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Role of Nitric Oxide in the Remodelling of Extracellular Matrix in Myxomatous Mitral Valve Degeneration of Dogs and Pigs

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Abstract

Myxomatous mitral valve degeneration (MMVD) or endocardiosis is a heart valve disease that occurs in many mammalian species, especially in humans, dogs and pigs. Nitric oxide (NO) plays an important role in the MMVD development. NO can be indirectly evaluated by the nitric-oxide synthase (NOS) expression and by the histochemical reaction of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d). The aim of this study was to evaluate NOS activity, by NADPH-d reaction, in the anterior leaflet of dogs with regular mitral valves and in those with MMVD, as well as in young swine and old females, comparing the reaction level with the degree of endocardiosis disease and also the histological alterations. Twelve mitral valves of dogs and 22 of swine were used for the research. All the valves were macroscopically analyzed for the occurrence or not of endocardiosis. They were fixed in a 4% paraformaldehyde, exposed to NADPH-d reaction, routinely processed and microscopically evaluated for the detection of mucopolysaccharides (MPS) deposition, collagen degeneration, fibrosis and level of endocardiosis. In dogs, relation was observed between higher intensity of the NADPH-d reaction, higher endocardiosis degree, MPS deposition as well as the collagen degeneration. No alteration in color was observed in pigs´ valves during NADPH-d reaction. In conclusion, NO works in canine mitral valve remodeling extracellular matrix and plays an important role in endocardiosis disease. In swine, the lack of reaction reinforces the absence of macroscopical endocardiosis lesions, suggesting restrict NO action or major differences in the structures of swine valves.

Key Words: endocardiosis, NOS, NADPH, valve, dog, swine

Introduction

The myxomatous mitral valve degeneration (MMVD) is a dystrophic and degenerative process that occurs in the valvular endocardium (1, 3, 10) and it is characterized by an accumulation of mucopolysaccharides (MPS) in the extracellular matrix (ECM) of the leaflets of mitral valve (1), being observed at higher rates in human, canine and swine species (21, 22).

Myxomatous mitral valve disease (MMVD) is the most prevalent heart disease in dogs (18, 20), and it affects a large percentage of the geriatric population of this species, presenting high levels of morbidity and mortality (17). It is directly related to age, being more regular in dogs between eight and 11 years old, and it shows higher incidence in small animals (18).

The incidence and severity of MMVD in swine are also strongly related to age. The occurrence is lower in young swine and the valvular changes are moderate,

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while in swine from three to four years of age, the occurrence of the disease may reach 90% or more (3).

The common macroscopic aspect of the leaflets of the mitral valve with myxomatous degeneration is characterized by thickening, opacity and several degrees of leaflet retraction, with nodes at the valve's ends and ***elongated*** **chordae tendineae** (4, 14, 17). The valve becomes larger and insufficient as the disease evolves (13, 20).

The progressive myxomatous valvular shift possibly represents a response to the repeated impact on leaflets of the valve, leading to an endothelial dysfunction (8, 20). The MMVD changes start primarily at the free edge of the valvular leaflet and are apparently expressed in the areas of insertion of the *chordae tendineae*. As the disease progresses, the degenerative lesions extend from the free edge to the remaining portions of the valvular leaflet (6, 17).

The nitric oxide (NO) is a gas-like free radical, produced from L-arginine, through catalysis, by the nitric oxide synthase (NOS), which uses nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor (7, 12, 15). In the NADPH diaphoresis (NADPH-d) histochemical reaction, the deposition of blue granules shows the presence of NOS in formaldehyde-fixed tissues (5, 11). Olsen et al. (20) observed an increase of the NADPH-d reaction in the mitral valve of dogs with myxomatous changes, suggesting a growth of NOS in these valves and a possible involvement of NO in the genesis of the canine MMVD.

The aim of this work was to verify the role of NO in the remodeling of the ECM and in the pathogenesis of MMVD in dogs and swine, submitting the mitral valves, both normal and with endocardiosis, of canine and young swine as well as female swine to the NADPH-d reaction and comparing it with the rate of endocardiosis and with the microscopic alterations, especially the mucopolysaccharides deposition, the collagen degeneration and fibrosis.

Material and Methods

Animals

Dogs: 12 mitral valves from adult to aged animals, without breed nor sex restrictions, were obtained during the necroscopy examination in the Animal Pathology Sector of the Veterinary School, at Universidade Federal de Goiás (Federal University of Goiás), Goiânia, GO, Brazil. The valves were randomly collected, and those that presented normal macroscopic morphology and endocardiosis were selected.

Swine: The mitral valves were collected from ten young animals of both sexes, aged between seven and eight months, as well as from 12 females aged between five and six years old, totaling 22 valves. All swine were half-breeds (Large White - Landrace) and the valve collection was carried out during the heart's inspection, after the slaughtering in a cold storage. Mitral valves with or without macroscopic signs of endocardiosis were selected.

Experimental design

The mitral valves of all the animals were collected within, at most, one hour after death. To avoid touching the leaflets of the endothelium during dissection, incisions were performed in the papillary muscles and in the valvular ring, obtaining the complete mitral apparatus. ***Each valve was initially evaluated for the presence or absence of*** endocardiosis and, subsequently, fixed in a 4% paraformaldehyde for 24 hours, at temperatures between 4° and 8°C. After fixing, the valves were bathed in a Tris/HCL 0.1mM (pH7.2) solution, then the anterior leaflet of each mitral was separated and photographed.

Afterwards, each leaflet was submitted to a NADPH-d reaction, through incubation in a Tris/HCL 0.1M (pH 7.2) solution, containing 1mM of β-NADPH (Sigma, N-1630), 0.2mM of blue ***nitrotetra***-zolium, triton X100 0.2% and ***0.53 mg/mL monosodium*** malate (***Sigma, M-1125***), for 1h30min at 38ºC (20, 25). After this period, the leaflets were once again bathed in Tris/HCL 0.1mM (pH 7.2) solution and photographed to evaluate the intensity of the NADPH-d reaction. The following staining scores were attributed: no staining (0), light blue (+), dark blue (+ +) and (+++) black (20).

The next stage consisted of a perpendicular cut-off of the mitral valve anterior leaflet (FAVM) from the valvular ring to the apex of the leaflet, for processing, inclusion in paraffin and confection of the slides. After that, Hematoxylin, Eosin, Alcian blue and ***Masson's trichrome*** stainings were carried out for histological analysis, collagen degeneration, MPS deposition and fibrosis, respectively (Table 1) (20).

The endocardiosis rate in canine mitral valves was also microscopically evaluated, measuring the apex of the leaflet thickness, through the software ImageJ Launcher (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/), according to criteria adapted from Olsen et al. (20) (Table 1).

Statistical analysis

Descriptive data and Spearman's coefficient test were used for the evaluation of the groups, because they comprise non-parametric data. This data was processed by the statistical software SPSS (Statistical Package for the Social Science) 16.0 version, with significant results when $p<0.05$.

TABLE 1 – Criteria of microscopic evaluation of dogs and swine mitral valve

Microscopic alterations in the mitral valve anterior leaflet in dogs and swine
Mucopolysaccharide deposition
No signs of deposition
Discrete deposition on the spongeous and/or fibrous layer with intact leaflet's layers
Moderate deposition on the spongeous and/or fibrous layer with disruption of the leaflet's layers
Accentuated deposition on the spongeous and/or fibrous layer without a structure of layers
Collagen degeneration
No signs of collagen degeneration with intact collagen bundles on the fibrous layer
Small area containing collagen degeneration on the fibrous layer
Large area containing collagen degeneration on the fibrous layer, however still distinctive
Destruction of the fibrous layer with degeneration of the collagen fibers arranged in whirls
Fibrosis
No sign of fibrosis
Small area containing fibrosis
Large area containing fibrosis, though dissociated/separated
Large area containing coalesced fibrosis
Endocardiosis rate on the canine mitral valve anterior leaflet
Discrete – thickening of the leaflet and/or nodules <1.5mm
Moderate – thickening of the leaflet and/or nodules between 1.5 and 3mm
Accentuated – thickening of the leaflet and/or nodules >3mm

Results

Dogs

Five normal canine valves and seven showing signs of endocardiosis were obtained through macroscopic evaluation (Figs. 1A e 1E). The NAPH-d reaction showed different staining scores on the canine mitral valves studied. Light blue, dark blue and black valves were verified (Figs. 1B, 1D and 1F). Mitral valves without staining were not observed.

The comparison among the intensity of the NAPH-d reaction, the MPS deposition and the collagen degeneration of the fibrous layer revealed that, as the staining intensity increases, MPS deposition and collagen degeneration ($p<0.05$) also increase (Table 2). On the other hand, there was no difference in the comparison between the intensity of the NAPH-d reaction and fibrosis. Fig. 2 represents the MPS deposition and the collagen degeneration in the canine anterior mitral leaflet.

Relations between the intensity of NADPH-d reaction, characterized by staining scores, and the endocardiosis rate ($p<0.05$) were observed in the canine valves, showing that a higher intensity of the reaction produces a greater mitral valve degenerative process (Table 3), considering the thickness of the apical portion of the valve's anterior leaflet. The microscopic aspect of the canine mitral valve with or without endocardiosis is presented in Fig. 3.

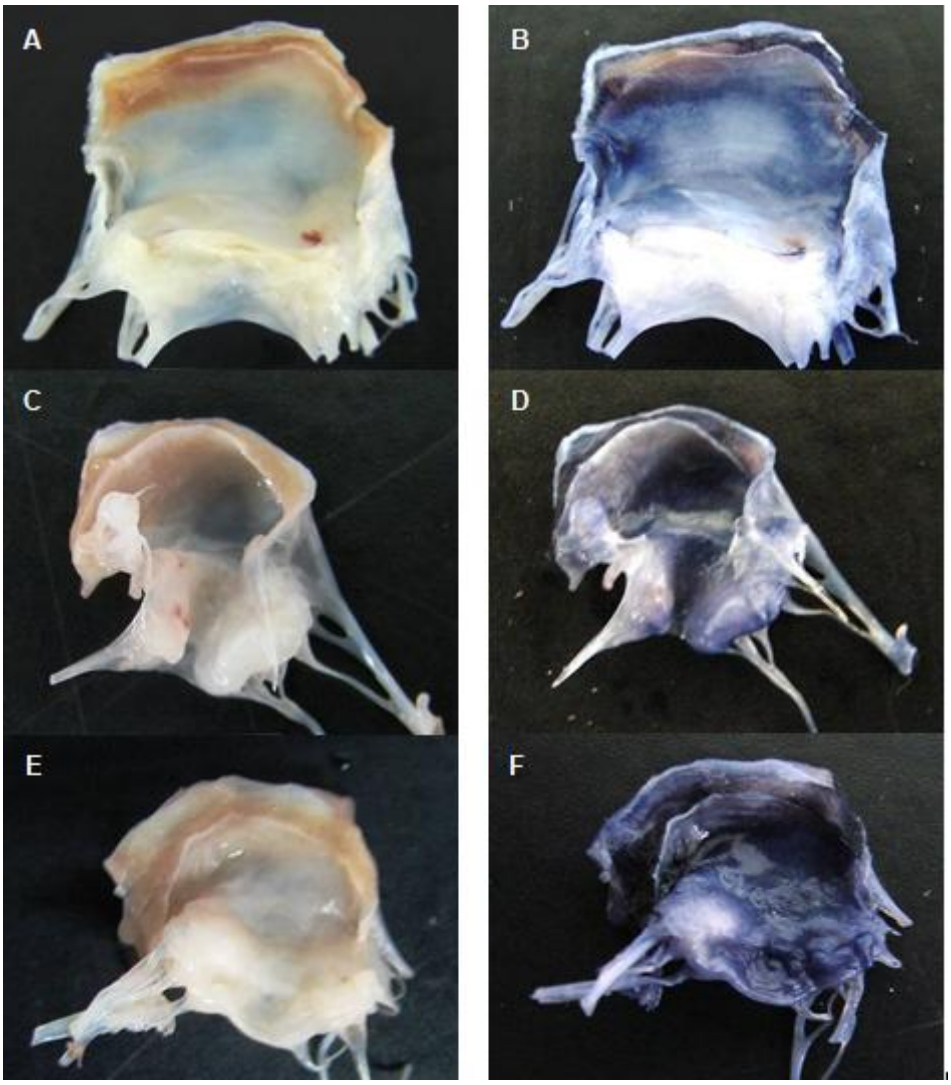


Figure 1 - Canine mitral valve. A), C) and E) Normal anterior mitral leaflet, with moderate and with discrete endocardiosis, respectively, before the NAPH-d reaction. B), D) and F) Normal anterior mitral leaflet (light blue), with moderate (dark blue) and with discrete (black) endocardiosis, respectively, after the NAPH-d.

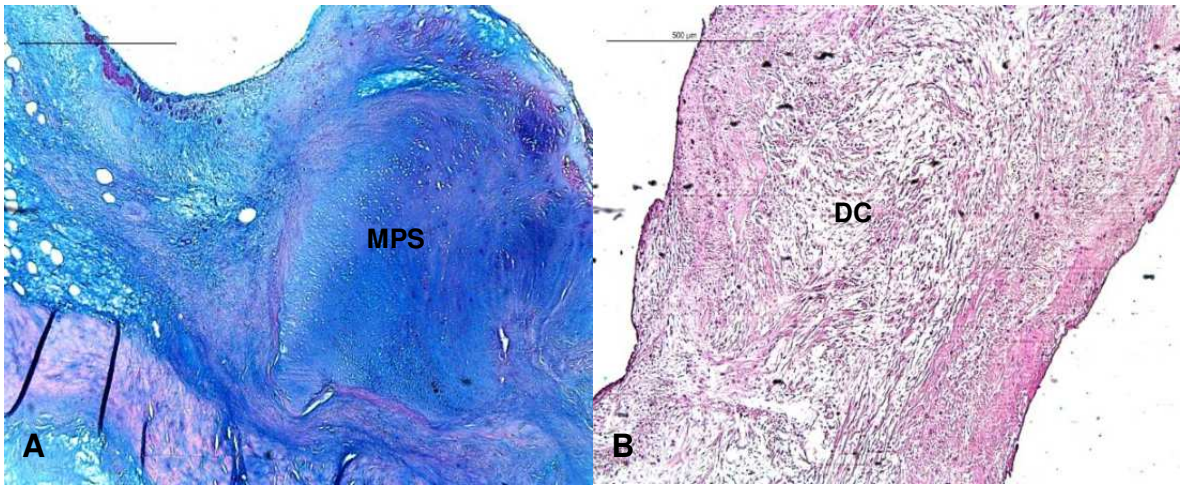


Figure 2 - Photomicrographs of the mitral valve of dog with endocardiosis. (A) Accentuated MPS deposition (MPS). Alcian blue. (B) Collagen degeneration (DC). HE.

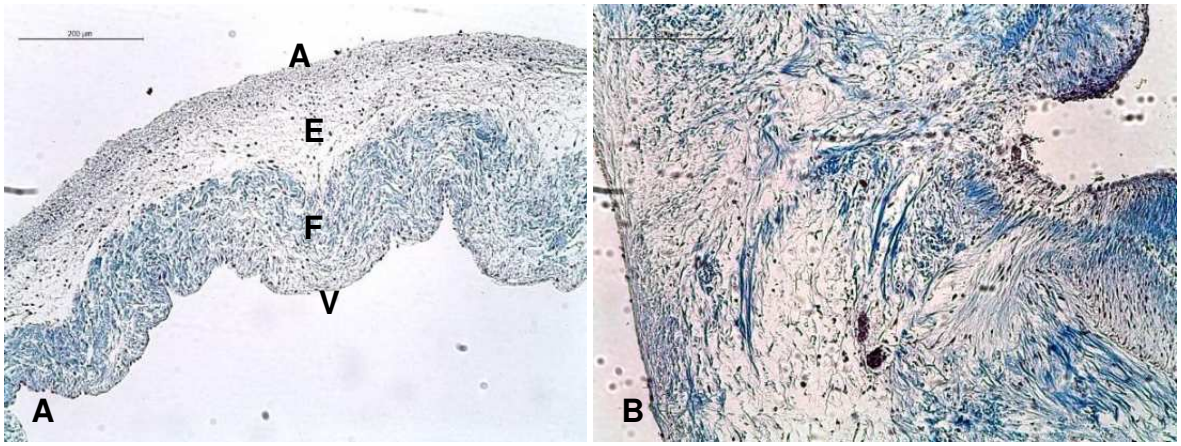


Figure 3 - Photomicrographs of the mitral valve of dog. A) Normal: atrial layers (A), spongeous (E), fibrous (F), and ventricular (V). B) Endocardiosis: absence of the valvular structure in layers. ***Masson's trichrome.***

TABLE 2 - Comparison among MPS deposition, collagen degeneration and the NADPH-d reaction in the canine anterior mitral leaflet

	NADPH reaction			
MPS Deposition	Light blue	Dark blue	Black	**Total**
Discrete	100% (3)	0% (0)	0% (0)	100% (3)
Moderate	25% (1)	50% (2)	25% (1)	100% (4)
Accentuated	0% (0)	80% (4)	20% (1)	100% (5)
Total	33.3% (4)	50% (6)	16.7% (2)	100% (12)

	NADPH reaction			
Collagen degeneration	Light blue	Dark blue	Black	**Total**
No areas of degeneration	100% (4)	0% (0)	0% (0)	100% (4)
Large area of degeneration	0% (0)	66.7% (2)	33.3% (1)	100% (3)
Total destruction of the ***fibrocartilage***	0% (0)	80% (4)	20% (1)	100% (5)
Total	33.3% (4)	50% (6)	16.7% (2)	100%(12)

TABLE 3 - Comparison between the intensity of the NADPH-d reaction and the endocardiosis rate in the anterior leaflet of the canine mitral valve

	NADPH reaction			
Endocardiosis rate	**Light blue**	**Dark blue**	**Black**	**Total**
Normal	80% (4)	20% (1)	0% (0)	100% (5)
Discrete	0% (0)	50% (2)	50% (2)	100% (4)
Moderate	0% (0)	100% (3)	0% (0)	100% (3)
Total	33.3% (4)	50% (6)	16.7% (2)	100% (12)

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Swine

All swine valves, ten from young swine and 12 from females, showed normal macroscopic aspects (Figs. 4A and 4C). Moreover, the swine's anterior mitral leaflet, from both swine aged between seven and eight months and females between five and six years, did not show staining to the NAPH-d reaction (Figs. 4B and 4D).

Despite the absence of macroscopic endocardiosis and of staining to the NAPH-d reaction, the swine valves showed various rates of MPS deposition and collagen degeneration by the microscopic evaluation, though fibrosis was not found in any of these valves (Table 4 and Fig. 5).

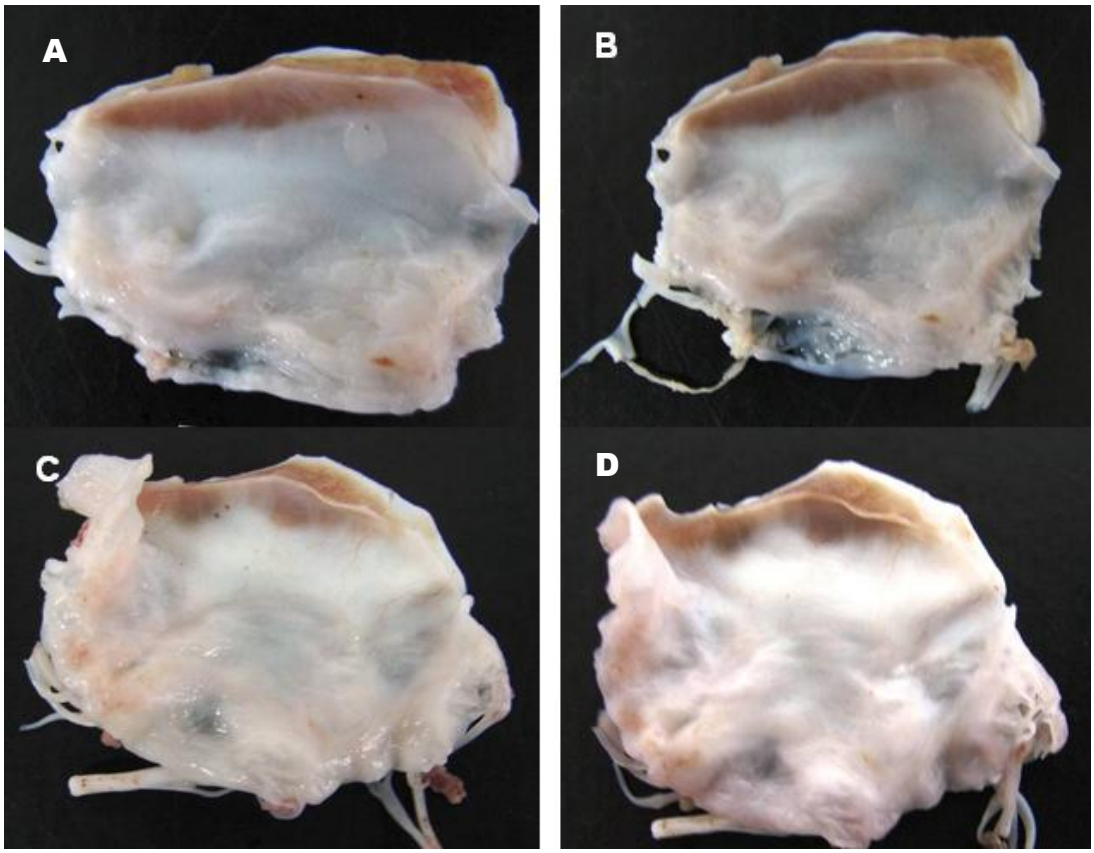


Figure 4 - Swine mitral valve. A) and C) Anterior mitral leaflets from young swine and from females, respectively, before the NADPH-d reaction. B) and D) Anterior mitral leaflets from the same animals of A) and C) without staining after NADPH-d reaction.

TABLE 4– Microscopic evaluation of the swine valves to HE, Alcian Blue and Masson stainings according to the age

Age	**MPS Deposition**			**Collagen degeneration**				**Fibrosis**
	Discrete	Moderate	Accentuaded	None	Small	Large area	Total destruction	None
7 to 8 m	30% (3)	70% (7)	0% (0)	20% (2)	40% (4)	30% (3)	10% (1)	100% (10)
Females	33.3% (4)	58.3% (7)	8.3% (1)	16.7% (2)	25% (3)	41.7% (5)	16.7% (2)	100% (12)
Total	31.8% (7)	63.6% (14)	4.5% (1)	18.2% (4)	31.8% (7)	36.4% (8)	13.6% (3)	100% (22)

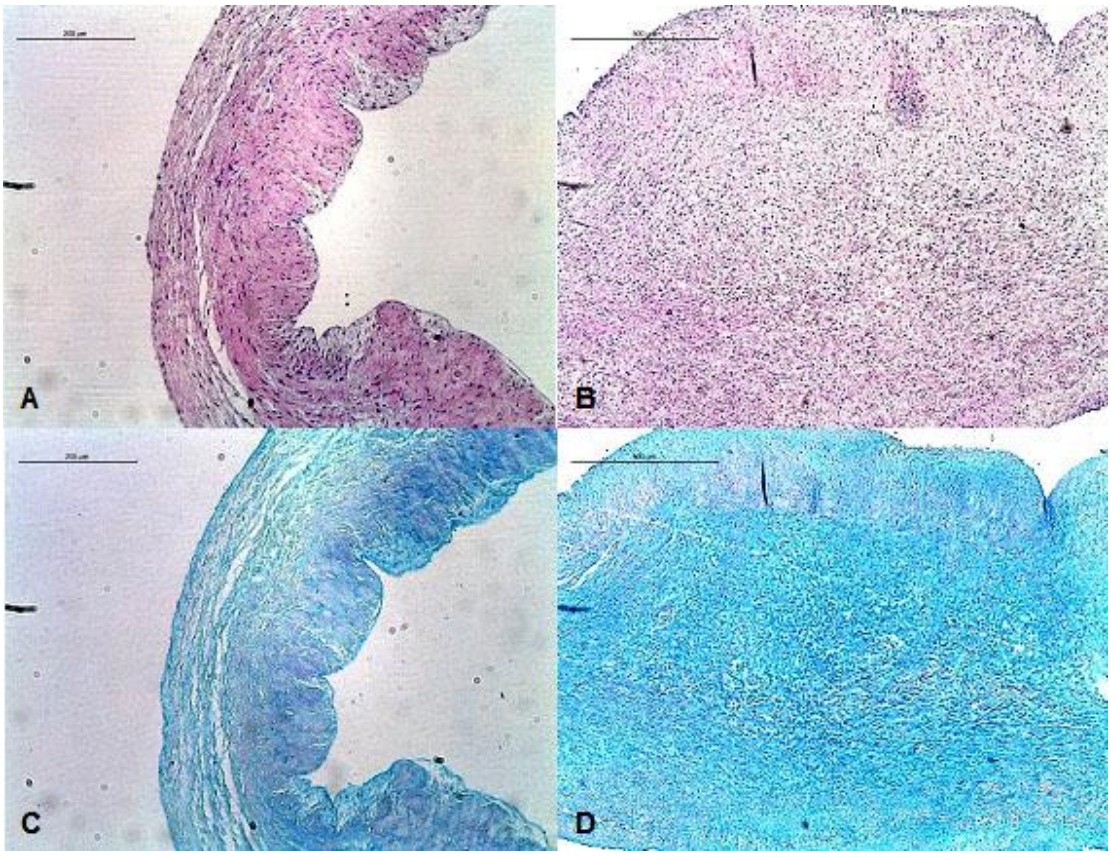


Figure 5 – Swine mitral valve. A) and C) Normal. Maintenance of the valvular structure in layers. From left to right, atrial, spongeous, fibrous and ventricular layers. HE and Alcian stainings, respectively. B) Collagen degeneration. HE. D) MPS deposition. Alcian blue.

Discussion

MMVD is a valvular disease with genetic association, especially in dogs and humans (20, 26), but little is known about the local valvular mechanisms involved in its genesis (20). However, chemical mediators such as NO and endothelin are found in the development of MMVD, since they are important metabolism regulators in the ECM (19, 27).

The diaphoresis nicotinamide adenine dinucleotide phosphate (NADPH) is a co-factor in the synthesis of NO via NOS catalysis (7, 12, 15). According to this principle, the NADPH-d reaction is commonly used to evaluate the expression of NOS and, indirectly, of NO (20), although it does not distinguish NOS isoforms (15). In this study, it was possible to measure the NOS activity in normal canine valves and in the ones with MMVD, with relations among the intensity of the reaction, the MPS deposition and the collagen degeneration, which confirms the hypothesis of the involvement of NO in the genesis of the valvular degenerative canine disease, as Olsen et al. (20) described.

Furthermore, the fact that all canine valves have reacted to NAPHD shows the NO activity in both the physiological remodeling of the mitral valve as in the degenerative disease. It is noteworthy that, in low concentrations, NO acts as a regulatory mediator in the cell signaling processes; however, in high concentrations, it may cause cellular damage, including apoptosis (7, 9, 16).

There were no relations between the intensity of the NADPH-d reaction and fibrosis, an evaluation criterion for diagnosing MMVD, which can be explained by the fact that only a few dogs showed secondary fibrosis (20). This was confirmed in this study, since of the 12 valves evaluated, eight were included in the lower scores of fibrosis (without fibrosis or a small area with fibrosis) and not a single mitral valve in the maximum score (large area of ***coalescing fibrosis)***. Thus, it seems clear that the increase in the thickness of the valve with myxomatous degenerative disease is not directly related to the deposition of fibrous connective tissue, but to MPS.

In the canine valves, relations between the intensity of NADPH-d reaction, characterized by staining scores, and the endocardiosis rate, measured by microscopic evaluation, were also found. The bigger the intensity of the reaction, the greater is the degenerative process of the mitral valve in the apex of the anterior leaflet, something similar to what was described by Olsen et al. (20), who measured the endocardiosis rate macroscopically.

No macroscopic signs of endocardiosis were found in the swine mitral valve leaflets and there was not staining to the NADPH-d reaction, in both young

and female animals, although Moesgaard et al. (15) have observed a spurious expression of eNOS (endothelial nitric oxide synthase), using the *western blotting* technique, mostly in the endothelial cells, which represent the smallest portion of total cells in the swine's mitral valve. In this context, it is suggested that the quantity of NOS has not been enough for the reaction to occur. Another fact that may have contributed to the absence of the reaction is the possible structural difference between swine and canine mitral valves, considering that, though it was not the purpose of this study, an apparently thicker endothelial layer of the swine valve than the one in dogs was observed, what somehow interferes in the NADPH-d reaction.

It is noteworthy that, in the studied literature, there were no quotes about the use of NADPH-d reaction as a NOS evaluation method in the swine valve, making this a pioneering work. Besides, even working with different species and methods, Aupperle et al. (2) described the possibility of structural and functional differences between the canine and human valves, suggesting here that this might also occur with the swine species´ mitral valve.

The absence of macroscopic signs of endocardiosis, despite age or number of animals evaluated (680 evaluated, 22 selected), associated to the absence of staining to the NADPH-d reaction brings questions about the myxamatous valvular degeneration in the swine species. Is the swine MMVD really common as the literature refers? (1, 3, 10, 23, 24). Or the pathogenesis of MMVD in this species differs from others? Considering the results in this study and some observations by other authors (2), differences seem evident regarding the incidence and the development of the disease, since, even without macroscopic signs, microscopic alterations involved in the mitral valve myxomatous degeneration process (MPS deposition, collagen degeneration and fibrosis) were found in both young and female swine.

Conclusions

The NO activity in the canine mitral valve can be evaluated, even if indirectly, through the NADPH-d reaction, since this biological mediator is involved in both physiological remodeling of the ECM and in the pathogenesis of the mitral endocardiosis in this specie. In the swine mitral valve, the absence of reaction to the NADPH-d confirms the absence of macroscopic lesions by endocardiosis, suggesting restrict NO activity in the ECM remodeling or structural differences in this valves.

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