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Xanthine oxidase is hyper-active in Duchenne muscular dystrophy

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Abstract

Generation of superoxide by xanthine oxidase can be stimulated under ischemic and aberrant calcium homeostasis. Because patients and mice with Duchenne muscular dystrophy (DMD) suffer from ischemia and excessive calcium influx, we tested the hypothesis that xanthine oxidase activity is elevated and contributes to disease pathology. Xanthine oxidase activity was measured by urinary isoxanthopterin in DMD patients at rest and in response to exercise. Urinary isoxanthopterin/creatinine was elevated compared to age-matched controls and Becker muscular dystrophy (BMD) patients. Concentrations were also increased after a six minute walk test in ambulatory patients. We also measured urinary isoxanthopterin in wildtype mice and a number of dystrophic mouse models; the DMD mouse model (*mdx*), *mdx* mice overexpressing a variety of transgenic miniaturized and chimeric skeletal muscle-specific dystrophins and utrophin and the β -sarcoglycan deficient (*Scgb^{-/-}*) mouse which represents type 2E human limb-girdle muscular dystrophy. *Mdx* and *Scgb^{-/-}* mice had greater urinary isoxanthopterin/creatinine than wildtype mice while *mdx* mice expressing dystrophin or utrophin linking the extracellular matrix to the actin cytoskeleton were not different than wildtype. We also measured higher levels of urinary *ortho*-tyrosine in humans and mice deficient for dystrophin to confirm elevated oxidative stress. Surprisingly, *mdx* had lower xanthine oxidase protein levels and higher mRNA in gastrocnemius muscle compared to wildtype mice, however, the enzymatic activity of skeletal muscle xanthine oxidase was elevated above wildtype and a transgenic rescued *mdx* mouse (Dys^{MTB}-*mdx*). Downhill treadmill running also caused significant increases in *mdx* urinary isoxanthopterin that

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⁵Competing Interests

All authors have no financial or personal conflict with other people or organizations that could inappropriately influence our work.

was prevented with the xanthine oxidase inhibitor allopurinol. Similarly, *in vitro* eccentric contraction-induced force drop of *mdx* muscle was attenuated by the allopurinol metabolite, oxypurinol. Together, our data suggests hyper-activity of xanthine oxidase in DMD, identifies xanthine oxidase activity as a contributing factor in eccentric contraction-induced force drop of dystrophin-deficient skeletal muscle and highlights the potential of isoxanthopterin as a noninvasive biomarker in DMD.

Keywords

Allopurinol; Duchenne muscular dystrophy; dystrophin; gene therapy; isoxanthopterin; mdx; oxidative stress; pterin; xanthine oxidase

1. Introduction

Reactive oxygen species (ROS) have been implicated in the pathophysiology of Duchenne muscular dystrophy (DMD) [1–4], a fatal muscle wasting disease caused by the loss of the protein dystrophin [5]. A significant source of ROS includes inflammatory cells, NADP(H) oxidase and mitochondria, all of which have abnormal function in DMD [6–8]. It has also been suggested that aberrations in calcium homeostasis result in activation of the xanthine dehydrogenase/oxidase system (XDH/XO) in DMD with the subsequent production of superoxide [9]. In healthy tissue, XDH/XO is normally found as XDH, which uses NAD⁺ as a co-factor. In diseased tissue, calcium-activated proteinases cleave XDH to XO [10], which instead uses molecular oxygen as a co-factor [11]. The conversion of XDH to XO is accelerated under ischemic conditions [12]. Because patients with DMD fail to localize neuronal nitric oxide synthase to the membrane [13], patients suffer from both central and peripheral ischemia [14]. Thus, ischemia-induced XO activity may provide a further mechanism contributing to the oxidative environment of dystrophic tissue.

Measurement of superoxide in biological systems remains challenging. An *in vitro* approach to measure XO activity utilizes the fluorescent conversion of pterin to isoxanthopterin. Isoxanthopterin is part of the pteridine family, which represents important co-factors utilized in several essential biological pathways. Isoxanthopterin has been used as a noninvasive detection and diagnostic biomarker for cancer [15–17] because of its relative ease of detection and measurement in human urine [18]. Changes in isoxanthopterin can represent both superoxide formation and activity of xanthine oxidase and thus may serve as a potential indicator of oxidative stress in DMD patients.

In our first study, we tested the hypothesis that xanthine oxidase is a source of oxidative stress in patients with DMD and the milder form Becker muscular dystrophy (BMD) by measuring urinary isoxanthopterin at rest and after the six minute walk test (6MWT). We separated urinary isoxanthopterin concentrations in DMD patients at rest based on age, ambulatory status, and use of corticosteroids at time of urine collection. We also measured urinary *ortho*-tyrosine as a control for oxidative stress. In our second study, we measured urinary isoxanthopterin in *mdx* mice at rest and in response to treadmill exercise. We also determined whether the hypothesized elevation in isoxanthopterin in *mdx* mice compared to healthy wildtype mice is corrected when dystrophin or utrophin expression is restored to

mdx skeletal muscle. Finally, because skeletal muscle contractions have been shown to activate XO-derived superoxide [19], we tested the effect of allopurinol on treadmill running and eccentric contraction-induced force drop in *mdx* mice.

2. Material and Methods

Ethical approval -

We made measurements in urine samples that had been collected from male DMD patients and healthy age-matched controls as previously reported [20]. The experimental protocol was approved by the Internal Review Board, University of Minnesota (1207M18081), and all participants were informed of the risks involved in the study before their written consent was obtained. The studies conformed to the standards set by the latest revision of the Declaration of Helsinki, except for registration in a database.

Experimental mice -

All animals were housed and treated in accordance with the standards set by the University of Minnesota Institutional Animal Care and Use Committee. Male *mdx* (C57BI/10ScSn-DMD^{mdx}) and C57BL/10 (C57BI/10ScSn) were generated using founders purchased from Jackson Laboratory (Bar Harbor, ME, USA). Generation of Dys^{MTBD}-*mdx* [21], Dys^{R4 23/ CT}-*mdx* [22], Dys^{R2-15/ R18-23/ CT}-*mdx* [22], Fiona-*mdx* [21], *Scgb*^{-/-} [23], Dp-116-*mdx* [24] were previously reported. All mice used in this study were 3 month old adult males. All mice were housed in groups of 3 – 4 per cage on a 14/10 h light/dark cycle with food and water provided *ad libitum*. All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee (1707-34941A).

Human participants -

Participants were male DMD patients (N = 47; 11.4 ± 0.6 yr), male BMD patients (N = 6; 19.7 ± 1.2 yr) and healthy unaffected male controls (N = 34; 13.9 ± 1.2 yr). Single catch urine samples were collected at several clinics and camps over three years. For 10 of the DMD patients, a repeat urine sample was collected at a different time point at least one year apart and incorporated into the total pool. At time of urine collection, participants completed a questionnaire which included information about age and ambulation but because of difficulties associated with ambulation, height and weight could not be collected. Because DMD patients are routinely administered corticosteroids, we also compared urinary isoxanthopterin concentrations based on known steroid use at time of collection. Each urine sample was collected mid-stream and placed on ice immediately before being transported to the laboratory, aliquoted and frozen at -80 °C until analysis.

Six minute walk test -

Urine samples were collected pre and post a 6MWT. The 6MWT for this study was similar to that performed by McDonald et al. [25]. Briefly, a 25-m taped line marked with 1-m intervals and cones at each end of the tape were used to mark the ambulatory path for patients with DMD. Continuous moderate verbal encouragement was allowed throughout the testing period. In addition, a “safety chaser” followed the child to keep him on the correct path, be in close proximity in case of a fall, to help initiate a standing position, or tend to the

child quickly if an injury occurred. Urine was collected immediately pre and within 1 h of completion of the test.

Ex vivo muscle preparation and physiology -

Baseline contractile functions of EDL muscles were assessed according to the methods of Moran et al. [26]. Mice were anesthetized with sodium pentobarbital (100 mg/kg body mass (BM) for WT and 75 mg/kg BM for *mdx*). EDL muscles were removed and mounted on a dual-mode muscle lever system (300B-LR; Aurora Scientific Inc., Aurora, ON, Canada) with 5-0 suture in a 1.2 mL bath assembly and filled with oxygenated (95:5 % O₂:CO₂) Krebs-Ringer bicarbonate (Krebs) buffer maintained at 25 °C. Muscles were adjusted to their anatomical optimal length (L₀) based on resting tension. Muscle length was then measured from myotendonous junction to myotendonous junction using digital callipers. Prior to performing eccentric contractions, muscles remained quiescent in the bath for 5 min before beginning a protocol for testing contractile function. Maximal isometric tetanic force (P₀) was measured every 2 min by stimulating the muscle to contract for 200 ms at 175 Hz until force plateaued (within 5 mN from one contraction to the next).

After plateau was attained, oxypurinol dissolved directly in Krebs buffer [19] was added to the bathing media (40 or 400 μM) and the muscle remained quiescent for 30 min as previously reported [20]. P₀ was then re-measured at 30 and 32 min after addition of oxypurinol. Next, 10 eccentric contractions were performed [20] and the force loss associated with each eccentric contraction was recorded. For each eccentric contraction the muscle was passively shortened to 95% L₀ and then stimulated for 200 ms while the muscle was simultaneously lengthened to 105% L₀ at 0.5 L₀/s. Each eccentric contraction was separated by 3 min of rest to prevent fatigue [27]. The force measured at each eccentric contraction was expressed as a percentage of the force produced during the first (“initial”) contraction.

Western blot analysis -

Gastrocnemius muscle was pulverized with mortar and pestle in liquid nitrogen. Tissue was then lysed with 1% SDS solution with added protease inhibitors (100 nM Aprotinin, 10 mg/ml E-64, 100 μM Leupeptin, 1 mM PMSF, 1 μg/ml Pepstatin) proportional to mass of the tissue pellet. Protein concentration was measured by A280 absorbance. Equal concentrations of lysate were then separated by electrophoresis at 150 V for 1 h and transferred to PVDF membrane at 100 V/0.7 Amp for 1.5 hrs. Membrane was blocked in 5% non-fat milk in PBS for 1 h and then incubated in blocking buffer with primary antibody overnight at 4 C. Primary antibodies used were anti-xanthine oxidase at 1:1000 (Ab109235, Abcam) and anti-GAPDH at 1:10,000 (Sigma G9545). Blots were then incubated in secondary antibodies anti-mouse and anti-rabbit IgG Dylight® 800 (Cell Signaling) at 1:10 000 in blocking buffer. Secondary antibody signal was visualized on Licor's Odyssey* Infrared Imaging System and band density calculated with Odyssey Software v2.1.

qRT-PCR —Total RNA was extracted from 3 mo old C57BL/10, *mdx* and Dys^{MTBD}-*mcix* gastrocnemius muscle samples using the Bio-Rad-Aurum Total RNA Mini Kit following the manufacturer's instructions. RNA concentration and purity (260/280 ratio)

were determined using a NanoDrop spectrophotometer (Wilmington, DE). First-strand cDNA was synthesized with a Bio-Rad iScript Advanced cDNA Synthesis Kit for qRT-PCR using the same initial RNA amount for all samples. Muscle samples were amplified in parallel with each specific qRT-PCR primer set using Bio-Rad SsoAdvanced Universal SYBR polymerase on the Bio-Rad CFX96 Real Time System C1000 Touch Thermal Cycler to measure xanthine oxidase transcript amount.

Treadmill assay -

We utilized a similar protocol to that previously reported as a “proof of concept” in *mdx* mice [28]. The protocol was modified because we found that *mdx* mice suffer from a stress-induced physical inactivity response and do not often voluntarily complete the proposed exercise prescription. Thus, 4–6 mice that were not familiarized with the treadmill were placed in individual running lanes. Over 5 min, the treadmill which was on a -10° decline, was increased in speed every minute from 10 to 15 m/min. Once at 15 m/min, the protocol began and mice were timed and distance calculated over 30 min. Mice either completed the protocol (450 m run) or became exhausted/unwilling to run which corresponded to termination. Urine was collected using a sterile 1.5 mL tube and gently massaging the lower back of the mouse while being elevated by the base of the tail immediately pre and at 5 time points post-exercise. Changes in urinary isoxanthopterin were measured as a percentage of initial. For *mdx* mice only, a separate cohort were anaesthetized with isoflurane and injected with 50 mg/kg allopurinol 1 h before treadmill exercise. A separate cohort was used to prevent any familiarization.

Xanthine oxidase activity assay-

Xanthine oxidase activity was measured according to the methods of Gomez-Cabrera [19]. Frozen gastrocnemius muscle or liver was pulverized with mortar and pestle in liquid nitrogen. Gastrocnemius muscle was chosen as the skeletal muscle for analysis because it possesses a variety of oxidative and glycolytic fibers [29]. Tissue was then lysed in 0.25M sucrose, 10 mM DTT, 0.2 mM PMSF, 0.1 mM EDTA, and 50 mM potassium phosphate, pH 7.4. Homogenates were centrifuged for 30 min at 14,000 *g*, and activities were measured in supernatants. The reaction was initiated by addition of pterin (100 $\mu\text{mol/l}$) as a substrate, and XO activity was obtained from the rate of increase in fluorescence due to the conversion of pterin to isoxanthopterin over 1 h. The reaction was stopped by addition of allopurinol (50 $\mu\text{mol/l}$). Protein concentration was measured by A_{280} absorbance.

High-performance liquid chromatography

Media collected following the xanthine oxidase activity assay were thawed on ice and an equal concentration of acetonitrile added, vortexed, and left on ice for 10 min. Samples were then spun down and the supernatant transferred to a microcentrifuge tube with a 3 kDa filter and spun at 14,000 *g* for 10 min. Thirty μL of the concentrate was transferred to a HPLC vial (Chromtech, MN, USA) and 10 μL directly injected onto the HPLC for isoxanthopterin and pterin measurement.

All human urine samples were thawed in the dark on ice immediately before analysis. Samples for pterin and isoxanthopterin were diluted 1 in 40 with 20 mM ammonium

phosphate (pH 2.5) and 10 μ L directly injected into the HPLC. Urine samples for *ortho*-tyrosine were prepared and measured with the same HPLC instrument and according to the methods of Molnar et al. as previously described [30].

Isoxanthopterin and pterin measurement was performed on a Shimadzu I-series 2030C with UV detector and auto-sampler, RF-20A fluorescence detector and on-line degasser with a 20 mM ammonium phosphate (pH 2.5) mobile phase, pumped isocratically at 1 mL/min as described previously [31]. Samples were maintained at 4 °C via the HPLC cooling unit and separated by positive charge using a Luna 5 μ m SCX 100 Å, 250 \times 4.6 mm column (Phenomenex, CA, USA). Pterin and isoxanthopterin (Schirck's Laboratory, Jona, Switzerland) were detected by fluorescence at 353 nm excitation and 438 nm emission. Creatinine was measured using at its natural absorbance of 234 nm. The concentration and identity of each pterin and creatinine was compared to their corresponding standard (freshly prepared each day and spaced evenly throughout each analytical run) and quantified by peak area using the software Shimadzu Class VP. We chose to correct urinary isoxanthopterin concentrations with creatinine because it is possible to accurately compare mouse to human data [20].

Statistics -

Differences between groups were analyzed by *t*-tests, paired *t*-test, one-way ANOVA, repeated measures two-way ANOVA or linear regression where appropriate. When a difference was measured between groups using a one-way or two-way ANOVA, *post-hoc* analyses using Tukey and Dunnett, respectively, were used to identify individual differences. All data are presented as mean \pm standard error of the mean (SEM) with significance set at $p < 0.05$.

3. Results

3.1 Urinary isoxanthopterin is elevated and associated with ambulation in DMD patients

We determined whether xanthine oxidase activity was elevated in DMD patients by measuring urinary isoxanthopterin. Compared to age-matched controls, DMD patients had greater urinary isoxanthopterin/creatinine (131 ± 11 vs. 251 ± 25 μ mol/mol creatinine; Fig. 1A; $p < 0.001$). Urinary isoxanthopterin was also significantly greater in age-matched DMD patients when compared to a group of 16–22 year old BMD patients (Fig. 1B; $p = 0.003$). Because DMD is a progressive disease where patients become wheelchair bound by their early teens, we also assessed urinary isoxanthopterin as a function of age and ambulation. Urinary isoxanthopterin/creatinine was elevated in non-ambulant compared to ambulant DMD patients (215 ± 19 vs. 377 ± 71 μ mol/mol creatinine; Fig. 1C; $p < 0.040$) but we did not measure a relationship between isoxanthopterin and age (Fig. 1D; $p = 0.099$). Next, we assessed urinary isoxanthopterin concentrations as a function of corticosteroid use because they are one effective therapy for DMD patients [32], We measured no effect of corticosteroid administration on urinary isoxanthopterin/creatinine concentrations (Fig. 1E). We also measured the response of urinary isoxanthopterin in nine DMD patients who performed the 6MWT because it has been utilized to test the efficacy of various therapies and treatments [33]. DMD patients completed 385 ± 31 m and urinary isoxanthopterin/

creatinine increased in 8 of the 9 participants from before to after the 6MWT (170 ± 39 vs. 202 ± 46 pmol/mol creatinine; Fig. 1F; $p = 0.044$). There was no association between distance completed during the 6MWT and percent change in urinary isoxanthopterin ($R^2 = 0.032$; $p = 0.623$).

3.2 Skeletal muscle-specific dystrophin and utrophin expression normalizes elevated urinary isoxanthopterin in dystrophin-deficient *mdx* mice.

Because the DMD mouse model (*mdx*) recapitulates numerous pathological and physiological manifestations observed in humans [34], we assessed whether the *mdx* mouse had elevated urinary isoxanthopterin compared to healthy wildtype mice (C57BL/10). Between the ages of 30 and 120 days, *mdx* mice had greater urinary isoxanthopterin/creatinine than C57BL/10 (Fig. 2). For comparison, we also measured urinary isoxanthopterin in a mouse model of human Type 2E limb girdle muscular dystrophy. The limb girdle muscular dystrophy model (*Scgb^{-/-}*) has C57BL/10 levels of dystrophin and utrophin but has a perturbed dystrophin-glycoprotein complex (DGC) because they are null for β -sarcoglycan [35,36]. *Scgb^{-/-}* also had elevated urinary isoxanthopterin/creatinine compared to C57BL/10 (Fig. 2).

Muscle specific transgenic expression of dystrophin or utrophin rescue nearly all known phenotypes of the *mdx* mouse [37,38]. Because DMD is primarily a skeletal muscle disease with associated cardiac and cognitive phenotypes, we tested the hypothesis that skeletal muscle-specific expression of therapeutically relevant miniaturized dystrophins and utrophin could ameliorate the elevation in urinary isoxanthopterin. Urinary isoxanthopterin/creatinine were normalized to C57BL/10 levels in *mdx* mice expressing two therapeutically-relevant miniaturized dystrophins, or utrophin, all of which provide a link between the extracellular matrix (ECM) and actin cytoskeleton (Fig. 2). Mice expressing the Dp116 isoform which is primarily expressed in Schwann cells [39] and restores the sarcolemmal glycoprotein complex [24], but fails to bind actin [24], had *mdx* concentrations of urinary isoxanthopterin/creatinine. There was also a significant effect of age for *mdx*, Dp116-*mdx* and *Scgb^{-/-}* mice ($p < 0.001$ for each genotype).

3.3 Urinary *ortho*-tyrosine in healthy and dystrophin-deficient humans and mice

To confirm an elevated level of oxidative stress in DMD patients compared to unaffected controls, we measured urinary *ortho*-tyrosine which is the hydroxyl-radical-induced oxidized product of tyrosine. Compared to age-matched controls, DMD patients had greater urinary *ortho*-tyrosine/creatinine (37 ± 7 vs. 71 ± 13 mmol/mol creatinine; Fig. 3A; $p < 0.039$). Next we compared urinary *ortho*-tyrosine levels between C57BL/10, *mdx* and transgenic mice. Urinary *ortho*-tyrosine/creatinine was restored to C57BL/10 levels in *mdx* mice that expressed miniaturized or chimeric dystrophins, or utrophin. Dp-116 and *Scgb^{-/-}* mice were not different than *mdx* (Fig. 3B; $p < 0.001$).

3.4 Hyper-activity of xanthine oxidase in *mdx* skeletal muscle

To understand the elevation in urinary isoxanthopterin in *mdx* mice (Fig. 2), we measured xanthine oxidase activity in gastrocnemius muscles from C57BL/10, *mdx* and Dys^{MTBD}-*mdx* mice. Dys^{MTBD}-*mdx* mice were chosen as the comparative mouse model because it is

rescued for all known skeletal muscle phenotypes [23] and urinary isoxanthopterin concentrations are not different from C57BL/10 (Fig. 2). Following administration of pterin to the muscle lysate, *mdx* gastrocnemius muscle generated greater isoxanthopterin concentrations/g protein than C57BL/10 ($p = 0.001$), while Dys^{MTBD}-*mcix* mice did not differ from C57BL/10 but lowered concentrations compared to *mdx* (Fig. 4A; $p = 0.009$).

Because xanthine oxidase activity was elevated in the *mdx* mouse (Fig. 4A), we measured xanthine oxidase protein levels in skeletal muscle and liver from C57BL/10, *mdx* and Dys^{MTBD}-*mdx* mice. Contrary to our hypothesis, xanthine oxidase protein levels in skeletal muscle of *mdx* mice were 71% lower than C57BL/10 (Fig. 4B). Dys^{MTBD}-*mdx* mice which are rescued for all other skeletal muscle phenotypes [23] normalized xanthine oxidase concentrations to C57BL/10 levels (Fig. 4B). To identify whether gastrocnemius muscle protein levels were affected by post-transcriptional change, we measured mRNA expression of xanthine oxidase in the same mouse strains. We measured a 4.9-fold increase in xanthine oxidase mRNA content in *mdx* compared to C57BL/10 that was normalized to C57BL/10 levels in Dys^{MTBD}-*mc/x* mice (Fig. 4C). We then measured liver xanthine oxidase activity to try and explain why systemic isoxanthopterin measured in the urine was elevated in *mdx* mice because xanthine oxidase is most abundantly expressed in the liver [40]. We measured no difference in xanthine oxidase activity in the liver between C57BL/10, *mdx* and Dys^{MTBD}-*mdx* (Fig. 4D), nor did we measure any difference in xanthine oxidase protein content (Fig. 4E).

3.5 Inhibition of xanthine oxidase attenuates exercise-induced isoxanthopterin increases in *mdx* mice

DMD patients presented with elevated urinary isoxanthopterin following the 6MWT (Fig. 1F). To determine if a similar effect occurred in *mdx* mice, we subjected C57BL/10, *mdx* and Dys^{MTBD}-*mdx* mice to treadmill exercise. Treadmill exercise did not cause any change in urinary isoxanthopterin/creatinine in C57BL/10 mice (Fig. 5). In the *mdx* mouse, we measured an immediate increase following exercise, which remained elevated up to 24 h post. As expected, Dys^{MTBD}-*mdx* mice were normalized to the C57BL/10 response. To confirm whether the increase in isoxanthopterin was directly associated with xanthine oxidase activity, we administered the XO inhibitor allopurinol to *mdx* mice 1 h before exercise. In response, urinary isoxanthopterin of *mdx* mice dramatically decreased immediately following exercise (to 51% of Pre) which remained depressed until 6 hours post exercise.

3.6 Inhibition of xanthine oxidase protects *mdx* skeletal muscle from eccentric contraction-induced force loss *in vitro*

Dystrophic skeletal muscle is extremely susceptible to eccentric contraction-induced force loss [41]. There is also evidence that skeletal muscle contractions induce xanthine oxidase superoxide production that is attenuated with the allopurinol metabolite, oxypurinol [19]. To test the hypothesis that eccentric contraction-induced force loss in dystrophin-deficient skeletal muscle is affected by xanthine oxidase-generated superoxide, we completed *in vitro* eccentric contractions in the *mdx* mouse. We completed eccentric contractions only in *mdx* mice because *extensor digitorum longus* (EDL) muscle from C57BL/10 mice is insensitive to

force loss in our assay [20,37], Force production in isolated EDL muscles was not affected by 40 or 400 μM oxypurinol as measured by peak twitch and tetanic forces (Table. 1; $p > 0.426$). During the eccentric contraction protocol, EDL muscle lost 75% eccentric force by the 5th contraction (Fig. 6). In the presence of 40 or 400 μM oxypurinol, EDL muscle lost less eccentric force (48 and 52%, respectively; $p < 0.001$) and preserved some of the post-ECC protocol isometric force at 40 μM only (Table. 1; $p = 0.036$).

4. Discussion

The current study was designed to assess the contribution of xanthine oxidase-derived ROS in DMD pathology. We focused on both patients and mice null for dystrophin and measured urinary isoxanthopterin at rest and in response to exercise. We also determined whether skeletal muscle-specific dystrophin or utrophin expression restored urinary isoxanthopterin concentrations in *mdx* mice. DMD patients had elevated urinary isoxanthopterin that was higher in non-ambulant patients and which increased in response to the 6MWT. Similarly, a DMD mouse model (*mdx*) had elevated isoxanthopterin, which increased further in response to treadmill exercise. Inhibition of xanthine oxidase prevented the exercise-induced increase in isoxanthopterin and attenuated eccentric contraction-induced force drop *in vitro*, while transgenic expression of miniaturized dystrophins, chimeric dystrophin and utrophin normalized urinary isoxanthopterin concentrations. We found that the difference in isoxanthopterin between healthy and *mdx* mice was associated not with xanthine oxidase protein content, but by the activity of the enzyme in skeletal muscle. Together the data suggests hyper-activation of xanthine oxidase in skeletal muscle of *mdx* mice which contributes to exercise-induced perturbations which can be mitigated by skeletal muscle specific dystrophin expression.

Oxidative stress is known to contribute to the pathophysiology of DMD [1,42]. Our data now suggests xanthine oxidase has increased activity in the skeletal muscle of the *mdx* mouse, corroborating elevated activity in dystrophic cardiomyocytes [43], and which contributes systemically to ROS-generation as indicated by elevated urinary isoxanthopterin in both patients and mice null for dystrophin. We further confirm the presence of oxidative stress in dystrophin null mice and humans by elevated urinary *ortho-tyrosine* which is produced through oxidation of tyrosine by hydroxyl radicals. While xanthine oxidase-derived ROS production seems to be due to skeletal muscle protein activity rather than protein content, the increased activity can be further amplified in response to a stress stimulus such as exercise. Interestingly, gastrocnemius muscle xanthine oxidase mRNA content was 5-fold higher in *mdx* than C57BL/10 yet the protein content was 4-fold lower. Evidence has shown that inflammatory and hypoxic conditions like that measured in DMD [14,44] can stimulate marked increases in xanthine oxidase mRNA and activity [45], thus potentially explaining the elevation in *mdx* mouse xanthine oxidase mRNA. It is therefore intriguing that the ischemic state and aberrant calcium perturbations associated with DMD [13,14], which are prominent in the conversion of xanthine dehydrogenase to xanthine oxidase [12], would not elevate xanthine oxidase protein levels above C57BL/10 - perhaps because of post-transcriptional regulation of xanthine oxidase in the *mdx* environment or perhaps the lack of correlations between mRNA and protein content [46]. Regardless, our data suggests dystrophin-deficiency increases activity of xanthine oxidase in *mdx* skeletal

muscle, thus contributing to the high oxidative environment of dystrophin-deficient tissue [1].

Under ischemic conditions and aberrant calcium regulation, XDH is converted to xanthine oxidase [9,12] which generates superoxide. Superoxide accelerates oxidative modification of essential biomolecules including DNA [47]. Because dystrophin-deficiency causes greater urinary isoxanthopterin and *mdx* skeletal muscle has increased xanthine oxidase activity which is exacerbated by exercise, inhibition of xanthine oxidase could provide a new avenue of therapeutic investigation. However, long term administration of allopurinol to DMD patients has been conducted with equivocal results. While some research shows no effect on functional impairment with allopurinol treatment [48–51], other clinical studies have found improvements in strength [52], muscular abilities according to the Zellwager and Harson clinical scale [53] and purine metabolism [54]. In contrast to studies in DMD patients, minimal research has been conducted to assess effects of allopurinol in the *mdx* mouse model of DMD [55], possibly because the *mdx* mouse had not been identified until after the equivocal clinical trials had been completed [34]. Interestingly, chronic administration of allopurinol can preserve cardiac function in a mouse model of cardiomyopathy [56]. Therefore, because DMD patients die from cardiac and respiratory failure and certain DMD mouse models suffer from cardiomyopathy [57], there is justification for examining the activity and inhibition of xanthine oxidase in DMD as a potential therapeutic treatment.

Eccentric contraction-induced force drop of dystrophic skeletal muscle is a robust phenotype impacted by cellular oxidative status [20,58]. There is also evidence that isometric contractions of healthy muscle generates superoxide by xanthine oxidase [19]. By inhibiting xanthine oxidase, we show that eccentric contraction-induced force drop can be attenuated in *mdx* skeletal muscle *in vitro*. Our data corroborates the role of ROS in eccentric force drop and now offers a new mechanism by which force drop is affected.

Recently neopterin and 7,8-dihydroneopterin were measured in DMD patients and found to be elevated above unaffected controls [20]. Although both neopterin and 7,8-dihydroneopterin were elevated, these metabolites did not correlate with age and only 7,8-dihydroneopterin was greater in non-ambulatory compared to ambulatory patients. Here we measured a greater concentration of urinary isoxanthopterin/creatinine in DMD patients, which increased in response to the 6MWT, and was greater in non-ambulatory than ambulatory patients. Because urinary isoxanthopterin is measureable by HPLC and can be collected by non-invasive and stress-free methods, isoxanthopterin may offer an alternative sensitive pterin biomarker for DMD that is capable of monitoring specific perturbations in DMD patients independent of BMD patients.

In summary, our data indicate that patients and mice deficient for the protein dystrophin have elevated urinary isoxanthopterin concentrations. Isoxanthopterin concentrations appear to be dependent on the stability of the DGC which may impact the activity of the xanthine oxidase enzyme in skeletal muscle and thus the level of oxidative stress in DMD patients. Encouragingly, skeletal muscle-specific dystrophin expression restores xanthine oxidase perturbations in *mdx* mice while inhibition prevents further stress-induced activation.

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Abbreviations:

| | |
|----------------------------------|---|
| 6MWT | six minute walk test |
| BMD | Becker muscular dystrophy |
| DMD | Duchenne muscular dystrophy |
| ECM | extracellular matrix |
| EDL | extensor digitorum longus |
| HPLC | high performance liquid chromatography |
| mdx | DMD mouse model |
| ROS | reactive nitrogen species |
| <i>scgb</i>^{-/-} | β -sarcoglycan knock out mouse and limb girdle muscular dystrophy model |
| XDH | xanthine dehydrogenase |
| XO | xanthine oxidase |

7. References

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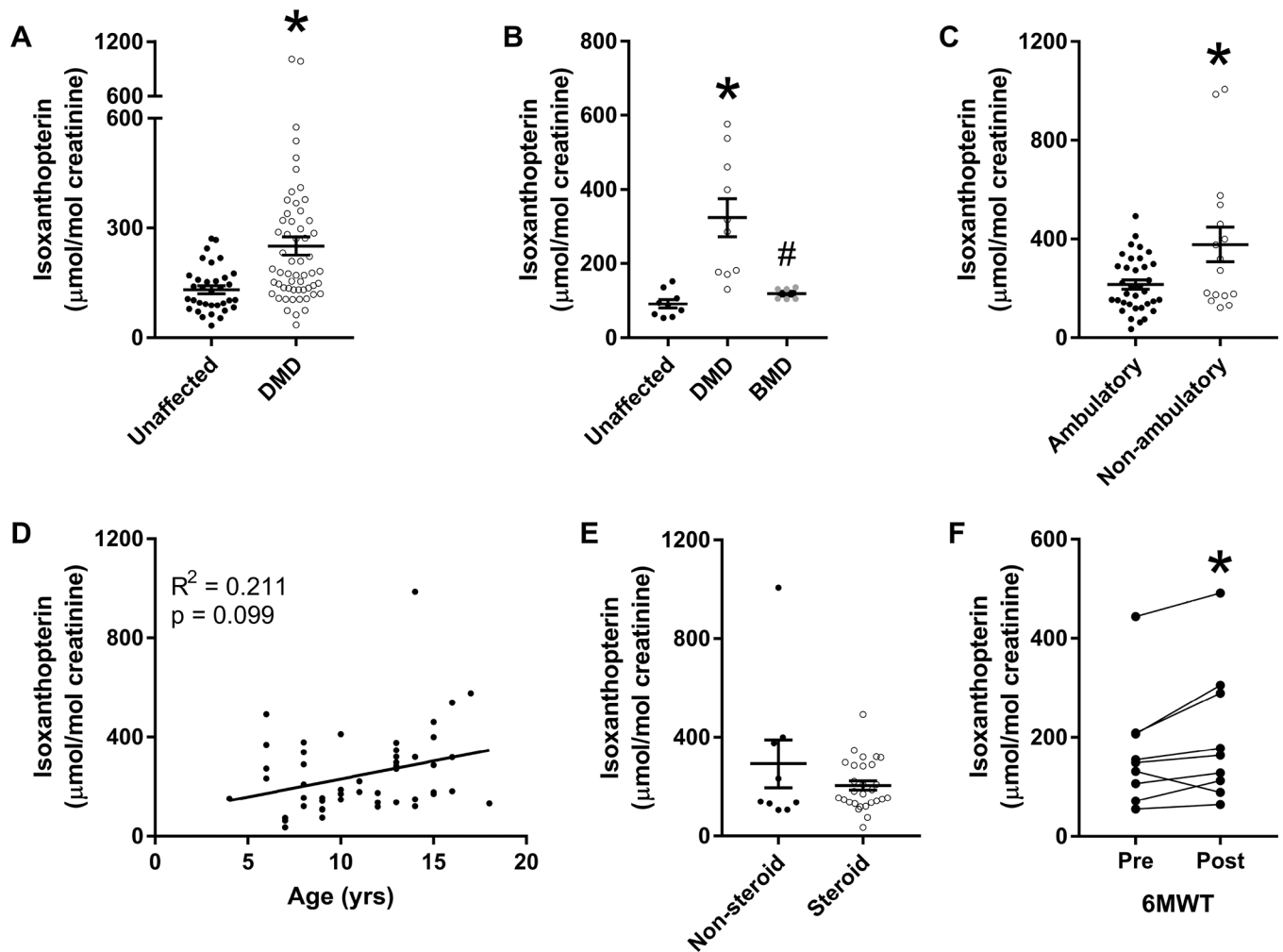


Figure 1.

(A) Urinary isoxanthopterin/creatinine in unaffected controls vs. DMD patients aged 4 –18 y. *different from unaffected. (B) Urinary isoxanthopterin/creatinine in unaffected controls, BMD and DMD patients aged 16 – 23 y; *different from unaffected,# different from DMD. (C) Urinary isoxanthopterin/creatinine in DMD patients separated by ambulation status; *different from ambulatory. (D) Urinary isoxanthopterin/creatinine relationship with age in DMD patients. (E) Urinary isoxanthopterin/creatinine in DMD patients separated by steroid use at time of urine collection. (F) Urinary isoxanthopterin/creatinine before and following the six-minute walk test (6MWT); *different from pre, n = 9.

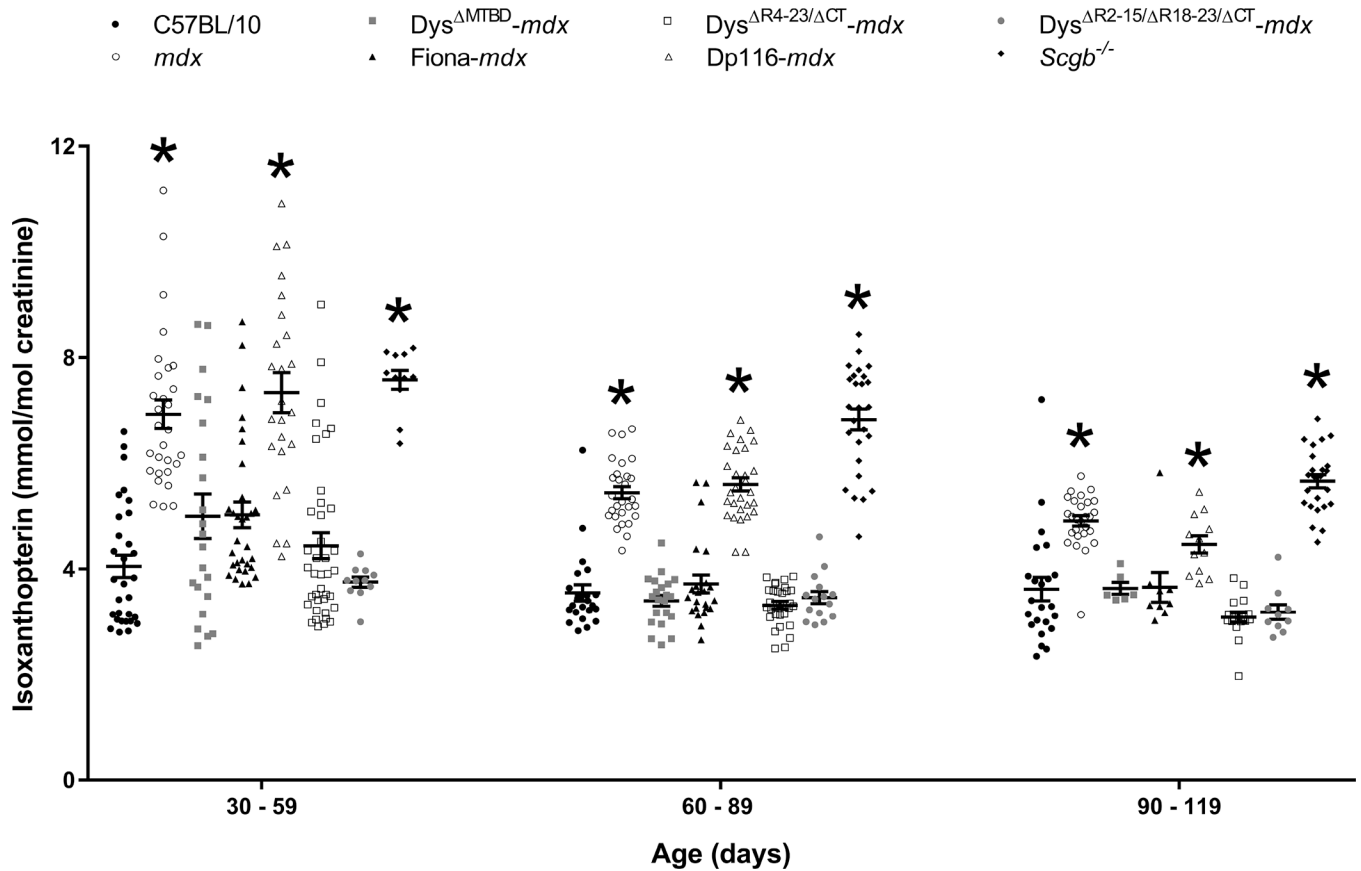


Figure 2.

Urinary isoxanthopterin/creatinine in C57BL/10, *mdx*, a mouse model of limb girdle muscular dystrophy (*Scgb^{-/-}*) and *mdx* mice expressing various truncated or chimeric dystrophins or utrophin. Data from mouse models are compared within each age range; *different from C57BL/10, n = 6/genotype.

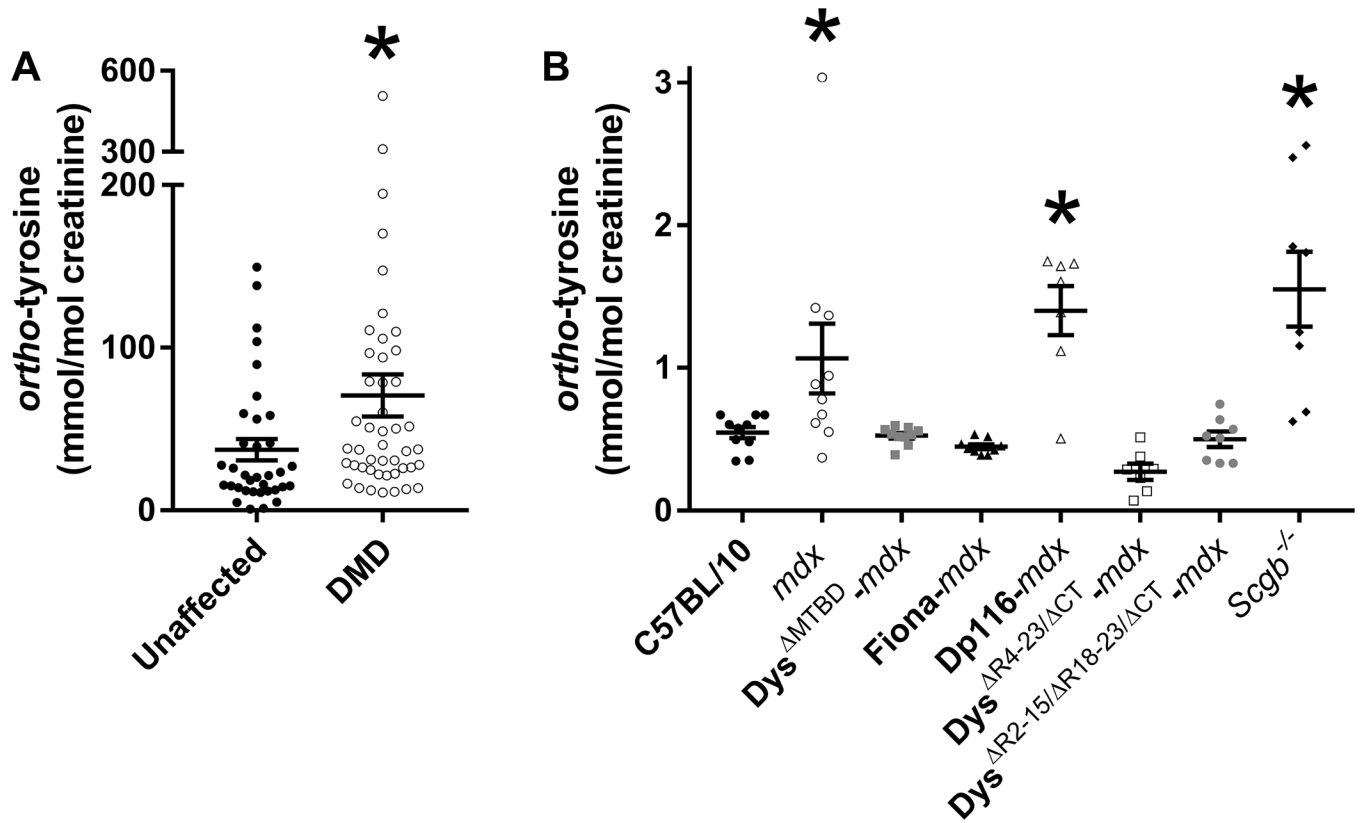


Figure 3.

(A) Urinary ortho-tyrosine/creatinine in unaffected controls vs. DMD patients aged 4–18 y. *different from unaffected. (B) Urinary ortho-tyrosine creatinine in C57BL/10, *mdx*, a mouse model of limb girdle muscular dystrophy (*Scgb^{-/-}*) and *mdx* mice expressing various truncated or chimeric dystrophins or utrophin. *different from C57BL/10, n = 7/genotype.

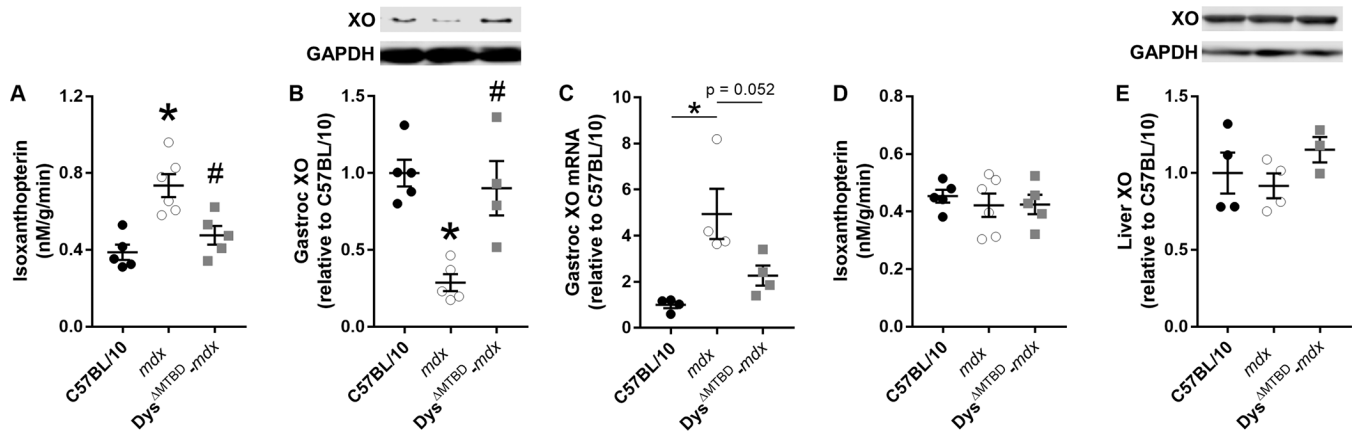


Figure 4.

(A) Isoxanthopterin concentrations in gastrocnemius muscle lysates following a 1 h incubation with 100 μ M pterin. (B) Gastrocnemius xanthine oxidase protein and (C) gene expression and (D) liver xanthine oxidase protein levels and (E) isoxanthopterin concentrations in liver lysates following a 1 h incubation with 100 μ M pterin in C57BL/10, *mdx* and *Dys*^{MTBD}-*mdx* mice; *different from C57BL/10, # different from *mdx*, n = 3/group. XO: xanthine oxidase.

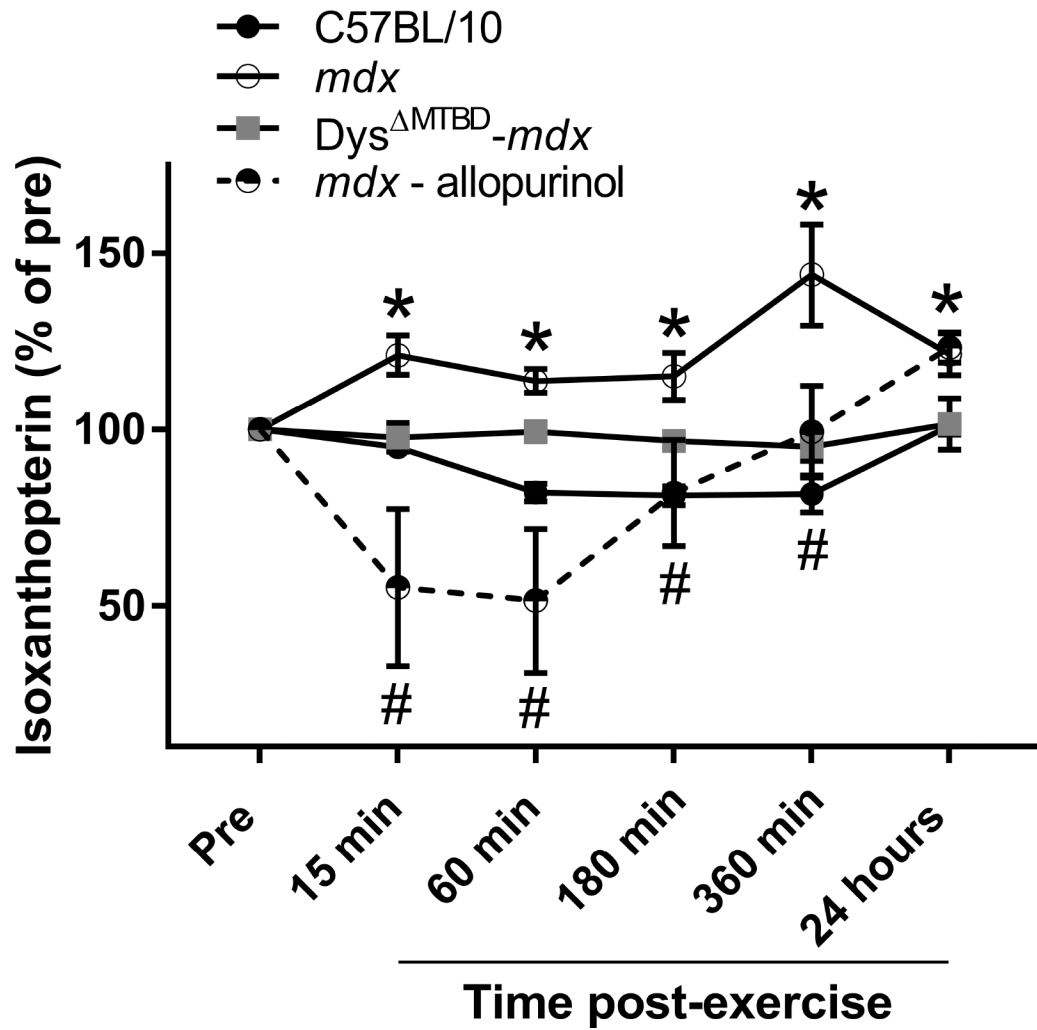


Figure 5. Urinary isoxanthopterin following forced downhill treadmill exercise in C57BL/10, *mdx* with or without allopurinol, and *Dys*^{MTBD}-*mdx* mice; * different from C57BL/10, # different from *mdx*, n = 4/group.

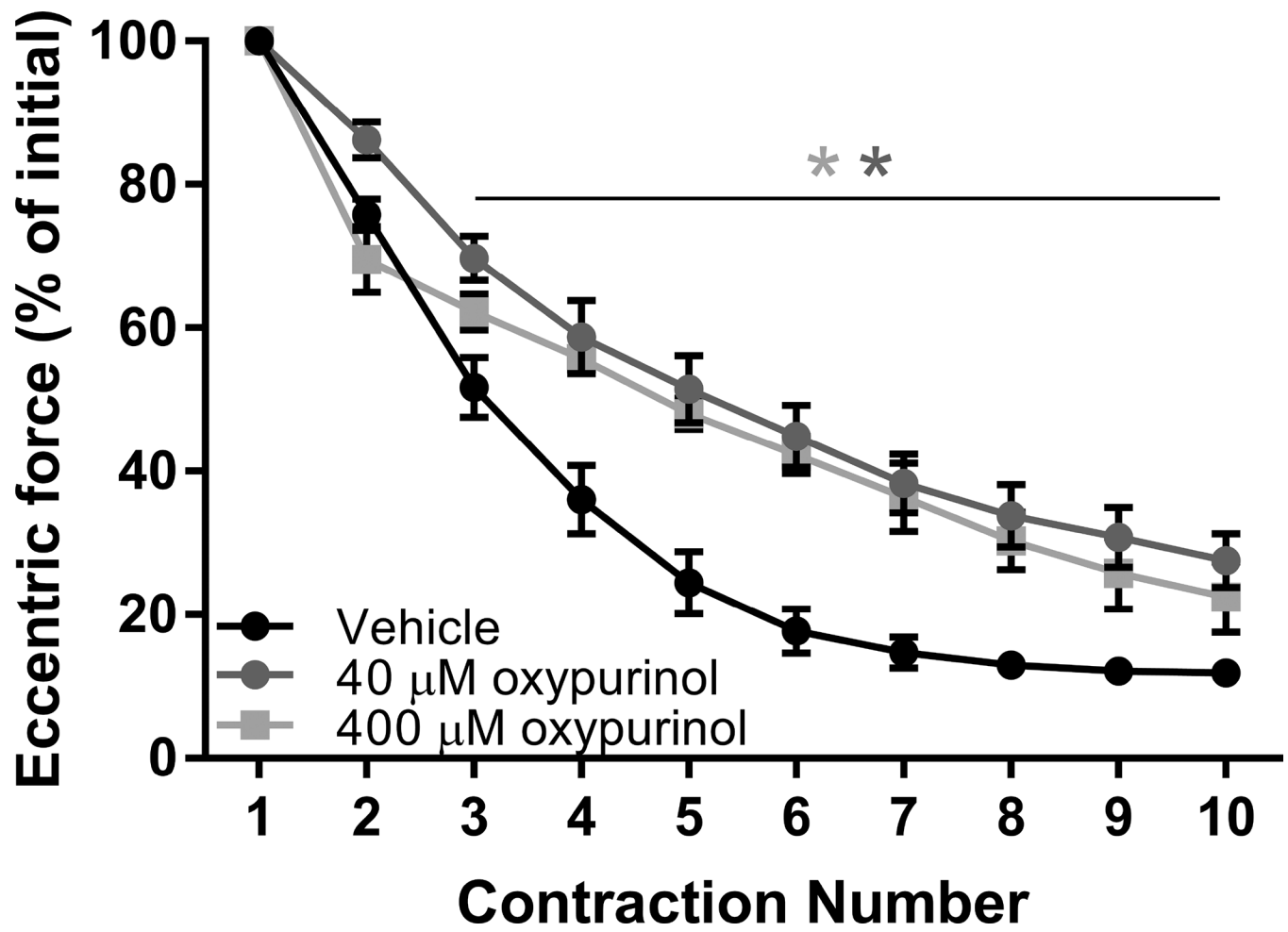


Figure 6. Eccentric force tracings of *mdx* EDL muscle *in vitro* during eccentric contractions with or without xanthine oxidase inhibition. *different from vehicle, n = 5/group.

Table 1.

Physiological parameters of isolated EDL muscles used in *ex vivo* force measurements.

| Parameter | Control | Oxyupurinol (μ M) | | p value |
|---|----------------|------------------------|----------------|---------|
| | | 40 | 400 | |
| Mouse mass (g) | 32.0 \pm 1.3 | 31.9 \pm 1.0 | 32.0 \pm 1.0 | 0.997 |
| EDL mass (mg) | 15.9 \pm 1.0 | 15.1 \pm 0.5 | 14.3 \pm 0.8 | 0.411 |
| L_o (mm) | 13.7 \pm 0.2 | 13.2 \pm 0.1 | 13.4 \pm 0.2 | 0.166 |
| CSA (mm^2) | 2.5 \pm 0.1 | 2.4 \pm 0.1 | 2.3 \pm 0.1 | 0.389 |
| Peak twitch (mN) | 139 \pm 8.5 | 137 \pm 5.8 | 129 \pm 11 | 0.701 |
| P_o (mN) | 484 \pm 12 | 451 \pm 21 | 442 \pm 36 | 0.426 |
| Specific P_o (N/cm^2) | 19.8 \pm 1.4 | 18.6 \pm 1.1 | 19.1 \pm 0.7 | 0.760 |
| P_o (%) | 88.6 \pm 1.0 | 74.9 \pm 3.9 * | 80.9 \pm 4.7 | 0.036 |
| Eccentric force loss (%) | 88.1 \pm 1.5 | 72.4 \pm 3.8 * | 77.6 \pm 5.0 | 0.020 |

Values are mean \pm SE.

L_o = optimal muscle length, CSA = physiological cross sectional area, P_o = maximal isometric tetanic force. P_o = change in P_o after eccentric contractions. n = 5 for each line. Statistics were performed using unpaired *t*-test analyses with $p < 0.05$ considered significant.

* different from control.