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Research Article

Characterization of Glycosaminoglycans in Atrioventricular Heart Valves of Healthy Dogs

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Abstract

In the present research, we describe the expression and type of oligosaccharides residues in each of the layers of canine atrioventricular valves. We used ten mitral and tricuspid valves of healthy dogs, which underwent histopathological and histochemical processing with lectins, to characterize quantity, type and distribution of oligosaccharides residues in each of the layers of canine atrioventricular valves. We observed increased positivity to α-D-GalNAc residues in mitral valve layers, particularly in the atrial layer. Furthermore, we found homogenous distribution of α-D-Man>α-D-Glc in both valves and increased GlcNAc>sialic acid expression in the atrial layer of mitral valve and of α-D-Gal residues in the atrial and ventricular layers of mitral valve. Statistically significant difference was found on the expression of aD-GalNAc in healthy patients ($p < 0.05$) between the atrial layer of the mitral and the tricuspid valve, with higher expression of this residue in the mitral valve. Although mitral and tricuspid valves share similar morphology, they have differences in the expression of GAGs. Further studies are required to link GAGs distribution with cardiovascular diseases affecting dogs.

ABBREVIATIONS

ECM: extracellular matrix; **HA:** hyaluronic acid; **CS:** chondroitin sulfate; **PG:** proteoglycans; **GAGs:** glycosaminoglycans; **HRP:** Horseradish Peroxidase

INTRODUCTION

Heart valves are dynamic structures that exhibit a complex architecture of highly specialized cells and extracellular matrix (ECM) (1-2). Canine atrioventricular valves are composed of atrial, spongy, fibrous, and ventricular layers (3-4). The atrial and ventricular layers correspond to the endocardial tissue, and are comprised of endothelial cells, collagen fibers, fibroblasts, and elastic fibers. The foam layer, which extends from the valve annulus to the free edge, is characterized by a thin, loose layer of collagen fibers, fibroblasts, and elastic fibers on a matrix bed of mucopolysaccharides, mostly, hyaluronic acid (HA) and chondroitin sulfate (CS). The fibrous layer, which occupies most of the valve area, consists of a dense set of well-organized collagen fiber bundles responsible for maintaining the mechanical integrity of the valve (5). The leaflets are supported by the chordae tendineae, which resist tensile and compressive loads on the valves, and are essential for efficient ventricular contraction (6).

The ECM of the heart valve contains endothelial stromal and valve cells, collagen fibers, elastic fibers, glycoproteins, proteoglycans (PGs) and complex polysaccharides such as glycosaminoglycans (GAGs) (7-9). Some studies demonstrate that collagen, elastin and proteoglycans content in human heart valves is about 60%, 10%, and 30% of the dry weight, respectively (10). These proportions have not yet been determined in dog valves. GAGs in human valves consist mainly of HA and CS. According to some researchers, there is not agreement on the classes of GAGs present in valves; they moderately agree about the general proportions of HA and chondroitin/dermatan sulfate (7,10).

GAGs are required for proper ECM binding as they interact with other important structural proteins and can play a role in interstitial cells and ECM interactions and also in elastin fibrogenesis (11).

Knowledge of GAGs and PGs composition and distribution seems to be essential for understanding the relationship between structure and mechanics of heart leaflets. Their relative amounts

and distribution have been reported to be different according to the type of mechanical load (1,12).

Mitral valves, both in humans and in dogs, usually contain various kinds of GAGs whose distribution in leaflet layers changes considerably. We have not found literature reports characterizing quantity, type, and distribution of GAGs in canine cardiac valves. The aim of this study was to establish the percentage and types of GAGs in the canine atrioventricular valves and determine whether there is an association or divergence between the different histological layers of the valves.

MATERIALS AND METHODS

This observational, descriptive, cross-sectional study was conducted with the endorsement of the Ethics Committee for Animal Experimentation of Universidad de Antioquia, Colombia (Act 69 of 2011).

Sample selection

Ten hearts of small breed dogs weighing less than 15 kg were used. The cause of death of the dogs was different from heart disease. Patients were provided by the zoonosis center of *Secretaria Distrital de Salud* in Bogotá. The owners of the patients who died of cardiac issues filled out and signed an informed consent to participate in this study. The hearts were collected between June 2011 and December 2012.

Sample Collection

Complete atrioventricular, tricuspid, and mitral heart valves were obtained post mortem to conduct histopathological and histochemical studies. Samples were immediately and individually fixed in Carnoj for 24 hours and sent to the histology laboratory of CES University for processing.

Immunohistochemistry

Samples were submitted to a post-fixation process by soaking them in a 2% calcium acetate solution and 4% paraformaldehyde (1:1 v/v) for three hours at room temperature. Samples were then subjected to dehydration in increasing alcohol concentrations, as follows: 70% alcohol for 12 hours, 80% alcohol for one hour, and finally in 95% alcohol for two hours, as described by Menghi (13). Then, samples were clarified in xylene and embedded in paraffin. Five 5 µm serial sections were obtained from tricuspid and mitral valves (10 sections per heart). One of these sections was stained with hematoxylin-eosin to characterize lesions; the remaining four sections were subjected to conventional histochemistry and lectins with enzymatic and chemical pretreatments, according to previously described procedures by Parillo et al., 2009 (14).

Histochemical processing with lectins

Oligosaccharide chains of glycoproteins present in the leaflets were characterized by histochemistry with four pure lectins (Table 1).

Chemical and enzymatic treatments

The sections were immersed in a 0.3% H2O2/methanol solution for 30 minutes to inhibit endogenous peroxidase activity. Samples were then rinsed in PBS and incubated in a humid chamber at room temperature with Horseradish Peroxidase (HRP) solution and with conjugated lectins in 0.1M PBS at pH 7.2 (0.1mM CaCl2, MgCl2 and MnCl2).

Then, the sections were rinsed with PBS to visualize peroxidase activity with 3-3' diaminobenzidine (DAB kit, D.B.A. Italia S.R.L., Milano, I) for 5 minutes.

At the end of the process, sections were rinsed in distilled water, cleared in xylene, hydrated through decreasing alcohol concentrations and mounted with resin (Eukitt Surgipath, Europe ltdventur,e Parkstirling Waybretton, Perborough, UK.)

An optical microscope (Nikon™ E100 Biological) was used to examine the samples. Histological evaluation included measuring thickness (microns) of valves and layers of atrioventricular valves using 10x magnification through Lucia Cytogenetics® software, version 4.84.

Oligosaccharide residues were quantified in ten fields, and the average was calculated. The percentage of cells labeled with the histochemical reaction with lectins was assessed semiquantitatively (Table 2).

Processing techniques and data analysis

The information gathered from primary sources was stored in an Excel spreadsheet (Microsoft Office©). Descriptive statistics were performed for all variables. Skewness and kurtosis were checked to assess normality of the data. A t-Student test was performed to determine significant differences between variable means before and after hydration. Data analysis was performed in STATA 10 software© (State Corp LP, Texas, USA) with a 95.0% confidence level (P <0.05).

RESULTS

Glycosaminoglycans expression was characterized in mitral

Table 1: Lectins used and their carbohydrate specificities are shown.

Source of lectin	Acronym	Oligosaccharides specificity	Lectin Concentration
Canavalia ensiformis	Con-A	α-D-Man>α-D-Glc	20 µg/ml
Triticum vulgaris	WGA	GlcNAc> acid sialic	20 µg/ml
Griffonia Bandeuraea	GSL-I	α-D-Gal	20 µg/ml
Dolichos biflorus	DBA	α-D-GalNAc	20 µg/ml

Abbreviations: Con-A: Canavalia ensiformis, WGA: Triticum vulgaris, GSL-I: Griffonia Bandeuraea, DBA: Dolichos biflorus, α-D-Man>α-D-Glc: alpha D Manose alpha D Glucose, GlcNAc> acid sialic: N-acetylglucosamine sialic acid, α-D-Gal: alpha D galactose, α-D-GalNAc: alpha D N-acetylgalactosamine.

Table 2: Qualification of lectins affinity by oligosaccharides.

Results	Percentage of positivity in 10 average fields	Reactivity brown coloration
(-)	0%	Nonreactivity
(+)	25 %	Weak
(++)	50%	Moderate
(+++)	75%	Marked
(++++)	100%	Strong

and tricuspid valve layers of normal dogs by histochemical evaluation with four pure lectins. Results showed homogeneous distribution of α-D-GalNAc and α-D-Man>α-D-Glc residues in mitral valves with increased accumulation of GlcNAc>sialic acid residues in both the spongy layer, and enhanced expression of α-D-Gal residues in spongy and fibrous layers (Figure 1). On the other hand, tricuspid valves showed enhanced expression of α-D-GalNAc residues in the spongy layer, low expression of GlcNAc>sialic acid residues in the atrial layer, and homogeneous distribution of α-D-Man>α-D-Glc and α-D-Gal residues in all layers. (Figure 2).

Expression of GAG residues was compared between layers of mitral and tricuspid valves. Increased positivity to α-D-GalNAc residues was observed in mitral valve layers, particularly in the atrial layer. Furthermore, homogenous distribution of α-D-Man>α-D-Glc was found in both valves, with increased GlcNAc>sialic acid expression in the atrial layer of mitral valve and of α-D-Gal residues in the atrial and ventricular layers of mitral valve (Table 3).

A significant increase was observed in the expression of GAGs such as N-acetyl glucosamine>sialic acid, α-D-Mannose>α-D-glucosamine, α-D-galactose, and α-D-galactose>sialic acid particularly in the fibrous layer of the tricuspid valve and to a lesser extent in the mitral valve.

Significant difference in the expression of α-D-GalNAc was found between the atrial layer of mitral and tricuspid valves in healthy dogs ($p<0.05$), with increased expression of this residue in the mitral valve.

Similar thickness was observed between mitral and tricuspid valves. However, the atrial and ventricular layer of the mitral valve were thinner with respect to the tricuspid valve (Table 4).

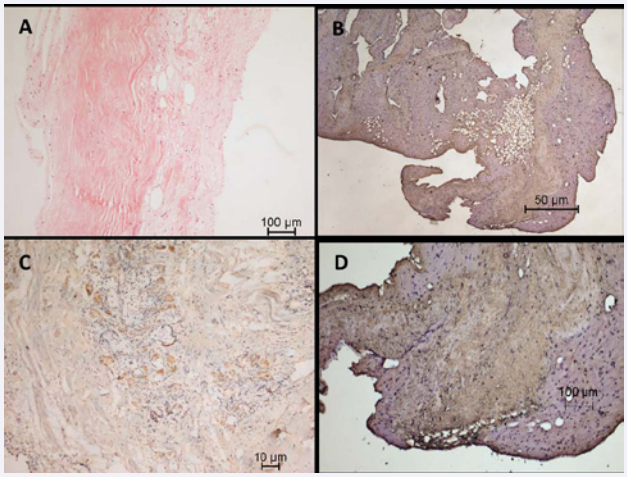

Figure 1 (A)- Canine mitral valve. Hematoxylin –eosin staining 4x. (B)- High residues of N-acetyl glucosamine> sialic acid in the ventricular layer and less in the fibrous layer evidenced by WGA positivity is observed. Counter-staining nuclei with hematoxylin, 10x magnification. (C)- high residues of α-D-Gal was observed in the spongy and fibrous layer positivity evidenced by GSL-I. Counter-staining nuclei with hematoxylin, 40x magnification. (D) Residues α-D-GalNAc are seen in the spongy and fibrous layer positivity evidenced by DBA. Counter-staining of the nuclei with hematoxylin 4x magnification.

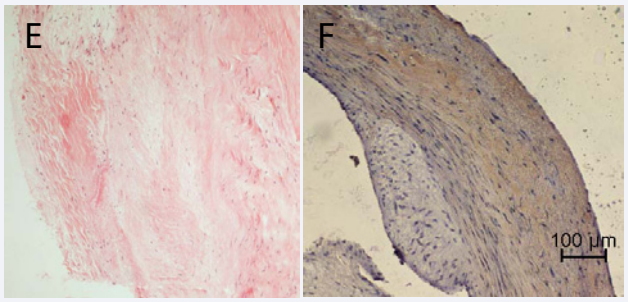

Figure 1 (E)- Canine tricuspid valve. Hematoxylin –eosin staining 4x. (F)- High residues of α-D-Man>α-D-Glc in the ventricular layer and fibrous layer evidenced by ConA positivity is observed. Counter-staining nuclei with hematoxylin, 40x magnification.

DISCUSSION

This is the first study to characterize glycosaminoglycan expression of atrioventricular valve layers in healthy hearts of dogs. Although both atrioventricular valves have similar morphology, this study found some differences in GAG expression between mitral and tricuspid valves.

Recent studies on the characterization of human GAGs (i.e. HA and chondroitin/dermatan sulfate) in the normal mitral valve showed tensile and compressive forces are consistent with the number and distribution of these proteins. In addition, it is also known that the relative percentage of the different GAGs and their total concentration can change significantly with age (10,11).

Mitral valve showed an accumulation of oligosaccharide chains, especially in the spongy layer, which is consistent with the histological description by (15). According to them, the spongy layer is composed primarily of proteoglycans and glycosaminoglycans distributed from the ring to the free edge of the leaflet. Additionally, a significant difference in α-D-GalNAc expression in the atrial layer of mitral valve (76%) relative to the tricuspid (8.8%) was obtained.

Increased positivity of Mannose>α-D-Glucosamine residues in the fibrous layer explains the observed changes in the normal structure of the fibrous layer, which has a more irregular collagen type.

Aupperle et al. (2010) characterized the distribution of some components of the extracellular matrix in tricuspid valves. According to them, proteoglycan accumulation starts in the spongy layer, which possibly favors pathophysiology and development of heart disease in small animals -considering that valvular degenerative disease occurs by alteration, increase, and destruction of these proteoglycans (16).

It is important to note that ECM is not static; it is constantly being remodeled to maintain a balance between synthesis of ECM components and their catabolism by different enzymes (3, 17). The ECM contains growth factors and proteins that bind to growth factors, playing an active role in their presentation and mobilization. This explains why any modulation of ECM structure

Table 3: Lectin positivity in canine mitral and tricuspid valve layers.

	DBA lectin		Con - A lectin		WGA lectin		GSL - I lectin	
	Mitral valve layers	Tricuspid valve layers	Mitral valve layers	Tricuspid valve layers	Mitral valve layers	Tricuspid valve layers	Mitral valve layers	Tricuspid valve layers
Atrial	76,1% +/- 20*	8,8% +/- 24*	41% +/- 23	42,7% +/- 47	47,8% +/- 39	51% +/- 37	5,5% +/- 10	22% +/- 27
Spongy	68% +/- 27	36,7% +/-30	46% +/- 24	33,9% +/-38	61,9% +/-33	53%+/- 40	12% +/- 19	14,7% +/- 26
Fibrous	68% +/- 21	13,2% +/-15	38% +/- 22	29,5% +/-22	44,5% +/- 35	52% +/- 39	11,6% +/- 14	20,58% +/- 20
Ventricular	73% +/- 21	7,35% +/-21	40,3% +/-19	38,3%+/-45	44,5% +/- 37	50% +/- 36	4,3% +/- 9.6	22% +/- 27

DBA: Dolichos biflorus, Con-A: Canavalia ensiformis, WGA: Triticum vulgaris, GSL-I: Griffonia Bandeuraea. *indicate significant statistical difference ($p<0.05$).

Table 4: Valves and layers thickness.

Atrioventricular valve layer	Mitral valve	Tricuspid valve
Atrial	7 µ +/- 3	12,1 µ +/-9.3
Spongy	467 µ +/- 93	468 µ +/- 78
Fibrous	242 µ +/-53	230 µ +/- 84
Ventricular	9 µ +/- 3.62	16 µ +/- 5.67
Valve	725 µ +/- 104	738 µ +/-120

or composition can have an impact on nearby cells. Interstitial cells that produce and repair the extracellular matrix populate the valve. It is also known that few blood vessels are present because the leaflets are thin enough to receive the necessary nutrition by diffusion only (18).

Preliminary studies suggest that appropriate proportions of GAGs play an important role in maintaining collagen fibers in a specific spatial order. According to those studies, an increase in the amount of GAGs not only alters the spatial distribution but also reduces the physical space, increasing the collagen depletion process (11).

CONCLUSION

This study is the first to describe the expression of oligosaccharide residues in atrioventricular valve layers of canines. Although mitral and tricuspid valves share similar morphology they have differences in the expression of GAGs. Further studies are required to link GAGs distribution with cardiovascular diseases affecting dogs.

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