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Short title: Glutathione resource in *mdx* mice

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ABSTRACT

Oxidative stress contributes to the pathogenesis of Duchenne muscular dystrophy (DMD). Although they have been used as a model for DMD, *mdx* mice exhibit a slowly developing cardiomyopathy. We hypothesized that the disease process was delayed owing to the development of an adaptive mechanism against oxidative stress, involving glutathione synthesis.

At 15-20 weeks of age *mdx* mice displayed 33% increase in blood glutathione level compared to age-matched C57BL/6 mice. In contrast, cardiac glutathione content was similar in *mdx* and C57BL/6 mice, as a result in *mdx* mice heart of the balanced increased expression of glutamate cysteine ligase catalytic and regulatory subunits ensuring glutathione synthesis, and that of glutathione peroxidase-1 utilizing glutathione. Oral administration from 10 weeks of age of the glutamate cysteine ligase inhibitor, L-buthionine(S,R)-sulfoximine (BSO, 5 mM), led to 33% and 50% drop in blood and cardiac glutathione, respectively, in 15-20-week-old *mdx* mice. Twenty-week-old BSO-treated *mdx* mice displayed left ventricular hypertrophy associated with diastolic dysfunction, discontinuities in beta-dystroglycan expression, micronecrosis and microangiopathic injuries. Examination of the glutathione status in 4 DMD patients provided evidence that 3 of them displayed systemic glutathione deficiency.

In conclusion, low glutathione resource hastens the onset of cardiomyopathy linked to a defect in dystrophin in *mdx* mice. This is relevant to the glutathione deficiency that DMD patients may suffer.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive disorder due to a defect in dystrophin, a sub-sarcolemmal structural protein of the cardiac and skeletal muscles that contributes to the integrity of the cellular membrane.[1] Dystrophin-deficient *mdx* mice are the most commonly used experimental model of DMD.[2-7] In DMD patients, as well as in *mdx* mice, dystrophin defect alters the sarcolemmal integrity of cardiac and skeletal myocytes,[8-10] with increased susceptibility to oxidative stress[11-13] leading to necrotic cell death and inflammation.[14, 15] Further evidence in support of oxidative stress as a cause of the dystrophic pathophysiology comes from *in vivo* studies demonstrating reduced signs of muscle damages in *mdx* mice given antioxidants.[16, 17]

The tripeptide glutathione (L-gamma glutamyl-cysteinyl-glycine) is a major antioxidant, which plays a central role in cell redox regulation and controls multiple cellular processes. In particular, glutathione, through the inhibition of the neutral sphingomyelinase-dependent apoptotic pathway,[18, 19] determines cardiac myocyte function and survival.[20-22] Alterations in glutathione homeostasis and metabolism are associated with inflammation[23] and characterize several human inflammatory chronic diseases.[24, 25] Thus, fibroblasts from DMD patients show a low capacity for glutathione synthesis.[26] In contrast, a noticeable feature of *mdx* mice dystrophic muscles is the up-regulation of glutathione peroxidase and glutathione reductase, which respectively utilizes and recycles reduced glutathione.[27] However, this protective mechanism does not entirely compensate the chronic oxidative challenge since glutathione level in 6-8-week-old *mdx* mice tibialis anterior muscles is decreased by 20% compared to control mice.[27] Accordingly, green tea polyphenols, which beyond their inherent antioxidant properties promote glutathione synthesis, reduce muscle damage and necrosis in *mdx* mice.[16, 17] This does not rule out that the intrinsic increased turnover of glutathione in *mdx* mice may delay the progression of the dystrophic disease, although it cannot fully protect against it.

Cardiac manifestations of DMD are recognized later than those of skeletal muscles, but are present in 90% of DMD patients by the age of 18 years, progressing to fatal heart failure in 20% of patients.[28] Similarly to DMD patients, *mdx* mice experience a progressive development of cardiac defects. However, the disease develops with less severity than in patients and in old mice only, young adult mice showing little echocardiographic signs of cardiomyopathy before 10 months of age.[7, 29] Glutathione plays a central role in protecting cardiac vasculature,[30] and cardiac glutathione deficiency is a common feature of cardiomyopathies in animal models[20, 21] as in patients.[21, 22, 31]

We hypothesized that the progression of cardiomyopathy in *mdx* mice was slow owing to a boost in glutathione synthesis and resource, set as an adaptation mechanism against oxidative stress. Mice administered oral L-buthionine sulfoximine (BSO), a specific inhibitor of glutamate cysteine ligase (GLCL), the rate-limiting enzyme of glutathione synthesis, are a suitable, well-characterized model for glutathione deficiency.[32] From 10 weeks of age *mdx* mice experience a continuing cycle of skeletal muscle fiber degeneration and regeneration.[33, 34] We chose this age to start a 10-week treatment with BSO added to the drinking water at a low dose (5 mM) devoid of toxicity.[32] At variance from 20-week-old untreated *mdx* mice receiving water, 20-week-old BSO-treated *mdx* mice displayed cardiac left ventricular (LV) structural abnormalities and hypertrophy together with altered diastolic function, reminiscent of the early stage of the cardiomyopathy in DMD patients prior to the manifestations of clinical symptoms.[35, 36] To get a first insight into the glutathione status of DMD patients, we measured blood glutathione in 4 DMD patients with mean age of 28 ± 2 years and on wheelchair dependency. We found that 3 of them demonstrated systemic glutathione deficiency.

MATERIALS AND METHODS

Patients

This study, carried out prospectively for a period of 7 months (September 2009 to March 2010) at the AP-HP, Groupe Hospitalier Cochin, Service de Cardiologie, Paris, France and at the Hôpital Raymond Poincaré, Service de Réanimation et Unité de Ventilation à domicile, Garches) was conformed to the Declaration of Helsinki and to our institutional ethics committee. All the participants gave informed consent. We enrolled 4 consecutive patients with DMD, (4 males with mean age of 28 ± 2 years), referred to our institutions for cardiac evaluation. Patients underwent biological tests including the measurement of whole blood glutathione and an echocardiography (ATL HDI 5000 system, ATL Ultrasound, Bothell, WA). Examinations were conformed to the recommendations of the American Society of Echocardiography. Left ventricular ejection fraction (LVEF) was determined according to the Simpson's method. Blood glutathione of DMD patients was compared to that of 4 matched healthy volunteers.

Animals and experimental design

Experimental procedures were performed in accordance with the European legislation on animal experimentation (L358-86/609/EEC). C57BL/6 (B6) and *mdx4Cv* (*mdx*) mice were used. The *mdx4Cv* mouse mutant, in which a C-to-T nucleotide transition generates a stop codon in exon 53 of the dystrophin gene, has almost no background of revertant fibers in skeletal muscle.[4, 6, 37] B6 and *mdx* mice were randomly assigned to two groups: 1) an untreated group receiving water; 2) a group receiving L-buthionine sulfoximine (BSO, Sigma) via drinking water at the concentration of 5mM (average dose of 185 mg/kg/ day) from 10 to 20 weeks of age. As previously stated by Watanabe et al.,[32] this dose of BSO did not produce either deaths or pathological signs. Blood was withdrawn from the queue at regular intervals from 3 weeks to 20 weeks of age. At 20 weeks of age, all mice were

subjected to echocardiography then euthanized by intraperitoneal injection of overdose anaesthetics (xyzaline (20 mg/kg) and ketamine (50 mg/kg). Blood was withdrawn and hearts were excised and frozen in liquid nitrogen cooled isopentane. All samples were stored at –80°C until use.

Echocardiography

Transthoracic 2D echocardiography measurements were performed in isoflurane-anesthesied mice using an echocardiograph (Vivid 7, General Electric) equipped with a linear 13 MHz transducer. Body temperature was maintained with a heating pad. Numeric images were stored and reviewed at a slower frame rate by a single observator blinded of the animal treatment and genotype status. Left ventricle end-diastolic diameter (LVEDD), inter-ventricular septal wall thickness in diastole (IVS) and posterior wall thickness in diastole (LVPW) were measured from long axis view by two-dimensional-guided M-mode. LV mass (LVM) was calculated using the formula: $LVM = 1.05 [(IVS + LVEDD + LVPW)^3 - (LVID)^3]$.[38] Left ventricular ejection fraction (LVEF) was measured using two-D parasternal long axis cine-loop.[39] Peak mitral early diastolic velocity (E), its deceleration time (DcT) and the peak velocity of the atrial contraction (A) were measured using the trans-mitral pulsed wave Doppler. All measurements were averaged on 3 consecutive cardiac cycles.

Biochemical analysis

Total glutathione was measured in patients and mice whole blood collected in EDTA tubes, and in mouse heart homogenates according to a modification of Tietze method[40] as previously described.[21, 22, 41] Results are given as means ± s.e.m of three separate determinations made in duplicates.

Histological and immunohistochemical analysis

Cardiomyocyte structure was studied in air-dried frozen heart sections from B6 mice, untreated or BSO-treated *mdx* mice. After blocking nonspecific staining with AffiniPure Fab

and Fc fragments (Jackson Immuno Research Laboratories),[17] sections were incubated with either a mouse monoclonal antibody to β-dystroglycan (Abcam; diluted 1:50) and the secondary goat anti-mouse Alexa-fluor 555-conjugated antibody (Invitrogen; diluted 1:1000) or a rat monoclonal antibody to mouse CD68 (Serotec; diluted 1:250) and the biotinylated sheep secondary anti-rat IgG antibody (Serotec; diluted 1:500). Slides were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen) or Immu-Mount (Thermo Electron Corporation) for β-dystroglycan or CD68 staining, respectively.

Electronic microscopy

Whole hearts were dissected and the apex of the LV was removed, and immediately placed in fix (4% paraformaldehyde, 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4) for 1h. The samples were transferred to 3% glutaraldehyde in phosphate buffer (0.1 M sodium phosphate, pH 7.4) overnight, then washed for 15 min in phosphate buffer, and post-fixed for 1.5 h in phosphate buffer added with in 1% osmium tetraoxide. Following osmium fixation, the samples were rinsed 3 to 5 min in phosphate buffer, dehydrated for 10 min in successive ethanol washes (50%, 70%, 80%, 95%, 100%, 100%), and rinsed for 15 min in propylene oxide. Finally, the samples were embedded in Lowecryl resin, overnight. Sections of 70–80 nm were cut on a Reichert UltracutE, stained with 2% uranyl acetate plus Reynold's lead citrate and viewed using a Phillips CM120 transmission electron microscope.

RNA isolation and Real-Time PCR analysis

Total RNA was extracted from frozen hearts using TRIzol reagent (Qiagen) and RT-PCR was performed as previously described.[42] Concentration of total RNA was dosed using a NanoDrop ND-1000 spectrophotometer (Labtech) and RNA quality was assessed by electrophoresis. cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was carried out on a Light Cycler (Roche Diagnostics), using the Quantitest SYBR green kit (Qiagen) with sense and anti-sense

oligonucleotides primers for the different genes (**Table 1**). The PCR-amplified products were controlled by sequencing. Relative levels of mRNA expression were calculated according to the $\Delta\Delta$CT method. [43] Individual mRNA expression values were normalized by comparison with 18S mRNA level.

Statistical analysis

Data are expressed as means ± s.e.m. Data were analyzed by Mann-Whitney test or Kruskal-Wallis test combined with Dunn post-test, as appropriate (Prism, GraphPad Software Inc.). Echocardiographic measurements were analyzed by a two-way repeated measure ANOVA followed by a multiple comparison test (Holm-Sidak method). Statistical significance was assumed at $p \leq 0.05$.

RESULTS

Blood level and cardiac content of glutathione in *mdx* mice

B6 mice displayed steady blood glutathione level from the age of 5 to 20 weeks, with an average value of 0.70 ± 0.03 mM. Until the age of 10 weeks, *mdx* mice had a similar blood glutathione level as B6 mice (**Fig. 1**). However, after 15 weeks of age, blood glutathione level in *mdx* mice increased by 33% to 0.93 ± 0.03 mM (**Fig. 1**). At variance from blood glutathione, cardiac glutathione content in 20-week-old *mdx* mice was not significantly increased as compared to B6 mice (**Fig. 2**), despite significant elevation in cardiac mRNA expression of both GLCL catalytic (GLCLC) and modulatory subunits (GLCLM) that ensure glutathione synthesis (**Fig. 3**). In fact, increased expression of GLCL subunits in the heart of 20-week-old *mdx* mice coped with that of glutathione peroxidase-1 (GPx1) that utilizes glutathione to reduce hydroperoxides (**Fig. 3**).

Effects of BSO treatment on blood and cardiac glutathione content in *mdx* mice

BSO was given at a low non-toxic dose (5 mM), i.e. a dose that did not produce either deaths or pathological signs.[32] BSO treatment, started at the age of 10 weeks, blunted the rise in blood glutathione level in 15- and 20-week-old *mdx* mice, maintaining it at 0.74 ± 0.04 mM (**Fig. 1**). Cardiac glutathione content in BSO-treated *mdx* mice was decreased by 50% as compared to untreated *mdx* mice given water, reaching a level not significantly different from that in B6 mice, receiving water or BSO (**Fig.2**). The decrease in cardiac glutathione content in BSO-treated *mdx* mice was related to a reduced expression of glutathione enzyme mRNAs as compared to untreated *mdx* mice, similar to that in B6 mice (**Fig. 3**).

BSO-treated *mdx* mice display LV hypertrophy and diastolic dysfunction

As compared to age-matched B6 mice heart, 20-week-old *mdx* mice heart was featured by a smaller body weight-indexed LV mass (iLVM) related to thinner posterior and

intraventricular septal wall thickness (LVPW and IVS, respectively) (**Fig.4A**). In other ways, *mdx* mice displayed normal LV systolic function, assessed by the LV ejection fraction (LVEF), and normal LV diastolic function, assessed by both the ratio between early diastolic filling wave (E) and filling wave illustrating atrial contraction (A), and the E-wave deceleration time (DcT) (**Fig.4A**). Ten week BSO-treatment induced LV hypertrophy in *mdx* mice, as demonstrated by the 21% increase in iLVM (**Fig.4A**). LV hypertrophy in BSO-treated *mdx* mice had no impact on LV systolic function, but was associated with impaired diastolic function as characterized by a concomitant E/A decrease and DcT prolongation (**Fig.4A**). Noteworthy, LV hypertrophy in *mdx* mice was correlated with cardiac glutathione deficiency (r = 0.66, *p* = 0.002; **Fig.4B**).

BSO-treated *mdx* mice display LV structure alterations

Immunohistochemical analysis on LV sections of 20-week-old *mdx* mice given water showed a regular expression of beta-dystroglycan along cardiac myocyte membranes, similar to that in LV sections of age-matched B6 mice (**Fig. 5A**). LV of *mdx* mice receiving water did not present either any histologically discernable abnormality (as assessed by hematoxylin eosin staining, not shown), or fibrosis (assessed by red-sirius staining, not shown), despite increased gene expression of 1a2, 3a1 and 6a1collagens as compared to B6 mice (**Fig. 5B**). In contrast, LV of BSO-treated *mdx* mice displayed destabilized beta-dystroglycan expression (**Fig. 5A**) with reduced collagen gene expression as compared to *mdx* mice receiving water (**Fig. 5B**). Foci of micronecrosis infiltrated by CD68+ macrophages (**Fig. 6A and 6B**) were also identified in LV sections of BSO-treated *mdx* mice.

Electron microscopy reveals focal ultrastructural lesions in LV of BSO-treated *mdx* mice

Electron microscopy analysis of LV of 20-week old *mdx* mice receiving water showed tightly packed contractile proteins, and did not show any ultrastructural abnormalities

(**Fig.7A**). In contrast, focal ultrastructural lesions were discernible in LV of BSO-treated *mdx* mice, with degenerative changes including intracellular amorphous accumulations separating contractile bundles, pronounced irregularities of sarcomere elements such as the Z-line (Z streaming), extreme dilation of smooth endoplasmic reticulum profiles and giant mitochondria (**Figure 7B**). These lesions were spatially associated with microangiopathic lesions characterized by edema in endothelial cells leading to partial obstruction of the lumen, and accompanied by edema in interstitium and neighboring cardiomyocytes (**Fig. 8**).

Glutathione status in DMD patients

Next, we intended to get a first insight into the glutathione status of DMD patients. Four DMD patients were enrolled, with mean age of 28 ± 2 years and on wheelchair dependency. They received an anti-angiotensin therapy, consisting in an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker, and showed a mean LVEF of 45 ± 5%. Compared to healthy volunteers, 3 out of the 4 DMD patients displayed 25% to 40% deficiency in systemic glutathione (**Fig.9**).

DISCUSSION

Our current findings are twofold. Firstly, the slow progression of cardiomyopathy in *mdx* mice is related to a boost in glutathione synthesis and resource. Conversely, administration of the GLCL inhibitor BSO to *mdx* mice from the age of 10 weeks leads to a decrease in glutathione resource in 20 week-old *mdx* mice, along with LV hypertrophy, diastolic dysfunction and structural alterations. Secondly, we point out the occurrence of glutathione deficiency in DMD patients.

Several studies have provided evidence that oxidative stress is implicated in the pathogenesis of the dystrophic disease in DMD patients[13, 44-46] and *mdx* mice,[12, 13] and that of cardiomyopathy in *mdx* mice.[47] Oxidative stress in the dystrophic muscles of *mdx* mice is counteracted by compensatory mechanisms involving up-regulation of glutathione cycle enzymes.[27, 45, 48] We now show a similar adaptation of glutathione cycle enzymes expression in the heart of *mdx* mice, together with increased systemic glutathione reserve from the age of 15 weeks, when mice undergo a continuing cycle of skeletal muscle fiber degeneration and regeneration.[34, 49] Such adaptive changes of glutathione metabolism in *mdx* mice are likely to lessen the severity and/ or delay the progression of dystrophic disease and cardiomyopathy. In fact, Dudley et al.[15] have shown that, as compared to age-matched B6 mice, *mdx* mice at the age of 14-days before the onset of fiber necrosis demonstrate normal glutathione content in tibialis anterior muscles whereas muscle necrosis in 6-8-week-old *mdx* mice is related to a 20% deficiency in glutathione content, occurring despite increased GPx activity.[15] We also found that glutathione content in gastrocnemius of 20-week-old *mdx* mice was depleted by 33% as compared to B6 mice (0.45 ± 0.06 *vs* 0.68 ± 0.07 nmol/ mg tissue, respectively, $p < 0.05$, data not shown). This is in keeping with the significant muscular weakness experienced by *mdx* mice from 1 month of age throughout their lifespan.[50, 51] At the difference from the muscular degenerative process, cardiac involvement in *mdx* mice is usually detected only above 29-weeks of age as an intermediate LV hypertrophic stage, prior to the manifestations of contractile dysfunction and dilated

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cardiomyopathy by the age of 42 weeks.[29] We now give evidence that the preservation of

cardiac structure and function in 20-week-old *mdx* mice is related to an increased

expression of cardiac GLCL and GPx1, likely to compensate for increased glutathione

demand ensuing from exacerbated oxidative challenge. This proposal is supported by the

finding that BSO-treated *mdx* mice, which are unable to boost glutathione supply, display an

earlier onset of cardiac symptoms than untreated *mdx* mice. Of note, at the dose of BSO

used, BSO-treated *mdx* mice have similar glutathione blood level and cardiac content as B6

mice. Thus BSO treatment essentially impinges on the adaptive increment of glutathione

synthesis, as further attested by the comparable level of glutathione recycling enzymes

gene expression in BSO-treated *mdx* mice and B6 mice.

The corollary of this statement would be that glutathione supplementation prevents the

development of cardiomyopathy. As a matter of fact, using a *mdx* mouse strain that has

overt cardiac fibrosis, inflammation and abnormalities in cardiomyocyte Ca^{2+} handling as

early as the age of 9 weeks, Williams and Allen reported reduced cardiac abnormalities in

the 9 week-old mice having received oral N- acetylcysteine (NAC), a glutathione precursor,

from weaning.[47] However, before 1 year-old most of the *mdx* mice from the different strains,

including ours, display little cardiac pathology.[29, 52] The small heart syndrome that we

identified in our 20-week-old *mdx* mice has been previously reported in 10-week-old *mdx*

mice,[53], and typifies patients with chronic fatigue.[54, 55] Interestingly, we found that NAC

treatment (1.4 mg/ 10 g body weight/ day), started from weaning, boosted blood glutathione

in young *mdx* mice (but not in older *mdx* mice with high blood glutathione) and prevented

the small heart syndrome in 20-week-old *mdx* mice (not shown).

From 6 months of age, *mdx* mice show LV hypertrophy.[29, 56] This is representative of

DMD development since most DMD boys display a hypertrophic stage prior to clinical

symptoms.[35, 57] An important finding of this study is that glutathione deficiency in BSO-

treated mice is associated with LV hypertrophy in 20-week-old *mdx* mice together with

diastolic dysfunction, structural and ultrastructural abnormalities. The presence of collapsed

vessels in BSO-treated *mdx* mice heart is compatible with compromised vascular supply and

cardiomyocyte injury due to ischemia. As a matter of fact, a main ultrastructural alteration in the heart of BSO-treated *mdx* mice consists of mitochondrial abnormalities in cardiomyocytes that are considered as stigmatic of oxidative damage.[58, 59] The blunting of cardiac collagen genes up-regulation in *mdx* mice treated with BSO, together with the appearance of necrosis and inflammation foci, are reminiscent of the increased inflammation associated with fibrosis reduction in the diaphragm of *mdx* mice treated with neutralizing antibody against transforming growth factor-beta1.[60] This may be indicative of a role of collagens in stabilizing the extracellular matrix and cellular membranes in *mdx* mice heart, before the development of fibrosis and subsequent disruption of the cardiac structure.

In conclusion, firstly, our study show that BSO-treated *mdx* mice provide a possible new experimental model for DMD cardiomyopathy, with defined endpoints (left ventricular hypertrophy, diastolic dysfunction, structural abnormalities) that could be used for translational studies to test various therapeutic agents. Secondly, our results bring to light an adaptive capacity of *mdx* mice to increase tissue glutathione resource, which faces oxidative stress associated with skeletal muscle degeneration/ regeneration cycles and is likely to delay dystrophic and cardiac disease progression. Finally, our preliminary insight into the glutathione status in DMD patients points out the possible occurrence of systemic glutathione deficiency in these patients. This is relevant with the previously reported altered glutathione metabolism in DMD fibroblasts.[26] One may also note that the degree of glutathione deficiency in DMD patients is akin to that previously reported in patients with cardiac diseases of non-genetic etiology, whose functional status and structural cardiac abnormalities were related to glutathione deficiency.[22] Obviously, more investigation is needed to prove that glutathione deficiency may determine the progression of the cardiac disease in DMD patients. However, these first results encourage further exploring blood glutathione status in DMD patients, at different stages of the disease process. In future, this might open new therapeutic perspectives with molecules targeting glutathione pathway, NAC in particular, which is being launched for the treatment of other inflammatory chronic diseases including idiopathic pulmonary fibrosis.[61, 62]

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DISCLOSURES

None

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Table 1: Primers used for quantitative RT-PCR

Gene	Primers	Fragment size (bp)	NCBI referenc
GLCLC	5' – ATCCTCCAGTTCCTGCACAT–3' 5' – TGTGAATCCAGGGCCTA-3'	390	NM_010295.1
GLCLM	5'-GCCACCAGATTTGACTGCCTTT-3' 5'-CAGGGATGCTTTCTTGAAGAGCTT-3'	119	NM_008129.2
GPX1	5'-GACTACACCGAGATGAACGA-3' 5'-CTTCATTCTTGCCATTCTCCT-3'	110	NM_008160.5
COL1A1	5'-ACCTGTGTGTTCCCTACTCA-3' 5'-GACTGTTGCCTTCGCCTCTG-3'	322	NM_007742.2
COL1A2	5'-TTAAGACTCAGCCACCCAGA-3' 5'-GAGCTGAGTTGCCATTTCCT-3'	267	NM_007743.2
COL3A1	5'-AATGGTGGTTTTCAGTTCAGC-3' 5'-TGGGGTTTCAGAGAGTTTGGC-3'	322	NM_009930.1
COL6A1	5'-AATTGCCCTGGTCATTACGG-3' 5'-CGATAAGCCTTGGCAGGAAA-3'	169	NM_009933.3
18S	5'-GTAACCCGTTGAACCCCATT-3' 5'-CCATCCAATCGGTAGTAGCG-3'	151	XO1117

Abbreviations: **GLCLC:** glutamate-L-cysteine ligase catalytic subunit, **GLCLM:** glutamate-L-cysteine ligase modulatory subunit, **GPX1:** Glutathione peroxidase 1, **COL1A1:** procollagen type I, alpha 1, **COL1A2:** procollagen type I, alpha 2, **COL3A1:** procollagen type III, alpha 1, **COL6A1:** procollagen type VI, alpha 1.

LEGENDS TO FIGURES

Figure 1. Evolution as a function of age of blood glutathione levels in B6 and _mdx_ mice treated or not with BSO. B6 mice received water; _mdx_ mice received either water or 5 mM BSO added to the drinking water from 10 to 20 weeks of age. Blood glutathione levels were measured at 5, 10, 15 and 20 weeks of age. N= 5 in each group. #$p < 0.05$ _vs_ 5-week-old _mdx_ mice.

Figure 2. Cardiac glutathione levels in 20-week-old B6 and _mdx_ mice treated or not with BSO. Glutathione was measured in cardiac samples of 20-week-old B6 and _mdx_ mice having received either water or BSO. N=4 to 10 in each group. #$p < 0.05$ _vs mdx_ mice receiving water.

Figure 3. Cardiac expression of glutathione recycling enzymes in 20-week-old B6 and _mdx_ mice treated or not with BSO. Quantitative real time PCR analysis of _GLCLC_, _GLCLM_ and _GPX1_ gene expression, encoding glutathione ligase catalytic subunit (_GLCLC_), glutathione ligase modulatory subunit (_GLCLM_) and glutathione peroxidase (_GPX1_), respectively, in the hearts of 20-week-old B6 and 20- _mdx_ mice having received water or BSO. Individual mRNA expression values were normalized relative to 18S mRNA. Results are expressed as fold change relative to mean cardiac mRNA expression in B6 mice. N = 4 to 6 in each group. #$p < 0.05$ _vs_ B6 mice; †$p < 0.05$ _vs_ untreated _mdx_ mice.

Figure 4. Echocardiography of 20-week-old B6 and _mdx_ mice treated or not with BSO. (**A**) Twenty-week-old B6 and _mdx_ mice have received either water or BSO. BW: body weight; HR: heart rate; IVS: intra ventricular septum thickness; LVPW: posterior wall thickness; LVEDD: LV end diastolic diameter; iLVEDD: LVEDD indexed to BW; LVM: LV mass; iLVM: LVM indexed to BW; LVEF: LV ejection fraction. E/A: ratio between early

diastolic filling wave (E) and filling wave due to atrial contraction (A); DcT: E-wave deceleration time. #$p < 0.05$ *mdx* vs matched B6 group; †$P<0.05$ BSO *mdx vs* H_2O *mdx* group. (**B**) LVM in *mdx* mice, having received water or BSO, was inversely linearly correlated with cardiac glutathione content (r = 0.66, $p = 0.002$).


Figure 5. Sarcolemmal β-dystroglycan immunostaining and cardiac collagen gene expression in 20-week-old B6 mice and *mdx* mice treated or not with BSO. (**A**) β-dystroglycan expression around cardiac myocyte membranes is regular in B6 mice and *mdx* mice receiving water, but is destabilized in BSO-treated *mdx* mice. (**B**) Quantitative real time PCR analysis of *COL1A2, COL3A1 and COL6A1* gene coding for 1a2, 3a1 and 6a1 collagen, respectively, in the hearts of 20-week-old B6 mice and *mdx* mice having received either water or BSO. Individual mRNA expression values were normalized relative to 18S mRNA. Results are expressed as fold change relative to mean cardiac mRNA expression in B6 mice. N = 4 to 6 in each group. #$p < 0.05$ *vs* B6 mice.


Figure 6. Presence of inflammatory micronecrotic foci in the hearts of 20-week-old BSO-treated *mdx* mice. (**A, B**) CD68 immunostaining showing typical foci of inflammatory infiltrates. (**B**) A necrotic cardiomyocyte encircled and phagocytized by CD68+ macrophages (original magnification x 400 for both).


Figure 7. Ultrastructural degenerative alterations in the hearts of 20-week-old BSO-treated *mdx* mice. (**A**) Hearts of 20-week-old *mdx* mice receiving water display normal sarcomere organization illustrated by tightly packed contractile proteins (original magnification x 11,500). (**B)** In contrast, **r**egions of 20-week-old BSO-treated *mdx* mice hearts show cardiomyocytes with cytoplasmic degenerative alterations: intracellular amorphous accumulations (*asterisks*) separating contractile bundles, pronounced sarcomere irregularities (Z streaming) (*arrow*), dilatation of smooth endoplasmic reticulum profiles (*arrowhead*) and giant mitochondria (original magnification x 11,700).

Figure 8. Vessel associated lesions in the hearts of 20-week-old BSO-treated *mdx* mice. Hearts of 20-week-old BSO-treated *mdx* mice display a gamut of vessel associated lesions. (**A**). Massive edema (*asterisk*s) and extravasated erythrocytes (*arrow*) of interstitium between 2 cardiomyocytes (*arrowheads*) (original magnification x 5,600); (**B** and **C**). Edematous endothelial cells (arrows) accompanied by edema in interstitium and neighboring cardiomyocytes (asterisks), incipient in **B** (original magnification x 7,100) and pronounced in **C** (original magnification x 5,600). (**D**) A lysing capillary endothelial cell characterized by a swollen cytoplasm, (*arrowhead*), a "ground glass" nucleus (*arrow*) and a reduced lumen (*asterisk*), and degenerated mitochondria spilling over from surrounding cardiomycytes (*double arrows*), suggesting a focus of micronecrosis (original magnification x 7 100).

Figure 9. DMD patients may demonstrate systemic glutathione deficiency. Total whole blood glutathione was measured in 4 DMD patients on wheelchair dependency (mean age of 28 ± 2 years) and in 4 matched healthy volunteers. Three DMD patients displayed 25% to 40% systemic glutathione deficiency as compared to healthy volunteers.
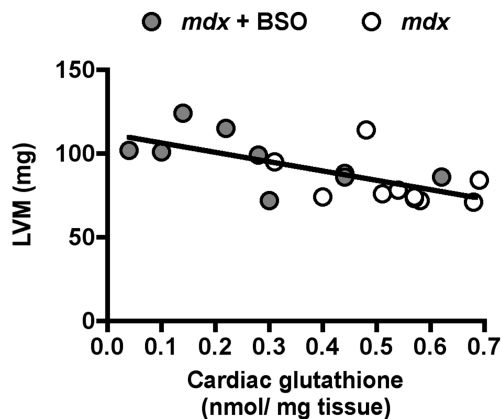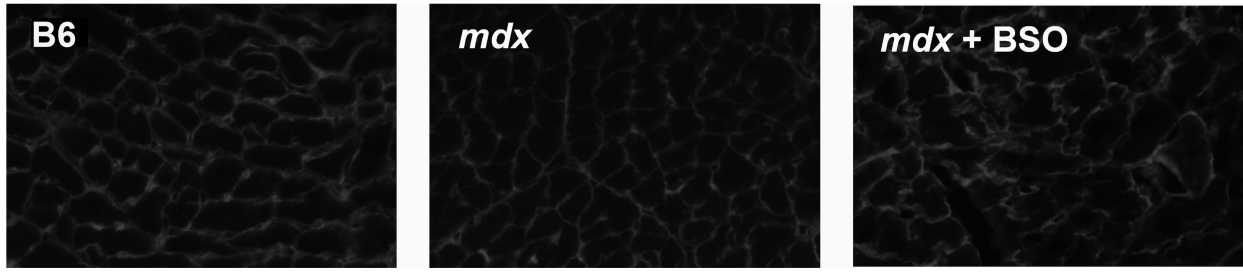
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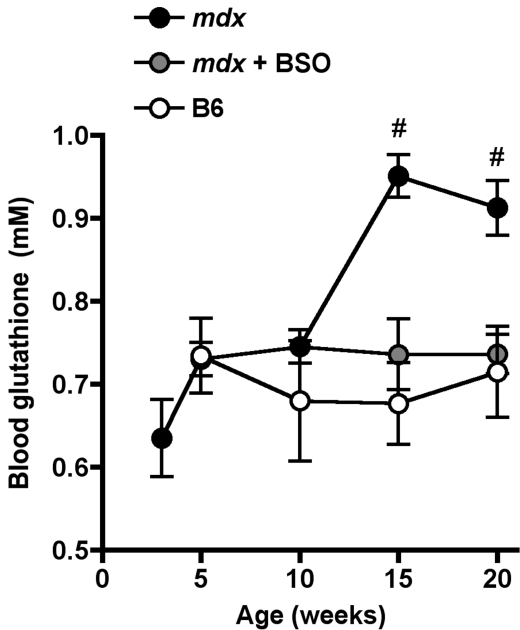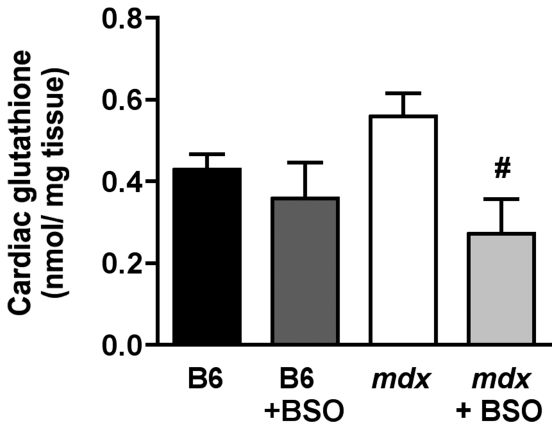
Glclc mRNA expression (fold change to B6)

B6   *mdx*   *mdx* + BSO
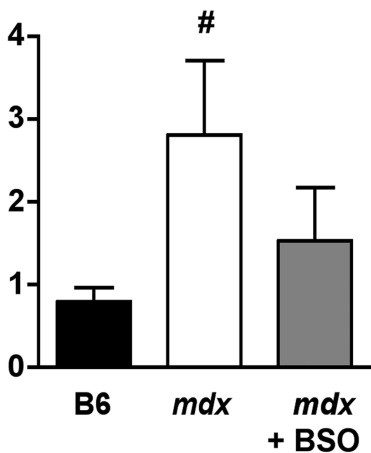
B

Gclm mRNA expression (fold change to B6)

B6   *mdx*   *mdx* + BSO
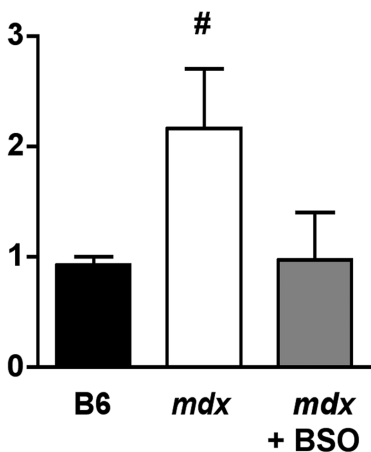
C

Gpx1 mRNA expression (fold change to B6)

B6   *mdx*   *mdx* + BSO
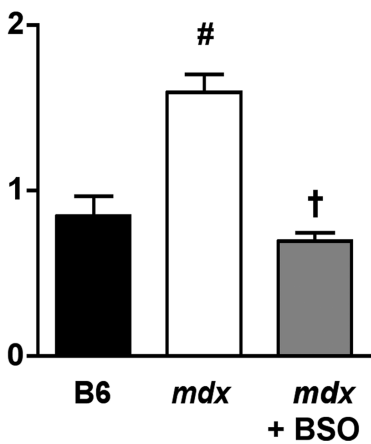
A

Subgroup	BW (g)	HR (bpm)	IVS (mm)	LVPW (mm)	LVEDD (mm)	iLVEDD (mm)	LVM (mg)	iLVM (mg)	LVE (%)	E/A	DcT (ms)
20-week B6 mice											
H₂O (n=10)	30±1	515±17	0.77±0.02	0.76±0.03	4.0±0.2	0.13±0.00	110±8	3.7±0.2	67±3	1.22±0.04	31±2
BSO (n=7)	28±1	522±18	0.75±0.02	0.76±0.03	3.9±0.2	0.14±0.00	104±3	3.8±0.1	65±3	1.25±0.03	31±2
20-week *mdx* mice											
H₂O (n=13)	27±1#	495±14	0.67±0.02#	0.62±0.02#	3.6±0.1#	0.14±0.01	74±3#	2.8±0.1#	72±2	1.38±0.05	26±2
BSO (n=8)	27±1	520±23	0.78±0.04†	0.80±0.07†	3.4±0.1#	0.13±0.00	94±7†	3.4±0.2†	66±2	1.20±0.04†	31±1†

B

A

B