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Effect of low-level laser therapy (GaAlAs - λ660 nm) on muscle function

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Abstract **Introduction:** Low-level laser therapy (LLLT) is effective in preventing fatigue and in stimulating the microcirculation and cellular activity. In this study, we examined the effect of LLLT on injured tibial muscle *in vivo* by assessing muscle function during fatigue. **Methods:** Twenty-four male mice were used. Each mouse received an injection of sterile 0.9% saline solution (50 μL) in the right tibialis anterior muscle, after which the tendon of the muscle was exposed, connected to an isometric transducer and subjected to a resting tension of 1 g. A bipolar electrode was attached to the tibial nerve for electrical stimulation. The mice were randomly allocated to one of two groups: G1 (control: 3 h – n=8 and 9 h – n=5) and G2 (treated with GaAlAs laser, λ660 nm, 35 mW, 0.6 J, 17 s: 3 h – n=6 and 9 h – n=5). **Results:** In G1 mice, the amplitude of the tetanic contracture in response to induced fatigue remained unchanged during six consecutive tetani. The amplitude of the tetanic contractions in response to electrical stimulation (4-8 mV) was also unchanged. These results indicated muscle intactness in response to the load imposed by tetanus. In G2 mice, there was an increase in the amplitude of contraction after 3 h and 9 h when compared to G1 at 83% tetanus. **Conclusion:** These results indicate that exposure of muscle to LLLT enhanced the contractile force and increased the resistance to muscle fatigue without causing morphological damage to cellular structures.

Keywords: Low-level laser therapy, Muscle activity, Muscle fatigue, Tetanus.

Introduction

Most studies of the effects of low-level laser therapy (LLLT) in laboratory animals have been done in rats and have shown that LLLT can reduce the weakness of local fatigue on muscle force and possibly reduce muscle damage after strenuous exercises (Albuquerque-Pontes et al., 2015; Caetano and Zanuto et al., 2013; Lopes-Martins et al., 2006; Santos et al., 2014). LLLT significantly enhances skeletal muscle performance and protects skeletal muscle tissue against damage when applied immediately (Santos et al., 2014). LLLT also causes a dose- and wavelength-dependent increase in cytochrome c oxidase expression in intact skeletal muscle tissue, indicating that muscular metabolism can be enhanced by phototherapy (Albuquerque-Pontes et al., 2015).

Compared to rats, little is known of the responses to LLLT in other species, although various reports have demonstrated the benefits of LLLT in improving muscle function in dystrophic (mdx) mice (Leal-Junior et al., 2014; Oron et al., 2014; Silva et al., 2015). Mice are extensively used in toxinology to study the local effects (pain, edema, hemorrhage and necrosis) of a variety of venoms. Myonecrosis caused by *Bothrops* snake venoms is a well-known phenomenon experimentally (Gutiérrez and Ownby, 2003) and clinically (França and Málaque, 2009; Warrell, 2004) and, in the latter case, can result in extensive tissue loss and permanent functional damage to the bitten limb. Experimental studies have shown that laser therapy can attenuate the local damage caused by snake venoms, as assessed histologically and based on the quantification of marker proteins or enzymes (Barbosa et al., 2008; 2009; Doin-Silva et al., 2009; Dourado et al., 2003; Nadur-Andrade et al., 2012; 2014). However, none of these investigations has provided a functional assessment of the potential improvement in the contractile activity of muscle injected with venom and treated with LLLT.

In view of the lack of detailed studies on the beneficial effects of LLLT on muscle function in normal mice, and the potential usefulness of such a model for studying venom-induced and other lesions, in this work we examined the effect of LLLT on normal (non-inflamed and non-lesioned) tibial muscle by assessing muscle function in response to tetanic stimulation. The standardization of this model should provide a useful basis for assessing the effects of LLLT on muscle function in a variety of situations in mice.

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Methods

Twenty-four Swiss mice (18-22 g; Animais de Laboratório - Anilab, Campinas, SP) were housed (six/cage) in the Laboratory of Physiology and Pharmacodynamics of the Institute of Research and Development at the Universidade do Vale do Paraíba (UNIVAP). The mice were maintained on a 12 light/dark cycle at 22-28 ºC and controlled humidity, with access to food and water *ad libitum*. The project was approved by the institutional Committee for Ethics in Animal Use at UNIVAP (protocol no. A001/CEUA/2012). The experiments were done within the ethical guidelines of the Brazilian Society for Laboratory Animal Science (SBCAL).

For use, the mice were randomly allocated to one of two experimental groups: G1 (control: 3 h and 9 h, n=8 and 5, respectively) and G2 (treated with laser: 3 h and 9 h, n=6 and 5, respectively). For the experiments, the right tibialis muscle of each mouse was injected with 50 µl of 0.9% NaCl and 30 min later the muscle of G2 mice was irradiated with a InGaAlP laser equipment (Twin Flex Evolution®, MMOptics), operated in indirect contact mode (parameters described in Table 1).

Three and nine hours after saline injection the mice were anesthetized with a mixture of xylazine hydrochloride (20 mg/kg, i.p.), ketamine chloride (100 mg/kg, i.p.), atropine (0.25 mg/kg, i.p.) and diazepam (5 mg/kg, i.p.) and fixed on a surgical table, after which the tendon of the tibialis anterior muscle and the right tibial nerve were exposed. In the insert region, near the metatarsal plantar region, the muscle was connected through the tendon to an isometric transducer and the nerve was connected to a bipolar electrode. The muscle was subjected to a constant tension of 1 g and indirectly stimulated by individual pulses from a stimulator GRASS Technologies S48® square pulse stimulator. Pulses of 4-8 mV, 0.2 Hz and 2 ms duration were applied for 3 min; these parameters were chosen based on the minimum and maximum voltages needed to induce muscle contraction with recruitment of all fibers (Doin-Silva et al., 2009; Lopes-Martins et al., 2006). Tetanic stimulation was done by increasing the frequency to 50 Hz for 10 s and the tetanic profile was recorded. Next, six tetani were applied at 3-min intervals while the stimuli for muscle contractions were maintained at the previously set parameters (4-8 mV, 0.2 Hz, 2 ms duration) to confirm the intactness of the muscle fibers. Muscle contractions and tetanic contractures were recorded using a Gemini 7070 physiograph (Ugo Basile®, Varese, Italy) and an Ugo Basile® model 7003 isometric force transducer. Tetani were analyzed by measuring the amplitude of the first movement of the physiograph pen up to the peak of each tetanus.

Table 1. Laser parameters.

Parameters	Values
Energy density (J/cm^2)	3
Energy (J)	0.6
Power (mW)	35
Irradiation time (s)	17
Spot (cm^2)	0.19
Wavelength (nm)	660±5

After completion of the records, the anesthetized mice were euthanized with an overdose of KCl (10%, i.c.) (Barbosa et al., 2009; 2010; Barbosa-Souza et al., 2011; Santos et al., 2010). The muscles were fixed in 4% formaldehyde for 24 h, followed by routine procedures for dehydration and embedding in paraffin or paraplast. Sections 5 µm thick were cut with a microtome, mounted on microscope slides and stained with hematoxylin-eosin (HE). The slides were examined and photographed to facilitate examination of the cell membrane intactness, the position of the nuclei and the intercellular spaces. In addition, tibial muscles were analyzed histomorphometrically 3 h and 9 h after treatment with saline or LLLT. Muscle fiber diameter was analyzed in longitudinal sections (n = 10 sections/animal) using ImageJ 1.49t software.

The results were expressed as the mean ± standard error of the mean (SEM). The significance of differences between the control group and laser-treated groups was determined by ANOVA followed by the Tukey-Kramer post-test for multiple comparisons. Values of $p<0.05$ were considered significant.

Results

Tetanus was induced by increasing the frequency of electrical impulses from 0.2 Hz to 50 Hz for 10 s. Figure 1a and 1b show the myographic profile of the six tetanic records 3 h and 9 h after the injection of 0.9% saline. Between each tetanus, the muscle was stimulated at 4-8 mV for 2 ms and 0.2 Hz for 3 min to assess the intactness of the fibers. In tetanus induction, muscle contraction was initially high but decreased slightly and persisted for 10 s until the stimulus was turned off (Figure 1a and 1b).

The control records showed that the amplitude of the electrically-induced contractions for 3 min between the tetani remained unchanged, indicating that the muscles were functionally intact and not damaged by the repeated tetani. The records of six tetani in the G2 muscles that received saline or LLLT at 3 h showed a significant increase in the amplitude of contraction

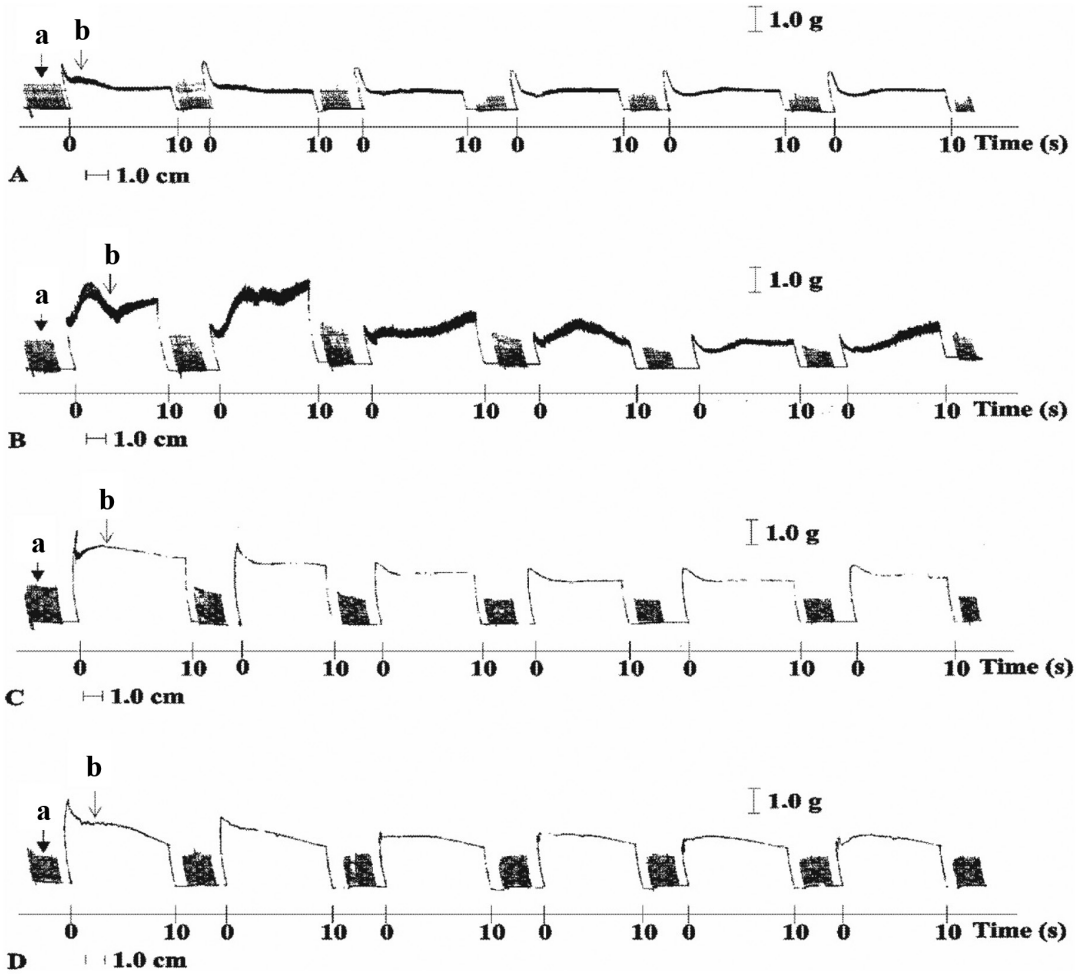

Figure 1. Muscle contractions (a) and tetanic contractures (b) were recorded using a physiograph. Myographic profiles of tetanic trains in tibial muscle stimulated via the nerve. A – Control 3 h (n = 8), B – Control 9 h (n = 5), C – Laser 3 h (n = 6), D – Laser 9 h (n = 5). Note the increase in the tetanic contracture of muscles treated with LLLT compared to the control muscle. The baseline remained stable throughout the experiment, indicating that the muscle did not go into spasm and was not injured during the procedure. The amplitudes were calculated based on the corresponding initial muscle contracture burst.

in the control group, indicating an increase in muscle contractile force (Figures 1c and 1d). In the control group, between the first and the sixth tetanus, the mean decrease in tetanus amplitude was 17.2 ± 6%. In muscles treated with LLLT, the decrease in tetanus amplitude was 34.0 ± 9.1%. Although the decrease in tetanus amplitude was greater in muscles treated with LLLT, these muscles showed a greater baseline amplitude and feedback when compared to control muscles. This was noted 3 h after irradiation. Figure 2 shows the development profile of the six tetani with and without LLLT. Three hours after laser application there was potentiation of the contractions and an increase in muscle strength. The contractile force developed in the first three tetani was larger than in subsequent tetani, indicating the onset of muscle fatigue, but was still above control values. Figure 3 shows the same situation 9 h after LLLT.

Histological analysis of the muscle fibers showed that the cell membranes of control and LLLT-treated muscles were intact after 3 h and 9 h. The nuclei also showed no alterations in either group. These results indicated that even if G2 had a greater muscle activity, this did not cause morphological changes (Figures 4 and 5).

Histomorphometric analysis showed a significant increase in fiber diameter in mice treated with LLLT (3 h) compared with saline; no change in diameter was seen after 9 h (Figure 6).

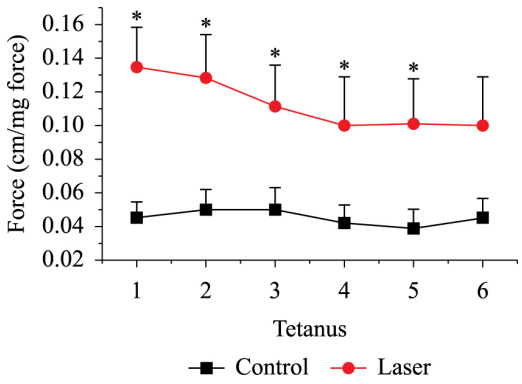

Figure 2. Muscle contractile force in the absence (control) and presence of LLLT (3 h post-LLLT). Note the greater contractile force in muscle treated with LLLT. Each point represents the mean of the measurements of the first muscle burst as explained in Figure 1. The data are expressed as the mean ± SEM (control n=8; LLLT n=6). *p<0.05 compared to control preparations.

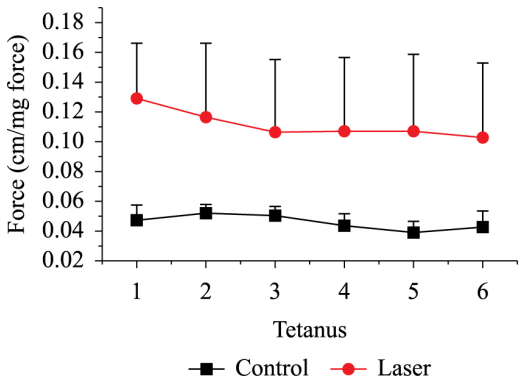

Figure 3. Muscle contractile force in the absence and presence of LLLT (9 h post-LLLT). Note the greater contractile force in muscle treated with LLLT. Each point represents the mean of the measurements of the first muscle burst, as explained in Figure 1. The data are expressed as the mean ± SEM (control n=5; LLLT n=5). There were no significant differences in the responses between the two groups of animals.

Discussion

In humans and rats, LLLT enhances the recovery of muscle contracture during tetanus. However, to date there has been no similar assessment in mice, a species widely used for testing drugs and toxins. In this study, we therefore sought to reproduce in mice the experimental technique previously used in rats. Thus, for example, the dose of LLLT used here has be extensively documented and standardized for rats, as described by Andrade et al. (2011), Caetano and Zanuto (2013), Lopes-Martins et al. (2006) and Santos et al. (2014). As shown in the present study, LLLT enhanced muscle contractility to tetanic stimuli in the first 3 h after laser application, but there was no enhancement after 9 h.

The benefits of LLLT in skeletal muscle include enhanced strength for everyday activities and sports, without damage to the fibers or reduced muscle functionality (Baroni et al., 2010; Caetano and Zanuto, 2013; Kelencz et al., 2010; Leal et al., 2009a; 2009b; Liu et al., 2009; Lopes-Martins et al., 2006; Maciel et al., 2013). The dose and form of LLLT application were selected to primarily enhance cellular activity (Andrade et al., 2011; Lopes-Martins et al., 2007; Caetano and Zanuto et al., 2013; Santos et al., 2014). The 660 nm wavelength laser used here corresponded to the electromagnetic spectrum absorbed in the respiratory cycle (Zhang et al., 2008). This wavelength stimulates the respiratory chain and increases mitochondrial ATP production to provide energy to the cell via the chemical bonds of cytochrome c (Lopes-Martins et al., 2006). The data reported here suggest that cell activity was increased, probably because of an improved blood supply coupled with enhanced ATP formation. This ATP is used by troponins in phosphorylation to promote the necessary cross-bridges required for muscle contraction (Sussai et al., 2010).

The inference of an enhanced post-irradiation energy intake was based on the increase seen in the amplitude of contractile force in the myographic recordings. Figure 1 shows that the peak of tetanus was greater in irradiated mice, with tetanus lasting for ~10 s followed by a decrease at the third tetanus that was maintained for the remainder of the series. A similar relationship between exposure to LLLT and the duration of tetanus was also observed by Lopes-Martins et al. (2006) and Santos et al. (2014) for rats.

The percentage decrease in amplitude between the first and sixth tetanic series in LLLT-treated mice was similar to that reported by Lopes-Martins et al. (2006), despite the different experimental conditions of the latter study. After three tetanic series, the degree of muscle fatigue was 34.0 ± 9.1% and probably reflected the restoration of muscle homeostasis after use of the energy provided by LLLT-induced ATP formation (Sussai et al., 2010).

Studies such as those of Doin-Silva et al. (2009) and Lopes-Martins et al. (2006), originally done to evaluate neuromuscular activity, demonstrated an increase in muscle contractile force after application of low intensity laser in rats. LLLT has been described as an adjunctive treatment for athletes in aerobic performance, adaptation and recovery of skeletal muscle (Sussai et al., 2010). The longer time required for the onset of muscle fatigue and the gain in the amplitude of the first tetanic series agreed with clinical studies describing greater torque in athletes treated

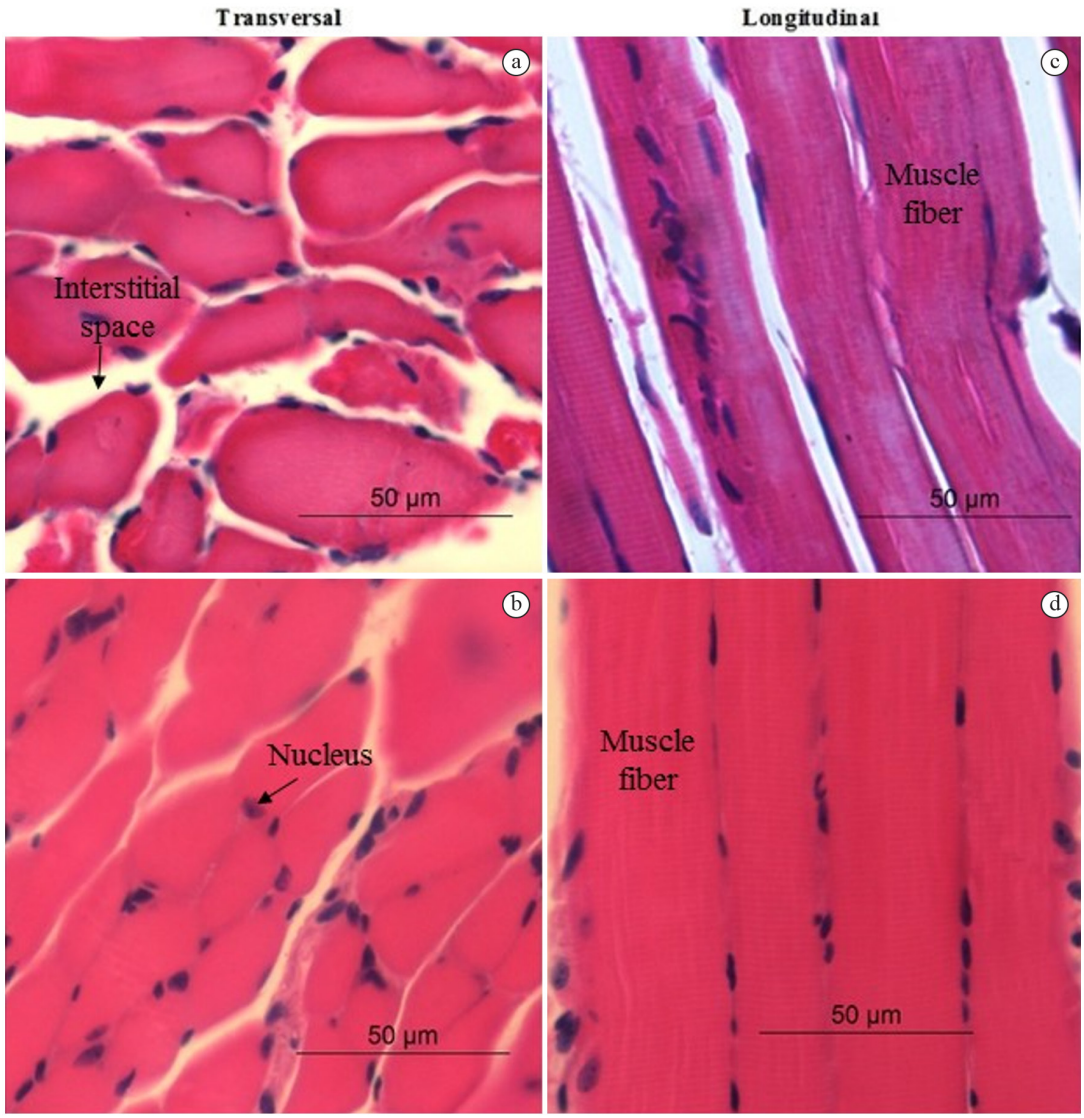

Figure 4. Histological sections of tibial muscle without LLLT (a) and (b) and 3 h after treatment with LLLT (c) and (d). (a) and (c) Cross-sections and (b) and (d) – Longitudinal sections. Note the normal appearance of the muscles, e.g., intact membranes and peripherally located nuclei. Scale bar = 50 µm.

with radiation different wavelengths (Andrade et al., 2011; Leal et al., 2010c; Lopes-Martins et al., 2007; Maciel et al., 2013; Place et al., 2010; Rocha and Barbanti, 2007).

Histological analysis of muscle fibers exposed to LLLT was done to examine whether LLLT could protect tissue from damage by subsequent tetanus. According to Sussai et al. (2010), stressful exercise can cause muscle injury, fiber breakdown, a decrease in performance, depletion of ATP and calcium imbalance. As shown here, muscle structure and organization were unaffected by LLLT, indicating that the gain in energy input involved intracellular biochemical alterations. Exposure to LLLT made the muscle more resistant to fatigue induced by indirect electrical stimulation, a finding in agreement with that of Leal et al. (2010a). Lopes-Martins et al. (2006) also observed a decrease in fatigue after application of LLLT in rat electrically-stimulated muscle. Leal et al. (2008; 2010b; 2010c) reported that LLLT increased the number and duration of contractions in high intensity, short duration exercises designed for volleyball players. Although LLLT apparently increased the muscle fiber diameter at 3 h, this enhancement probably reflected the lower fiber diameter in control muscles rather than an increase caused by LLLT; this

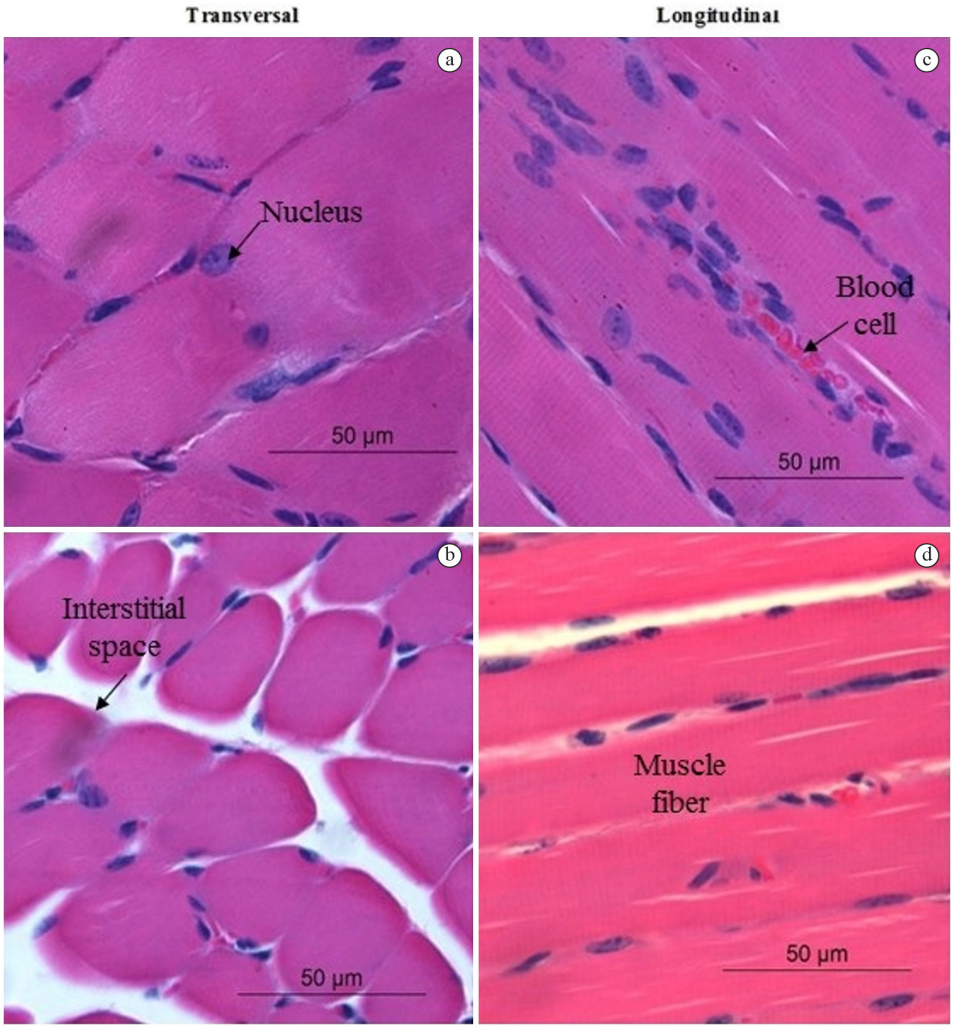

Figure 5. Histological sections of tibial muscle without LLLT (a) and (b) and 9 h after treatment with LLLT (c) and (d). (a) and (c) – Cross-sections and (b) and (d) – Longitudinal sections. Note the normal appearance of the muscles, e.g., intact membranes and peripherally located nuclei. Scale bar = 50 µm.

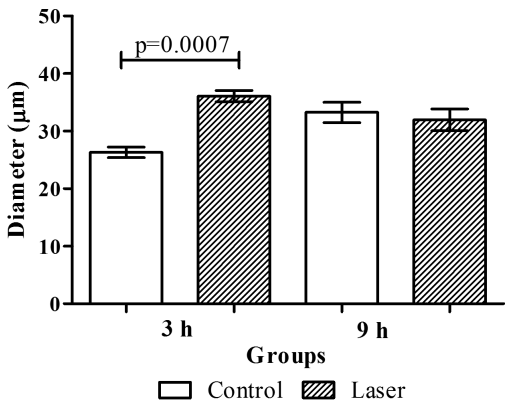

Figure 6. Histomorphometric analysis of control and LLLT-treated tibial muscles 3 h and 9 h after LLLT. Muscle fiber diameters were quantified in longitudinal sections (n=10 sections/mouse) and the columns represent the mean ± SEM.

conclusion is supported by the fact that the diameter of LLLT-treated muscles at 3 h was similar to the diameters of control and LLLT-treated muscles at 9 h. The reason for the lower fiber diameter in the 3 h control group is unclear.

In conclusion, the results of this study indicate that LLLT enhances the contractile force of mouse tibialis muscle without causing morphological damage and increases the resistance of this muscle to fatigue. This model should be useful for studying a variety of pathological conditions in mice.

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