Dystrophic animal models of diaphragm morphology for muscle ultrastructural analysis

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The Scanning Electron Microscopy (SEM) is a technique used for morphological analysis and provides a critical component that shows tridimensional and microscopic morphology. However dystrophic muscles like diaphragm are damaged on this technique during the material processing, making it difficult for visualization of the desired area. This has been observed in Golden Retriever Muscular Dystrophy (GRMD) and in the mdx mice that are animal’s models homologous to human Duchenne Muscular Dystrophy (DMD). These animals are widely used in preclinical trials of DMD and have severe impairment of the diaphragm. Facing this challenge, we have adapted a few steps of the conventional SEM protocol and scanning electron micrography in histology slides with 10 micrometres cuts. The muscle fascicles, the epimysium, endomysium and the organization of the diaphragm muscle were precisely identified and confirm the methodology and analysis results. In our work, a comparison of Canine and Mice X linked muscular dystrophy model was made showing main differences and also, critical points of fragility of this organ.

Keywords: histology; mdx; scanning electron microscopy

1. What is Duchenne Muscular Dystrophy?

Currently, Duchenne Muscular Dystrophy (DMD) is described as one of the most devastating forms of hereditary myopathies, rapidly evolving and with an incidence of 1 in 3,500 males born [1-2-3]. It is a recessive genetic disease, linked to the X chromosome, produced by a mutation of the gene which codes the dystrophin protein [4].

Dystrophin corresponds to only about 0.002% of the protein mass of striated muscle cell; it is located on the intracellular surface of sarcolemma and is associated to many integral membrane glycoproteins, forming the dystrophin-glycoprotein complex (DGC) [5]. The absence of dystrophin in the muscle cells promotes a gradual death as they undergo tension by muscle contractions [2-6].

The muscle weakness observed starts at two years of age in the pelvic girdle and it occurs afterwards in the shoulder girdle. Around 20 to 30 years of age, depending on the ventilation resources used, patients died due to impairment of respiratory muscles. Thus, 80% of the cause of death in patients with DMD is due to respiratory failure along with an infection or heart failure [4-7-8-9].

The main muscle involved in the breathing process is the diaphragm. In DMD, the diaphragm failure leads to a pulmonary impairment which is exacerbated by structural changes in the rib cage. Moreover, they are affected by structural deformities, the most prominent being scoliosis, which causes a dramatic decrease in lung functions and an increase in the mechanical load applied to each breath [8-10].

These data characterize DMD as a severe myopathy that, despite extensively studied, does not have a treatment able to stop or reverse the progression of the disease, yet.

2. What are the most frequently used clinical models?

An accurate identification of the DMD gene led to animal models of the disease [2]. Clarifying the pathogenesis, the degree of toxicity, and the efficacy of therapies for DMD through animal models is indispensable to assess the development of the therapies proposed.

The Golden Retriever dog model [11] and the murine mdx model are the most widely studied animals with regard to DMD (Fig. 1).

The Golden Retriever model of muscular dystrophy (GRMD) is characterized by progressive muscle weakness with posterior atrophy caused by a mutation in exon 7, resulting in the absence of dystrophin [3-11]. Clinical signs observed in GRMD occur gradually, with the loss of muscle mass, contractures, and skeletal deformities, assuming characteristics similar to those of human DMD [12].

The mouse mdx is characterized by a mutation in the dystrophin of exon 23 and skeletal muscle inflammation starts within three weeks of life, reaching its peaks between 8 and 16 weeks. Although having a smoother phenotype, these
animals present a severe impairment of the diaphragm and total absence of dystrophin in the skeletal muscle [13- 14- 15- 16].

Fig. 1– Canine (GRMD) and murine (mdx) models of muscular dystrophy.

3. How to analyze and process the diaphragm in the dystrophic models through scanning electron microscopy?

The morphology of diaphragm in dystrophic models may have its internal morphology analyzed by adapting some steps of the conventional processing of scanning electron microscopy (SEM). Membranous structures and muscles with a small amount of muscle mass in dystrophic animals may benefit from this technique obtaining more specific morphological data of DMD, which cannot be accurately assessed in the conventional processing of SEM.

3.1 Processing for inclusion in canine and murine diaphragm

The fragments of the diaphragm of GRMD and mdx models were collected and fixed in 4% paraformaldehyde for a 24-hour period.

In the GRMD model, the fragment collected was placed in an ascending series of ethanol (70% to 100%) for dehydration and in xylene for diaphanization. The time for each series of ethanol was 30 minutes, followed by 20 minutes for diaphanization. In the mdx model, the procedure for dehydration in an increasing series of ethanol (70% to 100%) and for diaphanization was carried out similarly, but with a time of 20 minutes for each series of ethanol followed by 15 minutes for diaphanization. Subsequently, the conventional procedure was used in both animals, for inclusion in Paraplast® (Leica/Germany), manufacturing 3 × 4 cm rectangular blocks.

The blocks were cut to 10 μm in a microtome Leica RM 2065 to obtain the slices and, then, deparaffinized in a stove at 60°C for 2 hours. Afterwards, the slices were placed in xylene for 20 minutes, in order to remove excess paraffin, and dried in the critical point apparatus Balzers CPD020 for 15 minutes.

3.2 Scanning electron microscopy

The 10 μm slices, after being removed from the critical point apparatus, were immediately covered with gold through the sputtering process (EMITEC H K550), and ultrastructural observation and photodocumentation were carried out through a scanning electron microscope LEO 435VP (Fig. 2).

Fig. 2– Process of scanning electron microscopy.
3.3 Analysis of the diaphragm of the canine and murine models of muscular dystrophy

The diaphragms of *mdx* and GRMD models were compared to a normal group. In the histological hematoxylin and eosin (HE) analysis with a 3 μm histological section (Fig. 3A) of the diaphragm of normal animals (BALB/C57), the presence of peripheral nuclei (indicated by a circle), endomysium (End) (indicated by an arrow), and the diameter of regular muscle fibers (Fb), grouped into fascicles, was observed. In a different manner, the *mdx* model (Fig. 3B), which presented areas of perimisial infiltrate (*), central nuclei (indicated by a triangle), and irregularities in the diameters of muscle fibers, with atrophic (At) and hypertrophic (Hp) fibers.

In the SEM analysis carried out with a 10 μm histological section, there were in the mice normal group (Fig. 3C) muscle fibers (Fb) grouped into fascicles with regular diameters, endomysium (Endo), and epimysium (Ep). The *mdx* model (Fig. 3D) showed a disorganized morphology of the diaphragm with irregular diameters, suggesting areas of fibrosis and/or perimisial inflammatory infiltrate (*).

![Fig. 3](image)

Fig. 3 – Cross section of the mouse diaphragm BALB/C57 (A and C) and *mdx* (B and D). A) (HE, 20x), one observes at the circle the peripheral nuclei (HE, 20x). It’s noticed in B) a perimisial inflammatory infiltrate (*), an endomysial infiltrate (End), indicated by an arrow; central nuclei (located within the triangle), atrophic muscle fibers (At), and hypertrophic muscle fibers (Hp). In C) and D), it’s observed, in the SEM, the mouse diaphragm BALB/C57 (C, bar: 10 μm) and *mdx* (D, bar: 10 μm). In (C), one observes the epimysium (Ep), the perimysium (Peri), and the muscle fibers (Fb), and, in (D), the perimysium (Pe).

The histological analysis, we found similarities between normal (dog and mice) and affect (GRMD and *mdx*). Similarly to *mdx*, GRMD presented a diaphragm with disorganized morphology and irregular diameters, suggesting areas of fibrosis and/or perimisial inflammatory infiltrate (Fig. 4).
Fig. 4 – Cross section of the normal dog diaphragm (A and C) and the dog affected by muscular dystrophy (B and D). In A) (HE, 20x), one observes at the circle the peripheral nuclei (HE, 20x). It’s noticed in B) a perimisial inflammatory infiltrate (*), an endomysial inflammatory infiltrate (End), indicated by an arrow; central nuclei (located within the triangle), atrophic muscle fibers (At), and hypertrophic muscle fibers (Hp). In C and D), it’s observed, in the SEM, the normal dog diaphragm (C, bar: 10 μm) and the dog affected by muscular dystrophy diaphragm (D, bar: 10 μm). In (C), one observes the epimysium (Ep), the perimysium (Peri), and the muscle fibers (Fb), and, in (D), the perimysium (Pe).

4. Conclusion

Using the conventional protocol of SEM for the morphology analysis of the diaphragm in dystrophic animal models does not allow visualizing the diaphragmatic morphology in detail. However, with the adaptation of the protocol for analysis in a histological slice of 10 μm cuts, we were able to identify characteristic structures of dystrophic animals that could not be identified through processing.

References


