Original article

Qualitative analysis of striated skeletal muscles in the dystrophynopathy of mdx mice submitted to physical activity

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Abstract

Duchenne's Muscular Dystrophy (DMD) is a recessive hereditary myopathy linked to the chromosome X, caused by a mutation in the dystrophin gene, which strengthens and stabilizes the sarcolemma during the stress of muscular contraction and, when absent, the sarcollema ruptures and allows calcium to enter, which causes the muscle fiber to necrotize. The object of the present study was to perform the morphologic analysis of the anterior tibial and the gastrocnemius muscles with (w/pa) or without (n/pa) physical activity for five weeks. We used 72 mice, divided in 12 experimental groups – 6 of them mdx and 6 control groups (C57/10J) aged 4, 7, and 10 weeks. The samples were collected, processed and stained with hematoxylin-eosin. They were analyzed by light microscopy, selected and photomicrographed. On the cross-sections of control animals aged 4, 7 and 10 weeks w/pa and n/pa, polygonal muscle fibers of many different sizes, ellipse-shaped and with several peripheral nucleuses were observed. In the mdx mice w/pa aged 4, 7 and 10 weeks, the muscle fibers showed different shapes, sizes and stain affinities, rounded edges, anuclear or centered nucleuses; hyalines and myofibrillas were highly contracted. Muscular regeneration and nectrotic areas with inflammatory infiltrates were identified in the mdx animals aged 4, 7 and 10 weeks w/pa, as well as in animals aged 10 weeks n/pa. With the progression of the disease in the animals submitted to physical activity, there was evidence of failure in the regeneration and muscular degeneration, intensified and characterized by the gradual replacement of the striated skeletal muscle tissue by fibroadipose connective tissue.

Keywords: dystrophinopathies, mdx, skeletal muscle, muscular dystrophy, duchenne.

1 Introduction

Emery (2002), on Duchenne's Muscular Dystrophy (DMD), reports that the first signs appear between 3 and 5 years of age, when the child has difficulties to move, climb stairs, run, stand up from the ground, among others. Gowers' sign can be observed, with frequent falls, increased calf girth (pseudo hypertrophy due to the replacement of the necrotized muscle fibers by fat and fibrous tissue), anserine walk, increased lumbar lordosis, and, later, kyphoscolliosis due to the atrophy of paravertebral musculature. At age 11, the child is unable to walk, and the diaphragm is usually affected by age 20, causing respiratory insufficiency, respiratory failure and/or respiratory infections.

Muscular dystrophies are a heterogeneous group of muscular pathologies, characterized by the atrophy and destruction of muscle fibers. Several genetic defects in the transmembrane muscular proteins, which link the cytoskeleton dystrophin to laminin-2 of the extracellular matrix, are responsible for muscular dystrophies. The most important muscular proteins involved are dystrophin, the dystroglycan complex, laminin-2 and the sarcoglycan complex. Dystrophin strengthens and stabilizes the sarcolemma during the stress of muscular contraction. When dystrophin is absent, the sarcolemma ruptures and allows the entrance of calcium, which causes muscular fibers to necrotize. Dystrophin deficiencies are characteristic of Duchenne's Muscular Dystrophy (DMD). Such a dystrophy is a recessive defect linked to the chromosome X, caused by a mutation in the dystrophin gene. Sudden episodes of vomiting are observed, caused by slow gastric emptying, in addition to abdominal pain. A typical laboratory data is the high serological level of creatine kinase. On the other hand, muscle biopsies reveal muscle destruction and absence of dystrophin, detected with immunohystochemical techniques (KIERSZEMBAUM, 2004).

Studies performed by Porter, Khanna, Kaminski et al. (2002) show that inflammatory cells, such as neutrophils, macrophages and mastocytes, contribute with the degeneration and regeneration cycles of the myofibers.

Anomalies in dystrophin or in some of the proteins associated to it, as a consequence of genetic alterations, result in diseases named muscular dystrophies, characterized by a progressive degeneration of the muscles, which may impair

the functions of the heart and lungs, resulting in death (DE ROBERTIS and HIB, 2001).

The *mdx* mouse is both a genetic and biochemical experimental model of muscular dystrophy in humans. Since it is easy to maintain and handle, the model has been widely used in the study of dystrophinopathies (COLLINS and MORGAN, 2003). This strain of mice appeared spontaneously in a C57BL/10J colony, the result of a point mutation that creates an ending translation codon on exon 23 of the dystrophin gene (BULFIELD, SILLER, WIGHT et al., 1984).

Mdx mice, aged approximately four weeks, present muscle degeneration due to the lack of functional dystrophin. In this period, the occurrence of instances of necrosis, significant amounts of regenerating myofibers with centered nucleuses and high serum concentrations of muscular creatine kinase can be observed. A mild case of myopathy associated to fibrosis remains throughout the animal's lifetime, and it only becomes acute with senility (WATCHKO, O'DAY, and HOFFMAN, 2002; COLLINS and MORGAN, 2003). Although it is apparently milder than the human type, the degeneration is intensified when the animals are submitted to physical activity (TSENG, ZHAO, PATTISON et al., 2002; DE LUCCA, PIERNO and LIANTONIO, 2003). In an attempt to regenerate the affected muscles, satellite cells are activated, undergoing cellular proliferation and fusion with myofibers (CHEN and GOLDHAMER, 2003).

Mutant mice have constitutive differences in muscle tissue, with higher amounts of connective tissue in the intercellular spaces when compared to normal animals (WATT, JONES and GOLDRING, 2004).

During the normal growth of the skeletal muscle, the total amount of DNA increases as muscular mass also increases. Due to the constance in the DNA content per nucleus within the same species, it should be concluded that the number of myonucleuses increases during the growth stage. Therefore, the first source of the aforementioned nucleuses seems to be a satellite cell, both in the normal growth of myofibrillas to produce new myofibers and the reparation of injured or diseased fibers. Satellite cells were first identified and named by Katz (1961 apud CAMPION, 1984) and Mauro (1961 apud CAMPION, 1984), who suggested that satellite cells would provide nucleuses for myofibers during the muscle regeneration process (CAMPION, 1984).

We set off to perform the present study by considering the data in literature and the need of morphological studies that could characterize Duchenne's Muscular Dystrophy more thoroughly.

2 Material and methods

The study was developed in the Research Laboratories of Universidade de Ribeirão Preto, in the Research Center for Muscular Dystrophy – *Centro de Pesquisas em Distrofia Muscular* (AADM, 2006), as well as the UNESP Electronic Microscopy Laboratory in Jaboticabal – *Laboratório de Microscopia Eletrônica de Varredura da Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal* – UNESP. The project was fully approved by the UNAERP (2006) Review Board, file ComÉt: #060/06.

In this study, 72 male mice were used, with 36 of them being controls (C57BL10) and 36 being mdx (X-linked muscular dystrophy). 24 animals were distributed according to age, according to Table 1.

The animals were kept in autoclavable polypropilene shelves organized in an isolating rack (Alesco), with a thermostat and a timer. Food and water were consumed ad libitum with 12 hour light cycles. At the beginning of the study, the characterization of the mice were performed with dosages of blood creatine kinase and hystologic evaluation of the skeletal striated muscles (right anterior tibial and right gastrocnemius). In order to perform these tests, 48 mice aged 4 and 7 weeks were anesthetized with pentobarbital (Nembutal, 40 $mg.kg^{-1}$ – ip) and sacrificed, with controls and *mdx* without physical activity and after 5 weeks of physical activity.

2.1 Dosage of creatine kinase (CK)

The blood levels of the creatine kinase (CK) enzyme of the animals without physical activity aged 4, 7 and 10 weeks used in the study were analyzed on a weekly basis. Blood samples (around 500 µL) were collected from each animal and kept at 4 °C during 12 hours for coagulation. The serum was removed and the CK activity was quantified with the creatine phosphotransferase kit (Doles brand).

2.2 Structural analysis

Skeletal muscle samples (right anterior tibial and right gastrocnemius muscles) of control and *mdx* animals, with and without physical activity, were collected from the same region, after the animal had been anesthetized. The anterior region of the right lower limb of each animal was cut open to collect the anterior tibial muscle sample, and the posterior region of the same limb for the sample of the right gastrocnemius muscle. After the collection had been performed, the samples were washed with saline solution and split in two parts.

The first part was fixated during 24 hours in a 4% formaldehyde solution, dehydrated in a crescent series of alcohols, diaphanized in a xylol solution and included in paraffin in a guided way, in order to obtain cross-sections. Microtomy was semi-seriated with a thickness of 5 µm. Staining was performed according to Gomori's trichrome technique, making the collagen fibers evident (ROMEIS, 1968; BURKITT, YOUNG and HEATH, 1994; GOMES, LIBERTI and DE SOUZA, 1997). The obtained histologic cross-sections were analyzed with a light microscope, selected

Table 1. Distribution of control and *mdx* animals.

	4 weeks		7 weeks		10 weeks	
	(C57BL/10J)	Mdx	(C57BL/10J)	Mdx	(C57BL/10J)	Mdx
w/pa	06	06	06	06	06	06
n/pa	06	06	06	06	06	06

w/pa – with physical activity; n/pa – no physical activity.

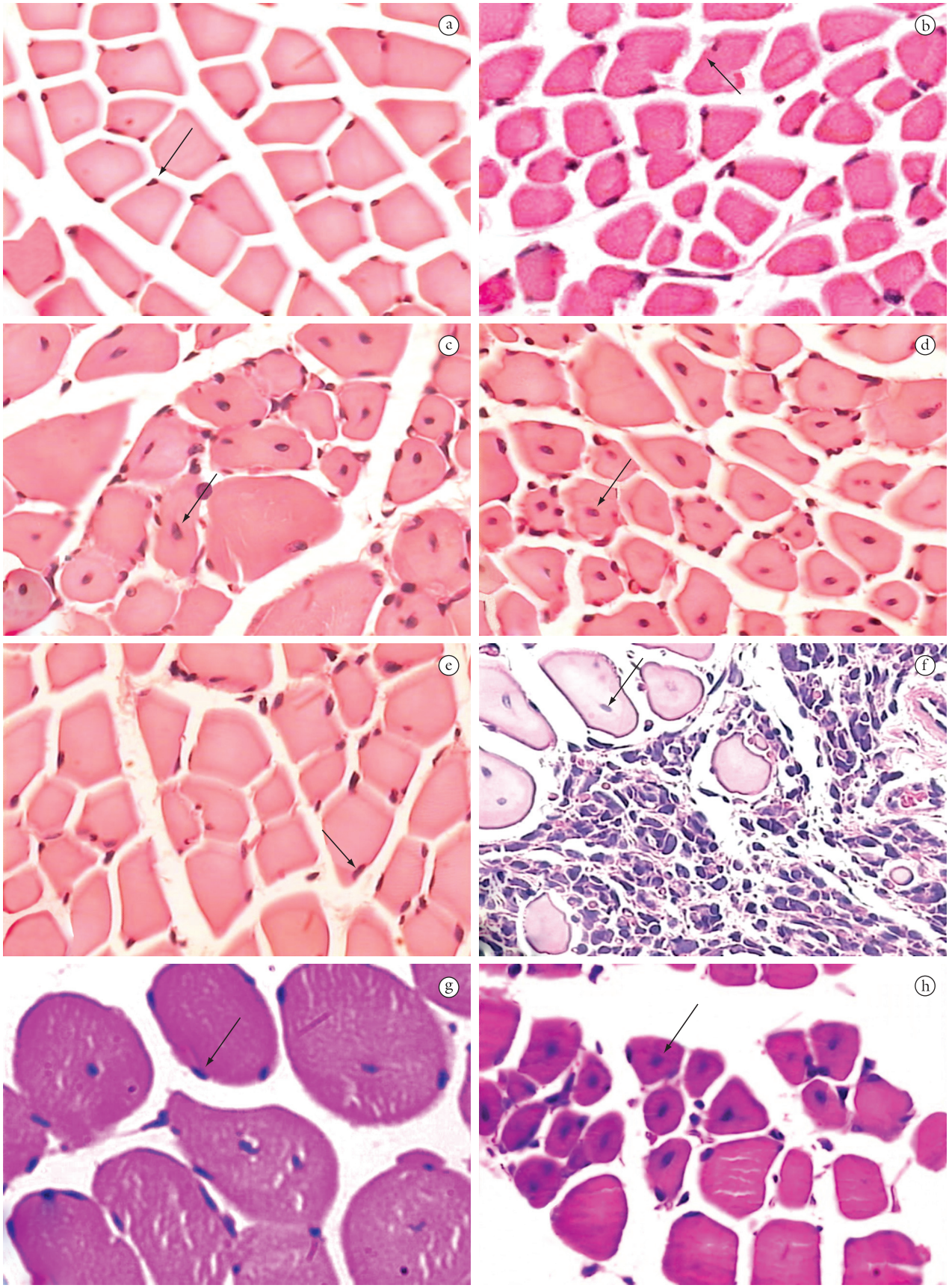

Figure 1. Photomicrographs M. gastrocnemius (a-d) animal control C57 4 weeks w/pa(a), n/pa(b), *mdx* animal w/pa(c) and *mdx* 7 weeks w/pa(d). M. tibialis anterior of control animals 4 weeks w/pa(e) and mdx w/pa(f). *Mdx* mice of 07 weeks w/pa(g) n/pa(h). HE-40X. Arrow indicates the nuclei.

and photomicrographed. The structure of each striated skeletal muscle was studied, focusing on its stain affinity, the position of the nucleuses, as well as their shape and size.

2.3 Image acquisition and analysis

The analyzed images were obtained with a Leica DFC 300FX digital video camera connected an Axiovert 135 binocular microscope, with a 40× objective. The image processing and analysis software Leica QWin was used for the analysis.

3 Results

This study found structural alterations in the muscular tissue of the mdx animals submitted to physical activity. These alterations were due to a higher intensity of the degenerative processes, different from those that occurred in the control animals (C57BL/10J) with physical activity.

3.1 Structural analysis

The striated skeletal muscle fibers of the gastrocnemius and anterior tibial muscles were found to be enlarged in the control mice (C57BL/10J), aged 4, 7 and 10 weeks, subjected to physical exercise for 5 weeks. Similar data regarding the increase of each muscle fiber were observed in the *mdx* animals of the same age submitted to physical activity during a 5-week period. Centered nucleuses were observed in these fibers, opposed to what was observed in the C57 mice. Fibers with different sizes and stain affinity were verified in both muscles. Fibers with irregular edges were observed in the gastrocnemius of the mdx animals submitted to physical activities. Some of them were wide and large, with centered nucleuses. Large muscle fibers, with small spaces in between, characterized the striated muscles of the mdx animals aged 7 weeks submitted to physical activity. Different fiber sizes occurred in the striated muscles of animals aged 4, 7 and 10 weeks, with the larger differences being observed in the gastrocnemius. Necrotized fiber occurrences with inflammatory infiltrates were found in the striated muscles of 10-week-old *mdx* animals submitted to physical activity. Occurrences of regenerating, smaller fibers with large centered nucleuses, with basophilic cytoplasm were more frequent in the mdx animals aged 4 and 7 weeks. (Figure 1)

Such findings agree with Watchko, O'day and Hoffman (2002) and Collins and Morgan (2003), as well as Tseng, Zhao, Pattison et al. (2002) and De Lucca, Pierno and Liantonio (2003), which also found centered nucleuses in the muscle fibers of the dystrophic mice, in addition to the intensification of the degenerative process in the animals submitted to physical activity.

In the striated muscles (tibial and gastrocnemius) of the mdx mice that were not submitted to physical activity were observed focal inflammatory infiltrates, with the presence of macrophages and lymphocytes. The infiltrates were considered moderate in both the gastrocnemius and tibial muscles. In the animals submitted to physical activity, a moderately-diffuse inflammatory infiltrate was evident with the predominance of neutrophils, fiber edema with reduced interfascicular spaces, anuclear fibers (degenerating) and some necrosis spots for all the striated skeletal muscles studied. Moderate occurrences of regenerating fibers were observed both in the gastrocnemius muscle of *mdx* mice submitted to physical activity and in those mice not submitted to it, as well as moderate occurrences of regenerating fibers, with a higher number of such fibers in the animals with physical activity (Figure 1).

4 Conclusion

Dystrophinopathy progresses according to the age in the *mdx* animals submitted to physical activity, being characterized both by the increased number of occurrences of necrotized fibers with infiltrates. The *mdx* phenotype increases with physical activity. It can be concluded that the number of connective (collagen) fibers increases due to physical activity, which was proven later with the morphometric results.

Acknowledgements: This work was supported by UNAERP and CNPq/PIBIC.

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Received June 17, 2010
Accepted September 30, 2010