Myxomatous mitral valve disease (MMVD) is a cardiac condition commonly found in older dogs. The disease process can lead to heart failure (HF). In HF, an increase of reactive oxygen species (ROS) and abnormal mitochondrial activity, as well as apoptosis, have been reported. Humanin (HN) is a polypeptide that has a cardioprotective effect against apoptosis and oxidative stress. The purposes of this study were (1) to investigate the potential role of plasma HN as a cardiac biomarker to predict disease progression of MMVD, and (2) to compare plasma HN concentrations with plasma NT-proBNP concentrations. Thirty-one dogs were included in the study. The dogs were separated into four groups: Group 1 was healthy dogs (n = 8), Group 2 was MMVD class B (n = 8), Group 3 was MMVD class C (n = 8), and Group 4 was MMVD class D (n = 7). All dogs were given a physical examination, thoracic radiography, echocardiography, and samples of their blood were collected for hematology and blood chemistry analysis. Levels of plasma HN and plasma NT-proBNP were also investigated. The results showed that plasma HN levels were lower in the dogs with MMVD and that lower plasma HN levels were associated with greater severity of MMVD-induced HF. It was possible to observe changes in plasma HN levels at a less severe disease stage than plasma NT-proBNP in dogs with MMVD. These findings suggest that a decreased plasma HN level can be used as a biomarker to identify dogs with MMVD-induced HF.

**Key words:** Humanin, myxomatous mitral valve disease, heart failure, NT-proBNP, ROS

Co-respondent to: C. Boonyapakorn, e-mail: boonyapakorn@gmail.com
Introduction

Myxomatous mitral valve disease (MMVD) is a pathological degeneration of the mitral valves. It is a cardiac condition commonly found in older small- and medium-sized dogs (Aupperle and Disatian 2012, Fox 2012). With MMVD, malcoaptation of thickening valves causes regurgitation of blood to the left atrium (LA) and a decrease in cardiac output (CO). Heart failure (HF) is a common outcome of the disease processes (Mann and Bristow 2005). There is growing evidence that reactive oxygen species (ROS) is a critical mediator, inducing myocardial damage in MMVD-induced HF (Olsen et al. 2003, Reimann et al. 2014, Li et al. 2015). Previous studies have demonstrated that the apoptotic process is one of the mechanisms leading to cardiac dysfunction and ultimately HF (Narula et al. 1996, Olivetti et al. 1997, Sabbah 2000). Excessive ROS production is one of the activators that initiates the apoptosis cascade of myocytes in HF (Harada et al. 2004, Hashimoto et al. 2009, Widmer et al. 2013, Thummasorn et al. 2017). Widmer et al. (2013) demonstrated that levels of plasma endogenous HN were significantly decreased in patients with coronary endothelial dysfunction when compared with patients with normal endothelial function. This finding is consistent with another study which reported that plasma endogenous HN was significantly decreased in rats with myocardial ischemia/reperfusion (I/R) injury (Thummasorn et al. 2017). The existence of a canine equivalent (caninein) has not yet been definitively demonstrated. However, a PCP ELISA kit is available which is designed specifically for research on caninein.

Humanin (HN) is a polypeptide which was first discovered by Hashimoto and colleagues (Hashimoto et al. 2001a, b). HN is encoded by the MT-RNR2 gene, the mitochondrial gene which encodes ribosomal RNA (rRNA) which is part of mitochondrial 16s rRNA (Lee et al. 2013). Two forms of HN have been identified, the cytoplasmic form and the mitochondrial form (Guo et al. 2003, Paharkova et al. 2015). The cytoplasmic form consists of a 24 amino acid molecule that is encoded in the nuclear genome and is translated within the cytoplasm (Hashimoto et al. 2001b, Yamagishi et al. 2003), whereas the 21 amino acid molecule is a mitochondrial form that is encoded and translated within the mitochondria (Maximov et al. 2002). Many studies have established that HN exerts a cytoprotective effect against apoptosis via binding to specific receptors (such as formylpeptide receptor-like-1, trimeric complex receptors) that cause downstream signaling cascades to begin (Harada et al. 2004, Hashimoto et al. 2009, Charununtakorn et al. 2016). Antiapoptotic properties have also been found to be the result of the inhibition of the pro-apoptotic protein’s translocation to the mitochondria. Under normal physiological conditions, endogenous HN can be produced by numerous tissues in the body such as the heart, brain, skeletal muscle, and liver (Gong et al. 2014, Charununtakorn et al. 2016). The endogenous HN is then secreted into the circulatory system and transported to HN receptors in several target cells, especially cardiomyocytes, inhibiting apoptosis (Fischer and Hilfiker-Kleiner 2008, Muzumdar et al. 2010, Cittadini et al. 2012, Klein et al. 2013, Thummasorn et al. 2017). Previous studies have demonstrated that a decrease in plasma endogenous HN level was correlated with cardiac injury (Widmer et al. 2013, Thummasorn et al. 2017). Previous studies have also found that a decrease in plasma HN levels is correlated with increased cardiac filling pressure (van Wamel et al. 2000, Roncon et al. 2006). In clinical studies, increases in plasma NT-proBNP have been used mostly as a prognostic marker in cases of heart failure (Greco et al. 2003, Prosek et al. 2007, Januzzi et al. 2008).

The present study investigated both the plasma HN levels in dogs with MMVD-induced HF as well as the potential of HN as a biomarker for detection of HF in dogs with MMVD. In addition, comparison of changes in the levels of plasma HN and of plasma NT-proBNP in dogs with MMVD-induced HF was conducted. The primary outcome of this study was a change in plasma HN levels in the dogs with MMVD-induced HF. We hypothesized that a decrease in plasma HN levels is correlated with an increase in the severity of HF in dogs with MMVD.

Materials and Methods

Animal preparation: All procedures involving animals were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University (Permit: No. R2/2558). Dog owners were informed about the study protocol, and consent forms were obtained from them before starting the study.

Experimental design: Thirty-one dogs were obtained from the cardiology clinic at the Small Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University (FVM-CMU). Dogs that had systemic diseases or other heart diseases were excluded from the
plasma samples were collected and stored at −80°C until the plasma HN and NT-proBNP levels were measured.

Measurement of plasma HN levels: In this study, plasma HN levels were analyzed using a commercial Canine Putative Caninein Peptide (PCP) ELISA test kit (catalog number MB5754707) (MyBioSource, San Diego, California, USA). The test was performed following the manufacturer instructions. Plasma (100 μL) was added to a microtiter plate. Conjugate (100 μL) was then added to each well, and the sample was then incubated for 60 min at 37°C. After incubation, the microtiter plate was washed using a washing solution. Substrate A (50 μL) and substrate B (50 μL) were added to each well, and the plate was then covered and incubated for 10-15 min at 20-25°C. A stop solution (50 μL) was then added to each well. Finally, the concentration of plasma HN was measured at a wavelength of 450 nm using a spectrophotometer.

Measurement of plasma NT-proBNP levels: Plasma NT-proBNP levels were analyzed using commercial canine proBNP ELISA test kits (catalog number CN0015) (Neo Scientific, Woburn, Massachusetts, USA). The test was performed as per the manufacturer’s instructions. Enzyme solution (50 μL) was added to each well and the plate was incubated for 60 min at 37°C in a humid chamber. After incubation, the microtiter plate was washed using a washing solution. Substrate A (50 μL) and substrate B (50 μL) were added to each well and incubated for 10-15 min at room temperature after which a stop solution (50 μL) was added to each well. Finally, the plasma NT-proBNP concentration level was measured at a wavelength of 450 nm using a spectrophotometer.

Statistical methods: All data are presented as mean (SD). Differences in between-group means of all parameters were analyzed using ANOVA. If the assumptions of ANOVA, including the normality of residuals and homogeneity of variance, were not met, then the Kruskal–Wallis test was used. The normality assumption was evaluated by Q-Q plot and the Anderson–Darling test. In addition, Levene’s test was used to test the homogeneity of variance assumption. Tukey’s test was performed for multiple comparisons. A value of p<0.05 was considered indicative of statistical significance for all statistical tests. All statistical analyses were done using the R program (version R-3.3.1).

Results

Characteristics, physical examination results, and radiographic findings

In this study, the dogs were grouped as follows: Group 1 (n = 8; 8 Poodles) included healthy dogs with-
out heart murmur, Group 2 (n = 8; 4 Poodles, 2 Shih Tzus, 1 Pomeranian, and 1 Chihuahua) was dogs with class B MMVD, Group 3 (n = 8; 4 Poodles, 1 Shih Tzu, 2 Pomeranians, and 1 Chihuahua) was dogs with class C MMVD, and Group 4 (n = 7; 4 Poodles and 3 Shih Tzus) was dogs with class D MMVD. Results of the physical examinations are shown in Table 1. The average age of the dogs in Group 4 was higher than in the other groups (Groups 1, 2 and 3). Additionally, the heart rate (HR) in Group 4 was higher than Groups 1 and 2, although the HR in Group 4 was not significantly different from Group 3. There was no significant difference in body weight among the four groups. Investigation of the severity of MMVD found that the dogs with class B MMVD (Group 2) had murmur intensity in the range of 3/6 – 4/6, while the murmur intensity in the dogs with class C MMVD (Group 3) and class D MMVD (Group 4) met grade 6/6. Results of the radiographic investigation showed that the VHS in the dogs with class C (Group 3) and class D (Group 4) MMVD was significantly higher when compared with the healthy dogs (Group 1).
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Echocardiographic results

Echocardiographic results (Table 2) showed that both LVIDd and EDV in all dogs with MMVD (Groups 2, 3, and 4) were higher than in healthy dogs (Group 1). The SV in Groups 3 and 4 were significantly higher compared with Group 1. Moreover, the LA:Ao ratios in Groups 3 and 4 were also significantly increased compared with Group 1. However, we found that only FS in Group 4 was significantly increased when compared with Group 1 and Group 2 (Table 2).

Levels of plasma HN and plasma NT-proBNP in dogs with MMVD

Our results showed that the plasma HN levels were highest in Group 1 and lowest in Group 4 (Table 3). The plasma HN levels were observed to have a decreasing trend from Group 1 through Group 4, although only the plasma HN levels in Groups 3 and 4 were significantly lower when compared with Group 1 (Table 3). Similarly, plasma NT-proBNP levels were observed to have an increasing trend from Group 1 through Group 4. However, only the plasma NT-proBNP levels in Group 4 were significantly increased when compared with Group 1 (Table 3).

Discussion

Our study found that plasma HN levels were lower in dogs with MMVD. Additionally, our results demonstrated for the first time that a significant change in plasma HN level can be observed at a less severe disease stage than with plasma NT-proBNP (a detection biomarker for HF). We found that plasma HN levels were significantly lower in dogs with class C MMVD and class D MMVD when compared with healthy dogs, whereas the plasma NT-proBNP levels were significantly higher only in dogs with class D MMVD. These findings suggest that lower plasma HN levels are related to increased cardiac injury and that decreased plasma HN levels can be used as an early detection marker for dogs with MMVD-induced HF.

Cardiac hypertrophy is one of the compensatory outcomes of the pathological processes of MMVD (Mann and Bristow 2005). Cardiac hypertrophy can induce myocardial damage and lead to HF via increased oxidative stress (von Harsdorf et al. 1999, Seddon et al. 2007, Tsutsui et al. 2011) and NOX-derived ROS production increased during the development of cardiac hypertrophy. In addition, ROS such as xanthine oxidase-derived O2− caused left ventricle contractile impairment in HF, increased myocardial oxygen consumption, and reduced cardiac efficiency (Seddon et al. 2007, Sag et al. 2014). It is well known that oxidative stress is the result of an imbalance between antioxidant and ROS production (Heusch and Schulz 2011, Tsutsui et al. 2011). Studies have demonstrated that ROS levels are markedly increased in HF (Freeman et al. 2005, Tsutsui et al. 2011, Munzel et al. 2015). An increase in ROS levels induces myocardial damage by activating apoptotic processes, leading to cardiac cell death (von Harsdorf et al. 1999). It has been established that the mitochondria is the major organelle producing ROS within cardiac cells (Akhmedov et al. 2015, Sabbah 2016), suggesting that trying to reduce ROS and apoptosis is a promising therapeutic strategy for attenuating cardiac dysfunction in patients with MMVD-induced HF. Growing evidence has indicated that one of the cytoprotective properties of HN is an anti-apoptotic effect by reducing ROS levels (Klein et al. 2013, Paharkova et al. 2015, Thummasorn et al. 2016). Recent studies have demonstrated that a change in HN level can be used as a detection marker for cardiovascular disease (Widmer et al. 2013, Thummasorn et al. 2016). In the present study, we first showed that plasma HN levels were significantly lower in dogs with MMVD-induced HF. This suggests plasma HN is a potential cardiac biomarker for detection of HF, something that should be investigated further in clinical studies.

Regarding the mechanism of reduction of plasma HN levels after MMVD-induced HF, it is possible that with lower plasma HN, endogenous HN in circulating blood is recruited into the heart against HF-induced ROS production levels to reduce myocardial damage. This hypothesis is consistent with a previous study which reported that plasma endogenous HN levels were decreased against reduced I/R injury by allowing endogenous HN from the circulating blood into the...
damaged myocardium (Thummasorn et al. 2017). In that study, the plasma HN level was significantly decreased following I/R injury. That study also reported that the endogenous HN level in the ischemic myocardium was significantly increased after I/R injury when compared to no I/R injury. Thus, it is possible that the decrease in plasma HN levels observed in the present study might have been a result of recruiting endogenous HN from the circulating blood into the heart to protect against MMVD-induced myocardial damage. A study of levels of humanin in different age groups reported that humanin can decline with age (Bachar et al. 2010). This pattern might exist in animals as well, although the pattern could be affected by the significantly shorter lifespan of dogs. Further studies are needed. In a human study, there was no statistical difference in humanin levels between males and females (Lytvyn et al. 2015). That is consistent with this study which found no statistical difference between male and female dogs. A larger sample size would be needed to identify the affect of gender on variation in HN levels in dogs.

NT-proBNP is one of the cardiac biomarkers commonly used in clinical practice (Greco et al. 2003, Januzzi et al. 2008, Oyama et al. 2008, Tarnow et al. 2009). Previous studies have demonstrated that NT-proBNP has the ability to determine the severity of heart failure in dogs (Oyama et al. 2008). In this study, we compared the efficacy of HN and NT-proBNP for the detection of MMVD-induced HF. Our results showed that plasma NT-proBNP levels tend to increase commensurate with the severity of the heart disease. However, we also observed that only dogs with class D MMVD had levels of plasma NT-proBNP significantly higher than in healthy dogs. This contrasts with the finding that plasma HN levels in dogs with class D MMVD as well as dogs with class C MMVD were significantly lower when compared with healthy dogs. These findings suggest that a decreased plasma HN level may be used as a detection biomarker for dogs with MMVD-induced HF. However, future studies with larger populations are needed to warrant its use.

Limitations of this study include the small sample size, variation in sex ratio, and recording of weight only rather than body condition scores. There were only 31 dogs, and the heart disease groups had a markedly different sex ratio than the control group. Thus it is possible that the findings are the result of sample size/sex ratio rather than exclusively actual group differences. A larger study, including a much larger normal reference range, would provide much stronger evidence for this biomarker. Use of body condition scores would allow evaluation of differences among groups based on body score.

Conclusions

Plasma HN levels are reduced in dogs with MMVD and that reduction is associated with increased severity of MMVD-induced HF. To the best of our knowledge, this is the first reported demonstration that changes in plasma HN level can be observed earlier than changes in plasma NT-proBNP. These findings suggest that a decreased plasma HN level can be used as a detection biomarker for dogs with MMVD-induced HF.

Acknowledgements

This study was supported by the National Science and Technology Development Agency, Thailand (NC). The author would like to thank Dr. Savitree Charununtakorn for valuable advice. The results related to humanin levels were previously presented as an Abstract at the 16th Chulalongkorn University Veterinary Conference, Bangkok, 22-24 March 2017.

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