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Low-intensity aerobic training reduces macrophages infiltration and improves morphological characteristics of soleus skeletal muscle from mdx mice

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Abstract: Duchenne Muscular Dystrophy (DMD) is characterized by the absence of the dystrophin protein. The absence of this protein determines recurrent injuries in muscle tissue progressing to necrosis and generalized weakness, leading to the patient's death due to respiratory and/or cardiac failure. There is no cure for DMD. However, some exercise programs could minimize the disease's progression. Low-intensity training has been used as a rehabilitation program for dystrophic muscles, although the effects are still unclear. This study aimed to analyze the effects of low-intensity aerobic training on the general morphological aspects of the skeletal muscle of mdx mice. Eighteen male mice were divided into three groups with six animals each: (mdx sedentary, mdx trained, and wild-type sedentary). The low-intensity training was performed on a treadmill running during the 37 sessions. After the experiments, the animals were euthanized, and the soleus muscle was excised for histological and immunofluorescence analyses. The training (37 sessions) showed an improvement intoning the morphological aspects of soleus mdx mice and a reduction of macrophage infiltration. The low-intensity training can minimize the inflammatory process and reverse morphological alteration in the soleus muscle of the mdx mice.

O treinamento aeróbico de baixa intensidade, reduz a infiltração de macrófagos e melhora as características morfológicas do músculo sóleo de camundongos mdx

Resumo: A Distrofia Muscular de Duchenne (DMD) é caracterizada pela ausência da proteína distrofina. A ausência desta proteína determina lesões recorrentes no tecido muscular que progridem para necrose e fraqueza generalizada, levando à morte do paciente por insuficiência respiratória e/ou cardíaca. Não há cura para a DMD, mas existem programas de exercícios que podem minimizar a progressão da doença. O treino de baixa intensidade tem sido utilizado como programa de reabilitação dos músculos distróficos, mas os seus efeitos ainda não são claros. O objetivo deste estudo foi analisar os efeitos do treino aeróbico de baixa intensidade nos aspectos morfológicos gerais do músculo esquelético de camundongos mdx. Dezoito camundongos machos foram divididos em três grupos com 6 animais em cada grupo: (mdx sedentário; mdx treinado e wild-type sedentário). O treino de baixa intensidade foi praticado em esteira durante 37 sessões. Após o treinamento, os animais foram eutanasiados e o músculo sóleo foi excisado para análises histológicas e de imunofluorescência. O treinamento (37 sessões) mostrou uma melhora nos aspectos morfológicos do sóleo de camundongos mdx e uma redução da infiltração de macrófagos. O treinamento de baixa intensidade pode minimizar o processo inflamatório e reverter a alteração morfológica no músculo sóleo dos camundongos mdx.

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Introduction

Duchenne Muscular Dystrophy (DMD) is a severe muscle degenerative, progressive, and fatal disease that affects one in every 3500 to one in every 5000 live born boys (RYDER *et al*., 2017; WANG *et al*., 2020; KARIYAWASAM *et al*., 2022). The DMD is involved by a mutation in the X chromosome (Xp21.2 region), responsible for dystrophin production. The mutation results in a dystrophin absence, promoting a series of injuries leading to muscle degeneration that can eventually become premature death by cardiac failure. The dystrophin protein is responsible for maintaining fiber stability during muscle contraction. The first group of muscles to be affected are those related to gait and standing posture support, such as the soleus, anterior tibial, psoas, gastrocnemius, and those related to life maintenance like the diaphragm and cardiac muscle (SANTOS *et al*., 2006; DUAN *et al*., 2021).

The injuries activate a cascade of events in the attempt to regenerate those damaged fibers. Among them is the activation of two agents, satellite cells and macrophages. Macrophages are known to participate in the effective repair process of muscle tissue (CHAZAUD *et al*., 2009; DUAN *et al*., 2021). The initial inflammatory process is characterized by an early increase in M1 macrophages and expansion of M2 macrophages, cytokines produced by M2 macrophages, such as IL-4 and IL-10, increase the expression of myogenin, which is necessary for cell differentiation of satellite cells and tissue repair. The acute inflammatory response is associated with an adequate regeneration of skeletal muscle. However, the chronic inflammation observed in dystrophic muscles is associated with compromised satellite cell function and, consequently macrophage imbalance (PERANDINI *et al*., 2018; FUKADA *et al*., 2022). In DMD, macrophage infiltration is maintained, leading to progressive fibrosis, which worsens the disease (KHARRAZ *et al*., 2013; DUAN *et al*., 2021).

Even though DMD has no cure, studies are focusing there on rehabilitation. The pharmacological treatment with glucocorticoids is the most used to increase the survival of patients with DMD. However, the prolonged use of these drugs has several collateral effects (GLOSS *et al*., 2016; COWEN *et al*., 2019; MORENA *et al*., 2019). Therefore, it is necessary to carry out studies that evaluate alternative methods, such as aerobic physical exercise, which is known to improve muscle strength and endurance in healthy individuals, but is still controversial in DMD (GRANGE; CALL, 2007; BOPPART *et al*., 2013). Studies with *mdx* mice, an experimental model for DMD, have shown improvement in muscle morphology and function after low-intensity aerobic training (FRINCHI *et al*., 2021; PEDRAZZANI *et al*., 2021; SIGOLI *et al*., 2022).

Our group of researchers has observed in the psoas muscle of *mdx* mice that low-intensity aerobic exercise improved several morphological characteristics and muscle regeneration (PEDRAZZANI *et al*., 2021; SIGOLI *et al*., 2022). The glycolytic muscles are more susceptible to injuries in DMD, but oxidative muscles are also affected by the disease evolution. Our group investigated the soleus muscle of *mdx* mice, and the study showed that low-intensity eccentric exercise could improve the soleus muscle function, after 21 treatment sessions (PEDRAZZANI *et al*., 2021).

Our current study shows additional information that the low-intensity aerobic exercise applied over a long period may promote an incremental cytoarchitecture rearrangement. These findings showed that low-intensity aerobic exercise could be an adjunct in treating DMD patients, leading them to a better quality of life.

Materials and Methods

This project was approved by the Federal University of São Carlos's Ethics Committee (CEUA n° 4740230518)

Animals

Eighteen male mice were used and divided into three groups: *mdx* C57BL/10-Dmdmdx *(n=6* Sedentary animals – *mdx*Sed; body weight 18.33 ± 1.49 g*), (n=6* Trained animals – *mdx*Tr; body weight 18.33 ± 1.49 g) and *wild type* C57BL/10 *(n= 6* Sedentary animals – wtSed; body weight 19 g ± 0.0 g). The animals were obtained from CEMIB (Multidisciplinary Center for Biological Investigation on

Laboratory Animal Science, UNICAMP, Campinas, Brazil) and maintained in cages in an environment with an inverted light/dark cycle (12/12 hrs) and supplied with food *ad libitum* and water. The mice were six weeks old at the beginning of the experiments because, at this age, it is possible to identify crucial morphological modifications in dystrophic muscles (LOWE *et al*., 2006).

The trained group was submitted to 37 low-intensity training sessions on a flat treadmill (SUPPLEMENTARY MATERIAL) three times/week (Monday, Wednesday, and Friday). Initially, all animals underwent a warm-up period of 2 min at a speed of 7m/min. The training sessions were performed at a speed of 9-10 m/min for 30 min (GAIAD *et al*., 2017). The animals were euthanized after the end of the last session (37th), and the soleus muscle was excised and frozen in liquid nitrogen for histological and immunofluorescence analysis.

Histology

Frozen soleus muscle was sectioned (6 µm of thickness) using a Leica CM 1850 UV Cryostat at -25°C. The histological slides were stained with hematoxylin and eosin to analyze morphological features: nuclear centralization, necrosis, basophilia, inflammatory infiltrate, and others (CORNACHIONE *et al*., 2008; CORNAHCIONE, 2013). The semiquantitative analyses were performed using images captured by a light microscope (KASVI/MOTIC k112l, São José do Pinhais, Brazil) (40x lens).

Immunofluorescence

The frozen section of the soleus muscle was immunostained to quantify the presence of macrophages and dystrophin protein. The slides were blocked with M.O.M. (mouse on mouse, Vector Laboratories, Burlingame, CA, USA) and incubated in primary antibodies for macrophages (CD68, 1:200; Abcam, IgG1, Cambridge, UK), dystrophin (ab15277, 1:400; Abcam, Cambridge, UK) and laminin (1:200; ab11575; Abcam, Cambridge, UK) in 1% of BSA (Bovine Serum Albumin; Sigma Aldrich, San Luis, Missouri, USA) for 45 min at 37°C. Following the washing in PBS and incubation in secondary antibodies Alexa Fluor® 488-green (1:200; 115-545-205, Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor® 647-red (1:200; ab-2535812, Invitrogen, Waltham, MA, USA). The slides were mounted with FluoroQuest Mounting Medium with 4'6-diamidino-2- phenylindole (DAPI, nuclei staining; cat#20004; AAT Bioquest; Sunnyvale, CA, USA). Images acquired by the ImageXPress XLS System microscope (Molecular Devices, San Jose, CA, USA) (magnifications 10x and 20x). The macrophages were analyzed using the Image J software (version 1.52a, Bethesda, MA, U.S.A.) in the whole muscle section. The macrophages were counted only when double-stained with the nucleus.

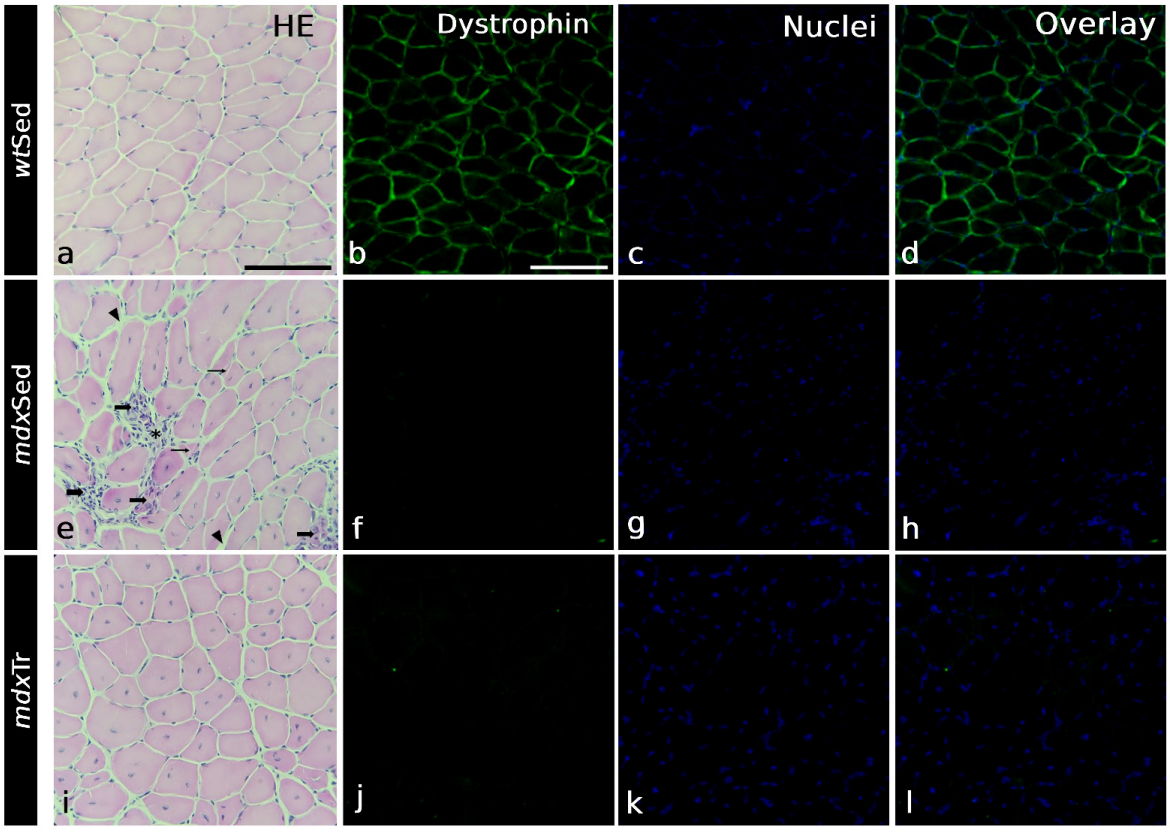
Statistics

All quantitative comparisons between groups were performed through analysis of variance using the Student's t-tests (non-parametric test). The significance level considered was 5% (α=5%) with a confidence interval of 95% (CI=95%); ($p < 0.05$). The analyzes were performed using the Graph Pad Prism 8.0.2.263 statistical program.

Results and Discussion

The Immunofluorescence technique confirmed the absence of dystrophin protein in the sarcolemma of mdx mice (Figure 1f, 1h, 1j, 1l), while it was present in the sarcolemma of soleus fibers in wild-type mice (green color - Fig. 1b and 1d). The semi-quantitative analysis from the sections stained by Hematoxylin and Eosin (HE) showed that the disease led to a substantial morphological alteration in soleus fibers such as centralized nuclei, inflammatory infiltrate, increase in connective tissue, variation in fiber size, necrosis, basophilic cells and splitting (Figure. 1e, Table I). The animals from the mdxTr group presented moderate morphological alteration when compared with *mdx*Sed (Figure 1i). There was a reduction of variation in fiber size, basophilic cells, necrosis, and splitting (Table I). The wtSed group showed a normality pattern in skeletal muscle morphology (Figure 1a).

Figure 1 - Photomicrographs of soleus muscle stained with hematoxylin and eosin (HE) (a, e, i) and immunolabelled by dystrophin antibody (dystrophin protein shown in green and nucleus is shown in blue) (b, c, d, f, g, h, j, k, l).



Source: Done by the author.

It is possible to see dystrophin protein only in wild-type animals. **a** - wtSED sample showed a healthy muscle morphology; **e –** *mdx*Sed presented substantial morphological alterations, such as nucleus centralized, necrosis (thick arrow), splitting (thin arrow), infiltrate inflammatory (*), increase of connective tissue (arrowhead). **i –** *mdx*Tr showed an improvement in the morphology of tissue after 37 sessions of low-intensity aerobic training. Groups: wtSed, wild-type sedentary; *mdx*Sed, *mdx* sedentary control; *mdx*TR, *mdx* trained during 37 sessions. (scale bar: 100 µm)

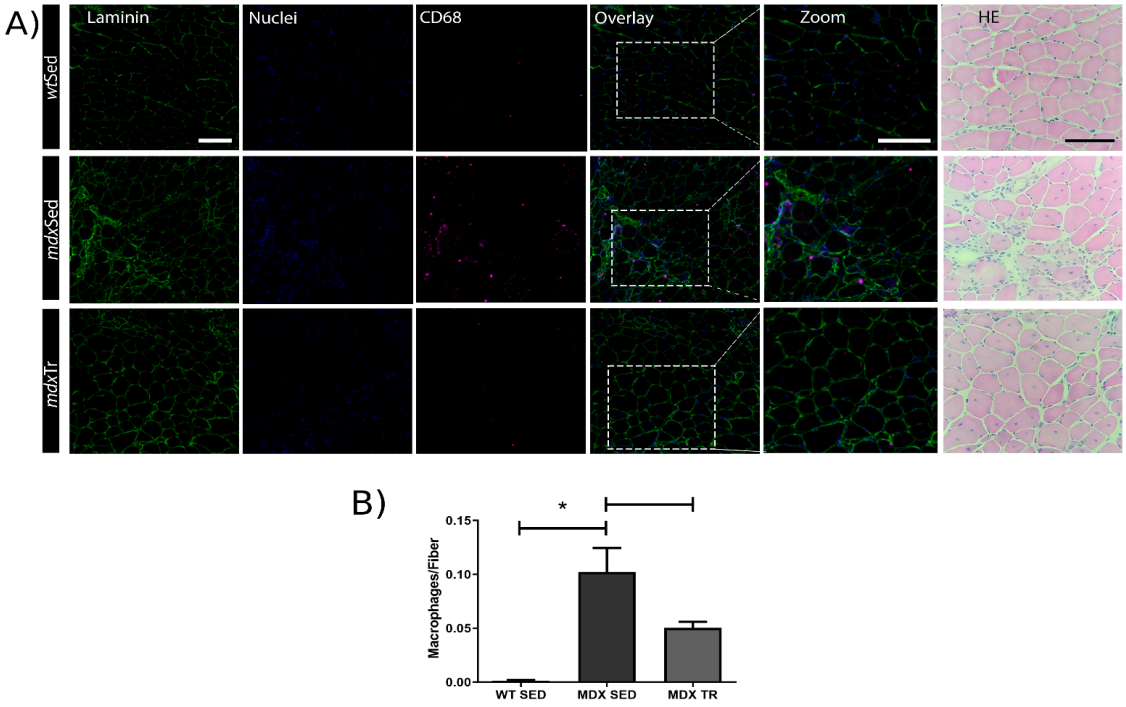
Table 1 - Semiquantitative analysis of morphological alterations in soleus muscle identified by hematoxylin and eosin staining.

Morphological alteration	wtSed	mdxSed	mdxTr
Centralized nuclei	0%	100%	100%
Inflammatory infiltrate	66,6%*	100%	100%
Connective tissue	16%*	100%	100%
Variation fibers size	33,3%*	83%	66,6%*
Necrosis	0%	66,6%*	33,3%*
Basophilic cells	0%	33,3%*	16%*
Splitting	0%	66,6	50%*

Groups: wtSed, wild-type sedentary; *mdx*Sed, *mdx* sedentary control; *mdx*TR, *mdx* trained during 37 sessions. % Refers to the number of rats that presented the anomaly in the group. *<5% of the cells.

Through the immunostaining quantification analysis is evident that the presence of macrophages in *mdx* muscle (*mdx*Sed) is enlarged by the disease showing a significant difference ($p<0.05$) when compared to the sedentary wild type. After 37 sessions of low-intensity exercise (*mdx*Tr) there was noticed a decrease in macrophage number when compared to the sedentary mice ($p<0.05$) (Figure 2A, B).

Figure 2 - Immunostaining of CD68 in skeletal muscle. **A** .



Source: Done by the author.

Photomicrographs of samples processed by CD68 antibodies and HE stain. Laminin (green), nuclei (blue), and CD68/macrophages (magenta) (40 magnification). Laminin, nuclei, CD68, and overlay panels (scale bar = 100µm). Zoomed areas from merged panels were done to visualize better macrophages (thin arrow) (scale bar = 50µm) . HE stained panels show the same area from the merged panel (scale bar = 50µm). **B** – *mdx*Sed group has a higher number of macrophages when compared to the *wt*Sed group (* $p < 0.05$). *mdx*TR group showed an improvement in tissue morphology and a lower number of macrophages compared to the *mdx*Sed group ($p < 0.05$). Groups: *wt*Sed, wild-type sedentary; *mdx*Sed, *mdx* sedentary control; *mdx*TR, *mdx* trained during 37 sessions.

The evolution of DMD promotes injuries in skeletal muscle, exacerbating intense inflammatory processes which determine important morphological changes and interfere with muscle function. The most common morphological alteration are centralized nuclei, inflammatory infiltrate, connective tissue increase, necrosis, basophilic cells, splitting, and variation in fiber size (CAMPBELL, 1995; RYDER *et al.*, 2017; PEDRAZZANI *et al.*, 2021; SIGOLI *et al.*, 2022). Our study showed that low-intensity aerobic training, when applied during rehabilitation (37 sessions) can reduce macrophage infiltration and improve histological characteristics of soleus muscle from *mdx* mice.

The mechanism of inflammation caused by injuries is promoted by defense cells such as macrophages. The presence of macrophages is significant for starting the repair process because the M2 macrophages stimulate the satellite cells' (CS) differentiation in muscle regeneration (TRIPODI *et al.*, 2021; FUKADA *et al.*, 2022). This study showed a significant presence of CD68 marker, a protein present in macrophages membrane, indicating an increase of inflammatory response in

soleus muscle from sedentary animals. It is possible to notice in Figure 2 that the region where the macrophages are located is equivalent to the region indicated as inflammatory infiltrate and necrosis, suggesting that an increase of CD68 (macrophages) may be an indicator of increased inflammation.

The nucleus centralization observed in the dystrophic muscle of mdx mice also indicates the cycle of muscle degeneration and regeneration. The satellite cell is an essential precursor of myogenesis and, when activated, proliferates, and differentiates into myoblasts and fuses at the lesion focus. The repaired cell will present the centralized nucleus until the maturation occurs and the nucleus migrates to the periphery. The chronic injuries observed in dystrophic muscle can impair regeneration and increase the connective tissue. The basophilic is another morphological change that can indicate an increase in inflammation. The lack of dystrophin makes the membrane fragile, breaking it easily. The membrane disruption will facilitate the influx of ion calcium into the cytoplasm, which triggers degenerative reactions that increase the inflammation, contributing to chronic damage and degeneration of dystrophic cells (DECONINCK; DAN, 2007; BURNS *et al*., 2017; PEDRAZZANI *et al*., 2021).

Low-intensity training has been the focus of studies by our research group. This type of therapeutic strategy is a promising noninvasive and nonpharmacological tool to treat DMD, because it has been showing improved muscle morphology and function of dystrophic muscles (KACZOR *et al*., 2007; PINTO *et al*., 2018; FERNANDES *et al*., 2019; FRINCHI *et al*., 2021; PEDRAZZANI *et al*., 2021; SIGOLI *et al*., 2022). Our present results corroborated with previous studies. The morphology analysis showed a reduction of inflammatory infiltrate, basophilic cells, necrosis, and splitting after 37 sessions of low-intensity training concomitant with a reduction in the number of macrophages in the soleus muscle of *mdx* mice. Hyzewicz *et al*. (2017) evaluated the effects of low-intensity exercise in the gastrocnemius muscle of the *mdx* mice for four weeks and observed that training decreases M1 in *mdx* mice.

The reduction of CD68 staining in immunofluorescence indicates a decrease in the inflammatory process, and this finding was also observed in our morphological analyses. The exercise possibly transformed M1 macrophages into M2, and M2 activated the proliferation of satellite cells that participated in the tissue repair process (TIDBALL; WEHLING-HENRICKS, 2014). Sigoli *et al*. (2022) showed an increase of CS in the psoas muscles of mdx mice after low-intensity training. The limitation of the present study was the non-identification of SCs in the soleus muscle.

Conclusion

Low-intensity training shows to reorganize the morphological aspects of the dystrophic soleus muscle of the mdx mice and reduces the number of macrophages. Our results suggest that the application of low-intensity exercise during a long training period can be a supporting therapeutic strategy in treating patients with DMD, minimizing the inflammatory process and delaying the evolution of the disease.

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Author contributions

Conceptualization RAA, ASC; Writing RAA, ES, ASC; Methodology RAA, ES, TOPdA, DLB; Software RAA; Visualization RAA, ES, ASC; Project administration ASC, Funding acquisition ASC; Supervision ASC. All authors read and agreed with the content of this article.

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