group), and the animals were sacrificed 7, 14, or 21 d after lesion. Histopathological findings revealed a lower inflammatory process in the laser-treated groups after 7 d. After 14 d, irradiated animals at both fluences showed higher granulation tissue, new muscle fibers, and organized muscle structure. After 21 d, full tissue repair was observed in all groups. Moreover, irradiated animals at both fluences showed smaller necrosis area in the first experimental period evaluated. MyoD immunoexpression was observed in both treated groups 7 d postinjury. Myogenin immunoexpression was detected after 7 and 14 d. The higher fluence increased the number of blood vessels after 14 and 21 d. These results suggest that LLLT, at both fluences, positively affects injured skeletal muscle in rats, accelerating the muscle-regeneration process.

**Key words:** animal model, cryolesion, gene expression, histopathological analysis, laser therapy, muscle regeneration, muscle tissue, MyoD, myogenin, rehabilitation.

## INTRODUCTION

Skeletal muscle injuries are extremely common in rehabilitation centers. They can occur through a variety of mechanisms, ranging from direct mechanical deformation (such as muscle laceration, strain, and contusion) to indirect damage related to ischemia and neurologic dysfunction [1–2]. Although muscle tissue can regenerate after injury, the process tends to be slow, often resulting in functional and structural muscle atrophy, contracture, pain, and reinjury [3–4].

Muscle regeneration is a highly orchestrated process that ideally leads to complete functional recovery. This process is characterized by inflammatory response, activation of muscle satellite cells, and formation of new myofibers [5]. The temporal and spatial interaction of the

**Abbreviations:** CG = control group, G10 = 10 J/cm<sup>2</sup> laser-treated group, G50 = 50 J/cm<sup>2</sup> laser-treated group, IgG = immunoglobulin G, LLLT = low-level laser therapy, MRF = myogenic regulatory factor, PBS = phosphate buffered saline, TA = tibialis anterior, US = ultrasound.

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different kinds of cells is regulated by a series of cell-signaling molecules and growth factors, which induce maturation of muscle cells to create a competent muscle structure at the injury site [6].

The family of myogenic regulatory factors (MRFs), such as myoD and myogenin, have an important role in this event. MyoD is markedly more effective in activation and differentiation of satellite cells, while myogenin is an important factor for terminal differentiation and fusion myoblasts in mature muscle fiber, restoring normal muscle architecture [7]. Furthermore, the formation of new vessels is necessary to ensure adequate blood supply during the healing process. This event is regulated by the vascular endothelial growth factor that exerts multiple effects on the vascular endothelium, including stimulation of endothelial cell proliferation, rapid induction of microvascular permeability, promotion of endothelial cell survival, stimulation of endothelial cell adhesion and migration, and subsequent connection between new vessels and the preexisting circulation [8].

Despite the excellent capacity of muscle tissue to regenerate after injury, in critical situations such as extensive myofibrillar degeneration or poorly vascularized injuries, the process of muscle healing can be delayed [8–9]. In this context, there is a need to develop treatments able to accelerate muscle cell proliferation and prevent fibrosis during the healing process, which would decrease the rehabilitation time and regenerative processes, producing a return to the previous level of function as quickly and thoroughly as possible [9–10].

In this context, low-level laser therapy (LLLT) seems to be an efficient resource for skeletal muscle recovery, and its effectiveness has been demonstrated over the years [11–12]. Several studies have shown that laser irradiation favors the regeneration of skeletal muscle in animal and human models, increasing the number of muscle fibers, mitochondrial density, angiogenesis, and myotube formation, thereby shortening the inflammatory phase and accelerating the proliferative and maturation phases of skeletal muscle regeneration [13–14].

Despite the stimulatory effects of laser on tissue repair, investigators have applied it at a wide variety of doses, which makes it difficult to compare published results and determine the best laser parameters for evoking the best tissue response [15–18]. Therefore, it is important to examine the effects of different fluences of LLLT to define its safety and efficiency.

These encouraging effects of LLLT on muscle metabolism formed the basis for the current *in vivo* study, which aimed to evaluate the biological response to laser irradiation in a model of cryolesion in rats. Cryolesion is an injury model well recognized to induce necrosis in a delimited area of skeletal muscle and regeneration [19–21]. This lesion produces myonecrosis, tissue disruption, edema, hypercontracted fibers, and inflammatory cell infiltration (especially neutrophils and macrophages) [22]. These characteristics are similar to those found after high-intensity eccentric exercise, which is also a valid method used to induce muscle damage, leading to muscle fiber disruption, infiltration by inflammatory cells, and swelling [23–24].

We hypothesized that laser irradiation could modulate the inflammatory infiltrate and increase neoangiogenesis, stimulating the expression of the myogenic immunorepression responsible for the regeneration process of skeletal muscle. In this context, the present study aimed to examine the influence of LLLT, comparing two different fluences (10 and 50 J/cm<sup>2</sup>) 7, 14, and 21 d after cryolesion on the tibialis anterior (TA) through morphology and muscle tissue structure analysis. Inflammatory process, granulation tissue, tissue structure, number of blood vessels, and presence of immunomarkers were evaluated histologically and by immunohistochemistry analysis.

## METHODS

### Experimental Groups

Sixty-three Wistar male rats (weighing  $300 \pm 20$  g) were used and maintained under a controlled temperature ( $22^\circ\text{C} \pm 2^\circ\text{C}$ ) and light-dark periods of 12 h and with free access to water and a commercial diet.

All animals were divided into a control group (CG) with injured animals and no treatment, injured animals treated with 10 J/cm<sup>2</sup> (G10), and injured animals treated with 50 J/cm<sup>2</sup> (G50). Each group then formed three different subgroups ( $n = 7$  per group) of animals sacrificed 7, 14, or 21 d after injury. Treatments started 48 h after surgery and were performed every 24 h for 5, 10, and 15 sessions.

### Experimental Design

#### Surgery

Surgical procedures (cryolesion) were performed based on those described by Miyabara et al. [20], under

anesthesia with 40 mg/kg ketamine (Dopalen, Vetbrands; São Paulo, Brazil) and 20 mg/kg xylazine (Anasedan, Vetbrands). After anesthesia, the skin around the right TA muscle was shaved and cleaned. Then, a transversal cut (about 1 cm) of the skin over the middle of the muscle was carried out, exposing the muscle. A flat top end ( $0.5 \times 0.5$  cm) of rectangular iron bar, precooled in liquid nitrogen, was then kept for 10 s on the center of the muscle. The procedure was repeated twice consecutively, with a time interval of 30 s. Finally, the skin was sutured, and thereafter, animals were kept for several hours on a warm plate ( $37^{\circ}\text{C}$ ) to prevent hypothermia.

#### *Low-Level Laser Therapy Protocol*

A 660 nm laser (Ga-Al-As) (MM Optics, São Carlos Equipment; São Paulo, Brazil) was used in this study. The following parameters were used: continuous wavelength, 4.0 mm<sup>2</sup> beam diameter, with 10 J/cm<sup>2</sup> (20 mW, 20s, 0.4 J total energy per point) and 50 J/cm<sup>2</sup> (40 mW, 50 s, 2 J energy per point). Irradiation was performed through the punctual contact technique on one point above the area of the injury. The treatments started 48 h postsurgery and were performed 5 times/wk (each 24 h), followed by an interval of 48 h. Rats were sacrificed by CO<sub>2</sub> suffocation on days 7, 14, and 21 postinjury.

#### *Histopathological Analysis*

Muscles were submitted to a standard protocol. Muscle samples were fixed in 10 percent buffered formalin (Merck; Darmstadt, Germany) and embedded in paraffin. Longitudinal axis sections (5  $\mu\text{m}$ ) were cut using a microtome (Leica Microsystems SP 1600; Nussloch, Germany). Five sections of each sample were stained with hematoxylin and eosin (HE stain, Merck) and analyzed. Histopathological evaluation was performed through a light microscope (Olympus, Optical Co Ltd; Tokyo, Japan) with 40 $\times$  magnification by a pathologist who was blinded to the treatment. Inflammatory process, granulation tissue, necrosis area, focal or diffuse myofibrillary degeneration, and tissue structure were considered [10].

#### *Morphometry of Injured Area*

For morphometric evaluation, one histological cross-section of each TA muscle located in the central region of muscle injury was chosen to measure the cross-sectional area of both injured and uninjured muscle, using software for morphometry (Axiovision 3.0.6 SP4, Carl Zeiss; Jena, Germany). Images were used to reconstruct the total muscle cross-sectional area, allowing the identifica-

tion and measurement of both injured and uninjured areas. A double-blind procedure was used for both muscle cross-section image selection and injured and uninjured muscle area measurements.

#### *Number of Blood Vessels*

For determination of the number of blood vessels at the injured area, five fields from different regions following the injured area were obtained by light microscope (Olympus, Optical Co Ltd) at a magnification of 40 $\times$ . The number of blood vessels was counted in each field by morphometric software (Axiovision 3.0.6 SP4). This was performed in five histological sections for each animal, and then the mean number of blood vessels per animal and per group was calculated [25].

#### *Immunohistochemistry*

Serial longitudinal muscle sections of 4  $\mu\text{m}$  were deparaffinated in xylene, rehydrated in graded ethanol, and then pretreated by microwave (Brastemp; São Paulo, Brazil) with 10 mM citric acid buffer (pH = 6) for 3 cycles of 5 min each at 850 W for antigen retrieval. The material was preincubated with 0.3 percent hydrogen peroxide in phosphate buffered saline (PBS) for 5 min for inactivation of endogenous peroxidase and then blocked with 5 percent normal goat serum in PBS solution for 10 min. The specimens were then incubated with anti-MyoD and antimyogenin antibodies (Santa Cruz Biotechnology, Inc; Dallas, Texas) at a concentration of 1:400. Incubation was carried out overnight at  $4^{\circ}\text{C}$  within the refrigerator and followed by two washes in PBS for 10 min. The sections were then incubated with biotin-conjugated secondary antibody (anti-rabbit immunoglobulin G [IgG]) (Vector Laboratories; Burlingame, California) at a concentration of 1:200 in PBS for 1 h. The sections were washed twice with PBS before the application of preformed avidin biotin complex conjugated to peroxidase (Vector Laboratories) for 45 min. The bound complexes were visualized by the application of a 0.05 percent solution of 3–3'-diaminobenzidine and counterstained with Harris hematoxylin. For control studies of antibodies, the serial sections were treated with rabbit IgG (Vector Laboratories) at a concentration of 1:200 in place of the primary antibody. Additionally, internal positive controls were performed with each staining bath.

Immunohistochemical data were evaluated by an experienced pathologist (DAR) under subjective morphologic analysis as established in previous studies conducted by our group [10].

## Statistical Analysis

The results are given as means and standard deviations. Data for the morphometry of the injured area and number of blood vessels analysis were evaluated by two-way analysis of variance, followed by the post hoc Student-Newman-Keuls method. Level of statistical significance was defined as  $p < 0.05$ . Statistical evaluation was carried out using STATISTICA 7 (StatSoft, Inc; Tulsa, Oklahoma).

## RESULTS

### Histopathological Analysis

In the CG, there was an intense inflammatory reaction 7 d postinjury composed mainly of mononuclear inflammatory cells (**Figure 1(a)**). After 14 d postinjury, the histological analysis revealed the presence of granulation tissue and newly formed muscle fibers (**Figure 1(b)**). After 21 d, full tissue repair was observed (**Figure 1(c)**).

In the experimental groups exposed to LLLT, some remarkable differences were detected at both fluences ( $10 \text{ J/cm}^2$  and  $50 \text{ J/cm}^2$ ) when compared with the CG. However, no histological differences were found between the two fluences. After 7 d, inflammatory infiltrate was observed but less intense than in the CG (**Figure 1(d)** and **1(g)**). In addition, granulation tissue and some newly muscle fibers were noticed in this period (**Figure 1(d)** and **1(g)**). Following 14 d postinjury, muscle tissue presented a higher amount of granulation tissue, newly formed muscle fibers, and an organized muscle structure at the area of injury (**Figure 1(e)** and **1(h)**). At 21 d postinjury, full tissue repair was noticed in both groups (**Figure 1(f)** and **1(i)**).

### Immunohistochemistry

#### Myogenin Expression

Immunohistochemical data for myogenin and MyoD are summarized in **Figures 2** and **3**, respectively. Immunostaining for both markers was detected in the nucleus of muscle cells and circumjacent areas.

In the CG, no immunoexpression of myogenin was detected in any experimental period, indicating a negative expression for this group (**Figures 2(a)–(c)**). Nevertheless, in the group exposed to LLLT at  $10 \text{ J/cm}^2$ , a positive myogenin immunoexpression was noticed 7 and 14 d postinjury (**Figure 2(d)** and **2(e)**, respectively). Fol-

lowing 21 d, a negative immunoexpression was detected (**Figure 2(f)**). Regarding animals treated at  $50 \text{ J/cm}^2$ , the same picture was observed; i.e., myogenin was detected 7 and 14 d postinjury (**Figures 2(g)** and **2(h)**, respectively). Negative immunoexpression was observed in the last period evaluated (**Figure 2(i)**).

#### MyoD Expression

In the CG, myoD immunoexpression was not detected in any experimental analyzed in this study (7, 14, and 21 d postinjury) (**Figure 3(a)–(c)**), indicating negative immunostaining in this group for all periods evaluated in this setting. Nevertheless, a positive myoD immunoexpression was noticed in the group exposed to LLLT at  $10 \text{ J/cm}^2$  only at 14 d postinjury (**Figure 3(e)**). A negative immunoexpression was observed 7 and 21 d postsurgery in this fluence (**Figure 3(d)** and **3(f)**, respectively). Regarding laser therapy at  $50 \text{ J/cm}^2$ , the same picture was observed; i.e., myogenin was detected 14 d postinjury (**Figure 3(h)**) and negative immunoexpression was observed at 7 and 21 d (**Figure 3(g)** and **3(i)**, respectively).

### Morphometry of Injured Area

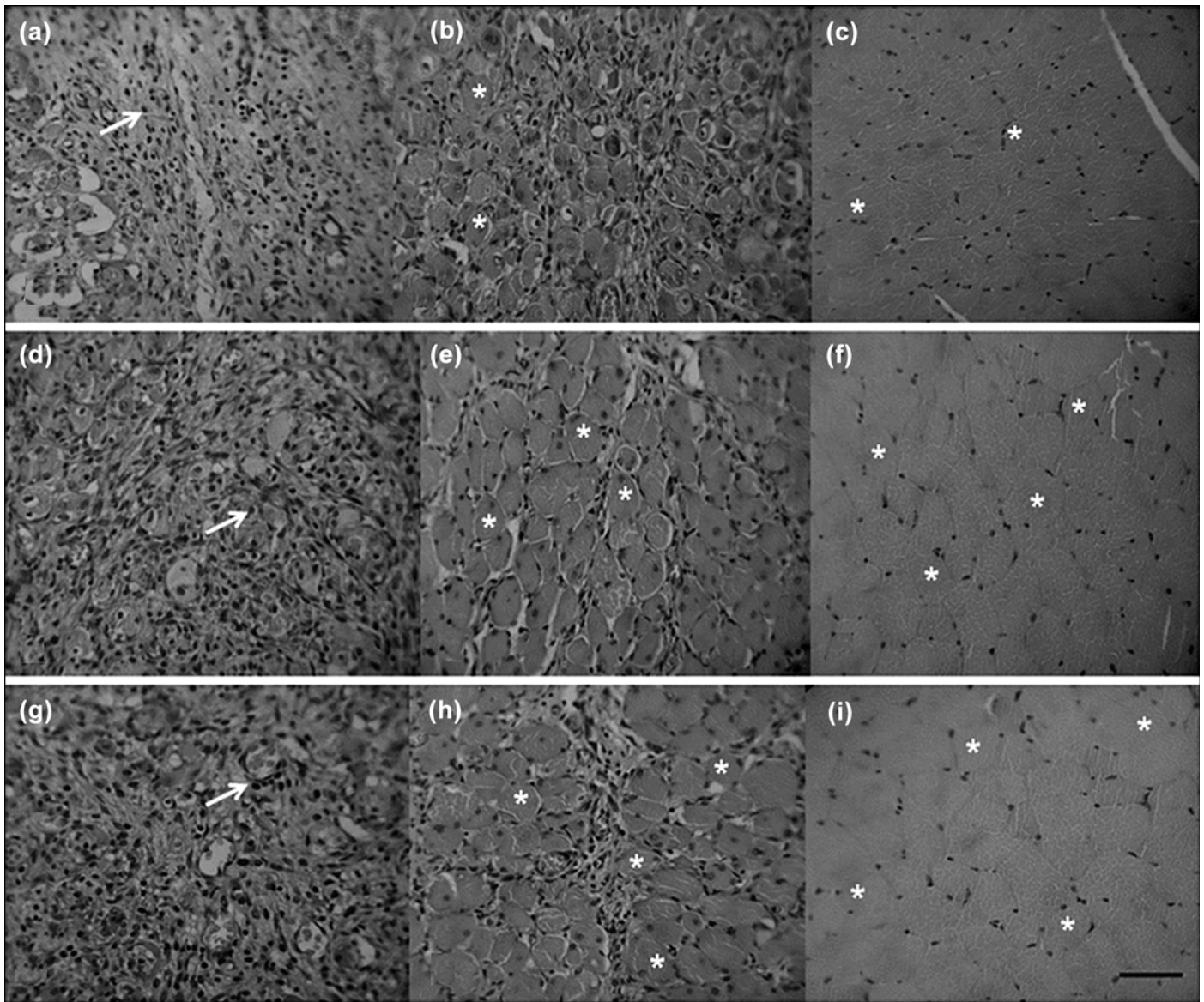
**Figure 4** shows the morphometry of the injured area in the three experimental periods. At the first experimental period, the 660 nm laser at both fluences produced a significant decrease in the injury area compared with the CG (CG vs G10:  $p < 0.001$ ; CG vs G50:  $p < 0.001$ ). Similar injury area results were found for all the groups in the other two experimental periods.

### Number of Blood Vessels

Seven days postsurgery no statistical difference was found between the CG and treated groups. At the second experimental period, a statistically higher number of blood vessels was found in G50 ( $p < 0.02$ ) than in CG and G10. Similarly, 21 d after surgery, the number of blood vessels was also significantly increased in G50 ( $p < 0.03$ ) compared with CG and G10 (**Figure 5**).

## DISCUSSION

The present study evaluated the in vivo response of the application of LLLT in a rat TA cryolesion model. We hypothesized that laser therapy would enhance muscle metabolism, accelerating the process of healing. The main findings demonstrated that the irradiated groups, at both



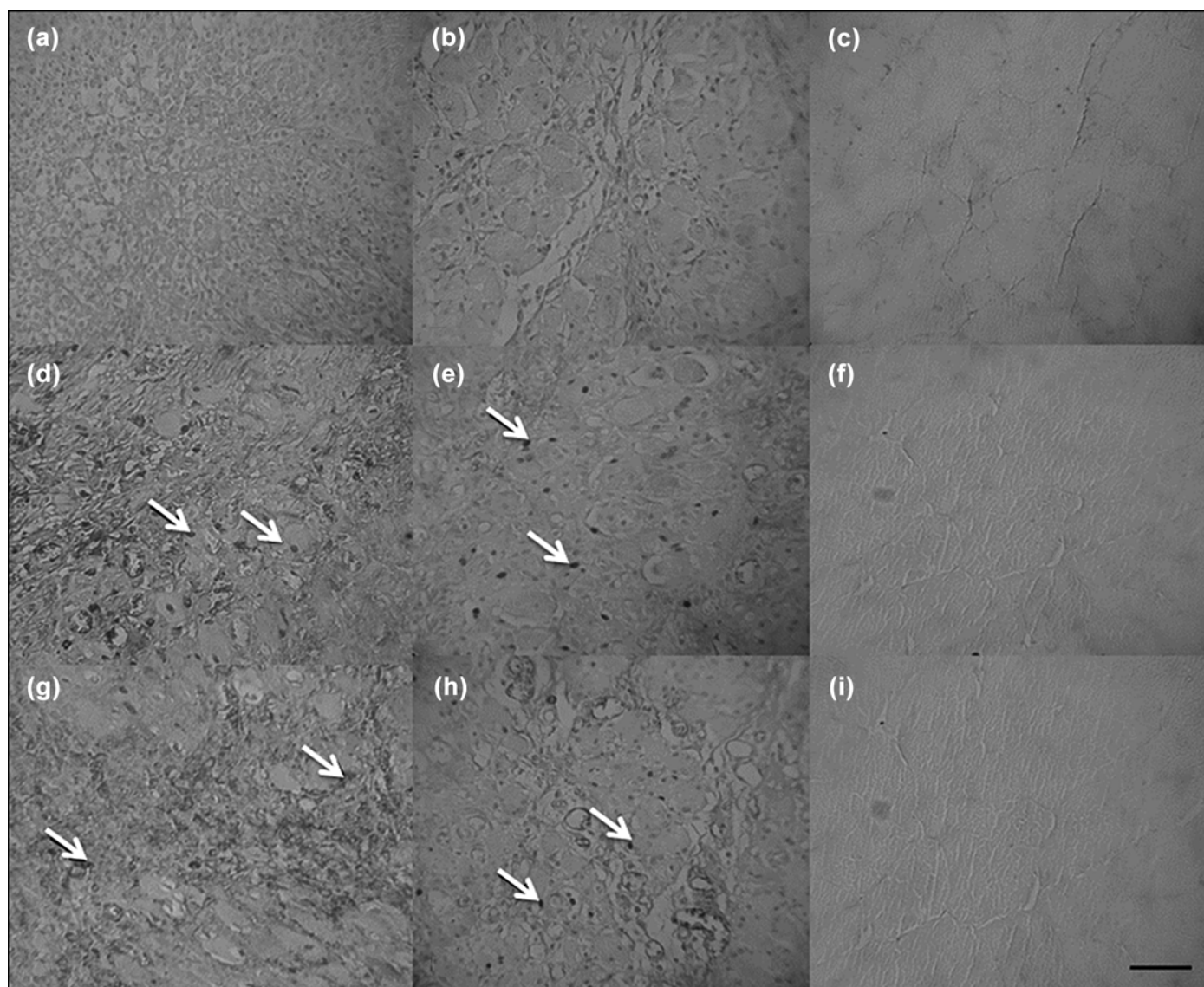
**Figure 1.**

Histological sections of injured muscle. **(a)–(c)** Control group 7, 14, and 21 d after surgery, respectively. **(d)–(f)** 10 J/cm<sup>2</sup> laser-treated animals 7, 14, and 21 d after surgery, respectively. **(g)–(i)** 50 J/cm<sup>2</sup> laser-treated animals 7, 14 and 21 d after surgery, respectively. Asterisks indicate inflammatory infiltrate and granulation tissue. Arrows indicate new muscle fiber. Hematoxylin and eosin stain. 10 $\times$ .

fluences, presented a better histological pattern, with earlier recruitment of cell infiltrate, higher amount of newly formed muscle fiber, and a more organized muscle tissue structure at the lesion. Furthermore, our results demonstrated a smaller necrosis area in the laser-treated groups 7 d postsurgery. The immunohistochemical analysis revealed an upregulated expression of myogenin in both

irradiated groups at 7 and 14 d and an upregulated expression of myoD at 14 d. In addition, LLLT at 50 J/cm<sup>2</sup> produced a significant increase in the number of blood vessels at the site of the injury.

Laser phototherapy is based on the photobiostimulation of cells and tissues [11]. Furthermore, many studies have investigated the effects of LLLT in a series of different



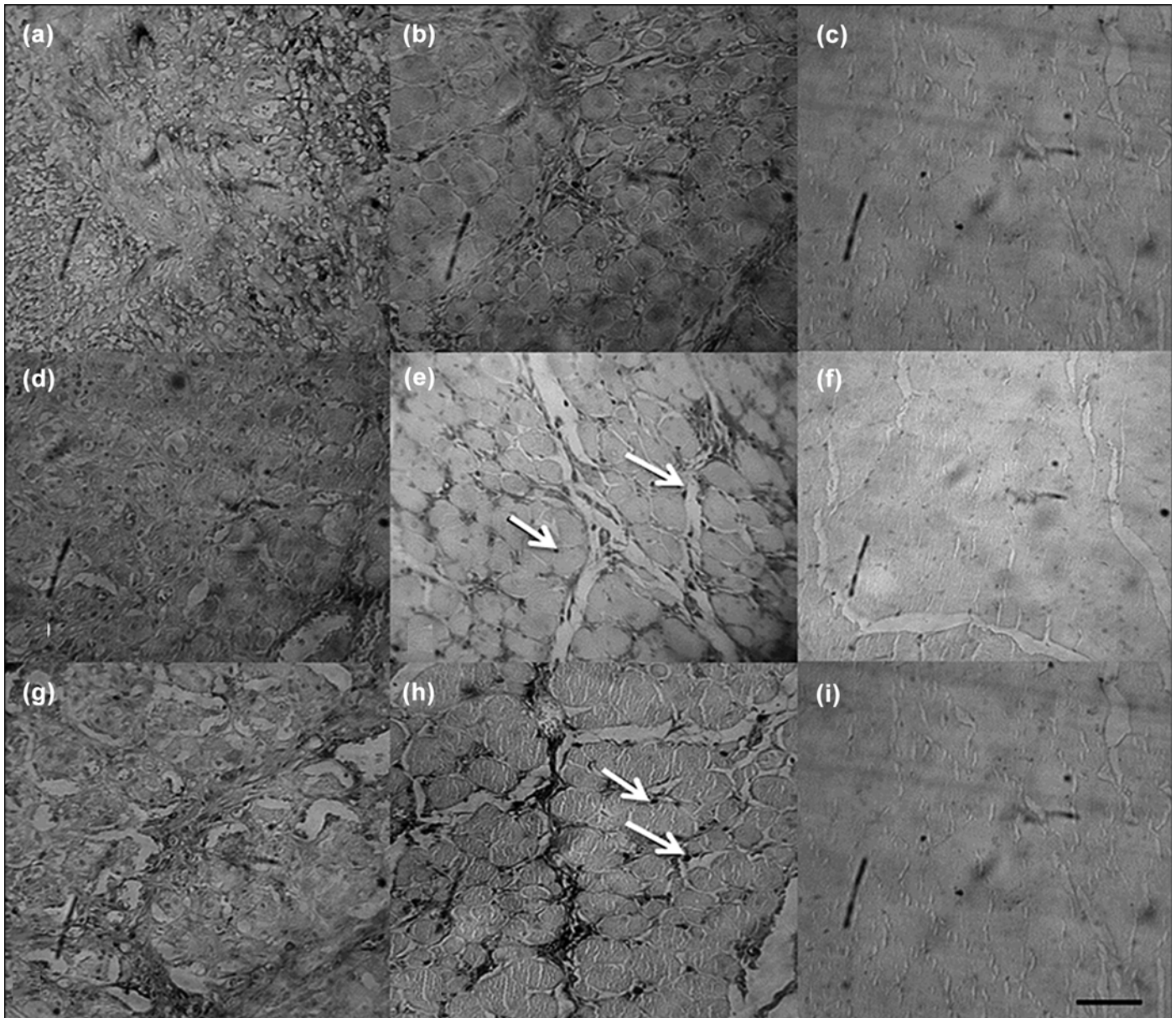
**Figure 2.**

Immunohistochemical staining for myogenin. **(a)–(c)** Control group 7, 14, and 21 d after surgery, respectively. **(d)–(f)** 10 J/cm<sup>2</sup> laser-treated animals 7, 14, and 21 d after surgery, respectively. **(g)–(i)** 50 J/cm<sup>2</sup> laser-treated animals 7, 14 and 21 d after surgery, respectively. Arrows indicate myogenin immunoexpression. 10 $\times$ .

pathological conditions to stimulate tissue repair [26–28]. Pires et al. showed that laser therapy (780 nm; 7.7 J/cm<sup>2</sup>; 75 s) reduced interleukin 6, COX-2, and transforming growth factor beta in an experimental model of tendinitis in rats [26]. Demidova-Rice et al. demonstrated that a single laser exposure stimulated the healing of wounds in mice [27]. In addition, Pallotta et al. demonstrated that LLLT (810 nm) reduced the inflammatory process in an experimental model of knee osteoarthritis in rats [28].

The histological and morphometric analysis showed that laser therapy attenuated the pathological features induced by cryolesion. Such findings may reflect the positive effects of laser irradiation on muscle regeneration, evidencing its myogenic potential and capacity to activate satellite and myogenic cells, culminating in better muscle fiber organization at the site of the injury. This is in agreement with Melo et al. [29], who affirmed that laser therapy decreased the number of inflammatory cells and increased





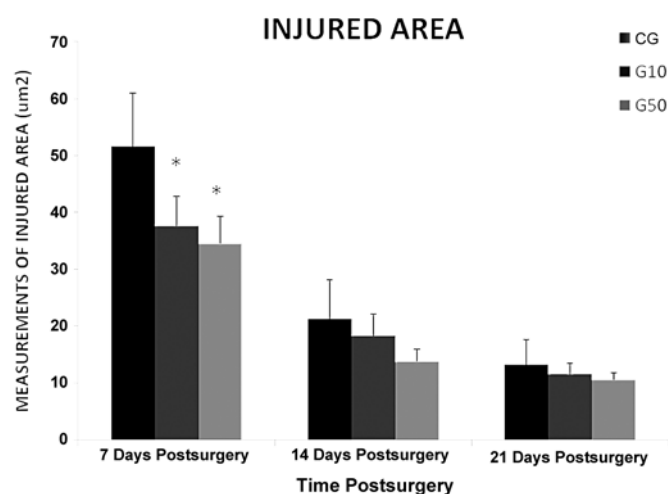
**Figure 3.**

Immunohistochemical staining of MyoD. **(a)–(c)** Control group 7, 14, and 21 d after surgery, respectively. **(d)–(f)** 10 J/cm<sup>2</sup> laser-treated animals 7, 14, and 21 d after surgery, respectively. **(g)–(i)** 50 J/cm<sup>2</sup> laser-treated animals 7, 14 and 21 d after surgery, respectively. Arrows indicate MyoD immunoexpression. 10 $\times$ .

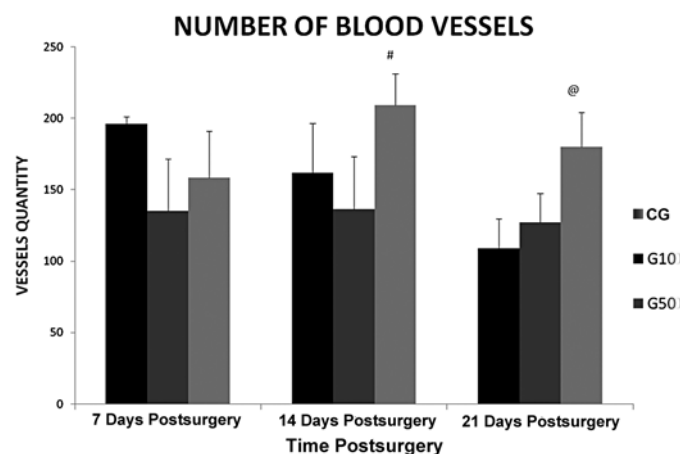
the amount of elastic fibers in the wound-healing process, which could explain the positive effect of laser on accelerating tissue repair. Also, our group, comparing the effects of 830 nm laser therapy and low-intensity pulsed ultrasound (US) on muscle healing, observed that the laser-irradiated animals presented minor degenerative changes of muscle tissue when compared with control and US-treated animals

[10]. The same results were found by Demir et al. [30], who observed that laser irradiation (904 nm; 1 J/cm<sup>2</sup>; 6 mW) was more effective than US in accelerating wound healing.

In addition, immunohistochemistry analysis showed that laser therapy, at both fluences, produced an upregulation of myogenin and MyoD expression during the process of muscle healing. Interestingly, this analysis



**Figure 4.** Mean and standard deviation of injury area. CG = control group, G10 = 10 J/cm<sup>2</sup> laser-treated animals, G50 = 50 J/cm<sup>2</sup> laser-treated animals.



**Figure 5.** Mean and standard deviation of number of blood vessels. CG = control group, G10 = 10 J/cm<sup>2</sup> laser-treated animals, G50 = 50 J/cm<sup>2</sup> laser-treated animals.

demonstrated a negative expression in the CG. MyoD and myogenin play a key role during neonatal myogenesis and have a regulatory function in the processes of muscle plasticity, adaptation, and regeneration in adult muscle [31–33]. Both myogenin and MyoD are responsible for satellite cell differentiation [34]. The arrival of satellite cells is crucial during muscle regeneration, because these cells can transform into myoblasts to substitute for the damaged muscle fibers [33–34]. When LLLT is applied

to tissue, the light is absorbed and can modulate cell biochemical reactions, increasing the synthesis of DNA, RNA, and cell-cycle regulatory proteins [35–36]. Therefore, the cited effects of LLLT may have induced increased myogenin and myoD gene expression, which culminated in upregulated expression of both MRFs. Therefore, the better organization of the muscle fibers and minor levels of inflammatory cells during the first periods after irradiation may be related to the higher levels of MyoD and myogenin expression.

Furthermore, the present study demonstrated a stimulatory effect of LLLT, mainly at the higher fluence, on blood vessel growth. An adequate blood perfusion is essential to guarantee injured tissue regeneration and, consequently, the success of the repair procedure [37]. Vascular photomodulation can be associated with the reduction of inflammatory cells and stimulation of macrophages, T-lymphocytes, endothelial cells, and fibroblast migration during the healing process, resulting in accelerated tissue healing [38]. Gonçalves et al. showed that the GaAsAl laser applied with an energy density of 60 J/cm<sup>2</sup> was more effective in stimulating neoangiogenesis than 30 J/cm<sup>2</sup> [37]. Furthermore, Corazza et al. reported that a dose of 20 J/cm<sup>2</sup> effectively increased fibroblast proliferation and neoangiogenesis in skin wounds [39].

The parameters of LLLT for optimal stimulation still need to be determined, and the molecular details involved in tissue repair have to be investigated [10]. These affirmations highlight the importance of studies exploring the effects of different parameters. Interestingly, in the present study similar beneficial effects were observed at both dosages.

## CONCLUSIONS

This study demonstrates that the LLLT had positive effects on injured skeletal muscle in rats, accelerating the muscle regeneration process by increasing the number of blood vessels and upregulation of myogenin and MyoD immunoexpression, which may have resulted in better tissue organization at the site of the injury. Although further clinical studies using functional tests are required, the findings of this work point to a promising utilization of such therapeutic modalities for tissue repair.



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