Histopathologic Evolution of Cardiomyopathy in a Canine Model of Duchenne Muscular Dystrophy

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a recessive X-linked disorder characterized for mutation in dystrophin gene and manifested by progressive degeneration and necrosis of skeletal and cardiac muscle with replacement leading to generalized muscular weakness and atrophy. The dog Golden Retriever Muscular Dystrophy (GRMD) is the best experimental model for DMD, with genotypic and phenotypic manifestations closely of human disease. Similar to patients with DMD, heart failure is a major cause of death in GRMD animals. The objective of this study was to evaluate the pathological progression of myocardial lesions from GRMD dogs in different ages in order to clarify the pathogenesis of Duchenne´s cardiomyopathy. Fragments of left and right ventricle and interventricular septum, from 18 GRMD dogs between 6 to 51 months were collected, fixed, dehydrated, clarified, and finally embedded in paraffin. Five micrometer thick serial sections were obtained and stained with Hematoxylin-Eosin (HE), Picrosirius red, and Von Kossa. Histological analyses were performed at the light microscopy. Myocardial lesions were observed in all GRMD dogs and the sequence of cardiac lesion classified according to according to the age included: abnormal calcium accumulation, myofibrillar necrosis, proliferation of granulation tissue, endomysial and perimysial fibrosis, and finally myocardial fatty infiltration. Interestingly, several Anitschkow cells, the hallmark of rheumatic carditis, were detected in inflammatory infiltrate present at granulation tissue. Our results demonstrate the sequence of cardiac lesions that determine the cardiomyopathy in Golden Retriever dogs affected by DMD and exhibit, for the first time, the Anitschkow cells in the histological findings of this cardiomyopathy. These results are relevant for to clarify the pathogenesis of cardiomyopathy in dogs and humans affected by DMD.

Introduction

Duchenne muscular dystrophy (DMD) is a recessive X-linked disorder characterized for mutation in dystrophin gene and manifested by progressive degeneration and necrosis of skeletal muscle with replacement by fat and connective tissue leading to generalized muscular weakness and atrophy (Valentine *et al*. 1989; Collins and Morgan, 2003). Dystrophin is the largest (2.2 Mb) gene in the human genome and essential member of the dystrophin glycoprotein complex (DGC), which protects the sarcolemma from mechanical stress during repeated cycles of muscle contraction and relaxation (Lapidos *et al*,. 2004; Allikian and McNally, 2007; Banks and Chamberlain, 2008; Kaspar *et al*., 2009). At the molecular level, the absence of dystrophin results in striking destabilization with delocalization of the rest of the DGC. Therefore, the mechanical link between the sarcolemma and the extracellular matrix is undermined leading to sarcolemmal fragility (Lapidos *et al*., 2004; Deconinck and Dan, 2007). Thus, the skeletal and cardiac

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muscle that lacks full-length dystrophin and the DGC is abnormally susceptible to damage from contraction leading to small tears in the membrane (Lapidos *et al.*, 2004; Deconinck and Dan, 2007; Kaspar *et al.*, 2009; Miyazato *et al.* 2011a).

In most cases of DMD, patients die during the third decade of life from respiratory or cardiac failure (Valentine *et al.*, 1989; Collins and Morgan, 2003; Kaspar *et al.*, 2009).

The use of animal models, mainly canine, in the research of DMD have provided valuable clues in elucidation of the pathogenesis and development of prospective therapies (Collins and Morgan, 2003; Miyazato *et al.*, 2011b; Kornegay *et al.*, 2012). In this sense, the Golden Retriever muscular dystrophy (GRMD) dog has been the most extensively studied and well characterized model (Valentine *et al.*, 1989; Collins and Morgan, 2003; Kornegay *et al.*, 2012). Both DMD and GRMD have progressive clinical signs and severe myopathy with early fiber necrosis and regeneration together with endomysial and perimysial connective tissue proliferation (Valentine *et al.*, 1990; Lanfossi *et al.*, 1999; Miyazato *et al.*, 2011b, Kornegay *et al.*, 2012). In addition, adult dogs also have a body mass comparable to DMD patients. Thereby, the GRMD dog is clinically, pathologically and genetically an excellent model of DMD (Cooper *et al.*, 1988). Similar to patients with DMD, heart failure is the leading cause of death in GRMD dogs (Moise *et al.*, 1991; Chetboul *et al.*, 2004). Previous study established the dog affected by muscular dystrophy as an animal model for study of Duchenne's cardiomyopathy (Moise *et al.*, 1991).

The aim of this study was to demonstrate the pathological progression of the myocardial lesions in GRMD dogs, seeking to a better understanding of Duchenne´s cardiomyopathy.

Materials and methods

Animals

A total of 18 male GRMD dogs between 5 and 51 months of age (5-8 months n=4, 9-11 months n=5, 13-18 months n=6, 20-51 months n=3) were examined in the present study. There were coming from the Brazilian GRMD colony at University of São Paulo. Five dogs with ages between 2 to 20 months were used as controls. The phenotype was initially determined based on the elevation of serum creatine kinase and confirmed by PCR. All animals died spontaneously from cardiac or respiratory arrest. This research was certified by the Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" adopted by "Ethic Committee in the use of animals" of the São Paulo State University.

Histopathology

Samples of the heart (left ventricle, interventricular septum, and right ventricle) were collected, fixed by immersion in phosphate-buffered 10% formalin, and after dehydrated, clarified, and embedded in paraffin. Five micrometer thick serial sections were obtained and stained with hematoxylin and eosin (HE) (morphological study), picrosirius red (interstitial fibrosis analysis), and Von Kossa (calcium deposits evaluation). The sequence of cardiac lesions including: mineralization, necrosis, granulation tissue formation, fibrosis, and fatty infiltration were semi-quantitatively evaluated using the following grading scale: 0 (absent) = none; 1 (minimal) = approximately 1–10% of the section; 2 (mild) = approximately 11–20% of the section; 3 (moderate) = approximately 21–30% of the section; 4 (marked) = more than 30% of the section (Kane *et al.*, 2013). Photomicrographs were taken with a Zeiss Axio Scope A1 (Carl Zeiss, Göttingen, Germany), videocamera (AxioCam MRc, Carl Zeiss, Göttingen, Germany) and a computer online.

Results

Gross Pathology

At the gross necropsy, signs of congestive heart failure including chamber heart enlargement and thoracic and abdominal fluid accumulation were found in 14 GRMD dogs (77.7%) between 6 to 51 months.

Histopathology and lesion score

Five main microscopic lesions were observed in cardiac muscles from GRMD dogs according to the age: abnormal calcium accumulation, myofibrillar necrosis, granulation tissue formation, endomysial and perimysial fibrosis, and myocardial fatty infiltration, as show in Table 1. Non cardiac lesion was

observed in controls dogs.

Table 1. Cardiac lesion score in GRMD dogs according to age

GRMD dogs ages	Microscopy lesions
6-8 months	Abnormal calcium accumulation
8-9 months	Myofibrillar necrosis
9-11 months	Granulation tissue formation with Anitschkow cells
11-21 months	Endomysial and perimysial fibrosis
20-51 months	Myocardial fatty infiltration

A total of 4 GRMD dogs (22.2%) between 6 to 8 months of age presented moderate foci of calcium accumulation in the septum, left and right ventricles evidenced by von Kossa staining (Fig. 1A). Marked foci of myocardial necrosis were verified in 5 GMRD dogs (27.7%) at 8 to 9 months of age, mainly in the septum and left ventricle. These necrotic cardiomyocytes exhibited degeneration, hyperacidophilic cytoplasm, loss of cross-striations and cytoplasmic detail, and nuclear pyknosis or karyolysis (Fig. 1B). A marked replacement of cardiomyocytes with a highly vascularized granula-
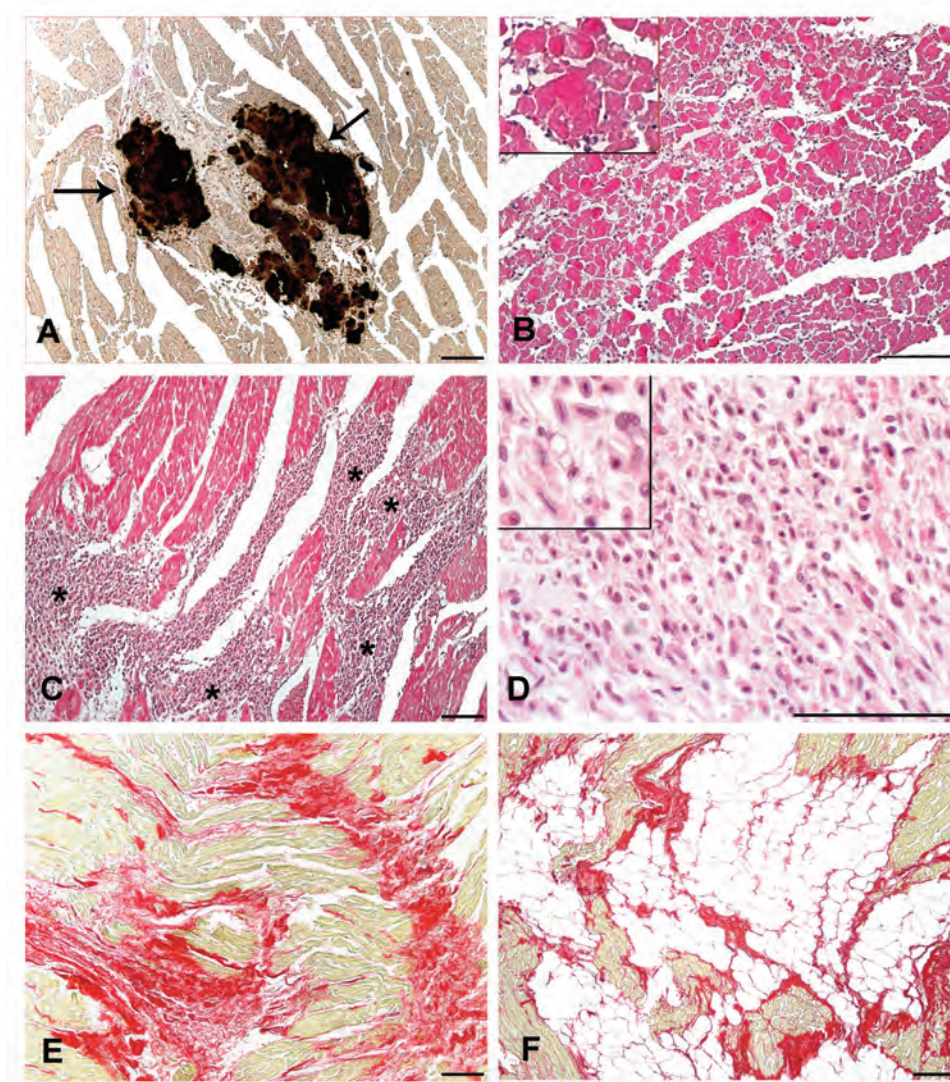

Fig. 1. A) Left ventricle from a 6-months-age GRMD dog. Abnormal accumulation of calcium salts (arrow). Von Kossa staining, magnification X10, Bar 100 µm. B) Interventricular septum from a 8-months-age GRMD dog. Foci of myocardial necrosis. Myofiber hyperacidofilia and loss of cytoplasmic detail. Inset: nuclear pyknosis or karyolysis. Hematoxylin and eosin staining (HE), magnification X20, Bar 100 µm. C) Left ventricle from an 11-months-age GRMD dog. Extensive cardiomyocyte replacement with granulation tissue (asterisk) composed of large number of capillaries and mononuclear cells. HE, magnification X10, Bar 100 µm. D) Left ventricle from an 11-months-age GRMD dog. Several Anistchkow cells at the granulation tissue. Inset: Characteristic "caterpillar-like" configuration of nuclear chromatin at Anistchkow cells. HE, magnification X40, Bar 100 µm. E) Left ventricle from an 18-months-age GRMD dog. Extensive perimysial fibrosis replacing the myocardium (red). Picrosirius red staining, magnification X10, Bar 100 µm. F) Left ventricle from a 51-months-age GRMD dog. Replacement of the myocardium by foci of fibrofatty tissue. Picrosirius red staining, magnification X10, Bar 100µm.

tion tissue composed by rich network of capillaries and moderate inflammatory infiltrate, mostly composed of mononuclear cells, was observed in the septum, left and right ventricles of 4 GRMD dogs (22.2%) at 9 to 11 months of age (Fig. 1C). Interestingly, this granulation tissue presented several Anitschkow cells (Fishbein *et al*., 1978) with characteristic "caterpillar-like" configuration of nuclear chromatin (Fig. 1D). As a consequence, a diffuse marked increase in both endomysial and perimysial fibrosis were detected in 10 hearts from GRMD dogs (55.5%) since early 11 to 21 months, mainly in the septum, left and right ventricles (Fig. 1E). These lesions were characterized by widespread replacement of the myocardium by large foci of fibrocollagenous connective tissue, evidenced by picrosiruis red staining, with remaining adjacent cardiomyocytes frequently exhibiting degeneration and fragmented sarcoplasm. As the end stage of the cardiac lesions, moderate foci of fibrofatty replacement of ventricular myocardium was observed in both right and left ventricles, in 4 GRMD dogs (22.2%) at 20 to 51 months (Fig. 1F).

Discussion

The critical importance of dystrophin, a key structural component in the muscle fiber that connects the cytoskeleton to the extracellular matrix and contributes to cell shape and mechanical resistance in cardiomyocytes, is easily verified in Duchenne cardiomyopathy according to striking findings of morbidity and mortality in consequence of heart diseases in both patients and dogs affected by muscular dystrophy (Valentine *et al*., 1989; Kaspar *et al*., 2009). So, Duchenne´s muscular dystrophy is a model of progressive cardiac dysfunction with clinical course ending in death. Many studies including histological, echocardiographic and electrocardiographic findings have been demonstrating that the cardiac involvement of the GRMD dogs would be similar to Duchenne´s cardiomyopathy in humans (Valentine *et al*., 1989; Moise *et al*., 1991; Chetboul *et al*., 2004).

Previous morphological studies involving experimental models of muscular dystrophy suggested that the initial event in the pathogenesis of this degenerative disease could be the sarcolemmal dysfunction, mainly due to dystrophin mutation (Valentine *et al*., 1989; Kaspar *et al*., 2009; Miyazato *et al*., 2011a). In this context, our results

evidenced foci of calcium accumulation, classified as moderate, in the cardiomyocytes from GRMD animals as early as 6 months of age indicating that abnormal calcium influx could be a primary lesion observed in GRMD cardiomyopathy. Importantly, the degree of mineralization decreased with age, fact that could be explained due to clearance of macrophages and the progressive pattern of cardiac lesion according to Valentine *et al*. (1990). However, sustained increase in cytosolic calcium, mainly at the early stage of the disease, would lead to activation of proteases, particularly calpains, resulting in the destruction of membrane constituents, increase of calcium entry and cardiomyocytes death (Kaspar *et al*., 2009). In this context, we observed diffuse foci of myofibrillar necrosis mainly in the septum and left ventricle from hearts of GRMD dogs, mostly in animals with 8 months of age, possibly as a consequence of increased calcium influx observed in cardiomyocytes at the early stage of the disease. At the light microscopy, cardiomyocytes exhibited degeneration, hypereosinophilic cytoplasm with loss of striations and nuclear pyknosis and karyolysis, lesions characteristics of coagulative necrosis. The next phase of cardiac disease was characterized by an extensive replacement of the cardiomyocytes by granulation tissue composed by rich network of capillaries and moderate mononuclear inflammatory infiltrate since 9 to 11 months of age. It has been demonstrated that the death of the cardiomyocytes would initiate an inflammatory response where macrophages would migrate to clear the damage cells and recruit the fibroblasts that start the collagen deposition (Kaspar *et al*., 2009). In addition, the granulation tissue cells and the extracellular matrix network are important to provide mechanical stability to the injured tissue after cardiomyocytes death. Thus, preservation of the collagenous matrix is important to minimize infarct expansion (Frangogiannis, 2006). Interestingly, our findings demonstrated that granulation tissue formed after diffuse foci of myofibrillar necrosis presented several cells with characteristic "caterpillar-like" configuration of nuclear chromatin known as Anitschkow cells. The Anitschkow cells accompanying caterpillar and owl-eyed nuclear configurations are a well-known histological hallmark of rheumatic heart (Satoh and Tsutsumi, 1999). It is currently believed that the caterpillar-like appearance of chromatin in Anitschkow cells

indicates cellular immaturity and undifferentiation, rather than any specific cell type, and it has been shown to be a characteristic of human fetal cardiac myocytes (Pontén *et al.*, 2003). These cells have controversial origin, cardiac muscle and histiocytes have been suggested as possible sources (Fishbein *et al.*, 1978). Previously, it was demonstrated the occurrence of Anitschkow cells in collagenous scar tissue in healing myocardial infarcts suggesting that these cells could have the capacity to differentiate in fibroblasts and may be observed when formation of collagen is occurring (Fishbein *et al.*, 1978). This statement corroborate with our observations, once that the occurrence of Anitschkow cells in the cardiac granulation tissue from GRMD dogs was detected immediately before of scar phase. In our knowledge, this is first study that describes the occurrence of Anitschkow cells in the histological findings of cardiomyopathy that frequently accompanies the muscular dystrophy.

In this context, our results demonstrated an extensive replacement of the myocardial tissue by connective tissue in the progression of the GRMD cardiomyopathy. It was observed diffuse endomysial and perimysial fibrosis in the hearts of GRMD dogs from 11 to 21 months of age. It has been documented that the fibrotic region is gradually stretched, and thus become thinner, lose contractility what results in dilated cardiomyopathy (Kaspar *et al.*, 2009). In addition, myocardial fibrosis can result in loss of cardiac contractility due the fact of fibrotic tissue is very inflexible comparable to normal tissue and thus restricts the efficiency of myocardial contraction leading to heart failure (Valentine *et al.*, 1989; Kaspar *et al.*, 2009).

Finally, our study evidenced moderate foci of fibrofatty replacement in myocardium of older GRMD animals, since 20 up 51 months, indicating that this structural change could be a final point of the cardiac lesions that determine GRMD cardiomyopathy. Previous study indicated that the myocyte necrosis could be the main factor responsible to trigger sequences of events that lead to fibrofatty replacement of the normal myocardium (Basso *et al.*, 2012). So, fatty tissue infiltration leads to progressive structural and functional abnormalities of the myocardial tissue. These changes can cause electrical instability resulting in life-threatening ventricular arrhythmias and chronic heart failure (Agudelo *et al.*, 2013).

Conclusion

These results demonstrate the sequence of the myocardial lesions that determine the cardiomyopathy in GRMD dogs and exhibit, for the first time, the Anitschkow cells in the histological findings of this cardiomyopathy. In our study, increased calcium influx, due to sarcolemmal dysfunction, could be the initial event followed by myofibrillar necrosis, proliferation of granulation tissue with participation of Anitschkow cells, endomysial and perimysial fibrosis, and finally fibrofatty infiltration. These findings shed light and on the pathogenesis of GRMD cardiomyopathy and contribute to better knowledge of Duchenne's cardiomyopathy.

Acknowledgement

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