pISSN 1598-298X / eISSN 2384-0749
J Vet Clin 36(2) : 102-105 (2019)
http://dx.doi.org/10.17555/jvc.2019.04.36.2.102

Journal of Veterinary Clinics
www.ksvc.or.kr

Serum Concentration of Nitrotyrosine as Indicator of Disease Progress in Dogs with Myxomatous Mitral Valve Disease

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(Received: January 09, 2019 / Accepted: March 18, 2019)

Abstract : Nitrotyrosine was found to be dependent on the severity of myxomatous mitral valve disease (MMVD). However, a correlation of serum nitrotyrosine concentration in dogs with MMVD and the progression of the disease has not been investigated. This study compared changes in serum nitrotyrosine concentration with the progression of MMVD. Nine client-owned dogs were recruited for the study. Dogs were classified by measuring the amount of regurgitation using echocardiography into mild, moderate, or severe MMVD groups. Serum nitrotyrosine concentration was measured by an enzyme-linked immunosorbent assay test. Serum nitrotyrosine concentration was significantly higher at 180 days than at 0 day ($P < 0.05$). However, serum nitrotyrosine concentration at 360 days was lower than that at 180 days ($P < 0.05$). Serum nitrotyrosine concentration at 540 days was lower than at 180 days ($P < 0.05$). There was no correlation between serum nitrotyrosine and left atrial to aortic root diameter ratio (LA/Ao ratio) ($n = 33$, $R^2 = 0.003$, $P = 0.759$). Also, there was no correlation between serum nitrotyrosine and vertebral heart score (VHS) ($n = 33$, $R^2 = 0.026$, $P = 0.368$) and left ventricular end-diastolic diameter, normalized for body weight by the formula (LVEDDN) ($n = 33$, $R^2 = 0.053$, $P = 0.196$). The results of the study suggest that the progression of MMVD is correlated with changes in serum nitrotyrosine concentration, which shows potential for use as a cardiac biomarker which can be used to analyze the progression of disease in MMVD.

Key words **:** MMVD, nitrotyrosine, iNOS, peroxynitrite, LVEDDN, canine**.**

Introduction

Myxomatous mitral valve disease (MMVD) due to chronic valve degeneration is one of the representative cardiac diseases in old dogs. Classification according to the state is necessary to manage the disease. Depending on this classification, the use of drugs may vary and the disease management method can differ (1,14).

The progression of heart disease is variable, and there are many ways to monitor progression of chronic heart failure. A variety of biomarkers, such as NT-proBNP, Troponin I, and serotonin, can be used, depending on physical examination, radiography, and echocardiography (4,17). Nitric oxide (NO) is produced as free radical molecules in blood vessels and acts on the regulation of blood pressure and blood vessel tension. The NO produced is called NO synthases (NOS) and exists in three isoforms. Constitutive NOS (nNOS and eNOS) usually appear at physiological conditions and inducible NOS (iNOS) is expressed by inflammatory cytokines and endotoxins. Experimental results have shown that excessive NO production due to inflammatory stimuli has a negative effect on cardiac function (8,11).

Studies in humans have shown that NO has negative inotropic and cytotoxic effects on heart disease (7). This is because iNOS in NO is stimulated by inflammatory cytokines in cardiac myocytes, resulting in a negative contraction effect (2,7,18). In fact, humans with heart disease have a high NO concentration (6). The exact mechanism by which NO has an effect on animal heart disease has not yet been confirmed. There is a correlation between serum nitrotyrosine concentration and MMVD severity (15). Serum nitrotyrosine concentration increases in dogs with chronic valve degeneration (15,16). The purpose of this study is to investigate a correlation of MMVD progression with serum nitrotyrosine concentration using enzyme-linked immunosorbent assays (ELISA).

Materials and Methods

Animals and sampling

Nine client-owned dogs were recruited at Veterinary Medical Teaching Hospital of Chungnam National University between April 2015 and September 2017. Nine dogs comprised the ranged in size from small to medium (weight: 3.2 to 9.1 kg), were between 4 and 16 years of age. An owner information agreement was obtained. As inclusion criteria for this study, dogs exhibited signs of MMVD during physical, radiographic and echocardiographic exams. Dogs suffering from congenital heart disease or severe systemic disease were excluded from this study. The examination included physical examination, blood collection, radiography and echocardiography. All examinations were performed in a quiet examination room without sedation or anesthesia. Three milliliters of blood were obtained through the jugular vein. Blood samples were placed at room temperature for 30 minutes and then

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centrifuged. Separated serums were transferred to an Eppendorf tube and immediately stored in a −80ºC freezer for batch analysis.

Sandwich ELISA assay

Serum nitrotyrosine concentration was measured by a commercially available enzyme-linked immunosorbent assay, ELISA (Nitrotyrosine ELISA Kit, CELL BIOLABS Inc., USA) according to the manufacturer's instructions.

Sample grouping

Echocardiography with an iU22® (Phillips, Bothell, WA, USA) was performed to diagnose size of the heart, measure the amount of regurgitation, and determine the severity of MMVD. The diagnosis of MMVD was based on the following factors: mitral valve leaflet prolapse, valve thickening, and mitral regurgitation (MR) on color Doppler mapping echocardiography from the left apical 4-chamber view. The left atrial to aortic root ratio (LA/Ao ratio) was measured by the right 2-dimentional short axis view. Vertebral heart score (VHS) was measured based on radiography. LVEDDN is a formula that normalizes the left ventricular diastolic diameter to body weight: LVIDd/Body Weight$^{(1/3)}$. Estimation of MMVD severity was based on color Doppler echocardiographic mapping obtained using a 2.5 MHz electronic sector transducer. MMVD in dogs was classified as follows: mild MMVD (< 30%), moderate MMVD (30-50%), or severe MMVD (> 50%).

Statistical analysis

Statistical analysis was performed by a commercially available computer based software program (IBM SPSS Statistics 24.0.0, SPSS Inc., USA). Serum nitrotyrosine concentration is presented as the average values of each group with standard deviation. A P value of < 0.05 was considered significant. In order to investigate overall association between serum nitrotyrosine concentration and the MMVD severity groups, one-way ANOVA was used. If a significant association ($P < 0.05$) was detected, a pair-wise comparison was performed by use of an independent t-test. Correlation between each dog's characteristics and echocardiographic measurement with serum nitrotyrosine concentration was determined by Pearson's correlation coefficient.

Table 1. Breed composition of MMVD dogs

Breed	MMVD dogs
Maltese	4
Shih Tzu	3
Yorkshire Terrier	1
Schnauzer	1
Total	9

MMVD; myxomatous mitral valve disease.

Results

Nine dogs, consisting of 4 male dogs (4 neutered) and 5 female dogs (3 neutered), were included in this study. The breeds, arranged in descending order, are as follows: Maltese (n = 4; 45%), Shih Tzu (n = 3; 33%), Yorkshire Terrier (n = 1; 11%), and Schnauzer (n = 1; 11%) (Table 1). On day zero of the study, all nine dogs were classified as having moderate MMVD based on their mitral regurgitation profiles. The severe group increased to 3 dogs in 180 days, 5 dogs in 360 days, and 6 dogs in 540 days. Dog characteristics and serum nitrotyrosine concentrations of the four period groups are presented in Table 2. Compared to day zero, dogs at 180 days had a significantly higher serum nitrotyrosine concentration ($P < 0.05$). The serum nitrotyrosine concentration in dogs at 360 days was significantly lower than that in dogs at 180 days ($P < 0.05$), and at 540 days it was significantly lower than that in dogs at 180 days ($P < 0.05$). However, serum nitrotyrosine concentrations were not significantly different between dogs at 360 days and at 540 days or between dogs at 0 days and 360 days (Fig 2). There was no correlation between serum nitrotyrosine and LA/Ao ratio (n = 33, $R^2 = 0.003$, $P = 0.759$). Also, there was no correlation between serum nitrotyrosine and VHS (n = 33, $R^2 = 0.026$, $P = 0.368$) or LVEDDN (n = 33, $R^2 = 0.053$, $P = 0.196$) (Fig 1).

Discussion

In animals, studies on the relationship between heart disease and functional impairment due to oxidative damage of the heart caused by NO in vivo, such as mouse and dog mod-

Table 2. Dog characteristics and serum nitrotyrosine concentrations of the four periods groups (mean ± SD)

	0 day	180 days	360 days	540 days
VHS	10.75 ± 0.34	11.15 ± 0.32	11.02 ± 0.34	11.26 ± 0.50
LA/Ao ratio	2.13 ± 0.15	2.00 ± 0.16	2.06 ± 0.14	2.24 ± 0.14
LVEDDN	1.46 ± 0.11	1.59 ± 0.10	1.58 ± 0.10	1.68 ± 0.13
MMVD				
Moderate	9	6	4	3
Severe	0	3	5	6
Serum nitrotyrosine (nM)	3,343.08 ± 174.19	4,220.12 ± 181.71*	3,460.98 ± 164.25**	3,319.47 ± 152.59***

MMVD; myxomatous mitral valve disease, LA/Ao ratio; left atrial to aortic root ratio, LVEDDN; left ventricular diastolic diameter to body weight, VHS; vertebral heart score.
*Represent a significant difference between 0 day and 180 days ($P < 0.05$).
**Represent a significant difference between 180 days and 360 days ($P < 0.05$).
***Represent a significant difference between 180 days and 540 days ($P < 0.05$).

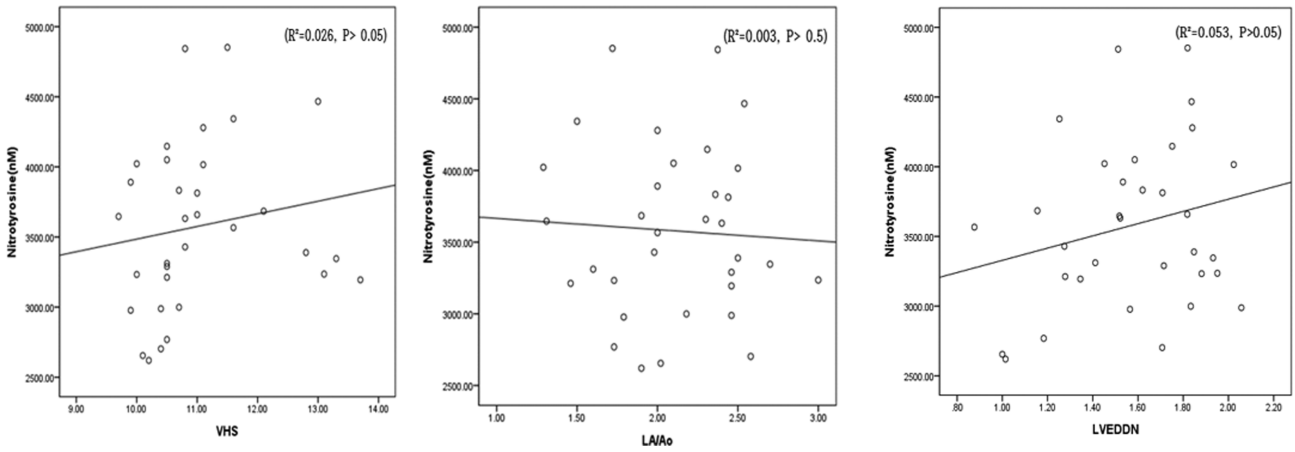

Fig 1. The relationship between serum nitrotyrosine concentration and VHS, LA/Ao ratio and LVEDDN with 4 periods groups. There were no significant difference at all.

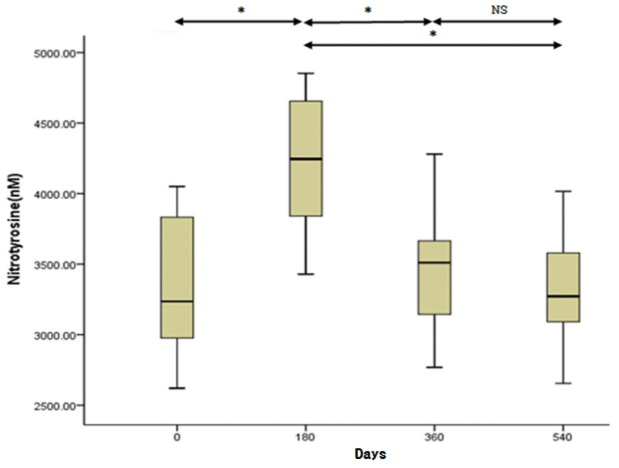

Fig 2. Average of serum nitrotyrosine concentrations in 4 periods groups. NS; no significant difference. *$P < 0.05$; significant difference between the groups.

els, are actively being carried out (16,19). Similar to humans, iNOS was measured using PCR, microscopic examination, immunoblot test, etc. to measure NO indirectly (5,8,15). Nitrotyrosine is a marker that is formed when nitration and tyrosine production conditions of tyrosine residues increase due to an oxidizing agent derived from nitric oxide called peroxynitrite (ONOO-) (13). It is possible to indirectly measure NO using a stable end product nitrotyrosine. This study measured serum nitrotyrosine concentration in MMVD patients over time and focused on their potential for use as biomarkers in connection with the progression of heart disease.

Our results show that, at 180 days into the study, serum nitrotyrosine concentration was significantly higher than at day zero. This finding is similar with that of a previous study measuring nitrate and nitrite, the end products of NO metabolism (8). Serum nitrotyrosine concentration from dogs with MMVD disease were also higher in mild and moderate MMVD (10). These are thought to be the result of over-expression of iNOS. There is also a study indicating that increased NO activity may induce iNOS over-expression, and that NO produced may be involved in the denaturation of leaflets of canine MMVD (15). Thus, an increase in serum nitrotyrosine concentration at 180 days is thought to result from the over-expression of NO by iNOS. However, compared to 180 days, serum nitrotyrosine concentration decreased at 360 and 540 days. Our results were similar to those obtained when serum nitrotyrosine concentrations were decreased to moderate and severe stages when classified according to the mitral regurgitation stage (10). In the study of NO metabolites in dogs with untreated MR, NO metabolites decreased as MR severity increased (16). Various factors, such as response to drugs, mechanical stress, and changes in membrane receptor type due to changes in myocyte composition, may influence the down-regulation of serum nitrotyrosine concentration. The rate of progression of heart disease can vary depending on age, overall health, and cardiac condition. Over time, the number of subjects in the severe group (based on MMVD) increased compared to the moderate group. Patients with heart failure will exhibit decreased cardiac function over time and should be treated with medications such as inotropic, diuretics, and ACEi. Drugs used in treating congestive heart failure in MMVD can function to regulate inflammatory mediators and regulate iNOS associated with the expression of NO (3,9).

However, the reasons for these phenomena have not been explained sufficiently. Mechanisms related to NO and its effects on heart disease are complex and have not been fully described (12). The correlation between serum nitrotyrosine concentration and LA/Ao ratio, VHS, and LVEDDN was low. All three were used for cardiac assessment, and the relationship between LA/Ao ratio and serum nitrotyrosine concentration was poorly correlated with the serum nitrotyrosine concentration measurement according to the mitral regurgitation stage (10). In this study, it was concluded that VHS and LVEDDN are also not correlated with serum nitrotyrosine concentration. There are some limitations to this study. The first was that the number of dogs included in the study was small. In addition, there was no strict control over diseases other than heart disease during this study period.

In conclusion, the study suggests that serum nitrotyrosine concentration as end-products of the tyrosine nitration may be used as a potential biomarker for measuring the progres-

sion of MMVD in dogs.

Acknowledgements

This work was supported by research fund of Chungnam National University (2018-0692-01).

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