Int. J. Morphol.,
29(4):1357-1363, 2011.

Effect of *Citrus aurantium* L. Essential Oil on Muscle Regeneration in Mdx Mice

Efecto del Aceite Esencial de *Citrus aurantium* L. en la Regeneración Muscular en Ratones Mdx

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DE SOUZA, P. A. T.; MARQUES, M. J.; LIMA, C. A. H. & MATHEUS, S. M. M. Effect of *Citrus aurantium* l. essential oil on muscle regeneration in mdx mice. ***Int. J. Morphol., 29(4)***:1357-1363, 2011.

SUMMARY: Duchenne muscular dystrophy (DMD) is a severe X-linked recessive disorder characterized by the progressive loss of muscular strength. Mdx mutant mice show a marked deficiency in dystrophin, which was related to muscle membrane stability. The aim of this study was to verify the possible protective anti-inflammatory effect of citrus oil on mdx muscle fibers. Thus, adult male and female mdx mice (014/06-CEEA) were divided into control and citrus-treated. After 60 days of treatment, one ml of blood was collected for creatine kinase (CK) test. Diaphragm, sternomastoideus, anterior tibial and gastrocnemius muscles were removed and processed according to histological routine methods. The observed alterations indicate a direct effect of citrus. Recent studies have improved the diagnosis of muscular diseases but with no definitions of efficient treatments. Intervention with several therapies is important to many patients presenting muscular dystrophy, which enables them to live longer and be more active, while there is no development of gene therapies.

KEY WORDS: Muscle fiber; Duchenne muscular dystrophy; *Citrus aurantium* L.; mdx mouse, Muscle regeneration; Central nuclei; Creatine kinase.

INTRODUCTION

Muscular dystrophies are genetically determined primary progressive myopathies (Robbins *et al*., 1989; Engel, 2003). Duchenne type is the most frequent and severe form of dystrophy since it is progressive, lethal and affects male children (Tidball &Wehling-Henricks, 2004). The child presents delayed motor development, frequent falls, difficulties to run, and increased muscular volume or calf pseudohypertrophy. Frequently, by 12 years old, the child cannot walk and is only able to move fingers, tongue and respiratory muscles (Engel *et al.*).

Duchenne muscular dystrophy (DMD) is an X-linked myopathy caused by a mutation in the dystrophin gene (Takeda, 2001). Dystrophin is a protein present on the cytoplasmic surface of sarcolemma.The complex dystrophin-glycoproteins, expressed at high concentrations in the striated muscle, connects the muscle fiber cytoskeleton (actin) to the extracellular matrix and is composed of sarcolemmal proteins (Campbell & Kahl, 1989, Ervasti & Campbell, 1991). Linked to integrating proteins, dystrophin stabilizes and prevents failures in the sarcolemma during cycles of muscle contraction-relaxation, keeping the muscle fiber integrity (Engel *et al*.; Gramolini & Jasmin, 1998).

Mdx mutants are the preferred animal models for DMD studies (Torres & Duchen, 1987). In mice, muscular degeneration begins around the third week of age and persists until nearly one month, with muscle loss compensated by regeneration cycles (Marques *et al.*, 2004). Despite the differences between mdx and human phenotypes, experiments in mdx mice have provided incalculable information about DMD pathogenesis (Marques *et al*.).

Serum enzymes such as creatine kinase (CK), creatine kinase MB (CK-MB), aldolase (ALD), and lactate dehydrogenase (LDH) present increased activity under DMD conditions during the first three years of life, which indicates muscular degeneration (Pernice *et al*., 1988). The activity

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Grant Fundunesp proc. nº 01268/11-DFP.

of these muscle enzymes is important for the diagnosis of muscular diseases. CK is more marked at the initial stages of the disease and its activity decreases together with the muscular activity (Engel *et al*.).

As a new therapeutic resource, phytotherapy has become increasingly popular among people in the whole world. Popularly known as sour orange (Santos *et al.*, 1988; Sanguinetti, 1989; Vieira, 1992), *Citrus aurantium* L. (Rutaceae) originated from Southeast Asia, India and China. This species is used to treat disorders of the gastrointestinal tract (Carvalho-Freitas & Costa, 2002; Pultrini *et al.*, 2006). Citrus fruits are rich in flavonoids, which have been considered modifiers of the biological response; most of the latter act as antioxidants, and some have notable anti-inflammatory properties (Theoharides *et al.*, 2001).

Thus, the aim of the present work was to identify a possible anti-inflammatory action of *Citrus aurantium L.* in order to minimize the effects of muscle fiber degeneration in mdx mice.

MATERIAL AND METHOD

Essential oil. A plant sample of the specie *C. aurantium* was collected and the exsiccates deposited in the Herbarium "Irina D. Gemtchujnicov"—BOTU, Department of Botany at Unesp, under nº BOTU 23123. After the fruits were collected at Unesp-Botucatu, fresh fruit peels of *C. aurantium* L. were submitted to extraction of essential oil by water vapor with the aid of a Clevenger type device (Marconi, Brazil). The vegetal material (fruits peels) was mixed inside a glass balloon (5 L) with distilled water and put on a heated pad. The essential oil (OEC) obtained was stored in an amber bottle at 5ºC temperature until the accomplishment of the pharmacological experiments and phytochemical analyses (Moraes *et al.*, 2009).

Animals. Ten mice of both sexes, strain C57BL/10, were used. After weaning, these animals were brought from the animal facility of the Department of Anatomy, Institute of Biology, State University of Campinas (UNICAMP), Campinas Municipality, São Paulo State, Brazil, to the animal facility of the Department of Immunology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu Municipality, São Paulo State, Brazil, where they were kept in individual cages with water and food *ad libitum*. The animals were divided into one control (n=5) and one treated group (n=5). The latter received essential oil of citrus (50 mg /kg) diluted in Tween 8% through gavage for 60 days. The essential oil was provided and elaborated by the Laboratory of Natural Products of the Department of Physiology, Institute of Biosciences, UNESP Botucatu, Brazil.

During the same 60 days, the control group received distilled water through gavage. The animals were kept in individual cages, under suitable environmental conditions (12h dark/light), with food and water *ad libitum* (014/06-CEEA).

Anesthesia, sacrifice and muscle collection. After 60 days of treatment, the animals were anesthetized with Hypnol 2%; then, around 1ml blood was collected by intracardiac puncture and frozen until CK test. Afterward, animals were euthanized by receiving an excessive anesthetic dose and were dissected for the removal of the following muscles: sternomastoideus, gastrocnemius, anterior tibial and diaphragm, which were fixed in 10% buffered paraformaldehyde. After around 48h, this material was washed in tap water and prepared for inclusion in paraplast. Sections of 5μm thickness were obtained and HE-stained.

Analysis procedure. The histological sections (5μm thickness) of control(n=5) and treated(n=5) muscles were observed under a light microscope (Axioplan 2) with objective of 20X magnification attached to a digital camera (AxioCam HR) to capture the image linked to the computer Pentium® 4.0. The number of fibers presenting central nucleus was evaluated since it indicates the regenerated muscle fibers (Torres & Duchen). The number of normal and regenerated fibers was obtained by using the software AxioVision 4.0.

Creatine kinase test. After collection, the blood was centrifuged and subjected to the biochemical assay of creatine kinase according to the kinetic method (Moura, 1982), in which the speed of creatine formation from creatine phosphate was recorded. Spectrophotometric absorbance readings were done by using a microplate reader (μQuant, BioTek Instruments, Inc.) at 340 nm and were recorded after 1-, 2- and 3-min intervals. The speed of creatine release was proportional to the creatine kinase activity in the sample.

Statistical analysis. Results were expressed as Standard Deviation and statistical significance the T-test was used to compare means between control and treated animals test with $P < 0.05$ defined as significant. As regards central nucleus count, a logarithmic transformation was done for all variables (number of central nuclei in different muscle groups).

RESULTS

The fibers of the studied muscles had oval, round or polygonal shape and different diameters. Each muscle fiber is involved by a loose connective tissue named endomysium. The grouping of several fibers form muscle fascicles that are delimited by a relatively dense connective tissue named perimysium.

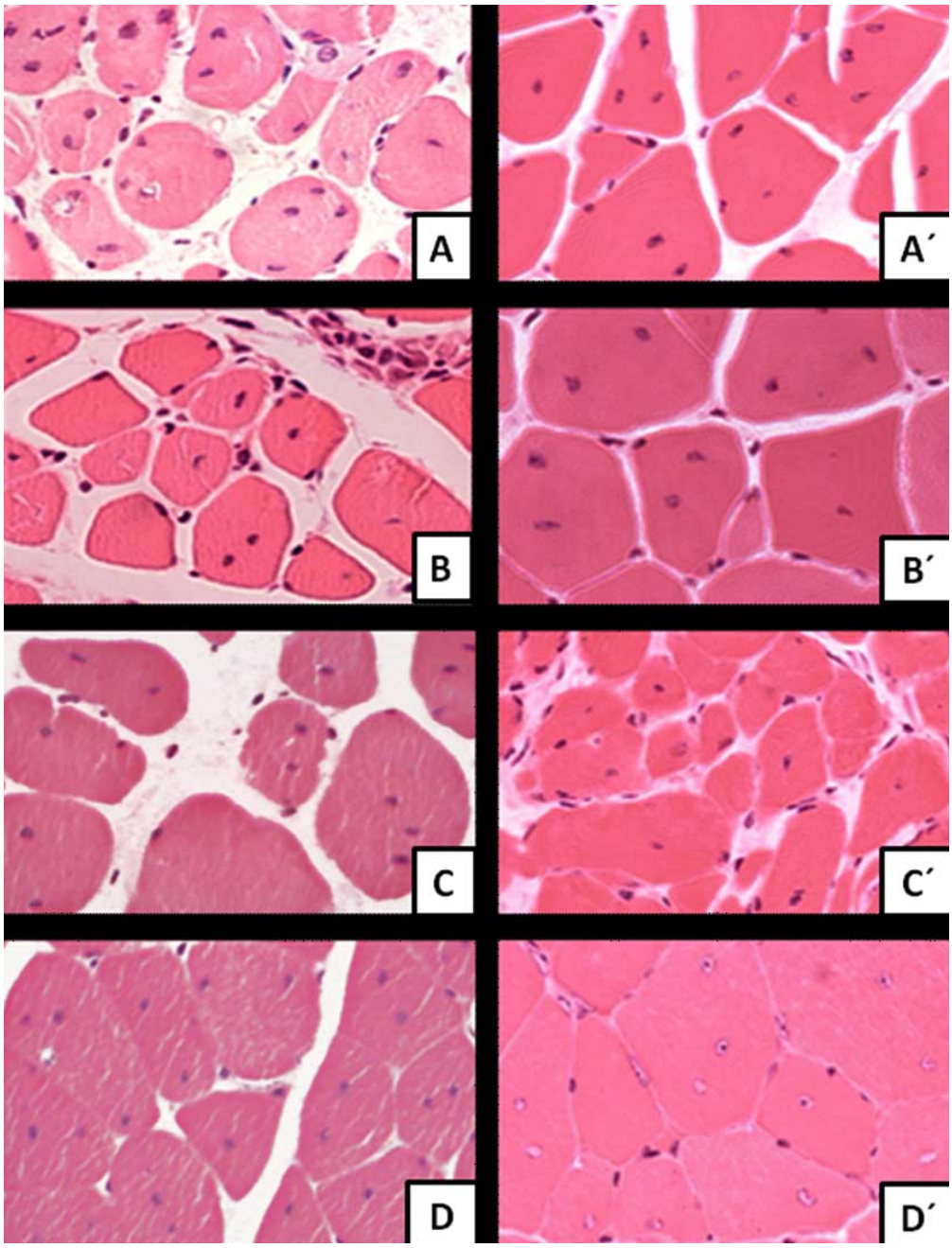

Fig. 1. Photomicrography of fibers muscular dystrophy with central nuclei in mdx mice. Transversal sections of the studied muscles. (A): Anterior Tibial – control, (A'): Anterior Tibial – treated, (B): Sternomastoideus – control, (B'): Sternomastoideus – treated, (C): Diaphragm – control, (C'): Diaphragm – treated, (D): Gastrocnemius – control, (D'): Gastrocnemius – treated.

Histological evaluations did not indicate any morphological differences between muscles from control (Fig. 1A´, B´, C´, D´) and those from treated animals (Fig. 1A, B, C, D). Thus, there were fibers with peripheral nucleus, degenerating fibers, and fibers at different regeneration stages in both groups. Fibers with peripheral nucleus had polygonal shape and were juxtaposed to other fibers with the same characteristic. Regenerated fibers had centralized and condensed nucleus; some fibers had up to two central nuclei and eosinophilic cytoplasm, and their diameter was similar to that of muscle fibers with peripheral nucleus. On the other hand, degenerating fibers presented irregular contours, were weakly HE-stained and apparently fragmented.

The number of central nuclei, which is used to verify muscle regeneration, indicated that the fibers underwent regeneration, since central nuclei were observed in all studied muscles from the treated group; some fibers had up to two central nuclei.

The muscles AT and GASTR had a larger number of central nuclei, as shown in Figure 1. Significant

differences could not be verified for this variable. Thus, *Citrus aurantium L*. can be assumed not to impair the muscle regeneration process.

The anterior tibial muscles, diaphragm and gastrocnemius showed larger number of central nuclei compared to control values as plotted in the figure 2, which indicates no significant difference between groups. P-value <0.05 was adopted for the t-test.

The CK test according to the kinetic method showed that the treated group had high levels of this enzyme, with acute anti-inflammatory effect. In this case, *Citrus aurantium L.* did not have the expected action, as shown in Figure 3.

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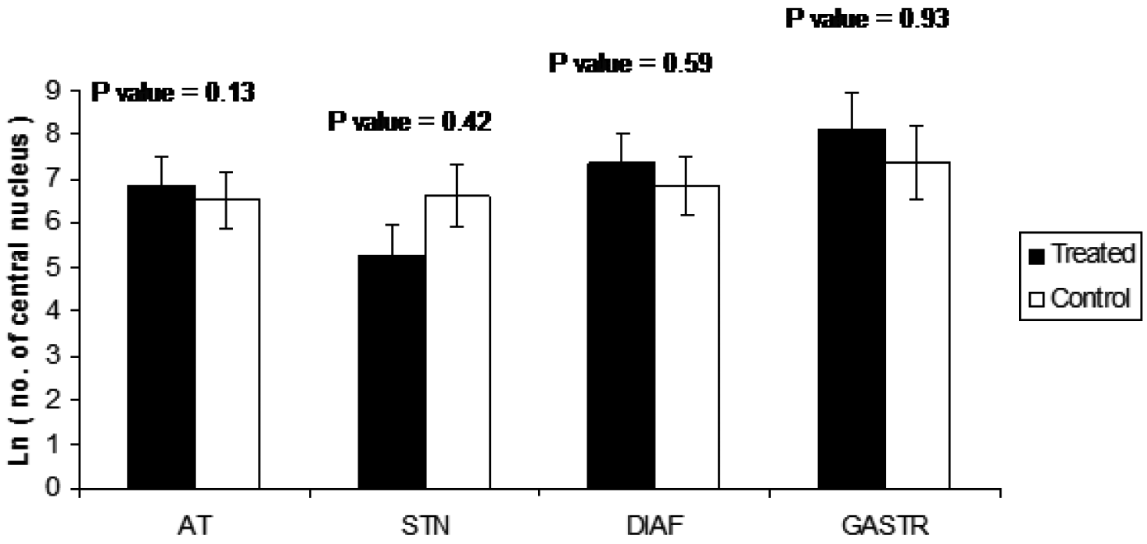

Fig. 2. Logarithmic transformation for all variables (number of central nuclei in different muscle groups) to compare means between control and treated animals according to the t-test.

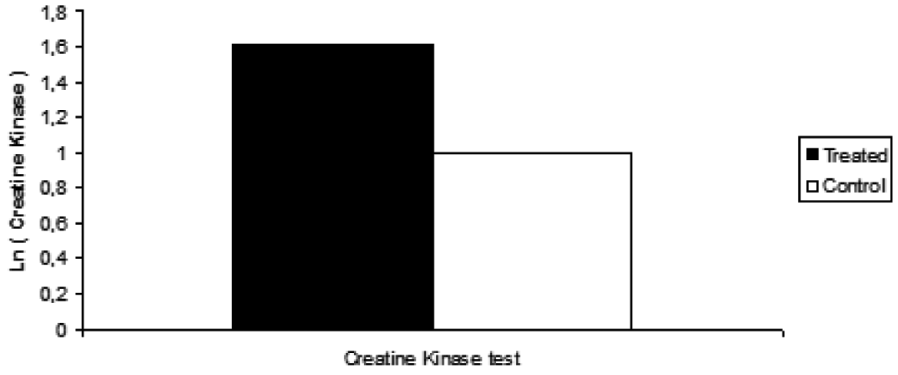

Fig. 3. Creatine kinase (CK) test according to the kinetic method to evaluate CK activity after treatment with citrus.

DISCUSSION

In the treatment of DMD, the pharmacological strategies are mainly aimed at decreasing the muscle involvement, minimizing the functional damages due to the disease (Porter *et al.*, 2004). Anti-inflammatory corticosteroids have been widely used as an attempt to reduce the disease progress; however, their side effects are severe (Muntoni *et al.*, 2002, Parreira *et al.*, 2007, Radley *et al.*, 2007, Manzur *et al.*, 2004). Alternative pharmacological strategies such as the use of nonsteroidal anti-inflammatory drugs (NSAIDs) have become an option, since these drugs cause less severe side effects. In addition, medicinal plants can be also used by means of phytotherapy. According to the Board of Directors resolution n.48/2004 from the National Health Surveillance Agency – ANVISA, herbal medicines are exclusively elaborated with medicinal plants or part of them (roots, barks, leaves, fruits or seeds), which have properties for healing, prevention, diagnosis or symptomatic treatment of diseases (Arnous *et al*., 2005). The use of plants to treat and heal diseases is as old as humankind and has great importance nowadays in the attempt to join folk and scientific knowledge. Known as sour orange, *Citrus aurantium* L. has been used by the folk medicine in Brazil and other countries to treat anxiety, hysteria, depression and insomnia; it also presents other important properties. Its chemical constituents include some flavonoids that show anti-cancer and anti-inflammatory actions (Manthey *et al*., 2001).

Under pathological conditions, such as myopathies and dystrophies, an excessive inflammatory response can lead to myonecrosis in addition to the lesion caused by an excess of the ion calcium inside muscle fibers. The inhibition of inflammatory cells can reduce muscle tissue degeneration and necrosis. Studies with anti-inflammatory drugs showed a significant reduction in myonecrosis in skeletal muscles from mdx mice (Tidball & Wehling-Henricks; Marques *et al*.). Myonecrosis was not observed in the present study; however, several degenerating regions and oval-shaped atrophied fibers were detected, which characterizes this pathology.

In some studies, muscle regeneration was impaired due to the use of NSAIDs (Huard, 2003), whereas in other works muscle regeneration occurred normally (Järvinen *et al*., 2005).

In the present study, there was no significant difference in the central nucleus count between the control and the treated group. A tendency toward an increase in central nuclei was observed for anterior tibial and gastrocnemius muscles. Thus, the beneficial effect of *Citrus aurantium* L. as anti-inflammatory cannot be ruled out. Albuquerque (2008) reported that the anti-inflammatory drugs naproxen and nimesulide had different actions in the studied muscle groups. After treatment with naproxen, anterior tibial and sternomastoideus muscles presented a different behavior relative to that of the saline-treated control group; furthermore, central and peripheral nuclei decreased in the anterior tibial muscle. On the other hand, nimesulide did not significantly change the proportions of central and peripheral nuclei from anterior tibial and sternomastoideus muscles.

Citrus has been proven to present anti-inflammatory action against ulcer by inhibiting H+ release and leading to increased gastric mucus secretion (Moraes *et al*., 2007; 2009). Nevertheless, components isolated from citrus still must be tested before their anti-inflammatory action on the striated muscle is ruled out.

Expectations of treatments for DMD remain uncertain; however, several studies involving gene therapy (De Luca *et al.*, 2008) and pharmacological treatments (Pierno *et al.*, 2007) have been published in the attempt to find solutions for this type of dystrophy (Yoshida *et al.*, 2006). The disease evolves with progressive muscle loss, replacement of muscle fibers for fibroadipose tissue and increased interstitial connective tissue (Seixas *et al*., 1997).

In mdx mice, up to three months old, there is an increase in the number of fibers with central nucleus undergoing degeneration-regeneration cycles, whereas spontaneous regeneration is followed by muscle fiber necrosis (Torres & Duchen). The diaphragm and smooth muscles have different time of response to the dystrophin deficiency (Porter *et al*.). Anterior tibial muscle degenerates between 3 and 4 weeks after birth and presents disorganized cytoskeleton and sarcolemma at 18 days postnatal, tending to increase over time. It has few central nuclei at 18 days and increased myofibril diameter in one month (Reed & Bloch, 2005).

As regards CK test, the high level of this enzyme may be due to factors of the studied microenvironment, where the activity of enzymes such as CK is lower, associated with increased production of inflammatory cytokines (Maegaki *et al.*, 1999). Tissue repair cycles are monitored by a sequence of biochemical and cell events organized to restore the tissue integrity after the lesion. There is participation of the immune system acting in the protection of injured tissues against possible infections and in the secretion of growth factors such as cytokines, which metabolically change the morphological state of animal

tissues (Park & Barbul, 2004; Gawronska-Kozak *et al*., 2006). Oral supplementation of NaCl decreases CK activity, but whether the inhibition of mdx muscle degeneration occurs before fiber necrosis remains unknown. Mdx mice treated with saline doses present a difference in CK activity between males and females during the days of treatment. In the control group, the animals treated with naproxen and nimesulide for 15 days had higher plasmatic levels (Albuquerque). The diagnosis and progress of neuromuscular diseases should not be based solely on CK values, which must be used as complementary data for the disease diagnosis. In DMD carriers, these values may be 50 to 100 fold higher than the minimum reference value (Engel *et al*.). In mdx mice, CK serum levels are high during their whole life due to the successive muscle degeneration-regeneration cycles (Yoshida *et al*.).

Studies related to factors that promote and act on the inflammatory process typical of this dystrophy will probably enable the identification and elucidation of some still obscure aspects of this pathology.

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RESUMEN: La distrofia muscular de Duchenne (DMD) es una enfermedad grave ligada al cromosoma X, trastorno recesivo que se caracteriza por la pérdida progresiva de fuerza muscular. Mdx ratones mutantes muestran una marcada deficiencia en la distrofina, que está relacionada con la estabilidad de la membrana muscular. El objetivo de este estudio fue comprobar el posible efecto protector, antiinflamatorio del aceite de cítricos en las fibras musculares mdx. Los ratones mdx adultos machos y hembras (014/06-CEEA) se dividieron en control y cítricos tratados. Después de 60 días de tratamiento, un ml de sangre fue recogida para cuantificar la creatina quinasa (CK) de prueba. Fueron retirados y procesados los músculos diafragma, esternomastoideo, tibial anterior y gastrocnemio de acuerdo con los métodos de rutina histológica. Las alteraciones observadas indican un efecto directo de los cítricos. Estudios recientes han mejorado el diagnóstico de enfermedades musculares, pero sin definiciones de tratamientos eficaces. Intervención con varias terapias es importante para muchos pacientes que presentan distrofia muscular, lo que les permite vivir más y ser más activos, mientras no exista desarrollo de terapias génicas.

PALABRAS CLAVE: Fibra muscular; Distofia muscular Duchenne; *Citrus aurantium L.*; Ratón mdx; Regeneración muscular; Núcleo central; Creatina kinasa.

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Received: 28-04-2011
Accepted: 04-08-2011