Effect of *N*-acetylcysteine plus deferoxamine on oxidative stress and inflammation in dystrophic muscle cells

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Objectives: Oxidative stress and inflammatory process play an important role in the pathogenesis of Duchenne muscular dystrophy (DMD). We investigated whether deferoxamine (DFX) improves the antioxidant effects of *N*-acetylcysteine (NAC) on primary cultures of dystrophic muscle cells from *mdx* mice, the experimental model of DMD.

Methods: Primary cultures of skeletal muscle cells from *mdx* mice were treated with either NAC (10 mM), DFX (5 mM), or NAC plus DFX for 24 hours. The muscle cells of C57BL/10 mice were used as controls.

Results: Production of hydrogen peroxide (H₂O₂) and levels of 4-hydroxynonenal (4-HNE), tumor necrosis factor alpha (TNF-α), and nuclear factor kappa-B (NF-κB) were significantly higher in *mdx* muscle cells than in C57BL/10 muscle cells. Treatment with NAC, DFX, or NAC plus DFX significantly decreased H₂O₂ production (24, 58, and 72%, respectively), and levels of 4-HNE-protein adducts (62, 33, and 71%, respectively), TNF-α (32, 29, and 31%, respectively), and NF-κB (34, 38, and 52%, respectively) on dystrophic muscle cells.

Discussion: This study demonstrates that *mdx* muscle cells are able to produce key oxidative stress and inflammatory markers, without the interference of inflammatory cells, and shows that NAC plus DFX reduced the inflammatory and oxidative stress indicators, mainly H₂O₂ production and NF-κB levels by dystrophic fibers.

Keywords: Deferoxamine, Duchenne muscular dystrophy, Dystrophic fibers, Inflammatory markers, *mdx* Mice, *N*-acetylcysteine, Oxidative stress

Introduction

Duchenne muscular dystrophy (DMD) is a lethal degenerative muscular disorder caused by the absence of dystrophin protein, and affects about 1 in 3500 male births.¹ There is evidence that the pathological mechanisms triggered by the loss of dystrophin in DMD include exacerbated inflammatory response and increased oxidative stress.^{2–6}

The dystrophin-deficient muscle fibers of patients with DMD and of the *mdx* mice (an experimental model of DMD) display elevated levels of oxidative stress markers and lipid peroxidation by-products.^{6–9} In addition, dystrophin-deficient cells appear to be more susceptible than normal cells to cellular injury when exposed to reactive oxygen species

(ROS) *in vitro*, in particular to hydrogen peroxide (H₂O₂).⁶

Previous studies showed that the antioxidant *N*-acetylcysteine (NAC) provided considerable protection against the ongoing muscle degeneration and the elevated inflammatory processes in *mdx* mice.^{7–10} Previous studies have demonstrated that a combination of NAC and deferoxamine (DFX) is an effective treatment for several oxidative stress and inflammatory diseases.^{11–15} DFX is a potent iron chelator with antioxidant properties under several conditions.^{16,17} Iron deprivation decreases muscle necrosis in the *mdx* mice, suggesting that iron-catalyzed free radical reactions have a role in the pathology of dystrophin-deficient muscle.¹⁸

We investigated whether DFX synergically improves the antioxidant effects of NAC in dystrophic muscle cells. Using an *in vitro* primary culture of dystrophin-

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deficient muscle fibers of the *mdx* mice, we evaluated the effects of NAC plus DFX on oxidative stress markers, such as H₂O₂ production and the levels of 4-hydroxynonenal (4-HNE), a major product of lipid peroxidation. Considering that oxidative stress greatly contributes to the inflammatory response in dystrophic muscles,¹⁹ we also determined whether NAC plus DFX decreases the levels of nuclear factor kappa-B (NF-κB), a transcription factor that regulates the expression of pro-inflammatory cytokines,²⁰ and tumor necrosis factor alpha (TNF-α), a key cytokine that stimulates the inflammatory cell response in *mdx* mice.³

Materials and methods

Animals

Both *mdx* and C57BL/10 (control) mice were obtained from a breeding colony maintained by our institutional animal care facility and were used in all the experiments. The mice were housed according to the institutional guidelines and had access to food and water *ad libitum*. The animal experiments described here were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; process #2128-1) and the guidelines set forth by our institution.

Muscle cell cultures

Primary culture of skeletal muscle cells was performed following the method described by Rando and Blau.²¹ Hind limbs were removed from young *mdx* and C57BL/10 mice (15 days old) and the quadriceps femoris, tibialis anterior, extensor digitorum longus, gastrocnemius, soleus, and plantaris muscles were used to prepare primary myoblast culture. Muscles were triturated using a pair of scissors and enzymatically digested with collagenase and trypsin solutions at 37°C. The satellite cells (5×10^4 cells/cm²) were plated in 1% MatrigelTM-coated dishes (MatrigelTM – BD Biosciences, Bedford, MA, USA). The myoblasts were cultured in a proliferation and growth medium containing Dulbecco's Modified Eagle Medium (DMEM) with glucose (5.5 mM), L-glutamine (2 mM), fetal bovine serum (10% v/v), horse serum (10% v/v), and penicillin/streptomycin (1% v/v) for 2 days. Myogenesis (myotube differentiation) was induced by the addition of a fusion medium that consisted of DMEM with glucose (5.5 mM), L-glutamine (2 mM), and horse serum (10% v/v). The culture was maintained at 37°C and 5% CO₂ and the differentiated muscle cells with contractile properties were observed at 7–8 days of culture in the fusion medium.

Experimental cell groups

Skeletal muscle cell cultures at 7–8 days were used in all experiments and all measurements were obtained

from triplicate cultures. The following groups were studied: (1) Ctrl (myotubes from C57BL/10 mice that did not receive any treatment), (2) CtrlN (myotubes treated with 10 mM NAC for 24 hours), (3) CtrlD (myotubes treated with 5 mM DFX for 24 hours), (4) CtrlND (myotubes treated with 10 mM NAC and 5 mM DFX for 24 hours), (5) *mdx* (myotubes that did not receive any treatment), (6) *mdx*N (myotubes treated with 10 mM NAC for 24 hours), (7) *mdx*D (myotubes treated with 5 mM DFX for 24 hours), and (8) *mdx*ND (myotubes treated with 10 mM NAC and 5 mM DFX for 24 hours).

Cell viability

Cell viability was assessed by morphology and by tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT (Sigma) assay (Saint Louis, MO, USA). Skeletal muscle cells were washed in PBS, treated with MTT solution (5 mg/ml, tetrazolium salt), and incubated for 4 hours at 37°C. After 4 hours, the cell supernatants were discarded, MTT crystals were dissolved with acid isopropanol, and the absorbance was measured at 570 nm. All assays were performed in triplicate. Percentage viability was defined as the relative absorbance of treated versus untreated control cells. Plates were analyzed in a multi-mode microplate reader model Synergy H1M (Bio Tek Instruments, Washington, DC, USA) at 570 nm with a 655 nm reference wavelength.

Determination of H₂O₂ level

Skeletal muscle cells were maintained in phenol red-free culture medium and muscle-derived ROS were determined using a fluorescence assay. The Amplex UltraRed reagent (50 μM) and horseradish peroxidase (0.1 U/ml) were added for 60 minutes (Amplex UltraRed – Invitrogen, Eugene, Oregon, USA). Amplex reacts with H₂O₂, in the presence of horseradish peroxidase, to produce resorufin, a stable red fluorescent and stable compound. The fluorescence signal of resorufin was determined at 530 (excitation) and 590 nm wavelength (emission). Measurements of ROS were previously calibrated using exogenous 10 μM H₂O₂ (positive control). All measurements were performed in phenol red-free culture medium (1 ml), pH 7.4, at 37°C.

Western blotting

Proteins were extracted in a buffer containing Tris-HCl (100 mM), pH 7.5; EDTA (10 mM), pH 8.0; sodium pyrophosphate (10 mM); sodium fluoride (0.1 mM); sodium orthovanadate (10 mM); PMSF (2 mM); and aprotinin (10 μg/ml). The cell extracts were sonicated for 30 seconds at 4°C. The homogenates were centrifuged at 11 000 g for 20 minutes at 4°C and the supernatants were treated with Triton X-100 (1%) and transferred to a –80°C freezer until they were used

for Western blotting analysis. An aliquot from the supernatant was used to determine the total protein content by the Bradford method. Samples of 30 µg of total protein homogenate were loaded onto 6–15% SDS-polyacrylamide gels. Proteins were transferred from the gels to a nitrocellulose membrane using a submersion electrotransfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 2 hours at room temperature with 5% skim milk/Tris-HCl buffer saline-Tween buffer (TBST; 10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20). The membranes were incubated with the primary antibodies overnight at 4°C, washed in TBST, incubated with the peroxidase-conjugated secondary antibodies for 2 hours at room temperature, and developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA). To control protein loading, Western blot transfer, and nonspecific changes in protein levels, the blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Band intensities were quantified using ImageJ 1.38X (National Institutes of Health, Bethesda, MD, USA) software. The following primary antibodies were used for Western blotting: (1) dystrophin (mouse monoclonal; Vector Laboratories, ON, Canada); (2) NF-κB (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (3) TNF-α (rabbit anti-mouse polyclonal; Chemical, Temecula, CA, USA); (4) 4-HNE (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (5) GAPDH (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody used was peroxidase-labeled affinity purified mouse or rabbit IgG antibody (KPL, Gaithersburg, MD, USA).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical analysis for direct comparison between means of two groups was performed by the Student's *t*-test. For multiple statistical comparisons between groups, analysis of variance (ANOVA) was used, followed by the Bonferroni test used for multiple statistical comparisons between groups. Values of $P \leq 0.05$ were considered statistically significant.

Results

Primary cell cultures from normal (C57BL/10) and dystrophic muscles (*mdx*) showed the typical progression of proliferation to differentiation and fusion into thick, branching myotubes (Fig. 1A and B). Treatment with NAC, DFX, and NAC plus DFX did not cause any morphological alteration and did not affect cell viability as compared with untreated

cultures (Fig. 1C). The presence or absence of dystrophin was ascertained using Western blots (Fig. 1D).

To test whether the treatments affected ROS in dystrophic muscle cells, we analyzed H₂O₂ production and 4-HNE-protein adducts levels. We initially verified that treatment with NAC, DFX, or NAC plus DFX did not change the H₂O₂ production and 4-HNE-protein adducts levels in control (C57BL/10) myotubes. Production of H₂O₂ was significantly higher in *mdx* myotubes than in control muscle cells ($P < 0.05$, Fig. 2). Treatment with NAC, DFX, or NAC plus DFX significantly decreased H₂O₂ production (by 24, 58, and 72%, respectively) in *mdx* myotubes (Fig. 2).

Representative immunoblots and quantification of 4-HNE-protein adducts are shown in Fig. 3. Bands of 4-HNE-protein adducts from 17 to 170 kDa were detected in control and in dystrophic muscle cells. Proteins of approximately 170, 55, 43, 22, and 17 kDa exhibited elevated 4-HNE binding in the dystrophic myotubes compared with control cells. Levels of 4-HNE-protein adducts were significantly reduced by NAC, DFX, and NAC plus DFX (by 62, 33, and 71%, respectively; $P < 0.05$, ANOVA followed by Bonferroni test) in *mdx* myotubes compared with untreated dystrophic muscle cells.

To address whether NAC and DFX attenuated inflammation, we analyzed the levels of TNF-α and NF-κB. In control muscle cells, the treatment with NAC, DFX, or NAC plus DFX did not affect the TNF-α and NF-κB levels. Already, in the dystrophic myotubes, immunoblotting analysis demonstrated a marked increase in TNF-α and NF-κB levels compared with control cells (Fig. 4A and B). In contrast, the treatment with NAC, DFX, and NAC plus DFX reduced the TNF-α levels in dystrophic myotubes (by 32, 29, and 31%, respectively; $P < 0.05$, ANOVA followed by Bonferroni test; Fig. 4A). Levels of NF-κB were markedly reduced after NAC (34.2%) and DFX (38.3%) treatment (Fig. 4B); NAC plus DFX reduced the NF-κB levels in *mdx* muscle cells (by 52.2%; Fig. 4B).

Discussion

We showed that dystrophic myotubes from *mdx* mice (15 days old), in comparison with normal myotubes from C57BL/10 mice, at the same age, present increased H₂O₂ and 4-HNE-protein adducts levels, with significantly higher H₂O₂ levels than those observed for 4-HNE (600% H₂O₂ increase versus 31% 4-HNE increase, in comparison with its respective controls). In addition, high levels of NF-κB and TNF-α were detected in *mdx* myotubes in relation to their controls, with NF-κB showing the highest values (41% TNF-α versus 75% NF-κB, compared with their respective controls). These results reinforce previous studies showing that dystrophic fibers *per se* can contribute to the production of ROS and

Figure 1 Morphology of normal (A) and dystrophic (B) myotube cultures. In (C), cell viability was assessed by measurement of MTT assay in the muscle cells from C57BL/10 mice (Ctrl) and *mdx* mice (*mdx*). Myotubes were treated for 24 hours with *N*-acetylcysteine (NAC), deferoxamine (DFX), NAC plus DFX (ND), or did not receive any treatment (untreated). In (D), Immunoblot analysis of dystrophin and graph showing protein level in the muscles cells from C57BL/10 mice (Ctrl) and *mdx* mice (*mdx*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. All the experiments were performed in triplicate, and; the relative value of the band intensity was quantified and normalized by the corresponding Ctrl. 'a' versus Ctrl. Error bars, SD.

inflammatory cytokines,¹⁹ and thereby to the progress of necrosis *in vivo*.

The *in vitro* treatment of dystrophic fibers with NAC was effective in reducing 4-HNE and TNF- α . This is in agreement with our previous study, in which we demonstrated that *in vivo* NAC treatment reduces 4-HNE-protein adducts and TNF- α levels in the diaphragm muscle of *mdx* mice.⁷ Regarding the 4-HNE-protein adducts, the soluble form of TNF ($\approx$ 17 kDa),²² the small heat shock protein HspB8

Figure 2 Quantification of H₂O₂ production in the muscle cells from C57BL/10 mice (Ctrl) and *mdx* mice (*mdx*). Myotubes were treated for 24 hours with *N*-acetylcysteine (NAC), deferoxamine (DFX), NAC plus DFX (ND), or did not receive any treatment (untreated). All the experiments were performed in triplicate and all values are expressed as mean $\pm$ SD. Results are expressed relative to Ctrl. 'a' versus Ctrl untreated, Ctrl NAC, Ctrl DFX, and Ctrl ND; 'b' versus mdx; 'c' versus *mdx* NAC.

Figure 3 Immunoblot analysis shows several bands of 4-HNE-protein adducts, ranging from 17 to 170 kDa. Graphs show protein level in the muscle cells from C57BL/10 mice (Ctrl) and *mdx* mice (*mdx*). Myotubes were treated for 24 hours with *N*-acetylcysteine (NAC), deferoxamine (DFX), NAC plus DFX (ND), or did not receive any treatment (untreated). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. All the experiments were performed in triplicate, and; the relative value of the band intensity was quantified and normalized by the corresponding Ctrl. 'a' versus Ctrl untreated, Ctrl NAC, Ctrl DFX, and Ctrl ND; 'b' versus *mdx*. Error bars, SD.

Figure 4 Immunoblot analysis showed several bands of TNF- α (A) and NF- κ B (B). Graphs show protein level in the muscle cells from C57BL/10 mice (Ctrl) and *mdx* mice (*mdx*). Myotubes were treated for 24 hours with *N*-acetylcysteine (NAC), deferoxamine (DFX), NAC plus DFX (ND), or did not receive any treatment (untreated). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. All the experiments were performed in triplicate, and the relative value of the band intensity was quantified and normalized by the corresponding Ctrl. 'a' versus Ctrl untreated, Ctrl NAC, Ctrl DFX, and Ctrl ND; 'b' versus *mdx*; 'c' versus *mdx* NAC and *mdx* DFX. Error bars, SD.

(≈ 22 kDa),²³ actin (≈ 43 kDa),²⁴ the mitochondrial protein (55 kDa),²⁵ or calcium-binding proteins, such as the calsequestrin-like proteins (170 kDa)²⁶ could be potential proteins modified by 4-HNE and protected by NAC, DFX, and NAC plus DFX treatment. However, further studies are necessary to confirm these proteins as new targets of oxidative stress in dystrophic muscle cells. In this study, we further demonstrated that NAC also reduces the production of H_2O_2 by dystrophic cells *in vitro*. Concomitantly, NF- κ B is also decreased by NAC. These results are in line with others *in vivo* studies showing the beneficial effects of NAC on different muscles of the *mdx* mice.^{10,27,28} Overall, these results indicate that molecular markers of oxidative stress (mainly represented here by H_2O_2) and of inflammation (mainly represented here by NF- κ B) can be detected in the muscle cell cultures obtained from *mdx* at an early stage of the disease.

DFX enhances the beneficial effects of NAC. This was mainly observed in relation to the production of H_2O_2 and NF- κ B levels, the two factors that were present in the highest levels in our *in vitro* dystrophic muscle cell model. Previous *in vivo* studies have shown that NAC plus DFX display antioxidant activities.^{11,15} By using an *in vitro* cell culture of dystrophic fibers, we were better able to evaluate the production of H_2O_2 by the dystrophin-deficient fibers *per se* specifically, without the interference of other cells normally present in the whole tissue, such as inflammatory cells, that contribute to H_2O_2 production. While other *in vitro* studies have shown that NAC has antioxidant effects,^{29,30} these studies did not use dystrophic fibers from the *mdx* mice. Prevention of H_2O_2 production is an important outcome, because H_2O_2 in

the presence of iron may lead to the formation of highly reactive hydroxyl radicals that can intensify the lipid peroxidation process.³¹ Although this study does not aim to elucidate the mechanisms through which NAC plus DFX affected ROS formation, it has been suggested that NAC, by increasing glutathione, reduces H_2O_2 production and, subsequently, DFX reduces the formation of the hydroxyl radicals.³²

The action of NAC plus DFX in reducing the levels of NF- κ B seen here and previously demonstrated with NAC under different experimental conditions^{33,34} may be related to the anti-inflammatory properties of DFX^{32,35} and/or to the antioxidant effects of NAC and DFX. NF- κ B is the major transcription factor involved in mediating the inflammatory response in the dystrophic muscle cell³⁶ and ROS increases the NF- κ B pathway.²⁰ Thus, the combination of NAC plus DFX is effective in decreasing the production of key inflammatory mediators by the dystrophic muscle fibers.

In conclusion, this study demonstrates that *in vitro* dystrophic cells from *mdx* mice are able to produce key oxidative stress and inflammatory markers, without the interference from inflammatory cells. This suggests that the dystrophic fiber *per se*, at least in part, can influence the progression of the disease. This highlights the importance of drug therapy that affects the muscle fiber directly, which we were able to address in this *in vitro* study by showing that NAC plus DFX effectively reduced H_2O_2 production and NF- κ B levels by dystrophic fibers. Overall, considering the well-known side effects of NAC and DFX and their use in other human diseases,³⁷ these results suggest that NAC and DFX might be a suitable complementary treatment for dystrophinopathies and

recommend clinical studies of their effectiveness in DMD patients.

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Disclaimer statements

Contributors L.H.R.M. conducted the study. L.H.R.M., R.C.B., and D.S.M. analyzed the data and performed the statistical analysis. E.M. participated in the design of the study and coordination. E.M., L.R.S., and M.J.M. helped to draft the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest None.

Ethics approval The animal experiments were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; process #2128-1) and the guidelines set forth by our institution.

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