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ORIGINAL ARTICLE

Pre-exercise low-level laser therapy improves performance and levels of oxidative stress markers in mdx mice subjected to muscle fatigue by high-intensity exercise

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Abstract This study was designed to determine if the levels of oxidative stress markers are influenced by low-level laser therapy (LLLT) in *mdx* mice subjected to high-intensity exercise training on an electric treadmill. We used 21 C57BL/10ScSn-Dmdmdx/J mice and 7 C57BL/10ScSn mice, all aged 4 weeks. The mice were divided into four groups: a positive control group of normal, wild-type mice (WT); a negative control group of untreated *mdx* mice; a group of *mdx* mice that underwent forced high-intensity exercise on a treadmill (*mdx* fatigue); and another group of *mdx* mice with the same characteristics that were treated with LLLT at a single point on the gastrocnemius muscle of the hind paw and underwent forced high-intensity exercise on a treadmill. The *mdx* mice treated with LLLT showed significantly lower levels of creatine kinase (CK) and oxidative stress than *mdx* mice that underwent forced high-intensity exercise on a treadmill. The activities of the antioxidant enzyme superoxide dismutase (SOD) were higher in control *mdx* mice than in WT mice. LLLT also significantly reduced the level of this marker. LLLT had a beneficial effect also on the skeletal muscle performance of *mdx* mice. However, the single application of LLLT and the dose parameters used in this study were not able to change the morphology of a dystrophic muscle.

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Introduction

Duchenne muscular dystrophy (DMD) is caused by mutations in the gene encoding dystrophin, a subsarcolemmal protein functioning within the dystrophin-associated glycoprotein complex [1–3]. The dystrophin-associated glycoprotein complex is a macromolecular structure of membrane-associated proteins that includes dystrophin and the sarcoglycan proteins (α-, β-, δ-, and γ-sarcoglycan), which maintain fiber integrity and protect against contraction-induced muscle damage [4].

The absence of dystrophin in the skeletal muscle makes the muscle more susceptible to free radical-induced damage than normal muscular injuries. In addition, there is a negative relation between dystrophin expression and susceptibility to oxidative damage, which causes lower dystrophin content and greater muscle protein oxidation [5].

The physiologic symptoms of DMD become apparent within the first few years of life, and muscle strength quickly deteriorates by puberty, usually resulting in early death. The common diagnostic signs of DMD include increased concentrations of blood creatine kinase (CK), increased rates of myofiber regeneration, centrally located myofiber nuclei, mitochondrial swelling, and ultimately myofiber necrosis [6]. Reactive oxygen species (ROS) and oxidative stress may cause cellular damage by direct and irreversible damage to macromolecules such as proteins, membrane lipids, and DNA. Another major consequence of cellular ROS is the reversible modification of thiol side chains of proteins that may affect many aspects of molecular function [7]. Dystrophin-

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deficient myotubes are highly susceptible to cell damage, particularly due to the loss of membrane integrity when exposed to ROS, which causes muscle fatigue and other comorbidities in persons with muscular dystrophy [5]. Acute fatigue can result from short-term excessive workload in myopathic patients after an eccentric exercise. Often, rupture of the sarcolemma and loss of sarcoplasmic enzymes, e.g., CK [8], occur. An imbalance between ROS production by oxidation and ROS reduction by antioxidant systems induces intracellular oxidation, leading to the initiation and progression of age-related stress diseases, including diabetes mellitus, hypertension, atherosclerosis, osteoporosis, and neurodegenerative diseases [9].

The mechanisms by which ROS play a role in the development of fatigue are not fully understood; however, it is known that oxidative stress leads to a reduction in muscle contractile function resulting in muscle fatigue [10]. Several studies have demonstrated that low-level laser therapy (LLLT) has analgesic effects [11–13], modulates the levels of inflammatory mediators, and has anti-inflammatory effects [14–16]. Furthermore, recent animal studies [17–19] and human studies [10, 20, 21] have hinted at the possibility of LLLT in lowering the levels of CK, other muscle damage markers, and oxidative stress after exercise. Therefore, this study was designed to determine whether the levels of oxidative stress markers could be influenced by LLLT in *mdx* mice subjected to high-intensity exercise training on an electric treadmill. Therefore, we investigated whether LLLT could produce changes in the levels of oxidative stress markers (protein carbonyl content) and the antioxidant enzyme superoxide dismutase (SOD) in the serum of *mdx* mice, as well as changes in CK levels and in the histology of the soleus and gastrocnemius muscles.

Materials and methods

Ethics statement

All experimental procedures were performed in accordance with the standards of the Brazilian College of Animal Experimentation (COBEA), and the Research Ethics Committee of the Universidade Nove de Julho-UNINOVE approved the research protocol (no. AN 0023–2011).

Animals

The cohort consisted of 28 mice (*Mus musculus*); 7 wild-type mice (C57BL/10ScSn) and 21 C57BL/10ScSn-Dmdmdx/J mice, all about 4 weeks of age were studied [22, 23]. The animals were obtained from the Breeding Center of Laboratory Animals (Cecal), Oswaldo Cruz Foundation (Fiocruz), Manguinhos, Rio de Janeiro and kept in a vivarium (UNINOVE/Unit Vergueiro).

Experimental design

The animals were divided into four experimental groups as follows: (1) one positive control group of normal wild-type mice (WT group), (2) one negative control group of *mdx* mice called "*mdx control*" that was not treated with LLLT and was not submitted to exercise, (3) one group of *mdx* mice called "*mdx* fatigue LLLT group" comprising animals that received laser therapy and were submitted to exercise protocol, and (4) one group of *mdx* mice called "*mdx* fatigue group" comprising animals that do not received laser therapy and were submitted to exercise protocol and were manipulated in the same way as those in the laser group but with the equipment turned off.

Figure 1 shows the experimental time for each group according to lineage. The term refers to basal blood collection performed before initiating any type of animal experimentation. The *mdx* fatigue LLLT group received LLLT for three consecutive days, and the *mdx* fatigue group was manipulated by simulating the treatment but with the power turned off. On the last day of therapy, after the application of LLLT, the animals were subjected to a forced exercise task on a treadmill, and another blood test was performed on the same day. After 24 h, the animals were anesthetized for blood collection and were euthanized for the removal of the gastrocnemius and soleus muscles.

Forced exercise protocol

The animals were made to run on a treadmill (Inbrasport Model KT-4000 Cl adapted for mice; Porto Alegre, Brazil). For the effectiveness of the method, the following protocol was carried out: every 2 min, the speed was increased by 0.1 km/h until the mouse was exhausted (which was determined as when the animal was unable to continue running at the predetermined speed and/or fell), and the experiment was repeated when the animal stood still for >10 s thrice in a row [24].

Low-level laser application and parameters employed

We used an aluminum gallium arsenide laser, and the data regarding to the parameters used are summarized in Table 1. The laser was applied before exercise protocol, transcutaneously to a single point, on the foreleg in the posterior region of the gastrocnemius muscle on three consecutive days according to the modified protocol [17].
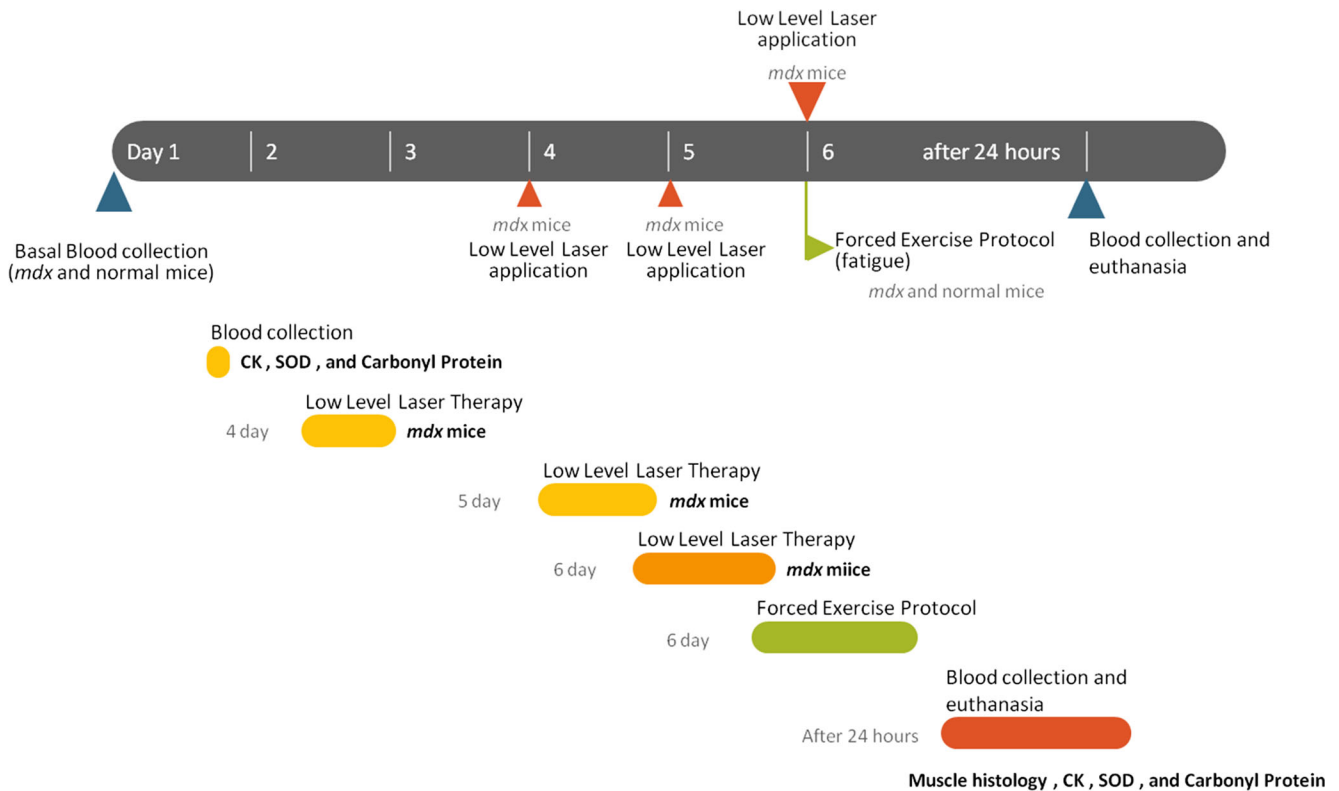
Fig. 1 Flowchart of experimental design and distribution of the groups

Blood collection and euthanasia

Twenty-four hours after laser application, blood was collected in glass tubes through the orbital venous plexus (microhematocrit) after the animals were given one drop of anesthetic eye drops (Allergan, SP, Brazil), as described by Morton et al. [22]. The mice were euthanized by intraperitoneally injecting an overdose (100 mg/kg) of thiopental (Thiopentax; Cristália, Itapira, SP, Brazil) and 10 mg/mL lidocaine (Xylestesin; Cristália, Itapira, SP, Brazil). The blood samples were separated by centrifugation at 3000 rpm for 15 min at 4 °C in a refrigerated centrifuge (5804R; Eppendorf, Hamburg, Germany).

Collection of muscle tissues

The gastrocnemius and soleus muscles of the right hind paw were collected and stored in 10 % buffered formalin for histological processing, and then hematoxylin and eosin staining was performed as a routine method. Slides were photographed, and the morphology of the skeletal striated muscle fibers was analyzed (Eclipse E-200; Nikon, Tokyo, Japan).

Creatine kinase

Quantification of the total CK activity in plasma was performed using the CK-NAC kit. The working reagents were used according to the amount of plasma obtained during blood collection. We used a 96-pore quartz plate and reduced the amount of plasma used to 40 μL, and the amount of the AOS working reagent (250 μL) used ranged from 10 μL to 1 mL, according to the manufacturer's instructions (Laborlab; Guarulhos, SP, Brazil) [17].

Protein carbonyl

The assay for the detection of protein carbonyl groups is one of the techniques used to determine the presence of oxidatively modified proteins. This assay is based on the reaction of oxidized proteins in blood plasma with 2,4-dinitro-phenyl-hydrazine (DNPH) in an acid medium, followed by successive

Table 1 Summary of the laser parameters

Wave length (nm)	Output power (mW)	Power density (W/cm^2)	Spot size (cm^2)	Energy density (J/cm^2)	Energy per point (J)	Irradiation time per point (sec)	Total energy delivered over treatments (J)
808	30	1071	0.028	107	3	100	9

washes with acid and organic solvents and a final incubation with guanidine. The carbonyl absorbance was measured using a spectrophotometer Cary; Varian (Palo Alto, CA, USA) at 360 nm, in a reaction medium containing the following reagents: 6 M guanidine hydrochloride in 2.5 M hydrochloric acid (HCl), pH 2.5; 2,4-DNPH in 2.5 M HCl; 20 % trichloroacetic acid (TCA); 10 % TCA; ethanol-ethyl acetate 1:1 (v/v). The plasma samples were then diluted with 25–75 μL of saline. A protein standard curve with albumin as the standard was created using absorbance values that were obtained at 280 nm using a BIOESPECTRO spectrophotometer.

Superoxide dismutase

The technique used for the detection of SOD was based on the inhibition of the reaction of superoxide by pyrogallol. As this technique cannot be used to determine the concentration of the enzyme or its activity in terms of the amount of substrate consumed per unit time, quantification was done in relative units. One unit of SOD was defined as the amount of enzyme that inhibits the rate of oxidation of the detector (pyrogallol) by 50 %. The oxidation of pyrogallol results in the formation of a colored product, which was detected spectrophotometrically at 420 nm (Biospectro) for 2 min. SOD activity was determined by measuring the rate of formation of oxidized pyrogallol. The reaction medium consisted of 50 mmol/L tris buffer (pH 8.2), 24 mmol/L pyrogallol, and 30 μmol/L catalase. This curve was used as a blank. In addition, a standard curve was constructed using three different concentrations of SOD (0.25 U, 0.5 U, and 1 U), from which the equation of the line used for the calculations was obtained [24].

Statistical analysis

Data were tabulated using Microsoft Excel 2007 and initially assessed for normality using the Shapiro-Wilk test. As normal distribution was observed, ANOVA using the Tukey post hoc test was used for comparisons between the groups. All data are expressed as mean and standard deviation values. The software program GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used; a significant difference was considered when $p < 0.05$.

Results

Treadmill running

The WT control mice ran on the treadmill for an average duration of 40.3±3.41 min, at an average speed of 1.6 mph. The *mdx* control group showed clinical signs of exhaustion after running for 5.6±0.7 min, which is 13.89 % of the running time of the positive control group (WT), presenting a

statistical difference of $p < 0.001$. The group that received LLLT remained on the mat for 9.5±1.4 min, which indicated a 69.64 % improvement in the time spent before reaching exhaustion compared to *mdx* fatigue group; a statistical difference of $p < 0.05$ compared with the *mdx* fatigue group was observed (Fig. 2).

Quantitative assessment of muscle histology

Dystrophic *mdx* animals of both the control group and no fatigue with fatigue and *mdx* group treated with LLLT presented with high levels of myofiber necrosis, characterized by inflammatory cell infiltration and degeneration of hypercontracted myofibers with a fragmented sarcoplasm. The control mice (WT) showed normal myofibers with peripheral nuclei intact, as well as non-fragmented nuclei and sarcolemma (Figs. 3 and 4).

Serum CK

The serum CK level at baseline was statistically different between the normal mice (2.009±500 IU/L) and the *mdx* control group (4.154±1.020 IU/L) ($p < 0.05$). The group that was subjected to forced exercise (*mdx* fatigue) showed a higher increase in serum CK (5.577±1.821 IU/L) than the *mdx* controls, whereas mice that were treated with LLLT showed a reduction in CK levels to a value that was almost equal to the baseline value (4.076±1.575 IU/L). At 24 h after the protocol of treadmill running, mice in the *mdx* fatigue group showed a serum CK level of 5.076±610.5 IU/L, i.e., a slight decrease compared with the baseline level, with a statistical difference when compared with the *mdx* control group ($p < 0.05$). When we analyzed the CK levels of the *mdx* fatigue LLLT group, a marked decrease was observed (2.367±
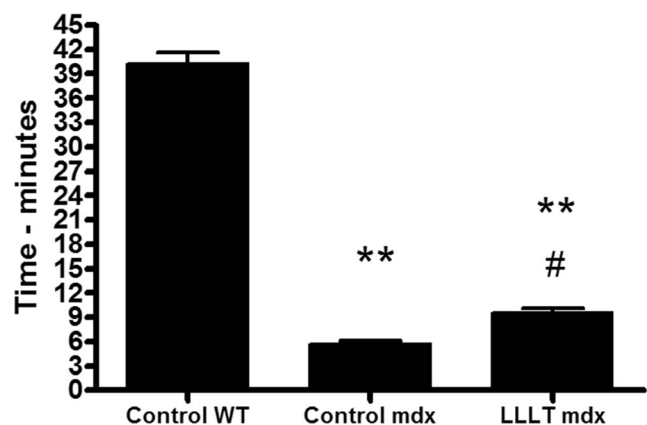
Fig. 2 Comparative analysis of the effect of treadmill running between the control (WT), *mdx* control, *mdx* fatigue, and *mdx* fatigue with low-level laser therapy (LLLT) groups. **$p < 0.001$, Tukey test compared with the control (WT) group. #$p < 0.05$, Tukey test compared with control *mdx*. Results are expressed mean (± standard deviation) values

Fig. 3 Representative gastrocnemius skeletal muscle sections stained with hematoxylin-eosin from wild-type and *mdx* mice. In **a** and **b**, no apparent morphological changes can be observed in control wild-type (WT) mice, note the positioning of nuclei present that within the normal limits (*asterisk*). In **c** and **d**, large necrotic areas can be observed in control *mdx* mice (N), note the positioning of nuclei have centralized that displaced (*arrowhead*). In **e** and **f**, observed in *mdx* mice fatigue at all points in time examined, note the immature fibers central nuclei, cytoplasm rounded shape (*arrowhead*). In **g** and **h**, observed in LLLT fatigue *mdx* mice, note the immature fibers central nuclei, cytoplasm rounded shape (*arrowhead*). Scale bar, 50 μm
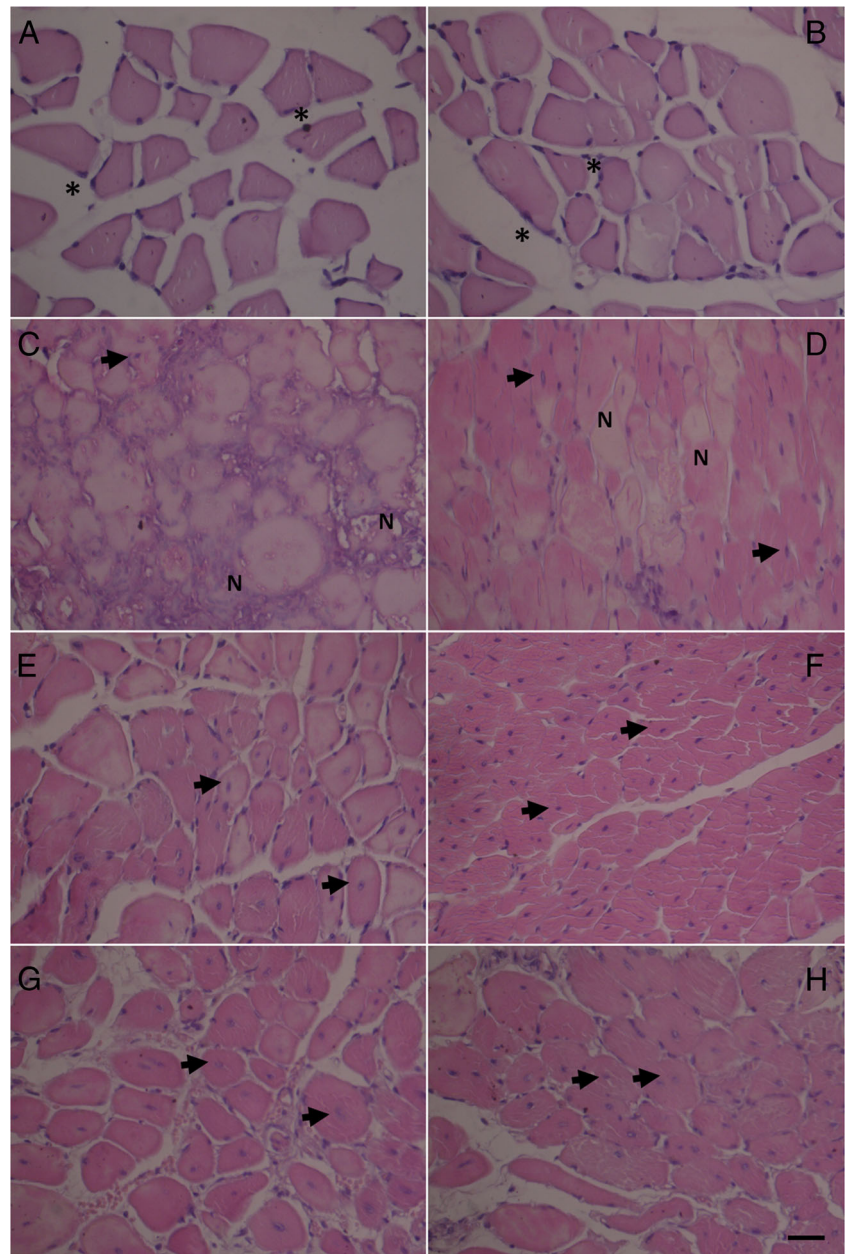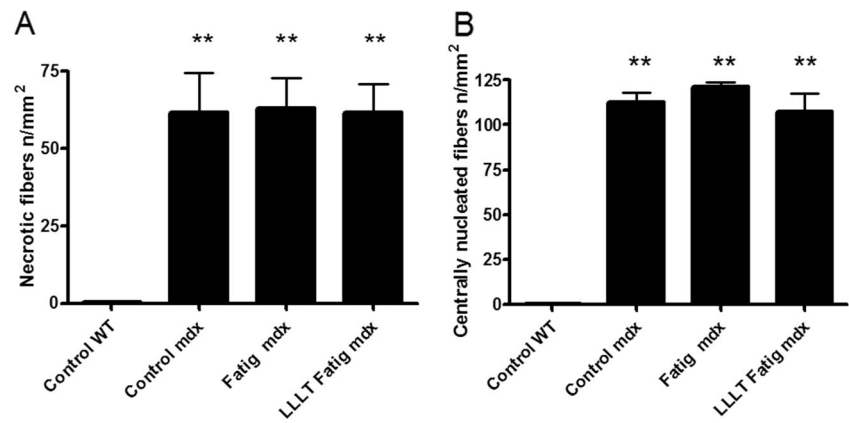

Fig. 4 Effect of low-level laser therapy (LLLT) on muscle damage. **a** Number of necrotic fibers per area. **b** Number of internalized myonuclei per muscle fiber area (no./mm^2). **p$<$0.001, Tukey test compared with the control (WT) group. Results are expressed mean (± standard deviation) values

983.8 IU/L), presenting a statistical difference compared with the *mdx* fatigue group ($p < 0.001$) (Fig. 5).

Protein carbonyl

The protein carbonyl level at baseline was 0.8214 ± 0.3030 nmol/mg protein in normal mice compared with 2.229 ± 0.6009 nmol/mg protein in the *mdx* control group, showing a statistical difference ($p < 0.05$). The group that was subjected to forced exercise (*mdx* fatigue) showed a higher level than the *mdx* controls (3.134 ± 0.6223 nmol/mg protein), whereas animals that were treated with LLLT showed a reduction in carbonyl level (2.310 ± 0.7132 nmol/mg protein) close to the baseline value (Fig. 6a). Twenty-four hours after performing the protocol of treadmill running, the mice in the *mdx* fatigue group showed a carbonyl level of 3229 ± 1214 nmol/mg protein, with a statistical difference when compared with the mice in the *mdx* control group ($p < 0.05$). In the *mdx* fatigue LLLT group, a marked decrease in the level of carbonyl was observed (2.174 ± 0.6640 nmol/mg protein), presenting a statistical difference compared with the *mdx* fatigue group ($p < 0.05$) (Fig. 6b).

Superoxide dismutase

The SOD level at baseline was 2.402 ± 0.2342 U/mg total protein in normal mice and 2.988 ± 0.1763 U/mg total protein in the *mdx* control group, showing a statistical difference ($p < 0.05$). The group that was subjected to forced exercise (*mdx* fatigue) showed an increase in SOD levels (3.540 ± 0.3725 U/mg total protein) compared with the level in the *mdx* control group, whereas animals that were treated with LLLT showed a decrease in SOD level (2.552 ± 0.2422 U/mg total protein) (Fig. 7a). Twenty-four hours after performing the protocol of treadmill running, animals of the *mdx* fatigue group showed an SOD level of 3.528 ± 0.3577 U/mg total protein, with a

statistical difference when compared with the *mdx* control group ($p < 0.05$). In the *mdx* fatigue LLLT group, a sharp decrease in SOD level (2.534 ± 0.4167) was observed, presenting a statistical difference compared with the *mdx* fatigue group ($p < 0.05$) (Fig. 7b).

Discussion

The results of this study provide evidence for the beneficial effects of LLLT against oxidative stress in *mdx* mice subjected to high-intensity exercise training on a treadmill, leading to remarkable recovery in the levels of CK, protein carbonyl, and SOD, and of functional capacity immediately after the exercise and after 24 h during recovery. Nevertheless, LLLT did not produce an improvement in the muscle morphology of the exercised *mdx* mice, which was in contrast to the strong change in biochemical markers of oxidative stress and muscle damage. One interpretation of this result could be that a single application of LLLT is not sufficient to cause structural changes in muscle morphology but could cause changes in oxidative stress in exercised mice. Therefore, this study provides further evidence that LLLT could be used for achieving functional recovery of dystrophic muscle in the same way as in healthy individuals, and thereby decreases the impact of muscle fatigue and oxidative stress in patients with muscle dystrophy [20, 21].

It is well established in the literature that *mdx*, possessing a mutation in the dystrophin gene, is a mouse model for DMD. Without dystrophin, there is a loss of neuronal nitric oxide synthase, resulting in a reduction of nitric oxide signaling capacity and decreased stability of the sarcolemma. Sarcolemmal disruption leads to pathological accumulation of intracellular calcium, which may activate apoptosis and necrosis and increase muscle protein degradation [5].
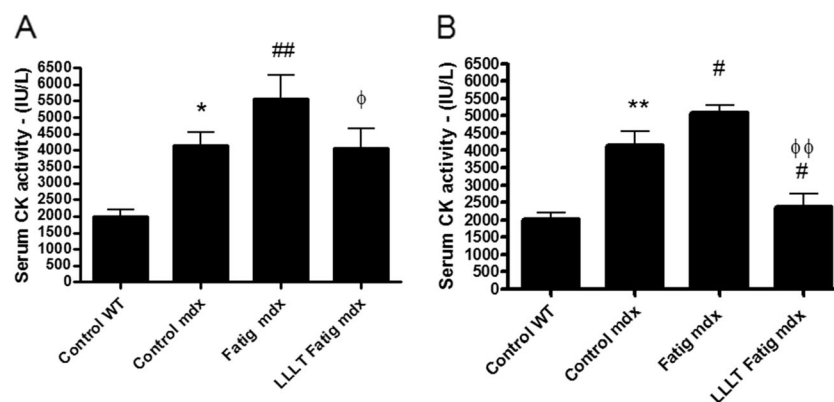

Fig. 5 Comparison of mean±standard deviation values of level of creatine kinase (CK) between groups (treated with LLLT and untreated). **a** CK levels after the completion of high-intensity exercise. *$p < 0.05$, Tukey test compared with the control (WT) group. ##$p < 0.001$, Tukey test compared with the *mdx* control group. $^{\phi}p < 0.05$, Tukey test compared with the *mdx* fatigue group. **b** CK levels 24 h after the completion of high-intensity exercise. **$p < 0.001$, Tukey test compared with the control (WT) group. #$p < 0.05$, Tukey test compared with the *mdx* control group. $^{\phi}p < 0.05$, Tukey test compared with the *mdx* fatigue group. Results are expressed the mean (± standard deviation) values
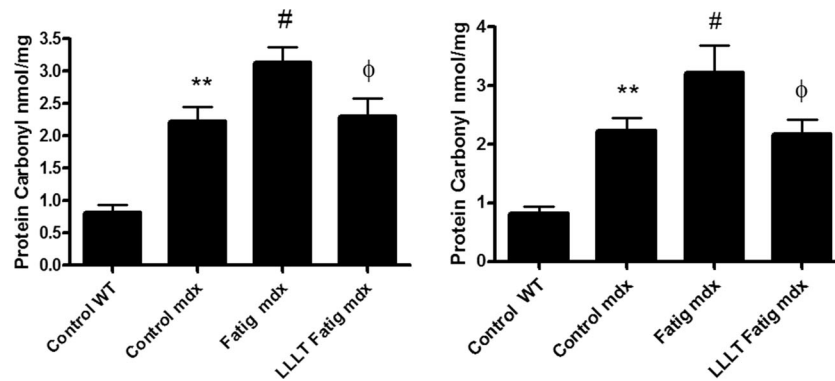
Fig. 6 Comparison of protein carbonyl level between groups (treated with LLLT and untreated). **a** Values of protein carbonyl immediately after the completion of high-intensity exercise. **$p<0.001$, Tukey test compared with the control (WT) group. #$p<0.05$, Tukey test compared with the mdx control group. $p<0.05$, Tukey test compared with the mdx fatigue group. **b** Protein carbonyl values 24 h after the completion of high-intensity exercise. **$p<0.001$, Tukey test compared with the control (WT) group. #$p<0.05$, Tukey test compared with the mdx control group. $p<0.05$, Tukey test compared with the mdx fatigue group. Results are expressed the mean (± standard deviation) values

We showed that mdx mice, compared to WT mice, have higher levels of protein carbonyl and CK when they are at rest. We also demonstrated that high-intensity physical exercise is able to further increase the levels of carbonyl and CK; this type of exercise has the potential to increase oxidative stress in these animals that previously already have high levels of those markers. Compared with the WT animals, we assert that LLLT was able to mitigate the presence of carbonyls, even after the completion of high-intensity exercises. Our results are in agreement with other studies that reported higher levels of lipid, protein, and DNA oxidative damage in mdx mice [5, 7, 25], as well as studies that have shown that LLLT causes changes in levels of oxidative stress markers, muscle damage, and muscle fatigue in healthy tissue [17–20].

In this study, we performed biochemical analysis of SOD. We observed that mdx mice had higher levels of SOD than normal mice (WT), and that immediately after high-intensity exercise, these levels increased further. On the other hand, animals that received LLLT showed values of SOD comparable to that of the normal control group that did not perform treadmill running. These results were in contrast to those in other reports [5, 26, 27].

According to Kaczor et al. [5], oxidative stress is involved in the pathogenesis of the muscle in mdx mice, and this process triggers a cellular response causing a compensatory up-regulation of antioxidant defense enzymes. The findings of lower levels of free radical damage markers after LLLT application in mdx mice together with a significant increase in antioxidant enzyme activities and protein content imply that LLLT lowered ROS production in this study. Recent studies under normal and pathological conditions in animals have presented results indicating improved cell balancing and attenuation of oxidative stress.

Therefore, our findings suggest that the reduction of oxidative stress is related to skeletal muscle. Furthermore, LLLT delays fatigue and protects against exercise-induced damage as well improves the redox state since the LLLT-treated mdx mice in this study were able to run for longer on the treadmill during the high-intensity exercise; that is, muscle fatigue was delayed in these animals. Moreover, another previous study
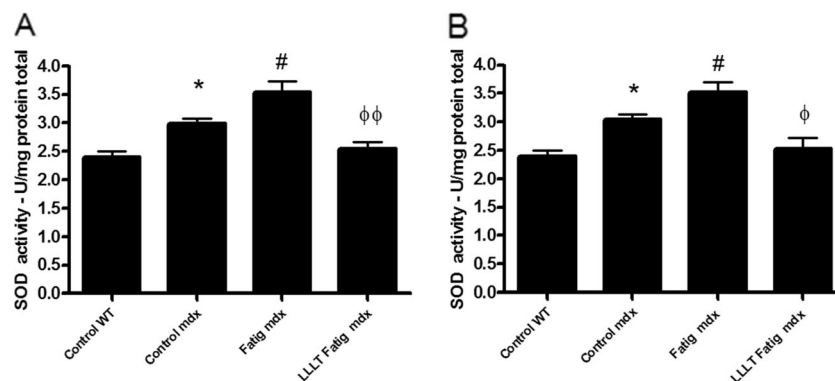

Fig. 7 Comparison of superoxide dismutase (SOD) level between groups (treated with LLLT and untreated). **a** Values of SOD immediately after the completion of high-intensity exercise. *$p<0.05$, Tukey test compared with the control (WT) group. ##$p<0.001$, Tukey test compared with the mdx control group. $p<0.001$, Tukey test compared with the mdx fatigue group. **b** Values of SOD 24 h after completion of the high-intensity exercise. $p<0.05$, Tukey test compared with the control (WT) group. #$p<0.05$, Tukey test compared with the mdx control group. $p<0.001$, Tukey test compared with the mdx fatigue group. Results are expressed mean (± standard deviation) values

[10] also suggests that the effects of LLLT include increasing microcirculation, potentially providing anti-inflammatory activity, and improving mitochondrial function.

Although other studies [28, 29] suggest that LLLT could improve muscle tissue repair and minimize muscle structural damage, the present study did show those observations. However, this is perhaps because only a single application of LLLT may not be sufficient in effecting such mechanisms; also, the time of observing the morphological changes in the dystrophic muscle of the animals, which still had large amounts of necrotic tissue and an altered position of internalized myonuclei, may be too short.

Finally, it is important to mention that very recently, a study showed that irradiation of LLLT on successive days five times per week for 14 weeks decreased morphological changes, skeletal muscle damage, and inflammation in *mdx* mice [30], which indicates that LLLT has the potential to decrease progression of Duchenne muscular dystrophy [30, 31]. Therefore, in this perspective, LLLT rises as a new and promising therapeutic tool in treatment of symptoms of DMD. However, mechanisms trough LLLT acts on this disease need to be better understood, and the findings of the present manuscript can help to elucidate such mechanisms.

In conclusion, oxidative stress markers were significantly higher in the muscle of *mdx* mice than in WT mice, and LLLT was able to reduce these markers even in conditions of muscle fatigue. The activity of the antioxidant enzyme SOD was higher in *mdx* mice than in WT mice. LLLT also reduced the levels of SOD markers significantly. LLLT had a beneficial effect on the skeletal muscle of *mdx* mice and improved the performance of these animals. However, the single application of LLLT and the dose parameters used were not able to cause changes in the morphology of dystrophic muscles.

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