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ARTIGO ORIGINAL

Efeitos de terapia combinada de antioxidante e quelante de ferro no músculo esquelético de camundongos mdx

Effects of antioxidant and iron chelator combined therapy in the skeletal muscle of mdx mice

Palavras-chave: antioxidantes, quelante de ferro, músculo esquelético, camundongos endogâmicos mdx
Key words: antioxidants; iron chelator; muscle,s; mice, inbred mdx

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ABSTRACT

Antioxidant treatments showed beneficial effects in dystrophic skeletal muscles in Duchenne muscular dystrophy patients and in the experimental model, the mdx mice. Due to the fact that studies suggest that a combination of N-Acetylcysteine (NAC) antioxidant and Deferoxamine (DFX) iron chelator is an effective treatment for several oxidative stress diseases, in this study our aim was to investigate in vivo the potential effect of NAC and DFX combined therapy in the quadriceps muscle of mdx mice. Mdx mice received intraperitoneal injections of NAC combined with DFX daily for 14 days, followed by the removal of the quadriceps muscle. C57BL/10 mice were used as a control group. The results showed that NAC and DFX combined therapy protected against the loss of muscle strength and muscle damage, indicated by the reduction in the creatine kinase levels, Evans blue dye positive fibers and fibers with central nuclei. The treatment also significantly reduced the dystrophic inflammatory process, reducing the inflammatory area. Considering the results obtained, we suggest that the beneficial effects of NAC and DFX combined therapy are probably due to the antioxidant properties of these drugs. However, further studies with other parameters are needed to confirm this hypothesis.

RESUMO

Tratamentos com antioxidantes mostraram efeitos benéficos nos músculos esqueléticos de pacientes com distrofia muscular de Duchenne e de camundongos mdx, modelo experimental da DMD. Devido ao fato de estudos sugerirem que

a combinação do antioxidante N-acetilcisteína (NAC) e do quelante de ferro deferoxamina (DFX) constitui um tratamento eficaz para várias doenças de estresse oxidativo, neste estudo, o nosso objetivo foi investigar in vivo, o potencial efeito da terapia combinada de NAC e DFX no músculo quadríceps de camundongos mdx. Camundongos mdx receberam injeções intraperitoneais de NAC e DFX diariamente, durante 14 dias, seguido pela remoção do músculo quadríceps. Camundongos C57BL/10 foram utilizados como grupo controle. Os resultados mostraram que a terapia combinada de NAC e DFX protege contra a perda da força muscular e de danos musculares, indicados pela redução dos níveis de creatina quinase, de fibras positivas ao azul de Evans e de fibras com núcleo central. O tratamento também reduziu significativamente o processo inflamatório distrófico, reduzindo a área de inflamação. Considerando os resultados obtidos, sugerimos que os efeitos benéficos da terapia combinada de NAC e DFX são provavelmente devido às propriedades antioxidantes destes medicamentos. No entanto, são necessários mais estudos com outros parâmetros para confirmar esta hipótese.

INTRODUCTION

In the group of muscular dystrophies, Duchenne muscular dystrophy (DMD) is classified as the most common and devastating, with a 1 per 3500 live male birth prevalence. DMD is a recessive genetic disease triggered by a mutation on the X chromosome, resulting in the absence of dystrophin protein expression. During disease development, the dystrophic patient has progressive muscle weakness, manifesting clinical signs such as: postural changes, difficulty to walk, falls and difficulty to get up. DMD progression leads to a loss of independent ambulation and, later, to cardiorespiratory failure, which leads to death[1].

The absence of dystrophin in the muscle fiber disrupts the communication between the cytoskeleton cell and extracellular matrix, making the sarcolemma unstable which allows the increase of intracellular calcium ions and triggers a series of mechanisms that contribute towards muscular necrosis[2]. One of these mechanisms is oxidative stress, since the excess calcium ions are captured by the mitochondria and converted into reactive oxygen species (ROS). ROS contribute to the formation of a pro-oxidizing environment, contributing to the degeneration of muscle fibers and myonecrosis in DMD[3-5].

Several studies showed the beneficial effects of antioxidants in the skeletal muscles of dystrophic patients and mdx mice, the most common experimental model of DMD6-9. Among these studies, N-Acetylcysteine (NAC) treatment is considered to be the most promising. NAC is a potent antioxidant, acting as a precursor of glutathione, one of the main components of the antioxidant system. Experiments with mdx mice have shown that treatment with NAC prevents the increase in membrane permeability, reduces power deficit associated with muscular damage induced by stretching, and reduces strength deficit and the ROS levels[6,9,10].

Despite the beneficial effects of NAC in the pharmacological DMD treatment, studies have shown limitations in their responses, since NAC easily interact with the iron and may have pro-oxidant effects[11]. An alternative to this preventing NAC effect is associated to its use as an iron chelator, such as Deferoxamine (DFX). DFX has the ability to inhibit generating free radical reactions catalyzed by iron, which makes it a powerful iron chelator[12, 13]. Previous studies have shown that a combination of NAC

and DFX is an effective treatment for several oxidative stress diseases[14, 15]. An in vitro study of our research group revealed that the combination of NAC and DFX effectively reduced oxidative stress markers and inflammatory process in mdx muscle cells[16]. So in this study, our aim was to investigate in vivo the potential effect of NAC and DFX combined therapy in the quadriceps muscle of mdx mice.

MATERIAL AND METHODS

Animals

C57BL/10 mice (C57BL/10ScCr/PasUnib) and mdx mice (C57BL/10-Dmdmdx/PasUnib) were housed in accordance with institutional guidelines, with food and water being available ad libitum and the experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; process #2128-1).

Combined therapy of NAC and DFX

Mdx mice (14 days old) received daily intraperitoneal injections of NAC (Sigma-Aldrich, Inc., St. Louis, MO) combined with DFX (Sigma-Aldrich, Inc., St. Louis, MO) at a dose of 150 mg/kg body weight either diluted in 0.1 ml saline. Each animal was weighed daily so that the oil dose could be adjusted accurately. C57BL/10 mice untreated and mdx mice treated with saline for 14 days were used to control.

Grip strength determination

The forelimb muscle strength was evaluated by grip strength meter (New Primer, Sao Paulo, Brazil), as previously reported (Mauricio et al., 2013). The measurements were obtained from all experimental groups (n= 05 animals per group) at the beginning (14 days of age) and at the end (28 days of age).

Measurement of creatine kinase

For biochemical evaluation of muscle fiber degeneration, control mice (n=05) and NAC+DFX-treated (n=05) and saline-treated (n=05) 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 samples were collected by cardiac puncture. The samples were microcentrifuged at 936g 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).

Morphological analysis

For morphological analysis (fibers in degeneration; fibers regenerated and inflammatory areas) were used 05 animals per group.

For quantification of muscle fiber damage, the animals were injected intraperitoneally with Evans blue dye (EBD). Twelve hours later, the mice were anesthetized as described above and the quadriceps 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 regenerated and normal fibers and areas with 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). The regenerated fibers were identified by the presence of central nuclei and the normal fibers for presenting peripheral nuclei. The number of central nucleated fibers and fibers with peripheral nuclei, expressed as a percentage of the total number of fibers, was determined in each cross-section (4-5 sections per muscle). 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.

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 t-test and ANOVA was used for multiple statistical comparisons between groups. P≤0.05 was considered statistically significant.

RESULTS

Longitudinal comparison showed an increase in body weight in all animals at the end of the experiment, showing that NAC and DFX combined therapy did not interfere with the growth rate of young mdx mice (Table 1).

Table 1:

	Body weight (g)		Force/Body weight (g/g)		Gain Force / period (%)
	Time 1	Time 2	Time 1	Time 2	
Ctrl	8.3±0.6	12.7±1.4	3.0±0.3	2.5±0.3	-16%
***mdx*S**	9.6±1.1	12.0±1.9	2.4±0.4[a]	1.8±0.4[a]	-25%
***mdx*N+D**	7.8±0.4	11.6±0.3	2.7±0.1	2.8±0.3[b]	+3.7%

Body weight (g) was measured at the beginning (time point 1) and after 2 weeks (time point 2) of Cilostazol treatment. Forelimb muscle strength was assessed by taking measurements of force at time points 1 and 2, normalized by body weight (g/g). Gain Force/period: Percentage the muscular force gain on the treatment period (%). Experimental groups: C57BL/10 mice (Ctrl), saline-treated *mdx* mice (*mdx*S), and N-Acetylscysteine and Deferoxamine-treated *mdx* mice (*mdx*N+D). All values are shown as mean±standard deviation (SD). [a]$P \leq 0.05$ compared with Ctrl group, [b]$P \leq 0.05$ compared with *mdx*S group (one-way ANOVA with Tukey's post-hoc test).

There were a 16% and 25% decrease in muscle strength in control (C57BL/10) mice and saline-treated mdx mice, respectively, over the study period (Table 1). On the other hand, there was a 3.7% increase in muscle strength in mdx mice treated with NAC and DFX (Table 1).

To analyze the fibers in degeneration of the dystrophic quadriceps muscle, we determined the CK levels and EBD-positive fibers. The CK levels significantly increased in saline-treated mdx mice compared to control mice. NAC and DFX combined therapy significantly reduced this enzyme levels in mdx mice (by 51%) compared to the saline-treated mdx mice (Table 2).

Table 2:

	CK (U/L)
Ctrl	51.3±2.98
***mdx*S**	1381.9±260.9[a]
***mdx*N+D**	669.7±131.7[ab]

Experimental groups: C57BL/10 mice (Ctrl), saline-treated *mdx* mice (*mdx*S), and N-Acetylscysteine and Deferoxamine-treated *mdx* mice (*mdx*N+D). All values are shown as mean±standard deviation (SD). [a]$P \leq 0.05$ compared with Ctrl group, [b]$P \leq 0.05$ compared with *mdx*S group (one-way ANOVA with Tukey's post-hoc test).

In the saline-treated mdx mice, a larger number of EBD-positive fibers was observed (Figure 1). NAC and DFX combined therapy treatment caused a decrease in EBD staining (by 83%) in the quadriceps muscle of mdx mice (Figure 1).

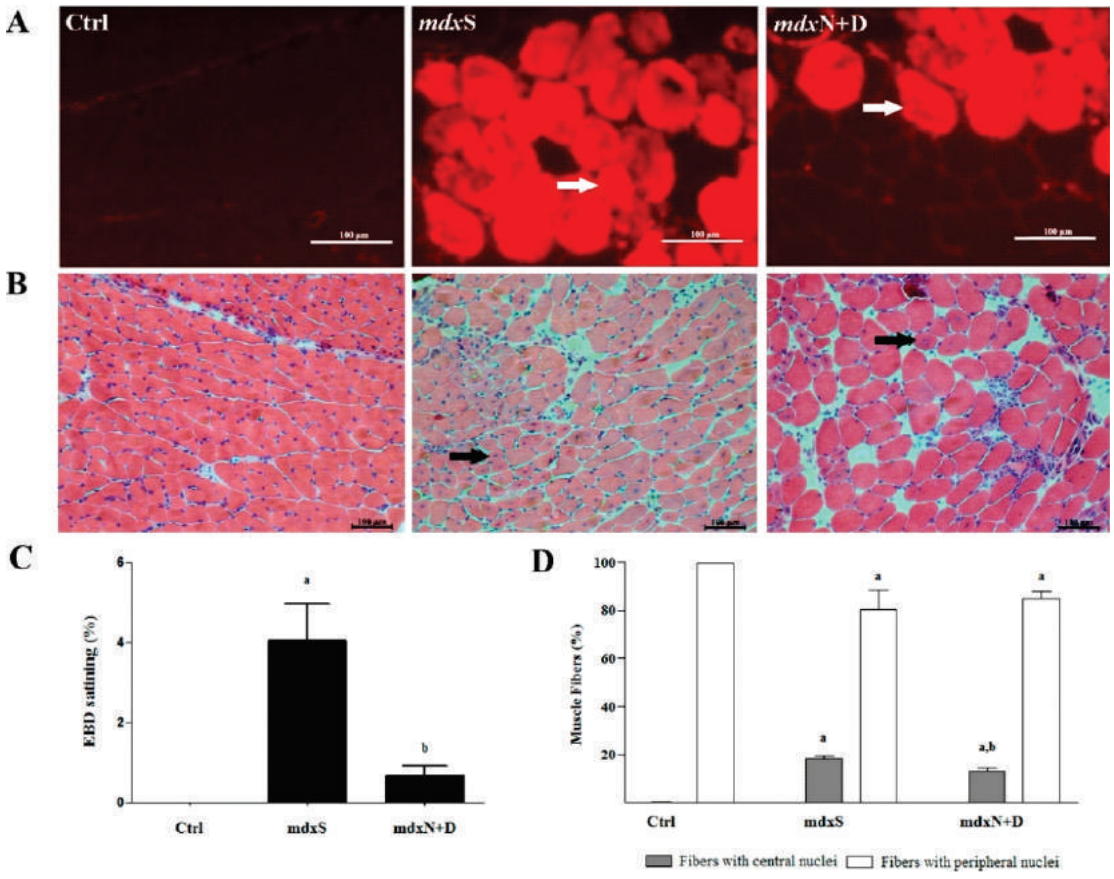

Figure 1: In (A) EBD-positive myofibers (white arrow) indicate sarcolemmal leakage and in (B) central nucleated fibers (black arrow) indicate regenerated muscle fibers in quadriceps muscle fibers of C57BL/10 mice (Ctrl), saline-treated mdx mice (mdxS), and N-Acetylscysteine and Deferoxamine-treated mdx mice (mdxN+D). In (C) graphs showing quantification of EBD-positive myofibers in the quadriceps muscle fibers of Ctrl, mdxS and mdxN+D groups. In (D) graphs showing quantification of fibers with centrally located nuclei and peripheral nuclei in the quadriceps muscle fibers of Ctrl, mdxS and mdxN+D groups. Values are expressed as the percentage of the total number of fibers in quadricesp muscle. aP 0.05 compared with Ctrl group, bP 0.05 compared with mdxS group (one-way ANOVA with Tukey's post-hoc test).

In the cross sections, the muscle fibers from control mice were round or roughly polygonal with rounded angles and their nuclei were in a peripheral location directly under the sarcolemma (Figure 2).

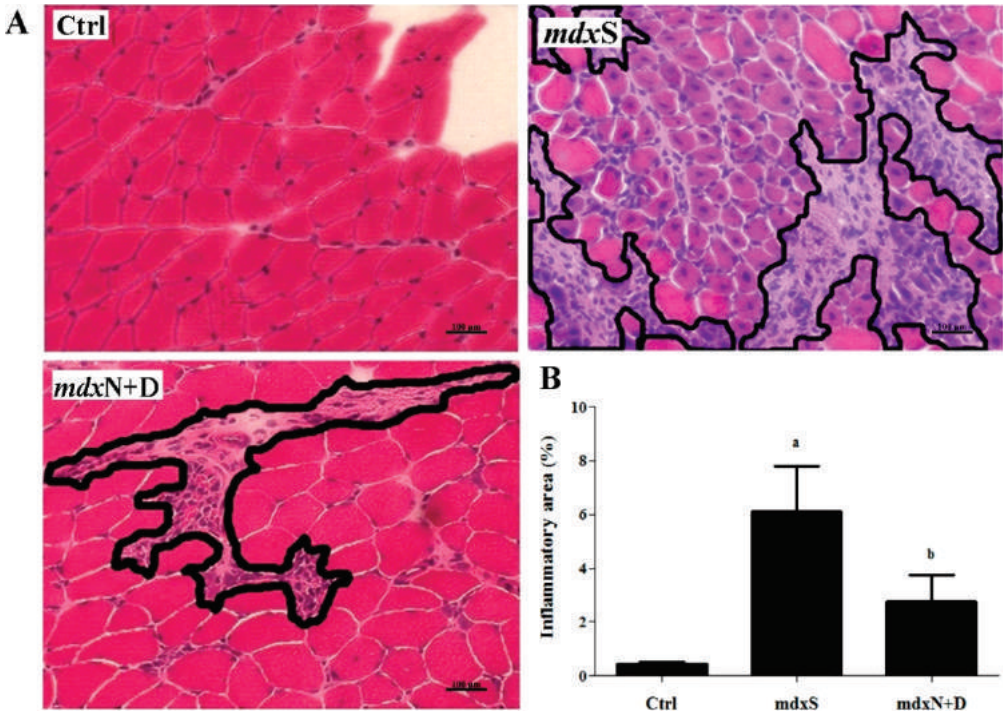

Figure 2: (A) The outline indicates the representative area of inflammation in mdx mice. (B) The graph show the inflammatory area (%) in the quadriceps muscle fibers of C57BL/10 mice (Ctrl), saline-treated mdx mice (mdxS), and N-Acetylscysteine and Deferoxamine-treated mdx mice (mdxN+D). Values are expressed as the percentage of the total number of fibers in quadricesp muscle. aP 0.05 compared with Ctrl group, bP 0.05 compared with mdxS group (one-way ANOVA with Tukey's post-hoc test).

DISCUSSION AND CONCLUSION

NAC and DFX combined therapy showed beneficial effects in the dystrophic skeletal muscle of mdx mice, with regard to the parameters analyzed in this study.

Grip strength analysis is a simple non-invasive test used to evaluate mice muscle force in vivo and it is widely used in mdx experiments to functionally evaluate the drug therapy effects[17]. The forelimb muscle strength was the first parameter analyzed in our conditions and showed that the combined drugs promote gain in muscle strength in dystrophic mice. This finding probably results from reduction of muscle degeneration and inflammation also observed in the dystrophic muscle after NAC and DFX treatment.

High CK levels in DMD patients, attributed to muscle damage, provide an index which is widely used as a diagnostic marker for muscular dystrophy1, [18]. In addition, several experimental studies used the EBD infiltration to reveal an increase in sarcolemma permeability in dystrophic muscle fibers[19, 20]. These two parameters were used to determine the degeneration muscle process in mdx mice in the present study and found the protective effect of NAC and DFX combined therapy against myonecrosis. Contributing to this result, we also verified a reduction of regenerated muscle fibers indicated by the decrease of fibers with central nuclei.

Inflammatory processes are highly associated with DMD pathogenesis[21]. After NAC and DFX combined therapy, an expressive reduction in inflammatory processes in the dystrophic muscle was morphologically observed. NAC and DFX administered alone or in combination showed a reduction of the inflammation in other diseases[6, 16, 22, 23]. In the in vitro study, we also observed the anti-inflammatory effect of NAC and DFX combined therapy in dystrophic muscle cells[16].

Considering that the dystrophic inflammatory process and the other parameters analyzed here are likely to be associated with elevated oxidative stress in mdx mice[24], we suggest that the beneficial effects of NAC and DFX combined therapy are probably due to the antioxidant properties of NAC and DFX. However, further studies with other parameters are needed to confirm this hypothesis.

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