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Muscle Damage, Metabolism, & Oxidative Stress in *mdx* Mice: Impact of Aerobic Running

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Abstract

Purpose—We tested how a treadmill exercise program influences oxygen consumption, oxidative stress and exercise capacity, in the *mdx* mouse, a model of Duchenne muscular dystrophy.

Methods—At age 4 weeks *mdx* mice were subjected to 4 weeks of twice-weekly treadmill exercise. Sedentary *mdx* and wild-type mice served as controls. Oxygen consumption, time-to-exhaustion, oxidative stress, and myofiber damage were assessed.

Results—At age 4 weeks there was a significant difference in exercise capacity between *mdx* and wild-type mice. After exercise, *mdx* mice had lower basal oxygen consumption and exercise capacity, but similar maximal oxygen consumption. Skeletal muscle from these mice displayed increased oxidative stress. Collagen deposition was higher in exercised versus sedentary mice.

Conclusion—Exercised *mdx* mice exhibit increased oxidative stress, as well as deficits in exercise capacity, baseline oxygen consumption, and increased myofiber fibrosis.

Keywords

Exercise; MDX; Oxidative Stress; Treadmill; DMD; Oxygen Consumption

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INTRODUCTION

Duchenne muscular dystrophy (DMD), a progressive, debilitating neuromuscular condition, remains among the most common adolescent genetic muscle diseases with a prevalence of approximately 1 in every 5,000 male births ¹. Skeletal and cardiac muscles of patients with DMD lack the protein dystrophin, which is responsible for attaching the muscle extracellular matrix to the cytoskeleton and acts as a force-transducer to protect muscle from repeated contraction-induced injury ^{2,3}. Patients afflicted with DMD typically do not live past their late 20s with an average life expectancy of 19 to 25 years ⁴. The genotypic animal model for the disease, the *mdx* mouse, has been studied extensively in an attempt to produce outcome measures useful for monitoring disease progression and advance potential treatments that might translate to extending quality and quantity of life of DMD patients ⁵⁻⁸.

Important disease features of dystrophic muscle include the development of fibrotic lesions within muscle ^{9,10}, increases in oxidative stress ¹¹, and perturbed oxygen utilization ¹². Additionally, dystrophic muscle fibers are known to be more vulnerable to exercise-induced muscle damage ^{3,13,14} making it important to study how they adapt to changes in physical activity, and if the adaptations are different than those observed in non-diseased skeletal muscles.

Specific monitored exercise protocols present a platform to study how varying amounts of muscular contraction influence the progression of disease pathology in dystrophin-deficient muscle. Experiments done using a specific quantifiable treadmill exercise protocol in *mdx* mice have demonstrated increases in muscle damage and protein oxidation as a result of the running ^{15,14}.

Previous research using *mdx* mice and the exercise protocol we used in this study has shown a link between exercise workload and adverse events, including increases in oxidative stress and serum creatine kinase, and deficits in forelimb strength ¹⁶. We have examined how these parameters might interplay with oxygen consumption, exercise ability, and muscle fibrosis and oxidative stress in other skeletal muscles. The understanding of how oxidative stress is linked to DMD is still unclear. The accumulation of oxidized protein is an important outcome of interest for several diseases related to cardiovascular and skeletal muscle biology, including stroke, atherosclerosis, diabetes, amyotrophic lateral sclerosis, and essential hypertension ^{17,18}. Exercise has been shown to improve the degree of oxidative stress ^{19,20} and exercise capacity in normal skeletal muscles but might further increase muscle damage due to contraction-induced injury in the context of dystrophic muscles. Investigating systemic, whole-animal basal and maximal oxygen consumption would also shed light on oxygen metabolism in this DMD model as it responds to exercise. Evaluating these outcome measures is important as improvements in treatment strategies for DMD allow for greater amounts of physical activity and may exacerbate negative aspects of the disease that require monitoring.

Prior research utilizing what has become a standard operating procedure treadmill exercise for *mdx* mice has gaps where data may be informative for designing and monitoring therapeutic interventions. Abdominal muscles, which are particularly critical for respiratory

function in dystrophy where the diaphragm muscle is severely compromised, have never been evaluated for the oxidative or fibrotic consequences of exercise. Although a recent study has begun to characterize alterations in further exercise ability after treadmill exercise, these studies did not correlate time to exhaustion with oxygen consumption²¹. Our aim was to determine how a quantifiable, specific, and monitored treadmill exercise protocol would influence several facets of DMD disease pathology in the *mdx* mouse in an attempt to identify additional outcome measures of the disease. We tested the hypothesis that *mdx* mice maladapt to exercise via increases in muscle damage, fibrosis, oxidative stress, and blunted exercise capacity on a graded exercise test. We subjected *mdx* mice to twice-weekly treadmill exercise, and measurements of muscle fibrosis and oxidative stress were taken to determine the extent of muscle disease, oxidative stress, and exercise capacity as the mice respond to treadmill exercise.

MATERIALS AND METHODS

Ethics Statement

All experiments were approved by the Institutional Animal Care and Use Committee of The Ohio State University (OSU) and conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. All research personnel were blinded throughout all phases of this study.

Animal procedures and groups

All experiments were performed on 4- to 8-week-old male and female dystrophic *mdx* mice and non-dystrophic control C57Bl/10 mice. The 3 experimental groups were: 1) Sedentary C57BL10 (n=5); 2) Sedentary *mdx* (n=10); and, 3) Exercised *mdx* (n=7). For identification purposes, mice from each litter were given numbered ear tags at age 3 weeks. Groups subjected to exercise were started at age 4 weeks and continued until age 8 weeks. Animals were housed under standard conditions with *ad libitum* access to food and water, and a 12:12-hour light:dark photocycle.

All mice were sacrificed by cervical dislocation at age 8 weeks following the metabolic measurements. Heart, quadriceps, diaphragm, extensor digitorum, tibialis anterior, and abdominal muscle were excised immediately. Half of each tissue sample was immediately flash frozen in liquid nitrogen for biochemical and oxidative stress measurement, while the other half was embedded in optimal cutting temperature (OCT) cryoprotectant and frozen in liquid nitrogen-cooled isopentane for preparation of histological sections.

Exercise protocol

The TREAT-NMD recommended protocol “Use of treadmill and wheel exercise for impact on *mdx* mice phenotype M.2.1_001” (<http://www.treat-nmd.eu/research/pre-clinical/SOPs>) exercise regimen consisting of 30 minute treadmill running at a speed of 12 m/min was used. The rodent treadmill was an Exer 3/6 from Columbus Instruments (USA). Running lanes were separated with clear dividers so that mice could see one another while exercising. The treadmill remained horizontal (0 percent incline), and *mdx* mice were run in groups of 2 or 3, with each mouse in an individual lane.

Exercise bouts covered 4 weeks and were performed twice-weekly at 12 m/min with no more than 48–72 hours between bouts, for a total of 8 bouts. The electrical shock grid was not used. If at any time during the 30 min exercise session a mouse fatigued and could no longer run, the procedure was as follows: the speed of the treadmill belt was turned down to 6 m/min for 2 minutes, then increased to 12 m/min for the remainder of the 30 minute bout. Mice that could not perform all bouts of exercise were excluded from the study. All exercise bouts were performed on this specialty rodent treadmill, while metabolic and maximal exercise tests were performed on another specialty treadmill, described below.

Metabolic measurements

To determine basal and maximal oxygen (VO_2) and carbon dioxide (VCO_2) consumption, animals were first housed for a period of 5 minutes inside the clinical laboratory animal monitoring system (CLAMS) for resting measurements, and they were then run until exhaustion inside the CLAMS. The CLAMS system consists of a single-lane rodent treadmill enclosed inside an airtight cage that allows for all gas entering and exiting to be measured. All mice completed a maximal exercise test at age 4 weeks (prior to the start of the exercise bout) and again at age 8 weeks (at the conclusion of the 4 week exercise program). The maximal exercise test consisted of a 5-minute stabilization period where the treadmill remained motionless inside the chamber. After the first 5 minutes, the speed was increased to 6 m/min while remaining at 0% grade for 7 minutes. At the 12 minute mark the incline was increased to 20% grade and remained there for duration of the test, and speed was increased 1 m/min each minute until exhaustion. Exhaustion was defined as the inability to remain on the treadmill belt and resting on the electrical shock grid for more than 3 seconds, a plateau of VO_2 , and/or a respiratory exchange ratio (RER) of ≤ 0.95 . During the test measurements of VO_2 , VCO_2 , and heat were taken every 15 seconds.

Hydroxyproline analysis

Quantification of quadriceps, diaphragm, abdominal, and cardiac hydroxyproline content was performed using the method of Reddy and Enwemeka²². Whole muscle samples, representative of the entire muscle, were weighed, homogenized, and hydrolyzed for 24 hours in 2N sodium hydroxide at 100 degrees C. Hydrolyzate (20 μL) was mixed with 450 μL of 0.056M chloramine T reagent (Sigma-Aldrich) in citrate buffer and oxidized at room temperature for 25 minutes. 500 μL of 1M Ehrlich reagent [15 g of 4-(dimethylamino) benzaldehyde (Sigma-Aldrich), 33 mL n-propanol, 66 mL perchloric acid] was added to each sample, mixed, and incubated at 65 degrees C for 20 minutes, followed by spectrophotometric measurements taken at 550 nm. A standard curve (0–5,000 nM, trans-4-hydroxy-L-proline; Sigma-Aldrich) was included for each assay. Measurements of hydroxyproline were normalized to tissue weight, and results are reported in μg hydroxyproline/mg tissue.

Oxidative stress measurements

To evaluate oxidative stress levels and redox state of heart, abdominal, and quadriceps tissue, we measured the reduced glutathione (GSH) ratio with oxidized glutathione (GSSG) using a GSH-sensitive nitroxide probe, RSSR (Enzo life sciences, Farmingdale, NY) as detected by electron paramagnetic resonance (EPR) spectroscopy. The EPR spectra of RSSR is

characteristic of biradicals exhibiting intramolecular spin exchange between 2 radical fragments resulting in the appearance of broad triplet spectral components in addition to the conventional sharp triplet spectral pattern of the mononitroxide. In the presence of GSH, the RSSR probe reacts with GSH resulting in decrease of the broad spectral component and increase in the sharp spectral component. The ratio of the intensity between the broad and sharp components was measured and expressed as the RSSR/RSH ratio, therefore, allowing for quantification of the reduced and oxidized glutathione levels. Hence, a higher intensity ratio of the oxidized probe (RSSR) compared to the reduced glutathione product (RSH) (*i.e.*, RSSR/RSH) indicates lower relative amounts of GSH, higher amounts of oxidized protein, and more oxidative stress from ROS. Relative amounts of glutathione oxidation have been shown to be a major indicator of cellular redox state with lower GSH, higher GSSG, and a lower GSH/GSSG ratio being found in many different disease pathologies^{23,24}.

EPR (Bruker Biospin, Ettlingen, Germany) measurements were carried out on an X-band spectrometer with HS resonator at room temperature. General instrument settings are as follows: microwave power, 10 mW; modulation amplitude, 0.5 G; receiver gain $3.17\text{--}3.56 \times 10^5$, time constant, 82 ms, time sweep 42 s, number of scans: 5. The entire muscle was homogenized, and 5–10 mg of pulverized muscle was incubated in Newcastle buffer (4 M urea, 75 mM Tris, pH 6.8, 3.8% SDS) for 35 minutes. An analogue of Ellman reagent, a disulfide biradical of imidazoline (RSSR) was used as the probe for GSH. Isolated muscle cellular proteins were incubated with 10 μM RSSR and loaded into the EPR spectrometer. The same volume amount of protein isolate was used for all samples. EPR scans were analyzed using WinEPR software. The relative spectral peak intensities of both the monoradical and biradical products were obtained.

Histology

Heart and skeletal muscle tissue samples were embedded in optimal-cutting-temperature medium and frozen on liquid-nitrogen cooled isopentane for histological sections. Eight μm muscle cryosections were stained for intracellular immunoglobulin G (IgG) and Collagen I. Immunostaining was performed using a CY3-conjugated goat anti-mouse IgG antibody (1:100, Jackson research laboratories) as previously described²⁵ with co-staining with a rabbit anti-Collagen I antibody (Abcam, 1:150), followed by an Alexa 488-conjugated goat anti-rabbit secondary (Life Technologies, 1:200). The percentages of IgG and Collagen I stained pixels in the cross section of the heart were quantified using Image J software.

Statistical Analysis

All values are presented as means $\pm$ SEM. Oxygen consumption and exercise capacity at age 4 weeks, oxidative stress, immunohistology, and hydroxyproline content were analyzed with Student *t*-tests. Oxygen consumption and exercise capacity at age 8 weeks was analyzed using ANOVA with Bonferroni *post hoc* tests. A *P*-value of <0.05 was used to determine significance level.

RESULTS

Oxygen Consumption and Exercise Capacity

At age 4 weeks, prior to the start of the exercise protocol, there were no significant differences between sedentary *mdx* (Mdx4) mice and sedentary wild-type C57BL/10 mice (WT4) in body weight, basal, or maximal oxygen consumption. Sedentary *mdx* mice exhibited highly blunted exercise capacity when compared with WT4 mice, reaching similar maximal oxygen consumption values at much earlier TTEs (Figure 1A, $P=0.0045$).

At age 8 weeks following the exercise intervention, exercised *mdx* (Mdx8Ex) mice had significantly lower exhaustion times (Figure 1C, $P=0.043$) and basal oxygen consumption (Figure 1D) compared with sedentary *mdx* (Mdx8Sed) mice. There were no significant maximal oxygen consumption differences between groups at age 8 weeks (Figure 1E).

Following the 4 weeks of treadmill running the Mdx8Ex mice showed similar maximal oxygen consumption as Mdx8Sed mice (Figure 1E) in spite of the Mdx8Ex mice having lower basal oxygen consumption (Figure 1D) and earlier exhaustion times (Figure 1C).

Oxidative Stress

Chronic exercise has been shown to improve oxidant levels, specifically ROS, and to increase endogenous buffering of oxidant molecules systemically in normal animals^{19,26}. We evaluated the redox state of several muscles to determine whether this advantageous exercise adaptation was present in dystrophic mouse muscle. Quadriceps muscle from Mdx8Ex mice had significantly increased oxidative stress when compared with Sed *mdx* mice (Figure 2, $P=0.0361$). Abdominal muscles from Mdx8Ex *mdx* mice also showed significantly higher oxidative stress than Mdx8Sed *mdx* mice (Figure 2, $P=0.0020$).

Hydroxyproline Content

Contraction-induced skeletal muscle injuries, like those associated with treadmill running, may be a factor affecting the degree of fibrotic scarring seen in dystrophic muscles¹⁰. Hydroxyproline content can be measured to determine the amount of collagen in a tissue. Assays performed on skeletal and cardiac muscle showed that Mdx8Ex mice had significantly higher hydroxyproline deposition in both quadriceps (Figure 3, $P=0.0397$) and heart tissue (Figure 3, $P=0.0047$) when compared with Mdx8Sed mice. Abdominal muscle hydroxyproline content was not significantly different between Mdx8Ex and Mdx8Sed mice (Figure 3), and could potentially be due to the differences in how this muscle may be utilized during treadmill exercise when compared to the heart and quadriceps.

Immunohistology

Representative sections from Mdx8Ex heart muscle showed large increases in collagen scarring compared to Mdx8Sed (Figure 4), and further quantification of these histological sections from Mdx8Ex mice indeed showed significantly higher collagen-I scar amounts (Figure 5B, $P=0.0325$) than Mdx8Sed mice, confirming the hydroxyproline results and indicating that exercise increases formation of these non-functional scars. The accumulation of these pathogenic scars in cardiac muscle might ultimately manifest as the exhaustion time

deficits in Mdx8Ex mice (Figure 1C). Levels of dying myocytes, as indicated by the amount of intracellular IgG accumulation, were higher in Mdx8Ex hearts compared to Mdx8Sed, however the difference was not significant (Figure 5A, 0.27% versus 2.20%, $P=0.0958$). To investigate the amounts of ongoing myofiber damage in limb muscles, we quantitated the percentage of quadriceps muscle histological sections with accumulation of intracellular IgG. Analysis of IgG staining in quadriceps was not different between groups ($P=0.6773$ for IgG).

DISCUSSION

These results support our working hypothesis that dystrophic mice adapt to a specific treadmill exercise deleteriously, as expected, by exacerbating disease pathology and altering exercise capacity. The goal of this study was to examine comprehensively how a specific monitored treadmill intervention affected the *mdx* mouse with regard to myofiber damage and fibrosis, oxygen consumption, exercise capacity, and oxidative stress, in an attempt to discover better outcome variables for disease management and further study of how increases in physical activity affect dystrophy. It is known that this specific treadmill regimen may exacerbate disease pathology, however the effects on other parameters, namely oxygen consumption, remain unknown.

We observed that forced treadmill exercise increased the severity of dystrophic pathology as evidenced by increases in limb muscle damage and deficits in exercise capacity, which is supported by aspects of other studies utilizing this exercise protocol ^{15,16}.

The results show that after treadmill exercise, *mdx* mice had increased fibrotic scarring in both heart and quadriceps muscles, as well as blunted exercise capacity on a graded treadmill test. Additionally, exercised *mdx* mice showed more oxidative stress in skeletal muscle and a similar trend in the heart. Therefore, diseased skeletal muscles may adapt unfavorably to certain aspects of exercise, specifically to the exercise response of oxidative stress. **(sentence omitted)** Dystrophic mice have shown deficits in muscle force ²⁷, and their diminished capacity on a graded maximal exercise stress test supports deficits in cardiorespiratory fitness as well as skeletal muscle function.

Several studies using voluntary wheel-based running have shown exercise to be beneficial in *mdx* mice ^{28–30} even given the large distances these animals ran, in some cases up to 4km a day ²⁹. In our study, the animals ran a total of 2.7km over the course of 4 weeks, a significantly shorter distance than many other studies utilizing exercised *mdx* mice, and resulted in large increases in fibrotic muscle scarring and deficits in exercise capacity and oxygen metabolism, as expected. The varying results do not appear to be explained by the differences in running distances. Rather, it appears to be the intensity and modality of exercise that play a key role in how exercise affects dystrophic muscle. The specific prescribed running protocol used here was developed to exacerbate the skeletal muscle pathology of young *mdx* mice (<http://www.treat-nmd.eu/research/pre-clinical/SOPs>), but the effects on oxygen consumption and comparison between effects in limb, abdominal, and cardiac muscle fibrosis and ROS had not previously been carried out.

Maximal oxygen consumption remains among the best predictors of all-cause mortality³¹ and serves as an important marker of skeletal muscle and cardiovascular function. The exercise protocol used here had no effect on maximal oxygen consumption, but a rather large effect on baseline oxygen consumption. Thus the truly novel contributions of this study were that we saw a dampening of basal oxygen consumption, which could indicate that exercise was producing disruptive changes in oxygen utilization. This finding supports our finding that treadmill exercise influences exercise capacity deleteriously, and also confirms results from previous research²¹. After several weeks of exercise, *mdx* mice were utilizing similar levels of oxygen as sedentary and wild-type mice, but at significantly lower workloads, in line with research which showed that *mdx* mice have increased energy expenditure and protein turnover compared to wild-type controls³².

Myofiber necrosis and collagen scar formation remain hallmark features of dystrophic disease pathology. Necrotic muscle fibers become replaced with non-contractile connective tissue and cause a blunting in muscle function. Interestingly, exercise has shown to have anti-fibrotic effects in normal muscle by enhancing the microenvironment vascular supply in the muscle, promoting the secretion of favorable growth factors, and limiting fibrotic scar formation resulting from an acute muscle injury in muscle-derived stem-cells³³. However, we observed that exercise increases fibrotic scar damage in quadriceps limb muscles, abdominal muscles, and heart in dystrophic mice. One weakness of this study design was that no control conditions were used to assess the possibility that *mdx* mice incurred muscle damage during the maximal exercise test, which might further confound indices of muscle damage. It should be noted however, that all mice, either sedentary or exercised, were subjected to the maximal test, thus increasing internal validity.

Another key finding indicated the influence of exercise on the oxidative environment of multiple dystrophic muscles via ROS analysis. ROS play an important role in several cellular processes in healthy tissue. Healthy tissue responds favorably to oxidative stress after chronic bouts of exercise by increasing endogenous antioxidant system activity to maintain redox homeostasis³⁴. We hypothesized that *mdx* mice would adapt in an unfavorable manner to exercise by having increased ROS levels in both skeletal and cardiac muscle. Our EPR analysis indicated this to be correct: multiple muscles from exercised *mdx* mice had significantly increased oxidative stress, suggesting diseased muscle does not adapt favorably to exercise with regard to oxidant buffering and redox homeostasis. Due to the normally observed increase in oxygen utilization of the heart during steady-state aerobic exercise, one would predict that cardiac muscle follows the same trends as skeletal muscle with regard to redox homeostasis. However, cardiac muscle from Mdx8Ex mice compared with Mdx8Sed *mdx* mice was not significantly more oxidized, although it showed a similar trend (Figure 2, $P=0.2170$).

This diminishment in oxidative homeostasis from exercise confirms research from previous studies^{15,16}, even when using a different technique to examine oxidative stress. We utilized entire-muscle preparations rather than plasma or histological indicators of oxidative stress. Oxidative stress is an important outcome measure for evaluating the progression of dystrophic pathology as *mdx* mice respond to exercise, and could also be harnessed to examine outcomes based on different treatment strategies.

In order to optimize the potential of exercise as a therapeutic strategy for muscle disease, future studies should investigate differing levels of exercise intensity and modality, and in turn determine how they affect different parameters of muscle disease. Additionally, future advances in treatment strategies for DMD will ultimately improve muscle function and would allow patients to undergo increases in physical activity. Understanding how changes in physical activity affect disease pathology, as well identifying novel outcome measures to monitor disease status, remains crucial to achieve the best outcomes for DMD patients.

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Acronyms and/or abbreviations used

DMD	Duchenne Muscular Dystrophy
OSU	The Ohio State University
OCT	Optimal Cutting Temperature
CLAMS	Clinical Laboratory Animal Monitoring System
RER	Respiratory Exchange Ratio
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
EPR	Electron Paramagnetic Resonance
IgG	Immunoglobulin G

References

1. Mendell JR, Shilling C, Leslie ND, Flanigan KM, al-Dahhak R, Gastier-Foster J, Kneile K, Dunn DM, Duval B, Aoyagi A, Hamil C, Mahmoud M, Roush K, Bird L, Rankin C, Lilly H, Street N, Chandrasekar R, Weiss RB. Evidence-based path to newborn screening for Duchenne muscular dystrophy. *Annals of neurology*. 2012; 71(3):304–313. [PubMed: 22451200]
2. Petrof BJ, Shrager JB, Stedman HH, Kelly AM, Sweeney HL. Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90(8):3710–3714. [PubMed: 8475120]
3. Weller B, Karpati G, Carpenter S. Dystrophin-deficient mdx muscle fibers are preferentially vulnerable to necrosis induced by experimental lengthening contractions. *Journal of the neurological sciences*. 1990; 100(1–2):9–13. [PubMed: 2089145]
4. Schram G, Fournier A, Leduc H, Dahdah N, Therien J, Vanasse M, Khairy P. All-cause mortality and cardiovascular outcomes with prophylactic steroid therapy in Duchenne muscular dystrophy. *Journal of the American College of Cardiology*. 2013; 61(9):948–954. [PubMed: 23352781]
5. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science*. 1989; 244(4912):1578–1580. [PubMed: 2662404]
6. Tanabe Y, Esaki K, Nomura T. Skeletal muscle pathology in X chromosome-linked muscular dystrophy (mdx) mouse. *Acta neuropathologica*. 1986; 69(1–2):91–95. [PubMed: 3962599]

7. Head SI, Williams DA, Stephenson DG. Abnormalities in structure and function of limb skeletal muscle fibres of dystrophic mdx mice. *Proceedings Biological sciences/The Royal Society*. 1992; 248(1322):163–169. [PubMed: 1352891]
8. Vainzof M, Ayub-Guerrieri D, Onofre PC, Martins PC, Lopes VF, Zilberztajn D, Maia LS, Sell K, Yamamoto LU. Animal models for genetic neuromuscular diseases. *Journal of molecular neuroscience : MN*. 2008; 34(3):241–248. [PubMed: 18202836]
9. Zhou L, Lu H. Targeting fibrosis in Duchenne muscular dystrophy. *Journal of neuropathology and experimental neurology*. 2010; 69(8):771–776. [PubMed: 20613637]
10. Klingler W, Jurkat-Rott K, Lehmann-Horn F, Schleip R. The role of fibrosis in Duchenne muscular dystrophy. *Acta myologica : myopathies and cardiomyopathies : official journal of the Mediterranean Society of Myology/edited by the Gaetano Conte Academy for the study of striated muscle diseases*. 2012; 31(3):184–195.
11. Terrill JR, Radley-Crabb HG, Iwasaki T, Lemckert FA, Arthur PG, Grounds MD. Oxidative stress and pathology in muscular dystrophies: focus on protein thiol oxidation and dysferlinopathies. *The FEBS journal*. 2013; 280(17):4149–4164. [PubMed: 23332128]
12. Faist V, König J, Hoyer H, Elmadfa I. Decreased mitochondrial oxygen consumption and antioxidant enzyme activities in skeletal muscle of dystrophic mice after low-intensity exercise. *Annals of nutrition & metabolism*. 2001; 45(2):58–66. [PubMed: 11359030]
13. Brussee V, Tardif F, Tremblay JP. Muscle fibers of mdx mice are more vulnerable to exercise than those of normal mice. *Neuromuscular disorders : NMD*. 1997; 7(8):487–492. [PubMed: 9447605]
14. De Luca A, Pierno S, Liantonio A, Cetrone M, Camerino C, Fraysse B, Mirabella M, Servidei S, Ruegg UT, Conte Camerino D. Enhanced dystrophic progression in mdx mice by exercise and beneficial effects of taurine and insulin-like growth factor-1. *The Journal of pharmacology and experimental therapeutics*. 2003; 304(1):453–463. [PubMed: 12490622]
15. Radley-Crabb H, Terrill J, Shavlakadze T, Tonkin J, Arthur P, Grounds M. A single 30 min treadmill exercise session is suitable for 'proof-of concept studies' in adult mdx mice: a comparison of the early consequences of two different treadmill protocols. *Neuromuscular disorders : NMD*. 2012; 22(2):170–182. [PubMed: 21835619]
16. Burdi R, Rolland JF, Fraysse B, Litvinova K, Cozzoli A, Giannuzzi V, Liantonio A, Camerino GM, Sblendorio V, Capogrosso RF, Palmieri B, Andreetta F, Confalonieri P, De Benedictis L, Montagnani M, De Luca A. Multiple pathological events in exercised dystrophic mdx mice are targeted by pentoxifylline: outcome of a large array of in vivo and ex vivo tests. *Journal of applied physiology*. 2009; 106(4):1311–1324. [PubMed: 19131478]
17. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *The Journal of biological chemistry*. 1997; 272(33):20313–20316. [PubMed: 9252331]
18. Tsai N-W, Chang Y-T, Huang C-R, Lin Y-J, Lin W-C, Cheng B-C, Su C-M, Chiang Y-F, Chen S-F, Huang C-C, Chang W-N, Lu C-H. Association between Oxidative Stress and Outcome in Different Subtypes of Acute Ischemic Stroke. *BioMed Research International*. 2014; 2014:7.
19. Abruzzo PM, Esposito F, Marchionni C, di Tullio S, Belia S, Fulle S, Veicsteinas A, Marini M. Moderate exercise training induces ROS-related adaptations to skeletal muscles. *International journal of sports medicine*. 2013; 34(8):676–687. [PubMed: 23325712]
20. Criswell D, Powers S, Dodd S, Lawler J, Edwards W, Renshler K, Grinton S. High intensity training-induced changes in skeletal muscle antioxidant enzyme activity. *Medicine and science in sports and exercise*. 1993; 25(10):1135–1140. [PubMed: 8231758]
21. Cozzoli A, Capogrosso RF, Sblendorio VT, Dinardo MM, Jagerschmidt C, Namour F, Camerino GM, De Luca A. GLPG0492, a novel selective androgen receptor modulator, improves muscle performance in the exercised-mdx mouse model of muscular dystrophy. *Pharmacological research : the official journal of the Italian Pharmacological Society*. 2013; 72:9–24.
22. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. *Clinical biochemistry*. 1996; 29(3):225–229. [PubMed: 8740508]
23. Swartz HM, Khan N, Khramtsov VV. Use of electron paramagnetic resonance spectroscopy to evaluate the redox state in vivo. *Antioxidants & redox signaling*. 2007; 9(10):1757–1771. [PubMed: 17678441]

24. Roshchupkina GI, Bobko AA, Bratasz A, Reznikov VA, Kuppusamy P, Khramtsov VV. In vivo EPR measurement of glutathione in tumor-bearing mice using improved disulfide biradical probe. *Free radical biology & medicine*. 2008; 45(3):312–320. [PubMed: 18468522]
25. Rafael-Fortney JA, Chimanji NS, Schill KE, Martin CD, Murray JD, Ganguly R, Stangland JE, Tran T, Xu Y, Canan BD, Mays TA, Delfin DA, Janssen PM, Raman SV. Early treatment with lisinopril and spironolactone preserves cardiac and skeletal muscle in Duchenne muscular dystrophy mice. *Circulation*. 2011; 124(5):582–588. [PubMed: 21768542]
26. Bayod S, Del Valle J, Lanza JF, Sanchez-Roige S, de Luxan-Delgado B, Coto-Montes A, Canudas AM, Camins A, Escorihuela RM, Pallas M. Long-term physical exercise induces changes in sirtuin 1 pathway and oxidative parameters in adult rat tissues. *Experimental gerontology*. 2012; 47(12):925–935. [PubMed: 22940286]
27. Sacco P, Jones DA, Dick JR, Vrbova G. Contractile properties and susceptibility to exercise-induced damage of normal and mdx mouse tibialis anterior muscle. *Clinical science*. 1992; 82(2):227–236. [PubMed: 1311662]
28. Baltgalvis KA, Call JA, Cochrane GD, Laker RC, Yan Z, Lowe DA. Exercise training improves plantar flexor muscle function in mdx mice. *Medicine and science in sports and exercise*. 2012; 44(9):1671–1679. [PubMed: 22460476]
29. Hayes A, Williams DA. Beneficial effects of voluntary wheel running on the properties of dystrophic mouse muscle. *Journal of applied physiology*. 1996; 80(2):670–679. [PubMed: 8929614]
30. Call JA, McKeen JN, Novotny SA, Lowe DA. Progressive resistance voluntary wheel running in the mdx mouse. *Muscle & nerve*. 2010; 42(6):871–880. [PubMed: 21104862]
31. Kodama S, Saito K, Tanaka S, Maki M, Yachi Y, Asumi M, Sugawara A, Totsuka K, Shimano H, Ohashi Y, Yamada N, Sone H. Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: a meta-analysis. *JAMA : the journal of the American Medical Association*. 2009; 301(19):2024–2035. [PubMed: 19454641]
32. Radley-Crabb HG, Marini JC, Sosa HA, Castillo LI, Grounds MD, Fiorotto ML. Dystrotophology increases energy expenditure and protein turnover in the mdx mouse model of duchenne muscular dystrophy. *PloS one*. 2014; 9(2):e89277. [PubMed: 24586653]
33. Ambrosio F, Ferrari RJ, Distefano G, Plassmeyer JM, Carvell GE, Deasy BM, Boninger ML, Fitzgerald GK, Huard J. The synergistic effect of treadmill running on stem-cell transplantation to heal injured skeletal muscle. *Tissue engineering Part A*. 2010; 16(3):839–849. [PubMed: 19788347]
34. Ji LL. Exercise-induced modulation of antioxidant defense. *Annals of the New York Academy of Sciences*. 2002; 959:82–92. [PubMed: 11976188]

Figure 1. Exercise and metabolic testing at ages 4 and 8 weeks

A: Exhaustion time on a graded maximal exercise test was significantly shorter in *mdx* mice and at a much lower workload when compared to wild-type controls. * $P < 0.05$. B: *mdx* and wild-type mice reach similar levels of oxygen consumption at maximal exercise output.

WT4: 4 week old C57BL/10 mice, $n=3$; *mdx*4: 4 week old *mdx* mice, $n=7$. C: Mdx8Ex mice had significantly shorter exhaustion times compared to Mdx8Sed mice on a graded maximal exercise test. * $P < 0.05$ (ANOVA) D: Exercise negatively impacted basal oxygen consumption in *mdx* mice, causing a decrease in resting oxygen consumption compared to non-exercised *mdx* mice. * $P < 0.05$ (ANOVA). E: Even after exercise intervention WT8Sed, Mdx8Ex, and Mdx8Sed mice all reach similar levels of maximal oxygen consumption. WT8Sed: 8 week old C57BL/10 mice, $n=3$; Mdx8Sed: 8 week old sedentary *mdx* mice, $n=7$; Mdx8Ex: 8 week old exercised *mdx* mice, $n=6$. (the axis labels are very tiny and hard to read – can you increase the font size?) Axis label font size has been increased.

Figure 2. EPR spectral intensity ratios used to investigate oxidative stress

A higher spectral intensity ratio of the probe compound (RSSR) compared to the bound reduced glutathione product (RSH) indicates higher relative amounts of oxidized GSH from ROS. Quadriceps muscle from Mdx8Ex mice was significantly more oxidized than Mdx8Sed mice. * $P < 0.05$. Abdominal muscle from Mdx8Ex mice followed the same trend to a higher degree. † $P < 0.01$. Mdx8Sed: 8 week old sedentary *mdx* mice, $n=10$; Mdx8Ex: 8 week old exercised *mdx* mice, $n=4-7$. (the dollar sign is somewhat incongruous/distracting here – please substitute the † symbol) † symbol has been inserted instead of the dollar sign.

Figure 3. Hydroxyproline deposition in several muscles of exercised (Ex) and sedentary (Sed) *mdx* mice

Four weeks of treadmill running caused a significant increase in muscle hydroxyproline content within quadriceps and cardiac muscle. Abdominal muscle appeared largely unaffected. Hydroxyproline is a main constituent of collagen scarring in DMD as a result of muscle fibrosis. * $P < 0.05$ versus corresponding control. Mdx8Sed: 8 week old sedentary *mdx* mice, $n=10$; Mdx8Ex: 8 week old exercised *mdx* mice, $n=7$.

Figure 4. Hematoxylin and Eosin (H&E) and Collagen I immunostaining of heart sections from exercised *mdx* mice show greater amounts of collagen scarring at age 8 weeks
Treadmill running increased the formation of collagen scarring in Mdx8Ex mice as detected by immunofluorescence. Mdx8Sed mice do not show obvious collagen scarring by age 8 weeks and appear only slightly worse than WT8 mice. WT8Sed: Sedentary 8 week old C57BL/10 mice; Mdx8Ex: Exercised *mdx*; Mdx8Sed: Sedentary *mdx*. Bar = 100 μ M. (these figures are unimpressive in black and white – could you submit them in color?) The author's agree this figure may look better in color, however, due to budgetary constraints we are unable to pay for color printing of this image.

Figure 5. Quantification of cardiac tissue damage and fibrosis by histological analysis

A: Cardiac myofiber damage in Mdx8Ex mice, while higher, was not quite significant ($P=0.0958$). B: Quantification of collagen I staining indicates that Mdx8Ex mice had significantly higher cardiac muscle fibrosis compared to Mdx8Sed mice. $*P<0.05$.

Mdx8Sed: 8 week old sedentary *mdx* mice, $n=7$; Mdx8Ex: 8 week old exercised *mdx* mice, $n=6$.