Original article

# Morphologic features from *mdx* mice spleens, used for duchenne muscular dystrophy studies

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## Abstract

The *mdx* mice model is widely used for Duchenne muscular dystrophy (DMD) studies, which is present in a high percentage from newborns human males. Therefore, the aim of this study was to evaluate possible morphological changes from spleens in these mice and to compare with normal mice (*Mus musculus*) in contribution to DMD understanding and its consequences on immune system by affected individuals. The study was performed by light and scanning electron microscopy (SEM) techniques beyond immunohistochemistry. Was found microscopically an increased number of lymph nodes and decreased in red pulp region by *mdx*, beyond a larger VEGF-C (vascular endothelial growth factor C) expression stimulates lymphangiogenesis in red pulp region from spleen. These findings suggest a spleen adaptation in order to supply immunological demand due upper respiratory infection, which are common in individuals affected by Duchenne muscular dystrophy.

**Keywords:** lymph nodes, lymphocytes, muscular dystrophy, red pulp, respiratory inflammation.

## 1 Introduction

The Duchenne Muscular Dystrophy (DMD) is a neuromuscular hereditary disease, which is character-linked recessive X chromosome. This disease is found only in boys at rate one to each 3500 newborns. Muscular dystrophies linked to X chromosome are described in other species such as mice (*mdx*), dogs (GRMD) and cats (HFMD). Thus, because these animals are homologous to DMD, it has been widely used as a model for the disease (FADIC, 2005).

The absence of dystrophin protein gene in dystrophic diseases provides increased permeability in muscle membranes, increasing calcium concentration, leading enzymes activation that cause breakdown in muscle cells (BERGMAN, INZANA, MONROE et al., 2002).

The loss of ability to regeneration could be a result from myogenic cells exhaustion caused by an excessive degeneration and regeneration cycles (LUZ, MARQUES and SANTO-NETO, 2002).

In this regard, the *mdx* mice are a widely used model for human DMD studies due easier reproduction, genetic uniformity, economy and convenience for laboratory experiments (SEIXAS, LAGROTA-CANDIDO, WILSON et al., 1997; LYNCH, HINKLE and FAULKNER, 2001; GOSSELIN, BARKLEY, SPENCER et al., 2003).

Therefore, the aim here was to verify possible morphophysiological changes due muscular dystrophy from *mdx* mice spleen and thus contribute to the clinical, surgical and pathological anatomy, since there are few studies related to the changes of the immune system by Duchenne muscular dystrophy affected individuals.

## 2 Material and methods

### *2.1 Animals*

Were utilized five *mdx* mice males and five normal mice (*Mus musculus*) aged five months from Science Biomedical Institute (ICB) by São Paulo University, São Paulo, Brazil.

This research was approved by the bioethics committee of the Faculty of Veterinary Medicine and Animal Science at São Paulo University (FMVZ/USP).

### *2.2 Euthanasia and collects*

The animals were euthanized by anesthetic ketamine 50 mg/kg (Ketamin-S®, Cristália) associated with 2 mg/kg xilazina hydrochloride (Calmiun®, Union Agener) by intraperitoneal way. The spleens were macroscopically analyzed, measured, collected and fixed on paraformaldehyde 4%.

### *2.3 Laboratories*

To the performance this work we used Histology, Embryology and Electron Microscopy Laboratories of Faculty of Veterinary Medicine and Animal Science at São Paulo University, São Paulo-SP.

### *2.4 Light microscopy*

Samples were dehydrated in a graded ethanol series (60-100%), cleared in xylene, and embedded in paraffin. Sections of 5 µm were obtained on a microtome (Leica RM 2155) and stained with Hematoxylin-eosin, Picrosirius and Masson's trichrome. Microscopy slides were mounted with Entellan (Historesin Merck). We utilized a microscope (Leica DM 2000) coupled with an image capture system to study tissue morphology.

### *2.5 Scanning electron microscopy*

Tissue fragments were fixed in glutaraldehyde (Propylene oxide EM Grade-Polysciences, Inc., USA), washed in 0.1 M phosphate buffer at a pH of 7.4, and post-fixed in 1% osmium tetroxide (Spurr's Kit – Electron Microscopy Sciences Co., USA). Then, tissue fragments were dehydrated in a graded ethanol series (50%, 70%, 90%, and 100%) and dried in a critical point dryer (Balzers PCD 020). Tissues fragments were fixed in metal supports (stubs) and then sputter coated with gold (Emitech K550). The analysis was performed on an electron microscope, model Leo 435 VP.

### *2.6 VEGF-C immunohistochemistry (vascular endothelial growth factor)*

The material fixed on 4% paraformaldehyde were processed by routine paraffin embedding technique, which samples were obtained in 5 µm on histology blades and processed by VEGF-C immunohistochemistry protocol: citrate buffer in a microwave, followed by peroxidase block, protein block (Dako biotechnology, Carpinteria, CA, USA) and overnight incubated with primary antibody VEGF-C (AbCam, Cambridge, MA, USA) under 1:400 dilution. In the following day, the samples are incubated with secondary antibody and revealed with DAB (Dako biotechnology, Carpinteria, CA, USA). The samples are counterstained with hematoxylin.

### *2.7 Nomenclature*

We used nomenclature established by the International Committee on Veterinary Histological Nomenclature (INTERNATIONAL..., 1994) and the International Committee on Veterinary Gross Anatomical Nomenclature (INTERNATIONAL..., 2005).

## 3 Results

### *3.1 Macroscopic analysis*

The spleen is contained in left cranial part in abdominal cavity, related with stomach greater curvature from both experimental models groups in this study (Figure 1).

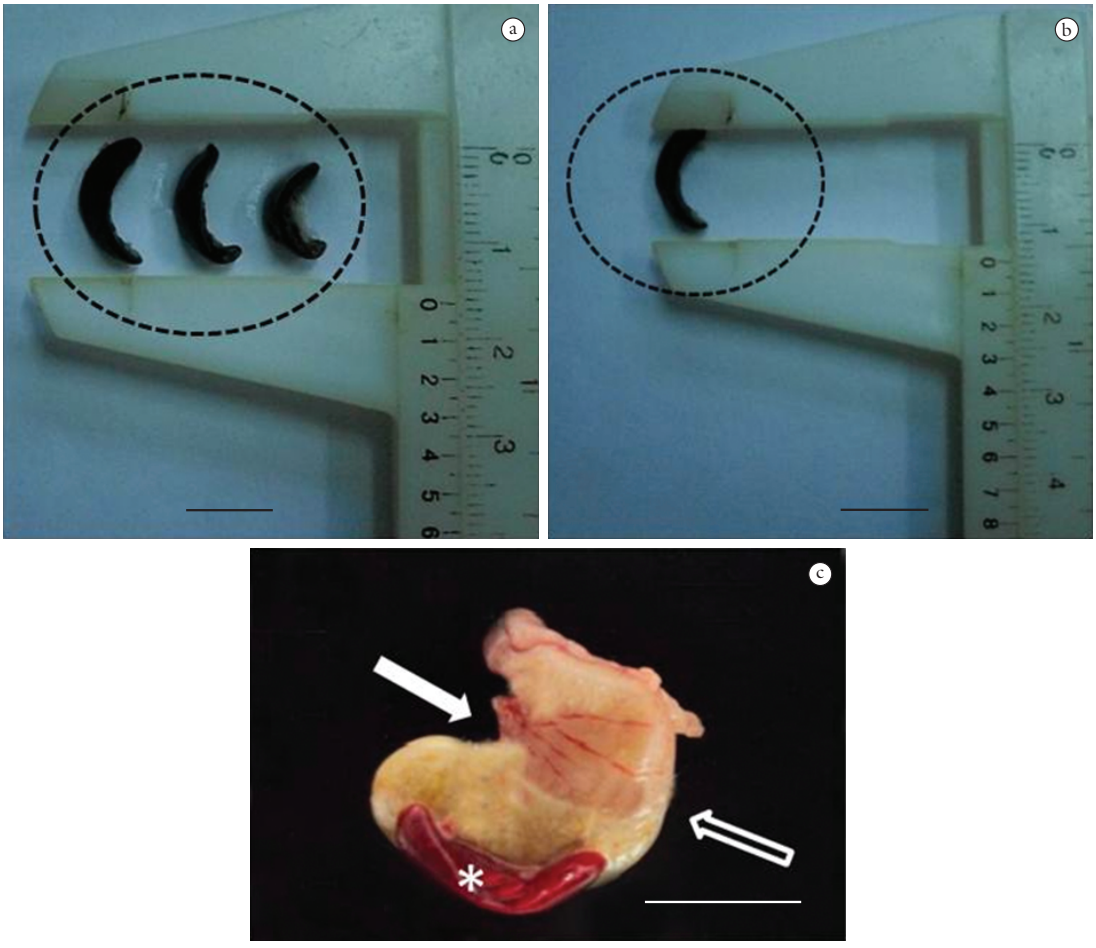


**Figure 1.** Picture from dystrophic spleens (*mdx*) and normal spleens (*Mus musculus*). a) *mdx* mice spleens; b) *Mus musculus* spleen (circle). c) stomach greater curvature (empty arrow), stomach minor curvature (full arrow), spleen (star). Bar: 1cm.

The above mentioned organ has two faces: a diaphragm and other visceral, which is present the hilum, which gives passage to vascular-nervous elements of the organ.

In this study there is a not large variation in shape, morphology and topography from spleens (Figure 1). Data regarding morphology spleens are summarized in Table 1.

### *3.2 Microscopic analysis*

By light and scanning electron microscopy, it might observe that spleens of both mice groups have a dense connective tissue capsule which sends a few trabeculae into organ parenchyma, where there are reticular and muscular fibers, beyond vessels and nerves (Figures 2 and 3).

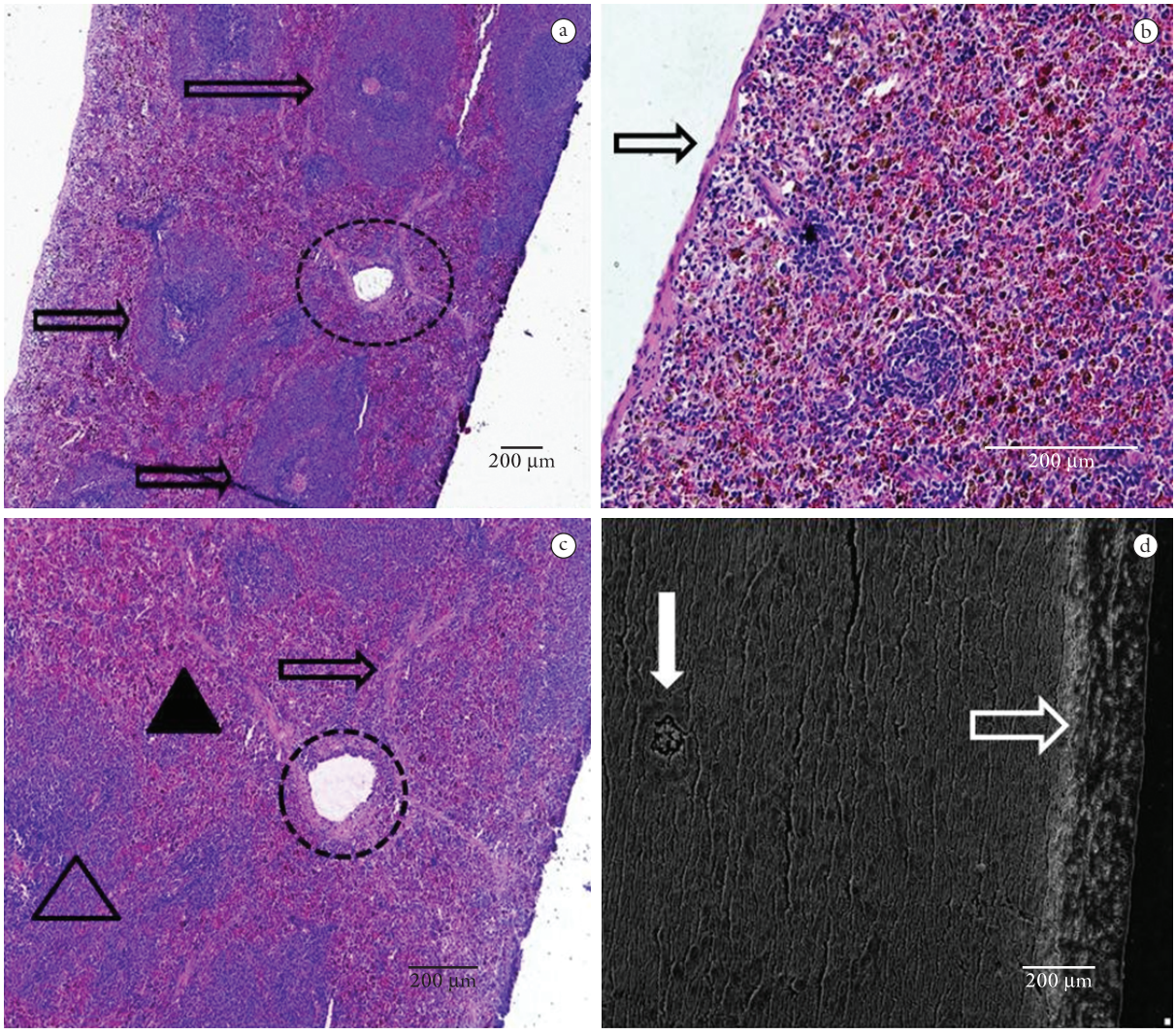


**Figure 2.** Photomicrography by light and scanning electron microscopy (SEM) from normal spleen (*Mus musculus*). a) lymph nodes (arrows) and trabecular arteries (circle). H.E. Bar: 200 µm; b) connective tissue capsule (arrow). H.E. Bar: 200 µm; c) White pulp region (empty arrowhead), red pulp region (full arrowhead), trabeculae (arrow) and trabecular arteries (circle). H.E. Bar: 200 µm; d) dense connective tissue capsule (empty arrow) and arteriole (full arrow). (MEV). Bar: 200 µm.

**Table 1.** Weight, length and thickness spleens measurements from normal and dystrophic mice, highlighting the average and standard deviation.

| Samples | Dystrophic mice (*mdx*) | | | Normal mice (*Mus musculus*) | | |
|---|---|---|---|---|---|---|
| | Weight (g) | Length (cm) | Thickness (cm) | Weight (g) | Lenght (cm) | Thickness (cm) |
| An.1 | 108 | 1,3 | 0,3 | 110 | 1,3 | 0,4 |
| An.2 | 105 | 1,2 | 0,2 | 108 | 1,3 | 0,3 |
| An.3 | 98 | 1,1 | 0,2 | 99 | 1,2 | 0,2 |
| An.4 | 91 | 1,1 | 0,1 | 95 | 0,9 | 0,1 |
| An.5 | 89 | 0,9 | 0,1 | 84 | 0,9 | 0,1 |
| **Average** | **98,20** | **1,12** | **0,18** | **99,20** | **1,12** | **0,22** |
| **Deviation** | **±8,35** | **±0,15** | **±0,20** | **±10,52** | **±0,20** | **±0,13** |

The parenchymal supporting structure contained in spleen of both species is divided into red and white pulp, where is possible to see clearly that the spleens of dystrophic mice has a greater amount of white pulp when compared to normal mice (Figures 2 and 3).

The white pulp is divided into focus called lymph nodes. Where in dystrophic mice these lymph nodes are presented in greater numbers and less organized when compared to normal mice spleen (Figure 3).

The red pulp on the other hand, consists in series with spaces occupied by blood vessels and an enormous concentration of blood cell elements that provide the red characteristic featuring the name given to those regions of the spleen.

The spleen irrigation is by splenic artery, which sends branches entering the hilum of the spleen and forms the trabecular arteries (Figures 2 and 3).

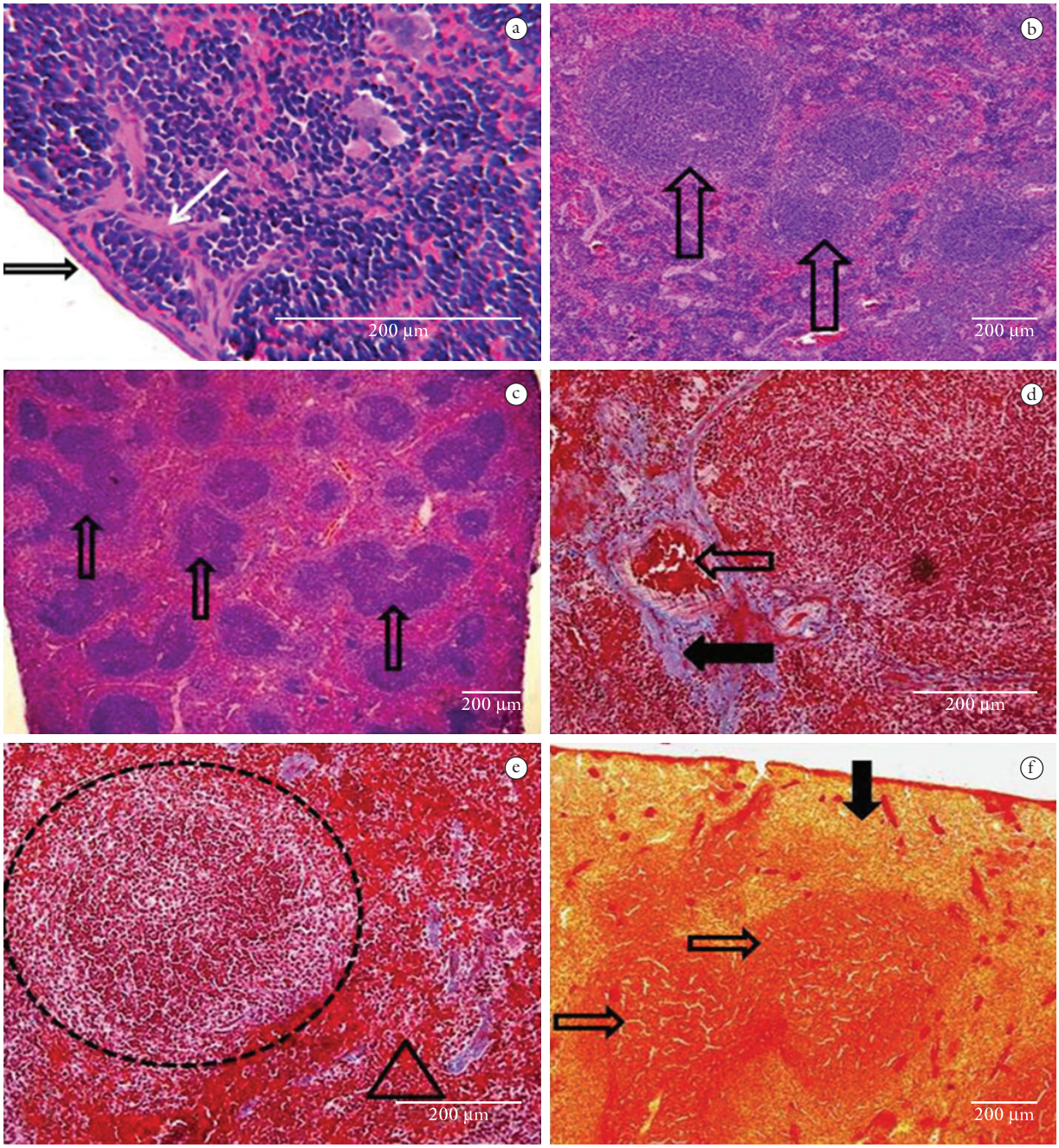


**Figure 3.** Light photomicrograph from dystrophic spleen (*mdx*). a) connective tissue capsule (thick arrow) that sends inner trabeculae from parenchymal organ (thin arrow). H.E. Bar: 200 µm; b) lymph nodes (arrows). H.E. Bar: 200 µm; c) lymph nodes dispersion into parenchymal organ (arrows). H.E. Bar: 200 µm; d) trabecular arteries (empty arrow) and elastic fibers trabeculae. Masson Trichrome. Bar: 200 µm; e) white pulp region (lymph nodes) (circle) and red pulp region (arrowhead) differences. Masson Trichrome. Bar: 200 µm; f) Collagen fibers differences: type III collagen (full arrow) into white pulp region and type I collagen (empty arrows) from red pulp region. Picrosirius. Bar: 200 µm.

These trabecular arteries turn branches to form central arteries or nodular found in lymph nodes in both species of this study.

In spleens is not possible to show a cortical and medullar area, but by light microscopy, is possible to see that highest concentration of white pulp area (lymph nodes) is at the center from organ, while the area of red pulp predominates the ends organ and interspersing the lymph nodes in normal mice, already in dystrophic mice has not found the organization since the lymph nodes are more scattered and larger quantities, however are apparently smaller and deformed shapes nodules (Figures 2 and 3).

Moreover, in histological sections by Picrossírius stain are significant differences in types of collagen fibers between white pulp regions with type III collagen and red pulp region with type I collagen in both species of this study (Figure 3).

### *3.3 VEGF-C immunohistochemistry*

The test results by immunohistochemistry show strong VEGF-C expression in red pulp region from *mdx* mice spleen, whereas the control group by normal mice shows less intensity expression in the same region of spleen (Figure 4).

In white pulp region does not occur VEGF-C expression from both groups of animals (Figure 4).

## 4 Discussion

The spleens topography to mice dystrophic (*mdx*) compared with normal mice (*Mus musculus*) follows the standard for domestic animals and humans (AGUIAR, BARRETO, MORAIS et al., 2008).

Small morphometric differences from both species studied are as a result of different body measurements of the specimens, which can vary in size, shape and measured in same species and between different species as related in domestic animals and humans (AGUIAR, BARRETO, MORAIS et al., 2008).

Therefore the morphometric variations found herein are not intended to diagnose abnormalities resulting from muscular dystrophy in *mdx* mice. Early research

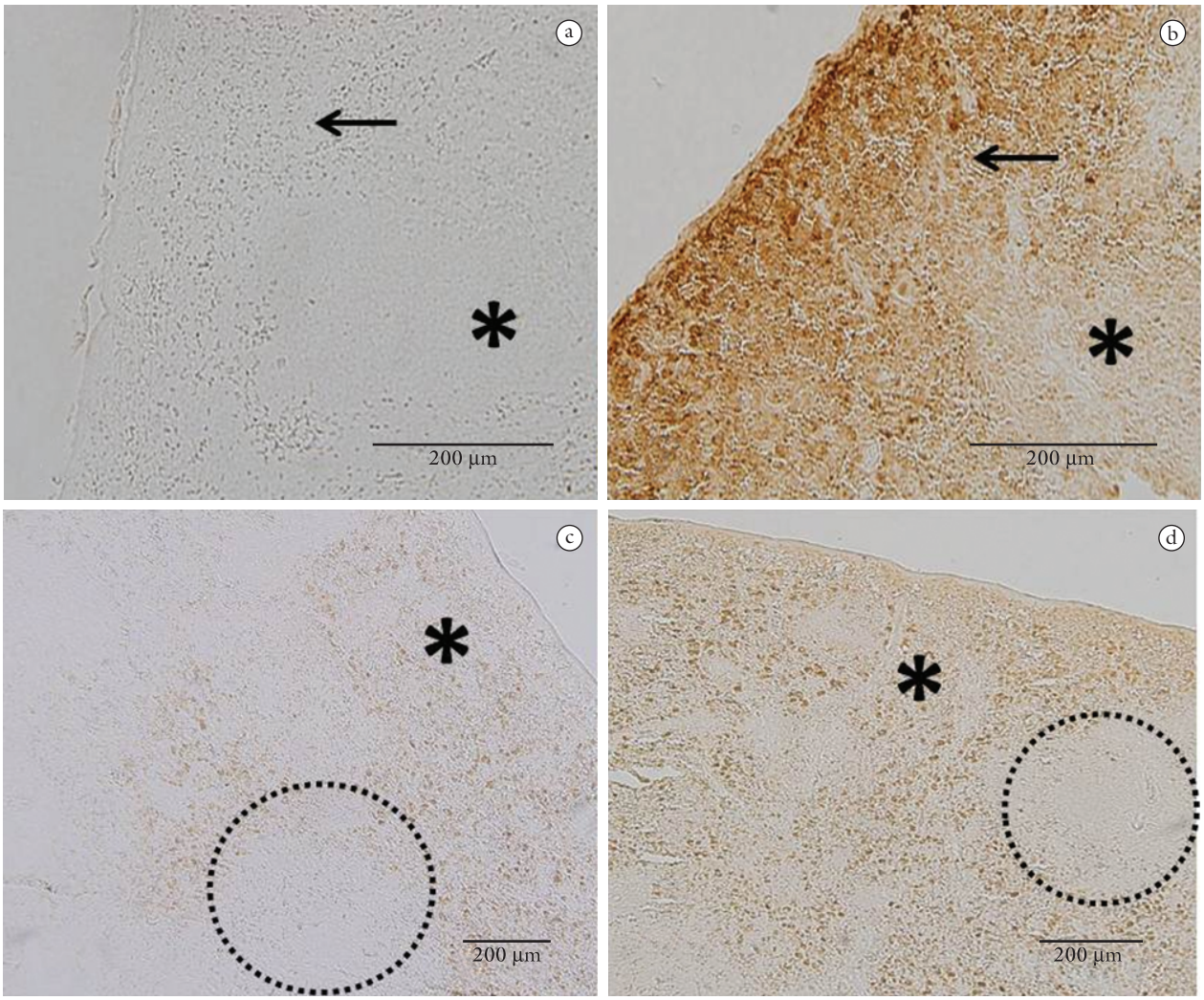


**Figure 4.** VEGF-C immunohistochemistry from dystrophic spleens (*mdx*) and normal spleens (*Mus musculus*). a) control for *mdx* spleen: red pulp region (arrow) and white pulp region (star). Bar: 200 µm; b) *mdx* spleen with strong VEGF-C expression in red pulp region (arrow) and VEGF-C absence in white pulp region (star). Bar: 200 µm. c) control for normal mice spleen: white pulp region (circle) and red pulp region (star). Bar: 200 µm. d) Normal mice spleen: red pulp region (star) and White pulp region (circle). Bar: 200 µm.

with mice and human patients with *Chagas* disease, the spleen undergoes a slight increase in weight, which could be consistent not only the hemodynamic factor, but also as systemic response to *T. cruzi* infection (PEREIRA, CORRÊA, MINICUCCI et al., 1999).

In another study, rat's spleen tissue removed appears to increase physical performance of these, possibly by a mechanism related to splenic metabolism and endocrine sexual mediators (CALDEIRA, ROCHA, ALBERTI et al., 2005).

Ioshii, Sakamoto, Machuca et al. (2004) argues that human's spleen may vary due injuries, often caused by lymphomas, hematomas, abscesses or heart attack. On the other hand, pseudomotors inflammations splenic are rare in literature and clinical characteristics of this disease are abdominal pain, fatigue, weight loss and night sudoresis, which fever and leukocytosis are associated. The histological features from pseudomotors inflammation are cancer spread, tissue metastasis and organ weight changes, often related to imbalances in vascular endothelial growth factor expression (VEGF-C) (ORPANA and SALVEN, 2002).

In our results for both species, are not found cellular changes caused by metastases and no significant changes in weight of spleen when compared normal and dystrophic mice.

The muscular loss also leads oxygenation decrease in muscular fibers, but from mice dystrophic spleens, signs of metastases which is also linked to melanoma and patients with acute anemia were not present (ORNELLAS, LANZONI and TOLEDO, 2000).

Trotte, Santos, Menezes et al. (2010) describes that several factors might be related to malignancies development, such as, age, lineage, population, studied period, geographical location and nutrition for the animals.

Functionally, the red pulp region from spleen is responsible to blood filtering, while white pulp area composed of dense lymphoid tissue, are responsible for immune defense (PEREIRA, CORRÊA, MINICUCCI et al., 1999). These different regions that comprise the spleen, lead important functional implications, particularly in relation to antigenic stimulus and removal of hemoglobin and iron of red blood cells for subsequent phagocytosis (CORTEZ, BENEDICTO, AGRESTE et al., 2009).

Microscopic variations found here about relationship by differences between white and red pulp composition for both species seems have no implications as a result from animals nutrition, since Cortez, Benedicto, Agreste et al. (2009) researching normal and diabetic rats does not narrate variations in white and red pulp distribution.

Moreover the variations between a larger white proportion pulp region related to the red pulp region observed in dystrophic mice compared with normal mice, might be related to reduced oxygen demand fact due to muscle activity dystrophy caused by dystrophin expression absence, which affects this experimental model (SEIXAS, LAGROTA-CANDIDO, WILSON et al., 1997) and Duchenne muscular dystrophy from humans (FONSECA, MACHADO and FERRAZ, 2007).

However, results test by immunohistochemistry showed a strong VEGF-C expression in red pulp from *mdx* mice spleens. The vascular endothelial growth factor C is directly involved in lymphangiogenesis, what suggesting a strong neovascularization in red pulp region from spleen of these animals (ORPANA and SALVEN, 2002), indicating the occurrence of a compensation for decreased red pulp region from spleen, by an increased vascularity.

Another factor that must be taken into account regards spleen to serve as a blood cells reservoir and undergo adjustments when the organ is relaxed or contracted, or in stress or physical activity situations (AGUIAR, BARRETO, MORAIS et al., 2008).

Moreover, muscular dystrophy affecting several organs and stimulate the development of several inflammatory processes (SEIXAS, LAGROTA-CANDIDO, WILSON et al., 1997; FONSECA, MACHADO and FERRAZ, 2007).

Therefore, the most likely among from differences found in white and red pulp regions, is that the increase in regions of white pulp from spleens mice affected by Duchenne muscular dystrophy are adaptations to attend most demand for cytotoxic T lymphocytes due to increased injuries and respiratory infections in individual holder such a Duchenne muscular dystrophy, as reported in human patients affected by the same type of muscular dystrophy (FONSECA, MACHADO and FERRAZ, 2007; CAMMARATA-SCALISI, CAMACHO, ALVARADO et al., 2008).

Finally, trabecular artery located in splenic trabeculae found in both species from this study are narrated in conformity by other authors for other not affected by muscular dystrophy mammalian species (CORTEZ, BENEDICTO, AGRESTE et al., 2009).

Maybe immunohistochemical and gene expression studies from older *mdx* mice or higher muscular dystrophy stages might indicate different from those in this research or even be able to diagnose the functionality due to increased lymphangiogenesis and lymph nodes observed in *mdx* mice.

## 5 Conclusion

We conclude that morphological findings suggest a spleen adaptation in order to supply immunological demand due upper respiratory infection, which are common in individuals affected by Duchenne muscular dystrophy.

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