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Supplementation action with ascorbic acid in the morphology of the muscular layer and reactive acetylcholinesterase neurons of ileum of mdx mice

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ABSTRACT

The Duchenne Muscular Dystrophy (DMD) is a genetic disorder characterized by the absence of dystrophin protein, causing severe myopathy from increases of oxidative stress. Injuries of intestinal muscle can compromise the myenteric plexus. This study aimed to evaluate the disorders occurred in the muscular layer and in the acetylcholinesterase myenteric neurons (ACHE-r) of ileum of *mdx* mice, and the effects of supplementation with ascorbic acid (AA) in both components. 30 male mice C57BL/10, and 30 male mice C57BL/10Mdx were separated according to the age and treatment (n = 10/group): 30-days-old control group (C30); 30-days-old dystrophic group (D30); 60-days-old control group (C60); 60-days-old dystrophic group (D60); 60-days-old control group supplemented with AA (CS60); and 60-days-old dystrophic group supplemented with AA (DS60). The animals were euthanized and the ileum was collected and processed. Semi-serial sections were stained by Masson's trichrome, and acetylcholinesterase histochemical technique in whole-mounts preparations to identify the myenteric neurons. The muscular layer thickness and the area of smooth muscle of ileum were lower in dystrophic groups, especially in D30 group. The DS60 group showed the muscular layer thickness similar to C60. The density of ACHE-r neurons of myenteric plexus of ileum was lower in D30 animals; however, it was similar in animals of 60-days-old without treatment (C60 and D60) and, higher in DS60. The cell body profile area of ACHE-r neurons was similar in C30-D30 and C60-D60; however, it was higher in DS60. DMD caused damage to the ileum's musculature and myenteric plexus, and the AA prevented the ACHE-r neuronal loss.

1. Introduction

The Duchenne Muscular Dystrophy (DMD) is a recessive X-linked form of muscular dystrophy characterizing a genetic disorder, locus Xp21, and the results in a severe myopathy, characterized by progressive muscle degeneration that affects the life quality and life expectancy of the individual (Abdel-Salam et al., 2009).

Patients with DMD show a decrease or even lack of dystrophin protein expression, which leads to reduction of protein complex linked to it, causing disorders and/or destruction among the cytoskeleton and extracellular matrix, determining the rupture and necrosis of muscle fibers (Alves et al., 2014).

Dystrophin is located in the sarcoplasm (Miyatake et al., 1991) and it is believed that it exerts a structural function, since it represents a link

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between actin of cytoskeleton and the extracellular matrix, which strengthens the sarcolemma during the stress of muscle contraction (Alves et al., 2014) and can operate in the maturation of membrane receptors at neuromuscular junctions (Pilgram et al., 2010), and even perform a smaller role, but meaningful, with respect to contractile response to intestinal muscle (Mulè et al., 2010; Alves et al., 2014).

Studies on the digestive system components in patients with DMD show that these individuals have difficulty swallowing in the first decades of life, which tends to get worse with age (Pane et al., 2006).

Worsening swallowing problems contributes to dysphagia framework in patients with DMD, outcome eating disorders due structural and neurological disorders involved in the complex process of swallowing, and the decrease in strength of masticatory muscles (Ueki et al., 2007).

The DMD also compromises the gastrointestinal tract (GIT) function, mainly affecting the smooth muscles of their walls, causing serious motility disorders (Abdel-Salam et al., 2009).

In addition to the problems related to swallowing other symptoms that suggest injuries in the structures of the GIT have been described (Pane et al., 2006). According to Mulè et al. (2010) muscular dystrophy is not only in the skeletal striated muscle, but also the cardiac muscle and the smooth muscle, the latter being components of the intestinal wall. The relationship between smooth muscle changes and dystrophies has been reported since the 1960s (Huvos and Pruzanski, 1967).

Differences studies with dystrophic patients and animal models has been performed with the stomach (Borrelli et al., 2005; Mulè et al., 2006, 2010), small intestine (Mulè et al., 1999; Zizzo et al., 2003; Alves et al., 2014) and large intestine (Vannucchi et al., 2003; Mulè et al., 2010) describes the changes in the function of these segments of the GIT.

As the smooth muscle control of GIT occurs by the components of the enteric nervous system (ENS), especially by the myenteric plexus (Furness, 2012), it is believed that motility disorders of GIT in patients with DMD can be related not only to muscle lesions, but also disorders in the myenteric neurons, since they are located between the longitudinal and circular layers of the muscular layer.

Different hypotheses have been postulated in an attempt to justify the appearance of muscle lesions in DMD. Among them, the highlight the increase of Ca^{2+} concentrations inside the muscle fibers (Brookes et al., 2004) activated by mechanosensitive ion channels (Whitehead et al., 2006). According to Whitehead et al. (2006) the increase of Ca^{2+} levels multiply the production of reactive oxygen species (ROS), which explains the damage detected in the muscle fibers.

According to Godin et al. (2012), the pathogenesis of DMD is associated with the increased ROS and abnormal levels of Ca^{2+} with mitochondrial disorder and failure in energy metabolism.

Some studies suggest the use of antioxidants to minimize the deleterious effects of oxidative stress (Veit and Zanoni, 2012; Ferreira et al., 2013; Melo et al., 2013). Ascorbic acid (AA) is the 2.3-enediol-L-gulonic acid, an antioxidant soluble in water and absolute ethanol (Mandl et al., 2009), found in a wide variety of foods and supplements formulations.

AA has electron donating ability, thus, it neutralizes a huge variety of free radicals and ROS (Araujo, 2015), which in high levels, induce the collapse of the mitochondrial membrane, compromises the repair of injured (Vila et al., 2016) and potentially lead to cell apoptosis (Siciliano et al., 2007).

Thus, this study aimed to evaluate the effects of DMD in the muscular layer morphology of ileum of *mdx* mice, and analyze its relation with the acetylcholinesterase-reactive neurons population (ACHE-r) of myenteric plexus of ileum of animals supplemented with AA.

2. Material and methods

All procedures described herein followed the principles adopted by the Brazilian College of Animal Experimentation and approved by the Commission of Experimentation and Animal Use of the Biomedical Sciences Institute of São Paulo University (Process n° 164/2011 CEUA/ICB/USP).

2.1. Groups of animals and supplementation with AA

Sixty 30-days-old male mice were used, being 30 *mdx* mice (C57BL/10-Dmd^{mdx}) and 30 normal mice (C57BL/10), from the Animal's House of the Biomedical Sciences Institute of São Paulo University (C57BL/10) and from the Animal's House of Oswaldo Cruz Foundation in Rio de Janeiro (C57BL/10-Dmd^{mdx}).

During trial period of 30 days, the mice were weighed and isolated in polypropylene boxes (five animals per box) provided with watering and feeding place, maintained in controlled temperature (24 ± 2 °C) and lighting (cycle of 12 h in the light/12 h in the dark) fed with ration (Nuvital®) and ad libitum water.

Therefore, the animals were randomly divided into six groups (n = 10/group), according to the age they would have at the end of the experiment. Thus, the groups were classified as 30-days-old control group (C30); 30-days-old dystrophic group (D30); 60-days-old control group (C60); 60-days-old dystrophic group (D60); 60-days-old control group supplemented with AA (CS60); and 60-days-old dystrophic group supplemented with AA (DS60).

CS60 and DS60 groups were daily supplemented via gavage with ascorbic acid diluted in 1 mL of water, at a dose of 200 mg/kg of body weight, from 31-day to 60-day of age (Tonon et al., 2012).

2.2. Animal's euthanasia and jejunum-ileum collect

After weighing, the animals were euthanized in a carbon dioxide chamber (CO_2) at the end of the experiment, 30-days-old for C30 and D30 groups, and 60-days-old for the other groups, in the Animal's Center of the Anatomy Department – ICB/USP. The mice were laparatomized and their ileum was removed.

Each group had five animals with histological analysis of the ileum and other five animals had a quantitative and morphometric analysis of ACHE-r myenteric neurons of ileum.

2.3. Determination of the jejunum-ileum area and obtaining of the ileum

Right after abdominal cavity removal, the jejunum-ileum was placed on a sheet of graph paper and photographed. The images were analyzed by software Image-Pro Plus version 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA) for area determination (cm^2).

Later, the ileum was separated from jejunum by cross-section, where the ileocecal fold defined the cranial limit, and the ileocecal junction defined the caudal limit.

2.4. Histological processing of ileum

Each obtained ileum (n = 5/group) was fixed in Bouin's solution and processed by following the histological routine, and embedded in paraffin. Masson's trichrome-stained cross sections (semi-serial sections) were prepared at 6 μm thickness (Gerger et al., 2013). Thirty microscope fields of ileum were analyzed for each animal.

2.5. Histochemistry in whole mounts of ileum

The acetylcholinesterase histochemical method was used to identify the ACHE-r myenteric neurons of ileum (Karnovsky and Roots, 1964). Therefore, the lumen of each ileum (n = 5/group) was washed with PBS (0.1 M, pH 7.4), filled with 4% buffered paraformaldehyde, and immersed in the same fixative solutions for 30 min at 4 °C. The edges of the ileum were tied with suture material to keep the filling.

Then, the ileum was immersed in a solution A for 12 h, which is