Attempting to Compensate for Reduced Neuronal Nitric Oxide Synthase Protein with Nitrate Supplementation Cannot Overcome Metabolic Dysfunction but Rather Has Detrimental Effects in Dystrophin-Deficient mdx Muscle

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Title

Attempting to compensate for reduced nNOS protein with nitrate supplementation cannot overcome metabolic dysfunction but rather has detrimental effects in dystrophin-deficient *mdx* muscle

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Running Head

Nitrate supplementation in the *mdx* mouse

Summary

Duchenne Muscular Dystrophy arises from the loss of dystrophin and is characterised by Ca^{2+} dysregulation, muscular atrophy and metabolic dysfunction. The secondary reduction of neuronal nitric oxide synthase (nNOS) from the sarcolemma reduces NO production and bioavailability. As NO modulates glucose uptake, metabolism and mitochondrial bioenergetics, we investigated whether an 8 week nitrate supplementation regimen could overcome metabolic dysfunction in the *mdx* mouse. Dystrophin-positive control (C57BL/10) and dystrophin-deficient *mdx* mice were supplemented with sodium nitrate (85mg.L^{-1}) in drinking water. Following the supplementation period, extensor digitorum longus and soleus were excised and radioactive GU was measured at rest (basal) and during contraction. Gastrocnemius was excised and mitochondrial respiration was measured using the Oroboros Oxygraph. Tibialis anterior was immunohistochemically analysed for the presence of dystrophin, nNOS and nitrotyrosine and histologically analysed to assess areas of damage and regeneration. Basal and contraction-induced glucose uptake was lower in dystrophic muscle and could not be rescued with nitrate supplementation. The mitochondrial utilisation of substrates was also impaired in *mdx* gastrocnemius during phosphorylating and maximal uncoupled respiration and nitrate could not improve respiration in *mdx* muscle. Although nitrate supplementation reduced mitochondrial hydrogen peroxide emission, it induced mitochondrial uncoupling in red gastrocnemius, increased muscle fibre peroxynitrite (nitrotyrosine) and promoted skeletal muscle damage. Our novel data suggests that despite lower nNOS protein expression and likely lower NO production in *mdx* muscle, enhancing NO production with nitrate supplementation in these mice has detrimental effects on skeletal muscle. This may have important relevance for those with DMD.

Keywords

Duchenne Muscular Dystrophy, nitrate supplementation, metabolism, glucose uptake, mitochondria

Introduction

Duchenne Muscular Dystrophy (DMD) is a progressive X-linked [1] neuromuscular disease affecting 1 in 3,500-5,000 live male births [2], which arises from the ablation of the cytoskeletal protein, dystrophin [3]. Dystrophin deficiency causes alterations to the myofibre architecture leading to membrane lesions, calcium (Ca^{2+}) accumulation, muscular weakness and cyclic bouts of degeneration and regeneration until the regenerative capacity of the muscle is unable to match demand for repair [4]. Damaged muscle is eventually replaced with fibrous and/or fatty connective tissue leading to a decrease in muscle function, with cardiorespiratory failure ensuing by the third decade of life [5].

Mitochondrial and metabolic dysfunction have been increasing implicated in the pathogenesis of DMD although it is not known if these abnormalities are associated with dystrophin deficiency, the pathophysiological sequelae caused by dystrophin deficiency, or completely independent of dystrophin deficiency [6]. Indeed, the only obvious physical link between dystrophin and the intracellular metabolic pathways is via neuronal nitric oxide synthase (nNOS) whereby ablation of dystrophin from the sarcolemma induces the secondary loss of the dystrophin-associated proteins [7] including nNOS [8, 9]. nNOS produces NO, a key signalling molecule in skeletal muscle that regulates various biological processes including blood flow, contraction, mass, satellite cell activation, Ca^{2+} handling and glucose uptake (GU), in addition to mitochondrial metabolism, gene expression and reactive oxygen species (ROS) production [10]. In dystrophic muscle, the dissociation of nNOS from the sarcolemma results in reduced nNOS content [11, 12, 13, 14], activity [9, 15, 16] and NO production [17, 18, 19]. Importantly, this loss of nNOS has been shown to contribute to the progression of the dystrophic condition and to the deficits in metabolic function. For example, nNOS is a positive allosteric regulator of phosphofructokinase (PFK), the rate limiting enzyme of the glycolytic pathway [20], and therefore plays a critical role in regulating glucose metabolism. Interestingly, DMD is not only associated with impairments in glycolysis [20, 21, 22] but also in β-fatty acid oxidation, the tricarboxylic acid cycle (TCA) and the electron

transport system (ETS) (for detailed reviewed see [6]). Collectively, these metabolic impairments result in reduced energy production [23], with reports of ATP content being 50% lower under resting conditions [24, 25]. Given that nNOS localisation and NO signalling are known to be important for metabolic control, the loss of nNOS and NO bioavailability might be key to metabolic deregulation in dystrophic skeletal muscle. Therefore, increasing NO availability has the potential to be of therapeutic benefit.

In an attempt to normalise NO production, several studies have reintroduced nNOS into dystrophic skeletal muscle which demonstrably reduces muscle damage and inflammation [26, 27]. As gene therapy for nNOS transfection is not yet available in humans, other strategies to restore NO availability have been investigated. Several studies have shown that supplementation with NO donors, often combined with anti-inflammatory drugs, results in reduced damage, necrosis and inflammation and improved muscle blood flow, function/strength and repair [28, 29, 30, 31, 32, 33, 34, 35] in dystrophin-deficient skeletal muscle. While these findings may suggest a positive effect of increasing NO availability, it is difficult to control the delivery of NO to the skeletal muscle with pharmacological donors and also to separate the effects of the NO donor from those of the anti-inflammatory co-treatment. Another approach to increase NO availability has been to supplement with the nNOS substrate, L-arginine [19]; however, the potential for L-arginine to increase NO production is limited by the lowered nNOS protein in dystrophic skeletal muscle. An alternative method to increase NO availability, that is independent of nNOS activity, is supplementation with nitrate (NITR). Specifically, dietary NITR can be reduced to nitrite by commensal bacteria of the oral cavity and gastrointestinal tract, with nitrite being subsequently reduced to NO via several enzymatic pathways in the blood and tissues [36]. This mechanism is complementary to NOS-derived NO production and, importantly, represents a pathway that could be exploited to increase NO availability in dystrophic muscle.

To date, no studies have investigated the effect of NITR supplementation on metabolic function in dystrophic muscle; however, recent studies suggest that NITR supplementation has the potential to improve metabolic function in skeletal muscle. For example, Larsen *et al.* [37] demonstrated that NITR supplementation in healthy, young males led to increased plasma NO concentration and subsequently, downstream metabolic adaptations including increased mitochondrial efficiency, reduced proton leak and ultimately increased ATP production capacity. Similar data has been derived in rats during fatty acid oxidation [38]. In addition, there is some evidence that NITR supplementation can increase exercise efficiency in humans [39, 40, 41] and exercise capacity in some disease conditions such as peripheral arterial disease, where NO production is reduced [42]. Most pertinently, increasing NO bioavailability through administration of sodium nitrite mitigates functional ischemia in Becker Muscular Dystrophy patients [43] suggesting that expansion of the NITR-nitrite-NO pool in DMD may also be beneficial. The results from these studies prompted us to investigate whether increasing NO availability via NITR supplementation, which has been previously proven to increase plasma [37, 44] and skeletal muscle [38] NO levels and elicit beneficial mitochondrial adaptations at the skeletal muscle level [37, 44], would improve mitochondrial function and rectify energy homeostasis dysregulation in dystrophic muscle. Therefore, we investigated whether an established dietary NITR supplementation regimen [44] could improve GU, mitochondrial function, ROS emission and muscle architecture in healthy (control; CON) and dystrophic (*mdx*) mouse models. We hypothesised that NITR supplementation would (1) increase GU in the contracting muscles from CON and *mdx* mice; (2) improve mitochondrial function in *mdx* mice and; (3) improve the muscle architecture of *mdx* mice.

Materials and Methods

Ethical approval

All experimental procedures were approved by the Victoria University Animal Ethics Experimentation Committee and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animals and supplementation

Three week-old male C57Bl/10ScSn (normal wild-type strain; CON) and C57Bl/10*mdx* (*mdx*) mice were purchased from Animal Resources Centre (Western Australia, Australia) and housed at the Western Centre for Health, Research and Education (Sunshine Hospital, Victoria, Australia) on a 12:12 hour light-dark cycle with *ad libitum* access to food and water. Following a one week acclimatisation period, mice were randomly assigned into four groups: unsupplemented (CON UNSUPP and *mdx* UNSUPP) and supplemented (CON NITR and *mdx* NITR). Mice in the supplemented groups were given 85 $mg.L^{-1}$ (1mM) sodium NITR [44] *ad libitum* in drinking water for 8 weeks and mice in the unsupplemented groups were given drinking water without NITR. The dose of NITR is comparable to doses studied in human experiments, is achievable through a normal diet [45], and is proven to increase the plasma NITR-nitrite-NO pool [37, 44].

Materials and antibodies

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). 2-Deoxy-D-[1,2-^{3}H]glucose and D-[^{14}H]mannitol were purchased from Perkin Elmer (Waltham, MA, USA). Dystrophin (ab15277), nNOS (ab1376), nitrotyrosine (ab42789) and Total OXPHOS (ab110413) primary antibodies were purchased from Abcam (MA, USA). Secondary antibodies for immunohistochemistry were purchased from Jackson Immunoresearch Laboratories (PA, USA) and for western blotting from Vector Laboratories (Burlingame, CA, USA).

159 **Muscle dissection and contraction protocol**

160 Mice were deeply anaesthetised via intraperitoneal injection of sodium
161 pentobarbitone (60mg/kg) and the white extensor digitorum longus (EDL) and red soleus
162 (SOL) muscle proximal and distal tendons in each hind limb were tied with 4/0 surgical silk.
163 Both EDL and SOL were surgically dissected tendon-to-tendon and placed into individual
164 muscle baths containing Krebs basal buffer (118.5mM NaCl, 24.7mM $NaHCO_3$, 4.74mM
165 KCl, 1.18mM $MgSO_4$, 2.5mM $CaCl_2$, 8mM mannitol, 2mM Na pyruvate, 0.01% BSA; pH 7.4)
166 bubbled with carbogen (95% O_2, 5% CO_2) at 30°C. The proximal tendon was attached to a
167 force transducer and muscles were rested for 20 minutes to equilibrate in the bath. Muscles
168 were stimulated via square wave electrical pulses delivered by platinum electrodes flanking
169 the muscles, and subsequent recording of the force output were obtained from a custom-
170 built muscle analysis system (Zultek Engineering, Victoria, Australia). Following
171 determination of optimal length (L_o) for each muscle via a succession of isometric twitch
172 contractions, the left EDL and SOL were stimulated to contract for a total of 10 minutes
173 (pulse durations of 350msec and 500msec for EDL and SOL, respectively at a frequency of
174 60 Hz). This protocol maintains muscle viability and maximises GU [46]. The right EDL and
175 SOL were not stimulated in order to measure basal GU.

176 **Glucose uptake**

177 Following 5 minutes of contraction, the Krebs basal buffer was exchanged for Krebs
178 buffer with 2-Deoxy-D-[1,2-^{3}H]glucose (0.128µCi/mL) and D-[^{14}H]mannitol (0.083µCi/mL) in
179 both resting and contracting muscles. At the end of the 10 minute contraction protocol,
180 muscles were immediately submerged in ice-cold Krebs basal buffer to stop further glucose
181 uptake, blotted on filter paper and snap frozen in liquid nitrogen. Whole muscles were
182 weighed frozen, digested for 10 minutes at 95°C in 135µL of 1M NaOH, neutralised with
183 135µL of 1M HCl and centrifuged for 5 minutes at 13,000g. 200µL of supernatant was added
184 to 4mL of inorganic scintillation fluid (UltimaGold, Perkin Elmer) and radioactivity was

185 measured in a β-scintillation counter (Tri-Carb 2810, Perkin Elmer). GU was calculated [47]
186 and corrected for sPo as described previously [48].

187 **Mitochondrial respiration and hydrogen peroxide emission measurements**

188 Left and right gastrocnemius were excised from the anaesthetised mice, separated
189 into RG and WG portions and immediately placed into ice-cold BIOPS (7.23mM K_2EGTA,
190 2.77mM CaK_2EGTA, 5.77mM Na_2ATP, 6.56mM $MgCl_2$-$6H_2O$, 20mM taurine, 15mM
191 phosphocreatine, 20mM imidizole, 0.5mM dithiothreitol, 50mM K^+-MES; pH 7.1). Muscle
192 fibres were mechanically separated from a small portion of muscle in ice-cold BIOPS to
193 maximise fibre surface area and transferred into ice-cold BIOPS supplemented with saponin
194 (50µg/mL) for 30 minutes. Separated fibres were agitated to permeabilise the sarcolemma
195 and allow diffusion of subsequent assay substrates, and then washed three times via
196 agitation in ice-cold respiration buffer (110mM K^+-MES, 35mM KCl, 1mM EGTA, 5mM
197 K_2HPO_4, 3mM $MgCl_2$-$6H_2O$, 0.05mM pyruvate, 0.02mM malate, 5 mg/mL BSA; pH 7.4).
198 Fibre bundles were then divided and weighed on a microbalance (2-4 mg each) for
199 subsequent respirometry analysis in duplicate.

200 ETS respiration, OXPHOS and H_2O_2 emission were measured by the Oxygraph O2k
201 high resolution respirometer (Oroboros Instruments, Innsbruck, Austria) via a substrate,
202 uncoupler, inhibitor titration (SUIT) protocol at 37°C in MIR05 respiration medium while
203 stirring at 750 rpm as previously described [49, 50], with minor modifications in order to
204 assess H_2O_2 emission. Briefly, after fibres and oxygen (O_2) were added to the respiration
205 chamber, the SUIT protocol commenced with titrations of the complex I (CI) substrates
206 malate (2mM final concentration) and pyruvate (10mM), followed by the complex II (CII)
207 substrate succinate (10mM) to determine leak (state 4) respiration. Titrations of ADP (0.25, 1
208 and 5mM) assessed OXPHOS (state 3) capacity, addition of cytochrome *c* (10µM) tested
209 mitochondrial membrane integrity and titrations of FCCP (0.025µM) determined uncoupled
210 respiration. Complex-specific respiration was inhibited by the addition of rotenone (1µM) and

antimycin A (5µM) to CI and complex III (CIII), respectively. Finally, complex IV (CIV) capacity was measured during oxidation of TMPD (0.5mM) with ascorbate (2mM). The O_2 flux due to autoxidation of these chemicals was determined after inhibition of CIV with sodium azide (75 mM) then subtracted from the raw CIV O_2 flux.

Mitochondrial H_2O_2 emission was simultaneously measured in the respiration chamber throughout the SUIT protocol via optical sensors (O2k-Fluorescence LED-2 Module, Oroboros, Austria) as previously described [51, 52, 53]. Superoxide (O_2^-) produced during the SUIT protocol was converted to H_2O_2 due to the presence of a saturating concentration of O_2^- dismutase (2.5 $U.mL^{-1}$), and the subsequent H_2O_2 generation was quantified via the reaction of Amplex UltraRed (25 µM; Molecular Probes, Invitrogen) with horseradish peroxidase (2.5 $U.mL^{-1}$) at excitation/emission 565/600 nm wavelength. The H_2O_2 detection chemicals were added to the chambers containing MIR05 respiration medium at the beginning of the experiment, prior to the addition of the muscle fibres.

Chamber O_2 concentration was maintained between 300-450 $nmol.ml^{-1}$. Mass specific O_2 flux and H_2O_2 emission was determined from steady-state flux normalised to tissue wet weight and adjusted for instrumental background and residual O_2consumption. Respiratory control ratios were calculated (complex specific O_2 flux relative to maximal uncoupled ETS respiration) to investigate intrinsic mitochondrial function independent of mitochondrial density.

Western blot analysis of mitochondrial respiratory chain proteins

Frozen tissues were homogenised for 20 seconds in ice-cold WB buffer (40 mM Tris, pH 7.5; 1 mM EDTA; 5 mM EGTA; 0.5% Triton X-100; 25 mM β-glycerophosphate; 25 mM NaF; 1 mM Na3VO4; 10 µg/ml leupeptin; and 1 mM PMSF), and the whole homogenate was used for further analysis. Sample protein concentrations were determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts of protein (15µg) from each sample were dissolved in Laemmli buffer, heated for 5 min at 37^oC and

subjected to electrophoretic separation on SDS-PAGE acrylamide gels. Following electrophoretic separation, proteins were transferred to a PVDF membrane, blocked with 5% powdered milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with primary antibody dissolved in TBST containing 1% bovine serum albumin (BSA). The Total OXPHOS Antibody Cocktail (1:1000), which detects representative proteins from each of the five mitochondrial respiratory chain complexes, was obtained from Abcam. This cocktail consisted of primary antibodies against the following proteins: NADH Dehydrogenase (Ubiquinone) 1 Beta Sub-complex, 8 (NDUFB8; CI), Succinate Dehydrogenase Assembly Factor 4 (SDH8; CII), Ubiquinol-Cytochrome-C Reductase Complex Core Protein 2 (UQCRC2; Complex III; CIII), Mitochondrially Encoded Cytochrome C Oxidase I (MTOC1; Complex IV; CIV) and Mitochondrial ATP Synthase Subunit Alpha (ATPSA; Complex V; CV). After overnight incubation, the membranes were washed for 30 min in TBST and then probed with a peroxidase-conjugated secondary antibody (1:10000, anti-mouse, Vector Labs) for 1 h at room temperature. Following 30 min of washing in TBST, the blots were developed with a DARQ CCD camera mounted to a Fusion FX imaging system (Vilber Lourmat, Germany) using ECL Prime reagent (Amersham, Piscataway, NJ, USA). Once the images were captured, the membranes were stained with Coomassie Blue to verify equal loading of total protein in all lanes. Densitometric measurements were carried out using Fusion CAPT Advance software (Vilber Lourmat, Germany).

Citrate Synthase Activity

Homogenised RG and WG samples were utilised to measure citrate synthase (CS) activity as a marker of mitochondrial density [54, 55]. Homogenised samples were added to reagent cocktail (100 mM Tris Buffer, 1 mM DTNB, 3 mM Acetyl CoA) and CS activity was measured spectrophotometrically (412nm at 25°C) for 5 minutes following the addition of 10mM oxaloacetate as described previously [23]. CS activity was calculated using the

extinction coefficient of 13.6 [56]. CS activity was normalised to whole muscle protein concentration.

Histological Analysis

Following excision, the right TA was frozen in liquid nitrogen-cooled isopentane and optimal cutting temperature compound (Sakura Finetek). Embedded TA's were cryo-sectioned (10µm) at -20°C using a Leica (CM1950) cryostat and mounted onto glass slides (Menzel-Glaser).

Dystrophin and nNOS Immunolabelling

Slides were fixed using a Cytofix/Cytoperm Plus kit (BD Biosciences) for 5 minutes. After incubation with blocking serum (0.1M PBS, 0.1% triton, 10% FBS) for 1 hour at room temperature, sections were labelled with primary antibodies: rabbit anti-dystrophin (1:400) and goat anti-nNOS (1:200) overnight at room temperature. After washing three times with a 0.1M PBS and 0.1% triton X solution, samples were incubated with secondary antibodies: donkey anti-rabbit Alexa 488 (1:200) and donkey anti-goat Alexa 594 (1:200) for 2 hours at room temperature. Tissues were washed three times with a 0.1M PBS and 0.1% triton X solution and mounted with fluorescent mounting medium (DAKO, Australia). Confocal microscopy was performed on an Eclipse Ti confocal laser scanning system (Nikon, Japan). Fluorophores were visualized using a 488 nm excitation filter for Alexa 488 or FITC and a 559 nm excitation filter for Alexa 594 or Rhodamine Red. Z-series images were acquired at a nominal thickness of 0.5µm (512x512 pixels). The density of dystrophin and nNOS immunoreactivity in TA sections was measured from eight randomly captured images (total area size 2mm^2) per animal at 20x magnification. All images were captured under identical acquisition exposure time conditions and calibrated to standardised minimum baseline fluorescence. Images were converted from red, green, and blue (RGB) to grayscale 8 bit then to binary; changes in fluorescence from the baseline were measured using Image J

289 software (NIH, USA). The area of immunoreactivity was then expressed as a percentage of
290 the total area examined. Quantitative analyses were conducted blindly.

291 *Nitrotyrosine, CD45 and IgG Immunolabelling*

292 Slides were fixed with 4% formaldehyde for 30 minutes at room temperature. After
293 incubation with blocking serum (0.1M PBS, 0.1% triton, 10% donkey serum) for 1 hour at
294 room temperature, sections were then labelled with primary anti-S-nitrotyrosine (rabbit;
295 1:200; Millipore), anti-CD45 (pan-leukocyte marker; rat; 1:200) and anti-IgG isotype control
296 (hamster; 1:200) primary antibodies overnight at room temperature. After washing three
297 times with a 0.1M PBS and 0.1% triton X solution, samples were incubated with the
298 appropriate secondary antibody (anti-rabbit Alexa Fluor 647, 1:100, Abacus ALS, UK; anti-
299 rat Alexa Fluor 488, Jackson Immuno Research; anti-hamster Alexa Fluor 594, Jackson
300 Immuno Research) for 2 hours at room temperature. A pan nuclei marker DAPI (4',6-
301 diamidino-2-phenylindole) was added to the tissue sections and incubated for 2 minutes at
302 room temperature, tissues were washed three times with a 0.1M PBS and 0.1% triton X
303 solution and mounted with an anti-fade fluorescent mounting medium. Excitation
304 wavelengths were set to 640nm for Alexa 647, 406nm for Alexa 405, 408.8nm for Alexa
305 Fluor 488 and 561.8nm for Alexa Fluor 594. The confocal microscope was calibrated to
306 standardise the minimum baseline fluorescence for imaging nitrotyrosine, CD45 and IgG
307 immunoreactivity in the TA cross sections. At time of analysis all files were converted to
308 thresholded 8-bit binary images using ImageJ software from eight randomly captured images
309 per animal. Images were analysed through the 'analyze particles' function, recording the
310 counts (to determine the number of DAPI positive nuclei) and relative nitrotyrosine
311 expression recorded as percentage area fraction in arbitrary units. Green pseudocolor
312 images of nitrotyrosine (Alexa Fluor 647; magenta) were generated using ImageJ software
313 for publication only.

314 *Haematoxylin & Eosin Staining*

Slides were air-dried and stained using a haematoxylin and eosin (H&E) staining protocol including a 30 second incubation in haematoxylin and a 1 minute and 45 second incubation in eosin. Slides were imaged using a Zeiss Axio Imager Z2 microscope at 20x magnification. Fibre size, damaged area (areas of myofibril demise and inflammatory cell infiltration [57] and fibres with centralised nuclei were determined using ImageJ software.

Statistics

Results are presented as mean ± standard error of the mean. For all data, except for GU, a two-way ANOVA was utilised to detect between strain/genotype (CON vs. *mdx*) and supplementation (UNSUPP vs. NITR) differences. For GU data, a three-way ANOVA was performed for each of EDL and SOL to detect between strain, supplementation and GU type (basal vs. contraction). When a main effect or an interaction was detected, unpaired T-tests were used to determine differences between individual groups using SPSS (version 21). An α value of 0.05 was considered significant.

Results

Effect of NITR supplementation on body weight, food and water consumption and muscle weights

Throughout the 8 week supplementation period, greater weight gains were observed in the *mdx* groups compared to CON ($p<0.0001$; Figure 1A), with NITR having no effect in *mdx* mice ($p>0.05$). NITR did, however, stimulate weight gain in CON ($p<0.05$). No significant difference in food or water consumption was observed between any group over the supplementation period (Figure 1B & C respectively; $p>0.05$) except at week two where food consumption was greater in CON UNSUPP compared to all groups ($p<0.05$, Figure

1B). Overall, individual hind limb muscle weights were greater in the *mdx* compared to CON strain (p<0.0001; Table 1 and 2) with NITR having no effect (p>0.05).

Immunolabelling of dystrophin and nNOS

To confirm the deficiency of both dystrophin and nNOS in *mdx* skeletal muscle, the presence of dystrophin and nNOS protein (Figure S1) was determined in the TA. Indeed, dystrophin was only evident in CON TA (Figure S1A and C) and was absent from *mdx* TA except for a few spontaneously revertant fibres (p<0.0001). Similarly, nNOS was only evident in CON TA (Figure S1A^{I} and C^{I}) and was completely absent from dystrophin-deficient *mdx* fibres (p<0.0001). Co-localisation of dystrophin and nNOS was only observed in CON (Figure S1A^{II} and C^{II}) and NITR had no effect on either dystrophin or nNOS expression (p>0.05).

Effect of NITR supplementation on glucose uptake

NO has been proposed to play a role in contraction-stimulated GU and as such, we first investigated the effect of NITR supplementation on GU in CON and *mdx* muscles. This is the first instance of contraction-induced GU being measured in the *mdx* mouse and we demonstrated no difference in basal- or contraction-induced GU between CON and *mdx* UNSUPP EDL (p>0.05, Figure 2A). As expected, contraction induced an increase in GU in the EDL's of CON UNSUPP (55%), CON NITR (61%), *mdx* UNSUPP (35%) and *mdx* NITR (51%) compared to basal conditions (p<0.05, Figure 2A). NITR supplementation significantly increased contraction-induced GU in CON EDL muscles (p<0.05; Figure 2A) however in contrast, NITR reduced both basal- and contraction-induced GU in *mdx* muscles (p<0.05; Figure 2A). Contrary to the EDL, contraction did not stimulate further GU beyond that observed in basal conditions for any group in the SOL (all less than 20%, p>0.05, Figure 2B). While NITR had no effect on basal or contraction-induced GU in CON SOL muscles (p>0.05; Figure 2B), NITR further reduced both basal and contraction-induced GU in *mdx* SOL muscles (p<0.05). Combined, these data suggest that NITR supplementation has a

negative effect on GU in both *mdx* EDL and SOL which may lead to impairments in downstream glycolysis and oxidative metabolism.

Effect of NITR supplementation on mitochondrial function

Respirometry

Next, we examined the effect of NITR on parameters of mitochondrial function. First, we measured state 4 leak respiration which, in the absence of ADP, indicates the contribution of proton leak to respiration. In the presence of pyruvate and malate (CI), state 4 leak respiration was significantly lower in *mdx* white (WG) (p<0.05; Figure 3A) and red gastrocnemius (RG) (p<0.01; Figure 3C) muscles compared to their respective controls. In the presence of pyruvate, malate and succinate (CI+II), state 4 leak respiration was significantly higher than CI respiration across all groups, in both WG and RG muscles (p<0.0001; Figure 3A and 3C, respectively). NITR supplementation had no effect on either CI or CI+II state 4 leak respiration in CON or *mdx* muscles (Figure 3A and 3C).

Next, the effect of NITR on coupled OXPHOS capacity was examined in WG and RG muscles by assessing maximal ADP-stimulated state 3 respiration in the presence of excess malate, pyruvate and succinate (complex I and II (CI+II) substrates). As shown in Figure 3, state 3 respiration was significantly depressed in *mdx* WG by ~15% (p<0.05; Figure 3A) and in *mdx* RG by 25% (p<0.001; Figure 3C) compared to CON. NITR supplementation, however, had no effect on State 3 respiration in either muscle (Figure 3A and 3C).

Maximal ETS capacity was then assessed by the addition of the uncoupling agent FCCP, which dissipates the mitochondrial membrane potential ($\Delta\Psi$). This parameter gives an indication of the maximal respiration in the uncoupled state. FCCP-induced maximal uncoupled respiration was significantly lower in *mdx* WG (p<0.05; Figure 3A) and RG (p<0.001; Figure 3C) compared to their respective controls; however, there was no effect of NITR on this parameter.

Next, we measured the activity of CIV (cytochrome C oxidase), the terminal oxidase of the ETS and the site of O_2 reduction to water. As shown in Figure 3, CIV activity was not different between UNSUPP CON and *mdx* WG muscles (Figure 3A); however, in the RG muscles, CIV activity was significantly lower in *mdx* UNSUPP compared to CON UNSUPP (p<0.01; Figure 3C). NITR induced a significant increase in CIV activity in CON WG muscles (p<0.01; Figure 3A) but reduced CIV activity in both CON and *mdx* RG muscles (p<0.01; Figure 3C).

Finally, the respiratory control ratio (state 3 respiration divided by state 4 respiration; RCR) was calculated. The RCR is an indicator of the extent to which O_2 consumption is coupled to ATP production and therefore mitochondrial efficiency, with a higher RCR indicating better coupling. No difference in RCR was observed between CON and *mdx* WG (p>0.05, Figure 5B). In *mdx* RG respiring on CI+II, the RCR was significantly lower compared to CON (p<0.0001) and NITR decreased the RCR further (p<0.01, Figure 3D). This highlights that in oxidative red muscle at least, *mdx* mitochondria are more uncoupled and that this uncoupling is exacerbated by NITR.

Electron Transport Chain Complex Expression

To determine whether the genotypic differences and NITR supplementation-induced changes in respiration parameters were associated with differences in mitochondrial ETS complex densities, the abundance of representative proteins from each of the five ETS complexes were measured using semi-quantitative Western blotting (Figure 4 and 5). In WG muscles, despite state 3, state 4 and maximal uncoupled respiration being lower in *mdx* muscles (Figure 3A), the relative abundance of representative proteins from complexes I to V were not lower. In fact, to the contrary, proteins from CII, CIII, CIV and CV were significantly elevated in *mdx* UNSUPP WG muscles compared to CON UNSUPP muscles (Figure 4). Interestingly, the NITR-induced increase in CIV respiratory activity (Figure 3A) was not associated with a significant increase in the abundance of the CIV protein (Figure

4D). NITR supplementation did, however, lead to an increase in representative proteins in WG muscles for CI, CII, CIII and CV in CON but not *mdx* muscles (Figure 4).

Unlike the WG muscles, the lower state 3, state 4, uncoupled respiration and CIV activity found in RG *mdx* muscles (Figure 3C) was accompanied by a reduction in representative proteins for CI, CII, CIV and CV compared with CON; however, NITR supplementation had no effect on any of these proteins in either CON or *mdx* RG muscles (Figure 5).

Citrate Synthase Activity

Finally, we measured citrate synthase (CS) activity in WG and RG muscles as a co-marker of mitochondrial content alongside mitochondrial ETC proteins [58] (Figure 4F and 5F, respectively). As shown in Figure 4F, there was a trend for CS activity to be higher in *mdx* UNSUPP compared to CON UNSUPP WG muscles. Moreover, NITR increased CS activity in both CON and *mdx* WG muscles. In the RG muscles there was no difference in CS activity between UNSUPP CON and *mdx* mice; however, NITR increased CS activity in RG muscles from *mdx* mice. Overall, NITR did not improve the capacity to phosphorylate ATP or maximal respiratory capacity in dystrophic muscle despite increasing CS activity, suggesting that NITR may have an alternative effect on mitochondrial function such as ROS generation.

Effect of NITR supplementation on ROS production in red and white gastrocnemius

The effect of NITR supplementation on the production of the mitochondrial ROS superoxide (O_2^-), was measured in intact and permeabilised fibres from WG and RG simultaneously with respiration. In the presence of excess O_2^- dismutase, O_2^- is converted to hydrogen peroxide (H_2O_2), which reacts with Amplex Red to produce the red fluorescent product, resorufin. During state 3 respiration, no differences in H_2O_2 emission was detected between CON and *mdx* UNSUPP WG (p>0.0 5; Figure 6A) with NITR having no effect in

444 either strain (p>0.05; Figure 6A). NITR did, however, induce a decrease in H_2O_2 emission
445 during state 4 leak respiration in CON WG muscle fibres respiring on CI substrates (p<0.05;
446 Figure 6A). When respiring on CI+CII substrates, there was significantly greater H_2O_2
447 emission in all groups during state 4 leak respiration compared to CI substrates only in WG
448 fibres (p<0.0001; Figure 6A). Importantly, NITR significantly decreased H_2O_2 emission in
449 both CON and *mdx* WG muscles respiring during state-4 while on CI+II substrates (p<0.05;
450 Figure 6A). There was no difference in H_2O_2 emission in WG between any groups during
451 FCCP-stimulated maximal uncoupled respiration (p>0.05; Figure 6A).

452 In *mdx* RG fibres, there was significantly less H_2O_2 emission during state 3
453 respiration (p<0.05; Figure 6B) compared to CON fibres; however, there was no effect of
454 NITR on this parameter (p>0.05). Similar to WG fibres, H_2O_2 emission was higher when
455 respiring on CI+CII substrates compared to CI substrates across all groups during state 4
456 leak respiration (p<0.0001), however, NITR only reduced H_2O_2 emission in *mdx* fibres
457 (p<0.001; Figure 6B). NITR also reduced H_2O_2 emission in *mdx* RG fibres during FCCP
458 uncoupled respiration (p<0.05; Figure 6B). While our data suggests that NITR reduces
459 mitochondrial ROS production in dystrophic muscle, it is possible that increased NO
460 bioavailability may sequester O_2^- from the O_2^- dismutase reaction to increase reactive
461 nitrogen species (RNS).

462 **Effect of NITR supplementation on peroxynitrite production, CD45-positive(+)**
463 **infiltration and IgG immunolabelling**

464 NO is known to rapidly react with O_2^- resulting in the production of the highly RNS,
465 peroxynitrite ($ONOO^-$), and given that elevated ROS is present in *mdx* muscle [59], we
466 investigated whether $ONOO^-$ production could account for the reduced H_2O_2 emission
467 observed in our study. Increased $ONOO^-$ can result in increased protein nitration of tyrosine
468 residues, potentially leading to altered protein function. Therefore, as an indirect marker of
469 oxidative/nitrosative stress, we measured the effect of NITR on levels of nitrotyrosine via

immunohistochemical staining of TA muscles. *Mdx* muscles had significantly higher nitrotyrosine staining compared to CON muscles (p<0.0001) and NITR increased nitrotyrosine staining in both CON (p<0.05) and *mdx* (p<0.0001) TA (Figure 7A). Importantly, NITR inducing a dramatically greater increase in nitrotyrosine production in *mdx* muscles (2775% increase compared to 82% increase in CON). Additionally, NITR further increased the presence of DAPI-stained nuclei in NITR supplemented *mdx* TA (p<0.0001, Figure 7B) . To assess if the increased nitrotyrosine staining was associated with increased inflammation, we measured CD45+ immune cell infiltration and IgG via immunolabelling. In *mdx* TA, both CD45+ and IgG+ area was elevated compared to CON muscles (p<0.001 and p<0.01 respectively, Figure 7C and D respectively). In contrast to nitrotyrosine staining, NITR had no effect on the CD45+ and IgG+ area in either strain (p>0.05).

Effect of NITR supplementation on muscle architecture

Finally, we assessed the effect of NITR on muscle fibre histopathology. As expected, intact *mdx* muscle fibres were significantly larger than fibres from CON muscles (Figures 8A and C) which is representative of pseudohypertrophy, a hallmark histopathological feature of dystrophin-deficient muscle. Interestingly, there was a strong trend for NITR supplementation to increase the number of fibres between 6000 and 7499 μm^2 (p=0.068; Figure 8A) and increase total mean fibre size (p=0.093; Figure 8C). The area of damage, as indicated by areas of inflammatory cell/nuclei infiltration, was significantly higher in *mdx* (p<0.01; Figure 8D) compared to CON TA sections and NITR significantly increased the damage area in *mdx* muscle (p<0.01). Centronucleated fibres, a marker of muscle cell regeneration, were significantly higher in *mdx* muscle (p<0.0001; Figure 8E) with NITR further increasing regeneration area in *mdx* sections (p<0.01). These results show that NITR supplementation enhances muscle damage, but also regeneration, in *mdx* TA but not in CON, which seems reflective of the increased $ONOO^-$ production.

Discussion

This is the first study to date to investigate NITR supplementation as a potential therapy for DMD and we show that the metabolic perturbations in dystrophin-deficient skeletal muscle could not be overcome by enhancing nNOS-independent NO production. Instead, our data suggests that chronically increasing NO bioavailability without restoring nNOS protein expression and its regulatory role on metabolism, in fact, promotes pathological muscle damage, potentially via a peroxynitrite ($ONOO^-$)-dependent mechanism.

In the first instance we investigated if impaired macronutrient uptake may be a contributing factor to the mitochondrial dysfunction in dystrophic muscle, as compromised transport of substrates across the sarcolemma could be a consequence of the loss of dystrophin and nNOS from the membrane. Specifically, we have investigated glucose uptake (GU) as it is well established that GU during rest and contraction is regulated by NO [46]. The secondary loss of dystrophin-associated nNOS was confirmed in *mdx* TA via immunolabelling. Concurrently, we have demonstrated that both basal- and contraction-induced GU in both *mdx* UNSUPP EDL and SOL is comparable to CON. We have demonstrated in our study that NITR increases contraction-induced GU in CON EDL but has no effect in CON SOL. Indeed, we have shown previously that there are greater effects of NOS inhibition on EDL than SOL [46], likely because of a greater comparative nNOS expression in fast-twitch versus slow-twitch muscles [46, 60] and the higher antioxidant enzymes in slow-twitch muscles which may buffer the effects of NO [61]. In both CON EDL and SOL muscles, however, NITR did not affect basal GU rate. This could infer that the NITR dosage administered in our study is sufficient to modulate non-cGMP-dependent contraction-induced GLUT-4 mediated GU [62] but perhaps not cGMP-dependent GLUT-1 basal [63] events. A notable limitation of our study is that we did not quantify cGMP levels in EDL and SOL muscles. However, in light of a recent study which demonstrated that even low dose (0.35mM) NITR therapy for ~2 weeks (in comparison to the 1mM NITR dosage for 8 weeks administered in our study) was sufficient to induce ~3-fold increases in cGMP levels

523 in rat skeletal muscle, this seems unlikely. Rather, basal GU is likely regulated in the first
524 instance by glucose utilisation, thus increasing NO signalling without the normal
525 simultaneous increase in muscle work (and thus glucose utilisation) results in an unchanged
526 basal GU. Unexpectedly and in contrast to CON EDL, NITR reduced basal GU in *mdx* EDL
527 and SOL. Taken with the fact that NITR stimulated contraction-induced GU in CON but
528 further depressed it in *mdx* muscle, our data suggests that NITR-derived NO is being
529 diverted away from its bio-modulatory effects on GU. Presumably, this is because in *mdx*
530 muscle, in which O_2^- production is notoriously increased [59], NITR-generated NO is being
531 sequestered into $ONOO^-$ production instead of cGMP activation, thus reducing the
532 proportional NO available to GU signalling. despite an increased NITR-nitrite-NO pool.

533 We have assessed various indices of mitochondrial respiratory function in
534 permeabilised red (RG) and white (WG) gastrocnemius fibre bundles. Permeabilisation of
535 intact muscle bundles and delivery of optimal substrate concentrations allows for the
536 measurement of the mitochondrial capacity independent of substrate delivery capacity.
537 Indeed, even in this optimised environment, we demonstrate a reduced capacity (up to 25%
538 of CON) to phosphorylate ADP in both WG and RG from the *mdx* mouse. This is consistent
539 with others [64, 65] who have reported similar depressions in ADP-stimulated
540 phosphorylating respiration in *mdx* skeletal muscle fibres. NITR did not improve
541 phosphorylating or maximal uncoupled respiration in either CON or *mdx* skeletal muscle but
542 did decrease CIV activity in both CON and *mdx* RG. CIV inhibition is an established effect of
543 reversible competitive binding of NO to heme-copper sites *in lieu* of O_2 on CIV, in addition to
544 CI and CIII [66, 67, 68]. Despite the inhibitory effect of NITR on CIV activity and therefore
545 ETS respiratory capacity, the lack of effect on phosphorylating and maximal uncoupled
546 respiration was unexpected, since NITR has been previously shown to improve various
547 mitochondrial properties through stimulation of mitochondrial biogenesis and improved
548 coupling of O_2 consumption to ATP production [37]. Since NO is a highly reactive molecule
549 that, to exert its biological role, must be produced in close proximity to its effector targets, the

550 exogenous NO source afforded by NITR supplementation in our study may not be
551 penetrating the muscle fibres sufficiently, or in sufficient concentration, to modulate
552 mitochondrial function. This is particularly true of the mitochondrial function governed by
553 nuclear gene regulation such as mitochondrial biogenesis and uncoupling. Aquilano *et al.*
554 [69], for instance, have demonstrated that the loss of nNOS-generated NO production
555 nearby the nucleus is a causative factor of the impairment of mitochondrial biogenesis in
556 skeletal muscle. Thus, while we have evidence of NITR-derived NO penetrating the
557 mitochondria to induce regulatory adaptations such as inhibition of CIV activity, overall
558 respiratory capacity which is dictated predominantly by mitochondrial density and coupling is
559 seemingly unaffected, even in CON mice. This is likely due to the chronic supplementation
560 period and particular dosage employed in our study. For example, similar to our study, Hezel
561 *et al.* [70] did not observe any changes in mitochondrial parameters following 17 months of
562 NITR supplementation in healthy mice. In contrast, others have shown beneficial
563 mitochondrial modulation following much shorter supplementation periods [37, 71]. Ashmore
564 *et al*. [38] have recently demonstrated that NITR dosage is also important to the control of
565 the nuclear signalling of mitochondrial biogenesis in which low (0.35mM), medium (0.7mM)
566 and high (1.4mM) dose NITR therapy (for 15-18 days) in rats had differential effects on
567 PPARα/β/δ signalling, PGC-1α expression, citrate synthase activity and mitochondrial fatty
568 acid oxidation. These data highlight that the promotion of mitochondrial biogenesis might be
569 an acute, dose-specific response to shorter-term increases in skeletal muscle NO signalling
570 which may switch off or become desensitised in response to more chronic, prolonged
571 increases in NO production.

572 The reduced capacity for *mdx* skeletal muscle to phosphorylate ADP and to ramp up
573 respiration during times of metabolic stress may be reflective of uncoupled respiration. In our
574 study, state 4 respiration was significantly less in both WG and RG of *mdx* mice and the
575 RCR was was lower in *mdx* RG respiring on CI+II substrates highlighting that respiratory
576 control is compromised in the muscle that is most dependent upon mitochondrial oxidative

ATP production (i.e. red oxidative muscle). When considered in context of a depressed state 3 and 4 respiration, tighter respiratory control would be required to maintain the ΔΨ and drive for ATP synthesis, especially given the heightened energy requirements of dystrophic muscle. Indeed, our observations of a depolarised ΔΨ in isolated *mdx* mitochondria (C.A. Timpani, A. Hayes and E. Rybalka, unpublished observations) indicate insufficient coupling to maintain the drive for ATP synthesis in red muscle at least. Intriguingly, NITR decreased the RCR only in *mdx* muscles, indicative of mitochondrial uncoupling. Uncoupling may be a beneficial adaptation to ETS dysfunction, to prevent potential hyperpolarisation of the ΔΨ which is an initiator of mitochondria-mediated apoptosis [72]. Certainly, the role of NITR-derived NO in the regulation of mitochondrial coupling efficiency is unclear since some studies have demonstrated an enhanced coupling efficiency of human skeletal muscle [37] while others have shown a reduced coupling efficiency of rodent skeletal muscle [38]. Despite the obvious species differences between these studies, these data highlight that NITR-derived NO has a modulatory role on the expression of uncoupling protein 3 (through increased PPAR-α activation [38]) and adenine nucleotide translocase expression, and seemingly regulates the leakiness of several respiratory complexes – all of which contribute to the coupled state of skeletal muscle mitochondria. However, this role requires further elucidation.

A reduced mitochondrial pool (particularly viable mitochondria) could also explain the decreased OXPHOS capacity of dystrophic skeletal muscle in our study. We saw no genotype or muscle-specific differences in CS activity (a marker of mitochondrial density) in our study, however we did see differential expression of ETC complex proteins in *mdx* RG and WG whereby complex proteins generally decreased in RG but increased in WG. This suggests a reduced respiratory capacity despite increased/unchanged mitochondrial density in *mdx* RG in particular. We [23] and others [22, 64, 73] have previously reported this, highlighting that a reduced mitochondrial functional and/or physical density does not account for the decreased mitochondrial respiration associated with dystrophin-deficiency but rather,

that the mitochondrial pool is intrinsically defective. While NITR had no effect on complex expression in RG from either strain, most complexes (except CIV), were upregulated in NITR-supplemented CON but not NITR-supplemented *mdx* WG. In fact, the only observed effect of NITR in WG that was consistent across strains was an increased CS activity, and this was reproducible in the RG from *mdx* but not CON mice. Our finding is curious since Ashmore *et al*. demonstrated that a high (1.4 mM) NITR diet increases CS activity in red SOL muscle from healthy rats, albeit a low (0.35mM) and medium (0.7mM) diet did not [38]. In context, we supplemented our mice with 1mM NITR. Our data thus suggests that there are variations in the response of different fibre types to NITR-derived NO dosages, in which type II fibres are more responsive to a lower NO concentration. Irrespective, changes in mitochondrial CS activity and ETC complex expression induced by NITR did not translate to improved mitochondrial respiration in either CON or *mdx* muscles in our study.

We found in various respiratory states that NITR reduced H_2O_2 production in *mdx* but not CON skeletal muscle. This would immediately seem to be beneficial, as ROS production is elevated in dystrophic muscle [59] and NO reduces oxidative stress at the level of the ETS [74]. However, excessive NO can lead to the generation of RNS in the presence of O_2^-. In addition to the inhibition of CIV, NO inhibits electron transfer at CI and CIII of the ETS [75], producing O_2^- anions that interact with NO to produce $ONOO^-$ which can induce cellular damage [76]. In our study, we have demonstrated elevated nitrotyrosine content in *mdx* TA muscles, which is consistent with increased $ONOO^-$ production, and this was dramatically exacerbated by NITR (2775% increase). Nitrotyrosine labelling corresponded with an increased area of damage in NITR-supplemented *mdx* TA sections. In previous studies, NO donor therapy has been shown to reduce the area of damage in dystrophic muscle, but as NO donors are typically given in combination with anti-inflammatories [29, 33], our data suggests that the anti-inflammatory component of these co-compounds is perhaps the more pertinent effector. NITR also increased the proportion of centronucleated fibres in *mdx* muscles, which has been previously observed with NO donors [29, 30] and is reflective of an

enhanced regenerative capacity in response to NITR-induced damage. NO is a known stimulator of satellite cell proliferation, which is crucial to skeletal muscle regeneration following damage [77] and is notably defective in dystrophin-deficient muscle [78, 79]. Since dystrophic muscle is in a state of enhanced oxidative stress superfluous NITR-derived NO bioavailability appears detrimental to dystrophic muscle by promoting excess $ONOO^-$ formation which, in turn, may exceed antioxidant buffering capacity to promote muscle damage and escalate pathology. This effect may be more evident in predominantly white fast-twitch glycolytic muscles (such as TA) due to the lower endogenous antioxidant content and therefore NO handling capacity, however further investigation is required to elucidate if this is true. It is also possible that the absence of nNOS protein expression, its translocational capacity to deliver NO to specific intracellular sites and the metabolic modulatory effects it exerts, may account for the deleterious effect that NITR had on dystrophic muscle histopathology in our study, since breeding transgenic overexpressing nNOS mice with the *mdx* strain results in significant improvements to dystrophic muscle architecture [26, 27]. NITR therapy, however, might be beneficial for the stimulation of satellite cell replication and dystrophic skeletal muscle regeneration as we observed elevated presence of DAPI-positive nuclei in NITR-treated *mdx* TA. While we did not stain for Pax-7 (a satellite cell marker), we did label CD45+ immune cell infiltrate and IgG deposition within the muscle cross-sections – neither of these measures were affected by NITR SUPP suggesting that the NITR-dependent increase in nuclei content is most likely reflective of an enhanced satellite cell pool. Therefore NITR therapy could be beneficial especially if mitochondrial O_2^- production could be pharmacologically attenuated and RNS-induced damage prevented (such as with antioxidant therapy).

In summary, our study is the first to demonstrate that an 8 week supplementation regimen of NITR in drinking water cannot overcome the metabolic dysfunction observed in the *mdx* mouse model of DMD. We are the first to examine contraction-induced GU in the *mdx* model and to demonstrate that NITR supplementation reduces otherwise normal GU in

mdx muscles and cannot positively modulate mitochondrial function. Although NITR supplementation reduced mitochondrial H_2O_2 emission, it induced mitochondrial uncoupling in RG, increased muscle fibre nitrosylation (and therefore $ONOO^-$ radicals) and promoted skeletal muscle damage. Our data is consistent with recent literature linking NO to muscle soreness [80]Together this suggests that enhancing endogenous NO production via exogenous NITR therapy is contraindicative for the treatment of DMD. This is potentially due to the fact that there is no concomitant increase in nNOS protein expression and its regulatory role over metabolic flux control, and, that excessive ROS promotes RNS production which actually reduced NO bioavailability.

There were some limitations to our study that are worthy of mention. In the first instance, we did not quantify cGMP levels in EDL and SOL muscles, and thus cannot confirm that in the presence of heightened O_2^- production, NO is diverted away from intracellular signalling pathways (i.e. cGMP production, nucleus signalling of mitochondrial biogenesis) and into RNS formation. This was because whole EDL and SOL was required for our primary measure being radioactive GU, and that our other tissues were not immediately snap frozen, thus cGMP was heavily degraded beyond detectable levels. Secondly, while simultaneous measurement of O_2 flux and H_2O_2 emission has previously been well characterised and reported [51, 52, 53], a potential limitation of this assay is that the supra-physiological chamber pO_2 used to overcome O_2 diffusion limitations of permeabilized muscle fibre preparations, may lead to non-physiological rates of H_2O_2 emission [81]. Therefore, it should be acknowledged that the H_2O_2 measured using the present assay may not completely recapitulate *in vivo* mitochondrial ROS emission rates.

Our data is in stark contrast to previous findings of significant improvements in the dystrophic condition following NO donor therapy, and in Becker patients following nitrite supplementation, suggesting that long-term NITR/NO supplementation requires better characterisation, particularly in conditions of heightened oxidative and/or metabolic stress such as in DMD. While the precise myopathological mechanisms of NITR has not been fully

elucidated in the present study, our data is of particular importance considering NITR therapy is currently in clinical trials for the treatment of DMD patients.

Table 1. Weights of EDL and SOL used in GU experimentation.

	Left EDL (mg)	Right EDL (mg)	Left SOL (mg)	Right SOL (mg)
CON UNSUPP	12.3 ± 0.8	12.4 ± 1.2	9.0 ± 0.9	9.8 ± 0.9
CON NITR	11.3 ± 0.3	12.6 ± 0.8	10.3 ± 0.5	10.3 ± 0.6
***mdx* UNSUPP**	14.8 ± 0.8$^{\#\#\#\#}$	15.5 ± 1.4$^{\#\#\#\#}$	13.5 ± 1.3$^{\#\#\#\#}$	14.9 ± 1.4$^{\#\#\#\#}$
***mdx* NITR**	14.7 ± 0.9$^{\#\#\#\#}$	16.0 ± 0.8$^{\#\#\#\#}$	13.7 ± 0.8$^{\#\#\#\#}$	13.3 ± 0.6$^{\#\#\#\#}$

Irrespective of supplementation, both EDL and SOL weight were significantly higher in *mdx* mice compared to CON mice. There was no effect of NITR. $^{\#\#\#\#}$ Significant difference from CON mice $p<0.0001$. CON UNSUPP $n=14$; CON NITR $n=16$; *mdx* UNSUPP $n=12$; *mdx* NITR $n=13$.

Table 2. Weights of the left gastrocnemius used in mitochondrial respiration experimentation, determination of CS activity and western blotting of mitochondrial complexes and the right TA used for immunohistochemistry.

	Gastrocnemius (mg)	TA (mg)
CON UNSUPP	145.18 ± 4.4	46.9 ± 1.7
CON NITR	145.2 ± 3.3	46.5 ± 1.5
***mdx* UNSUPP**	169.9 ± 4.2$^{\#\#\#\#}$	68.9 ± 2.1$^{\#\#\#\#}$
***mdx* NITR**	170.2 ± 4.5$^{\#\#\#\#}$	66.9 ± 4.3$^{\#\#\#\#}$

Irrespective of supplementation, gastrocnemius was significantly higher in *mdx* mice compared to CON mice. There was no effect of NITR. Similarly, TA was significantly higher

in *mdx* mice compared to CON mice with no effect of NITR observed in either strain. #### Significant difference from CON mice $p<0.0001$. CON UNSUPP $n=13$; CON NITR $n=16$; *mdx* UNSUPP $n=12$; *mdx* NITR $n=13$.

Abbreviations

ADP: adenosine diphosphate; ATP: adenosine triphosphate; Ca^{2+}: calcium; cGMP: cyclic guansione monophosphate; CI-V: mitochondrial ETS complexes I-V; CS: citrate synthase; CON: control c57BL/10 (mouse); DMD: Duchenne Muscular Dystrophy; EDL: extensor Digitorum longus; ETS: electron transport system; GLUT: glucose transporter; GU: glucose uptake; *mdx*: muscular dystrophy x-linked on c57BL/10 background (mouse); H_2O_2: hydrogen peroxide; $NaNO_3$: sodium nitrate; NITR: nitrate; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; $O_2^{\cdot}$ oxygen; O_2^{-}: superoxide; $ONOO^{-}$: peroxynitrite; PFK: phosphofructokinase; PGC1-α: PPARγ-coactivator 1α; PKG: cGMP-regulated protein kinase; PPAR: peroxisome proliferator-activated receptor; RCR: respiratory control ratio; RNS: reactive nitrogen species; ROS: reactive oxygen species; SOL: soleus; TA: tibilalis anterior; TCA: tricarboxylic acid; WG: white gastrocnemius.

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Conflict of Interest

The authors declare no conflict of interest.

Figure Legends

Fig 1. Body weight and average food and water consumption of unsupplemented and NITR-supplemented mice over the supplementation period. Changes in body weight are shown as a percentage of pre-supplementation weight (A). Overall, *mdx* mice gained more weight over the 8 week supplementation period compared to CON ($p<0.0001$, A). NITR had no effect on *mdx* weight gain but did increase weight gain in CON compared to CON UNSUPP ($p<0.05$). Over the 8 week supplementation period, food (B) and water consumption (C) did not differ between unsupplemented and supplemented animals ($p>0.05$) except for food consumption during week two where CON UNSUPP consumed more than all groups ($p<0.05$). CON UNSUPP $n=16$; CON NITR $n=17$; *mdx* UNSUPP $n=14$; *mdx* NITR $n=18$.

Fig 2. GU in isolated EDL and SOL from unsupplemented and NITR supplemented CON and *mdx* mice. In all groups, contraction-induced GU significantly compared to basal conditions ($p<0.05$, A). NITR increased contraction-induced GU in CON EDL ($p<0.05$) but in contrast, reduced both basal- and contraction-induced GU in *mdx* EDL ($p<0.05$). For the SOL, both basal- and contraction-induced GU (B) were comparable ($p>0.05$). NITR reduced basal GU in *mdx* SOL ($p<0.05$) but had no effect in CON SOL. CON UNSUPP $n=9$-13; CON NITR $n=11$-; *mdx* UNSUPP $n=11$; *mdx* NITR $n=10$-12.

Fig 3. Mitochondrial function in intact, permeabilised muscle fibres from the white and red portion of gastrocnemius from unsupplemented and NITR-supplemented CON and *mdx* mice. State 4 leak respiration (A) is significantly reduced in *mdx* compared to CON WG, irrespective of substrate combination ($p<0.05$). ADP-stimulated state 3 respiration (A) is significantly reduced in *mdx* compared to CON WG ($p<0.05$) with NITR having no effect. FCCP-stimulated uncoupled respiration (A) is significantly reduced in *mdx* WG compared

with CON (p<0.05) with no difference in CIV activity detected between CON and *mdx* WG (p>0.05). The respiratory control ratio (RCR; B), an indicator of the coupling of O_2 consumption and ATP production at the ETS, was comparable between CON and *mdx* WG, although a trend for *mdx* to be lower was detected (p=0.083). When respiring on CI+II-substrates, the RCR was significantly lower across all groups (p<0.0001). State 4 leak respiration (C) is significantly reduced in *mdx* compared to CON RG, irrespective of substrate combination (p<0.01). ADP-stimulated state 3 respiration (C) is significantly reduced in *mdx* compared to CON RG (p<0.001) with NITR having no effect on phosphorylating respiration. FCCP-stimulated uncoupled respiration (C) is significantly reduced in *mdx* compared with CON (p<0.001). CIV activity (C) is significantly reduced in *mdx* UNSUPP compared to CON UNSUPP (p<0.01) with NITR inducing a significant decrease in both CON and *mdx* (p<0.01). The RCR (D) in *mdx* RG during CI+II-stimulated respiration is lower compared to CON (p<0.0001) with NITR decreasing the RCR in *mdx* during CI+II-stimulated respiration (p<0.001 respectively). CON UNSUPP n=12-13; CON NITR n=12-13; *mdx* UNSUPP n=10-11; *mdx* NITR n=11-12.

Fig 4. Mitochondrial respiratory chain complex proteins, and citrate synthase activity, from the white portion of gastrocnemius from unsupplemented and NITR-supplemented CON and *mdx* mice. In *mdx* UNSUPP WG, expression of CII (p<0.05; B), CIII (p<0.01; C), CIV (p<0.05; D) and CV (p<0.01; E) were greater compared to CON UNSUPP. NITR induced an increase in CI (p<0.01; A), CII (p<0.01; B), CIII (p<0.05; C) and CV (p<0.05; E) subunits in CON WG but not in *mdx* WG. NITR also increased CS activity (F) in both CON and *mdx* WG (p<0.05) with a trend for CS activity to be higher in *mdx* UNSUPP compared to CON (p=0.07). Representative western blots of proteins from each of the five mitochondrial respiratory complexes (G) with coomassie blue stains of the respective western blots to demonstrate equal loading of the total protein (H). n= 8 per group.

Fig 5. Mitochondrial respiratory chain complex proteins, and citrate synthase activity, from the red portion of gastrocnemius from unsupplemented and NITR supplemented CON and *mdx* mice. Overall, expression of CI, CIII, CIV and CV subunits were decreased in *mdx* RG compared to CON (p<0.05; A, C, D, E respectively). NITR increased CS activity in *mdx* RG but not in CON (p<0.05; F). Representative western blots of proteins from each of the five mitochondrial respiratory complexes (G) with coomassie blue stains of the respective western blots to demonstrate equal loading of the total protein (H). n= 8 per group.

Fig 6. H_2O_2 emission in intact fibres from the white (A) and red (B) portions of gastrocnemius from unsupplemented and NITR-supplemented CON and *mdx* mice. In WG, NITR induced a decreased H_2O_2 emission during state 4 leak respiration (A) in CON during CI-stimulated respiration and in both CON and *mdx* during CI+II-stimulated respiration (p<0.05). In WG, no significant difference was detected in H_2O_2 emission during ADP-stimulated state 3 respiration (A). There was no significant difference in H_2O_2 emission during FCCP-stimulated uncoupled respiration (A) in WG. In RG (B), NITR induced a decrease in H_2O_2 emission during state 4 leak respiration in *mdx* muscle during CI+II-stimulated respiration (p<0.001). In *mdx* RG (B)**,** H_2O_2 emission during ADP-stimulated state 3 respiration was significantly less compared to CON WG (p<0.05) with NITR having no effect (p>0.05). While there was no differences in H_2O_2 emission during FCCP respiration between CON UNSUPP and *mdx* UNSUPP RG (p>0.05, B), NITR reduced H_2O_2 emission in *mdx* RG compared to *mdx* UNSUPP (p<0.05). CON UNSUPP n=12-13; CON NITR n=12-13; *mdx* UNSUPP n=10-11; *mdx* NITR n=11-12.

Fig 7. Immunohistological analysis of TA from unsupplemented and NITR supplemented CON and *mdx* mice. Nitrotyrosine expression was higher in *mdx* TA compared to CON (p<0.0001, A) with NITR supplementation elevating nitrotyrosine

822 expression in both CON (p<0.05) and *mdx* (p<0.0001). Nuclei content was higher in *mdx* TA
823 compared to CON (p<0.0001, B) with NITR further increasing nuclei content in *mdx* TA
824 (p<0.0001). CD45 infiltration (C) and IgG staining (D) was elevated in *mdx* TA compared to
825 COM (p<0.001 and p<0.01 respectively) and NITR supplementation had no effect in either
826 CON or *mdx* TA (p>0.05). Scale bars= 100 µm. CON UNSUPP n=3-4; CON NITR n=3-4;
827 *mdx* UNSUPP n=3-4; *mdx* NITR n=3-4.

828

829 **Fig 8. Histological analysis of TA from unsupplemented and NITR supplemented CON**
830 **and *mdx* mice.** The frequency histogram (A) indicates an increase in fibre size of *mdx* TA
831 with fibres more frequent from 6000-12000 µm (p<0.0001). NITR had no effect on the
832 distribution of CON or *mdx* fibres but there was a trend for an increased number of fibres
833 around 6000 µm (p=0.068). Mean fibre size (C) was significantly greater in *mdx* TA (p<0.01)
834 with a trend for NITR to increase fibre size in *mdx* TA (p=0.093). Damaged area (D) and
835 percentage of centronucleated fibres (E) was significantly higher in *mdx* TA (p<0.01 and
836 p<0.0001 respectively) with NITR stimulating further damage and regeneration (p<0.01).
837 CON UNSUPP n=11; CON NITR n=12; *mdx* UNSUPP n=11; *mdx* NITR n=10.

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