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Short-term treatment with *Uncaria tomentosa* aggravates the injury phenotype in *mdx* mice

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ABSTRACT

Introduction: *Uncaria tomentosa* (Willd. ex Roem. & Schult.) DC. (Rubiaceae) or UT is a medicinal plant with antiviral, antimutagenic, anti-inflammatory and antioxidant properties. Duchenne muscular dystrophy (DMD) is a severe muscle wasting disease caused by mutations in the dystrophin gene; this deficiency leads to sarcolemma instability, inflammation, muscle degeneration and fibrosis. **Objective:** Considering the importance of inflammation to dystrophy progression and the anti-inflammatory activity of UT, in the present study we evaluated whether oral administration of UT extract would ameliorate dystrophy in the mdx mice, a DMD model. **Methods:** Eight-week-old male *mdx* mice were submitted to 200 mg/kg body weight daily UT oral administration for 6 weeks. General histopathology was analysed, and muscle tumor necrosis factor α, transforming growth factor-β, myostatin and osteopontin transcript levels were assessed. The ability of mice to sustain limb tension to oppose their gravitational force was measured. Data were analysed with the unpaired Student's *t*-test. **Results:** Morphologically, both untreated and UT-treated animals exhibited internalised nuclei, increased endomysial connective tissue and variations in muscle fibre diameters. Body weight and muscle strength were significantly reduced in the UT-treated animals. Blood creatine kinase was higher in UT-treated compared to untreated animals. In tibialis anterior, myostatin, transcript was more highly expressed in the UT-treated while in the diaphragm muscle, transforming growth factor-β transcripts were less expressed in the UT-treated. **Conclusion:** While previous studies identified anti-inflammatory, antiproliferative and anticarcinogenic UT effects, the extract indicates worsening of dystrophic muscles phenotype after short-term treatment in *mdx* mice.

Keywords: *Uncaria tomentosa*; Duchenne muscular dystrophy; *mdx* mice; neuromuscular; myotoxic.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe and degenerative muscle wasting disease caused by frame-shift mutations, mainly deletions, in the dystrophin gene (located on Xp21); it occurs in 1 in 3,600-6,000 newborn boys[1]. Dystrophin, the product of the dystrophin gene, is a 427 kDa subsarcolemmal protein that assembles with some transmembrane and cytosolic proteins to form the dystrophin-associated glycoprotein complex (DAGC). DAGC mediates interactions among the cytoskeleton, membrane and extracellular matrix and promotes mechanical stability during muscle contraction. Additionally, dystrophin acts as a scaffold in different signalling pathways. Therefore, the absence of dystrophin disrupts DAGC, causes sarcolemma instability and elevates calcium influx, inflammation and muscle degeneration[2]. DMD symptom onset usually occurs between 2 and 5 years of age. Initially, proximal muscle weakness is commonly observed, and during the toddler years, patients usually present delays in motor milestones, toe-walking, difficulty in rising from the floor (Gowers maneuver) and frequent falls. Loss of ambulation, scoliosis and cardiac and respiratory complications usually occur in the second decade of life, and as the disease progresses, patients usually die due to cardiac and/or respiratory failure[3]. Muscle dysfunction and a progressive decrease in force generation are the main consequences of the classical hallmarks of muscular dystrophy, inflammation and fibroses[4]. However, the chronicity of the inflammation is potentially responsible for enhancing muscle damage and reducing regenerated muscle fibres[5].

Currently, dystrophic animal models serve an important role in DMD preclinical applications. The mdx mouse (X-chromosome-linked muscular dystrophy[6]; is the most widely used animal model for investigating DMD. It exhibits a single point mutation in exon 23 of the dystrophin gene; this change prevents full-length dystrophin expression[1]. Although this genetic defect resembles human DMD, mdx mice present phases where muscle degeneration

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is replaced by regeneration. Due to this feature, their dystrophic phenotype is considered to be milder compared to humans, and researchers must pay close attention when considering the age of the mdx mice used for studies. At 2 weeks old, mdx skeletal muscles are no different from normal mice. Alterations usually appears at 3 to 6 weeks of age, with inflammation, necrosis and fibrosis. After this age, most skeletal muscles become more stable due to significant muscle fibre regeneration; however, the diaphragm exhibits progressive degeneration[7].

TNF-α is an up-regulated pro-inflammatory cytokine in DMD and acts as a chemotactic factor attracting inflammatory cells to the injured area[8]. Transforming growth factor-β (TGF-β1) and osteopontin are also increased in dystrophic skeletal muscle and participate in extensive disorganization and structural remodeling of the extracellular matrix as part of the fibrotic process[9]. Therefore, these are important biomarkers to be evaluated in studies that propose new therapeutic approaches for DMD. Myostatin, a muscle growth inhibitory protein, is also touted as an important biomarker for neuromuscular diseases[10].

Uncaria tomentosa (Willd. ex. Roem. & Schult.) DC. (Rubiaceae), usually known as "Cats claw" or "uña de gato" and heretofore abbreviated as UT, is a vine located in the Peruvian Amazon and other South and Central American tropical areas. UT aqueous extract and decoctions are traditionally used to treat cancer, heart and inflammatory diseases by Ashaninka Indians. UT has pertinent antiviral, antimutagenic and antioxidant properties. Additionally, UT can regulate the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α), namely by inhibiting its secretion by lipopolysaccharide (LPS)-activated THP-1 monocytic cells and also by preventing nuclear factor-kappa B (NF-κB) activation[11,12] UT extract is composed of a mixture of quinovic acid and glycosides and pentacyclic or tetracyclic oxindole alkaloids, including pteropodine, speciophylline, uncarine F, mitraphylline, isopteropodine and isomitraphylline[13].

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Considering the potential benefit of UT in inflammatory processes and the challenge in controlling the dystrophic hallmarks in DMD, the purpose of this study was to evaluate potential benefits of UT extract over muscle force and histopathology of the mdx mouse. The Kondziela test, histologic analysis and quantitative real-time polymerase reaction (q-RTPCR) for TNF-α, TGF-β, osteopontin and myostatin of the tibialis anterior (TA) and diaphragm (DIA) muscles were performed to examine the effect of UT extract.

METHODS

UT extract

We used UT aqueous extract at a dose of 200 mg/kg body weight[14] which had standardized total alkaloid contents corresponding to 5.0% – 0.5% of mitraphylline, as measured by chromatography, and was produced by the Herbarium Laboratory (São Paulo, Brazil). The extract derived from U. tomentosa root bark. UT powder was dissolved in 0.9% saline and stored in a refrigerator throughout the study.

Experimental mice

All protocols were approved by the Animal Care and Use Committee of the Centro Universitário FMABC, protocol 13/2014 and were performed in accordance with National Institutes of Health guidelines. Male 8-week-old mdx mice were utilised (untreated *n*=6; UT-treated *n*=10). The untreated animals received 0.2 mL saline and the treated animals received 0.2 mL aqueous UT extract (200 mg/g body weight) administered daily via gavage for 6 weeks. All animals were weighed weekly on a digital scale, and the weight was recorded. All quantifications were made blinded.

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Muscle strength assay

The muscle strength assay was performed with the four limb hanging test to measure the efficacy of the UT treatment (untreated *n*=6; UT-treated *n*=10). The test was performed according to the TREAT-NMD protocol (DMD_M.2.1.005) "The use of four limb hanging tests to monitor muscle strength and condition over time"[15] (George Carlson, last reviewed 29 June 2016). Briefly, the animals were placed individually in the centre of a wire grid. This grid was inverted and raised 30 cm above a box full of wood chips and the length of time that the animal was able to stay on the grid without falling was recorded.

Holding impulses (gm sec): body weight (grams = gm) x the time the animal remained suspended (seconds = sec).

The Holding impulses is used to oppose the gravitational force as an attempt to correct for the negative effects of body mass on the Hang Time.

Analysis of creatine kinase

After the treatments, the animals (untreated *n*=6; UT-treated *n*=10) were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight). Blood samples were collected caudal vena cava puncture and used to determine CK activity. The samples were centrifuged (Sigma® 3-18K refrigerated centrifuge) at 3000 rpm, for 10 minutes at 4 °C. The serum obtained was used to determine the amount of CK using the Roche / Hitachi cobas c 701/702, cobas® 8000 ISE analyzers systems.

Histological analysis

After the 6-week treatment, the animals were anesthetised as described in item 2.4 (untreated *n*=6; UT-treated *n*=10). A general morphological analysis was performed on the frozen *tibialis anterior* (TA) and *diaphragm* (DIA) muscles from one side randomly. These

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muscles were chosen because they are differentially affected in mdx mice. DIA muscle is severely affected, while TA muscle displays considerable regeneration[7]. The samples were stained with hematoxylin and eosin (H&E) and the total number of fibers with a central nucleus (indicative of regenerated muscle fibers) and fibers with a peripheral nucleus (characteristic of normal fibers) were counted. Two sections of each muscle were analyzed by light microscopy (Micro Nikon eclipse E200) connected to a camcorder (Moticam 1000) in 10X objective, using ImageJ software (ImageJ, http://rsb.info.nih.gov/ij/index.html). Each section muscle was dived into 10 fileds that were randomized photography. All the fibers of the 10 fields (normal and regenerated fibers) were counted to estimate the total population of fibers of each muscle. The percentage of normal and regenerated fibers of the studied animals and the average number of cells of each filed were obtained. The cross-sectional area of individual myofibres (measured as Feret's diameter) was determined from digitised images using Image J (http://rsb.info.nih.gov/ij/index.html).

qRT-PCR

Gene expression for TGF-β1, TNF-α, osteopontin and myostatin was determined with qRT-PCR using SYBR Green Master Mix (Invitrogen) on the TA and DIA muscles from one side randomly (untreated n=6; UT-treated n=10). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The reaction (20 µL total volume) was performed with 1 µL (20 ng) diluted complementary DNA (cDNA), 10 µL SYBR Green Master Mix, 0.5 µL each of 10 µM forward and reverse primers and 8 µL RNase-free water. The thermocycling protocol included an initial incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s and 72°C for 15 s. Primer sequences appear in Table 1.

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Statistical analysis

Data are presented as mean ± standard deviation. Statistical differences between the groups were analysed with an unpaired Student's *t*-test and grouped comparisons were carried out using two-way analysis of variance (ANOVA), followed by Bonferroni post-test using Prism software (GraphPad, San Diego, CA). $p<0.05$ was considered statistically significant.

RESULTS

Effect of UT on body weight, muscle strength and serum CK levels

The treatment did not change the body weight of the mdx mice when compared over the five weeks. However, at the fourth week, UT-treated mdx mouse weight at this time was approximately 13.8% less compared to the untreated mouse body weight (Figure 1A).

Regarding the inverted grid test, mdx mice from both groups did not show significant changes in the mean maintenance time throughout the week. However, at the end of the fifth week, there was a significant reduction (46.5%) in the maintenance time for the UT-treated compared to the untreated mice (Figure 1B).

Blood CK levels were significantly increased in the UT-treated compared to the untreated animals (Figure 1C).

UT effect on the TA and DIA histopathological pattern

In TA muscles, there was significant decrease (23.8%) in the percentage of centrally nucleated muscle fibre count but not in cell count under 10x magnification in the UT-treated compared to the untreated group (Figure 2C-D). When analysing the minimum Feret's diameter, there was a significant increase in the 40-µm-diameter fibre population and a significant reduction in the 60-µm-diameter fibre population in the UT-treated compared to the untreated mice (Figure 2B).

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In DIA muscle, there was a significant increase (34.4%) in the percentage of centrally nucleated fibres in the UT-treated compared to the untreated group (Figure 3C), but when the number of cells per field (10x magnification) was counted, there was no statistical difference between the groups (Figure 3D). Quantification of the minimum Feret's diameter demonstrated a significant increase in the 60-µm-diameter fibre population and a significant reduction in the 90-µm-diameter fibre population in the UT-treated compared to untreated mice (Figure 3B).

Effect of UT on TGF-β1, TNF-α, osteopontin and myostatin gene expression in TA and DIA muscles

In the TA muscle, qRT-PCR revealed a significant increase in myostatin expression, in UT-treated compared to untreated mdx mice approximately 376.1% (Figure 2E). Comparatively, in the DIA muscle, there was a significant reduction in TGF-β1 expression and a nonsignificant reduction in myostatin expression in the UT-treated compared to the untreated group approximately 82.8% and 88.1%, respectively (Figure 3E-H).

DISCUSSION

The results of the present study demonstrated a worsening of dystrophic muscle injury after six weeks of administration of 200 mg/kg aqueous extract of UT root coat, based on molecular and histopathological markers (Figure 4).

Muscle fibre injury related to altered sarcolemma permeability is evidenced by the increased plasma CK level in dystrophic mdx mice[6]. As described by Maglara et al.[16], this finding reflects the intense and severe state of muscular injury in dystrophic animals. Thus, in the present study, UT administration indicated a myotoxic effect in the mdx mouse, as evidenced by the increase in blood CK concentration. Unfortunately, studies that evaluate any

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UT-mediated toxicity are scarce[17]. Additionally, the impaired development of mdx mouse body mass and muscle function after UT administration may be related to worsening muscle injury, as studies clearly demonstrate that decreased CK levels are associated with improvements in muscle function in DMD patients and mdx mice[18].

Studies demonstrated that osteopontin is an immunomodulator and regulates TGF-β1 expression. Mdx mice with deletion of the *SPP* gene, which encodes osteopontin, show marked reduction in fibrosis and improved muscle strength[19]. The administration of the oral proteasome inhibitor, ixazomib, demonstrated in the mdx mouse a reduction in osteopontin and TGF-β, associated with an improvement in the dystrophic phenotype of the DIA muscle[20]. Another study showed increased levels of TGF-β and OPN in the muscles of GRMD dogs, while TGF-β was shown to be positively associated with the degree of sartorius muscle hypertrophy[21]. Our findings do not demonstrate this relationship in the DIA muscle of animals that were treated with the aqueous extract of UT. The reduction of TGF-β1 seems to be associated with proliferative effects, considering the increase in the number of regenerated cells. Elevated level of TGF-β1 was shown in DIA muscle to be accompanied by development of fibrosis and muscle wasting in mdx mice[22]. Another study shows that TGF-β1 immunomodulation, despite reducing the proliferation of connective tissue in the DIA muscle of mdx mice, showed an increase in the inflammatory response in these animals[23]. Therefore, it is too early to say that the reduction in TGF-β1 observed in the present study corresponds to the beneficial effect of UT on the dystrophic muscle.

The muscles of mdx mice suffer intense necrosis around the fourth week of life, followed by muscle fibre regeneration and hypertrophy[21]. Given the susceptible cycles of degeneration/regeneration, this condition continues with muscle atrophy and elevated fibrosis[24]. As a method for evaluating muscle tropism, determining the minimum Feret's diameter is an advantageous analysis due to the small variability in this parameter[25]. Studies

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showed that prevention of muscle atrophy is associated with the effects of preventing myonecrosis and improving muscle function[26]. These parameters correspond with our findings in the DIA muscle by correlating the increase of fibers with central core with the increase of fibers of smaller diameter.

Myostatin belongs to the TGF-β superfamily, and it is an important factor that regulates muscle growth[27]. Increased myostatin inhibits the expression of myogenic modulators, including MyoD and myogenin, and this effect directly influences satellite cell proliferation and differentiation and thus impairs muscle regeneration and contributes to muscle atrophy[27]. This phenomenon was especially apparent in the TA muscle, which demonstrated increased myostatin expression in association with the smaller regenerated fibre population and the anticipation of muscle atrophy in the mdx mouse. This finding is consistent with the literature that describes myostatin as a negative modulator of muscle regeneration[28]. Data from the DIA muscle underscore this feedback loop; regeneration was not impaired in this muscle because the myostatin level was low. Our findings are consistent with Pasteuning-Vuhman et al.[29], who demonstrated an increase in regenerated areas and a significant reduction in muscle fibre size in mdx mice following treatment with an Alk4 functional blocker, the main receptor for the myostatin signaling pathway.

The worsening of the damage caused by UT administration in mdx mice was also denoted by increased TNF-α expression, a cytokine that promotes very pronounced anti-tumor and immunological responses in the mdx mouse and DMD patients[8]. UT treatment did not decrease TNF-α expression, as seen in other systems, possibly because that increased muscle injury *per se* may contribute to maintenance of the elevated TNF-α expression. The pentacyclic indole alkaloid mitraphylline is the main bioactive secondary metabolite of UT extracts and associated with cytotoxic effects on cancer cells in anti-proliferative, pro-apotocytic and immunoregulatory terms[14,30,31]. The UT extract increased the production of

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ROS in HepG2 cells, which resulted in a decrease in the level of GSH, leading to apoptosis of these cells through the activation of caspase-3 and caspase-7[32]. In certain cell types, ROS are the main mediators of the pathways that regulate the expression of TNF-α, from the modulation of kinases of the redox system, activation of transcription factors, intracellular alteration of Ca2+ and gene expression[33]. It is possible that in the present study, the dose of 200 mg/kg may be directly[14] associated with the antiproliferative effect of mitraphylline via a mechanism of overproduction of ROS, contributing to the maintenance of high expression of TNF-α39. Furthermore, Ermolova et al.[34] observed that the administration of a TNF-α blocker in the skeletal and cardiac muscle of mdx mice reduces the expression of myostatin and improves the phenotype of the disease, reinforce our findings.

Oxidative stress is considered a primary event of DMD[35]. Studies show that the UT ethanol extract has a more effective antioxidant action compared to the aqueous extract[36-38]. The use of hydroalcoholic extract of UT bark showed antitumor and antioxidant effects by partially regulating redox and metabolic homeostasis[39]. In contrast, Navarro-Hoyos et al.[40] suggest that the leaves constitute the most suitable part of UT for use in the elaboration of standardized phenolic extracts because they are rich in proanthocyanidins and with high antioxidant activity, either in aqueous or ethanol extracts. Based on the above, we believe that the use of aqueous extract of UT root bark at a dose of 200mg/kg was decisive for the results obtained in the present study, since it does not contain some antioxidant and anti-inflammatory properties reported in studies with the ethanol extract of the root of the UT, or other parts of the plant, being essential the accomplishment of new studies to consider its real effect.

Conclusion

In conclusion, the administration of aqueous extract of UT root bark at a dose of 200mg/kg did not reduce important molecular, biochemical and morphological markers in

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mdx mouse muscle. Despite the apparent evidence of its efficacy in immunomodulatory diseases and other experimental models, UT root bark at the chosen period and dose actually exhibited an aggravating potential for the mdx mouse dystrophic muscles. Future studies are needed to determine UT extract pharmacokinetic patterns to weigh potential modulations and appropriate therapeutic strategies to enable its use as a possible therapy for DMD.

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Tables and Figures Legends

Table 1: Forward and reverse primers used in quantitative real time PCR analyses

Primer	Sequence
TNF-α	
Forward	5’ ATGAGCACAGAAAGCATGATC 3’
Reverse	5´TACAGGCTTGTCACTCGAATT 3’
TGF-β	
Forward	5’ CCCCACTGATACGCCTGAGT 3’
Reverse	5’ AGCCCTGTATTCCGTCTCCTT 3’
Myostatin	
Forward	5’ AACCTTCCCAGGACCAGGAG 3’
Reverse	5’ CATCGCAGTCAAGCCCAAAG 3’
Osteopontin	
Forward	5’ GCTTGGCTTATGGACTGAGG 3’
Reverse	5’ CGCTCTTCATGTGAGAGGTG 3’
GAPDH	
Foward	5’ CCAGAACATCATCCCTGCAT 3’
Reverse	5’ GTTCAGCTCTGGGATGACCTT 3’

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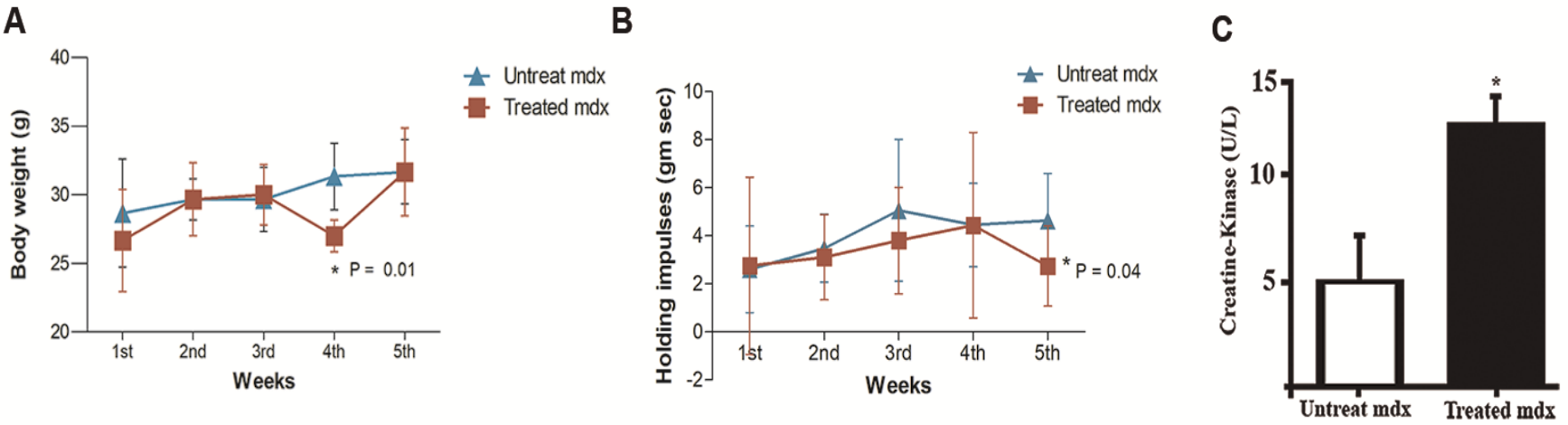

Figure 1: (A) Body weight (g) and (B) muscle strength in untreated (blue line) and UT-treated (red line) *mdx* mice. All values are expressed as mean ± standard deviation (SD). * compared with untreated *mdx* group, (unpaired Student's *t*-test). (C) Serum creatine kinase (CK) levels for untreated and UT-treated *mdx* mice. * compared with untreated *mdx* mice; (unpaired Student's *t*-test).

https://doi.org/10.7322/abcshs.2022018.2058

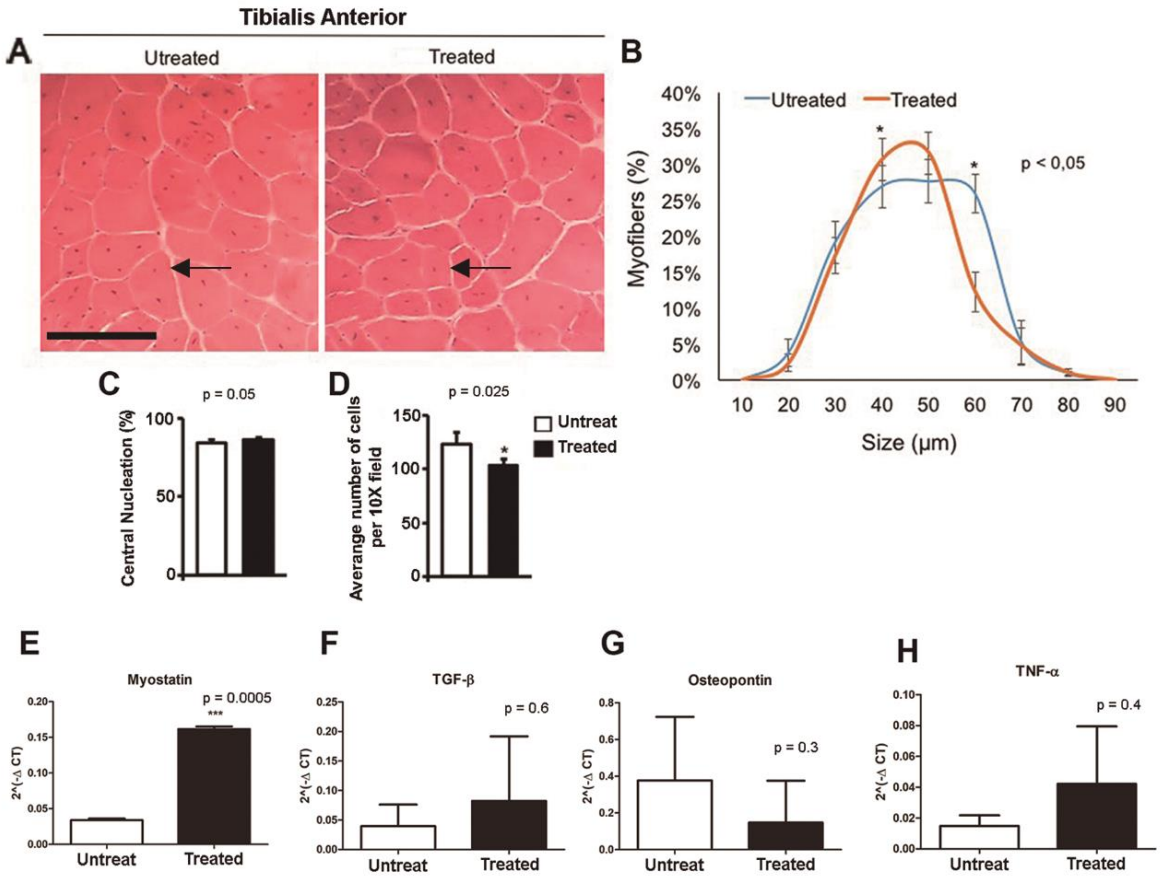

Figure 2: (A) Tibialis anterior (TA) muscle cross-sections that showing fibres with central nuclei in untreated and UT-treated *mdx* mice (arrow). Scale bar is 100 µm. (B) The graph shows analysis of the minimum Feret's diameter in the TA muscle of the untreated (blue line) and UT-treated (red line) *mdx* mice. (C and D) The graphs show the percentage of centrally nucleated muscle fibres (C) and the cell count (D) at 10x magnification in the TA muscle of untreated and UT-treated treated *mdx* mice. qRT-PCR results are shown for myostatin (E), TGF-β1 (F), osteopontin (G) and TNF-α (H) expression in TA muscle. All values expressed as mean ± standard deviation (SD). * compared with untreated *mdx* mice (unpaired Student's *t*-test).

https://doi.org/10.7322/abcshs.2022018.2058

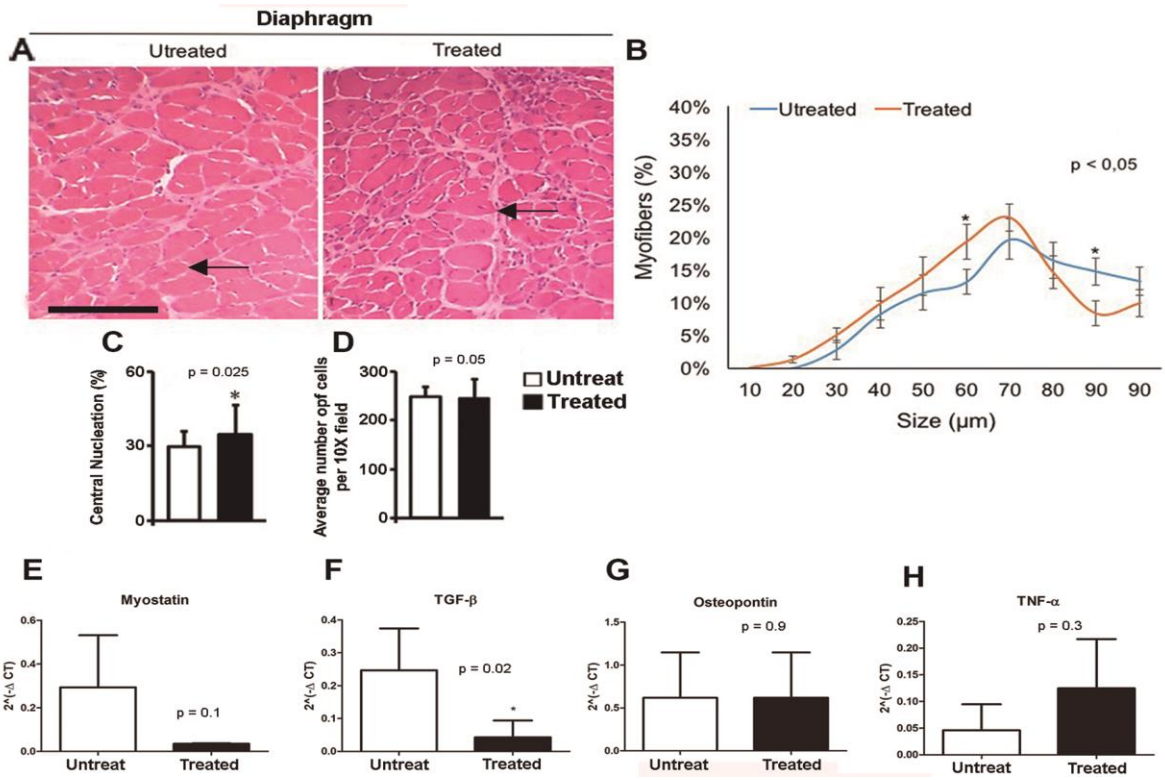

Figure 3: (A) Diaphragm (DIA) muscle cross-sections that show centrally nucleated fibres in the untreated and UT-treated *mdx* mice (arrow). The scale bar is 100 µm. (B**)** The graph shows the analysis of the minimum Feret's diameter in the DIA muscle of the untreated (blue line) and UT-treated (red line) *mdx* mice. (C and D) The graphs show the percentage of centrally nucleated muscle fibres (C) and the cell count (D) at 10x magnification in the DIA muscle. qRT-PCR results are shown for myostatin (E), TGF-β1 (F), osteopontin (G) and TNF-α (H) expression in DIA muscle. All values expressed as mean ± standard deviation (SD). * compared with untreated *mdx* mice (unpaired Student's *t*-test).

https://doi.org/10.7322/abcshs.2022018.2058

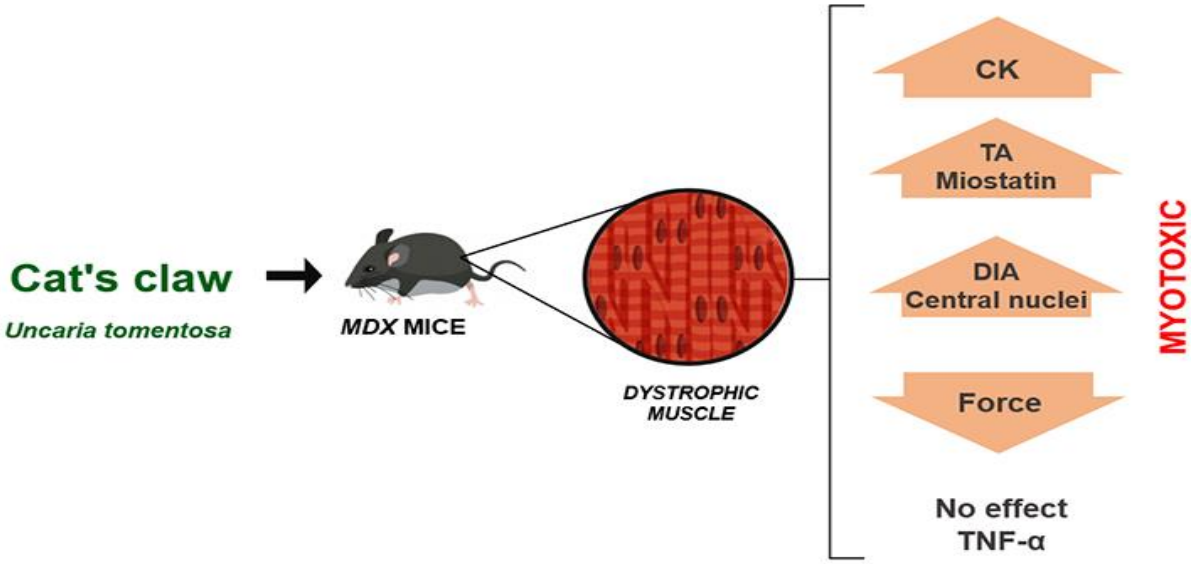

Figure 4: Graphical abstract - Representation of effects of UT on mdx mice. The illustrations are disponibilize free from freepik.com®.

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