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ORIGINAL ARTICLE

Photobiomodulation therapy protects skeletal muscle and improves muscular function of mdx mice in a dose-dependent manner through modulation of dystrophin

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Abstract

This study aimed to analyze the protective effects of photobiomodulation therapy (PBMT) with combination of low-level laser therapy (LLLT) and light emitting diode therapy (LEDT) on skeletal muscle tissue to delay dystrophy progression in mdx mice (DMD^{mdx}). To this aim, mice were randomly divided into five different experimental groups: wild type (WT), placebo-control (DMD^{mdx}), PBMT with doses of 1 J (DMD^{mdx}), 3 J (DMD^{mdx}), and 10 J (DMD^{mdx}). PBMT was performed employing a cluster probe with 9 diodes (1 x 905nm super-pulsed laser diode; 4 x 875nm infrared LEDs; and 4 x 640nm red LEDs, manufactured by Multi Radiance Medical®, Solon - OH, USA), 3 times a week for 14 weeks. PBMT was applied on a single point (tibialis anterior muscle—bilaterally). We analyzed functional performance, muscle morphology, and gene and protein expression of dystrophin. PBMT with a 10 J dose significantly improved ($p < 0.001$) functional performance compared to all other experimental groups. Muscle morphology was improved by all PBMT doses, with better outcomes with the 3 and 10 J doses. Gene expression of dystrophin was significantly increased with 3 J ($p < 0.01$) and 10 J ($p < 0.01$) doses when compared to placebo-control group. Regarding protein expression of dystrophin, 3 J ($p < 0.001$) and 10 J ($p < 0.05$) doses also significantly showed increase compared to placebo-control group. We conclude that PBMT can mainly preserve muscle morphology and improve muscular function of mdx mice through modulation of gene and protein expression of dystrophin. Furthermore, since PBMT is a non-pharmacological treatment which does not present side effects and is easy to handle, it can be seen as a promising tool for treating Duchenne's muscular dystrophy.

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Introduction

Duchenne muscular dystrophy (DMD) is a genetic disorder linked to the X chromosome, affecting 1 in 5000 male births [1], degenerative and rapidly progressive muscular disease caused by the absence of dystrophin protein [2]. It is a severe neuromuscular disorder, linked to the X chromosome, which belongs to a group of inherited conditions typically characterized by muscle weakening leading to increased disability [3, 4]. DMD which usually affects boys presents no signs at birth; they usually begin to occur between the ages of 3 to 5 [5]. The disease develops very quickly, and patients commonly need a wheelchair by the age of 10 [6, 7]. Muscular dystrophies are a heterogeneous group of genetic disorders that cause

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progressive loss of motor capacity. DMD in particular is associated with mutations in the dystrophin gene, resulting in reduced protein expression. Dystrophin serves as the intracellular link of the dystrophin-glycoprotein complex located in the muscular sarcolemma. It plays an important structural role in muscle fibers, binding the cytoskeleton to the extracellular matrix [8, 9].

An important function of the dystrophin complex is the mechanical stabilization of stresses during eccentric muscle contraction [10]. It is present in the skeletal muscle, more specifically in the sarcolemma. Dystrophin binds to actin F, located in the cytoskeleton, as well as to the dystroglycan complex [11]. The lack of dystrophin in DMD causes intracellular mechanical destabilization that weakens the sarcolemma and progressively promotes cell degeneration [10]. The absence of dystrophin in DMD also causes a redistribution of neuronal nitric oxide synthase (nNOS) from plasma membrane to the cytosol in muscle cells [11]. In addition, recent evidence suggests that dystrophin also plays an important role in the regulation of signaling pathways, particularly those that activate the production of reactive oxygen species (ROS), such as nitric oxide, and those that trigger the entry of calcium [9].

The most prevalent preclinical model for DMD studies is the C57BL/10ScSn-DMD^{mdx}/J mouse, which arose from a spontaneous mutation in a C57BL/10 mouse line. It is generally used in research due to the relative low maintenance costs and low individual variability [12]. In this animal model, muscular degeneration and regeneration are associated with histopathological features, such as centrally nucleated fibers, increased collagen and fibrosis expression, and the presence of phagocytic cells [12]. Inflammatory and immune responses play a key role in the pathogenesis of DMD [13]. As a result, the most prevalent pharmacological treatment for DMD is the administration of a subclass of anti-inflammatory glucocorticoids [14]. However, this treatment is clinically limited because corticosteroids produce serious side effects in long-term treatments and often need to be discontinued [15].

Photobiomodulation therapy (PBMT) is a clinical therapeutic tool in which light in a wavelength range of 600-1000 nm is used to stimulate physiological cellular function. PBMT has been tested in a wide range of disorders, and the studies have shown that it reduces pain, inflammation, and promotes tissue repair [16]. The PBMT acts through the absorption of photons by the natural chromophore cytochrome c oxidase, a terminal enzyme of the mitochondrial respiratory chain [17, 18]. It catalyzes the metabolic oxygen reduction reaction. As oxygen consumption increases, it elevates energy production through oxidative mitochondrial phosphorylation [19]. Besides stimulating an increase in energy production, it reduces the release of ROS and the activity of creatine phosphokinase, thereby increasing the production of antioxidants and heat shock proteins [20, 21].

PBMT can be performed using lasers and/or light-emitting diodes (LEDs) and is possible over a range of wavelengths; studies have demonstrated positive phototherapy results related to fatigue and muscle performance with infrared LEDs, red LEDs, low-level infrared lasers, and low-level red lasers [22, 23]. As different light sources and wavelengths have already been successfully used, we believe that the combination of these wavelengths and light sources may provide even more favorable results through optimized modulation of cytochrome c oxidase [24].

The pioneer study investigating the effects of PBMT on skeletal muscles of DMD^{mdx} mice was performed by Leal-Junior et al. [25], and it was observed that PBMT improved morphological aspects of skeletal muscles, decreased creatine kinase (CK) activity, and decreased gene expression of inflammatory markers.

Other recent studies also suggest that PBMT protects dystrophic muscle tissue. Macedo et al. [2] observed increased proliferation of dystrophic muscle cells, accelerated cell differentiation, and reduced oxidative and inflammatory stress. Silva et al. [26] showed reduced oxidative stress and delayed fatigue when the therapy was applied in a preventive manner. However, these studies did not analyze some important aspects such as functional performance, and only a limited set of parameters were tested in these previous studies [2, 25, 26]. Therefore, there is a clear need for the optimization of PBMT parameters for further use of PBMT in clinical trials involving DMD.

With these aspects in mind, this study aimed to test the effects of different doses of PBMT on muscle morphology, gene and protein expression of dystrophin, and on functional performance of DMD^{mdx} mice.

Methods

Animals

All experimental procedures were performed in accordance with the standards of the Brazilian College of Animal Experimentation (COBEA). All experimental protocols were submitted and approved by the Animal Experimentation Ethics Committee of our institution.

The study was conducted in accordance with policies and procedures of Brazilian laws and the Department of Health and Human Services in the USA.

Experimental groups

Twenty-five animals were randomly divided into five experimental groups with five animals in each group:

– *Wild type—WT (C57BL/10ScSn)*: Untreated

- *Placebo-control group—DMD^{mdx} mice*: The animals were treated with placebo-control PBMT (using a placebo PBMT probe).
- *1 J group—DMD^{mdx} mice*: The animals were treated with PBMT with dose of 1 J.
- *3 J group—DMD^{mdx} mice*: The animals were treated with PBMT with dose of 3 J.
- *10 J group—DMD^{mdx} mice*: The animals were treated with PBMT with dose of 10 J.

All treatments started with animals at 6 weeks of age. PBMT was applied with direct contact at skin on animals' hindlimbs on a single point (tibialis anterior muscle (bilaterally) 3 times per week (Monday, Wednesday, and Friday) for 14 weeks (Fig. 1).

Animals were euthanized at 20th week of age with an overdose of ketamine and xilazin 24 h after the last PBMT treatment. After the removal of skin and connective tissue, the tibialis anterior muscles (bilaterally) were removed and processed for further analysis.

Photobiomodulation therapy parameters

PBMT was used employing a cluster probe with 9 diodes (1 laser diode of 905 nm, 4 LED diodes of 875 nm, and 4 LED diodes of 640 nm—manufactured by Multi Radiance Medical™). The full description of PBMT parameters is summarized in Table 1. The optical power of the device was verified at every 2-week period by a researcher that was not involved in data collection and analysis, for such it was employed a Thorlabs® power meter (Model S322C, Thorlabs®, Newton, NJ, USA).

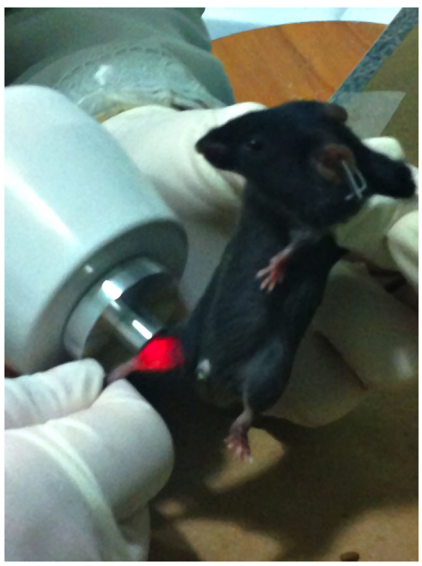


Fig. 1 Treatment procedure

Table 1 Parameters for photobiomodulation therapy (PBMT)

Number of lasers	1 Super-pulsed infrared
Wavelength (nm)	905 (± 1)
Frequency (Hz)	250
Peak power (W)	25
Average mean optical output (mW)	0.625
Power density (mW/cm^2)	1.42
Energy density (J/cm^2)	0.011, 0.033, or 0.11
Dose (J)	0.005, 0.0144, or 0.048
Spot size of laser (cm^2)	0.44
Number of red LEDs	4 Red
Wavelength of red LEDs (nm)	640 (± 10)
Frequency (Hz)	2
Average optical output (mW)—each	15
Power density (mW/cm^2)—each	16.66
Energy density (J/cm^2)—each	0.133, 0.383, or 1.283
Dose (J)—each	0.12, 0.345, or 1.155
Spot size of red LED (cm^2)—each	0.9
Number of infrared LEDs	4 Infrared
Wavelength of infrared LEDs (nm)	875 (± 10)
Frequency (Hz)	16
Average optical output (mW)—each	17.5
Power density (mW/cm^2)—each	19.44
Energy density (J/cm^2)—each	0.155, 0.447, or 1.497
Dose (J)—each	0.14, 0.4025, or 1.3475
Spot size of LED (cm^2)—each	0.9
Magnetic field (mT)	35
Irradiation time per site (s)	8, 23, 77
Total dose per site (J)	1.0, 3.0, or 10.0
Total dose applied in muscular group (J)	1.0, 3.0, or 10.0
Aperture of device (cm^2)	0.197
Device power density (mW/cm^2)	663.07
Device energy density (J/cm^2)	5.07, 15.23, or 50.76
Application mode	Cluster probe held stationary in skin contact with a 90-degree angle and slight pressure

Outcomes

Analyses of functional performance, histology, RT-PCR of dystrophin, and protein quantification of dystrophin through Western blotting were performed by a blinded researcher.

Functional performance Functional performance evaluation protocol consists in the exercise of climbing stairs with the following dimensions (1 × 0.09 m, distance between the steps of 0.5 cm and 80°) using the animal's body own weight (the ladder ends at a platform measuring 0.18 × 0.18 m). The

protocol began with the animal's familiarization, in two sessions with a 24-h interval between them. The aim of the sessions was to teach the mouse to move up the ladder and consisted of three repetitions to the top, with a 60 s interval to rest. If necessary, a clamp stimulus for the animal to start moving was applied. After this familiarization, the assessments were performed before the treatments begin and 24 h after the last treatment, in order to evaluate how many times each mouse was able to perform the climb up the stairs with or without tail clamp stimuli until the animal came to fatigue. Fatigue was identified when the mouse could no longer perform the activity.

Morphological assessment The tibialis muscles of the hind paw were collected and stored in 10% buffered formalin for histological processing; then, hematoxylin and eosin staining was performed in a routine method. Slides were photographed, and the morphology of the skeletal striated muscle fibers was analyzed (Eclipse E-200; Nikon, Tokyo, Japan). Images of all groups were obtained using the 400× magnification. The images were presented with a similar photographic pattern.

RNA isolation and real-time polymerase chain reaction (RT-PCR) analysis Firstly, muscles were thawed, and Trizol was immediately added (Gibco BRL, Life Technologies, Rockville, MD, USA, 1 ml/100 mg tissue). Then, muscles were homogenized for the recovery of total RNA, according to the manufacturer's instruction. DNase I was employed to digest DNA to obtain RNA purification and the integrity of RNA was verified by agarose gel electrophoresis. Total RNA (2 mg) was used for first-strand cDNA synthesis [reverse transcriptase (RT)] using SuperScript II. RNaseOUT was also added to protect the RNA during this process. Three pooled RNA aliquots were routinely sham reverse transcribed (i.e., reverse transcriptase omitted) to ensure the absence of DNA contaminants. Diluted RT samples (1:10) were submitted to real-time PCR amplification using Platinum Sybr QPCR Supermix-UDG and specific oligonucleotides (designed using http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer used was dystrophin anti-sense: AGGTCTAGGAGGCG TTTTCC was used as an internal control b-actin sense: GGCTGTATTCCCCTCCATCG; b-actin anti-sense: CCAGTTGGTAACAATGCCAT GT. The conditions for PCR were as follows: 50 μC–2 min; 95 μC–2 min, followed by 30 cycles of 95 μC–15 s; 60 μC–1 min, followed by 72 μC–15 s. Cycle threshold (Ct) values were recorded for each gene, and the results of genes of interest were normalized to results obtained with the internal control gene. Delta-Delta-Ct (ddCt) values were calculated and results expressed as fold increases. All oligonucleotides and reagents utilized were purchased from Invitrogen Co. (USA).

Western blotting Frozen samples of tibialis anterior muscles were homogenized using ice-cold lysis buffer and proteinase inhibitor cocktail. Lysates corresponding to 30 μg of protein were subjected to 10% SDS-PAGE. Separated proteins were transferred to PVDF membrane (Amersham Biosciences, NJ, USA), and transfer effectiveness was examined with 0.5% Ponceau S. After blocking with 5% non-fat dry milk for 2 h at room temperature, PVDF membranes were probed with Abcam (Cambridge, MA, USA) primary antibodies for rabbit anti-dystrophin (1:5000) in overnight incubation. Membranes were then washed five times with PBS and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit (1:20,000; Zymed, CA, USA). Membranes were again washed five times with blocking buffer and then rinsed twice with PBS. Antibodies binding were detected by chemiluminescence reagents (Amersham Biosciences, NJ, USA), and images were captured using an Amersham Imager 600 system. Quantification of target proteins was normalized for the internal control glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

The Kolmogorov-Smirnov test was used to verify the normal distribution of data. Since data showed normal distribution, non-paired *t* test was used to verify statistical significance between WT and placebo-control groups regarding gene and protein expression of dystrophyn. The one-way ANOVA test followed by the Bonferroni post hoc test was performed to verify statistical significance between multiple groups regarding gene and protein expression of dystrophyn. For the functional analysis, the two-way ANOVA test followed by the Bonferroni post hoc test was performed to verify statistical significance among groups. The level of statistical significance was set at $p < 0.05$. Data analyses were performed using the mean values and standard deviation (SD). However, in graphs, data are presented as mean and standard error of the mean (SEM).

Results

Functional assessment

All the treatment groups, as well as the placebo-control group, had the same mean number of repetitions when subjected to the ascending ladder test before treatments begin. Therefore, the groups were homogeneous prior to treatment, indicating that meaningful comparisons could be made. In the post-treatment evaluation, the 10 J group showed a significantly increased number of repetitions, both when compared to the placebo-control group and to the other treatment groups, as shown in Fig. 2.

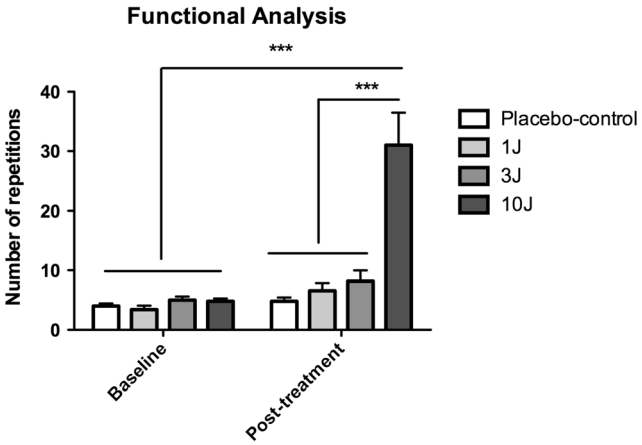


Fig. 2 Functional performance assessment performed in baseline and post-treatment time points. The *** indicates a significant difference ($p < 0.001$) compared with other experimental groups at baseline and at post-treatment as indicated in graph

Morphological analysis

Compared to wild-type mice (WT), the placebo-control group presented a marked amount of connective tissue, as shown in Fig. 3, with a decrease in the number and size of muscle fibers, less positioning of the nuclei at the center of the muscle fibers and fewer nuclear clusters. All of which indicate degeneration of the muscle tissue.

A histological comparison of the WT and placebo-control groups revealed that dystrophin-deficient DMD^{mdx} mice (placebo-control group) showed a reduction in the number of fibers and an expressive reduction in volume, indicating that the myofibers are persistently immature. The nuclei were grouped and centrally located, indicating attempted regeneration to counteract the degenerative process. However, compared to the placebo-control group, the groups treated with PBMT showed signs of improvement, though these changes were dose-dependent (Figs. 4, 5, and 6). Compared to the placebo-control group, 1 J group did not present marked morphological differences (Fig. 4). The 3 J group had practically no nuclei displaced to the center and no nuclear grouping, as shown in Fig. 5. This indicates a delay in the pathological progression of the disease. Compared to the control, 10 J group showed some minimization of the morphopathological aspects of the disease (Fig. 6). However, this decrease was slightly lower than observed in 3 J group. A semi-quantitative analysis of main histological findings in different experimental groups is summarized in Table 2.

Gene expression analysis

Dystrophin gene expression was significantly reduced in the placebo-control group compared to the WT group ($p < 0{,}01$), as presented in Fig. 7a. The 1 J group showed no significant difference when compared to the placebo-control group. The 3 J ($p < 0.01$) and 10 J groups ($p < 0.01$) showed statistically significant increases in dystrophin gene expression compared to placebo-control group as presented in Fig. 7b.

Protein expression analysis

The same pattern was found for dystrophin protein expression analysis, in which the placebo-control group showed

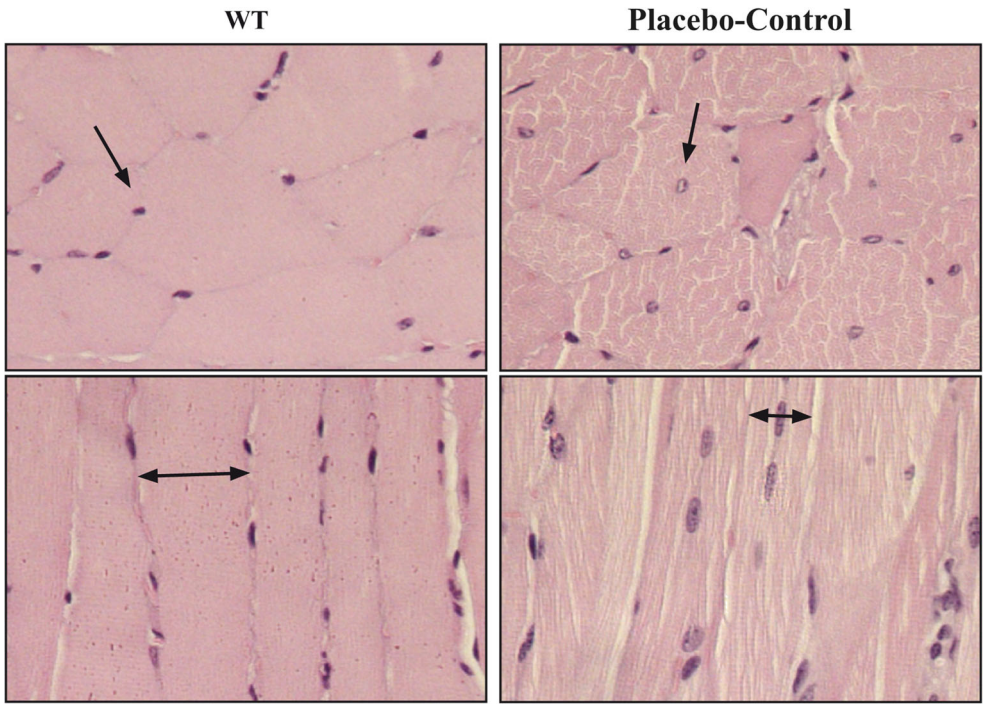


Fig. 3 Photomicrographs of histological muscle sections (longitudinal and transversal sections) of WT, and placebo-control groups (HE, original magnification × 400)

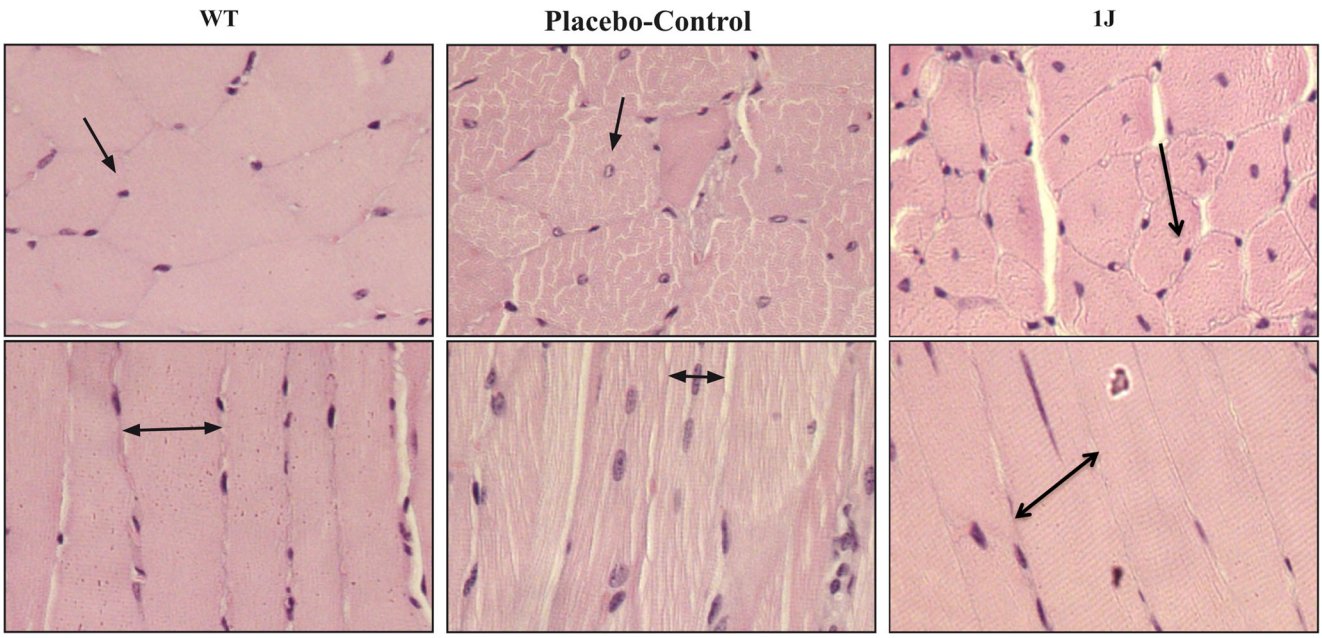


Fig. 4 Photomicrographs of histological muscle sections (longitudinal and transversal sections) of WT, and placebo-control, and 1 J groups (HE, original magnification × 400)

significant difference from the WT group ($p < 0.01$), as presented in Fig. 8a. The comparison between all treated groups showed that the 1 J group had no difference from the placebo-control group; however, the 3 J ($p < 0.001$) and 10 J groups ($p < 0.05$) presented an increase in dystrophin protein expression compared to placebo-control group, as shown in Fig. 8b.

Discussion

This is a pioneer study investigating the effects of PBMT, at different treatment doses, in an experimental model of DMD. This differs from previous research in which only one treatment dose was tested at each time. In addition, functional,

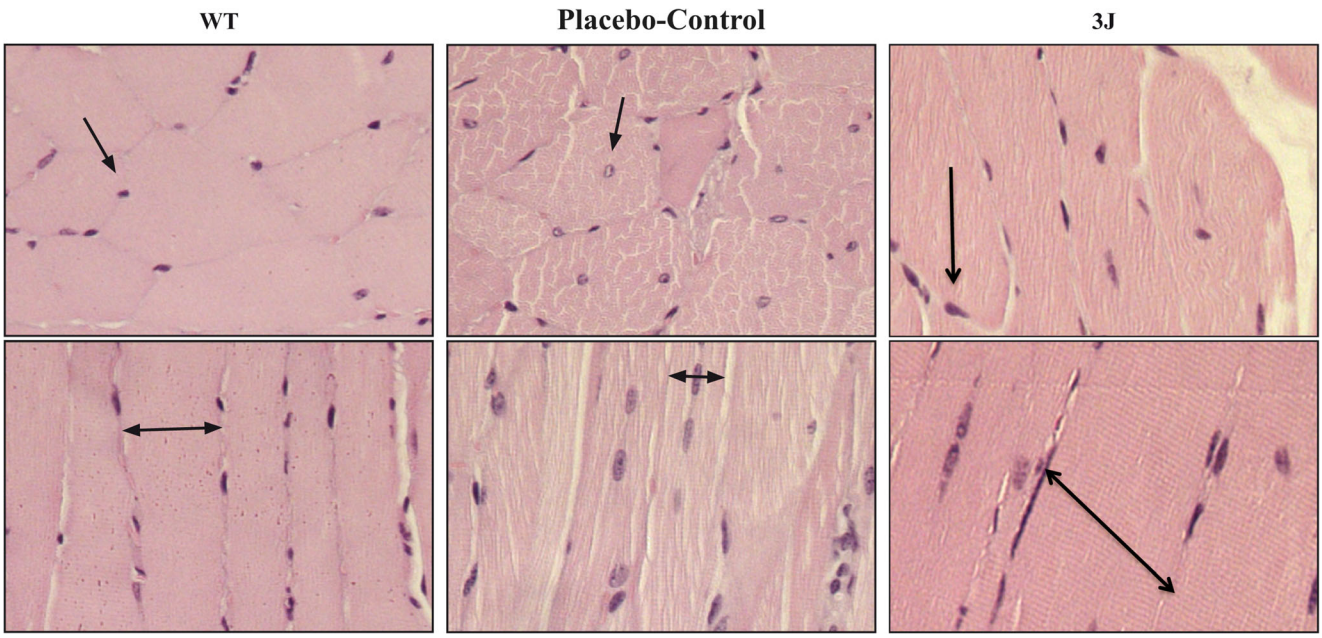


Fig. 5 Photomicrographs of histological muscle sections (longitudinal and transversal sections) of WT, and placebo-control, and 3 J groups (HE, original magnification × 400)

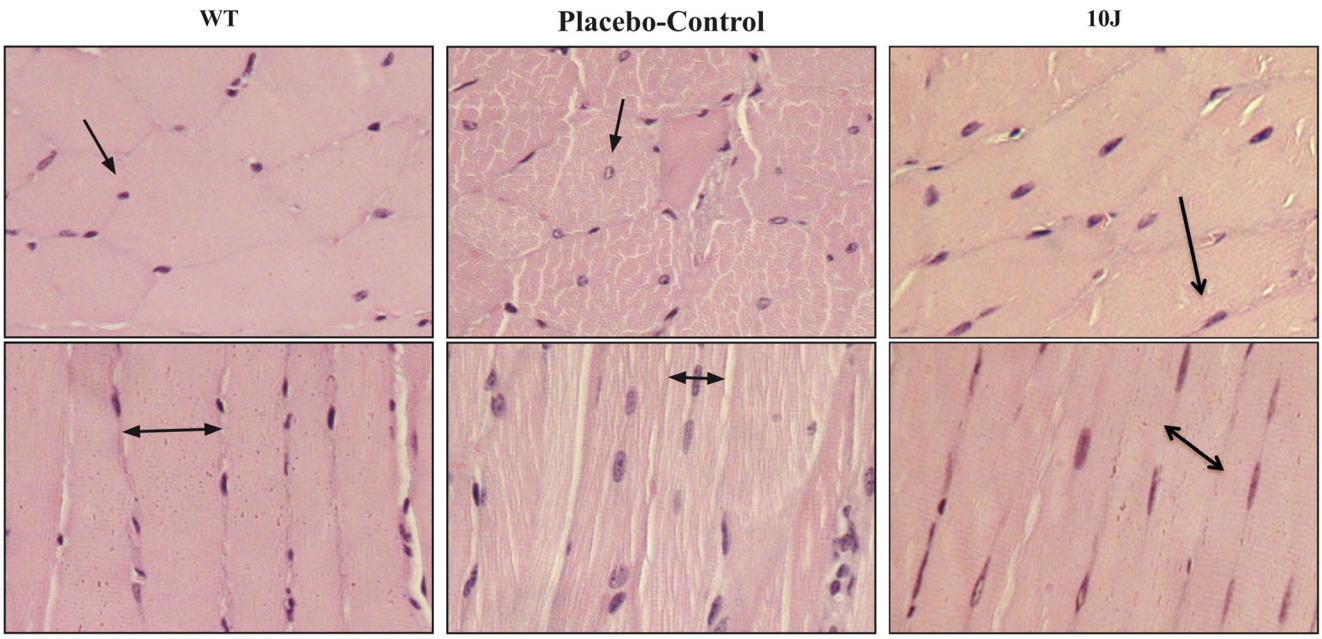


Fig. 6 Photomicrographs of histological muscle sections (longitudinal and transversal sections) of WT, and placebo-control, and 10 J groups (HE, original magnification × 400)

morphological, and biochemical findings were correlated, enabling a deep and critical comparison of the three tested doses.

Previous studies have found that PBMT using low-level laser therapy (LLLT) promoted anti-inflammatory effects and reduced muscle injury in dystrophic cells and tissues [2, 25]. Macedo et al. [2] also found increased proliferation and differentiation in dystrophic muscle cells. Silva et al. [26] demonstrated a reduced level of oxidative stress markers, such as superoxide dismutase (SOD) and carbonylated proteins. This decreased muscle injury resulting from the reduction of creatine kinase in DMD^{mdx} mice, suggesting that PBMT can be used for recovery in dystrophic muscle.

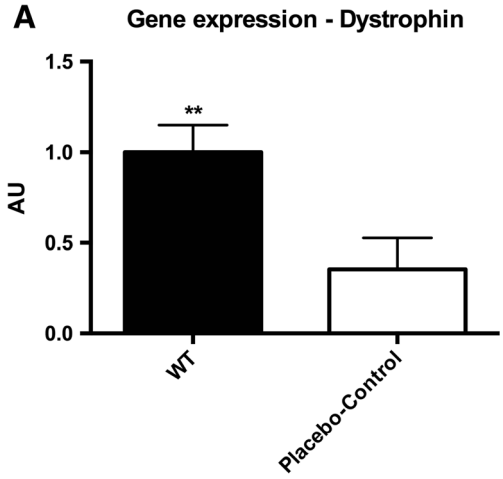
Considering the observed therapeutic potential and possible protective role of PBMT, especially when applied to skeletal muscle, we considered the possibility that this technique could be extended to improve DMD signs and symptoms.

Table 2 Semi-quantitative analysis of main histological findings in different experimental groups

	Change in size and quantity of fibers	Presence of fibrous tissue	Presence of centronuclear fibers	Presence of nuclear groupings (clusters)
WT	–	–	–	–
Placebo-control	+++	+	+++	++
PBMT 1 J	++	–	++	+
PBMT 3 J	+	–	–	–
PBMT 10 J	+	–	+	+

The *DMD*^{mdx} mice used in this study were chosen due to their relative ease of management and the wide variety of research that has already used this strain [27]. It is known that in *DMD*^{mdx} mice, the rates of protein syntesis, degradation, and deposition increase according to the aging, but in all ages, the protein syntesis and degradation are higher in *DMD*^{mdx} model than WT mice [28]. However, it is important to highlight that the animals of placebo-control group in our study were also *DMD*^{mdx} animals and that they were "treated" with a placebo PBMT over the same period (14 weeks) than the other PBMT groups. Therefore, the influence of aging of *DMD*^{mdx} animals on turnover of dystrophin protein was minimized. The characteristics of this model is worth noting, since untreated *DMD*^{mdx} mice are expected to show reduced dystrophin gene expression and, consequently, protein expression, when compared to WT mice. Thus, the histological findings also showed histopathological differences that characterize degenerative or regenerative processes. These aspects were observed herein, thereby ensuring the characteristics of the experimental model used by us.

Dystrophin deficiency in dystrophic muscle tissue results in an imbalance of the dystrophin-glycoprotein system [10], making the muscle fiber more susceptible to the mechanical stress imposed by normal muscle contraction [29]. These factors culminate in tissue degeneration and the loss of structural components, allowing increased calcium influx [29] and, consequently, tissue necrosis [30]. Muscle degeneration and regeneration in DMD^{mdx} are associated with histopathological characteristics, such as centrally nucleated fibers, increased



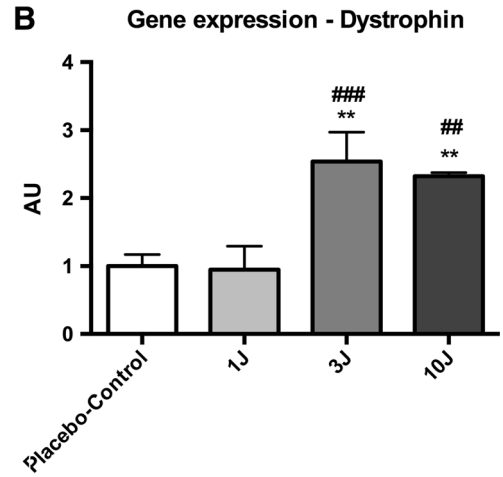
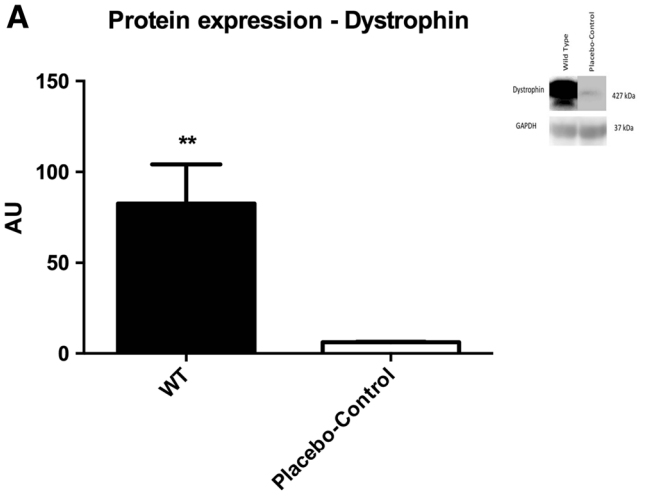


Fig. 7 **a** mRNA gene expression of dystrophin in WT and placebo-control groups (**p < 0.01). **b** mRNA gene expression of dystrophin in placebo-control and PBMT-treated groups. The ** indicates a significant difference compared with placebo-control group (p < 0.01), ## indicates a significant difference compared with 1 J group (p < 0.01), and ### indicates a significant difference compared with 1 J group (p < 0.001)



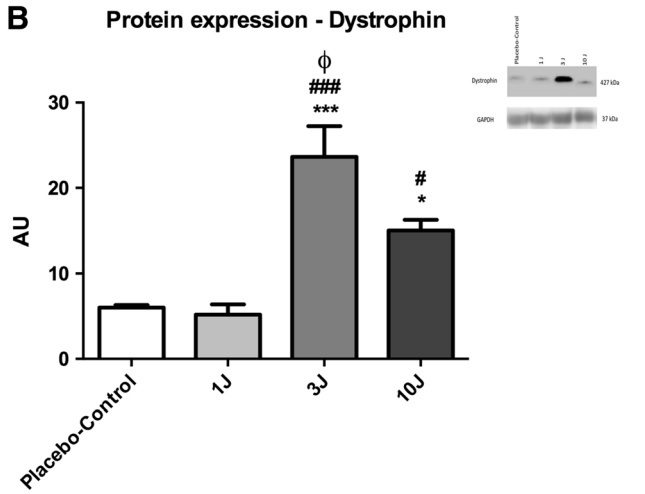


Fig. 8 **a** Protein expression of dystrophin in wild-type and placebo-control groups (**p < 0.01). **b** Protein expression of dystrophin in placebo-control and PBMT-treated groups. The *** indicates a significant difference compared with placebo-control group (p < 0.001), * indicates a significant difference compared with placebo-control group (p < 0.05), ### indicates a significant difference compared with 1 J group (p < 0.001), # indicates a significant difference with 1 J group (p < 0.05), and ϕ indicates a significant difference compared with 10 J group (p < 0.05)

expression of collagen and fibrosis, and variation in muscle fiber size [12]. These characteristics were observed in the placebo-control group, in which most fibers presented centralized nuclei and reduced muscle fiber caliber.

Previous studies suggested that PBMT induces the proliferation and differentiation of satellite cells, which might occur through the activation of their cell cycle promoters [31]. This suggests that the histopathological profile of the muscular tissue in this study corresponded to tissue regeneration that is closely linked to the action of satellite cells [32]. It is important to highlight that groups treated with PBMT with 3 and 10 J doses were morphologically improved when compared to the control group. These improvements were observed in the reduced number of centrally nucleated fibers and the decreased normotrophy of dystrophic muscle fiber. These findings suggest that PBMT may have acted to promote biostimulation of the dystrophic muscle tissue. The morphological improvement reflected in the improved functional performance of the animals as observed in the 10 J group.

In DMD, repeated cycles of muscular regeneration may lead to early loss of the proliferative potential of satellite cells in patients [32–34]. Satellite cells respond to harmful stimuli, such as injury or exercise. However, these stimuli are practically constant in this disorder due to the lack of dystrophin, which has a stabilizing role in the sarcolemma during muscle contraction [35]. The satellite cells have a regenerative capacity, keeping their number practically constant. This study did not assess the proliferative potential of satellite cells. However, in DMD is known to frequently exhaust the capacity for cell proliferation of the organism. Stimulating cell differentiation is believed to minimize this negative effect.

The exhaustion of satellite cells may be due to the interaction between the primary genetic defect and production of free

radicals. Neuronal nitric oxide synthase (nNOS) is a component of the dystrophin complex in skeletal muscle; the absence of dystrophin in both DMD and the DMD^{mdx} experimental model redistributes nNOS from the plasma membrane to the cytosol. This may induce the oxidation of free radicals, which is detrimental to the myofibrils [36–38].

Thus, the chronic imbalance of the cellular redox state is associated with several pathological processes [39]. Among other deleterious effects of this imbalance, the chronic disruption in the function of transcription factors is particularly notable, as it causes a global alteration of gene expression [40].

Kaczor et al. [40] reported that muscle pathogenesis in DMD^{mdx} mice is involved in oxidative stress. Meanwhile, Silva et al. [2] demonstrated that PBMT could reduce oxidative stress in dystrophic muscle tissue by reducing SOD and carbonylated protein levels, allowing a decrease in muscle injury. Other studies have also suggested PBMT as an efficient therapy to reduce oxidative stress and increase antioxidant levels [2, 26, 41]. Therefore, increased dystrophin gene expression, as seen in 3 and 10 J groups, and the consequent increase in dystrophin protein expression may help to balance redox states.

While the improvement in gene and protein expression for the 10 J group was smaller than 3 J group, it has proven effective in redistributing nuclei to the fiber periphery and adjusting the size of dystrophic muscle fibers. These changes, in turn, led to an improvement in the functional capacity of the DMD^{mdx} mice. We believe that this improvement allowed the protein to resume its normal functions, which are extremely important for homeostasis of the skeletal muscular tissue [10, 42].

In this manner, this study demonstrated that PBMT has biomodulatory effects capable of protecting the dystrophic skeletal muscle in DMD^{mdx} mice at morphological and functional levels, through modulation of gene and protein expression of dystrophin. As such, PBMT may contribute in future clinical practices as a therapeutic, non-pharmaceutical, non-invasive intervention that has no side effects reported and contributes positively to the prognosis of patients with DMD. However, other studies and clinical trials are needed before PBMT can be fully implemented in clinical treatment of DMD patients. Since it is a non-invasive, non-pharmacological intervention, PBMT arises as a promising therapy for the treatment of muscular impairments in DMD [43].

Conclusion

We conclude that PBMT had positive effects on dystrophic skeletal muscle and delayed disease progression. This indicates that this therapy may have a protective effect when applied at the onset of DMD.

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Compliance with ethical standards All experimental procedures were performed in accordance with the standards of the Brazilian College of Animal Experimentation (COBEA). All experimental protocols were submitted and approved by the Animal Experimentation Ethics Committee of our institution.

The study was conducted in accordance with policies and procedures of Brazilian laws and the Department of Health and Human Services in the USA.

Conflict of interests Professor Ernesto Cesar Pinto Leal-Junior receives research support from Multi Radiance Medical (Solon, OH, USA), a PBMT device manufacturer. The remaining authors declare that they have no conflict of interests.

Ethical aspects All experimental protocols were submitted and approved by the Animal Experimentation Ethics Committee of the University of Nove de Julho (UNINOVE) (Protocol AN0008.2014).

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