

Serum nitrotyrosine concentration in dogs with myxomatous mitral valve disease

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Abstract: The aim of this study was to compare serum nitrotyrosine concentrations in healthy dogs with those in dogs with myxomatous mitral valve disease (MMVD). Fifty client-owned dogs were included in this study. Based on echocardiographic results, dogs were categorized into healthy (control), mild-, moderate-, and severe-MMVD groups. Serum nitrotyrosine concentrations were determined from enzyme-linked immunosorbent assays. No significant difference between control dogs and dogs with mild MMVD was detected ($p=0.31$). However, dogs with moderate MMVD had significantly higher serum concentrations of nitrotyrosine ($p=0.04$) than that in controls, and dogs with severe MMVD had significantly lower serum concentrations of nitrotyrosine ($p=0.03$) than that in moderate MMVD dogs. There were negative correlations in the association of serum nitrotyrosine with age ($n=30$, $R^2=0.067$, $p=0.27$), left atrial-to-aortic root diameter ratio ($n=30$, $R^2=0.02$, $p=0.57$), and platelet count ($n=30$, $R^2=0.39$, $p=0.003$); however, only the platelet correlation was significant. Among dogs with MMVD, there was no significant difference in serum nitrotyrosine concentration between males and females. The results of this study suggest that tyrosine nitration end-products might be potential biomarkers for the detection of MMVD in dogs.

Keywords: biomarker, canine disease, peroxynitrous acid, serum nitrotyrosine

Introduction

Canine myxomatous mitral valve disease (MMVD) is the most common acquired cardiovascular disease in dogs, particularly in small breed dogs. The evaluation of cardiovascular disease can be accomplished by examining biomarkers, including N-terminal pro brain natriuretic peptide (NT-pro BNP), cardiac troponin I, and serotonin [1]. Nitric oxide (NO), an endothelium-derived relaxing factor, is a noxious, unstable gas and has three isoforms that can be subdivided into constitutive nitric oxide synthase (cNOS) and inducible iNOS [7, 14].

Nitrotyrosine is a marker that is formed when tyrosine nitrated by the NO-derived oxidant peroxynitrite (ONOO-) increased under conditions that produce NO [9]. In human cardiac diseases, such as dilated cardiomyopathy (DCM), ischemic heart disease, and valvular heart disease, increased iNOS expression has been reported [5, 10]. Two studies have reported that overexpressed iNOS causes ONOO- generation and leads to myocardial dysfunction in mice [6, 16]. In addition, Cesselli *et al.* [3] demonstrated that oxidative stress is a major determinant of dogs with ventricular dysfunction and DCM.

In human medicine, nitrotyrosine is markedly increased in atherosclerosis and is used as a biomarker of endothelial damage [12]. Nakazawa *et al.* [17] reported that nitrotyrosine concentration was related to cytokine-induced myocardial dysfunction in a canine model, while Cunningham *et al.* [4] reported that mono-nitrogen oxides (NOx) level was positively correlated with NT-pro BNP concentration in dogs with mitral valvular disease or DCM [4, 17]. Another study reported that NOx concentration was high in dogs with chronic valvular disease [19].

The aim of this study was to evaluate serum nitrotyrosine levels to assess its association with the severity of MMVD.

Materials and Methods

Study animals

Fifty client-owned dogs [21 male dogs (14 neutered) and 29 female dogs (16 neutered)] were prospectively recruited at the Veterinary Medical Teaching Hospitals of Chungnam National University from October 2013 to July 2015 (Table 1). Owner consent was obtained prior to evaluation and blood collection. Dogs were included in this study if they exhibited signs of MMVD during physical, radiographic and

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echocardiographic examination. Dogs with congenital heart disease or significant systemic disease were excluded from the study. All examinations included physical examination, blood collection, and echocardiography, and an owner interview. All dogs were examined without sedation in a quiet examination room.

Sample grouping

Echocardiography with an iU22 system (Phillips, USA) was performed to diagnose and determine the severity of MMVD. Diagnoses of MMVD were based on characteristic valvular lesions of the mitral valve apparatus (thickened and/or prolapsing mitral valve leaflets) and mitral regurgitation in color Doppler echocardiograms from a left apical 4-chamber view. Estimation of MMVD severity was based on color Doppler echocardiographic mapping obtained by using a 2.5 MHz electronic sector transducer. MMVD in dogs was classified as follows: mild MMVD (< 30%), moderate MMVD (30–50%), and severe MMVD (> 50%).

Sandwich enzyme-linked immunosorbent assay (ELISA)

Blood samples (5 mL) were collected from the jugular veins of the dogs and placed into serum tubes. Serum from the samples was separated by centrifugation and transferred into Eppendorf tubes. Serum samples were stored at -80°C prior to batch analyses. Serum nitrotyrosine concentrations were measured by using nitrotyrosine ELISA kit (OxiSelect Nitrotyrosine ELISA Kit; Cell Biolabs, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed by using a commercially available computer-based software program (SPSS 18.0.0; SPSS, USA). Serum nitrotyrosine concentrations are presented as average and SE values for each study group. A p value of < 0.05 was considered significant. In order to

investigate the overall associations between nitrotyrosine concentrations and the three MMVD severity groups, one-way ANOVA with *post hoc* analysis was used. If a significant association ($p < 0.05$) was detected, a pair-wise comparison was performed by using an independent *t*-test. Correlations between dog characteristics, echocardiographic measurements, and serum nitrotyrosine concentrations were determined by calculating Pearson's correlation coefficients.

Results

Breed composition and platelet counts

The breeds included in the study, arranged in descending order of abundance were: Maltese ($n = 18$; 36%), Shih Tzu ($n = 10$; 20%), Pomeranian ($n = 5$; 10%), Yorkshire Terrier ($n = 4$; 8%), Pekinese ($n = 3$; 6%), Cocker Spaniel ($n = 3$; 6%), Poodle ($n = 2$; 4%), mixed ($n = 2$; 4%) and Schnauzer, Chihuahua and Boxer (each, $n = 1$; 2%) (Table 1).

Platelet counts tended to increase with severity of MMVD severity. However, no significant difference in platelet count was detected between the control group and the three MMVD groups ($p = 0.322$) (Table 2).

Serum nitrotyrosine concentrations

Compared to healthy dogs (control group), dogs with moderate MMVD had a significantly higher serum nitrotyrosine concentration ($p = 0.04$) (Table 2; Fig. 1). The serum nitrotyrosine concentration in dogs with severe MMVD was significantly lower than that in dogs with moderate MMVD ($p = 0.03$). However, there was no significant difference in serum nitrotyrosine concentrations between healthy dogs and dogs with mild MMVD ($p = 0.08$) or between as dogs with mild MMVD and those with moderate MMVD ($p = 0.57$). Moreover, in dogs with MMVD, serum nitrotyrosine concentrations were not significant different between males ($n = 20$; $4,731.53 \pm 130.53$ nM) and females ($n = 22$; $4,676.27 \pm 100.24$

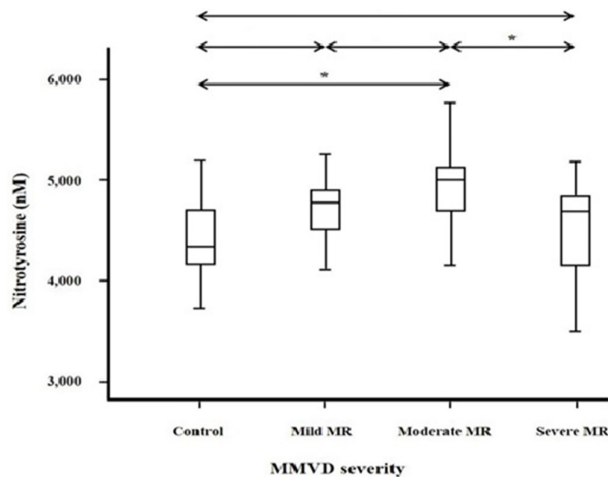
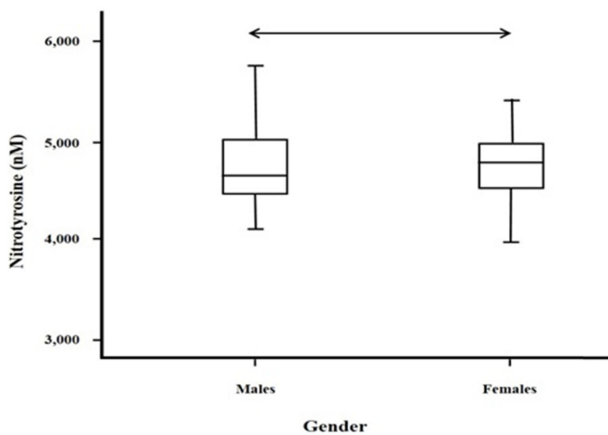
Table 1. Breed composition of control and three myxomatous mitral valve disease (MMVD) groups in this study

Breed	Control	MMVD		
		Mild	Moderate	Severe
Maltese	1	7	6	4
Shih Tzu	1	3	1	5
Pomeranian	4	–	1	–
Yorkshire Terrier	1	1	1	1
Pekinese	–	2	1	–
Cocker Spaniel	–	1	–	2
Poodle	1	–	–	1
Mixed	–	1	–	1
Schnauzer	–	–	1	–
Chihuahua	–	–	–	1
Boxer	–	–	–	1
Total	8	15	11	16

Table 2. Dog characteristics and serum nitrotyrosine concentrations of the control and three MMVD groups (mean \pm SE)

	Control (n = 8)	Mild MMVD (n = 15)	Moderate MMVD (n = 11)	Severe MMVD (n = 16)
Age (yr)	6.12 \pm 2.99	12.17 \pm 1.08	12.73 \pm 0.56	11.75 \pm 1.03
Sex (males/females)	3/7	8/7	5/6	7/9
Weight (kg)	4.91 \pm 1.56	5.13 \pm 0.79	4.37 \pm 0.80	4.18 \pm 0.88
Platelets ($10^3/\mu\text{L}$)	386.25 \pm 125.93	415.00 \pm 43.65	473.73 \pm 45.18	497.50 \pm 60.02
Serum nitrotyrosine (nM)	4,416.21 \pm 162.03	4,793.79 \pm 122.51	4,905.83 \pm 160.23*	4,477.36 \pm 124.96†

*Significant difference between control and moderate groups ($p < 0.05$). †Significant difference between moderate and severe groups ($p < 0.05$).

**Fig. 1.** Average of serum nitrotyrosine concentrations in controls and three MMVD groups. Significant difference was observed among groups (* $p < 0.05$).**Fig. 2.** Average of serum nitrotyrosine concentrations between males and females with MMVD.

nM) ($p = 0.74$) (Fig. 2). There were negative correlation between serum nitrotyrosine concentration and age ($n = 30$; $R^2 = 0.067$, $p = 0.27$), and between serum nitrotyrosine and the left atrial-to-aortic root (LA/Ao) ratio ($n = 30$; $R^2 = 0.018$, $p = 0.57$), but the correlations were not significant. However, the negative correlation between serum nitrotyrosine concentration and platelets ($n = 30$; $R^2 = 0.389$, $p = 0.003$) was significant (Fig. 3).

Discussion

In this study, the serum concentration of nitrotyrosine was significantly higher in the moderate MMVD group than in the control group. Our results, showing an increased nitrotyrosine in early stage MMVD, are similar to those reported for a previous study of dogs with spontaneous cardiac disease [13]. The high nitrotyrosine concentrations in mild and moderate MMVD dogs are thought to be the result of the upregulated iNOS in early stage MMVD in dogs. Many studies in humans and animals have reported that iNOS expression, which induce NO production, is increased in various cardiac diseases, such as myocardial infarction, DCM, ischemic heart disease, and valvular heart disease [3, 10, 11]. Umar *et al.* [21] reported that cardiac remodeling, resulting in ventricular hypertrophy, dilatation, and sudden cardiac death can be initiated by iNOS overexpression. Another report indicated that the left ventricular ejection fraction and nitrotyrosine concentration had a statistically significant negative linear relationship in cytokine-induced myocardial dysfunction of dogs [18]. Thus, the increased serum nitrotyrosine concentration in dogs with mild and moderate MMVD observed in this study is thought to have resulted from the overproduction of NO from iNOS.

We also found that the serum concentration of nitrotyrosine in dogs with severe MMVD was significantly low compared to that in dogs in the moderate MMVD group. This result is similar to that of Pedersen *et al.* [19] in which as MMVD severity increased serum nitrotyrosine levels decreased. Medications for congestive heart failure resulting in MMVD, such as pimobendan and ACE-inhibitors, may regulate inflammatory mediators and iNOS expressions. Mechanisms related to NO and its effects in heart disease are complex and have not been fully described [15].

Negative correlation were detected in the relationships of serum nitrotyrosine with age, LA/Ao ratio, and platelet counts by performing Pearson correlation analysis. However, of those three variables, the only variable with a significant negative correlation to serum nitrotyrosine concentration was platelet count. One explanation for a high platelet count may be platelet fragmentation resulting from shear stress due to mitral regurgitation [2]. In addition, increased platelet reactivity has been reported in some human patients with mitral

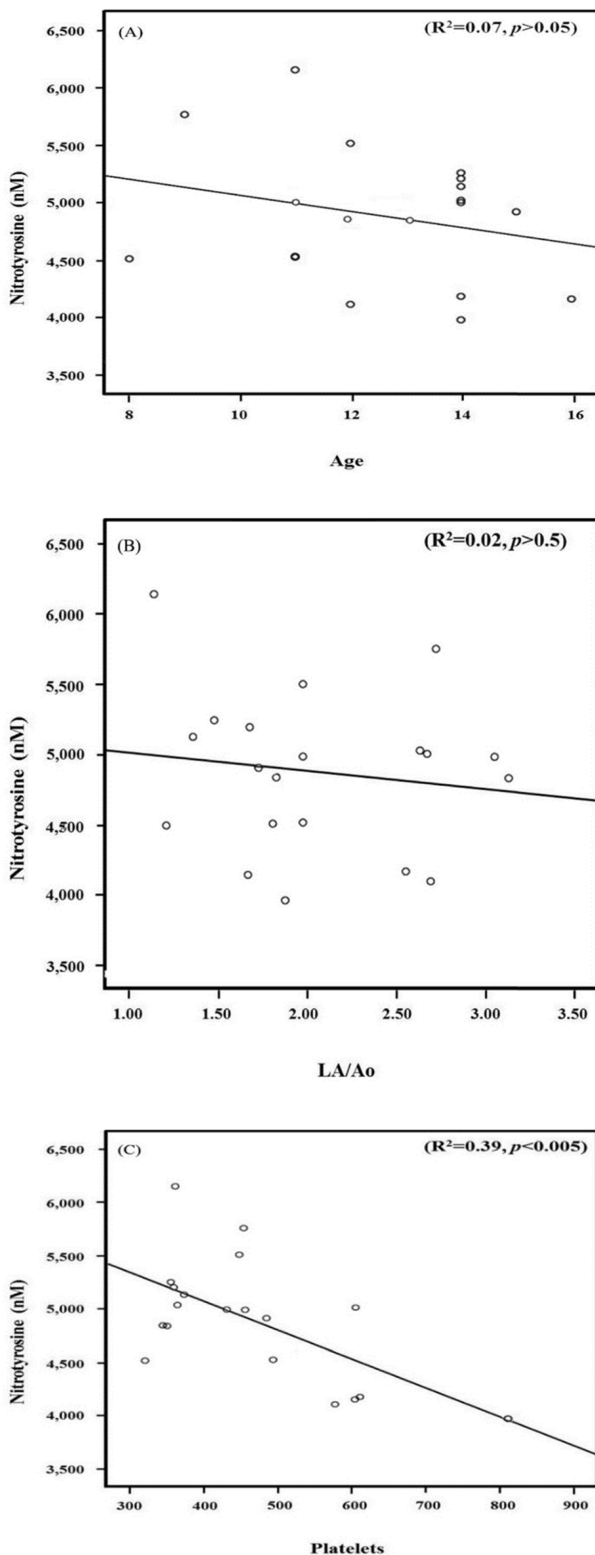


Fig. 3. Correlations of serum nitrotyrosine concentrations versus (A) age, (B) the left atrial-to-aortic root (LA/Ao) ratio, and (C) platelets with control and three MMVD groups. Significant negative correlation is observed between platelets count and serum nitrotyrosine concentration.

valve prolapse [22].

In patient with chronic heart failure, coagulation related factors such as fibrinogen, Von Willebrand factor, and soluble P-selectin levels were elevated in patients with congestive heart failure [8]. In another study, platelet counts were significantly higher in the moderate to severe MMVD group than in the mild MMVD group [20]. Those results are similar to our results in which a significant negative correlation was observed between platelet count and serum nitrotyrosine concentrations. Further study is needed to elucidate the relationship between platelet counts and serum nitrotyrosine concentrations. There are some limitations to this study. Although the prevalence of MMVD has been reported to be 90% in small breed dogs older than 8 years old, there was no breed standardization. In addition, each individual could not be measured the serum nitrotyrosine concentrations on progression of the disease.

In conclusion, this study showed that end-products of the tyrosine nitration were higher in moderate MMVD dogs compared to the levels in control dogs and were lowered in severe MMVD dogs than moderate MMVD dogs. This suggests that nitrotyrosine as an end-products of the nitration of tyrosine may represent a potential biomarker which can be used in the MMVD severity in dogs. Further studies are needed to demonstrate the breed-specific changes in nitrotyrosine concentration in dogs with cardiac dysfunction and increasing MMVD severity.

Acknowledgments

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