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

Hannah Radley-Crabb a,*, Jessica Terrill a,b, Thea Shavlakadze a, Joanne Tonkin a, Peter Arthur b, Miranda Grounds a

a School of Anatomy and Human Biology, The University of Western Australia, Crawley, Australia
b School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, Crawley, Australia

Received 11 March 2011; received in revised form 24 June 2011; accepted 11 July 2011

Abstract

The extent of muscle pathology in sedentary adult mdx mice is very low and treadmill exercise is often used to increase myofibre necrosis; however, the early events in dystrophic muscle and blood in response to treadmill exercise (leading to myofibre necrosis) are unknown. This study describes in detail two standardised protocols for the treadmill exercise of mdx mice and profiles changes in molecular and cellular events after a single 30 min treadmill session (Protocol A) or after 4 weeks of (twice weekly) treadmill exercise (Protocol B). Both treadmill protocols increased multiple markers of muscle damage. We conclude that a single 30 min treadmill exercise session is a sufficient and conveniently fast screening test and could be used in ‘proof-of-concept’ studies to evaluate the benefits of pre-clinical drugs in vivo. Myofibre necrosis, blood serum CK and oxidative stress (specifically the ratio of oxidised to reduced protein thiols) are reliable markers of muscle damage after exercise; many parameters demonstrated high biological variation including changes in mRNA levels for key inflammatory cytokines in muscle. The sampling (sacrifice and tissue collection) time after exercise for these parameters is critical. A more precise understanding of the changes in dystrophic muscle after exercise aims to identify biomarkers and new potential therapeutic drug targets for Duchenne Muscular Dystrophy.

© 2011 Published by Elsevier B.V.

Keywords: Mdx mouse; Treadmill exercise; Skeletal muscle damage; Myofibre necrosis; Creatine kinase; Inflammation; Oxidative stress

1. Introduction

Duchenne Muscular Dystrophy (DMD) is an X-linked, lethal muscle wasting disorder that affects mainly boys [1,2]. Impaired function or absence of the sub-sarcolemmal protein dystrophin, renders dystrophic myofibres susceptible to sarcolemma damage in response to contraction [3–7].

This initial damage can progress to myofibre necrosis and subsequent regeneration; repeated cycles of necrosis ultimately result in the replacement of myofibres with fat and/or fibrotic connective tissue [8]. A progressive loss of muscle mass and function in DMD leads to premature death often due to respiratory or cardiac failure [9]. While the genetic defect was identified over 20 years ago the specific cause of myofibre necrosis is still unknown, although increased levels (or dysregulation) of inflammation, oxidative stress and intracellular calcium are all heavily implicated [7,10–16].

Mdx mice (C57Bl/10ScSn-mdx/mdx), which lack dystrophin, are an animal model for DMD and are widely used...
in pre-clinical research [11,17]. In sedentary adult mdx mice, the extent of dystropathology is relatively mild, with usually <6% myofibre necrosis in the quadriceps muscle (expressed as % cross-sectional area CSA) and relatively low serum creatine kinase (CK) activity [11], thus exercise is routinely used to increase dystropathology [18–21] enabling potential therapeutic interventions to be more rigorously evaluated in vivo [22–29].

The term ‘exercise’ is used broadly and can cover whole body in vivo exercise such as voluntary wheel and treadmill running; it can also cover ex vivo stretching protocols or in vivo electrically stimulated eccentric contractions that require surgical intervention. This paper is focused on in vivo treadmill exercise as this closely represents the physiological situation and is a technique widely accessible by many research groups across the world.

In the past, our laboratory has used voluntary wheel exercise over 48 h to increase myofibre necrosis and histology to demonstrate the benefits of anti-inflammatory drugs on dystrophic muscle in vivo [26,27,30]. Muscle necrosis is roughly doubled (~6% to 12% CSA) in quadriceps muscle after 48 h of voluntary exercise, although other muscles such as the tibialis anterior (TA) are barely affected by voluntary wheel exercise [11,24,26].

A widely used alternative to voluntary wheel (usually nocturnal) exercise is controlled treadmill running (experiments usually conducted during the day). This occurs at a controlled speed for a pre-determined length of time, thus eliminating some of the behavioural variables experienced with voluntary exercise. A protocol of 30 min treadmill running on a horizontal treadmill at a speed of 12 m/min, twice a week for at least 4 weeks, causes a significant increase in the dystropathology of adult mdx mice and is widely used in pre-clinical research [20,22,25,31,32]. There is however some concerns regarding treadmill exercise because mdx mice can have problems coping and thus be reluctant to run, although a short warm-up period at a slower speed appears to help with treadmill running [23].

A single 30 min treadmill exercise session represents a precise amount of controlled exercise that allows the time-course of early cellular and molecular events to be measured. It is of fundamental interest to determine the extent of the initial skeletal muscle damage and associated molecular changes in response to a single 30 min exercise session (Protocol A) in unexercised mdx mice, compared with mice exercised for 4 weeks (Protocol B) which is a widely used exercise regime [20,22,25,31,32]. In the present study parameters measured included; histological quantification of myofibre necrosis, circulating blood CK activity, quadriceps muscle gene expression levels (mRNA) of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor (TNF) and quantification of oxidative stress (protein thiol oxidation ratio and malondialdehyde (MDA) quantitation in the quadriceps muscle.

The aims of the present study were to: (1) develop a short (30 min) and repeatable in vivo treadmill protocol to increase myofibre necrosis in adult mdx mice; (2) profile the time course of multiple indicators of muscle damage immediately after a single exercise session; (3) compare these responses after a single treadmill exercise session to responses after 4 weeks of treadmill exercise; (4) establish if a single 30 min exercise session is an appropriate protocol to increase muscle damage in adult mdx mice and thus be used in pre-clinical ‘proof-of-concept’ studies; (5) identify key parameters with potential as diagnostic biomarkers to rapidly monitor efficacy of pre-clinical drug treatments.

2. Materials and methods

2.1. Animal procedures

All experiments were carried out on 8- to 12-week-old (adult) male non-dystrophic control C57Bl/10 and dystrophic mdx mice; mice were obtained from the Animal Resource Centre, Murdoch, Western Australia. They were maintained at the University of Western Australia on a 12-h light/dark cycle, under standard conditions, with free access to food and drinking water. Mice of each strain were caged in groups of 3–4. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004) and the Animal Welfare act of Western Australia (2002) and were approved by the Animal Ethics committee at the University of Western Australia.

2.1.1. Establishing the 30 min treadmill protocol

Based on previous research [20,22,31] and as per the TREAT-NMD recommended standard 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/ a treadmill exercise regime consisting of 30 min treadmill running at a speed of 12 m/min was used. The rodent treadmill was an Exer 3/6 from Columbus Instruments (USA).

Treadmill setup: Individual running lanes were separated by clear Perspex dividers so that the mice could see each other while exercising. The treadmill was horizontal (0° incline) and mdx mice were run in groups of 3 or 4 as it is time consuming and inefficient to run mdx mice individually.

Exercise protocol: Groups of 3 or 4 mdx mice were all (1) settled for 2 min with the treadmill belt stationary, (2) then acclimatized with gentle walking for 2 min at a speed of 4 m/min, followed immediately by (3) a warm-up of 8 min at 8 m/min and then (4) the main exercise session for 30 min at 12 m/min. If during the 30 min exercise session a mouse fatigued and could no longer run, the procedure was as follows: turn the treadmill belt off and give all mice a 2 min rest, turn the belt on at 4 m/min for 2 min, increase the speed to 12 m/min and run for the remainder of the 30 min. Repeat this process if fatigue occurs again (up to 5 times for an individual mdx mouse).
Time-course and histological analysis

<table>
<thead>
<tr>
<th>Protocol (A)</th>
<th>Single 30 min treadmill session</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological analysis (archival)</td>
<td>Unexercised Mdx</td>
</tr>
<tr>
<td></td>
<td>Exercised – 24 h post Mdx</td>
</tr>
<tr>
<td></td>
<td>Exercised – 48 h post Mdx</td>
</tr>
<tr>
<td>Time-course analysis</td>
<td>Unexercised Mdx and C57Bl/10</td>
</tr>
<tr>
<td></td>
<td>Exercised – 0 min post Mdx and C57Bl/10</td>
</tr>
<tr>
<td></td>
<td>Exercised – 2 h post Mdx</td>
</tr>
<tr>
<td></td>
<td>Exercised – 24 h post Mdx</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protocol (B)</th>
<th>4 weeks treadmill exercise (8 sessions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-course and histological analysis</td>
<td>Unexercised Mdx</td>
</tr>
<tr>
<td></td>
<td>Exercised – 0 min post Mdx</td>
</tr>
<tr>
<td></td>
<td>Exercised – 24 h post Mdx</td>
</tr>
<tr>
<td></td>
<td>Exercised – 96 h post Mdx</td>
</tr>
</tbody>
</table>

2.1.2. Treadmill regime and animal sample groups

All experiments (exercise and sampling) were started at 8am and completed by 11am each day.

Exercise Protocol A (a single 30 min treadmill exercise session): 12-week-old (completely untrained) mdx and control C57Bl/10 mice were exercised for a single session on the rodent treadmill. All male mice were sampled at 12 weeks of age. The following three groups of male mdx mice were used for histological analysis: (1) unexercised, (2) mice subjected to 30 min treadmill exercise and sampled 24 h or (3) 48 h post exercise. Numerous treadmill exercise experiments have been conducted in our laboratory over the last year using 12-week-old male mdx mice and the histological data from all experiments were pooled to provide large group numbers (see Table 1). The time course study was conducted on a total of 32 mdx and 16 C57Bl/10 12-week-old mice representing six different groups with \( n = 8 \) for each group. The following groups were used: (1) unexercised C57Bl/10, (2) exercised C57Bl/10 sampled immediately (0 min) post exercise, (3) unexercised mdx, (4) exercised mdx sampled immediately (0 min) or (5) 2 h exercise or (6) 24 h post exercise (see Table 1).

Exercise Protocol B (4 weeks of treadmill exercise): mdx mice were exercised on the treadmill twice a week for 4 weeks, with a consistent 72 or 96 h break between each exercise session. The treadmill exercise started when mice were 8 weeks old and therefore all mice were 12 weeks old at time of sampling. For consistency all mice had a 72 h (3 day) break before the final (8th) exercise session and subsequent sampling. The 4 week treadmill exercise protocol was conducted on a total of 32 male mdx mice representing four different groups with \( n = 8 \) for each group. The following groups were used: (1) unexercised mdx, (2) 4 week exercised mdx sampled immediately (0 min) or (3) 24 h or (4) 96 h post exercise (see Table 1). C57Bl/10/10 mice were not included in Protocol B.

2.1.3. Forelimb grip strength

Mice from Protocol B were also assessed throughout the study for forelimb grip strength (measured 24 h prior to 1st, 5th and 8th treadmill exercise session). Grip strength was measured using a Chatillon Digital Force Gauge (DFE-002) and a triangle metal bar, as per the TREAT-NMD recommended standard protocol “Use of grip strength metre to assess limb strength of mdx mice – M.2.2_001” http://www.treat-nmd.eu/research/preclinical/SOPS/. In brief, the mouse was placed on the front of the triangle bar (attached to a force transducer) and pulled gently until release. Each mouse underwent five consecutive grip-strength trials; the grip strength value for each mouse was recorded as the average of the three best efforts. Average grip strength was then normalized for body weight [force (kg)/BW (g)]. Change in normalised grip strength was determined by subtracting normalised grip strength (8 weeks) from normalised grip strength (12 weeks) [25].

2.2. Tissue collection and image acquisition

All mice were sacrificed by cervical dislocation while under terminal anaesthesia (2%/v/v Attane isoflurane Bomac Australia). Various muscles were collected, some were immediately snap frozen in liquid nitrogen for molecular analysis (quadriceps) and some were prepared for histology (quadriceps, triceps, gastrocnemius, diaphragm, tibialis anterior and extensor digitorum longus). Limb muscles were fixed immediately in 10% BFS (Confix Australia Biostain AB1020) and remained in solution for at least 72 h. Tissues were placed into 70% ethanol, processed in a Shandon automatic tissue processor overnight, and paraffin embedded for sectioning. Transverse sections (5 μm) were cut through the mid-region of each muscle. Slides were routinely stained with Haematoxylin and Eosin (H&E) for morphological analysis of the histology. Non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross-section. Images were acquired using a Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Tiled images were taken at 10× magnification.

2.3. Histological image analysis

Histological analysis of muscle necrosis was carried out on whole muscle cross-sections. Muscle morphology was assessed using a light microscope (Leica DM 2500). Image analysis for histological groups was performed using Image Pro Plus 4.5.1 software. Tiled images were analysed using a software program

Please cite this article in press as: Radley-Crabb H et al., 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, Neuromuscul Disord (2011), doi:10.1016/j.nmd.2011.07.008
drawn manually by the researcher using Image Pro Plus 4.5.1 software. The area occupied by necrotic myofibres (i.e. myofibres with fragmented sarcoplasm and/or areas of inflammatory cells) was measured as a percentage (area) of the whole muscle section. All section analysis was done ‘blind’. Histological analysis was completed as per the TREAT-NMD recommended standard protocol “Histological measurements of dystrophic muscle – M.1.2_007” http://www.treat-nmd.eu/research/preclinical/SOPs/.

2.4. Blood collection and serum creatine kinase (CK) assay

While mice were under terminal anaesthesia, whole blood (approx. 0.5 ml) was collected via cardiac puncture using a 27.5 gauge tuberculin syringe (Sigma Z192082), into a 1.5 ml tube. Extensive experimentation revealed that storage of blood samples overnight at 4 °C to enable clotting leads to a false increase in serum CK levels and therefore blood samples were immediately spun down in a refrigerated centrifuge for 5 min (12,000g), serum was removed and aliquoted. Blood serum CK activity was determined in pure ethanol and resuspended in SDS buffer. Samples were washed using the CK-NAC kit (Randox Laboratories) and measured using a BioTek Powerwave XS Spectrophotometer using the KC4 (v 3.4) program. A minimum of 10 µl serum is required to complete this assay.

2.5. Measuring cytokine gene expression by RNA extraction and RT-PCR

Levels of mRNA for three inflammatory cytokines (IL-1β, IL-6 and TNF) was measured in the quadriceps muscle since this appeared to have the greatest amount of exercise-induced muscle damage, as indicated by the extent of myofibre necrosis (Figs. 4 and 5). RNA was extracted from one half of a snap frozen quadriceps muscles using Tri-reagent (Sigma T9424) and quantitated using a Nano Drop Spectrophotometer (ND 1000) and ND 1000 software version 3.5.2. The RNA was DNase treated using Promega RQ1 RNase free DNase (M610A), RQ1 RNase free 10× buffer (M198A) and RQ1 DNase stop solution (M199A). RNA was reverse transcribed into cDNA using Promega M-MLV Reverse Transcriptase (M3682), random primers (C1181) and 10 mM dNTPs (U1515) and the cDNA was purified using a MoBiL Clean up kit (12500-250). RT-PCRs were run on a Corbett 3000 (Corbett Research) using QIA-GEN quantifast SYBR green PCR mix (204054) and QIA-GEN Quantitect Primer Assays for IL-1β (QT01048355), IL-6 (QT00098875) and TNF (QT00104006), and standardised to a house-keeping gene; ribosomal protein L-19 (QT01779218) as per [33]. mRNA expression levels were calculated and standardised using Rotor-gene 6.1 and Microsoft Excel software.

2.6. Quantitation of oxidative stress

Oxidative stress in the quadriceps muscle was measured in two different ways:

2.6.1. As a ratio of oxidised (di-sulphide) to reduced (sulphhydryl) protein thiols – 2-Tag technique

Frozen quadriceps muscles were crushed under liquid nitrogen, before protein extraction with 20% trichloroacetic acid/acetone. Protein was washed with acetone and solubilised in 0.5% sodium dodecyl sulphate 0.5 M Tris (SDS buffer), pH 7.3 and thiols were labelled with the fluorescent dye BODIPY FL-N-(2-aminoethyl)maleimide (FLM, Invitrogen). Following removal of the unbound dye using ethanol, protein was resolubilised in SDS buffer, pH 7 and oxidised thiols were reduced with tris(2-carboxyethyl)phosphine (TCEP, Sigma) before the subsequent unlabelled reduced thiols were labelled with a second fluorescent dye, texas red maleimide (Invitrogen). The sample was washed in pure ethanol and resuspended in SDS buffer. Samples were read using a fluorescent plate reader (Fluostar Optima) with wavelengths set at excitation 485, emission 520 for FLM and excitation 595, emission 610 for texas red. A standard curve for each dye was created using ovalubumin and all results were expressed per mg of protein, quantified using Detergent Compatible protein assay (BioRad), as per [34,35].

2.6.2. Malondialdehyde (MDA)

A product formed via the decomposition of lipid peroxidation products [36] was quantitated using High Performance Liquid Chromatography (HPLC). Quadriceps muscles were ground under liquid nitrogen, homogenised in 10 × 5% perchloric acid and 150 µl of supernatant mixed with 150 µl of 40 mM Thiobarbituric acid. Samples were then incubated at 50 °C for 90 min and cooled on ice for 15 min. Butanol (250 µl) was added, before vortexing and centrifuging for 5 min. Twenty microliters of the upper butanol layer was injected into a C18 HPLC column (5 µl, 4.6 × 150 mm, Dionex) with an isocratic mobile phase of 60:40 50 mM KH2PO4:methanol. Samples were run at a flow rate of 800 µl/min for 7 min; the retention time was approximately 4.5 min. Fluorescent detection was achieved using the bandpass filters of 515 for excitation and 553 for emission. Tetraethoxypropane (Sigma) was used as a standard for absolute calculation. Approximately 30 mg of tissue was required for this assay.

2.7. Statistics

Statistical analysis was performed using Microsoft excel and SPSS 16.0. Data were checked for equal distribution and normality using Q–Q plots. Multiple variables were analysed by ANOVAs (one, two or three-way to account for exercise, sampling time and strain). All data are expressed as mean ± SEM unless otherwise stated.

3. Results

3.1. Ability of mice to run on the treadmill

Protocol A: Preliminary studies revealed that approximately 45% of 12-week-old untrained mdx mice could
not complete a full 30 min of treadmill exercise at 12 m/min (despite being rested 5 times during the 30 min exercise session), some exhibited severe fatigue after 10 min exercise. This inability to exercise on a treadmill is similar to previous reports [23,31,37]. For this reason the additional ‘warm-up’ period (8 min at 8 m/min) was included in the treadmill protocol to help the mdx mice to complete the treadmill exercise session, as per [23]. It is extremely important, especially when only conducting one single exercise session, to minimise biological variation in the experimental exercise protocol. Adding a ‘warm-up’ period significantly increased the ability of mdx mice (92%) to complete the treadmill exercise session. Out of the 24 mdx mice exercised in Protocol A, 2 mice did not fully complete the 30 min treadmill exercise; these 2 mice each completed approximately 26 min exercise but, since neither produced any outlying results, the data were included in all analyses.

C57Bl/10 mice were all capable of completing the 30 min treadmill exercise without any rests. Previous experiments in our laboratory indicate that C57Bl/10 mice are also capable of completing 30 min of treadmill exercise at 12 m/min without an extensive warm-up and can also run comfortably for 60 min (Grounds et al., unpublished data).

**Protocol B:** The average number of rests required to complete each exercise session reduced throughout the 4 week exercise period, particularly after the 3rd week, suggesting that mdx mice can improve their running ability with exercise training (Fig. 1). Untrained 12-week-old male mdx mice (Protocol A) required significantly (P < 0.01) more rests to complete a 30 min exercise session compared to 12-week-old male mice that had been ‘trained’ for 4 weeks (Protocol B) (Fig. 1). However, it must be noted that the running protocol used in this study involved resting all the mdx mice on the treadmill when only one was experiencing fatigue and this may have had an influence on mdx running ability overtime. There was no significant difference in the running ability of unexercised 8-week-old (1st session of Protocol B) and unexercised 12-week-old (Protocol A) mdx mice (Fig. 1).

### 3.2. Forelimb grip strength (Protocol B only)

After 4 weeks of treadmill exercise (Protocol B) the forelimb grip strength of 12-week-old mdx mice was significantly weaker than unexercised mice (Fig. 2) as shown by a significant (P < 0.01) decrease in both absolute forelimb grip strength.
strength (0.168 kg ± 0.007 unexercised vs 0.124 kg ± 0.005 exercised) and a significant ($P < 0.01$) decrease in normalised change (normalised strength at 12 weeks minus normalised strength at 8 weeks) in forelimb strength (0.002 kg/g ± 0.0004 unexercised vs 0.00038 kg/g ± 0.0002 exercised) (Fig. 2). This decrease in forelimb grip strength is similar to previous reports [31,32] and falls within the expected general range discussed in the TREAT-NMD recommended standard protocol “Use of grip strength metre to assess limb strength of mdx mice – M.2.2_001” http://www.treat-nmd.eu/research/preclinical/SOPs/.

3.3. Histological analysis of muscle necrosis

3.3.1. Biological variation

Protocol A: One striking feature of histological analysis was the high variation in the amount of myofibre necrosis (fragmented sarcoplasm and inflammatory cell infiltration) from both sedentary and exercised (age, sex and muscle matched) mdx mice. The variation in myofibre necrosis (% area) in quadriceps muscle from 12-week-old sedentary male mdx mice is demonstrated in Fig. 3 and ranges from 1.04% to 23.1% for each individual quadriceps muscle and 2.97–17.15% average per experimental group. When results from nine separate experiments were pooled together ($n = 60$ quadriceps) the average amount (% CSA) of myofibre necrosis in the quadriceps muscle of an unexercised 12-week-old male mice is 6.12%. Pooled histological data were also used for myofibre necrosis in unexercised triceps muscle – 8.5% ($n = 28$) and unexercised gastrocnemius muscle – 6.89% ($n = 11$).

3.3.2. Exercise induced myofibre necrosis

Protocol A: High variation in myofibre necrosis was again seen in response to a single 30 min treadmill exercise session and pooled histological data were also used for the various muscles sampled 24 h after exercise from four experiments ($n = 15–25$) and at 48 h after exercise from six experiments ($n = 13–43$) (Fig. 4). The quadriceps muscle showed the highest level of myofibre necrosis ($15.06 ± 6.01\%$) after a single bout of treadmill exercise when sampled 24 h, compared with triceps, gastrocnemius, (Fig. 4) tibialis anterior (TA) and extensor digitorum longus (EDL) (data not shown). Necrosis was significantly increased in treadmill exercised (compared with unexercised) quadriceps muscles when sampled at either 24 h ($P < 0.01$) or 48 h ($P = 0.04$) post exercise. Both the TA and the EDL muscle from 12-week-old mdx mice had a very low level of background myofibre necrosis (average < 3%) and both appeared unaffected by the single treadmill exercise session with no consistent or significant increase in myofibre necrosis (data not shown), in accordance with previous reports [24,26,27].

Unexercised C57Bl/10 mice show no myofibre necrosis in their skeletal muscles (0%). The C57Bl/10 mice from

Fig. 4. Myofibre necrosis (% CSA) in the quadriceps, triceps and gastrocnemius muscles of 12-week-old male mdx mice: a comparison of unexercised mice with mice subjected to a single 30 min exercise session (Protocol A). Myofibre necrosis in the quadriceps muscle is significantly increased in treadmill exercised mice when sampled both 24 h ($n = 25$) and 48 h ($n = 43$) after exercise in comparison to unexercised mice ($n = 60$). Bars represent standard error. $N = 28, 19, 12, 20, 11, 15, 12, 20$, respectively, for triceps and gastrocnemius muscle. *Significant difference ($P < 0.05$), in exercised quadriceps compared to unexercised quadriceps.

Fig. 5. Myofibre necrosis (% CSA) in the quadriceps, triceps, gastrocnemius, diaphragm and tibialis anterior muscles of 12-week-old male mdx mice: a comparison of unexercised mdx mice with mdx mice sampled after 4 weeks of treadmill exercise (Protocol B). *Significant increase in necrosis in exercised muscle in comparison to unexercised muscle (same muscle only). #Significant decrease in necrosis in exercised muscle in comparison to unexercised muscle (same muscle only). For unexercised mice $n = 60, 28, 11, 8, 8$ muscles, respectively. $N = 8$ for all other groups. Bars represent standard error and significant differences were determined by $P < 0.05$.

Please cite this article in press as: Radley-Crabb H et al., 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, Neuromusc Disord (2011), doi:10.1016/j.nmd.2011.07.008
Protocol A were sampled 0 min after treadmill exercise and thus do not show any myofibre necrosis (nor did we expect them to within 30 min of the start of the exercise). In order for precise comparison with mdx mice we examined C57Bl/10 mice sampled at 24 h after treadmill exercise from another experiment conducted within our laboratory (Radley-Crabb et al. unpublished data), again these C57Bl/10 mice show 0% myofibre necrosis after horizontal treadmill exercise.

Protocol B: Myofibre necrosis was significantly elevated in the quadriceps \((P = 0.04)\), triceps \((P = 0.05)\), diaphragm \((P = 0.04)\) and TA \((P = 0.05)\) muscles after 4 weeks of treadmill exercise training when mdx mice were sampled 24 h after the last exercise session. Necrosis was also significantly elevated in the diaphragm muscle \((P = 0.02)\) when sampled immediately \((0 \text{ min})\) after the last exercise session (Fig. 5), suggesting prolonged myofibre necrosis after the penultimate exercise session or a particular sensitivity to exercise induced damage in the diaphragm.

Necrosis was most elevated in the quadriceps \((2 \times \text{fold})\) and diaphragm \((3 \times \text{fold})\) muscle at 24 h after the last exercise session. The consistently elevated necrosis in the quadriceps is similar to that seen after a single exercise session \((\text{Protocol A})\). No significant increase in myofibre necrosis was seen in the exercised gastrocnemius muscle possibly due to high level of variation in this parameter (Fig. 5). Myofibre necrosis (fragmented sarcoplasm and inflammation) returned to unexercised levels \((\text{or below})\) in all muscles, within 96 h after exercise \((\text{i.e. when the next exercise session would be due})\). This indicates that dystrophic myofibres can regenerate muscle to replace necrotic sarcoplasm and inflammation in between each treadmill exercise session. This also emphasises the importance of sampling time when quantitating myofibre necrosis after treadmill exercise.

3.4. Blood serum CK levels as a measure of muscle leakiness

Protocol A: Serum CK levels are very low in control C57Bl/10 mice and no significant change was seen after 30 min of treadmill exercise in control C57Bl/10 mice sampled 0 min after exercise \((\text{unexercised } 183.3U/L \pm 53.8 \text{ vs } 0 \text{ min post exercise } 267.7U/L \pm 98.7)\) (Fig. 6). Additional data from another experiment conducted in our laboratory also show no change in serum CK levels at 24 h after treadmill exercise in C57Bl/10 mice \((\text{unexercised } 183.3U/L \pm 53.8 \text{ vs } 24 \text{ h post exercise } 167.9U/L \pm 54.9)\). However, serum CK levels were rapidly elevated in response to treadmill exercise in mdx mice and were significantly higher \((P = 0.01)\) when blood was collected immediately \((0 \text{ min})\) after exercise. The exercise induced increase in serum CK was transient and CK levels dropped rapidly down to unexercised level within 24 h (Fig. 6).

Protocol B: In mdx mice subject to 4 weeks treadmill exercise, blood serum CK levels were significantly \((P < 0.01)\) elevated at 24 h after the last exercise session and decreased to unexercised level within 96 h \((\text{Fig. 6})\). This is in marked contrast to the rapid elevation of CK in mdx mice subject to a single exercise session \((\text{Protocol A})\).

3.5. Inflammatory cytokine gene expression in the quadriceps muscle

Protocol A: IL-6 mRNA levels were significantly increased in the quadriceps muscle from C57Bl/10 mice after a single 30 min treadmill session \((\text{Fig. 7ii})\); although there was no significant change in IL-1\(\beta\) or TNF mRNA. Expression of all three inflammatory cytokines was significantly higher in unexercised mdx mice compared to unexercised C57Bl/10 mice \((\text{Fig. 7i–iii})\). In mdx mice, mRNA levels for both IL-1\(\beta\) \((2 \text{ h post exercise } P = 0.03)\) and IL-6 \((0 \text{ min post exercise } P = 0.05 \text{ and } 2 \text{ h post exercise } P = 0.05)\) were significantly elevated after a single 30 min exercise session compared to unexercised mice \((\text{Fig. 7i and ii})\). This rapid increase in gene expression returned to the unexercised level for both genes within 24 h. In contrast, levels of mRNA for TNF were significantly reduced after exercise \((0 \text{ min post exercise } P < 0.01, 2 \text{ h post exercise } P = 0.02 \text{ and } 24 \text{ h post exercise } P < 0.01)\) compared to unexercised mdx mice \((\text{Fig. 7iii})\).

Protocol B: In mdx mice there was no change in mRNA for IL-1\(\beta\) or IL-6 immediately after 4 weeks of treadmill exercise, although mRNA for both IL-1\(\beta\) and IL-6 was significantly \((P = 0.05)\) decreased for muscles sampled 96 h post exercise, compared to unexercised mdx mice \((\text{Fig. 8i and ii})\). TNF mRNA was significantly decreased in muscles sampled immediately \((0 \text{ min})\) after exercise compared to unexercised mdx mice \((\text{Fig. 8iii})\), but returned to unexercised level within 24 h.
3.6. Oxidative stress measurement in the quadriceps muscle

3.6.1. Ratio of oxidised (di-sulphide) to reduced (sulphydryl) protein thiols

Protocol A: Oxidative stress in the quadriceps muscle, specifically the ratio of oxidised to reduced protein thiols as measured by the novel 2-tag technique, was significantly (\( P = 0.01 \)) higher in mdx mice at 2 h post exercise compared to unexercised mdx mice. IL-6 was significantly increased in both C57Bl/10 and mdx mice immediately after treadmill exercise. TNF is significantly decreased after treadmill exercise at all times in mdx mice. Bars represent standard error. \( N = 7-8 \) mice per group. (A–C) Significant differences, groups with different letters are significantly different from each other (\( P < 0.05 \)).

3.7. Quantitation of malondialdehyde (MDA)

Protocol A: Oxidative stress in the quadriceps muscle with respect to irreversible lipid peroxidation, measured

![Graphs showing gene expression changes](image-url)

Fig. 7. Gene expression (mRNA) changes in the quadriceps muscle of non-dystrophic C57Bl/10 mice and dystrophic mdx mice in response to a single 30 min exercise session (Protocol A) for (i) IL-1\( \beta \), (ii) IL-6 and (iii) TNF. IL-1\( \beta \) mRNA is significantly increased in mdx mice at 2 h post exercise compared to unexercised mdx mice. IL-6 is significantly increased in both C57Bl/10 and mdx mice immediately after treadmill exercise. TNF is significantly decreased after treadmill exercise at all times in mdx mice. Bars represent standard error. \( N = 7-8 \) mice per group. (A–C) Significant differences, groups with different letters are significantly different from each other (\( P < 0.05 \)).

![Graphs showing gene expression changes](image-url)

Fig. 8. Gene expression (mRNA) changes in the quadriceps muscle of mdx mice in response to 4 weeks treadmill exercise (Protocol B) for (i) IL-1\( \beta \), (ii) IL-6 and (iii) TNF. Both IL-1\( \beta \) and IL-6 mRNA are significantly decreased after 4 weeks of treadmill exercise when mdx mice are sampled 96 h post exercise. TNF mRNA is significantly decreased at 0 min after exercise compared to unexercised mdx mice. Bars represent standard error. \( N = 8 \) mice per group. (A–C) Significant differences, groups with different letters are significantly different from each other (\( P < 0.05 \)).

0 min (\( P = 0.015 \)) and 2 h (\( P = 0.04 \)) after a single treadmill session compared to unexercised mdx mice (Fig. 9i). Protein thiol oxidation returned to unexercised level within 24 h.

Protocol B: Similarly, protein thiol oxidation was significantly increased (\( P = 0.02 \)) in mdx mice after 4 weeks of treadmill exercise when sampled immediately (0 min) after exercise (Fig. 9ii) and returned to unexercised level within 24 h.

Please cite this article in press as: Radley-Crabb H et al., 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, Neuromuscul Disord (2011), doi:10.1016/j.nmd.2011.07.008
as concentration of MDA, showed no significant difference between C57Bl/10 and mdx mice, and was unaffected by exercise even in mdx mice (Fig. 10). MDA levels were not measured for Protocol B.

4. Discussion

Treadmill exercise is widely used in pre-clinical experiments to increase the extent of dystropathology in mdx mice, yet the cellular consequences of a single 30 min treadmill exercise session (Protocol A) have not been described previously. The present study analysed the time-course of molecular and cellular changes after a single standardised 30 min treadmill exercise session (Protocol A). These data were compared to data from age matched mdx mice subjected to 4 weeks of twice weekly treadmill exercise (Protocol B) a protocol currently widely used for pre-clinical drug screening in mdx mice [11,20,22,25].

4.1. Running ability of mdx mice

Adding a short warm-up period for 8 min at a slower speed (8 m/min) produced much more consistent running by the mdx mice in both treadmill exercise protocols. With only a single exercise session it is important that all mice complete the exercise protocol to reduce variation. It is not recommended to remove the ‘non-running’ mice from sample groups as these may represent mice with the most severe dystropathology; in comparison to a full range of dystropathology being represented in unexercised control mice. Preliminary experiments showed that adding a warm-up period significantly increased the ability of mdx mice (from 45% up to 92%) to complete the 30 min treadmill exercise session.

In the present study, 4 weeks of treadmill exercise training (Protocol B) significantly improved the running ability of mdx mice (Fig. 1). Despite exhibiting a significant reduction in both absolute forelimb strength (kg) and change in normalised forelimb strength (kg/g bw) compared to unexercised mdx mice (Fig. 2), exercised mdx mice show a small increase in normalised forelimb strength after 4 weeks of treadmill exercise (Protocol B). This increase in normalised forelimb strength along with behavioural adaptation to exercise training may account for the improvement in treadmill running ability. However, it must be noted that during the treadmill exercise protocol mice were run in groups of 3 or 4 and if one mouse fatigued during the 30 min protocol all mice on the treadmill were rested and...
this may have impacted the results. Some studies document an improvement in the voluntary wheel exercise ability (distance run) of mdx mice over time, especially when exercise is started at a young age (reviewed in [40]). However, an improvement in running capacity on a treadmill overtime has not been previously reported for mdx mice.

4.2. Myofibre necrosis

Increased myofibre necrosis after both treadmill exercise protocols is transient (Figs. 4 and 5) and muscles must be sampled between 24 and 48 h after exercise to visualise the increase in this histological parameter. While dystrophic skeletal muscles appear fully capable of regenerating between repeat exercise sessions (Fig. 5) it must be noted that grip strength is significantly reduced in mdx mice subjected to repeated bouts of treadmill exercise compared with age-matched unexercised mdx mice. Skeletal muscle fibrosis was not measured in this study although it is likely that, due to exercise induced cycles of myofibre necrosis associated with inflammation (and regeneration), fibrosis is indeed progressively increased after 4 weeks of treadmill exercise and may impact negatively on forelimb grip strength.

Similar to histological results seen after voluntary wheel exercise, treadmill exercise induces a large amount of damage in the quadriceps muscle [24, 26, 27]. Large variation in the extent of myofibre necrosis is seen for most muscles; with the quadriceps muscle showing the highest increase in necrosis after a single 30 min exercise session (Protocol A – Fig. 4) and the quadriceps, triceps and diaphragm muscles all showing increased necrosis after 4 weeks of treadmill exercise (Protocol B – Fig. 5).

Four weeks of treadmill exercise (Protocol B) induced a more consistent increase in myofibre necrosis in many muscles (excluding the gastrocnemius) compared to a single 30 min exercise session (Protocol A). These data emphasise that large groups of mdx mice (at least 8 mice) are required for histological analysis, due to the notorious variation in mdx mice (reviewed in detail [11, 17]) and that the choice of muscle is critical to assess the impact of exercise induced damage.

There is no myofibre necrosis in unexercised C57Bl/10 control mice nor does horizontal treadmill exercise produce necrosis in these mice. The resistance of normal (non-dystrophic) muscle to treadmill exercise-induced necrosis is not surprising, especially since we have previously reported that even much more damaging eccentric contractions in vivo do not result in muscle necrosis in control C57Bl/10 mice (in marked contrast with mdx mice) [28]. Similarly, Roche et al. clearly showed no significant increase in myofibre necrosis after eccentric contractions (large strain injury) in normal control (A/WySnJ) mice [41].

4.3. Blood serum creatine kinase level

Serum CK activity is widely used as an indirect measure of muscle damage (sarcolemma leakiness) and levels are consistently increased in mdx mice after exercise [22, 26, 30, 31, 42]. There is no absolute correlation between the extent of dystrophopathy in an individual mdx mouse and CK activity [30], with many factors including stress and muscle mass influencing CK activity (reviewed in [11]). CK is an enzyme and has a short circulating half life of approximately 12 h [43], thus there is considerable interest in understanding the kinetics of CK release from dystrophic muscle after treadmill exercise.

Serum CK was strikingly increased immediately (0 min) after a single 30 min exercise session (Protocol A) and returned to baseline within 24 h post exercise (Fig. 6). This elevated level is similar to the transient elevation in mdx serum CK reported at 1 h after eccentric exercise (16° downhill, 10 m/min for 5 min) [19] and to the increased (10× fold) serum CK after 8 weeks of voluntary wheel exercise [26]. This immediate increase in serum CK after exercise indicates that Protocol A was sufficiently strenuous to render myofibres ‘leaky’ and allow the release of CK into circulation; however it was not damaging enough to induce widespread myofibre necrosis (Fig. 4) in many muscles with the exception of the quadriceps. This is in marked contrast to the blood serum CK levels in control C57Bl/10 mice which are very low and do not increase after treadmill exercise (Fig. 6).

Serum CK activity is a measure of muscle damage throughout the whole body, unlike histological analysis of myofibre necrosis which exclusively examines sections of an individual muscle. In addition to the results presented, some mice received Evans Blue Dye injections 24 h prior to the single 30 min exercise session to enable histological quantitation of ‘leaky’ myofibres; however this did not produce any consistent results (data not shown).

In contrast to a single exercise session (Protocol A), CK levels in mdx mice subjected to 4 weeks treadmill training (Protocol B) were significantly increased at 24 h after exercise, but not immediately after exercise (Fig. 6). This suggests either a delayed or sustained release of CK from leaky myofibres to account for the prolonged elevation of blood CK activity. Many factors including training and type of exercise can affect the level and duration of CK increase; for example in humans a single session of high intensity resistance exercise immediately increases blood serum CK which peaks at 24 h and begins to decline within 48 h [44]. Our result emphasises the importance of documenting the timing of such events after different exercise regimes in order to determine the optimal time for sampling (sacrifice and tissue collection) after exercise when measuring specific parameters.

4.4. Gene expression of inflammatory cytokines

IL-1β, IL-6 and TNF are three major pro-inflammatory cytokines; however it is also recognised that IL-6 is a myokine, with many important anti-inflammatory and metabolic effects, produced by and released from contracting myofibres in vivo (reviewed in [45, 46]). Accordingly, a
significant increase in IL-6 mRNA was seen in the quadriceps muscle from both C57Bl/10 and mdx mice after a single 30 min treadmill session (Fig. 7i). The increased IL-6 mRNA in C57Bl/10 mice after treadmill exercise does not coincide with any inflammation (no change in the expression level of IL1β or TNF – Fig. 7i and iii) or with muscle damage (no increase in blood serum CK level (Fig. 6) or muscle necrosis) and further demonstrates the capacity of normal muscle contraction to increase IL-6 production. The pronounced increase in IL-6 mRNA in mdx mice after a single 30 min exercise session (Protocol A) is presumably caused by both a response to myofibre contraction and exercise induced muscle damage. There was no significant increase in IL-6 mRNA in mdx mice after 4 weeks of treadmill exercise (Protocol B); this may reflect an adaptation (training) in response to treadmill exercise over time.

The level of TNF mRNA was significantly reduced after both treadmill exercise protocols in mdx mice. While there is strong evidence that TNF plays a major role in the dystrophopathology of mdx mice and blockade of TNF can reduce myofibre necrosis [26,30,42,47], increased IL-6 after exercise can inhibit TNF [48–50] and this may explain the transient reduction in TNF mRNA seen in response to exercise in the present study.

While there were transient changes in TNF, IL1β and IL-6 mRNA expression after exercise, changes and bioavailability at the protein level were not measured. This is in part because of issues with sensitivity of quantification [51]. Indeed, attempts were made to measure TNF protein levels in blood serum using a standard ELISA (Invitrogen, USA), however serum TNF levels for all mice were below the lowest standard (15.6 pg/ml) and therefore results were uninformative (data not shown). Another important factor to consider is that high levels of TNF (and other cytokine) protein are already present, yet sequestered, within resident and invading inflammatory cells (e.g. mast cells, neutrophils and macrophages) in dystrophic muscle [13,27,30,52–55]. Protein quantification makes no comment on the bioavailability or the re-distribution of these cytokines: for example these proteins are rapidly released from activated mast cells and other inflammatory cells (e.g. neutrophils and macrophages) that accumulate after myofibre damage. Thus it is likely that, despite a reduction in TNF mRNA after treadmill exercise, localised bioavailable TNF protein increases rapidly independent of gene transcription [56].

4.5. Oxidative stress

A significant increase in oxidative stress in unexercised mdx compared to unexercised C57Bl/10 quadriceps muscle was demonstrated by the increased ratio of oxidised to reduced protein thiols (reversible oxidative modification) as measured by the novel 2-tag technique [34,35]. This elevated oxidative stress in dystrophic muscle supports earlier reports that measured oxidative stress in dystrophic muscle (from both DMD patients and mdx mice) [16,25,57–60].

Our second measurement of oxidative stress, using a HPLC method to quantitate MDA and irreversible peroxidation of membrane lipids, showed no significant difference in oxidative stress between C57Bl/10 and mdx quadriceps muscle and MDA levels were not increased in mdx muscle after 30 min treadmill exercise (Protocol A). While previous studies have reported increased MDA in the hind limbs of young (<20 days) mdx mice [60] and the gastrocnemius muscle of 90 day old mdx mice [57], compared to age-matched C57Bl/10 mice, there are numerous reasons for this discrepancy in MDA results, including animal age and specific muscle examined. It is also important to note that many commonly used methods for measuring MDA have specificity issues and only HPLC based methods are recommended (reviewed in [61]).

Despite no evidence of increased lipid peroxidation in adult mdx quadriceps muscle, reversible changes in oxidative state were evident by the ratio of oxidised to reduced protein thiols in mdx muscle. Importantly this 2-tag method identified rapid and significant increases in protein thiol oxidation immediately (0 min) and 2 h after a single 30 min treadmill exercise session, emphasising the speed of such changes in vivo and the sensitivity of this specific assay for oxidative stress.

There is an increasing need for standardised experiments involving mdx mice in order to readily compare data between laboratory groups globally; this is the subject of recent reviews [11,17] that aim to establish a set of recommendations for pre-clinical mdx drug trials. The present study provides strong support for a single 30 min exercise session in adult mdx mice as an appropriate fast protocol to conduct preliminary ‘proof-of-concept’ testing of potential therapeutic drugs to reduce the severity of myofibre necrosis associated with muscular dystrophy. We have successfully conducted in vivo studies in adult mdx mice examining the potential benefits of N-acetylcysteine (NAC) using the 30 min treadmill exercise protocol established in this manuscript (Terrill et al., under review).

A single 30 min exercise session (Protocol A) results in a similar level of muscle damage (muscle necrosis, serum CK, oxidative stress) as 4 weeks of treadmill exercise (Protocol B) and thus the single exercise session appears suitable as a high through-put screening test. However a short term protocol does not allow for monitoring of running pattern or changes in normalised grip strength over time. It is noted that potential therapeutic drugs for DMD (identified in pre-clinical proof-of-concept short studies) should be tested chronically to examine efficiency, toxicity and also possible negative side effects e.g. on heart function.

We conclude that a single 30 min treadmill exercise session is a suitable screening protocol for assessing therapeutic interventions in adult mdx mice (proof-of-concept studies), that serum CK level, myofibre necrosis and oxidative stress in the quadriceps muscle are key endpoints which should be monitored when assessing the efficacy of drug treatments in combination with treadmill exercise (summarised in Table 2), and emphasise the importance
of specific muscle and sampling time (sacrifice and tissue collection). It is hoped that this efficient single exercise protocol will help accelerate pre-clinical drug trials in mdx mice and that further insight into the very early events that lead to myofibre necrosis will identify more precise and better targets for drug interventions to reduce the severity of the dystrophopathy.

Acknowledgements

The authors thank Greg Cozens and Griffin Grounds for excellent technical assistance. Research funding from the Australian National Health and Medical Research Council (M.G., T.S. and P.A.) and Australian Postgraduate Award Scholarships (H.R.-C. and J.T.) are gratefully acknowledged.

References


[56] Clark IA. How TNF was recognized as a key mechanism of disease. Cytokine Growth Factor Rev 2007;18:335–43.


