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Ascorbic acid protects the diaphragm muscle against myonecrosis in *mdx* mice

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ABSTRACT

Objective: Oxidative stress contributes to myonecrosis in the dystrophin-deficient fibers of *mdx* mice and in Duchenne's muscular dystrophy. We examined the effects of ascorbic acid (AA), an antioxidant and free radical scavenger, on the dystrophic diaphragm muscle.
Methods: *Mdx* mice (14 d old) received AA for 14 d. Control *mdx* mice received saline. The muscle damage was visualized by the penetration of Evans blue dye into myofibers and the extent of inflammation was assessed by histologic analysis. Creatine kinase levels were measured for the biochemical evaluation of muscle fiber degeneration. The levels of tumor necrosis factor-α (a proinflammatory cytokine) and 4-hydroxynonenal (a marker of lipid peroxidation) were analyzed by immunoblotting.
Results: Ascorbic acid decreased creatine kinase levels, myonecrosis, inflammation, and the levels of tumor necrosis factor-α and 4-hydroxynonenal.
Conclusion: The present results suggest that AA plays a protective role in dystrophic muscle degeneration, possibly by decreasing reactive oxygen species, and support further investigations of AA as a potential therapy for dystrophinopathies.

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Introduction

Duchenne's muscular dystrophy (DMD) is a progressive muscle-wasting disease resulting from the lack of dystrophin [1]. The *mdx* mouse also lacks dystrophin and is the most widely used animal model of DMD. *Mdx* mice exhibit increased serum creatine kinase (CK) levels [2] and muscle necrosis followed by muscle regeneration [3,4].

Several studies using the *mdx* mouse model have proposed a critical role for reactive oxygen species (ROS) in the pathogenesis of dystrophic muscle [5–8]. ROS have been suggested to exert their effects through a direct toxic action on target cells [8] or to contribute to the activation of transcription factor nuclear-κB (NF-κB) in the *mdx* muscle [9]. This factor regulates the expression of genes related to inflammation, such as interleukin-1β, cyclooxygenase-2, and tumor necrosis factor-α (TNF-α) [10,11]. All of these factors are involved in the pathogenesis of muscular dystrophy [12].

Tumor necrosis factor-α is a key cytokine that stimulates the inflammatory cell response. This cytokine is found to be elevated in several conditions [13], including DMD and *mdx* mouse muscles [14,15]. The blockade of TNF-α with the antibody infliximab (Remicade) in young *mdx* mice has a striking protective effect on dystrophic myofibers and suppresses the early acute phase of myofiber necrosis [16]. Etanercept, a soluble TNF receptor fusion protein, decreases inflammatory cell infiltration [17] and protects young dystrophic muscle against myonecrosis [18].

Ascorbic acid (AA; vitamin C) has been reported to be an effective antioxidant and free radical scavenger, which decreases the oxidative and free radical-induced damage to the DNA and membranes in biological systems under in vitro and in vivo conditions [19,20]. Studies using endothelial cells have shown that AA inhibits TNF-α–induced NF-κB activation [21]. In addition, AA supplementation decreased the detection of TNF-α in the lungs of mice exposed to different doses of cigarette smoke [22].

We hypothesized that in vivo treatment with AA would have beneficial effects on the muscle morphology and serum CK levels in *mdx* mice by decreasing oxidative stress and by inhibiting TNF-α–induced NF-κB activation.

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Materials and methods

Animals

Male and female *mdx* mice obtained from a breeding colony maintained by our institutional animal care facility were used in all experiments. The mice were housed according to institutional guidelines and had access to food and water ad libitum. Pregnant females were separated and monitored daily. The date of birth was designated postnatal day 0. AA treatment was initiated on postnatal day 14 before the onset of the muscle degeneration–regeneration cycles [23] to verify whether AA therapy would prevent or attenuate the development of the dystrophic phenotype during the early stages of the disease. The animals were weaned at 21 d of age. The present animal experiments were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; process 1217-1) and the guidelines set forth by our institution.

Drug administration

Males and females *mdx* mice ($n = 15$, 14 d old) received by oral gavage AA 200 mg/kg daily (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.1 mL of saline [24] or saline for 14 d. Each animal was weighed daily so that the drug dose could be accurately adjusted.

Analysis of creatine kinase

For the biochemical evaluation of muscle fiber degeneration, control C57Bl/10 mice ($n = 10$) and AA-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, TX, USA) and xylazine hydrochloride (6.8 mg/kg; 2% Virbaxil, Virbac), and blood samples (0.8 mL) were collected by cardiac puncture. After incubation at room temperature for 1 to 2 h to allow for clotting, the samples were microcentrifuged at 3000 rpm for 10 min and the supernatant (serum) was removed and used for analysis. The CK assay was performed using a commercially available kit (CK Cinético Crystal, Bioclin, Ireland) and a Thermo Electron Genesys 20 spectrophotometer (Fisher Scientific, Pittsburgh, PA, USA). Values are reported as international units per liter.

Evans blue dye staining

For morphologic visualization and the quantification of muscle fiber damage, AA-treated ($n = 5$) and saline-treated ($n = 5$) *mdx* mice were injected with Evans blue dye (EBD; Sigma-Aldrich). EBD is a sensitive and early marker of myofiber damage and is widely used to study cellular membrane permeability [25–27]. The animals received an intraperitoneal injection of 1% EBD in phosphate buffered saline (0.15 M NaCl, 10 mM phosphate buffer, pH 7.4) at a dose of 100 μL/10 g of body weight. The mice were visually examined for dye uptake.

Discoloration of all animals was observed within 1 to 2 h after the intraperitoneal injection of EBD. The successful injection of the dye was indicated by a blue color of the ears and paws. Twelve hours later, the mice were anesthetized with a mixture of ketamine hydrochloride (130 mg/kg; Francotar, Virbac) and xylazine hydrochloride (6.8 mg/kg; 2% Virbaxil, Virbac). The diaphragm muscle was dissected out, snap frozen in isopentane, cooled in liquid nitrogen, and stored at −70°C. Cryostat cross sections (7 μm thick) were incubated in ice-cold acetone for 10 min at 20°C, washed three times for 10 min in phosphate buffered saline, and mounted in DABCO (fluorescence mounting medium; Sigma-Aldrich). EBD emits a bright red color when analyzed under a fluorescence microscope. The number of EBD-positive muscle fibers was determined in all sections with a hand counter, and the fibers were photographed with a Nikon fluorescence microscope connected to a Hamamatsu video camera (Chiyoda-ku, Tokyo, Japan). The number of EBD-positive muscle fibers is expressed as the percentage of the total number of muscle fibers counted in each section.

Histopathologic analysis

Cryostat cross sections of the diaphragm muscle were stained with hematoxylin and eosin. Slides were examined under a Nikon Eclipse E400 microscope connected to a personal computer and a video camera (Nikon Express Series). Non-overlapping images of the entire muscle cross section were taken and tiled together using ImagePro Express software (Media Cybernetics, Silver Springs, MD, USA). Areas containing an inflammatory cell infiltrate were identified and quantified as described previously [28]. Briefly, inflammatory cells were identified in sections stained with hematoxylin and eosin based on nuclear morphology and cell size, showing basophilic nuclear staining and little cytoplasm. Areas containing densely packed inflammatory cells were measured with ImagePro Express software and were calculated as the percentage of total muscle area in each section studied (four to five sections per muscle). The sections were examined under a Nikon Eclipse E400 microscope equipped with a micrometer eyepiece at 200× magnification. All counts and measurements were performed by a blinded observer.

Western blot analysis

Tumor necrosis factor-α and 4-hydroxynonenal (4-HNE) were quantified by western blotting in control C57Bl/10 mice ($n = 10$) and AA-treated ($n = 10$) and saline-treated ($n = 10$) *mdx* mice. Muscles were lysed in assay lysis buffer containing freshly added protease and phosphatase inhibitors (1% Triton, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μg/mL aprotinin, 1 mM phenyl-methyl-sulfonyl-fluoride, and 0.25 mM Na_3VO_4). The samples were centrifuged for 20 min at 11 000 rpm, and the soluble fraction was resuspended in 50 μL of Laemmli loading buffer (2% sodium dodecylsulfate, 20% glycerol, 0.04 mg/mL bromophenol blue, 0.12 M Tris-HCl, pH 6.8, and 0.28 M β-mercaptoethanol). Thirty micrograms of total protein homogenate from C57BL/10 and saline- and AA-treated *mdx* diaphragm was loaded onto 12% to 15% sodium dodecylsulfate–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 h at room temperature with a 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 h at room temperature, and developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA). To control for protein loading, western blot transfer, and non-specific changes in protein levels, the blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Band intensities were quantified using ImageJ 1.38X software (National Institutes of Health, Bethesda, MD, USA). The following primary antibodies were used for western blotting: 1) 4-HNE (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 2) TNF-α (rabbit anti-mouse polyclonal; Millipore, Billerica, MA, USA), and 3) glyceraldehyde-3-phosphate dehydrogenase (rabbit polyclonal; Santa Cruz Biotechnology). The secondary antibody used was a peroxidase-labeled affinity-purified mouse or rabbit immunoglobulin G antibody (KPL, Gaithersburg, MD, USA).

Statistical analysis

All data are expressed as mean ± standard deviation. The statistical analysis for a direct comparison between the means of two groups was performed by Student's *t* test, and analysis of variance was used for multiple statistical comparisons between groups. $P \leq 0.05$ was considered statistically significant.

Results

No significant differences in weight gain were observed between AA- and saline-treated *mdx* mice during the study period. The mean body weights were 14.0 ± 1.1 g for AA-treated *mdx* mice and 14.8 ± 1.8 g for saline-treated *mdx* mice ($P > 0.05$, Student's *t* test). These results showed that the antioxidant treatment did not interfere with the growth rate of young *mdx* mice.

The CK levels were significantly higher in saline-treated *mdx* mice than in control C57Bl/10 mice (Table 1). Although the AA treatment significantly decreased the CK levels in *mdx* mice compared with saline-treated *mdx* mice, there was still a difference when compared with control C57BL/10 mice (Table 1).

The EBD-positive fibers were present as clusters or as single isolated fibers (Fig. 1A). The AA treatment caused a significant decrease of EBD staining (84%) in the *mdx* diaphragm muscle

Table 1
Effect of ascorbic acid on blood creatine kinase levels in *mdx* mice

	Creatine kinase (U/L)
Ctrl	119.2 ± 57.9
*mdx*SAL	1596.1 ± 305.5*
*mdx*AA	1099.6 ± 278.1†

Ctrl, C57BL/10 mice, control for *mdx*; *mdx*AA, ascorbic acid–treated *mdx* mice; *mdx*SAL, saline-treated *mdx* mice

Values are expressed as mean ± SD ($n = 10$ mice/group)

* Significantly different from Ctrl ($P \leq 0.05$, Student's *t* test).

† Significantly different from *mdx*SAL and Ctrl ($P \leq 0.05$, analysis of variance).

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(Fig. 1B). In addition, a significant decrease of the inflammatory area was observed in the AA-treated diaphragm muscle (Fig. 1C,D). The inflammatory area was defined by regions containing conspicuous, densely packed inflammatory cells among regenerated and degenerating muscle fibers (Fig. 1C). AA decreased the inflammatory area in the diaphragm muscle by 28% (Fig. 1D).

The 4-HNE was used as a marker of lipid peroxidation to determine whether the AA treatment decreased the levels of ROS in *mdx* muscles [29]. Representative immunoblots and the quantification of 4-HNE are shown in Figure 2A. Several bands of 4-HNE, ranging from 26 to 170 kDa, were detected in the diaphragm muscle of control C57Bl/10 mice and of saline-treated and AA-treated *mdx* mice. Considering the sum of the all bands (six bands), the 4-HNE levels in the saline-treated *mdx* group were only slightly higher than in the controls (Fig. 2A). However, considering the levels of 4-HNE separately, in most bands, the levels were clearly increased, supporting the physiologic significance of higher levels of lipid peroxidation in the untreated *mdx*. AA resulted in a significant decrease of 4-HNE levels in the diaphragm muscle (Fig. 2A).

Immunoblotting revealed that TNF-α levels were significantly increased in saline-treated *mdx* muscle compared with control muscle (Fig. 2B), and that AA treatment decreased the TNF-α levels in diaphragm muscle (Fig. 2B).

Discussion

Ascorbic acid is one of the most widely used dietary supplements with antioxidant [30] and anti-inflammatory [31,32] properties. The present study demonstrated that exogenously administered AA, before the onset of the disease, resulted in a dramatic decrease of EBD uptake in the diaphragm muscle of *mdx* mice. In addition, the concomitant decrease of CK levels further supports the lack of muscle degeneration. Taken together, these results show that AA treatment protects against myofiber breakdown in the diaphragm muscle of young *mdx* mice, thus attenuating the dystrophic phenotype at earlier stages of the disease.

The mechanism underlying the protective effect of AA is probably related to its antioxidative activity [30,33]. In the muscles of patients with DMD or *mdx* mice, an increase in the biological byproducts of oxidative stress and lipid peroxidation decreased cellular antioxidants, and increased concentrations of the antioxidant enzyme have been observed during the prenecrotic phase of the disease [8]. Further evidence in support of oxidative stress as a cause of the dystrophic pathophysiology comes from in vivo studies, in which *mdx* mice receiving antioxidants derived from green tea [6,34,35] or a low-iron diet, which decreases hydroxyl radicals [36], showed weaker signs of muscle damage.

In the present study, 4-HNE, a marker of lipid peroxidation [29], was found increased in the diaphragm muscle of *mdx* mice. Studies have suggested that oxidative stress may contribute to necrosis in the *mdx* diaphragm muscle, with most necrotic myofibers, myosatellites, and interstitial inflammatory cells in the *mdx* diaphragm muscle containing accumulated lipofuscin [37,38]. In the present study, AA treatment resulted in a significant decrease of 4-HNE. These findings are in agreement with previous studies showing that AA can decrease lipid peroxidation by inhibiting the generation of ROS [39,40] and to increase superoxide dismutase activity [41]. In addition, evidence have

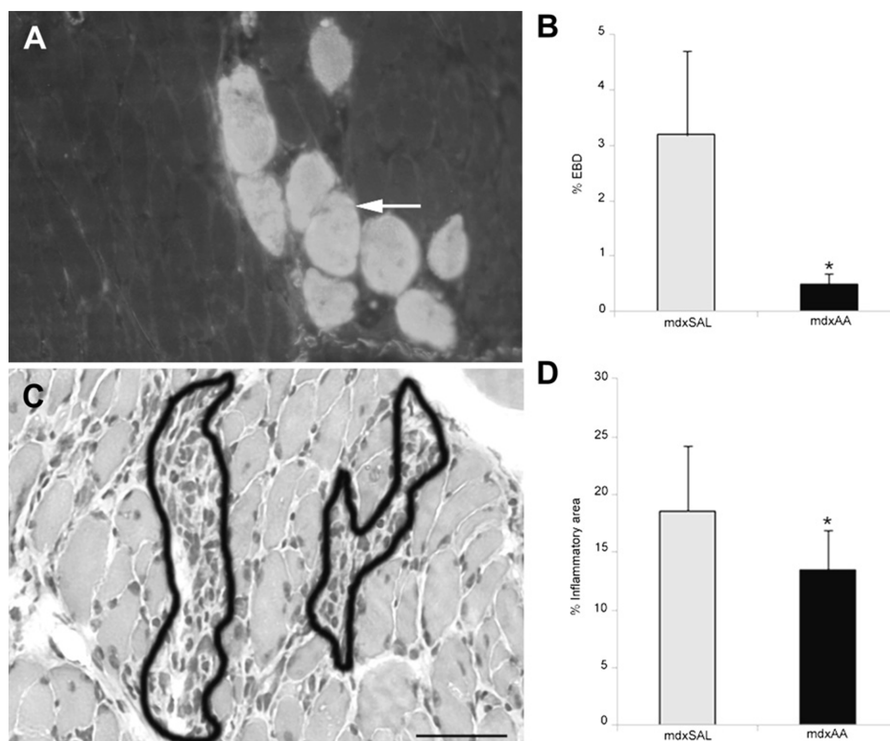

Fig. 1. (A) The EBD-positive myofibers indicate sarcolemmal leakage in saline-treated *mdx* diaphragm muscle fibers (arrow). (B) Quantitation of fibers stained with EBD in the diaphragm muscle from *mdx*SAL and *mdx*AA. Values are expressed as the percentage of the total number of fibers in the diaphragm muscle. (C) A representative inflammation/regeneration area is indicated by the outline. (D) Quantitation of the inflammatory area in the diaphragm muscle from *mdx*SAL and *mdx*AA. The inflammatory area is represented as a percentage of the total muscle area. * Significantly different ($P \leq 0.05$, Student's *t* test) from *mdx*SAL. Error bars indicate SD. Scale bars = 50 μm in A and C. EBD, Evans blue dye; *mdx*AA, ascorbic acid–treated *mdx* mice; *mdx*SAL, saline-treated *mdx* mice.

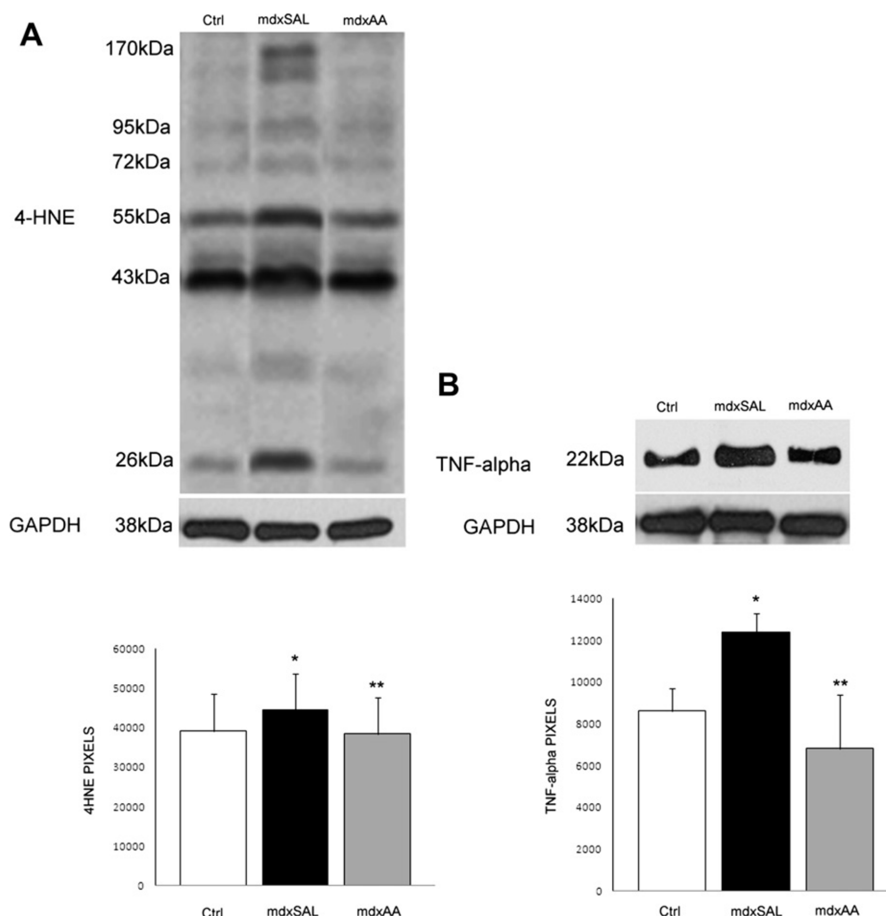

Fig. 2. Immunoblot analysis of 4-HNE (A) and TNF-α (B), with respective graphs showing protein expression in the crude extracts of diaphragm muscles from Ctrl, *mdx*SAL, and *mdx*AA. GAPDH protein was used as a loading control. * Significantly different ($P \leq 0.05$, Student's *t* test) from Ctrl. ** Significantly different ($P \leq 0.05$, analysis of variance) from *mdx*SAL. Error bars indicate SD. Ctrl, control C57Bl/10 mice; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 4-HNE, 4-hydroxynonenal; *mdx*AA, ascorbic acid–treated *mdx* mice; *mdx*SAL, saline-treated *mdx* mice; TNF, tumor necrosis factor.

suggested that oxidative stress is associated with an increased inflammatory cell infiltration in the dystrophic muscle, followed by an increase in the proinflammatory cytokine TNF-α [9]. TNF-α has been shown to be involved in mechanisms promoting muscle wasting in dystrophic muscle [16,42,43], and drugs that silence TNF-α have shown protective effects against inflammatory processes [13,16–18]. The present study confirmed the overexpression of TNF-α in the *mdx* muscle and demonstrated a strong decrease of this cytokine after AA treatment.

The molecular mechanisms whereby AA exerts its anti-TNF effects are still unclear. One possibility is that AA inhibits TNF-α activation by blocking the phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), a key step that permits the translocation of NF-κB, a proinflammatory transcription factor, to the nucleus for the activation of gene expression. The inhibitory effect of AA on TNF-α through the blockade of IκBα phosphorylation has been observed in several cell systems, including HeLa (human cervical adenocarcinoma), MCF7 (human breast adenocarcinoma), U937 (monocytic), and HL60 (myeloid leukemia) cell lines, and in primary cultures of human umbilical vein endothelial cells, demonstrating the generality of this effect [21].

In conclusion, the present study demonstrated that the administration of AA to dystrophic mice decreases myonecrosis, inflammation, lipid peroxidation, and TNF-α levels in the diaphragm muscle. AA is one of the most common vitamin supplements consumed daily [44]. The current recommended dietary allowance of AA for human adults is 60 mg/d [45]. In the present study, we used 200 mg/d because a high dose of AA has been shown to be necessary to decrease muscle soreness, to delay CK increases, and to prevent oxidative stress in human adults undergoing eccentric exercise [46]. High intakes of AA (2–4 g/d) are well tolerated biologically in healthy mammalian systems [47] and have been shown to exert preventive and therapeutic effects in several diseases [48]. The present results suggest the use of AA as a complementary treatment for dystrophinopathies. However, further studies are needed to understand better the mechanisms of action of AA in dystrophic muscles.

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