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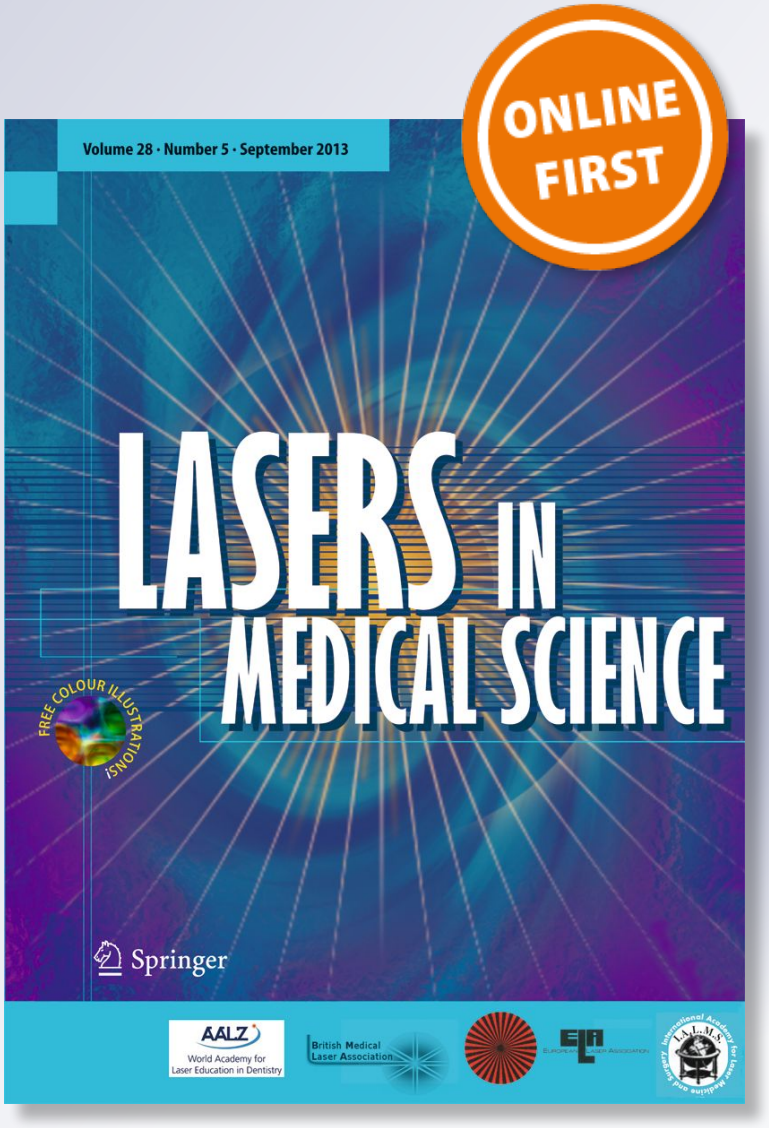

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ORIGINAL ARTICLE

Histomorphologic and ultrastructural recovery of myopathy in rats treated with low-level laser therapy

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Abstract The purpose of the present work was to study the effect of low-level laser therapy (LLLT): helium-neon (He-Ne) and gallium arsenide (Ga-As) laser on the histomorphology of muscle and mitochondria in experimental myopathy in rats. Thirty Suquía strain female rats were distributed in groups: (A) control (intact), (B) injured, (C) injured and treated with He-Ne laser, (D) injured and treated with Ga-As laser, (E) irradiated with He-Ne laser on the non-injured muscle, and (F) irradiated with Ga-As laser on the non-injured muscle. Myopathy was induced by injecting 0.05 mg/rat/day of adrenaline in the left gastrocnemius muscle at the same point on five consecutive days, in groups B, C, and D. LLLT was applied with 9.5 J cm^{-2} daily for seven consecutive days in groups C, D, E, and F. The muscles were examined with optic and electronic microscopy. The inflammation was classified as absent, mild, and intense and the degree of mitochondrial alteration was graded I, II, III, and IV. Categorical data were statistically analyzed by Chi-square and the Fisher-Irwin Bilateral test, setting significant difference at $p < 0.05$. The damage found in muscle and mitochondria histomorphology in animals with induced myopathy (B) was intense or severe inflammation with grade III or IV of mitochondrial alteration. They underwent significant regression ($p < 0.001$) compared with the groups treated with He-Ne (C) and Ga-As (D) laser, in which mild or moderate inflammation was seen and mitochondrial alteration grades I and II, recovering normal myofibrillar architecture. No differences were found between the effects caused by the two lasers, or between groups A, E, and F. Group A was found to be different from B, C, and D ($p < 0.001$). LLLT in experimental myopathy caused significant muscular and mitochondrial morphologic recovery.

Keywords LLLT · Helium-neon laser · Gallium-arsenide laser · Myopathy · Inflammation · Mitochondria

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Introduction

The term myopathy is defined as "muscle disease" affecting the muscle's structure, morphology, and biochemistry. It may arise from multiple causes and leads to changes in muscle tone and contraction with varying degrees of severity, including inflammatory myopathies, collectively known as myositis [1].

All the forms of focal myofiber damage and repair, involving plasma membrane, myofibrils, or myonuclei, are reversible processes not causing cell death, take place without obvious histological changes in myofibers and surrounding

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tissues, and do not involve inflammatory responses. However, more severe injuries due to traumatic lesions cause necrosis of whole myofibers or myofiber segments. Necrosis stimulates an inflammatory response with invasion of macrophages, followed by activation of satellite cells, which undergo proliferation, differentiation, and fusion to one another or to undamaged portions of the fiber. The formation of new myofibers or myofiber segments following necrosis is called muscle regeneration, a process that, in many but not all respects, recapitulates the sequence of events observed during embryonic myogenesis [2].

Myositis is a heterogeneous group of disorders clinically characterized by muscle weakness and by certain histopathological findings, including inflammatory infiltrates in muscle tissue [3], fiber necrosis, and degeneration and regeneration [4].

Mitochondrial dysfunction is considered to be the cause of certain myopathies and a number of multisystem disorders in humans. Muscle fiber morphology shows abnormally intensive oxidative enzyme reactions and numerically increased mitochondria in the fibers, which are often markedly enlarged and possess aberrant configurations of cristae. The mitochondrial matrix often contains lipid-like inclusions or shows vacuolation in the intracristal or intermembrane space [5]. A strong correlation between the severity of inflammation, degree of mitochondrial change, and atrophy implies the existence of a mechanistic link between these three parameters [6].

The presence of several cytokines and chemokines has been reported in muscle tissue of myositis patients. Cytokines have pleiotropic effects and may have pro- or anti-inflammatory properties. Several have effects on muscle fiber contractility and remodeling. The cellular source of cytokines in muscle tissue may be infiltrating inflammatory cells or other sources such as endothelial cells or muscle fibers. Chemokines have a role in attracting cells into the tissue inflammation, in this case into muscle tissue, and are also possible targets of therapy [7].

The current and traditional mainstay of treatment for idiopathic inflammatory myopathies has been therapeutics aimed at suppressing or modifying the immune system [8]. To counteract the effects caused by inflammation, nonsteroid anti-inflammatory drugs (NSAID) are used, despite the tolerance problems that they cause, especially from gastrointestinal and renal toxicity [9]. Ziltener et al. do not recommend their use for muscle injuries [10].

Since the mid-60s [11], the use of light energy as a therapy for inflammatory processes and cell trophism has opened up a new field in the interaction of electromagnetic energy with biological tissue. Photobiomodulation therapy is the specific term for the effective and important application of light [12]. Low-level laser therapy (LLLT) activates cellular functions without causing significant tissue-level heating. It can stimulate and/or inhibit a biological process depending on the tissue and the dose of irradiation. It is widely used in controlling inflammatory processes, repair of skin wounds, rheumatic diseases, osteoarthromyopathies, neuromuscular disorders, sports injuries, and oral pathologies, among others [13–18].

Another novel application is LLLT for muscle fatigue and muscle injury. Since agreement is being reached that mitochondria are the principal photoacceptors inside cells, and it is known that muscle cells are exceptionally rich in mitochondria, LLLT should be highly beneficial in the case of muscle injuries [19].

Although LLLT is widely used in rehabilitation, there are conflicting results in the literature and we believe that investigating the cellular and molecular events that occur when lasers of different wavelengths interact in myopathies could significantly contribute to support its effect and optimize its use in inflammatory muscle diseases. We set out to investigate the photobiomodulator effect of helium-neon (He-Ne) and gallium arsenide (Ga-As) laser in an experimental model of inflammatory myopathy, analyzing the possible morphologic recovery of myofibrillar and mitochondrial ultrastructure.

Materials and methods

Experimental groups

Thirty Suquía strain female rats (70 days old) weighing 200 ± 20 g were used, distributed in 6 groups ($n = 5$): (A) control (intact rats that received LLLT sham exposures), (B) injured, (C) injured and treated with He-Ne laser, (D) injured and treated with Ga-As laser, (E) irradiated with He-Ne laser on the non-injured muscle, and (F) irradiated with Ga-As laser on the non-injured muscle.

Experimental model

Myopathy induction: 0.05 mg/rat/day of adrenaline was injected in the left gastrocnemius muscle at the same point for five consecutive days in groups B, C, and D in order to produce muscle injury, inducing an inflammatory process [15, 20].

In all cases, adequate measures were taken to minimize animal discomfort or pain, and experiments were conducted following the guidelines of our institutional IACUC (FCM-UNC), Protocol N° 27/15, which are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Treatment with LLL

The treatment started 24 h after the fifth adrenaline injection in groups C and D, and in non-injured muscle in groups E and F [15]. LLLT parameters are presented in Table 1.

Table 1 Parameters of LLLT

Equipment model	Laser 851LO	Laser IR 170®
Manufacturer	Laseroptics SA	Meditea
Manufacturer's geographical location	Bs. As. Argentina	Bs. As. Argentina
Active medium	Helium-Neon	Gallium arsenide
Wavelength—color	632.8 nm—red	904 nm—infrared
Energy density (J/cm^2)	9.5	9.5
Power density (W/cm^2)	0.16	0.2
Power (W)	0.005	0.012
Time of irradiation (s)	60	47
Spot size (cm^2)	0.0314	0.06
Spot diameter (cm)	0.2	0.28
Emission mode	Continuous	Pulsed
Number of points	1	1
Area of application (cm^2)	0.0314	0.06
Contact or not	Contact	Contact
Frequency of treatment	One session per day	One session per day
Number of sessions	7	7
Accumulative dose (J/cm^2)	66.5	66.5

The power is measured with an Ophir Nova II Laser Power Meter.

Muscle tissue collection

The animals were sacrificed by decapitation after anesthesia with Ketamine 10 mg/kg/rat. The muscles were cut and placed in formaldehyde 10%, stained with Hematoxylin–Eosin (H&E) to see the amount of fibrous or connective tissue generated by inflammation and observed by optic microscopy. Groups B, C, and D were sacrificed 12 days after the first injection with adrenaline.

Optical microscopy studies

A 3-level classification was used to evaluate the changes in muscle morphology observed in the different experimental groups, analyzed with ×100 and ×400 objectives:

- Absence of inflammation, no infiltrate, edema, or vascular congestion.
- Mild to moderate inflammation, edema, and inflammatory infiltrate with neutrophils. Tissue structure is conserved. The percentage of the total field area occupied with inflammatory infiltrate is less than 50%.
- Severe inflammation: as above but with greater intensity and adding the presence of necrosis and marked architectural alteration. The percentage of the total field area occupied with inflammatory infiltrate is greater than 50%.

Electron microscopy studies

A millimeter square section from the left gastrocnemius muscle was fixed immediately after extraction in a Karnovsky solution (4% formaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer) for at least 2 h at room temperature. Then, the tissues were washed three times in cacodylate buffer and post-fixed in 1% osmium tetroxide for 1–2 h. After dehydration in a graded acetone solution (50, 70, and 90%), it was embedded in araldite 506 (48.5%), dodecenylsuccinic anhydride (48.5%), dibutyl phthalate (0.5%), and dimethylaminobenzene accelerator (2.5%). Thin sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome (Nikon, Tokyo, Japan) and examined in a Zeiss Leo 906-E electron microscope (Oberkochen, Germany) [21, 22].

In order to evaluate the changes in mitochondrial morphology observed in the different experimental groups (five micrographs of ×21,560 for each rats), a 4-level classification [23] was used:

Grade I: Normal structure.
Grade II: Normal size with dilated cristae.
Grade III: Normal size and/or altered shape. Intact membrane with few cristae.
Grade IV: Mitochondrial swelling.

Statistical analysis

The data obtained were analyzed by Chi-square and Fisher-Irwin Bilateral test for categorical variables. The Axiovision 4.8 program—Carl Zeiss Imaging Solutions GmbH (Hallbergmoos, Germany), referenced to a scale of 1 μm, was used to quantify the grade of mitochondria alteration. The significance level was set $p < 0.05$.

Results

Below are shown the histomorphological results separated according to the type of observation: (A) with optical microscope and (B) with electron microscope.

(A) Figure 1 shows three grades of inflammation in skeletal striated muscle found in the study groups.

Very similar features were seen in groups A (control), E (uninjured and treated with He-Ne), and F (uninjured and treated with Ga-As): longitudinal and cross striations, multiple and peripheral nuclei. No signs of inflammation were found, as shown in Fig. 1a.

In groups C (injured and treated with He-Ne) and D (injured and treated with Ga-As), conserved muscle fibers were seen with little vascular connective tissue,

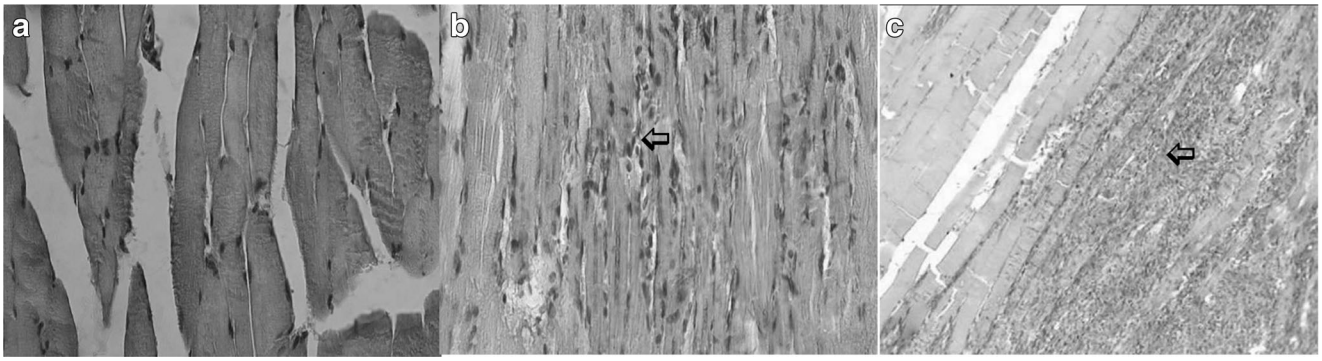

Fig. 1 Grades of inflammation in skeletal striated muscle. **a** Normal muscle, H&E (×400). **b** Muscle with mild to moderate inflammation, H&E (*arrow*) (×400). **c** Overall view of muscle with intense inflammation, H&E (*arrow*) (×100). In **b**, **c**, the inflammatory infiltrate was composed of neutrophils, lymphocytes, and plasma cells

cross striations, and peripheral arrangement of the nuclei, mild interstitial fibrosis, more restricted to epimysium. The percentage of the total area occupied in the field with inflammatory infiltrate was less than 20%. Myofibrillar structure was very similar to normal muscle architecture and similar to the control group, corresponding to the type of mild to moderate inflammation as shown in Fig. 1b.

In group B (injured without subsequent treatment), the percentage of the area occupied in the field with inflammatory infiltrate was well above 50%, located mainly in the perimysium and endomysium, encompassing muscle fibers. It is intense and of mixed type with polymorphonuclear neutrophils, lymphocytes, plasma cells, and edema, matching the degree of severe inflammation and tissue structure alteration, as shown in Fig. 1c.

(B) Figure 2 shows the four degrees of mitochondrial alteration.

In groups A (control), E (without injury and treated with He-Ne), and F (without injury and treated with Ga-As), very similar characteristics were observed, mostly normal sized mitochondria unaltered (grade I) and with conserved myofibrillar structure as presented in Fig. 2a.

In groups C (injured and treated with He-Ne) and D (injured and treated with Ga-As), similar features were observed, with a higher percentage of larger mitochondria, dilated cristae, and conserved myofibrillar architecture. In most of the sections, mitochondria were found with grade II alterations, as seen in Fig. 2b.

In group B (injured without subsequent treatment), no normal mitochondria were found, either in cristae arrangement or in size. The myofibrillar system was seen

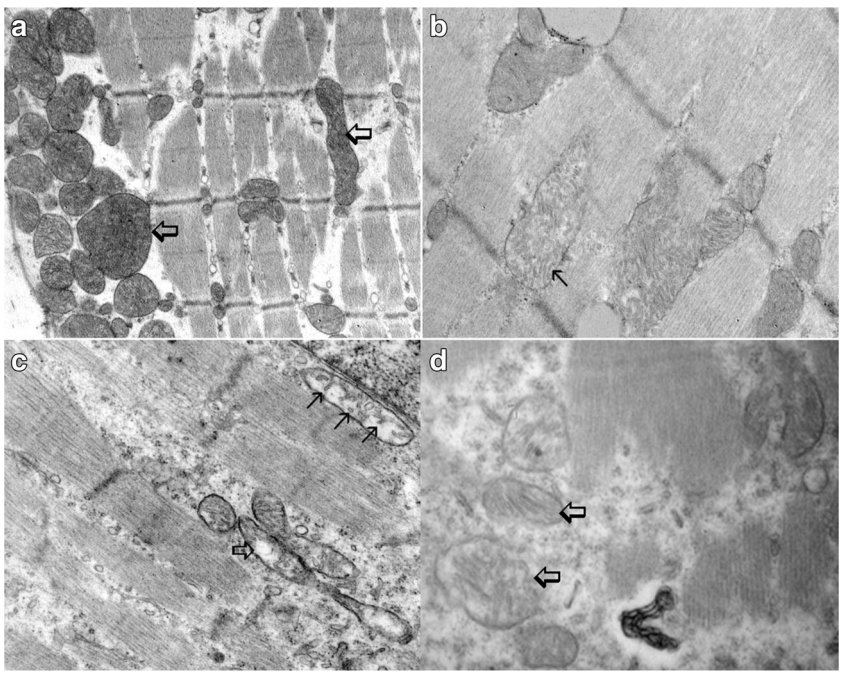

Fig. 2 Degrees of mitochondrial alteration. **a** Mitochondria with normal appearance, corresponding to grade I (*arrows*), without alterations (×21,560). **b** Mitochondria with grade II of alteration (*arrow*) (×21,560). **c** Mitochondria with grade III of alteration (*arrows*) (×21,560). **d** Mitochondria with grade IV of alteration (*arrows*)

to be completely altered, with loss of regularity of the arrangement of myofibrils and disruption of the sarcomeres. Mitochondria observed corresponded to grade III of alteration as shown in Fig. 2c: larger than normal, some with clarification of the matrix and some cristae or remains of these, and to grade IV of alteration: size well above normal, swelling, disordered, and with disruption of the cristae, as seen in Fig. 2d, with clarification of the matrix and abnormal separation of the inner and outer membrane.

Figure 3 shows the percentage of mitochondria found at each grade of alteration by study group from five photos chosen at random of ×21,560.

Significant differences were found when comparing the control group of intact rats (A) G_I: 85.45%, G_{II}: 14.55%, G_{III}: 0%, G_{IV}: 0% and the group of injured animals (B) G_I: 0%, G_{II}: 4.16%, G_{III}: 62.5%, G_{IV}: 33.34%; animals injured and treated with He-Ne laser (C) G_I: 30.36%, G_{II}: 55.36%, G_{III}: 10.71%, G_{IV}: 3.57% and animals injured and treated with Ga-As laser (D) G_I: 15.69%, G_{II}: 47.05%, G_{III}: 33.33%, G_{IV}: 3.93% ($p < 0.001$).

No significant differences were found between the control group (A) and uninjured groups treated with He-Ne laser (E) G_I: 73.08%, G_{II}: 26.92%, G_{III}: 0%, G_{IV}: 0% and with Ga-As laser (F) G_I: 97.7%, G_{II}: 2.3%, G_{III}: 0%, G_{IV}: 0%. Nor were differences found when comparing the groups injured and treated with each laser (C vs. D).

A significant difference ($p < 0.001$) was found between the injured group (B) and those injured and treated with each laser (C and D).

Discussion

This study evaluated the possible effects of He-Ne and Ga-As LLLT on the recovery of muscle tissue from mitochondrial alterations resulting from inflammatory myopathy induced in rats. The experimental model of myopathy was successfully reproduced. We used adrenaline to induce myopathy because the literature and previous studies showed that repeated injections of epinephrine can cause necrosis as a result of vascular constriction and reduce the oxygen tension of tissues at the injection site. Although in the development of this experimental model we have not observed necrosis in the injured group (B), there has been intense inflammation and mitochondrial morphological alteration. We cannot say whether these events are the result of the ischemia produced by adrenaline or the lack of energy supply resulting from mitochondrial alteration.

Significant edema and erythema of the limb was observed macroscopically with optic and electronic microscopy, in addition to the significant changes found in a previous work [15] in the concentration of inflammatory biomarkers associated with oxidative stress, such as plasma fibrinogen, nitric oxide, L-citrulline, and superoxide dismutase, using the same experimental model of myopathy.

The results of earlier work from our laboratory showed no significant histological structure changes [14] between a group of intact rats and a group of rats injected with saline, which also confirms that the induction of the inflammatory process is attributable only to adrenaline and not to the possible injury caused by the puncture.

Baez et al. using the same experimental model determined the plasma concentration of epinephrine in a group of rats injected with saline and in another group injected with

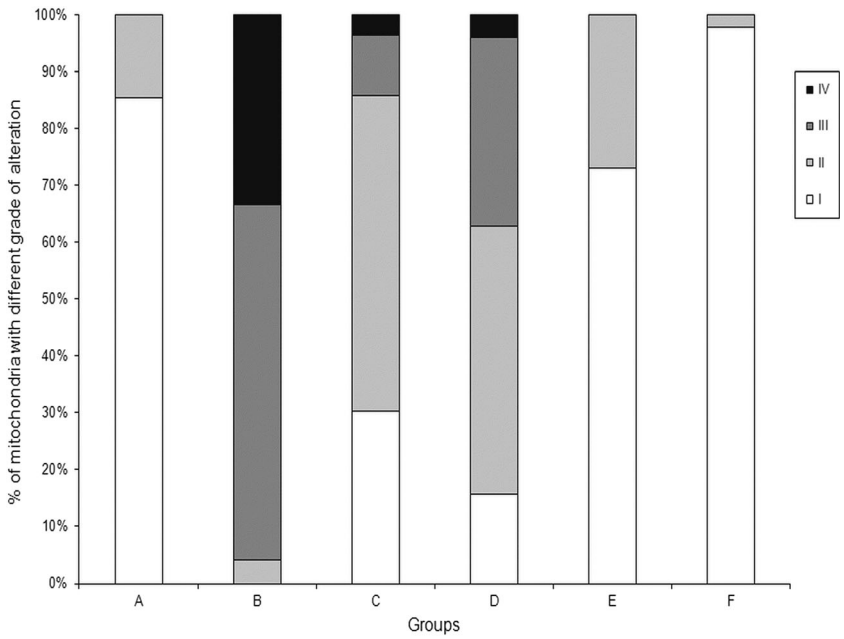

Fig. 3 Effect of LLLT on mitochondrial morphology represented in the percentage of mitochondria with each grade of alteration (I, II, III, IV) by study group. A vs B, C, and D = $p < 0.001$; B vs. C and D = $p < 0.001$

adrenaline, to rule out the possible effect of stress on the various organs and systems [20]. The results showed that its release by the suprarenal medulla was inhibited in the second group, as its concentration was significantly lower compared to the first group, possibly due to a down-regulation type system, probably from a decrease in the number of receptors available [24].

Rygiel et al. propose a role for inflammatory cells in the initiation of mitochondrial DNA damage, which, when accumulated, causes respiratory dysfunction, fiber atrophy, and ultimately muscle fiber degeneration [6].

Comparing the morphological results with those of a previous study [15], on which we relied for this research, the different stains used, trichrome Gomori in the previous work and H&E in this one, reveal the same degrees of inflammation. The percentage of inflammatory infiltrate quantified in that work in the myopathy group was 80.67%, which corresponds to the intense or severe inflammation found in group B of this study, as shown in Fig. 1c and decreases significantly in the groups with myopathy and treated with He-Ne (2.12%) and Ga-As (12.85%) laser, corresponding to mild to moderate inflammation as observed in groups C and D, recovering normal myofibrillar architecture as shown in Fig. 1b.

Neither of the lasers alone (groups E and F) caused disruption of normal tissue at morphological level, nor differed from controls, nor were histological changes observed (Fig. 1a), demonstrating that LLLT is a low-risk treatment and does not affect cell viability at therapeutic doses. Parra Lara et al. claims that the Ga-As laser has a hypertrophic effect on rat skeletal muscle, since, by morphometry, it detects an increase in muscle fiber area post irradiation, without change in the density of the volume of connective tissue [25], results that could not be confirmed through those of this study.

The choice of LLLT parameters was based on the revised bibliography. In tissue, there is an "optical window" (600 to 1100 nm) in which the effective penetration of light is maximized. LLLT is employed to treat diverse injuries or pathologies in humans or animals between 1 and 20 J/cm^2 and irradiance can vary depending on the light source and spot size [26].

Other authors, using treatment doses and days similar to our study (between 5 and 10 J cm^{-2}—between seven and ten treatment days) found positive histomorphological results in lesioned muscle. Iyomasa et al. showed that LLLT increases mitochondrial activity in lesioned muscular fibers, activates fibroblasts and macrophages, and stimulates angiogenesis [16]. Baptista et al. show that the collagen IV content is modulated in regenerating skeletal muscle under LLLT in rats after cryolesion [27]. Gigo-Benato et al. show that LLLT is able to accelerate neuromuscular recovery after nerve crush injury in rats, thus suggesting its effectiveness [13]. However, further studies are necessary to examine the effect of LLLT in a variety of conditions and times in different muscles. Karu T. explains why LLLT has different biological effects on different cells and why the biological effects are sometimes nonexistent. The cells whose overall redox potential is shifted to a more reduced state (e.g., certain pathological conditions) are more sensitive to irradiation. In contrast, cellular response is weak or absent when the redox potential of the irradiated cell is optimal or near optimal [28].

LLLT can activate myoblast proliferation and increase the expression of cell cycle-related proteins. This suggests that stimulating quiescent myoblasts to enter the proliferative stage may be an important cellular mechanism involved in the healing of skeletal muscle [29].

Analysis at mitochondrial level of skeletal muscle in the group with myopathy (B) showed disorganization of the mitochondrial cristae with electrodense granules, possibly due to the entry of the Ca^{++} ion, which generates deposits of insoluble salts and takes part in the modulation of mitochondrial nitric oxide synthase. This group shows a prevalence of grades III (Fig. 3c) and IV (Fig. 3d) of alteration, with cristae or remains of cristae and a condensation of the matrix. Associated with these alterations, an increase of the mitochondrial area is induced, corresponding to an increase in water in the matrix of this organelle, as a consequence of disruption of the integrity of the cell membrane directly altering its permeability. This leads to possible repercussions in the functionality of the mitochondrial respiratory chain, since this takes place in its inner membrane, where the largest amount of reactive oxygen species are generated, high quantities of which would provoke damage in this organelle from oxidative stress [30, 31].

In groups of animals which underwent muscle injury and subsequent treatment with each of the lasers separately (groups C and D), there was a marked decrease in mitochondrial alteration, as a predominance of mitochondrial alteration grade II was observed in both groups; ie, under the influence of laser, a reorganization occurs in the cristae, repair of muscle fibers, and recovery of their structure. These ultrastructural changes associated with structural changes could be due to stimulation of the microcirculation, increasing the speed of the blood stream, thus increasing the accumulation of exudate of O_2 of cellular defense elements in the affected tissue and of phagocytosis. In addition, other cellular phenomena have been described, including increased ATP production, protein synthesis, and activation of cell multiplication, which favor the speed of reparative phenomena [32, 33]. One possible explanation for the susceptibility of injured skeletal muscle to treatment with LLLT, demonstrated by the reconstitution of muscle fibers, may be related to its mitochondrial content and the presence of satellite cells. These cells, stimulated by LLLT [34], are located above the muscle fiber but beneath the sarcolemma, and are involved in skeletal muscle regeneration and participate in its growth, providing nuclei and cytoplasmic mass to the muscle fiber, which argues that LLLT promotes

recovery of muscle atrophy in association with satellite cell proliferation and angiogenesis [35, 36].

The principles of LLLT are based on photoreceptors of the mitochondrial respiratory chain, which change the membrane potential. Its effect is attributed to the formation of small amounts of ROS and antioxidants changing the cellular redox state, reducing oxidative stress [37] and leading to biological responses responsible for its therapeutic effects [38, 39], with the added benefit of having no adverse effects. LLLT (660 and 905 nm) can increase cytochrome c oxidase activity in intact skeletal muscle [19], decrease morphological changes, skeletal muscle damage and inflammation in mice (904 nm) [40], as well as reduce IL-1β, IL-6, and TNF-α levels in the acute inflammatory phase after muscle trauma [41]. This contributes to our understanding of how LLLT can protect skeletal muscles against tissue damage.

In a previous work [15], LLLT caused significant changes in inflammatory biomarkers and oxidative stress: decreased levels of fibrinogen, L-citrulline, and superoxide dismutase in rats with the same experimental model of myopathies, which helps to reinforce the anti-inflammatory effect of this treatment.

Hayworth et al. show that LLLT may enhance the oxidative energy metabolic capacity of different types of muscle fibers, and that LLLT may be used to enhance the aerobic potential of skeletal muscle [42]. Skeletal muscles can regenerate following injury, and the response is mediated by a specific type of stem cell, the satellite cell [2]. LLLT irradiation promotes proliferation of muscle satellite cells, angiogenesis, and expression of growth factors. Satellite cells, angiogenesis, and growth factors play important roles in the regeneration of muscle [35]. A recent study showed that to maximize the cell response, it is necessary to increase the power density and decrease the energy density [36].

The ability of LLLT to stimulate stem cells and progenitor cells means that muscle satellite cells may respond well to LLLT and help muscle repair. Furthermore, reducing inflammation and lessening oxidative stress is also beneficial in cases of muscle fatigue and injury [19]. There were no differences between the effects generated by either of the lasers, which behaved similarly in our experimental model of myopathy.

Other authors demonstrated that optimal doses are partly wavelength (1–3 J/660 nm and 1, 3, and 10 J/905 nm), specific, and, consequently, must be differentiated to obtain optimal effects on skeletal muscle fatigue and tissue preservation [43]. De Almeida et al. demonstrated that LLLT at all doses improved morphological aspects of muscle tissue, and decreased TNF-α, showing better results than injury groups treated with diclofenac [44]. LLLT (904 nm) pre-exercise decreased COX-2 mRNA expression in skeletal muscle, enhanced skeletal muscle performance, and decreased post-exercise skeletal muscle damage and inflammation [45].

Today, LLLT provides an easy, noninvasive, safe, nonionizing method to directly treat the injury site, the source of pain and inflammation, and a variety of diseases and pathologies. Millions of people worldwide have received help in treatment of the musculoskeletal system, as well as pain relief. LLLT is one important modality in rehabilitation. Basic research in LLLT will guide the application of LLLT in clinical practice.

In summary, our results indicate that morphological alterations of muscle and mitochondria in animals with experimental myopathy showed significant regression when they were treated with LLLT, showing its anti-inflammatory effect, repairing morphological structure and ultrastructure. No histomorphological differences were observed in outcomes between the groups treated with helium-neon and gallium arsenide LLLT equipment with different emission wavelengths and modes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Ethical approval Experiments were conducted following the guidelines of our institutional IACUC (FCM-UNC), Protocol N° 27/15, which are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Informed consent Not necessary for laboratory animals.

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