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Short communication

N-Acetylcysteine treatment reduces TNF-α levels and myonecrosis in diaphragm muscle of *mdx* mice

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SUMMARY

Background & aims: Duchenne muscular dystrophy (DMD) is a genetic muscle disease caused by the absence of dystrophin. An established animal model of DMD is the *mdx* mouse, which is unable to express dystrophin. Inflammation, particularly the proinflammatory cytokine tumor necrosis factor alpha (TNF-α), strongly contributes to necrosis in the dystrophin-deficient fibers of the *mdx* mice and in DMD. In this study we investigated whether the antioxidant N-acetylcysteine (NAC) decreases TNF-α levels and protects the diaphragm muscle of *mdx* mice against necrosis.
Methods: Mdx mice (14 days old) received daily intraperitoneal injections of NAC for 14 days, followed by removal of the diaphragm muscle. Control *mdx* mice were injected with saline.
Results: NAC reduced TNF-α and 4-HNE-protein adducts levels, inflammation, creatine kinase levels, and myonecrosis in diaphragm muscle.
Conclusions: NAC may be used as a complementary treatment for dystrophinopathies. However, clinical trials are needed to determine the appropriate dose for patients with Duchenne muscular dystrophy.

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal X-linked muscle disease resulting from a defect in the subsarcolemmal protein dystrophin. The disease affects 1 in every 3500 male births.[1] The *mdx* mouse is unable to express dystrophin and is widely used as an animal model of DMD.[2] In addition to the deregulation of calcium homeostasis, evidence indicates a crucial role of oxidative stress and inflammatory responses in the pathogenesis of the disease.[3,4]

There is currently no effective therapy for DMD, but a wide range of anti-inflammatory drugs and nutritional interventions have been tested in an attempt to reduce the severity of the disease. Previous studies have shown that N-acetylcysteine (NAC) can protect limb and heart muscles of *mdx* mice against degeneration and inflammation.[3,5] However, the effects of NAC on the most affected muscle in DMD, the diaphragm (DIA), were not investigated. Contrary to limb muscles, DIA muscles of *mdx* mice show progressive myonecrosis and functional loss that closely resemble the dystrophic phenotype seen in limb muscles of boys with DMD.[6] In addition, the production of reactive oxygen species (ROS) seems to be higher in *mdx* DIA than in *mdx* limb muscles, a fact that may contribute to the extensive fibrosis, weakness and fatigue of this muscle.[4]

In the present study, we investigated whether NAC would protect the DIA muscle of *mdx* mice against degeneration. Considering the anti-inflammatory properties of NAC,[3,7] we also determined whether this agent would decrease the levels of tumor necrosis factor alpha (TNF-α), a key cytokine that stimulates the inflammatory cell response in limb and DIA muscles of *mdx* mice.[8–10]

2. Materials and methods

Mdx mice (14 days old) received daily intraperitoneal injections of NAC (Sigma–Aldrich, Inc., St. Louis, MO) at a dose of 150 mg/kg body weight diluted in 0.1 ml saline, or only saline for 14 days. Some C57BL/10 mice (control mice) were used for focused experiments. The animal experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; process #1584-1).

For biochemical evaluation of muscle fiber degeneration, control mice ($n = 10$) and NAC-treated ($n = 10$) and saline-treated ($n = 10$) *mdx* mice were anesthetized with a mixture of ketamine hydrochloride (130 mg/kg; Francotar, Virbac, Fort Worth, Texas) and xylazine hydrochloride (6.8 mg/kg; 2% Virbaxil, Virbac), and blood

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samples were collected by cardiac puncture. After incubation at room temperature for 1–2 h to allow for clotting, the samples were microcentrifuged at 936 g for 10 min and the supernatant (serum) was removed and used for analysis. The creatine kinase (CK) assay was performed using a commercially available kit (CK Cinetico Crystal, Bioclin, Quibasa, Minas Gerais, Brazil) and a Genesys 20 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). Values are reported as international units (U/liter).

For morphological analysis and quantification of muscle fiber damage, NAC-treated ($n = 5$) and saline-treated ($n = 5$) *mdx* mice were injected intraperitoneally with Evans blue dye (EBD). Twelve hours later, the mice were anesthetized as described above and the DIA muscle was dissected out, snap-frozen in n-hexane, cooled in liquid nitrogen, and stored at −80 °C. Cryostat cross-sections were incubated in ice-cold acetone, washed with PBS, and mounted in DABCO (mounting medium for fluorescence microscopy; Sigma). EBD staining shows a bright red emission upon fluorescence microscopy. EBD-positive muscle fibers were counted with a hand counter in all sections and photographed under a Nikon fluorescence microscope connected to a Hamamatsu video camera. The number of EBD-positive muscle fibers is expressed as the percentage of the total number of muscle fibers.

Other sections were stained with hematoxylin-eosin for analysis of the inflammatory cell infiltrate. The slides were examined under a Nikon Eclipse E400 microscope connected to a personal computer and a video camera (Nikon Express Series). Nonoverlapping images of the entire muscle cross-section were taken and tiled together using the ImagePro-Express software (Media Cybernetics, Silver Springs, MD). Inflammatory cells were identified based on nucleus morphology and cell size characterized by basophilic nuclear staining and scarce cytoplasm. Areas containing densely packed inflammatory cells were measured with the ImagePro-Express software and were calculated as the percentage of total muscle area in each section studied (4–5 sections per muscle). All counts and measurements were done by a blinded observer.

TNF-α and 4-hydroxynonenal (4-HNE)-protein adducts (a biomarker of lipid peroxidation[11]) were quantified by western blotting in control mice ($n = 10$) and in NAC-treated ($n = 10$) and saline-treated ($n = 10$) *mdx* mice. Samples of the DIA muscle were lysed in lysis buffer containing freshly added protease and phosphatase inhibitors. The samples were centrifuged at 12581 *g* for 20 min and the soluble fraction was resuspended in 50 μl Laemmli loading buffer. Next, 30 μg total protein homogenate was loaded onto 12% SDS-polyacrylamide gels. Proteins were transferred from the gels to a nitrocellulose membrane using a submersion electrotransfer apparatus (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked for 2 h 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 TNF-α (rabbit anti-mouse polyclonal, Millipore, California, USA) or 4-HNE (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, California, USA) overnight at 4 °C, washed in TBST, incubated with the peroxidase-conjugated secondary antibodies (mouse or rabbit IgG antibody, KPL, Gaithersburg, Maryland, USA) for 2 h at room temperature, and developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL). To control for protein loading, blot transfer, and nonspecific changes in protein levels, the blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, California, USA). Band intensities were quantified using the ImageJ 1.38X software (National Institutes of Health, Bethesda, MD).

All data are expressed as mean ± standard deviation (SD). Statistical analysis for direct comparison between means of two groups was performed by the Student *t*-test and ANOVA followed by Bonferroni test was used for multiple statistical comparisons between groups. $P \leq 0.05$ was considered statistically significant.

3. Results

Body weight did not differ significantly between NAC-treated and saline-treated *mdx* mice during the study period. Longitudinal comparison showed an increase in body weight at the end of the experiment in all animals. The final mean body weight was 15.0 ± 0.8 g for NAC-treated *mdx* mice and 14.6 ± 1.5 g for saline-treated *mdx* mice ($P > 0.05$, Student *t*-test). These results show that NAC treatment did not interfere with the growth rate of young *mdx* mice.

The levels of CK were significantly increased in saline-treated *mdx* mice (1389.1 ± 121.6 U/l) when compared to control mice (111.3 ± 31.0 U/l). Administration of NAC markedly reduced the levels of this enzyme (184.8 ± 35.1 U/l; NAC *mdx* vs saline *mdx*, $P < 0.05$).

NAC caused a significant decrease of EBD staining, indicative of sarcolemmal leakage and myonecrosis, in DIA muscle of *mdx* mice (Fig. 1A,B). This reduction was accompanied by a significant decrease in the inflammatory area (Fig. 1C,D).

The levels of TNF-α were significantly higher in the dystrophic DIA muscle of saline-treated *mdx* mice compared to control mice (Fig. 2). NAC significantly reduced TNF-α levels in dystrophic DIA muscle (Fig. 2).

Several bands of 4-HNE-protein adducts ranging from 26 to 170 kDa were detected in the DIA muscle (Fig. 3). Proteins of approximately 170, 55 and 26 kDa exhibited elevated 4-HNE binding in the dystrophic DIA muscle of saline-treated *mdx* compared to control mice and NAC significantly reduced this increase (Fig. 3).

4. Discussion

NAC is a thiol-reducing agent that naturally occurs in vegetables. NAC acts as a cysteine donor and maintains the intracellular levels

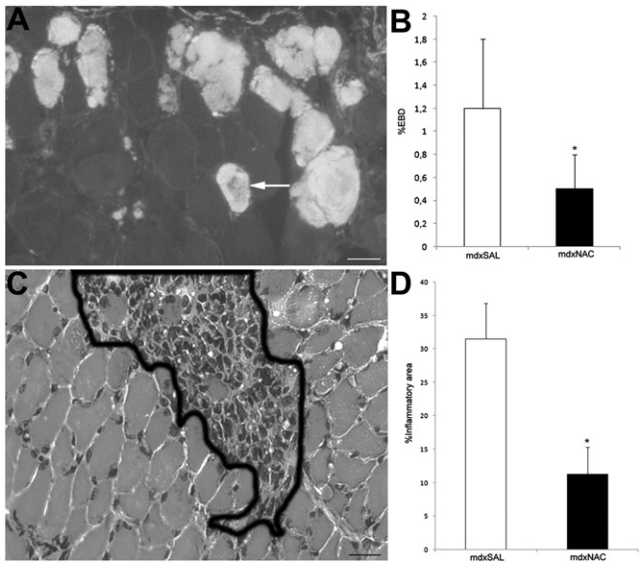

Fig. 1. In (A) EBD-positive myofibers (arrow) indicate sarcolemmal leakage in saline-treated *mdx* diaphragm muscle fibers. In (B), quantification of EBD-positive fibers in diaphragm muscle of saline-treated *mdx* mice (*mdx*SAL) and N-acetylcysteine-treated *mdx* mice (*mdx*NAC). Values are expressed as the percentage of the total number of fibers in diaphragm muscle. In (C), a representative inflammation/regeneration area is indicated by the outline in NAC-treated *mdx* mice. In (D), quantification of the inflammatory area in diaphragm muscle of *mdx*SAL and *mdx*NAC. All values are expressed as mean ± SD ($n = 5$, $^*P < 0.05$ versus *mdx*SAL). Scale bar: 50 μm (A,C).