

Zymographic and ultrastructural evaluations after low-level laser irradiation on masseter muscle of HRS/J strain mice

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Abstract Low-level laser therapy (LLLT) has been widely used in the treatment of the stomatognathic system dysfunction; however, its biological effect remains poorly understood. This study evaluated the effect of LLLT (GaAlAs, 780 nm, 20 J/cm², 40 mW) on masseter muscle of HRS/J mice after different numbers of laser irradiations (three, six, and ten) for 20 s in alternate days. Three experimental groups were defined according to the number of laser irradiations and three control groups ($n=5$) were used. On the third day after the last irradiation, all animals were killed and the masseter muscle was removed and processed for the following analysis: (a) transmission electron microscopy, (b) zymography, (c) immunohistochemistry for vascular endothelial growth factor (VEGF) and VEGFR-2. The results showed: (a) with six laser applications, a dilation of T tubules, and sarcoplasmic reticulum cistern, increased pinocytosed vesicles in the endothelium; with ten laser applications, few pinocytotic vesicles in the endothelium and condensed mitochondria. (b) Under the conditions of this study, the synthesis of other matrix metalloproteinases was not

observed, only the MMP-2 and -9. (c) After ten laser irradiations, immunostaining was observed only for VEGFR-2. We conclude that after six laser applications, ultrastructural changes may facilitate the Ca⁺² transfer to cytosol and increase the fluid transport from one surface to another. The ultrastructural changes and no immunostaining for VEGF with ten applications may decrease the metabolic activity as well as damage the angiogenic process, suggesting that an effective number of laser applications may be less than ten, associating to this therapy a better cost–benefit.

Keywords Low-level laser therapy · Masseter muscle · Transmission electron microscopy · Zymography · VEGF · VEGFR-2

Introduction

In medical practice, lasers are widely used for treatment of patients with temporomandibular disorders, involving the joints and masticatory muscles, with pain expression that radiates to the face, neck, and shoulders. The laser treatment has been successful due to its anti-inflammatory effect [1], its analgesic and muscular relax actions [2], ability to reduce fatigue during tetanic contractions [3], improves the skeletal muscle performance and decrease the skeletal muscle damage [4], delays muscle fatigue and decreases the blood lactate post-exercise in rat model [5]. In addition, laser reduces electromyographic activity and muscular symptoms [6], increases the bite force, and decreases the orofacial pain [7, 8]. Despite these studies demonstrating the efficacy of low-level laser therapy (LLLT), the biological effects on specific tissues are not well understood.

Non-surgical lasers use portions of the visible and invisible spectrum, with wavelengths between 700 and 980 nm, emitting

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pulsed or continuous wave [9], whose power reaches up to hundreds of milliwatts (mW). Therefore, an enormous variation in these used parameters was found in literature. For example, irradiation with He–Ne laser (632.8 nm) energy density of 7.2 J/cm^2 has a deleterious effect on the keratinocytes differentiation [10]. On the other hand, the LLLT with 15 mW output power and 3.8 J/cm^2 of energy density for 15 s, induced apoptosis during the tissue healing after three applications [11]. Moreover, it was observed that the 585 nm laser may be more harmful than the 595 nm in vascular tissue of normal human skin [12]. The changes on instrument type, semiconductor, wavelength, intensity, exposure time, and duration of LLLT used in different published studies make comparisons difficult. Each wavelength has different type of interactions, depending if the target is muscle or joint tissues [13, 14], considering that each one has different characteristics [15]. According to Rizzi et al. [16], LLLT decreased the iNOS protein and blocked the NF- κ B activation in injured gastrocnemius muscle.

Several previous studies analyzed the changes of muscle fibers in malocclusion induction by histoenzymologic methods, which identify the fibers according to the speed of fiber contraction and oxidative capacity [17, 18]. However, a fewer number of studies analyzed the laser effect on muscular ultrastructure in experimental animals [19].

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases capable of degrading protein structures such as collagen, elastin, and other proteins present in the extracellular matrix [20, 21]. These enzymes are primarily responsible for remodeling, degradation, or modification of the extracellular matrix [21]. Under physiological conditions, activated MMPs are controlled by tissue inhibitors of metalloproteinases and are usually expressed at low levels. However, the levels of some MMPs may be raised rapidly when the tissue is subjected to ultraviolet radiation (UVR) [22, 23] during inflammation, wound healing, and progression of cancer [24, 25]. However scarce studies are found involving laser on the extracellular matrix of muscle by zymography, which is an important method for MMPs detection.

Vascular endothelial growth factor (VEGF) is a glycoprotein that belongs to the family gene for vascular growth factors, involved in angiogenesis [26]; it is a protein located in the subsarcolemmal region of skeletal muscle fibers, in vascular smooth muscle and the wall of capillaries, being of the last mentioned structure, one factor that has an effect on endothelial cell proliferation [27]. VEGF acts at capillaries neoformation from pre-existing blood vessels, and there are five types of VEGF in mammals: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF (placental growth factor). Among its receptors, the VEGFR-1 and the R-2 are receptors for VEGF-A, and it is believed that the binding of VEGF-A receptor VEGFR-2 is the major signaling pathway for angiogenesis [28]. However, recent data show that VEGF exerts multiple non-angiogenic effects in various cell types including neurons, skeletal muscle,

and heart cells. One therapy that results in the formation of new blood vessels, by angiogenic gene action, seems to be a promising strategy [29]. In this context, the family of VEGF and VEGFR-2 may be considered an important molecular tool to analyze the effect of laser on the muscle.

Currently, despite the increasing use of phototherapy in the medical field, there are a small number of basic researches using LLLT. Literature shows the need for further studies using animal models, considering that the wavelengths, dosages, and appropriate conditions of laser irradiation are not well established [30], and phototherapy is a possible alternative treatment to reduce the need for invasive procedures [31]. Thus, to help clinicians in understanding the biological effect of lasers in clinical practice and improve the cost benefits associated with this treatment, this study examined the effect of three different laser applications (three, six, and ten) on masseter muscle of HRS/J hairless mouse, using the usually recommended parameters [32] for muscle pain and temporomandibular disorder therapy.

Materials and methods

Laser

The Twin Laser (MM Optics; São Carlos, São Paulo, Brazil) was used, 780 nm, with a semiconductor gallium–aluminum–arsenide, and the assessment of output power was made before this experiment research using this apparel by the manufacturer.

Animals

Male mice, strain HRS/J (hairless), 3 months old, weighing about 35 g, obtained from the vivarium of the Faculty of Dentistry of Ribeirão Preto, University of São Paulo were divided into groups according to the number of laser irradiations (three, six and ten). Each group was composed by control ($n=5$) and irradiated ($n=5$) animals. The animals were kept in polyethylene boxes under conditions of controlled room temperature between 24 and 25° C and 12 h of light per day. They received food and water ad libitum. All study procedures were approved by the Local Ethics Committee (number: 08.1.290.53.9) in accordance with international laws for animals use.

Experimental procedures

Animals of the experimental group were anesthetized with halothane and the left masseter muscle was irradiated with energy density of 20 J/cm^2 (continuous wave, 40 mW, 20s, 0.04 cm^2 of spot area) in alternate days [32]. The laser irradiation was performed in direct contact with the skin, on one point (Energy: 0.8 J/point, power density: 1 W/cm^2) in the

middle region of the left masseter muscle. The control group also received the same treatment but without irradiation. Seventy-two hours after the last application [33] the animals were anesthetized with intramuscular injection of xylazine (10 mg/kg) and ketamine (75 mg/kg) and the masseter muscle was dissected for transmission electron microscopy, immunohistochemistry and zymography analyses.

Samples preparation for transmission electron microscopy (TEM)

On the third day after the last irradiation, animals were anesthetized and the left masseter muscle was excised, reduced to small fragments, and immersed in modified Karnovsky solution containing 2.5 % glutaraldehyde and 2 % paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4 [34]. The fragments, post-fixed in a solution of osmium tetroxide at 4° C, were immersed in 5 % uranyl acetate aqueous, dehydrated in an ascending series of alcohol, treated with propylene oxide, infiltrated by mixture of pure resin and propylene oxide (1:1), and embedded in Spurr resin to complete polymerization. After identifying the interest area, in thick sections (300 nm), ultrathin sections (55 nm) were obtained, collected on 200 mesh networks, contrasted with uranyl acetate and lead citrate solutions [34], and examined using a transmission electron microscope Jeol 1010 (TEM).

Qualitative analysis of proteinases (MMP) by zymography

The substrate zymography included in sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was used to detect gelatinase activity of whether the masseter muscle irradiated or not. Masseter muscle dissected and frozen in dry ice (1:4 w/w dilution) was homogenized in 50 mM Tris–HCl (pH 7.4) containing 10 mM CaCl₂ and 1 % cocktail of protease inhibitors in a “Turax TE-102” (Turratec, São Paulo, Brazil). The total of homogenate muscle was centrifuged at 12,000×g and used to measure the levels of protein by the Lowry method [35]. Aliquots of supernatant (50 mL) were mixed with 100 mM Tris–HCl (pH 7.4) containing 4 % SDS, 20 % glycerol, and 0.001 % bromophenol blue. Proteolytic activity was assessed qualitatively, comparing the irradiated and control animals.

Immunohistochemistry for vascular endothelial growth factor and receptor-2 for vascular endothelial growth factor

Transversal histological sections, 10 µm thick, were obtained from muscle rapidly frozen in isopentane, cooled in liquid nitrogen, using the Leica cryostat (CM1850, Germany).

To inhibit nonspecific binding, slides were incubated with PBS-BSA (2 %) in an acrylic tank. The slides were incubated with primary antibody diluted in PBS-BSA (2 %), washed, and endogenous peroxidase activity was blocked by

immersion in a container with 30 % H₂O₂. The sample tissues were covered by drops of yellow link DAKO, transferred to an acrylic tub, and then covered by red link. After washing and drying, each slide was covered by the developer solution (Dako kit), and counter-stained by hematoxylin and mounted in Entellan. The images were captured using a Leica IM50 program on a computer connected to a Leica DC 300F camera adapted to the Leica microscope DMLB2.

Result

Ultrastructure of the masseter muscle

Under transmission electron microscopic analysis, muscular fibers with invaginations of the sarcolemma and dilated cisternae of sarcoplasmic reticulum (Fig. 1a) in relation to other groups was observed in the group with six laser irradiations. The group submitted to ten laser irradiations showed the mitochondrial cristae to be condensed and with dilated T tubules and sarcoplasmic reticulum cisternae (Fig. 1d). In relation to endothelial cells, the group with six irradiations (Fig. 1b) showed pinocytosis and macropinocytose vesicles, as well as cytoplasmic protrusions and endothelial cell budding, whose characteristics were more relevant than in groups with no irradiation (Fig. 1c) and ten irradiations (Fig. 1e).

Zymography

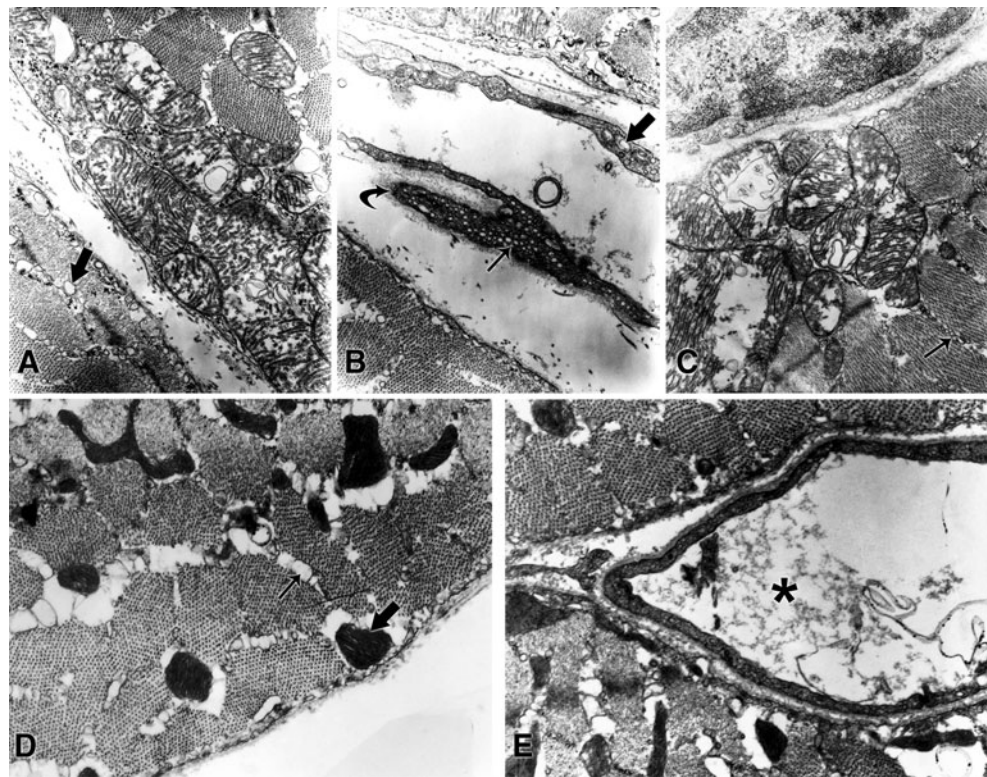
The representative data of two separate experiments with five animals showed that the LLLT did not change the proteinase activity profile of masseter muscle in this experimental animal model (Fig. 2). However, analyzing the electrophoretic profiles corresponding to irradiated groups, i.e., which received ten laser irradiations, it is possible to observe lower intensity of the bands with proteolytic activity when compared with the control group (Fig. 2, T3).

Immunohistochemistry for VEGF and VEGFR-2

VEGF immunostaining was diffusely found in connective tissue of the perimysium and endomysium, whose pattern was similar between the control and treated groups with three and six laser irradiations (Fig. 3a, b, c and d), however, without immunoidentification in the group that received ten laser irradiations (Fig. 3e and f). Figure 3g shows the negative control (VEGF) of irradiated masseter muscle.

Immunohistochemical expression of VEGFR-2 showed immunoidentification more localized in vascular endothelial cells, whose expression pattern was similar between the control and irradiated with three, six, and ten applications (Fig. 4a, b, c, d, e, f). Figure 4g shows the negative control (VEGFR-2) of irradiated masseter muscle.

Fig. 1 **a** Irradiated muscle (6) with dilated cisternae of sarcoplasmic reticulum (*arrow*). Magnification $\times 20,000$. **b** Irradiated endothelial cell (6) with numerous pinocytosis (*arrow*) and macropinocytose vesicles (*thick arrow*) and endothelial cell budding (*curved arrow*). Magnification $\times 20,000$. **c** Non irradiated muscle with normal T tubules and cisterns of sarcoplasmic reticulum (*arrow*). Magnification $\times 20,000$. **d** Irradiated muscle (10) with condensed mitochondrial cristae (*arrow*) and dilated T tubules and cisternae of sarcoplasmic reticulum (*thin arrow*). Magnification $\times 20,000$. **e** Irradiated (10) capillary (*) with thin wall and few pinocytic vesicles. Magnification $\times 20,000$



Discussion

The most relevant ultrastructural alterations of muscle fibers demonstrated in the treated group at six and ten laser irradiations, dilated invaginations of the sarcolemma (T-tubule) and sarcoplasmic reticulum cisterns. Considering that this organelle stores Ca^{+2} at high concentrations, these characteristics suggest that the low laser therapy interfere with the transfer mechanism of Ca^{+2} into the cytosol. With regard to the ultrastructure of mitochondria, the group that received ten laser irradiations showed a condensation mitochondrial cristae, whose characteristics may reveal that the number of radiation interfere in metabolic activity of the muscle fiber. These data strengthen the studies of Rizzi et al. [36] in which the increased metabolic activity revealed by the reaction of succinate dehydrogenase was evident by the number of six laser applications, but was lower with ten applications. It is well established that the chain of mitochondrial electron transport

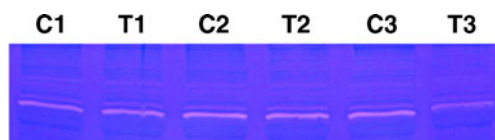


Fig. 2 Electrophoretic profile of the proteolytic activity in masseter muscle of hairless mice. *C1*, *2*, and *3* correspond to non irradiated groups. *T1*, *2*, and *3* correspond to groups with three, six, and ten irradiations (20 J/cm^2), respectively. The data are representative of two separate experiments ($n=5$)

is photosensitive to red and infrared light [37], being activated by LLLT [38, 39]. The literature described that a muscle using laser irradiation with an energy density of 5 and 10 J/cm^2 for seven consecutive days, showed an increase in the space between the outer and inner membrane of mitochondria and also dilated mitochondrial cristae [19]. With respect to endothelial cells, the group with six laser irradiations showed pinocytosis vesicles and macropinocytose, as well as cytoplasmic protrusions and endothelial cells budding, more relevant than to the groups with three and ten irradiations. The relationship of these vesicles is clear; with the ability of the cell membrane include fluids, transporting them from one surface to another, in both directions, suggesting that six irradiations stimulated the activity of endothelial cells. Although the molecular mechanisms involved in these biological effects are still little known, laser therapy improves the microcirculation [40]. Ten laser irradiations under the experimental conditions of this study caused undesirable damage on the ultrastructural morphology of mitochondria and endothelial cells, which may impair the metabolic activity of the muscle fiber. These data corroborate to the studies in which ten applications of laser (20 J/cm^2) altered the relative area of the basal and granulosum layers of the epithelium [25, 41]. Thus, studies aiming to find a number of the most effective laser irradiation will be very important, which may be smaller than the one used in this study. The present research evaluated the effects of laser irradiation on proteinases activity in masseter muscle of HRS/J mice. Using zymographic methods as it

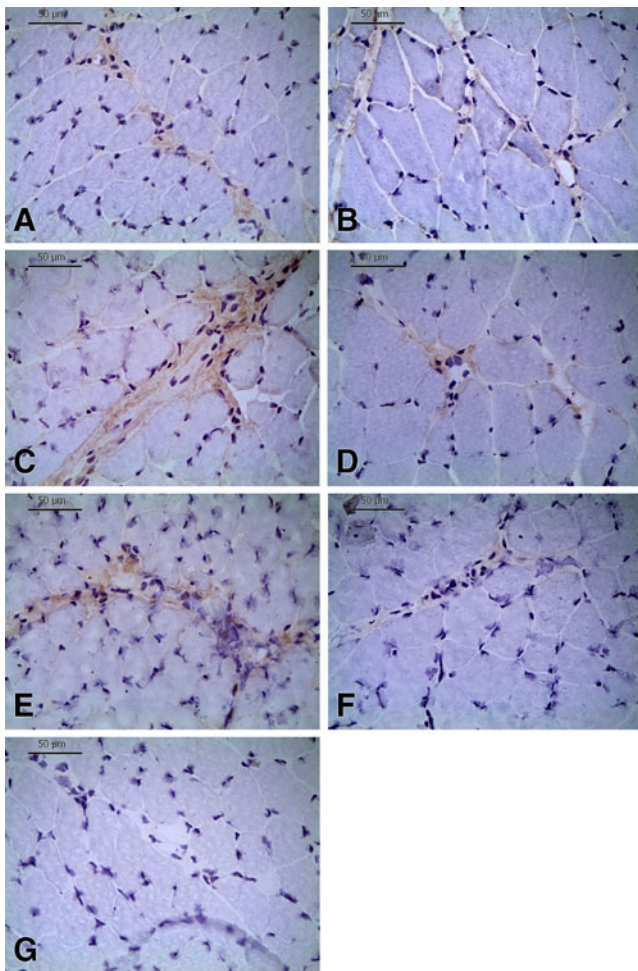


Fig. 3 Masseter muscle, VEGF immunostaining. **a** Control for the group that received three laser applications, $\times 40$ magnification. **b** Control for the group that received six laser applications, Magnification $\times 40$. **c** control for the group that received ten laser applications, $\times 40$ magnification. **d** Three laser applications, magnification $\times 40$. **e** Six laser applications, magnification $\times 40$. **f** Ten laser applications with weaker immunostaining, magnification $\times 40$. **g** Negative control, VEGF of irradiated masseter muscle, magnification $\times 40$

was used in the present study, acute exposure to ultraviolet radiation caused a dose-dependent induction (0.96 to 2.87 J/cm²) in the activity of MMP-2 (72 kDa of gelatinase) and MMP-9 (92 kDa of gelatinase) in the skin of hairless mice [42]. LLLT did not cause significant changes in proteinase activity profile on masseter muscle of HRS/J mice, different from the effect caused by the exposure to ultraviolet radiation. However, analyzing the electrophoretic profiles corresponding to the group that received ten laser irradiations, we found lower intensity of the bands indicating proteolytic activity, when compared with the control group. It is known that under normal conditions, MMP activity is required for tissue remodeling, but altered MMP activity has been reported in disease. When the masseter muscle of rats were irradiated with different doses of LLLT (0, 0.5, 1, 2.5, 5, 20 J/cm²), the groups irradiated with the

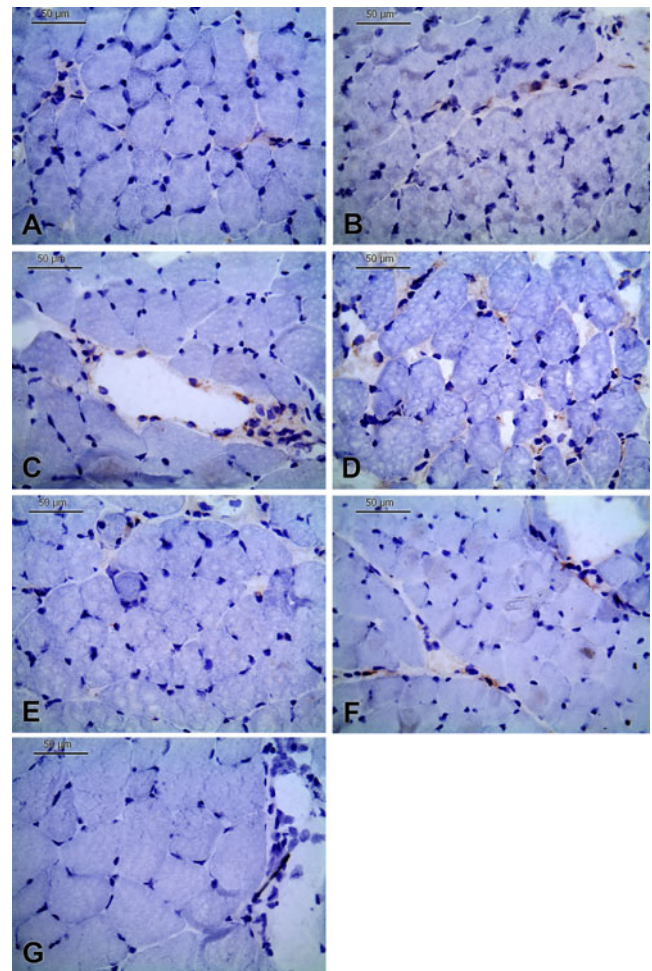


Fig. 4 Masseter muscle, VEGFR-2 immunoidentification. **a** Control for the group that received three laser applications, magnification $\times 40$. **b** Control for the group that received six laser applications, magnification $\times 40$. **c** Control for the group that received ten laser applications, magnification $\times 40$. **d** Three laser irradiations group, magnification $\times 40$. **e** Six laser irradiations group, magnification $\times 40$. **f** Ten laser irradiations group, magnification $\times 40$. **g** Negative control, VEGFR-2 of irradiated masseter muscle, magnification $\times 20$

highest doses revealed increased activity of MMP-2; and in all groups exposed to different energy densities of laser irradiation, MMP-9 activity [43] increased. The band of low intensity showed by zymography method suggests that ten laser applications may increase the risks for altered activity of MMP on healthy muscle. Thus, future studies using different parameters are needed aiming to clarify this issue.

In the present study, the VEGF immunostaining patterns were similar between the control and treated groups with three and six laser irradiations, but no immunostaining was found in the group with ten laser irradiations. VEGF is an essential mediator for angiogenesis in skeletal muscle [44]. Some studies show positive results between laser application and VEGF expression in various tissues and experimental conditions, as culture of odontoblast-like cells [45], psoriatic plaques [46],

neuropathic pain after chronic constrictive lesion [47], recovery of bone defects in rats with osteoporosis [48], healing of burns [49], and post-infarct hearts in rats [50]. Researches showing immunoreactivity for VEGF and VEGFR-2 antibodies in muscle are scarce in the literature; however, these factors had a positive identification on the masseter muscle when the rats were irradiated with different doses of LLLI (0, 0.5, 1, 2.5, 5, 20 J/cm²) [51]. After ten laser radiations in this research, as it was described by Dias et al. using highest energy density, no VEGF immunoidentification was found in mouse muscle, suggesting that angiogenesis may be impaired when compared to control groups and treated groups with three and six irradiations.

The patterns for VEGFR-2 immunostaining were similar between the control and treated groups with three, six, and ten irradiations. VEGFR-2 is mainly expressed in vascular endothelial cells, and it has strong tyrosine kinase activity, acting as a transducer for the primary differentiation and proliferation of positive endothelial cell precursor [52]. Thus, the similar VEGFR-2 expression between the groups suggests in the present research that the differentiation and proliferation of endothelial precursor cell was not modified by different number of laser irradiations.

In this experimental condition, the ultrastructural and molecular effects of the low-level laser therapy were evident. Although the large number of laser irradiations stimulated the muscle fiber activities, some characteristics suggested that the actual number of laser irradiations may be less than ten. Then, to associate the better cost–benefit to this therapy, besides the effectiveness and safety, future studies that correlate molecular effects and laser irradiations that are lower than ten irradiations are necessary.

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