

Ursolic Acid Increases Strength in mdx Mice Model and may Decrease Fibrosis Deposition by TGF- β Downregulation

El Ácido Ursólico Aumenta la Resistencia en el Modelo de Ratones mdx y Podría Disminuir la Deposición de Fibrosis Mediante la Regulación Decreciente de TGF- β

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SUMMARY: Dystrophin disfunction results in sarcolemma destabilization, leading muscle cell damage by continuous degeneration cycles and limited regeneration. In muscle dystrophy, caused by dystrophin dysfunction, inflammation, necrosis and fibrosis are pathophysiological muscle function loss characteristics. As a genetic disease, this muscle dystrophy has no cure, however, advances in drug therapy using glucocorticoids can decrease the disease progression. Subsequently, alternative therapies were studied, such as ursolic acid (UA), that inhibits muscle atrophy and increases muscle mass and strength. Herein, we used 10 mg/kg daily supplementation in mdx mice for 4 weeks to evaluate serum creatine phosphokinase (CPK), muscle strength (Kondziela test), muscular organization (histology) and expression of fibrosis related genes (TGF- β , TNF- α , mstn and osten). UA supplementation increased muscle morphological organization, motor strength and decreased muscular TGF- β expression. Altogether, the gene expression profile, histological organization and strength could suggest that UA treatment did not stop the fibrogenesis but decreased its progress.

KEY WORDS: Apple wax; Fibrogenesis; Muscular dystrophy; Natural supplementation.

INTRODUCTION

Some dystrophic disorders are related to mutation in a single muscular-related gene, that leads to low or no protein-specific expression, i.e. Duchenne Muscular Dystrophy (DMD), that is linked to mutations in dystrophin gene (Kunkel, 2005). Low dystrophin levels firstly (and induced by contraction) damages the sarcolemma, leading to necrosis and inflammatory cell invasion (Kramerova *et al.*, 2019). Even muscle stem cells compensating this cell loss, the muscle degeneration accumulates TGF- β , that increases collagen deposition and muscle replacement by adipose and connective tissues (Kramerova *et al.*). Consequently, there is progressive muscle degeneration, muscular function loss and premature death due to heart and/or respiratory failure (Nakamura & Takeda, 2011; Lessa *et al.*, 2012; McGreevy *et al.*, 2015; Aartsma-Rus *et al.*, 2016; Mah, 2016).

Among experimental models used for DMD the mdx mouse (X chromosome-linked muscular dystrophy mouse) which presents a dystrophin spontaneous mutation at the exon 23, forming a prematurely stopping codon (Sicinski *et al.*, 1989; Duncley *et al.*, 1998) altering protein function. In the first two weeks of mdx life, skeletal muscles show no differences when compared to normal mice. Necrosis is observed between 3 - 6 weeks. High levels of weakness and necrosis in limb muscles are observed around 8-16 weeks. After this period, these changes recede spontaneously, by increasing the regenerative capacity, except for the diaphragm muscle where the degeneration process remains progressive (Zhou *et al.*, 2008; Guerron *et al.*, 2010; McGreevy *et al.*). Overall its phenotype is less severe than in human (Sicinski *et al.*; Deconinck *et al.*, 1997; van Putten *et al.*, 2012).

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Recently, studies suggest that ursolic acid (UA) can inhibit skeletal muscle atrophy and increase muscle mass and strength in animal models, with anti-inflammatory effects (Bakhtiari *et al.*, 2016; Lee *et al.*, 2017). Aiming the potential of UA in the progressive muscle damage observed in dystrophin-deficient dystrophy, this study evaluated daily supplementation effects of UA in mdx mice by means of morphological, histopathological, strength and fibrogenic-related genes analysis.

MATERIAL AND METHOD

These experiments were conducted under rules of Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of University of São Paulo, Brazil (protocol number 6862041016) and ABC Medicine School (protocol number 07/2016).

Animals: Twenty male mdx mice (C57BL/10-mdx), between 6-8 weeks of age and 20-30 grams were randomly divided in three experimental groups: control group (received physiological solution, n=4), vehicle group (received corn oil, n=6) and ursolic acid (UA) group (received 10 mg/kg of ursolic acid, n = 10). All three groups were supplemented daily by gavage for four weeks (Sundaresan *et al.*, 2016). The group size was designed accordingly to TREAT-NMD Neuromuscular Network (www.treat-nmd.eu/research/preclinical/dmd-sops/) (Collins & Morgan, 2003; Denayer *et al.*, 2014; Perrin, 2014). Mice (one per cage) were maintained at ABC Medicine School animal facility under controlled temperature, ad libitum feeding and 12h day/night cycle.

The Inverted Screen (Kondziela) test: All mice from three groups were tested weekly using methodology adapted from (Deacon, 2013; Carlson, 2019). Twenty minutes before the test, mice were transferred to another room and untrained mice were placed individually on a wire square screen (7.5 x 7.5 cm), that was rotated 180°, and maintained for 5 minutes. When a mouse fell off before the limit time, two new attempts were made with 2 minutes in-between. The motor strength was measured by the maximum time to climb up normalized by mice weight, and results expressed by Newton-second (N.s).

Evaluation of serum creatine phosphokinase (CPK): At the end of the fourth week all animals were euthanized by intraperitoneal anesthetic overdoses of sodic thiopental (100 mg/kg). After, abdominal cavity was exposed and caudal cava vein were punctuated for serum CPK quantification to check muscular damage (Morgan *et al.*, 1981).

Muscle morphology analysis: After euthanasia, diaphragm (DIA), long finger extensor (LFE) and cranial tibial (CT) muscles were isolated, covered with talcum powder and frozen in liquid nitrogen. Slices of 5 μ m were produced in cryostat microtome, and transferred to silanized glass slides (StarFrost, Knitel Glasser, German). Slices were stained with hematoxylin and eosin (for histological analysis and central nuclei count). The central nuclei (regenerated muscle fibers) were measured using cell counter plugin from ImageJ software (version 1.39u).

Fibrogenic cytokines quantification: By qPCR four fibrosis-related genes (TNF- α , TGF- β , mstn and ostn) were analyzed using gapdh as endogenous gene. The total mRNA was extracted from muscle sample (same muscles used for histology) using Trizol reagent (#15596-026, Ambion, EUA) and converted to cDNA using Quanti-Nova Reverse Transcription Kit (#205410, Qiagen, EUA), both under manufacturer's instructions. Each real time qPCR reaction was composed by 7.5 μ L of SyBr Green PCR master mix (#4344463, Invitrogen, EUA), 1.0 mM of each primer; 2.0 μ L of cDNA and ultrapure water up to 15 μ L, under standard cycling program of 7500 Fast Real Time PCR System (Life Technologies, EUA).

Statistical analysis: all data were analyzed by least square means analysis of variance with GLM procedure of SAS (version 9.4). Comparison between groups was by ANOVA ($p \leq 0.05$), followed by Tukey test.

RESULTS

In general, UA group has more regular shape muscle fibers and decreased inflammatory infiltrate when comparing to vehicle and control groups. Those two groups have no significant morphological differences between them (Fig. 1). All three analyzed muscles in the UA group still have fibrosis sites (as shown in Figs. 1C, F and I), however thickness decreased when compared with the other two groups. In addition, the regenerating muscle fibers (that are characterized by central nucleus) increased in UA group, for all three muscles analyzed, when compared with control and vehicle groups (Fig. 2).

The Kondziela test, used to measure the motor strength after ursolic acid administration, showed increased strength during each week of treatment, with an almost 3-fold increase in motor strength at fourth week when compared to first week, and around 1.5-fold increase in average when compared with other two groups at the fourth week (Fig. 3).

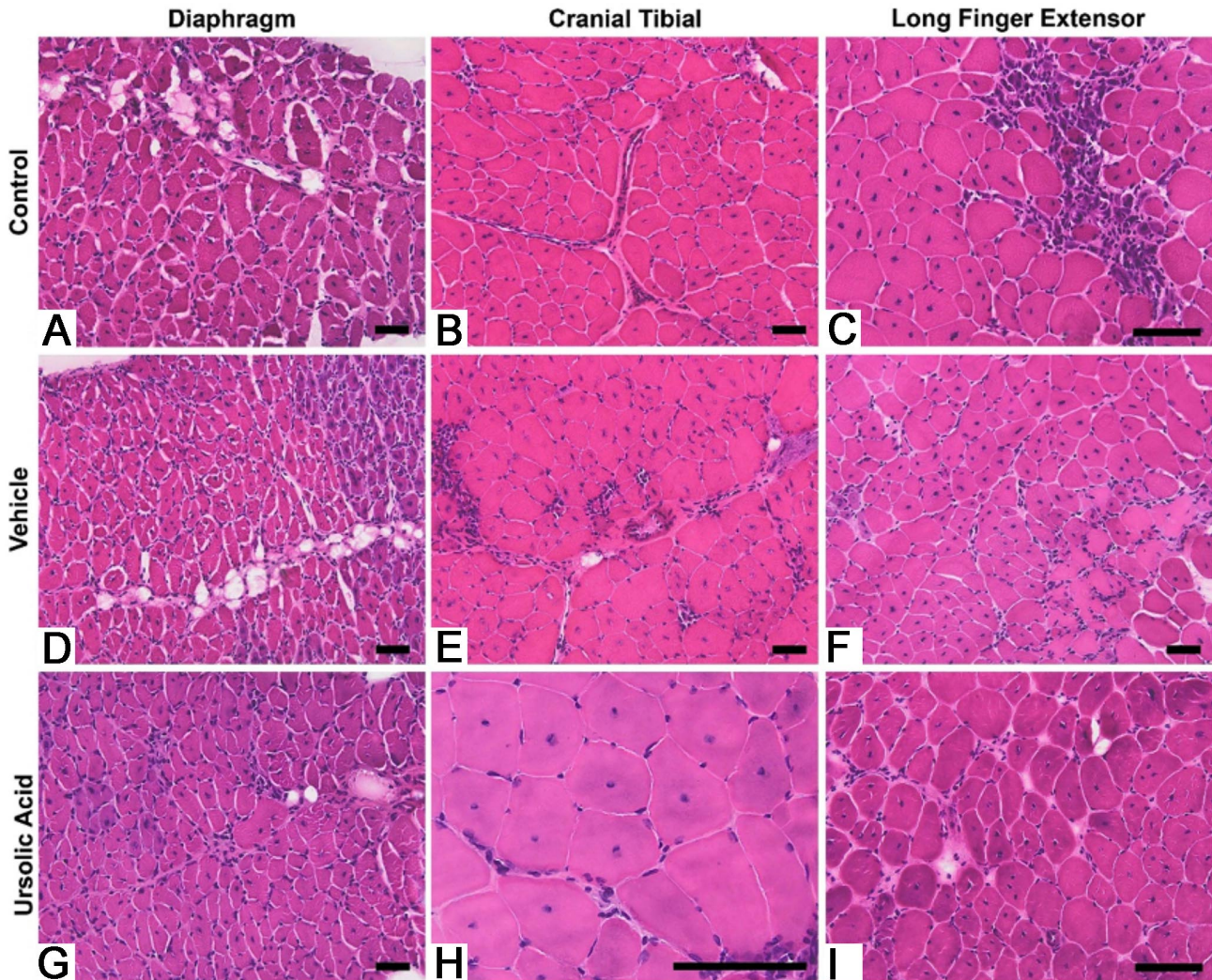


Fig. 1. Histology of mdx mice diaphragm (A, D and G), long finger extensor (B, E and H) and cranial tibialis (C, F and I) muscles. Observe that control (A-C) and vehicle (D-F) treatment are similar in-between. However, in UA treatment (G-I) muscles decreased adipose and connective tissue, such as inflammatory infiltrates. Hematoxylin-eosin stain. Bars = 50 μ m.

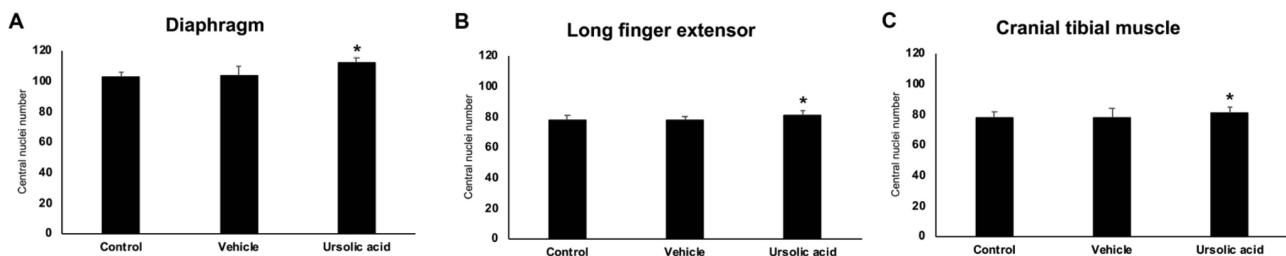


Fig. 2. Central nuclei count in mdx mice diaphragm (A), long finger extensor (B) and cranial tibialis (C) muscles, in control, vehicle and ursolic acid treated groups. Asterisk represents statistical difference ($p \leq 0.05$).

Even with higher muscle fiber organization and increased motor strength, no statistical differences in serum CPK was observed between groups (Fig. 4).

Inflammatory cytokine expression was analyzed and TGF- β was decreased in the UA group when compared to control and vehicle groups (Fig. 5), otherwise TNF- α , mstn

and osth showed no statistical differences between groups (data not shown).

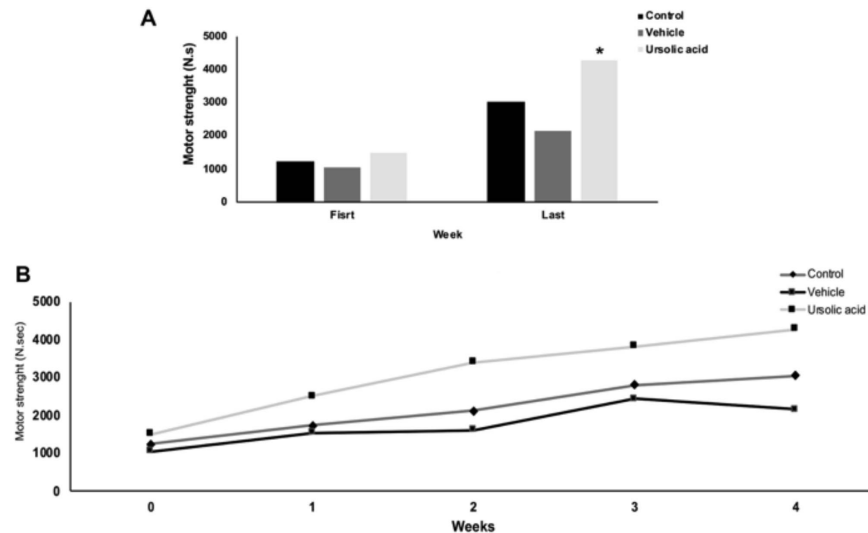


Fig. 3. Muscular strength of mdx mice measured by Kondziela test at first and fourth weeks (A) and during the four weeks (B) of treatment. Asterisk represents statistical difference ($p \leq 0.05$).

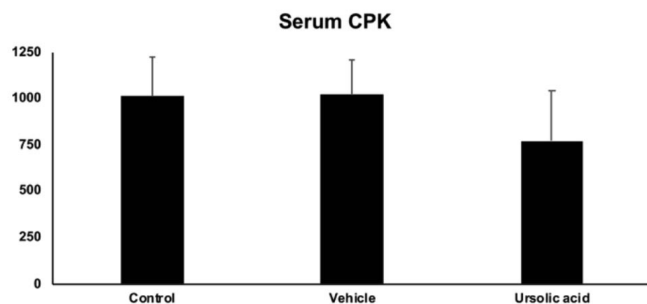


Fig. 4. Serum creatine phosphokinase (CPK) levels in control, vehicle and ursolic acid treated mdx mice groups.

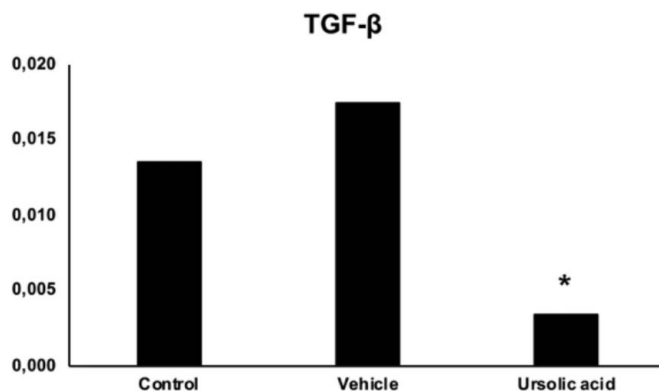


Fig. 5. mdx mice muscular TGF- β expression after ursolic acid treatment. Asterisk represents statistical difference ($p \leq 0.05$).

DISCUSSION

In our results, mdx mice after four weeks supplementation of daily 10mg/kg of ursolic acid increased muscle morphological organization, motor strength and decreased muscular expression of TGF- β with maintenance of TNF- α , msnt and osth expression. Those findings were seen even when the treatment started in the period that corresponds to peaks of necrosis, inflammation and muscle weakness (McGreevy *et al.*). Otherwise, no effects of UA treatment in mdx mice were observed, even with higher administration doses; i.e. 0.02 % or around 25mg/kg of UA (Verhaart *et al.*, 2019). However, using DBA/2J-mdx lineage, while we used a lineage with less intense dystrophic phenotype (C57BL/10-mdx).

The dystrophin deficiency in mdx, as other muscular dystrophies and chronic myopathies, is marked by a decrease of muscle strength and elasticity, namely as a result of increased fibrosis deposition (Zhao *et al.*, 2008) and severe muscle degeneration (Murphy & Ohlendieck, 2016; Piñol-Jurado *et al.*, 2017). However, we observed a reduction of fibrosis and better muscle fiber organization after 4 weeks of UA treatment through histological analysis.

The mdx strain is a dystrophin deficient mouse, and UA treatment is not able to reestablish dystrophin in the muscle but is may be used to decrease or recover the damage caused by dystrophin absence (Ogasawara *et al.*, 2013; Seo *et al.*, 2018). Serum CPK is used as marker of muscle damage (Morgan *et al.*), and in spite of muscle strength and organization increases, no differences in serum CPK levels were observed. Thus, may suggest that muscle strength and histological improvement were not a result of muscle damage decrease by dystrophin deficiency.

Otherwise, TGF- β is known as a pro-fibrotic cytokine, which acts inducing collagen (majorly type I) deposition, and as result increase fibrosis formation (Verrecchia & Mauviel, 2004). Also, this negatively affects muscle regeneration by inhibiting satellite cell proliferation, preventing myotubule fusion and myocyte differentiation (Burks & Cohn, 2011). Compensatory

replacement of muscle by fibrotic and adipose tissue occurs secondary to a lack of dystrophin and is followed by myonecrosis (Bernasconi *et al.*, 1995). In our results, after UA treatment, TGF- β expression decreased, suggesting a down-regulation of fibrosis signal, and a decrease in fibrosis deposition was confirmed by histology. In parallel other fibrosis-related genes studied (mstn, ostn and TNF- α) showed no alterations in their expression.

Miostatin (mstn) is a negative regulator of skeletal muscle development. As such mstn deficient animals show expressive muscle hypertrophy, known as double musculature (Zhu *et al.*, 2007). Also, mstn regulates myocyte development, such as muscle fibroblasts (Zhao *et al.*). In the early stages of injury, mstn and TGF- β show colocalization acting synergically as amplified signal to active fibrosis (Zhu *et al.*). The mstn deficient animal has augmented muscle regeneration and reduced fibrosis (Kornegay *et al.*, 2016). Analogously, osteopontin (ostn), also a negative myogenesis regulator, is activated after muscle damage and increases TGF- β and fibrosis, decreasing muscle regeneration and strength (Bello & Pegoraro, 2019; Kramerova *et al.*), being highly expressed in muscular dystrophy models (Uaesoontrachoon *et al.*, 2013). As both mstn and ostn maintained their expression level after UA treatment, another mechanism could regulate TGF- β and have led to fibrosis decrease in our model. Differently from TGF- β , the TNF- α is anti-fibrotic cytokine, that induces collagenase and inhibit collagen expression, resulting in diminishment of fibrosis deposition (Verrecchia & Mauviel). Also, TNF- α did not change their levels after UA treatment.

Altogether, these gene expression profiles, histological organization and strength could suggest that UA treatment did not stop the fibrosis deposition but decreased its progress.

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ETHICS APPROVAL

These experiments were conducted under rules of Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of University of São Paulo (protocol number 6862041016) and ABC Medicine School (protocol number 07/2016).

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RESUMEN: La disfunción de la distrofina resulta en la desestabilización del sarcolema, llevando al daño de las células musculares por ciclos continuos de degeneración y regeneración limitada. En la distrofia muscular, debido a la disfunción de la distrofina, la inflamación, la necrosis y la fibrosis, son características fisiopatológicas de la pérdida de la función muscular. Como enfermedad genética no es posible remediar esta distrofia muscular, sin embargo, los avances en la terapia de medicamentos con glucocorticoides pueden disminuir la progresión de la enfermedad. Se estudiaron terapias alternativas, como el ácido ursólico (UA), que inhibe la atrofia muscular y aumenta la masa y la fuerza muscular. En este estudio, utilizamos una suplementación diaria de 10 mg / kg en ratones mdx durante 4 semanas para evaluar la creatina fosfoquinasa (CPK) sérica, la fuerza muscular (prueba de Kondziela), la organización muscular (histología) y la expresión de genes relacionados con la fibrosis (TGF- β , TNF- α , mstn y ostn). La suplementación con AU aumentó la organización morfológica muscular, la fuerza motora y la disminución de la expresión muscular de TGF- β . El perfil de expresión génica, la organización histológica y la fuerza simultáneamente podrían sugerir que el tratamiento con AU no detuvo la fibrogénesis sino que disminuyó su progreso.

PALABRAS CLAVE: Cera de manzana; Fibrogénesis; Distrofia muscular; Suplementación natural.

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