

Diaphragm degeneration and cardiac structure in *mdx* mouse: potential clinical implications for Duchenne muscular dystrophy

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Abstract

We examined the effects of exercise on diaphragm degeneration and cardiomyopathy in dystrophin-deficient *mdx* mice. *Mdx* mice (11 months of age) were exercised (swimming) for 2 months to worsen diaphragm degeneration. Control *mdx* mice were kept sedentary. Morphological evaluation demonstrated increased fibrosis in the diaphragm of exercised *mdx* mice ($33.3 \pm 6.0\%$ area of fibrosis) compared with control *mdx* mice ($20.9 \pm 1.7\%$ area of fibrosis). Increased (26%) activity of MMP-2, a marker of fibrosis, was detected in the diaphragms from exercised *mdx* mice. Morphological evaluation of the heart demonstrated a 45% increase in fibrosis in the right ventricle ($8.3 \pm 0.6\%$ in sedentary vs. $12.0 \pm 0.6\%$ of fibrosis in exercised) and in the left ventricle (35% increase) in the exercised *mdx* mice. The density of inflammatory cells–degenerating cardiomyocytes increased 95% in the right ventricle (2.3 ± 0.6 in sedentary vs. 4.5 ± 0.8 in exercised) and 71% in the left ventricle (1.4 ± 0.6 sedentary vs. 2.4 ± 0.5 exercised). The levels of both active MMP-2 and the pro-fibrotic factor transforming growth factor beta were elevated in the hearts of exercised compared with sedentary *mdx* mice. The wall thickness to lumen diameter ratio of the pulmonary trunk was significantly increased in the exercised *mdx* mice (0.11 ± 0.04 in sedentary vs. 0.28 ± 0.12 in exercised), as was the thickness of the right ventricle wall, which suggests the occurrence of pulmonary hypertension in those animals. It is suggested that diaphragm degeneration is a main contributor to right ventricle dystrophic pathology. These findings may be relevant for future interventional studies for Duchenne muscular dystrophy-associated cardiomyopathy.

Key words: cardiomyopathy; Duchenne muscular dystrophy; fibrosis; metalloproteinases-2.

Introduction

Duchenne muscular dystrophy (DMD) is lethal and is the most common form of myopathy, affecting approximately 1 in 6000 live male births (Mendell et al. 2012). DMD is caused by the loss of dystrophin, which is a structural protein of the skeletal and cardiac muscle fibers. Loss of dystrophin results in necrosis and progressive weakness in appendicular and diaphragm (DIA) muscles (Engel et al. 1994; De Bruin et al. 1997; Humbertclaude et al. 2012).

In certain diseases, diaphragm weakness causes functional and structural changes in the cardiac muscle (Coats, 1996;

Voelkel et al. 2006; Versteegh et al. 2007; Alonso-Gonzalez et al. 2013). In DMD, diaphragm weakness is usually observed (De Bruin et al. 1997; Humbertclaude et al. 2012). More than 90% of DMD patients display cardiomyopathy, and nearly 10–20% die from heart failure (Finsterer & Stollberger, 2003; Fayssoil et al. 2010; Spurney, 2011; Mosqueira et al. 2013).

Understanding the natural history of progressive cardiomyopathy is relevant because it can influence the therapy to be used. In DMD, it is not clear whether DIA injury can contribute to cardiac damage. The high variability in the degrees of DIA involvement in DMD, in addition to the obvious risks in obtaining heart and DIA biopsies from these patients, has limited our understanding of the interplay between diaphragm and cardiac damage in this disease (Humbertclaude et al. 2012).

In this study, we used histological and histomorphometric methods to examine whether worsening degeneration in the dystrophic diaphragm by using physical exercise could affect cardiac structure in *mdx* mice, which is the experi-

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Accepted for publication 22 December 2015
Article published online 29 January 2016

mental model of DMD (Stedman et al. 1991). The levels of metalloproteinases and transforming growth factor beta, molecular markers of fibrosis and inflammation (Bernasconi et al. 1995; Gosselin et al. 2004; Kumar et al. 2010), were evaluated in dystrophic DIA and heart. Preliminary results were presented at the European Muscle Conference (Barbin et al. 2014).

Materials and methods

Animals

Male *mdx* (C57BL/10-*Dmd*^{*mdx*}/PasUnib) mice that were 11 months old at the beginning of experiments were used. Some male normal (C57BL/10) mice that were 11 months old were used for focused experiments. All experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; protocol #2377-1) and the guidelines set forth by our Institution.

Experimental design

Two groups of animals (exercised *mdx* and exercised normal) were made to swim for 60 min once a day, 6 days a week, for 2 months. Mice start swimming for 10 min (day 1) with a 10-min increase each day, reaching the 60-min session by the end of the first week. We used a swimming apparatus (Evangelista et al. 2003) in which the water was maintained at 30–32 °C. Another group of mice was kept sedentary (herein called sedentary *mdx* and sedentary normal mice). Twenty hours after the last swimming exercise session, the mice were anesthetized with a mixture of ketamine hydrochloride (130 mg kg⁻¹; Francotar, Virbac, São Paulo, Brazil) and xylazine hydrochloride (6.8 mg kg⁻¹, 2% Virbaxyl; Virbac). Hearts and diaphragm (DIA) muscles were dissected out.

Histological and morphometric analysis

We evaluated the following: (1) fibrosis, (2) inflammation-degeneration in the heart and DIA, (3) the wall/lumen ratio of the pulmonary trunk, and (4) the thickness of the right ventricle wall. Hearts and DIA of exercised ($n = 5$), sedentary ($n = 5$) *mdx* mice, and sedentary ($n = 3$) and exercised ($n = 3$) normal mice were flash-frozen in cooled isopentane before mounting in Tragacanth (Sigma-Aldrich, St. Louis, MO, USA). Cryostat transverse cross-sections (6 µm thick) of the whole DIA and heart were stained with Masson's trichrome for the observation of fibrosis. The total cross-sectional area and fibrosis area from the entire section were measured in dystrophic muscles using a microscope (Nikon Eclipse, Nikon Instruments Inc., Melville, NY, USA) with 10× and 20× magnification (hearts and DIA, respectively) attached to a personal computer. Measurements were taken using IMAGEPRO-EXPRESS software (Pereira et al. 2012). The mean fibrosis area was expressed as the percentage of the total cross-sectional area.

The density of patches of inflammatory cell-degenerating cardiomyocytes from dystrophic heart was evaluated directly using a hand counter on a microscope (Nikon Eclipse) fitted with a graduated eyepiece micrometer at 100× magnification. The results are expressed as patches per mm².

To estimate the wall/lumen ratio of the main pulmonary trunk, images of the pulmonary trunk were taken using the microscope (Nikon Eclipse) at 10× magnification. Measurements of the wall thickness, major axis (a) and minor axis (b) at right angles to the major axis of the lumen of the trunk were made using IMAGEPRO-EXPRESS software. The lumen diameter of the sections (D_i) was calculated from the equation $D_i = \sqrt{ab}$ (Komolafe et al. 2009). The wall to lumen ratio of the pulmonary trunk was calculated as the thickness of the wall/diameter of the lumen (Komolafe et al. 2009).

Zymographic analysis

We examined the following isoforms of metalloproteinases-2 (MMP-2): active MMP-2, pre MMP-2, and pro MMP-2. Hearts and DIA from both the exercised ($n = 6$) and sedentary ($n = 6$) *mdx* mice were flash-frozen in cooled isopentane. Heart and DIA were divided, half used to zymography and the other half used to Western blot. Zymography assays were performed on 7.5% polyacrylamide gels. Gelatinolytic bands were visualized by staining the gels with Coomassie Brilliant Blue dye (Sigma-Aldrich). Bands were quantified by densitometry (IMAGEJ 1.38X software; National Institutes of Health, Bethesda, MD, USA). We performed 2 pools samples, with 3 mice (3 half hearts or 3 half DIA) in each pooled sample. Zymographic analysis was repeated three times, for each pooled sample.

Western blot analysis

The levels of transforming growth factor beta (TGF-β; Sigma) were quantified using western blotting. As described above, we performed two pool samples, with three mice (three half hearts or three half DIA) in each pooled sample. Western blot was repeated three times for each pooled sample.

As previously described (Ferretti et al. 2011), heart and DIA muscles were lysed in lysis buffer (1% Triton, 10 mM sodium pyrophosphate, 100 mM NaF, 10 g mL⁻¹ aprotinin, 1 mM PMSF and 0.25 mM Na₃VO₄), the samples were centrifuged, and the soluble fraction was resuspended in Laemmli loading buffer [2% sodium dodecyl sulfate (SDS), 20% glycerol, 0.04 mg mL⁻¹ bromophenol blue, 0.12 M Tris-HCl, pH 6.8 and 0.28 M-mercaptoethanol]. An aliquot (60 µg) of the total protein homogenate from control and exercised *mdx* DIA and heart was loaded onto 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad Laboratories submer-sion electrotransfer apparatus, Hercules, CA, USA). After blocking with 5% skim milk-Tris-HCl-buffered saline Tween buffer (TBST; 10 mM Tris-HCl, pH 8, 150 mM NaCl and 0.05% Tween 20), the membranes were incubated with the primary antibody overnight at 4 °C, followed by a peroxidase-conjugated secondary antibody [peroxidase-labeled affinity-purified mouse IgG antibody (H+L, KPL, Gaithersburg, MD, USA), and developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA). Ponceau-stained membranes were used to control for protein loading as described previously (Fogagnolo Mauricio et al. 2013). The luminescent signal from the western blot bands was captured (G: Box iChemi camera; Syngene, Cambridge, UK), and band intensities were quantified using the analysis software provided by the manufacturer (GENE TOOLS Version 4.01, Syngene, Cambridge, UK).

Statistical analysis

All data are expressed as the means ± standard deviation (SD). Statistical analysis for direct comparison between the means of two

groups was performed using Student's *t*-test and ANOVA for comparison between the means of three groups or more, followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Diaphragm

In normal DIA, exercise did not lead to fibrosis (Fig. 1A). In exercised *mdx*, fibrosis was increased (Fig. 1C). Quantitative analysis (Fig. 1) showed a significant ($P < 0.05$) increase (60%) in the fibrotic area in DIA from exercised *mdx* mice (33.3 ± 6.0) compared with sedentary *mdx* mice (20.9 ± 6.1 ; values are expressed as percentage of total sectional area). Zymographic analysis (Fig. 2) revealed that the levels of pro MMP-2, pre MMP-2 and active MMP-2 in the DIA of exercised *mdx* mice were, respectively, 28, 15 and 26%, each higher than those observed in the sedentary group ($P < 0.05$).

Heart

In both right and left ventricles (Fig. 3A,B), myocardial fibrosis, as identified by Masson's trichrome stain (Fig. 3C, D), and patches of inflammatory cells–degenerating cardiomyocytes, represented by lymphomononuclear infiltrations and cardiomyocyte lysis (Fig. 3E), were observed in intramyocardial, subendocardial and subepicardial areas in both exercised and sedentary *mdx* mice.

Quantitative analysis (Fig. 3) revealed that the histological parameters differed between hearts from sedentary and exercised *mdx* mice. The percentage of myocardial fibrosis in total heart (Fig. 3) was 46% higher in the exercised *mdx* mice ($8.6 \pm 0.6\%$ of fibrosis) than in the sedentary mice ($5.9 \pm 0.7\%$ of fibrosis in sedentary). In the right ventricle (RV), fibrosis increased significantly by 45% ($8.3 \pm 0.6\%$ of

fibrosis in sedentary vs. $12.0 \pm 0.6\%$ in exercised *mdx* mice); in the left ventricle (LV), it was 36% higher ($5.6 \pm 0.7\%$ in sedentary vs. $7.6 \pm 0.8\%$ in exercised *mdx* mice). The density of patches of inflammatory cells–degenerating cardiomyocytes increased in hearts from exercised mice (Fig. 3). This increase was approximately 95% in the right ventricle (2.3 ± 0.6 patches mm^{-2} in sedentary vs. 4.5 ± 0.8 patches mm^{-2} in exercised *mdx* mice) and 71% in the left ventricle (1.4 ± 0.6 patches mm^{-2} in sedentary vs. 2.4 ± 0.5 patches mm^{-2} in exercised *mdx* mice). The thickness of the right ventricle wall was increased by 40% in the exercised *mdx* mice (exercised *mdx* mice: $0.203 \pm 0.015^*$ cm; exercised normal mice: 0.129 ± 0.027 cm; sedentary *mdx* mice: 0.145 ± 0.017 ; $*P < 0.05$ compared to exercised normal and sedentary *mdx* mice; ANOVA).

Figure 4 shows the histological features of the main pulmonary trunk in the *mdx* mice, focusing on medial wall proliferation. The wall thickness to lumen diameter ratio of the pulmonary trunk (Fig. 4) was significantly increased ($P < 0.05$) in the exercised *mdx* mice (0.11 ± 0.04 in sedentary *mdx* vs. 0.28 ± 0.12 in exercised *mdx*), which indicated a decrease in the relative lumen diameter in those animals. Exercise did not change the wall thickness to lumen diameter ratio of the pulmonary trunk in normal mice (0.076 ± 0.007 in sedentary normal vs. 0.075 ± 0.014 in exercised normal; $P > 0.05$).

Zymographic analysis (Fig. 5) showed that the expression of pro MMP-2, pre MMP-2 and active MMP-2 were, respectively 28% (7.4 ± 1.7 in sedentary vs. 9.4 ± 1.0 in exercised *mdx*), 21% (11.3 ± 0.4 in sedentary *mdx* vs. 13.3 ± 1.3 in exercised *mdx*) and 32% (6.7 ± 1.3 in sedentary *mdx* vs. 8.8 ± 1.8 in exercised *mdx*) higher in hearts from exercised *mdx* mice than in the sedentary *mdx*. The levels of TGF- β (Fig. 6) were significantly elevated (1.8 ± 0.4 in sedentary *mdx* vs. 3.7 ± 1.3 in exercised *mdx*; $P < 0.05$) in the hearts of the *mdx* mice from the exercised group.

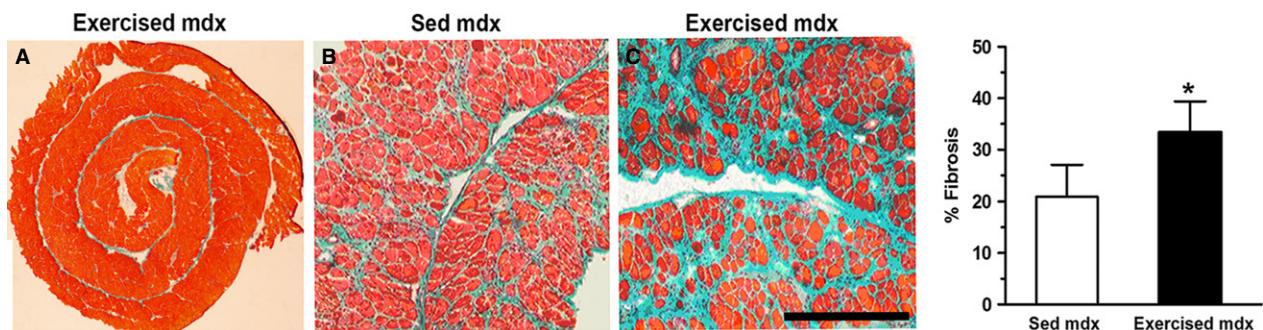


Fig. 1 Exercise-increased fibrosis in the dystrophic diaphragm. Representative transverse histological sections of diaphragm muscles from normal C57BL/10 (A), sedentary (B; Sed) *mdx* mice and exercised (C) *mdx* mice. Fibrosis (green, Masson trichrome) fills the spaces between muscle fibers mainly seen in the exercised *mdx* mice. Histomorphometric analysis (bar graph) revealed that the mean fibrosis area was significantly larger ($*P < 0.05$) in the diaphragms of exercised *mdx* mice than in those of sedentary *mdx* mice. Data are presented as the mean \pm SD. Scale bar (shown only in C): 1350 μm (A), 250 μm (B,C).

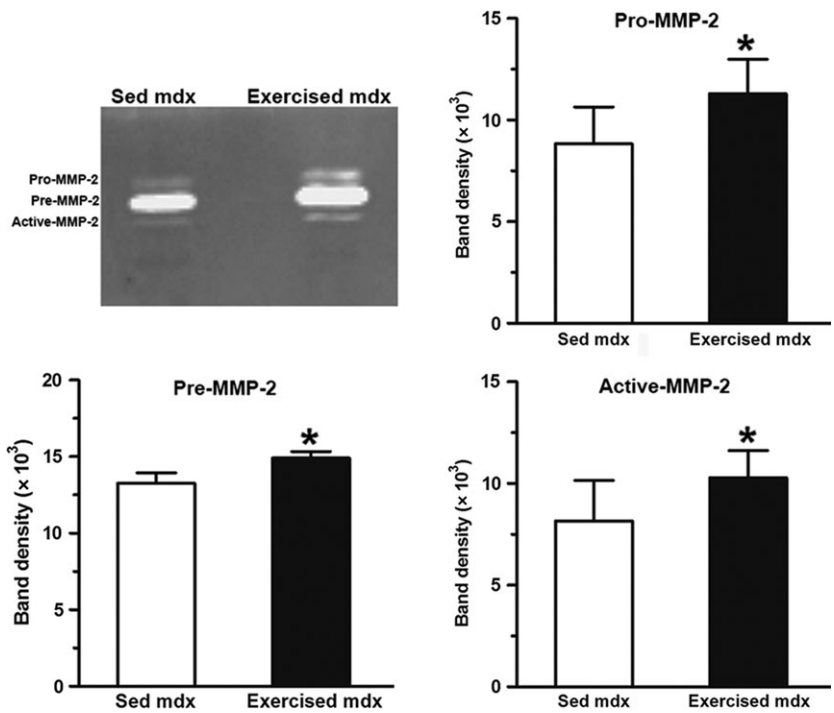


Fig. 2 Exercise-increased metalloproteinases in the diaphragms of *mdx* mice. Zymographic analysis of metalloproteinases obtained from the diaphragm muscles of control (sedentary, Sed) and exercised *mdx* mice. The graphs show the activity level of pro-MMP-2, pre-MMP-2 and active MMP-2. Data are presented as the mean \pm SD. *Significantly different from sedentary mice ($P < 0.05$).

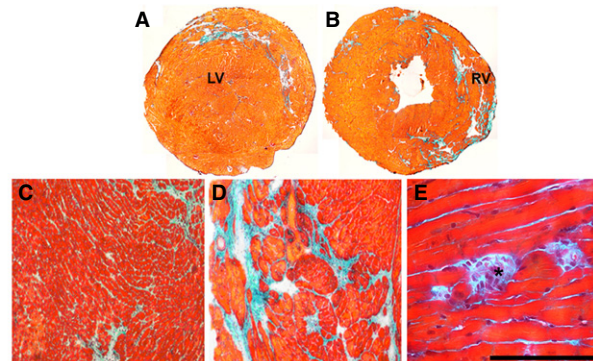
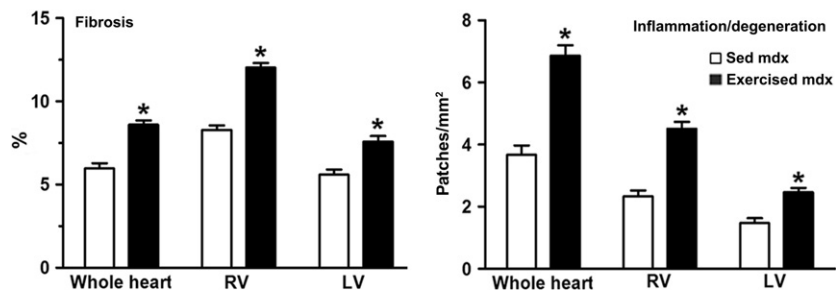


Fig. 3 Myocardial fibrosis and inflammation/degeneration were increased by exercise. (A, C) Sedentary *mdx*. (B, D, E) Exercised *mdx*. Fibrosis (Masson's trichrome; green) is seen among cardiomyocytes in whole heart sections (A, B) and in histological sections (C, D). Inflammation-degeneration (asterisk in E). Quantitative analysis (bar graph) revealed that exercise worsened the heart histopathological features, mainly seen in the right ventricle (RV). LV, left ventricle. Data are presented as the mean \pm SD. *Significantly different from sedentary mice ($P < 0.05$). Scale bar (shown only in E): 400 μ m (C, D), 100 μ m (E).



Discussion

Physical exercise exacerbated diaphragm degeneration

In many studies, physical exercise has been used to accentuate the dystrophic phenotype (Bouchentouf et al. 2006; De Luca et al. 2008; Radley-Crabb et al. 2012), although it has

been reported that physical exercise seems to benefit some skeletal muscles of the *mdx* mice (Dupont-Versteegden et al. 1994; Hayes & Williams, 1996; Call et al. 2010; Duong et al. 2011; Baltgalvis et al. 2012). In the present study, we observed a significant increase in the fibrotic area in exercised *mdx* compared with sedentary *mdx* mice. Concomitantly, the expression of the isoforms of MMP-2 was also higher in the DIA of *mdx* mice submitted to exercise.

MMP-2 is known to degrade basement membrane proteins, collagen, fibronectin and laminin after injury in cardiac and skeletal muscle fibers (Page-McCaw et al. 2007) and dysregulation of matrix metalloproteinases in *mdx* mice is associated with a worsened dystrophic phenotype (Li et al. 2009). Therefore, the exercise protocol used here was efficient in exacerbating diaphragm degeneration in *mdx* mice.

Diaphragm degeneration and cardiac structure in *mdx* mice

Although improvements in cardiac function have been observed after physical exercise in *mdx* mice, even in the presence of a failing diaphragm (Selsby et al. 2013), other studies have indicated that cardiac structure is negatively

affected by physical exercise (Nakamura et al. 2002; Costas et al. 2010). Cardiac damage in *mdx* mice is aggravated when voluntary activity is restored, possibly due to cardiac stress (Townsend et al. 2008). In the present study, we observed increased levels of the pro-fibrotic factor TGF- β in the cardiac muscle of exercised *mdx* mice, along with increased fibrosis and inflammatory areas. Therefore, the present exercise protocol also negatively affected the cardiac muscle.

In many conditions, diaphragm weakness produces apneic events and pulmonary vasoconstriction that leads to pulmonary hypertension and right-sided ventricular damage (Coats, 1996; Voelkel et al. 2006; Versteegh et al. 2007; Alonso-Gonzalez et al. 2013). Recently, using a biventricular cardiac catheterization technique to measure

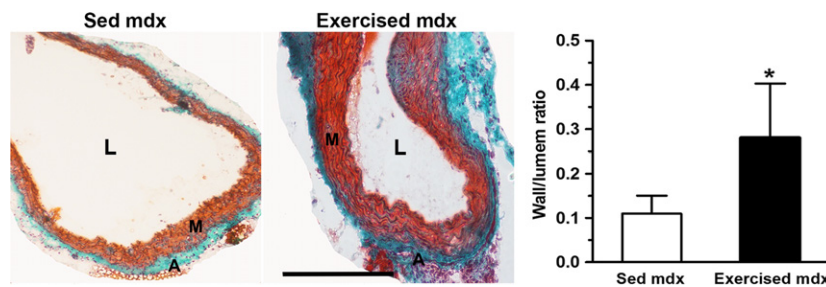


Fig. 4 Representative photomicrographs showing histological features of the pulmonary trunk from sedentary (Sed *mdx*) and exercised *mdx* mice with Masson staining. Quantitative analysis (bar graphs) of media wall thickness to lumen ratio of pulmonary arteries. Compared with the control (Sed *mdx*) group, the media thickness of the pulmonary arteries was markedly increased in the exercised *mdx* group. The development of medial wall thickening suggests the presence of accentuated diaphragm degeneration in the exercised *mdx* group compared with controls. The results are expressed as the mean \pm SD. * $P < 0.05$. A, adventitial layer; L, lumen; M, media layer. Scale bar: 1.2 μ m.

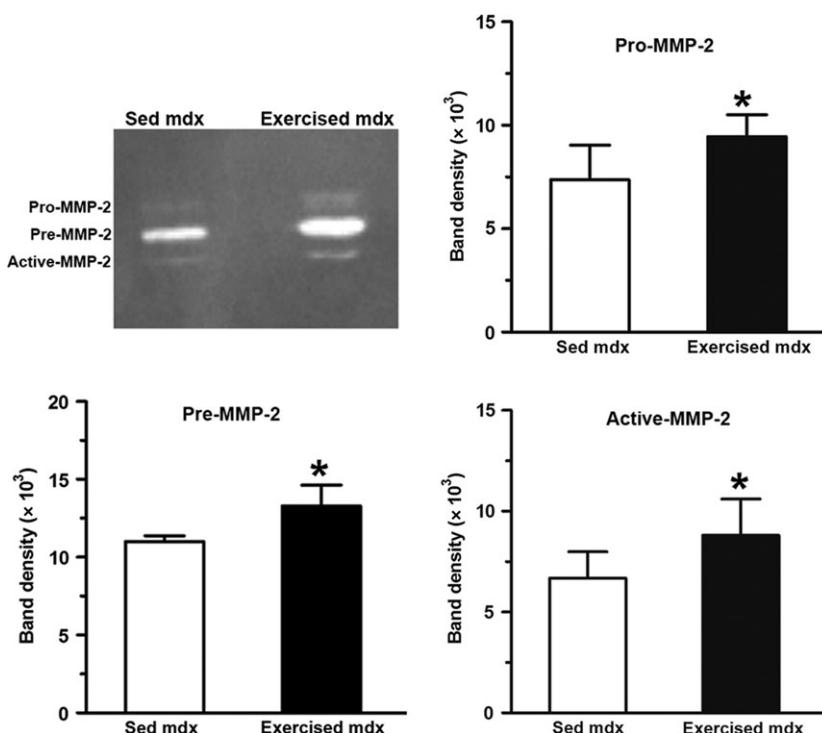


Fig. 5 Exercise-increased metalloproteinases in the hearts of *mdx* mice. Zymographic analysis of metalloproteinases obtained from the heart of control (sedentary, Sed) and exercised *mdx* mice. The graphs show the activity level of pro-MMP-2, pre-MMP-2 and active MMP-2. The results are presented as the mean \pm SD. *Significantly different from sedentary mice ($P < 0.05$).

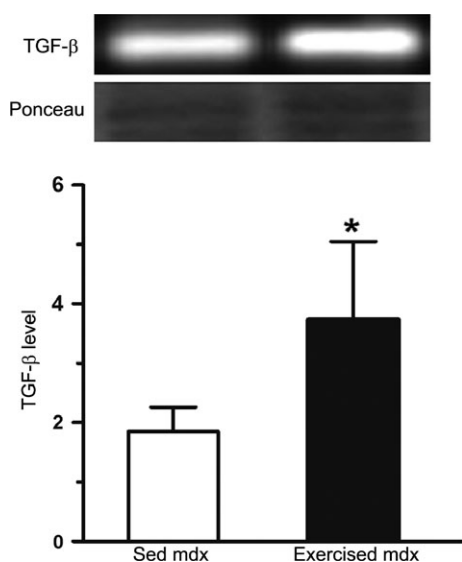


Fig. 6 Western blot analysis of transforming growth factor beta (TGF- β) in crude extracts of hearts from control (sedentary, Sed) and exercised *mdx* mice. Graphs show the relative value of TGF- β . The Ponceau-stained membranes were used to control for protein loading. Data are presented as the mean \pm SD. *Significantly different from sedentary mice ($P < 0.05$).

ventricular function, an early involvement of the right ventricle in the cardiomyopathy of the *mdx* mice was demonstrated, possibly related to increased constriction of the pulmonary vessels (Meyers & Townsend, 2015). Pulmonary vasoconstriction could be due to abnormal sympathetic activation or to hypoventilation related to respiratory dysfunction (Meyers & Townsend, 2015). In the present study, we demonstrate pulmonary artery remodeling concomitant with an increase in the right ventricle thickness wall, histological evidence of the possible presence of pulmonary hypertension only in the *mdx* mice. In normal exercised mice, no anatomical changes in DIA (no fibrosis in exercised normal) and heart were detected. Conversely, in *mdx* mice with worsened diaphragm degeneration (exercised group), histological analysis revealed (1) that RV was more affected than the left ventricle, (2) an increase in the thickness of the right ventricle and (3) an increase in the pulmonary trunk media thickness, as demonstrated by the higher wall thickness to lumen diameter ratio. Increasing media thickness is a histological hallmark predicting the occurrence of pulmonary hypertension (Durmowicz & Stenmark, 1999; Drexler et al. 2008; Sommer et al. 2008). Elevation of the pulmonary pressure induces remodeling of the pulmonary trunk, characterized by an increase in the media thickness (Durmowicz & Stenmark, 1999; Drexler et al. 2008; Sommer et al. 2008). The finding that diaphragm degeneration was concomitant with cardiac morphological changes (fibrosis) argues in favor of the suggestion that respiratory dysfunction and hypoventilation (Meyers & Townsend, 2015) are the main contributors to pulmonary hypertension in *mdx* mice.

Clinical significance

Although caution is required when the results obtained with the *mdx* mice are transposing to human dystrophy, the present findings may be of clinical importance. Regarding the time-course of the progressive cardiac damage, it has been mostly portrayed that the left ventricle is the primary site of injury in DMD, with the right ventricle being spared (Finsterer & Stollberger, 2003). However, a predominance of right ventricular failure with the left ventricle being only slightly affected has also been observed in DMD, even in terminal stages of the disease (Sanyal et al. 1978; Yotsukura et al. 1988; Nishimura et al. 2001). Furthermore, although some DMD studies have indicated that the risk of cardiac failure is inversely related to diaphragm damage, other studies noted that failure of the left heart correlates with diaphragm degeneration (Yotsukura et al. 1988; Backman & Nylander, 1992; FINDER et al. 2004). Interestingly, diaphragm weakness and restrictive respiratory insufficiency are highly heterogeneous in DMD (De Bruin et al. 1997; Humbertclaude et al. 2012). The present findings motivated us to hypothesize that the degree of diaphragm degeneration may play a role in the natural history of the cardiomyopathy associated with DMD. Possibly, in patients with a diaphragm more severely affected, the right ventricle may be compromised earlier and more intensely compared with the left ventricle.

The present findings may also be of interest for the treatment of DMD-associated cardiomyopathy. Adrenergic beta-antagonists and angiotensin-converting enzyme inhibitors have been the most commonly recommended as first-line treatments for all DMD patients, independent of their clinical signs (Fayssol et al. 2010). However, in patients with a more accentuated diaphragm dysfunction, targeting pulmonary hypertension should result in a better outcome for cardiac function. In a recent study, treatment with sildenafil citrate, which was successfully used as a pulmonary anti-hypertensive drug (Galie et al. 2005), failed to improve cardiac function in DMD patients. However, in that study there was a lack of information regarding diaphragm status, and in right ventricular function in particular (Leung et al. 2014). Therefore, the present results suggest that the evaluation of diaphragm degeneration and respiratory function is of great relevance when designing a better therapy for cardiomyopathy in DMD, which is in line with the concept that treatment of DMD-associated cardiomyopathy requires diaphragm preservation, as shown in the *mdx* mice (Townsend et al. 2008; Crisp et al. 2011; Wasala et al. 2013; Betts et al. 2015).

Concluding remarks

In this morphometric study, we demonstrated that swimming exercise induces structural changes in the heart of the *mdx* mouse. The cardiac changes could be due to a direct

effect of the exercise on the heart of the *mdx* mouse, since it is well described that swimming exercise induces physiological or pathological remodeling of the heart, mainly in the left ventricle, depending on the intensity of the exercise (Fernandes et al. 2015; Tosic et al. 2015). Alternatively, cardiac changes could be related to diaphragm degeneration, which was exacerbated in the exercised *mdx* mice. Supporting this alternative, we found that the right ventricle was preferentially affected, in agreement with a recent functional study (Meyers & Townsend, 2015). Furthermore, exercise did not affect the right ventricle of the normal mice, with no diaphragm degeneration. Although there is no direct evidence of changes in pulmonary pressure in *mdx* mouse, the significant increase in the thickness of the media wall of the pulmonary trunk and of the right ventricle wall, possibly related to hypoventilation due to diaphragm degeneration, may be consistent with increased pulmonary pressure in the *mdx*. These findings may be relevant for future interventional studies for DMD-associated cardiomyopathy.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grants 04/15526-9, 08/58491-1, 2021/03498-7 and 14/04782-6). H.S.N. and M.J.M. are recipients of fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grants 302831/2013-4, 303320/2013-3). J.A.P. was the recipient of a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes)-Proex fellowship.

Conflict of interest

None.

Author contributions

Concept/design (H.S.N., I.C.C.B.), acquisition of data (I.C.C.B., J.A.P., M.R., D.O.M.), data analysis/interpretation (H.S.N., M.J.M., I.C.C.B., J.A.P., D.O.M.), drafting of the manuscript (H.S.N., M.J.M.), critical revision of the manuscript (H.S.N., M.J.M., J.A.P.) and approval of the article (all authors).

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