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## Twenty-one days of low-intensity eccentric training improve morphological characteristics and function of soleus muscles of *mdx* mice

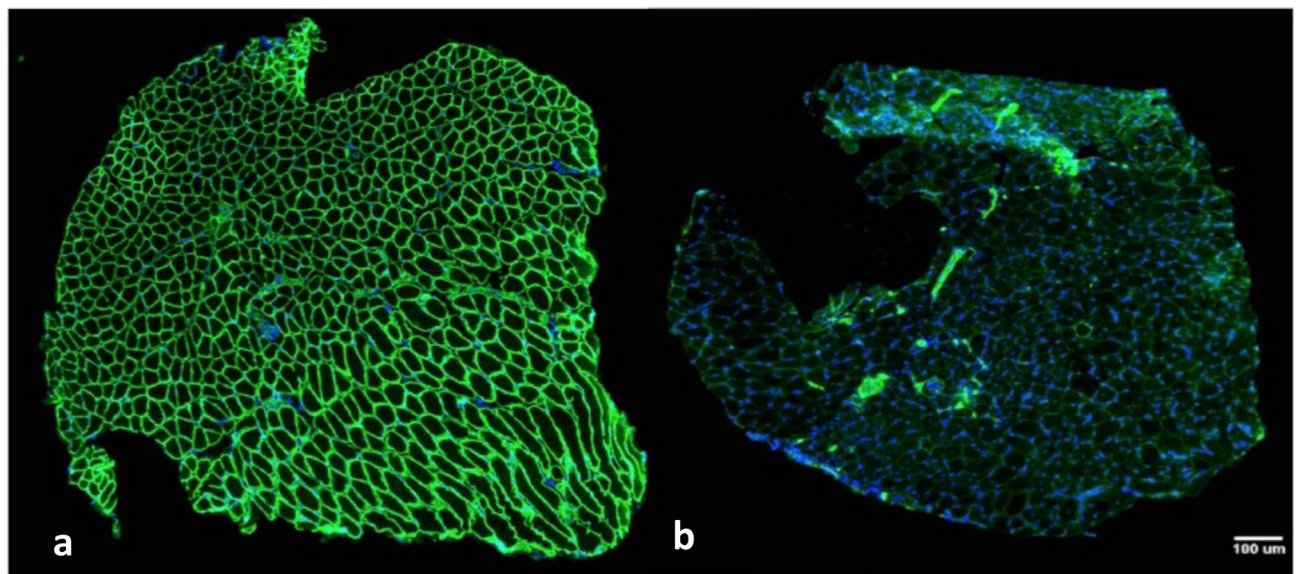
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Duchenne muscular dystrophy (DMD) is caused by the absence of the protein dystrophin, which leads to muscle weakness, progressive degeneration, and eventually death due to respiratory failure. Low-intensity eccentric training (LIET) has been used as a rehabilitation method in skeletal muscles after disuse. Recently, LIET has also been used for rehabilitating dystrophic muscles, but its effects are still unclear. The purpose of this study was to investigate the effects of 21 days of LIET in dystrophic soleus muscle. Thirty-six male *mdx* mice were randomized into six groups (n = 6/each): *mdx* sedentary group; *mdx* training group-3 days; *mdx* training group-21 days; wild-type sedentary group; wild-type training group-3 days and wild-type training group-21 days. After the training sessions, animals were euthanized, and fragments of soleus muscles were removed for immunofluorescence and histological analyses, and measurements of active force and Ca<sup>2+</sup> sensitivity of the contractile apparatus. Muscles of the *mdx* training group-21 days showed an improvement in morphological characteristics and an increase of active force when compared to the sedentary *mdx* group. The results show that LIET can improve the functionality of dystrophic soleus muscle in mice.

Duchenne muscular dystrophy (DMD) is a lethal pediatric muscle disorder caused by a mutation in the region Xp 21.2 (*dystrophin gene*) that leads to an absence of dystrophin protein. DMD affects one in every 5000 boys<sup>1</sup> and causes many deficiencies including an impaired mobility, wheel chair confinement, respiratory and cardiac failure. Dystrophin and the dystrophin-glycoprotein complex have an important role in the sarcolemma membrane integrity during muscle contraction. Lack of dystrophin in muscle fibers leads to mechanical damage, impaired Ca<sup>2+</sup> homeostasis, increased proteolysis and widespread cellular dysfunction<sup>2–4</sup>, resulting in weakness and loss of muscle function.

There is no cure for DMD, but there are rehabilitation exercise programs aimed to slow the progression of the disease<sup>5</sup>. Although the effects of exercise for DMD are still controversial<sup>6,7</sup>, there is an increasing interest in the potential benefits of physical training, since it is cost-effective and non-invasive. Currently, there is no consensus on the best type, frequency and intensity of exercise to be used with DMD<sup>7–9</sup>. Studies conducted with *mdx* mice, an animal model for DMD, have shown that physical training can improve muscle function without aggravating the disease. Low-intensity training (LIT) in treadmill running improves the overall function of *mdx* muscles, reduces oxidative stress and inflammation, while increasing their mitochondrial capacity<sup>10–12</sup> and oxidative capacity<sup>13,14</sup>. There are also significant improvements in strength, resistance to fatigue and muscle morphology after 10–16 weeks of treadmill training<sup>15–17</sup>. The most common types of training used in studies with dystrophic mice include running on a flat treadmill<sup>18</sup>, running on wheels, or swimming for long periods of time (10–16 weeks)<sup>15–17,19</sup>.

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**Figure 1.** Photomicrographs of soleus muscle immunolabelled by dystrophin antibody. **A** *wtSED* sample, with dystrophin protein shown in green and nucleus is shown in blue (DAPI). **B** *mdxSED* sample, with nucleus shown in blue, without the presence of dystrophin protein. Bars: 100  $\mu\text{m}$ . Abbreviations: wild-type Sedentary group (*wtSED*); *mdx* Sedentary group (*mdxSED*).

Eccentric exercise, which can lead to muscle injury<sup>19–22</sup>, has also been shown to partially re-establish some of the cytoarchitectural features in disused muscles, especially when incorporated in a training protocol conducted for long periods of time<sup>23–26</sup>. These studies include muscles that were immobilized and used low-intensity exercise, because it is known that a high-intensity eccentric training can lead to irreversible muscle damage. There are no studies in the literature that address low-intensity eccentric exercise in *dystrophic* muscles for a long period of time. Although dystrophic muscles are more vulnerable to injuries caused by eccentric contractions than healthy muscles<sup>27,28</sup>, many physiological tasks such as walking or sitting involve eccentric contractions.

The purpose of this study was to investigate if low-intensity eccentric training (LIET) applied during a prolonged period of time could improve the contractile function and morphology of the soleus muscle of *mdx* mice. We observed that 21 days of a LIET in treadmill induced positive adaptations in dystrophic muscles, suggesting that it may be a positive strategy to mitigate the effects of DMD in skeletal muscles.

## Results

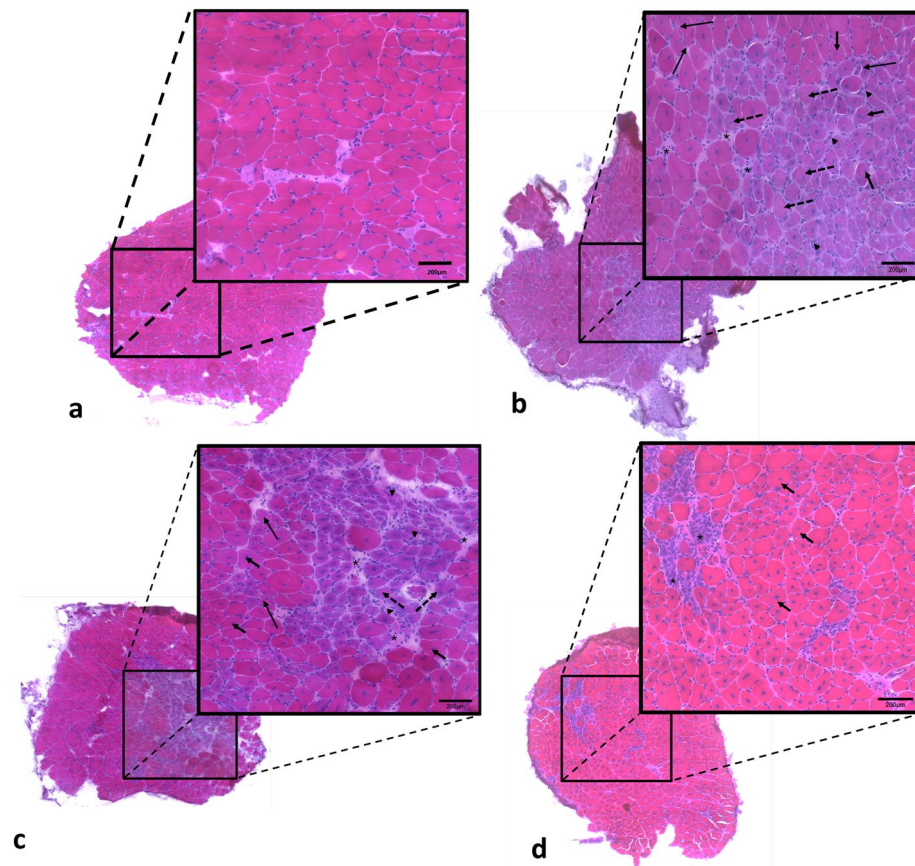
**Morphological alteration.** Immunofluorescence analysis confirmed the absence of dystrophin in *mdx* mice muscle. Figure 1 shows that dystrophin is present in the sarcolemma of soleus fibers in wild-type mice (green color—Fig. 1A), but is absent in *mdx* mice (Fig. 1B).

The morphological examination of sections from muscles stained by Hematoxylin and Eosin (HE) revealed a homogeneous pattern of muscle fibers in wild-type mice. Photomicrography shows polyhedral fiber and nuclei in the periphery of the fiber (Fig. 2A). The dystrophic muscles from the *mdxSED* group (Fig. 2B) showed a large variation in fiber size, nuclear centralization, basophilic fibers and necrosis. Although we have not quantified the amount of connective tissue in these muscles, which may limit a definitive conclusion, qualitative analysis showed an increase in connective tissue in dystrophic muscles, a result on line with previous studies<sup>29</sup>.

After eccentric low-intensity training during 3-days (*mdxTr<sub>3</sub>*) the dystrophic muscles showed a dramatic increase in the degree of morphological abnormalities (Fig. 2C). These alterations include an increase in necrosis and basophilic fibers, variation in fiber size and an increase of connective tissue when compared to animals of *mdxSED* group. Notably, the muscles from dystrophic animals trained during 21 days (*mdxTr<sub>21</sub>*) showed an improvement in their morphological characteristics. This group presented moderate abnormality when compared with *mdxTr<sub>3</sub>*. There is a significant decrease of basophilic fibers, degree of necrosis and connective tissue (Fig. 2D). These muscles of *mdxTr<sub>21</sub>* group still showed a high-centralized nucleus and with a more homogenous trophism of the fibers, leading to a small variation in fiber sizes (Fig. 2D).

**Minimal Feret's diameter of the different fiber types.** The absence of dystrophin protein caused a significant alteration in the trophism in muscle fibers of *mdx* mice. The muscle of *mdxSED* group showed a significant increase in the diameter of unmixed (FTI, FTIIA and FTIID) and hybrids fibers (FTIC and FTIIAD) when compared to animals of the wild-type group (*mdxSED* vs. *wtSED*;  $p < 0.05$ ). The results of the minimal Feret's diameter of the different fiber types are shown in Table 1.

The effects of LIET on the trophism of all conditions investigated in this study are summarized in Table 1. LIET was effective to improve the trophism of muscle fibers in the *mdx* mice. The 3-days of training already caused a reduction in the diameter of the dystrophic muscles, while causing only a reduction in the FTI fibers the

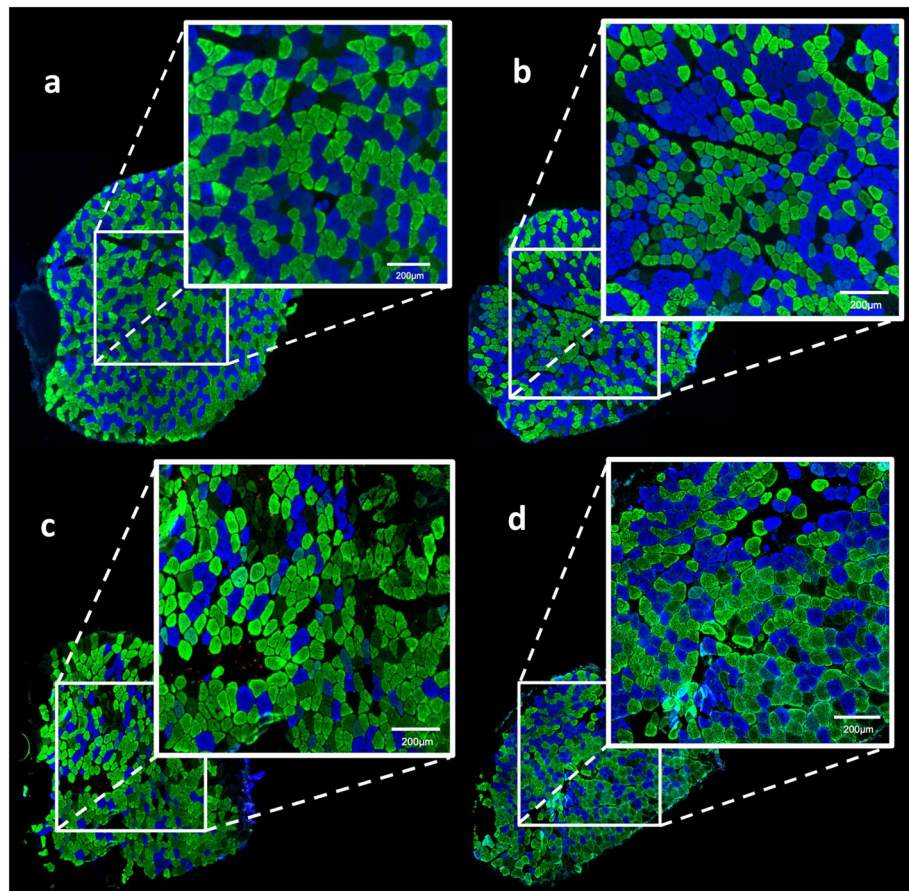


**Figure 2.** Photomicrographs of soleus muscle stained with hematoxylin and eosin (HE). **A** *wtSED* sample, with polyhedral fibers with peripheral nuclei; **B** *mdxSED* sample, showing variations in fiber size, increased of connective tissue (\*), nuclear centralization (thick arrow), splitting (thin arrow), necrosis (arrowhead), cytoplasmic rarefaction (dotted arrow), and basophilic cell (purple cell); **C** *mdxTR<sub>3</sub>* sample, showing variation in fiber size, increased of connective tissue (\*), nuclear centralization (thick arrow), cytoplasmic rarefaction (dotted arrow), and splitting (thin arrow); **D** *mdxTR<sub>21</sub>* sample, showing less variation in fiber size, in connective tissue (\*), and nuclear centralization (thick arrow). Bars: 200 μm. Abbreviations: wild-type Sedentary group (*wtSED*); *mdx* Sedentary group (*mdxSED*); wild-type Training group for 3 days (*wtTR<sub>3</sub>*); *mdx* Training group for 3 days (*mdxTR<sub>3</sub>*); wild-type Training group for 21 days (*wtTR<sub>21</sub>*); and *mdx* Training group for 21 days (*mdxTR<sub>21</sub>*).

	<i>wtSED</i>	<i>mdxSED</i>	<i>wtTR<sub>3</sub></i>	<i>mdxTR<sub>3</sub></i>	<i>wtTR<sub>21</sub></i>	<i>mdxTR<sub>21</sub></i>
FTI	32.10*	39.92	30.72 <sup>†</sup>	27.67*	32.6 <sup>^</sup>	31.68* <sup>‡</sup>
	31.7–32.4	39.01–40.83	30.34–31.1	27.16–28.18	32.13–33.10	31.17–32.20
FTIIC	23.65*	33.36	23.09	24.08*	25.28	27.35* <sup>‡</sup>
	23.1–24.20	32.19–34.54	22.39–23.80	23.61–24.54	24.14–26.42	26.67–28.03
FTIIA	25.87*	36.86	25.02	27.81*	29.76 <sup>^†</sup>	34.16* <sup>‡</sup>
	25.65–26.1	36.03–37.63	24.78–25.26	27.48–28.14	29.39–30.13	33.64–34.68
FTIIAD	27.52*	39.99	27.25	27.94*	31.75 <sup>^†</sup>	33.37* <sup>‡</sup>
	26.87–28.16	37.83–42.16	26.57–27.94	26.92–28.96	30.73–32.76	31.73–35.01
FTIID	30.65*	40.76	29.46	38.29*	33.88 <sup>^†</sup>	39.72
	29.89–31.41	38.74–42.77	28.65–30.28	36.62–39.97	32.94–34.82	37.62–41.83

**Table 1.** Mean of minimal Feret’s diameter (μm) and the respective 95% confidence intervals in soleus fibers type I, type IIC, type IIA, type IIAD and type D fibers in the different groups studied. \**p* < 0.05 compared to *mdxSED*; <sup>†</sup>*p* < 0.05 compared to *wtSED*; <sup>‡</sup>*p* < 0.05 compared to *mdxTR<sub>3</sub>*; <sup>^</sup>compared to *wtTR<sub>3</sub>*.





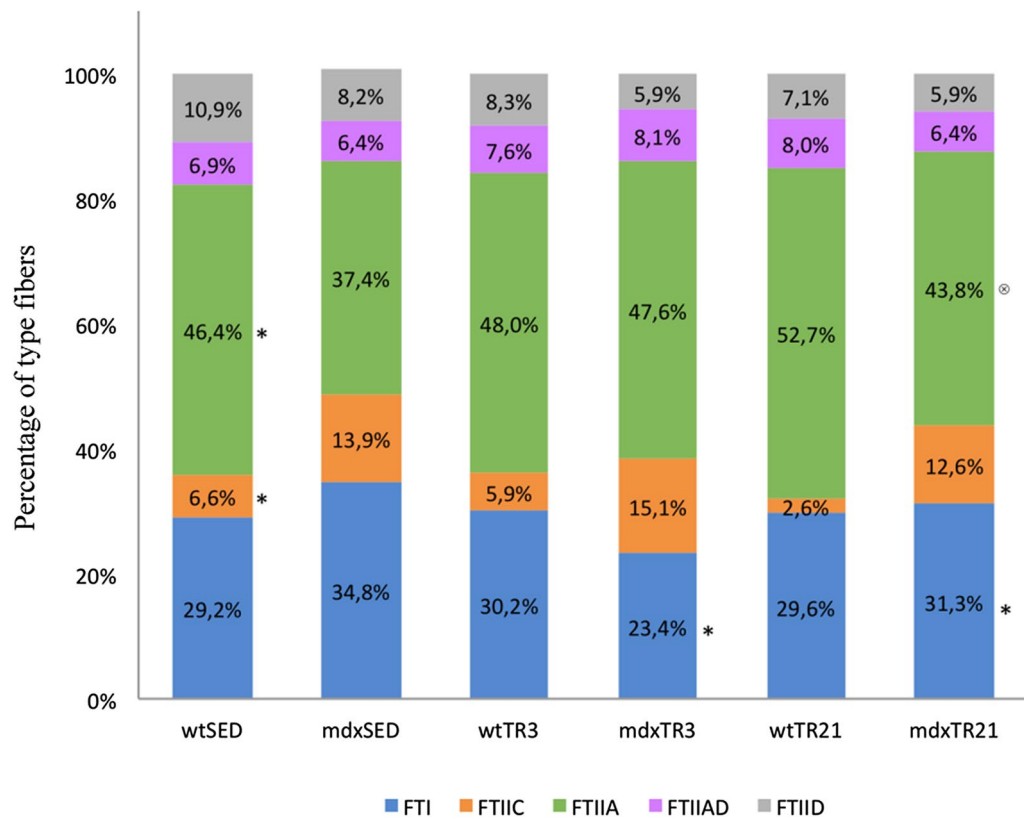
**Figure 3.** Photomicrographs of soleus muscle immunolabelled by different myosin heavy chain (MHC) antibodies. MHC I (FTI) stained in blue; MHC II A (FTIIA) stained in green; MHC II D (FTIID) did not stain (black color) and MHC II B (FTIIB) stained in red. There are not FTIIB in soleus muscle. **A** *wtSED* sample, showing a homogeneous distribution between FTI (blue color) and FTIIA (green color); **B** *mdxSED* sample, showing a grouping of fibers; **C** *mdxTR<sub>3</sub>* sample, showing heterogeneity in fiber distribution with an increase of FTIIA (green fibers) and FTIID (black fibers) and a decrease of FTI (blue fibers). **D** *mdxTR<sub>21</sub>* sample, showing a larger degree of homogeneity between FTI (blue fibers) and FTIIA (green fibers). Bars: 200  $\mu\text{m}$ . Abbreviations: wild-type Sedentary group (*wtSED*); *mdx* Sedentary group (*mdxSED*); wild-type Training group for 3 days (*wtTR<sub>3</sub>*); *mdx* Training group for 3 days (*mdxTR<sub>3</sub>*); wild-type Training group for 21 days (*wtTR<sub>21</sub>*); and *mdx* Training group for 21 days (*mdxTR<sub>21</sub>*).

wild-type trained. The maintenance of the training for 21 days caused an increase in the diameter of all fiber types of the muscles from dystrophic animals (*mdxTR<sub>21</sub>*) compared to animals trained for 3 days (*mdxTR<sub>3</sub>*). Nevertheless, the values were still lower when compared to sedentary dystrophic animals. Similar results were observed in the wild-type group that trained 21 days when compared to the wild-type group that trained for 3 days.

**Proportion of the different fiber types.** Morphometrics alterations were observed through analysis of the slides processed by immunofluorescence for different MHC isoforms. Figure 3A,B show slides from *wtSED* and *mdxSED*, respectively. Figure 3A shows a homogeneous distribution between FTI (blue color) and FTIIA (green color) fibers while Fig. 3B shows grouping of fiber in the fascicles, i.e., typing group. Figure 4 illustrates the distribution of fiber types in all groups tested in this study. As expected, the absence of dystrophin protein implied a significant reduction in the number of FTIIA fibers with a concomitant increased of FTIIC fibers in sedentary animals.

The short-term training applied to *mdx* mice (*mdxTR<sub>3</sub>*) did not cause a significant alteration in the proportion of different type fibers. Although it the *mdxTR<sub>3</sub>* showed a small increase of the number in FTIIA fibers and a reduction in FTI fibers (Fig. 4), the difference was not statistically significant. Figure 3C illustrates a predominance of glycolytic FTIIA (green color) in the *mdxTR<sub>3</sub>* group.

Training of *mdx* mice for 21 days caused a reduction of oxidative fibers (FTI) compared to the *mdx* sedentary group (*mdxSED* vs. *mdxTR<sub>21</sub>*;  $p < 0.05$ ). There was not a difference between the proportions of FTI in both trained groups (*mdxTR<sub>3</sub>* vs. *mdxTR<sub>21</sub>*;  $p > 0.05$ ), but there was a slight increase in these fibers (*mdxTR<sub>3</sub>* vs *mdxTR<sub>21</sub>*;  $p < 0.05$ ). Figure 3D shows a large homogeneity between FTI and FTIIA fibers after a long period



**Figure 4.** Percentage measurement of type I, IIC, IIA, IIAD and IID of muscle fibers in all groups studied. \* $p < 0,05$  compared to *mdxSED*, ⊗ $p < 0,05$  compared to *mdxTR<sub>3</sub>*. Abbreviations: wild-type Sedentary group (*wtSED*); *mdx* Sedentary group (*mdxSED*); wild-type Training group for 3 days (*wtTR<sub>3</sub>*); *mdx* Training group for 3 days (*mdxTR<sub>3</sub>*); wild-type Training group for 21 days (*wtTR<sub>21</sub>*); and *mdx* Training group for 21 days (*mdxTR<sub>21</sub>*).

of training. The use of low-intensity eccentric exercise did not affect the distribution of different fiber types in healthy animal muscles (Fig. 4).

**Total force of the single cell.** Figure 5 shows the force traces collected during contractions developed by single fibers dissected from mice from *wtSED*, *mdxSED* and *mdxTR<sub>21</sub>*. The isometric contractions were recorded during experiments in which fibers were maximally activated ( $pCa^{2+} 4.5$ ) at an initial sarcomere length of 2.5  $\mu m$ . The forces produced during maximal activation were lower in *mdx* fibers than wild-type fiber (black trace). After 21 days of low-intensity eccentric training the total force increased significantly in the *mdx* fibers, but was still lower than the force produced by wild-type fibers (Fig. 5). The force values were significantly different among the *wtSED* ( $241.5 \pm 33.8$ ), *mdxSED* (mean:  $67.20 \pm 17.24$ ) and *mdxTR<sub>21</sub>* ( $106.5 \pm 8.82$ ) groups.

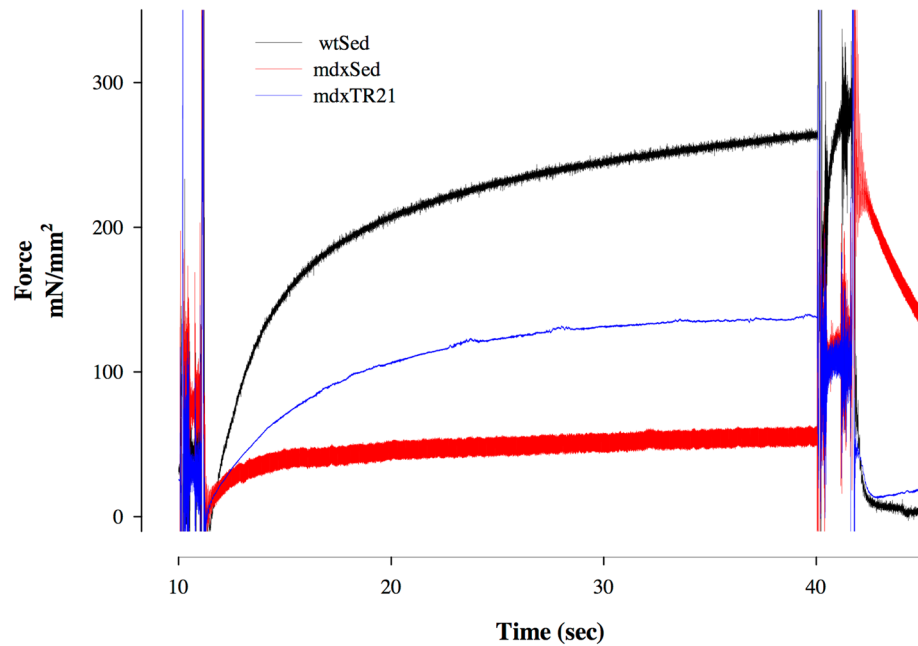
We also tested the force responses to different levels of  $Ca^{2+}$  activation, to construct a force-pCa curve and evaluate the  $Ca^{2+}$  sensitivity of the contractile apparatus (Fig. 6). A difference in  $Ca^{2+}$  sensitivity (measured by the  $pCa^{50}$ ) was not observed among groups, i.e.,  $Ca^{2+}$  sensitivity was not impaired in the *mdx* fibers investigated in this study (Fig. 6).

## Discussion

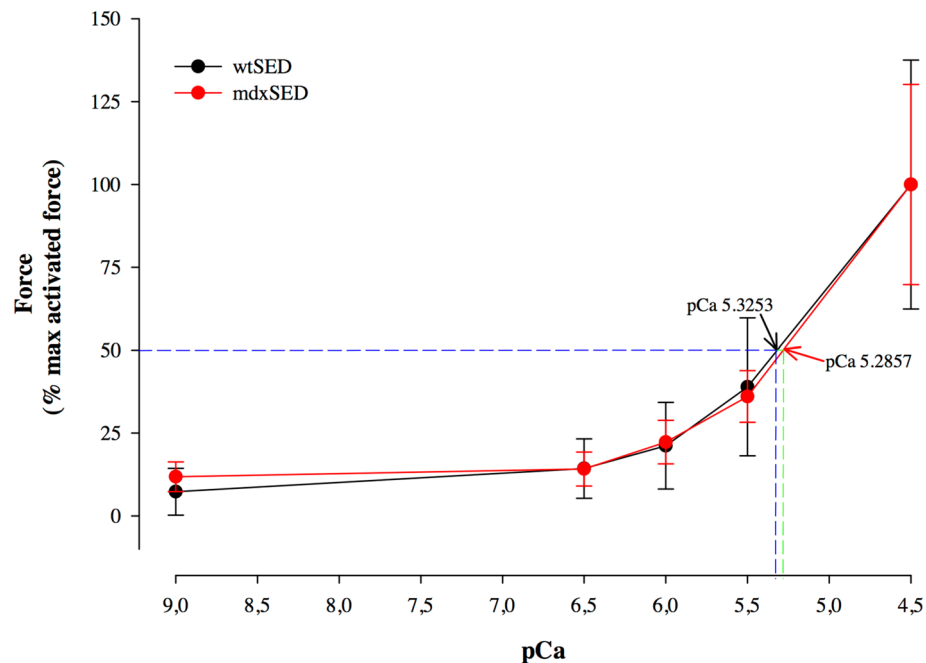
The results of this study demonstrate that LIET improved the general histology, trophism, the overall distribution of different fiber types and the contractile force of *mdx* mice. These findings suggest that a well-controlled LIET is a viable therapeutic model for improving muscle function in DMD.

Physical exercise has been studied as a therapeutic strategy to delay the progression of DMD. However, physical exercise must be carefully planned because it can also accelerate a degenerative process in DMD muscles. High-intensity exercise, for example, can increase muscle damage and degeneration<sup>18,30</sup>. Although our findings showed a worsening of the phenotype of *mdx* animals trained for 3 days with LIET, this result was reverted with 21 days of training. It has been shown that eccentric exercise when applied for a prolonged period may improve the muscle morphological characteristics<sup>14,31</sup>.

This study confirmed some of the main alterations induced by the absence of dystrophin in soleus muscle: nuclear centralization, splitting, necrosis, increased connective tissue and basophilic cells. The centralization observed in the dystrophic muscle of *mdx* mice is a result of muscle degeneration and regeneration<sup>32</sup>. During repair of skeletal muscle tissues, satellite cells, which are precursors of myogenesis, are activated and proliferate



**Figure 5.** Contractions produced by a single fiber dissected from the soleus muscle from a mouse from *wtSED* (black line), *mdxSED* (red line) or *mdxTR<sub>21</sub>* (blue line) groups. Abbreviations: wild-type Sedentary group (*wtSED*); *mdx* Sedentary group (*mdxSED*); and *mdx* Training group for 21 days (*mdxTR<sub>21</sub>*).



**Figure 6.** Force- $pCa^{2+}$  relation for the *wtSED* and the *mdxSED* groups. There was no difference in  $Ca^{2+}$  sensitivity, as the value for  $pCa^{50}$  was not different between the control (mean  $5.35 \pm 0.08$ ) and *mdx* (mean  $5.41 \pm 0.13$ ) groups. Abbreviations: wild-type Sedentary group (*wtSED*); *mdx* Sedentary group (*mdxSED*).

to the site of the lesion where they will merge into the focus of the lesion and subsequently differentiate into myoblasts. These newly repaired cells present the centralized nucleus until the maturation occurs, with subsequent migration of the nucleus to the periphery<sup>33</sup>. Other cytoarchitectural changes such as basophilia and splitting are characteristic of cell membrane damage and  $Ca^{2+}$  influx into the cytoplasm of the dystrophic cell<sup>34</sup>, which triggers degenerative reactions that increase the inflammation, contributing to chronic damage and degeneration of

dystrophic cells<sup>35</sup>. The degenerative process culminates with increased connective tissue that can lead to fibrosis and impairment of muscle function<sup>32,36,37</sup>.

There was an increase in minimal Feret's diameter of fibers of the *mdxSED* animals when compared to *wtSED* animals, as previously observed<sup>14,38–40</sup>. The successive degeneration/regeneration processes that occur in the dystrophic fiber exacerbate the inflammatory process in the cytosol, increasing the cell volume<sup>41–43</sup>. After 21 days of training, the soleus muscle cells of the *mdx* animals reduced their volume compared to the *mdx* sedentary; in it has been shown that low-intensity eccentric training applied during long period reduces inflammatory processes<sup>44</sup> and reduce the cross-section area (CSA) of *mdx* muscle fibers<sup>15</sup>.

The *wtSED* animals presented a homogeneous distribution between type I and type IIA fibers, as previously shown<sup>15,27</sup>. The absence of dystrophin promoted an imbalance in this distribution, as the proportion of FTIIA fibers was reduced while the proportion of FTI fibers was increased in *mdxSED* animals<sup>27,45,46</sup>. We observed that the LIET applied during a short period of training (3-days) reduced the number of FTI fibers. However, with the maintenance of the training for 21 days, the proportion of these oxidative fibers was increased, concomitant with a reduction in FTIIA fibers, in agreement with previous studies<sup>6,47–49</sup>. It is likely that low-intensity training promotes adaptations in the mitochondria thus increasing the oxidative capacity of the fibers and the resistance to fatigue.

The most striking symptom of DMD is the loss of muscle strength due to progressive degeneration of muscle fibers. In this condition, non-contractile tissues, such as adipose and connective tissue, replace the contractile tissue. Our results from experiments conducted with single fibers showed that DMD reduced the specific force of *mdx* muscle, confirming previous results<sup>27,50–54</sup>. The reduction in force in the *mdx* mice could be explained by a reduction of the sensitivity of the contractile system to  $Ca^{2+}$ , which would lead to a decrease in force produced at a given level of activation<sup>55,56</sup>. However, our results do not show an altered difference in  $Ca^{2+}$  sensitivity in fibers dissected from *mdx* mice, as previously observed<sup>57–59</sup>. Therefore, the decrease in force in *mdx* fibers may be a result of dysfunction in contractile proteins. In this regard, there are conflicting results in the literature. Canepari, et al.<sup>52</sup> observed an impairment of the myosin function in dystrophic muscles that was attributed to post-translational modifications, which could determine a change in its enzymatic or mechanical properties of myosin. On the other hand, Bates, et al.<sup>60</sup> did not observe a significant reduction in myosin activity or cross-bridge kinetics in *mdx* mice. The difference in results is difficult to explain, but it may be associated with differences in experimental protocols, and with the age of the *mdx* mice. In this study, we used 7-week-old mice, an age in the peak phase of the lesion, presenting many cells in degeneration/regeneration, as discussed earlier.

We are not aware of other studies investigating the effects of LIET on contractile properties of single fibers from dystrophic muscles. However our results are on line with studies showing an improvement in the forelimb and hindlimb strength of *mdx* mice after long periods of low intensity training on a flat treadmill<sup>11,44,61</sup>. There are studies showing an improvement in fatigue resistance and oxidative capacity after 12 weeks of low-intensity training<sup>47</sup>.

## Conclusion

LIET improved the morphological and functional characteristics of the dystrophic soleus muscle of mice by reducing cell degeneration, improving trophism and distribution of different types of fibers and increasing the contractile force of muscle fibers. LIET may be an important method to be used for rehabilitation of dystrophic muscles, with potentially far-reaching implications for the treatment of the disease.

## Methods

**Ethics statement.** This study was conducted in accordance with the Ethical Principles in Animal Research, adopted by the National Council for the Control of Animal Experimentation (CONCEA, Brazil) and was approved by the Ethics Committee on Animal Use in Ribeirão Preto Medical School (Brazil) (protocol number 173/2013).

**Animals.** Thirty-six male mice were used in this study. Eighteen *mdx* mice (C57BL/10-Dmd<sup>mdx</sup>/PASUnib; body weight 17.37 g;  $\pm 0.48$ ) and eighteen wild-type mice (background: C57BL/10; body weight 19.16 g;  $\pm 0.39$ ) were acquired from CEMIB (Multidisciplinary Center for Biological Investigation on Laboratory Animal Science, UNICAMP, Campinas, Brazil). The animals were bred in the Pasteur Institute (Paris, France).

The mice were maintained in cages on a light–dark cycle (12 h/12 h) at 22°C and supplied with water and food ad libitum. The *mdx* mice were randomized into three groups (n = 6 for each group): Sedentary group (*mdxSED*), Training group—3 days (*mdxTR*<sub>3</sub>) and Training group—21 days (*mdxTR*<sub>21</sub>). Wild-type mice were similarly randomized into three groups (n = 6 for each group): *wtSED*; *wtTR*<sub>3</sub> and *wtTR*<sub>21</sub>.

Training started when the animals were 6-weeks old, because at this age severe morphological alterations and signals of degeneration and/or regeneration are detected in dystrophic muscles<sup>62</sup>. The exercise protocol was performed during three days per week (Monday, Wednesday and Friday) for 3 days (1 week) or 21 days (7 weeks) of training. Therefore, at the end of training the animals of *mdxTR*<sub>3</sub> and *wtTR*<sub>3</sub> groups were 7-week-old and the animals of *mdxTR*<sub>21</sub> and *wtTR*<sub>21</sub> were 13-week-old. The sedentary animals (*mdxSED* and *wtSED*) were 7-week-old.

**Low-intensity training.** *Eccentric protocols.* The training protocols were performed on a declined (-16°) treadmill (EP 132C; Insight, Ribeirão Preto, Brazil). Before the training period, all animals underwent a period of adaptation 3 times/week (Monday, Wednesday, Friday), in which they run for 2 min, at a speed of 7 m/min. During the training period, the animals were also exercised 3 times/week (Monday, Wednesday, Friday), but for 10 min/day at a speed of 10 m/min.



**Histology and Immunofluorescence.** The soleus muscle of all animals was excised and frozen in liquid nitrogen for histological and immunofluorescence examination. Slides were prepared by sectioning the muscles (6  $\mu\text{m}$  in thickness) using a cryostat (Leica CM 1860, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) at  $-25\text{ }^{\circ}\text{C}$ . Hematoxylin and eosin (H&E, MERCK, Darmstadt, Germany) stains were used to analyze morphological features through light microscopy: nucleus centralized, basophilic cells, and necrosis<sup>23,63</sup>.

Immunofluorescence was applied to quantify different isoforms of myosin heavy chain (MHC) and dystrophin proteins. The slides with frozen sections were blocked with M.O.M. (mouse on mouse—Vector Laboratories, Burlingame, USA). Section were incubated in primary antibodies for dystrophin (MANDYS1, 1:5; DSHB—Developmental Studies Hybridoma Bank, Iowa, USA), MHC type 1 (BA-D5, 1:50; DSHB, Iowa, USA), MHC type 2A (SC-71; 1:100; DSHB, Iowa, USA), MHC type 2D (6H1, 1:100; DSHB, Iowa, USA) and MHC type 2B (BF-F3, 1:50; DSHB, Iowa, USA) in 1% of BSA (Bovine Serum Albumin—Sigma Aldrich, San Luis, Missouri, USA) for 30 min at  $37\text{ }^{\circ}\text{C}$ . The slides were washed with PBS and incubated in secondary antibodies (Alexa Fluor 488, 1:200; Alexa Fluor 594, 1:200; DyLight 405, 1:200; Jackson Immuno Research, Pennsylvania, USA). Sections were mounted in polyvinyl alcohol solution or in prolong gold with DAPI. The slides were analyzed with Image X-Press (Molecular Devices, San Jose, CA, USA) and fluorescence illumination. The minimal Feret's diameters—the minimum distance of the parallel tangents in opposing borders of the muscle fiber<sup>40</sup>—were measured using Image J software (version 1.50e, NIH, USA). The proportion of fiber types was quantified using whole muscle sections.

**Contractile measurements of single fiber.** The muscles were dissected, tie to wood sticks, and chemically permeabilized following standard procedures<sup>64–66</sup>. Briefly, the muscle samples were incubated in rigor solution (50 mM Tris, 100 mM NaCl, 2 mM KCl, 2 mM  $\text{MgCl}_2$ , and 10 mM EGTA pH7.0, Sigma Aldrich, San Luis, Missouri, USA) for 4 h, after which they were transferred to a rigor-glycerol (50:50) solution for 15 h. Then, the samples were placed in a fresh rigor-glycerol (50:50) solution with a cocktail of protease inhibitors (Roche Diagnostics, Basel, Switzerland) and stored in a freezer  $-20\text{ }^{\circ}\text{C}$  for at least 7 days.

**Single cell force measurements.** On the day of experiment, single fibers were carefully dissected in relaxing solution, and gripped at their ends with T-shaped clips made of aluminum foil. The fiber was transferred to a temperature-controlled chamber (802 D, Aurora Scientific, Aurora, Ontario, Canada) where it was attached between a force transducer (403A, Aurora Scientific, Aurora, Ontario, Canada) and a length controller (322C, Aurora Scientific, Aurora, Ontario, Canada).

Before the start of each experiment, the average sarcomere length (SL) was measured in relaxing solution (100 mM KCl, 2 mM EGTA, 20 mM imidazole, 4 mM ATP, and 7 mM  $\text{MgCl}_2$ , Sigma-Aldrich, San Luis, Missouri, USA) using a high-speed video system (HVSL, Aurora Scientific 901B, Aurora, Ontario, Canada). Images from a selected region of the fibers were used to calculate the SL by fast Fourier transform (FFT) analysis based on the striation spacing produced by dark and light bands of myosin and actin filaments. The fiber diameter and length were measured using a CCD camera (Go-3, QImaging; pixel size:  $3.2\text{ }\mu\text{m} \times 3.2\text{ }\mu\text{m}$ , Aurora Scientific, Aurora, Ontario, Canada), and the cross-sectional area was estimated assuming a circular geometry. Fibers were activated with different  $\text{Ca}^{2+}$  concentration (pCa 4.5; 5.5; 6.0; 6.5; 9.0) (20 mM imidazole, 14.5 mM creatine phosphate, 7 mM EGTA, 4 mM MgATP, 1 mM free  $\text{Mg}^{2+}$ , and free  $\text{Ca}^{2+}$  ranging from 1 nM (pCa<sup>2+</sup> 9.0) to 32 M (pCa<sup>2+</sup> 4.5) (Sigma-Aldrich, San Luis, Missouri, USA) at an initial SL of 2.5  $\mu\text{m}$ . The active force produced during the experiments was measured after force stabilized during the contractions.

**Statistical analysis.** All contractile data were compared among groups using a two-way analysis of variance (ANOVA) for repeated factors. When significant changes were observed, post hoc analyses were performed with Newman–Keuls tests. Data for the minimal Feret's diameter and the proportion of different type fibers were analyzed using mixed-effects linear models, and multiple comparisons were performed using diagonal contrasts. All statistical analyses were performed using the SAS software version 9.4, with the level of significance set at  $p < 0.05$ . The results are shown as means and standard error of the mean (SEM).

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## References

- Mah, J. K. *et al.* A systematic review and meta-analysis on the epidemiology of Duchenne and Becker muscular dystrophy. *Neuromuscul. Disord.* **24**, 482–491. <https://doi.org/10.1016/j.nmd.2014.03.008> (2014).
- Alderton, J. M. & Steinhardt, R. A. Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *J. Biol. Chem.* **275**, 9452–9460 (2000).
- Morris, C. A., Selsby, J. T., Morris, L. D., Pendrak, K. & Sweeney, H. L. Bowman–Birk inhibitor attenuates dystrophic pathology in mdx mice. *J. Appl. Physiol.* **109**, 1492–1499. <https://doi.org/10.1152/jappphysiol.01283.2009> (2010).
- Godin, R. *et al.* Peroxisome proliferator-activated receptor  $\gamma$  coactivator 1- $\alpha$  gene transfer restores mitochondrial biomass and improves mitochondrial calcium handling in post-necrotic mdx mouse skeletal muscle. *J. Physiol.* **590**, 5487–5502. <https://doi.org/10.1113/jphysiol.2012.240390> (2012).
- Haas, M. *et al.* European medicines agency review of ataluren for the treatment of ambulant patients aged 5 years and older with Duchenne muscular dystrophy resulting from a nonsense mutation in the dystrophin gene. *Neuromuscul. Disord.* **25**, 5–13. <https://doi.org/10.1016/j.nmd.2014.11.011> (2015).
- Carter, G. T., Abresch, R. T. & Fowler, W. M. Adaptations to exercise training and contraction-induced muscle injury in animal models of muscular dystrophy. *Am. J. Phys. Med. Rehabil.* **81**, 151–161. <https://doi.org/10.1097/01.PHM.0000029776.62399.B7> (2002).



7. Bushby, K. *et al.* Ataluren treatment of patients with nonsense mutation dystrophinopathy. *Muscle Nerve* **50**, 477–487. <https://doi.org/10.1002/mus.24332> (2014).
8. Cup, E. H. *et al.* Exercise therapy and other types of physical therapy for patients with neuromuscular diseases: a systematic review. *Arch. Phys. Med. Rehabil.* **88**, 1452–1464. <https://doi.org/10.1016/j.apmr.2007.07.024> (2007).
9. Case, L. E. *et al.* Rehabilitation management of the patient with duchenne muscular dystrophy. *Pediatrics* **142**, S17–S33. <https://doi.org/10.1542/peds.2018-0333D> (2018).
10. Kaczor, J. J., Hall, J. E., Payne, E. & Tarnopolsky, M. A. Low intensity training decreases markers of oxidative stress in skeletal muscle of mdx mice. *Free Radic. Biol. Med.* **43**, 145–154. <https://doi.org/10.1016/j.freeradbiomed.2007.04.003> (2007).
11. Hyzewicz, J. *et al.* Low intensity training of mdx mice reduces carbonylation and increases expression levels of proteins involved in energy metabolism and muscle contraction. *Free Radic. Biol. Med.* **82**, 122–136. <https://doi.org/10.1016/j.freeradbiomed.2015.01.023> (2015).
12. Hyzewicz, J. *et al.* Low-intensity training and the C5a complement antagonist NOX-D21 rescue the mdx phenotype through modulation of inflammation. *Am. J. Pathol.* **187**, 1147–1161. <https://doi.org/10.1016/j.ajpath.2016.12.019> (2017).
13. Faist, V., König, J., Höger, H. & Elmadfa, I. Decreased mitochondrial oxygen consumption and antioxidant enzyme activities in skeletal muscle of dystrophic mice after low-intensity exercise. *Ann. Nutr. Metab.* **45**, 58–66. <https://doi.org/10.1159/000046707> (2001).
14. Gaiad, T. P. *et al.* Low-intensity training provokes adaptive extracellular matrix turnover of a muscular dystrophy model. *J. Exerc. Rehabil.* **13**, 693–703. <https://doi.org/10.12965/jer.1735094.547> (2017).
15. Hayes, A., Lynch, G. S. & Williams, D. A. The effects of endurance exercise on dystrophic mdx mice I. Contractile and histochemical properties of intact muscles. *Proc. R. Soc. B Biol. Sci.* **253**, 19–25. <https://doi.org/10.1098/rspb.1993.0077> (1993).
16. Hayes, A. & Williams, D. A. Beneficial effects of voluntary wheel running on the properties of dystrophic mouse muscle. *J. Appl. Physiol.* **80**, 670–679 (1996).
17. Hayes, A. & Williams, D. A. Contractile function and low-intensity exercise effects of old dystrophic (mdx) mice. *Am. J. Physiol. Cell Physiol.* **274**, 1138–1144 (1998).
18. Capogrosso, R. F. *et al.* Contractile efficiency of dystrophic mdx mouse muscle: In vivo and ex vivo assessment of adaptation to exercise of functional end points. *J. Appl. Physiol.* **122**, 828–843. <https://doi.org/10.1152/jappphysiol.00776.2015> (2017).
19. Birnkrant, D. J. *et al.* Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and neuromuscular, rehabilitation, endocrine, and gastrointestinal and nutritional management. *Lancet Neurol.* **17**, 251–267. [https://doi.org/10.1016/S1474-4422\(18\)30024-3](https://doi.org/10.1016/S1474-4422(18)30024-3) (2018).
20. Evans, W. J. *et al.* Metabolic changes following eccentric exercise in trained and untrained men. *J. Appl. Physiol.* **61**, 1864–1868 (1986).
21. Liao, P., Zhou, J., Ji, L. L. & Zhang, Y. Eccentric contraction induces inflammatory responses in rat skeletal muscle: Role of tumor necrosis factor- $\alpha$ . *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, 599–607. <https://doi.org/10.1152/ajpregu.00480.2009> (2010).
22. Sonobe, T., Inagaki, T., Sudo, M., Poole, D. C. & Kano, Y. Sex differences in intracellular Ca<sup>2+</sup> accumulation following eccentric contractions of rat skeletal muscle in vivo. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **299**, 1006–1012. <https://doi.org/10.1152/ajpregu.00623.2009> (2010).
23. Cornachione, A. *et al.* Morphological comparison of different protocols of skeletal muscle remobilization in rats after hindlimb suspension. *Scand. J. Med. Sci. Sports* **18**, 453–461. <https://doi.org/10.1111/j.1600-0838.2007.00720.x> (2008).
24. Cornachione, A., Cação-Benedini, L. O., Martinez, E. Z., Neder, L. & Mattiello-Sverzut, A. C. Effects of eccentric and concentric training on capillarization and myosin heavy chain contents in rat skeletal muscles after hindlimb suspension. *Acta Histochem.* **113**, 277–282. <https://doi.org/10.1016/j.acthis.2009.10.009> (2011).
25. Cornachione, A. S., Cação-Benedini, L. O., Chesca, D. L., Martinez, E. Z. & Mattiello-Sverzut, A. C. Effects of eccentric exercise in rehabilitation of phasic and tonic muscles after leg immobilization in rats. *Acta Histochem.* **116**, 1216–1224. <https://doi.org/10.1016/j.acthis.2014.07.002> (2014).
26. McBride, T. A., Gorin, F. A. & Carlsen, R. C. Prolonged recovery and reduced adaptation in aged rat muscle following eccentric exercise. *Mech. Ageing Dev.* **83**, 185–200 (1995).
27. Lindsay, A. *et al.* Variable cytoplasmic actin expression impacts the sensitivity of different dystrophin-deficient mdx skeletal muscles to eccentric contraction. *FEBS J.* **286**, 2562–2576. <https://doi.org/10.1111/febs.14831> (2019).
28. Vilquin, J.-T. *et al.* Evidence of mdx mouse skeletal muscle fragility in vivo by eccentric running exercise. *Muscle Nerve* **21**, 567–576. [https://doi.org/10.1002/\(SICI\)1097-4598\(199805\)21:5%3C567::AID-MUS2%3E3.0.CO;2-6](https://doi.org/10.1002/(SICI)1097-4598(199805)21:5%3C567::AID-MUS2%3E3.0.CO;2-6) (1998).
29. Fernandes, D. C. *et al.* Low intensity training improves redox status and reduces collagen fibers on dystrophic muscle. *J. Exerc. Rehabil.* **15**, 213–223. <https://doi.org/10.12965/jer.1938060.030> (2019).
30. Schill, K. E. *et al.* Muscle damage, metabolism, & oxidative stress in mdx mice: impact of aerobic running. *Muscle Nerve* **54**, 110–117. <https://doi.org/10.1002/mus.25015> (2016).
31. Proske, U. & Morgan, D. L. Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. *J. Physiol.* **537**, 333–345 (2001).
32. Burns, D. P. *et al.* Restoration of pharyngeal dilator muscle force in dystrophin-deficient (mdx) mice following co-treatment with neutralizing interleukin-6 receptor antibodies and urocortin 2. *Exp. Physiol.* **102**(1177–1193), 1177–1193. <https://doi.org/10.1113/EP086232> (2017).
33. Itai, Y., Kariya, Y. & Hoshino, Y. Morphological changes in rat hindlimb muscle fibres during recovery from disuse atrophy. *Acta Physiol. Scand.* **181**, 217–224. <https://doi.org/10.1111/j.1365-201X.2004.01271.x> (2004).
34. Brussee, V., Tardif, F. & Tremblay, J. P. Muscle fibers of mdx mice are more vulnerable to exercise than those of normal mice. *Neuromuscul. Disord.* **7**, 487–492 (1997).
35. Evans, N. P., Misyak, S. A., Robertson, J. L., Bassaganya-Riera, J. & Grange, R. W. Dysregulated intracellular signaling and inflammatory gene expression during initial disease onset in duchenne muscular dystrophy. *Am. J. Phys. Med. Rehabil.* **88**, 502–522. <https://doi.org/10.1097/PHM.0b013e3181a5a24f> (2009).
36. Deconinck, N. & Dan, B. Pathophysiology of duchenne muscular dystrophy: current hypotheses. *Pediatr. Neurol.* **36**, 1–7. <https://doi.org/10.1016/j.pediatrneurol.2006.09.016> (2007).
37. Pelosi, L. *et al.* Increased levels of interleukin-6 exacerbate the dystrophic phenotype in mdx mice. *Hum. Mol. Genet.* **24**(6041–6053), 6041–6053. <https://doi.org/10.1093/hmg/ddv323> (2015).
38. Anderson, J. E., Bressler, B. H. & O'valle, W. K. Functional regeneration in the hindlimb skeletal muscle of the mdx mouse. *J. Muscle Res. Cell Motil.* **9**, 499–515. <https://doi.org/10.1007/BF01738755> (1988).
39. Schäfer, R., Zweyer, M., Knauf, U., Mundegar, R. R. & Wernig, A. The ontogeny of soleus muscles in mdx and wild type mice. *Neuromuscul. Disord.* **15**, 57–64. <https://doi.org/10.1016/j.nmd.2004.09.011> (2005).
40. Briguet, A., Courdier-Fruh, I., Foster, M., Meier, T. & Magyar, J. P. Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. *Neuromuscul. Disord.* **14**, 675–682. <https://doi.org/10.1016/j.nmd.2004.06.008> (2004).
41. Tinsley, J. *et al.* Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat. Med.* **4**, 1441–1444. <https://doi.org/10.1038/4033> (1998).
42. Yiu, E. M. & Kornberg, A. J. Duchenne muscular dystrophy. *J. Paediatr. Child Health* **51**, 759–764. <https://doi.org/10.1111/jpc.12868> (2015).

43. Niranjani, N. *et al.* Sarcoplasmic overexpression impairs myogenic differentiation in Duchenne muscular dystrophy. *Am. J. Physiol. Cell Physiol.* **317**, C813–C824. <https://doi.org/10.1152/ajpcell.00146.2019> (2019).
44. Frinchi, M. *et al.* Recovery of damaged skeletal muscle in mdx mice through low-intensity endurance exercise. *Int. J. Sports Med.* **35**, 19–27. <https://doi.org/10.1055/s-0033-1343405> (2014).
45. Deconinck, N. *et al.* Consequences of the combined deficiency in dystrophin and utrophin on the mechanical properties and myosin composition of some limb and respiratory muscles of the mouse. *Neuromuscul. Disord.* **8**, 362–370 (1998).
46. Banks, G. B., Combs, A. C., Odom, G. L., Bloch, R. J. & Chamberlain, J. S. Muscle Structure influences utrophin expression in mdx mice. *PLoS Genet.* **10**, 1–16. <https://doi.org/10.1371/journal.pgen.1004431> (2014).
47. Baltgalvis, K. A. *et al.* Exercise training improves plantar flexor muscle function in mdx Mice. *Med. Sci. Sports Exerc.* **44**, 1671–1679. <https://doi.org/10.1249/MSS.0b013e31825703f0> (2012).
48. Lynch, G. S., Hayes, A., Lam, M. H. C. & Williams, D. A. The effects of endurance exercise on dystrophic mdx mice. II. Contractile properties of skinned muscle fibres. *Proc. R. Soc. B Biol. Sci.* **253**, 27–33. <https://doi.org/10.1098/rspb.1993.0078> (1993).
49. Landisch, R. M., Kosir, A. M., Nelson, S. A., Baltgalvis, K. A. & Lowe, D. A. Adaptive and nonadaptive responses to voluntary wheel running by mdx mice. *Muscle Nerve* **38**, 1290–1293. <https://doi.org/10.1002/mus.21141> (2008).
50. Guiraud, S. *et al.* Embryonic myosin is a regeneration marker to monitor utrophin-based therapies for DMD. *Hum. Mol. Genet.* **28**, 307–319. <https://doi.org/10.1093/hmg/ddy353> (2019).
51. Coirault, C., Lambert, F., Pourny, J. C. & Lecarpentier, Y. Velocity of actomyosin sliding in Vitro is reduced in dystrophic mouse diaphragm. *Am. J. Respir. Crit. Care Med.* **165**, 250–253 (2002).
52. Canepari, M., Rossi, R., Pansarasa, O., Maffei, M. & Bottinelli, R. Actin sliding velocity on pure myosin isoforms from dystrophic mouse muscles. *Muscle Nerve* **40**, 249–256. <https://doi.org/10.1002/mus.21302> (2009).
53. Guellich, A., Negroni, E., Decostre, V., Demoule, A. & Coirault, C. Altered cross-bridge properties in skeletal muscle dystrophies. *Front. Physiol.* **5**, 1–9. <https://doi.org/10.3389/fphys.2014.00393> (2014).
54. Han, R., Rader, E. P., Levy, J. R., Bansal, D. & Campbell, K. P. Dystrophin deficiency exacerbates skeletal muscle pathology in dysferlin-null mice. *Skelet. Muscle* **1**, 1–11. <https://doi.org/10.1186/2044-5040-1-35> (2011).
55. Fink, R. H., Stephenson, D. G. & Williams, D. A. Calcium and strontium activation of single skinned muscle fibres of normal and dystrophic mice. *J. Physiol.* **373**, 513–525. <https://doi.org/10.1113/jphysiol.1986.sp016060> (1986).
56. Schneidereit, D. *et al.* Optical prediction of single muscle fiber force production using a combined biomechanics and second harmonic generation imaging approach. *Light Sci. Appl.* **7**, 1–14. <https://doi.org/10.1038/s41377-018-0080-3> (2018).
57. Williams, D. A., Head, S. I., Lynch, G. S. & Stephenson, D. G. Contractile properties of skinned muscle fibres from young and adult normal and dystrophic (mdx) mice. *J. Physiol.* **460**, 51–67. <https://doi.org/10.1113/jphysiol.1993.sp019458> (1993).
58. Wood, D. S., Sorenson, M. M., Eastwood, A. B., Charash, W. E. & Reuben, J. P. Duchenne dystrophy: abnormal generation of tension and Ca<sup>++</sup> regulation in single skinned fibers. *Neurology* **28**, 447–457. <https://doi.org/10.1212/wnl.28.5.447> (1978).
59. Schertzer, J. D., Van Der Poel, C., Shavlakadze, T., Grounds, M. D. & Lynch, G. S. Muscle-specific overexpression of IGF-I improves E-C coupling in skeletal muscle fibers from dystrophic mdx mice. *Am. J. Physiol. Cell Physiol.* **294**, 161–168. <https://doi.org/10.1152/ajpcell.00399.2007> (2008).
60. Bates, G. *et al.* Molecular, cellular, and muscle strip mechanics of the mdx mouse diaphragm. *Am. J. Physiol. Cell Physiol.* **304**, 873–880. <https://doi.org/10.1152/ajpcell.00220.2012> (2013).
61. Zelikovich, A. S., Quattrocchi, M., Salamone, I. M., Kuntz, N. L. & McNally, E. M. Moderate exercise improves function and increases adiponectin in the mdx mouse model of muscular dystrophy. *Sci. Rep.* **9**, 1–10. <https://doi.org/10.1038/s41598-019-42203-z> (2019).
62. Grounds, M. D., Radley, H. G., Lynch, G. S., Nagaraju, K. & De Luca, A. Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. *Neurobiol. Dis.* **31**, 1–19. <https://doi.org/10.1016/j.nbd.2008.03.008> (2008).
63. Cornachione, A. S., Cação-Benedini, L. O., Benedini-Elias, P. C. O., Martinez, E. Z. & Mattiello-Sverzut, A. C. Effects of 40 min of maintained stretch on the soleus and plantaris muscles of rats applied for different periods of time after hindlimb immobilization. *Acta Histochem.* **115**, 505–511. <https://doi.org/10.1016/j.acthis.2012.11.008> (2013).
64. Campbell, K. S. & Moss, R. L. History-dependent mechanical properties of permeabilized rat soleus muscle fibers. *Biophys. J.* **82**, 929–943. [https://doi.org/10.1016/S0006-3495\(02\)75454-4](https://doi.org/10.1016/S0006-3495(02)75454-4) (2002).
65. Minozzo, F. C. & Rassier, D. E. Effects of blebbistatin and Ca<sup>2+</sup> concentration on force produced during stretch of skeletal muscle fibers. *Am. J. Physiol. Cell Physiol.* **299**, 1127–1135. <https://doi.org/10.1152/ajpcell.00073.2010> (2010).
66. Cornachione, A. S. & Rassier, D. E. A non-cross-bridge, static tension is present in permeabilized skeletal muscle fibers after active force inhibition or actin extraction. *Am. J. Physiol. Cell Physiol.* **302**, 566–574. <https://doi.org/10.1152/ajpcell.00355.2011> (2012).

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## Author contributions

P.S.P., T.O.P.A., E.S., I.R.S. trained all animals and performed the experiments, analyzed the data and prepared the figures. D.L.R. analyzed the data of the minimal Feret's diameter and fiber type distribution. D.L.C. developed experiments with immunofluorescence technique. A.S.C. developed the experiments with single fibers, analyzed the data and prepared the graphs. A.S.C., D.E.R. wrote and edited the manuscript. All authors reviewed the manuscript.

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## Competing interests

The authors declare no competing interests.

### Additional information

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