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นางสาวธนวัน มังคละพฤกษ์

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CIRCULATING SEROTONIN CONCENTRATIONS IN DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE

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จากการศึกษาในคน เป็นที่ทราบกันดีว่าระดับซีโรโตนินในกระแสเลือดนั้นเกี่ยวข้องกับการเกิดโรคลิ้นหัวใจ แต่ระดับซีโรโตนินต่อโรคหัวใจไมทรัลเสื่อมในสุนัขนั้นยังไม่เป็นที่ทราบแน่ชัด การศึกษานี้มีจุดประสงค์เพื่อเปรียบเทียบระดับซีโรโตนินในกระแสเลือดระหว่างสุนัขปกติและสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม การศึกษาประกอบด้วยกลุ่มสุนัขปกติ 20 ตัว และกลุ่มสุนัขที่เป็นโรค 23 ตัว สุนัขทั้งหมดเป็นสายพันธุ์เล็กที่มีน้ำหนักตัวน้อยกว่าสิบกิโลกรัม และมีอายุมากกว่าเจ็ดปี เลือดจากสุนัขที่ทำการศึกษานำมาแยกส่วนของซีรั่ม พลาสม่า และเกล็ดเลือด จากนั้นทำการวัดระดับซีโรโตนินในส่วนต่างๆด้วยชุดทดสอบแบบอีไลซ่า (ELISA test) ค่ากลางของระดับซีโรโตนินในพลาสม่าและเกล็ดเลือดของสุนัขปกตินั้นเมื่อเปรียบเทียบกับสุนัขที่เป็นโรคพบว่าไม่แตกต่างกันอย่างมีนัยสำคัญ ส่วนค่ากลางของระดับซีโรโตนินในซีรั่มในสุนัขที่เป็นโรคนั้นน้อยกว่าสุนัขปกติอย่างมีนัยสำคัญ ($p < 0.01$) ระดับซีโรโตนินไม่มีความสัมพันธ์กับอายุ จำนวนเกล็ดเลือดและค่าต่างๆจากการอัลตราซาวน์หัวใจ จากผลการศึกษาพบว่าระดับซีโรโตนินในพลาสม่าและเกล็ดเลือดไม่มีความแตกต่างกันระหว่างสุนัขทั้งสองกลุ่ม ถึงแม้จะพบความแตกต่างในซีรั่ม แต่ภายในสภาวะร่างกายจริงแล้วซีรั่มไม่ได้เป็นตัวแทนของระดับซีโรโตนินในกระแสเลือดที่ดี ดังนั้นการทดลองนี้เห็นว่าระดับซีโรโตนินในกระแสเลือดนั้นไม่ได้เป็นแหล่งของซีโรโตนินที่เกี่ยวข้องกับการเกิดโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัข แหล่งของซีโรโตนินน่าจะมาจากภายในลิ้นหัวใจไมทรัลเป็นหลัก การศึกษาภายหน้าควรทำการศึกษาเกี่ยวกับแหล่งอื่นๆของซีโรโตนินในร่างกายหรือการยับยั้งซีโรโตนินเฉพาะส่วนที่สามารถชะลอการเกิดโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัข

ภาควิชา.....อายุรศาสตร์…….....ลายมือชื่อนิสิต.....

สาขาวิชา...อายุรศาสตร์สัตวแพทย์..ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…....

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5275582931 : MAJOR VETERINARY MEDICINE
KEYWORDS : DEGENERATIVE MITRAL VALVE DISEASE / DOGS / PLASMA / PLATELETS / SEROTONIN / SERUM

TANAWAN MANGKLABRUKS: CIRCULATING SEROTONIN CONCENTRATIONS IN DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE. ADVISOR : SIRILAK SURACHETAPONG, D.V.M., Ph.D., 66 pp.

Serotonin mediating valvular disease is widely known in human medicine, but it is still unclear in canine degenerative mitral valve disease (DMVD). The Purpose of this study was to compare circulating serotonin concentrations between normal dogs and dogs with DMVD. Twenty healthy and twenty-three newly confirmed DMVD dogs, small breed less than 10 kilograms and older than 7 years old, were collected for the blood samples. Serum, plasma and platelet serotonin concentrations were measured by the serotonin ELISA test. Median plasma and platelet serotonin concentrations were not significantly different between the normal dogs and dogs with DMVD. While, median serum serotonin concentration in dogs with DMVD was significant lower than the normal dogs ($p < 0.01$). Age, platelet count, and echocardiographic indices showed no significant correlation with the circulating serotonin concentration. From the plasma and platelet results, the serotonin concentrations were unchanged between normal and DMVD group. Even though the difference had found in serum serotonin concentration, the serum sample does not represent the real circumstance in the body. In conclusion, circulating serotonin is unlikely to be a major source of serotonin in DMVD. Local serotonin signaling is suggested in mediating canine DMVD. Further study about the local serotonin blockage or other serotonin pathways mediating DMVD is warranted.

Department :.....Veterinary Medicine……. Student's Signature .....
Field of Study :….Veterinary Medicine…… Advisor's Signature .....
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CHAPTER I
INTRODUCTION

Degenerative mitral valve disease (DMVD) or myxomatous mitral valve disease is the important disease in the dog. It is the most commonly found in veterinary practice. The prevalence is increased in small breed dogs such as Poodle, Shih Tzu, Dachshund, and Chihuahua as well as mixed breed dogs. In Cavalier King Charles Spaniels, the disease has been reported for the highest occurrence and the earliest onset. The evidence of DMVD is increased with age and slightly greater in male than in female (Ware, 2003). More than 30% of small breed dogs that older than 10 years develop DMVD. Importantly, DMVD is also the most common cause developing congestive heart failure in dogs. At the early stage of the disease, the affected mitral valve becomes nodular and thickened. Chordea tendineae are also elongated, thickened, and occasional rupture. Fifty-eight percent of dogs older than 9 years develop severe degenerative valvular changes, whereas 90% of mild degenerative valvular changes have been reported in postmortem evidence of dogs older than 13 years (Detweiler and Patterson, 1965). Affected mitral valves result in mitral regurgitation into left atrium. As the disease progresses, cardiac remodeling will develop. Dogs with DMVD will eventually develop congestive heart failure, which impairs the normal activities of dogs and decreases their quality of life. Despite the fact that DMVD is the important disease in old dogs, the truly etiology of valvulopathy in DMVD is still unknown. As a result, until now, DMVD cannot be prevented or decreased disease progression (Abbott, 2008).

Many researchers have tried to find the causes of mitral valve degeneration in order to reduce the disease occurring. Recently, the evidence of serotonin signaling mediating canine DMVD has been reported. Serotonin or 5-hydroxytryptamine is a neurotransmitter in animals. Serotonin functions in dogs are related with behaviors, metabolism, vasoconstriction, and platelet function (Muneoka et al., 1991). According to many previous

studies, serotonin is suggested to play an important role in naturally occurring DMVD in dogs. (Oyama and Chittur, 2006; Arndt et al., 2009; Connolly et al., 2009; Disatian and Orton, 2009; Oyama and Levy, 2009).

The roles of serotonin in canine DMVD are widely established, but the hypothesis that circulating serotonin might play the important roles in serotonin signaling in canine DMVD becomes controversial. In the circulation, the major storage of serotonin is in the platelets (Meyer et al., 1982), only less amount of serotonin circulates in the blood stream. To find the truly circulating serotonin, plasma serotonin should be measured because this serotonin portion directly reflects the real serotonin in the body of the dogs. Simultaneously, platelet serotonin should be evaluated as it is the major source of serotonin. When platelets aggregate during blood clotting process, serotonin will be released from the platelets. Therefore, serum serotonin in clot blood samples can over exaggerate the actual circulating serotonin. The aim of this study is to define circulating serotonin concentration in serum, plasma, and platelets in dogs with DMVD. This study will be another research elucidating involvement of serotonin signaling in canine DMVD. The short term goal of this research is to determine the relationship between circulating serotonin and canine DMVD. The researcher ultimate goal is to prove a role of serotonin in mediating canine DMVD. In case that serotonin is the cause of canine DMVD, serotonin blockage may reduce serotonin signaling and inhibit the happening of the disease.

Objectives of Study

To compare serum, plasma, and platelet serotonin concentrations in normal dogs and dogs with DMVD.

Hypothesis

Serum, plasma and platelet serotonin concentrations increase in dogs with DMVD.

Keywords: Degenerative mitral valve disease, Dogs, Plasma, Platelets, Serotonin, Serum

Advantages of Study

1. To be able to understand the etiology of DMVD in dogs.
2. To be able to find the way to prevent or prolong the occurring of DMVD in dogs.

CHAPTER II

LITERATURE REVIEWS

Degenerative mitral valve disease

Degenerative mitral valve disease (DMVD) or myxomatous mitral valve disease or endocardiosis is the most common disease in geriatric dogs. DMVD is also the most frequency cause of congestive heart failure in dogs. The predisposing breed is toy breed dogs such as Poodle, Dachshund, Chihuahua, Miniature schnauzer, and Shih Tzu. In Cavalier King Charles Spaniel, the disease can be clinically detected as young as four years of age (Beardow et al., 1993). In general, the disease incidence and progression are increasing in the advanced age. 30 – 35 percent of dogs over than 13 years old are clinically detected signs of DMVD (i.e. murmur heart sound). Moreover, 58 percent of dogs over than 9 years show grossly changes of mitral valve leaflets from the autopsy (Detweiler and Patterson, 1965). Males are affected more than females, with a 1.5:1 male: female ratio (Atkins et al., 2009).

The etiology of DMVD is still unknown; however, there are many factors including genetics, hemodynamic, and metabolic disorders proving to be the potential causes of DMVD. Serotonin is one of the possible causes for DMVD. Roles of serotonin in DMVD will be discussed later in this chapter.

The pathology of DMVD occurs mainly at the mitral valve leaflets. However, it can affect tricuspid valves also. Grossly, the normal valve leaflets are thin and smooth while the abnormal valve leaflets are nodular thickening, opaque, and redundant. Chordae tendineae also become thickening, elongated and sometimes rupture. Histologically, the normal valve tissue consists of 4 layers, which are nicely divided into atrialis, spongiosa, fibrosa, and ventricularis. In DMVD, the affected valve tissue is marked thickening secondary to the

extension of the spongiosa layer. The expansion of the spongiosa layer is causing by the accumulation of glycosaminoglycan (GAG). Moreover, the fibrosa layer losses of collagen organization. With the accumulation of GAG and collagen abnormality, the valve leaflets loss of its function. These changes are often seen in mitral valve, less in tricuspid valve, and only rarely in aortic and pulmonic valve (Buchanan, 1977).

The pathophysiology begins with the changing of the mitral valve leaflets. The abnormal mitral valve leads to mitral valve insufficiency, causing the other secondary changes. Due to mitral regurgitation, blood leaks into the left atrium. As the time progresses left ventricular dilatation and volume overload occur. When cardiac output is reduced, the neurohormonal activation is triggered, resulting in sodium and water retention in the cardiovascular system in order to maintain enough cardiac output, blood pressure, and tissue perfusion (Oyama, 2009). Effects of excessive neurohormonal activation including increased water retention and vasoconstriction eventually lead to cardiac volume overload and left sided congestive heart failure (Abbott, 2008).

DMVD is divided into asymptomatic and symptomatic stages. Dogs with asymptomatic DMVD have mitral valve thickening or sometimes cardiac remodeling without any clinical signs. Dogs with symptomatic DMVD show signs of coughing, exercise intolerance, dyspnea, weakness, syncope, and ultimately the signs of left sided congestive heart failure due to pulmonary edema. All of these clinical signs impair the normal activities of dogs and reduce their quality of life (Buchanan, 1977; Boswood, 2008). The time of disease progression from asymptomatic DMVD to symptomatic DMVD is unpredictable.

The diagnosis of canine DMVD is initially performed by the detection of the systolic murmur at mitral area. However, other clinical approaches are also important for the DMVD diagnosis including

- Signalment: Small breed and aging dogs are predisposing to the disease (Buchanan, 2004). Cavalier King Charles Spaniel could have DMVD at the young to middle age (Beardow and Buchanan, 1993). Dalmatian and mixed breeds are the medium breed dogs that commonly have DMVD. Large breed dogs with DMVD would develop heart failure faster than in small breed (Borgarelli et al., 2004).
- Complete history taking: previous clinical signs especially coughing, dyspnea, or syncope, previous medical treatments, and environment of habitat are useful information for the veterinarians to deal with the disease (Abbott, 2008).
- Physical examination: The detection of systolic murmur at mitral area caused by the mitral regurgitation is the most important information for diagnosis. The intensity of murmur reflects the severity of mitral regurgitation and can be used to predict the diseased severity (Desjardins et al., 1996).
- Complete blood count and blood chemistry: The blood profile is not significantly specific for DMVD (Abbott, 2008).
- Thoracic radiography: If cardiac remodeling develops, left atrial enlargement or sometimes together with left ventricular enlargement can be seen from the radiograph. The presentation of pulmonary edema can be seen if left sided congestive heart failure occurs (Abbott, 2008).
- Electrocardiography: No specific abnormal ECG is found in DMVD. Tachycardia or atrial fibrillation is occasionally seen (Abbott, 2008).
- Echocardiography: This method is a non-invasive gold standard technique to diagnose DMVD. The mitral valve leaflet changes, prolapsed, or regurgitation would be seen in echocardiogram. Moreover, echocardiography can signify many important cardiac structural and functional abnormalities such as dimension of left atrium and left ventricle, systolic function, and degree of mitral regurgitation. Degree of mitral valve regurgitation is classified by jet size occupying in left atrial chamber during ventricular systole assessed by color flow Doppler echocardiography.

Percentage of mitral regurgitation or jet size is divided into: mild, less than 15%; moderate, 15 -50%; severe, more than 50%. The severity of DMVD is related to the degree of valve regurgitation, pressure in the left atrium, and function of the left ventricle. The determination of disease severity is useful to determine prognosis and treatment protocols of DMVD (Fuentes, 2008; Bonagura and Schober, 2009).

The ideal treatment for DMVD is mitral valve replacement through the open-heart operation; however, this surgical procedure has been performed in a few dogs (Griffith et al., 2004). Therefore, the medical therapy is the best management for DMVD at this moment. American College of Veterinary Internal Medicine (ACVIM) classifies canine DMVD into 4 stages (Table 1) in order to be the guideline for the treatment and prognosis of canine DMVD.

Table 1: Classification system for dogs with DMVD

	Definition
Stage A	Dogs at risk for developing DMVD that have no identifiable cardiac structural disorder (e.g. Cavalier King Charles Spaniel, Dachshund)
Stage B1	Dogs with DMVD that have never developed clinical signs and have no radiographic or echocardiographic evidence of cardiac remodeling
Stage B2	Dogs with DMVD that have never developed clinical signs but have radiographic or echocardiographic evidence of cardiac remodeling (e.g. left-sided heart enlargement)
Stage C	Dogs with DMVD and past or current clinical signs of heart failure associated with structural heart remodeling
Stage D	Dogs with end-stage DMVD and heart failure that is refractory to standard therapy (e.g. furosemide, ACEI, pimobendan, or spironolactone)

Source: Borgarelli and Haggstrom, 2010

This classification is based on the risk factors and clinical presentation of dogs. Stage A is the predisposing dogs which are needed to be routinely monitored for DMVD. No treatment is needed at this stage. The goal management of dogs in stage B is to prolong asymptomatic period, to reduce the systemic hypertension and to reverse cardiac remodeling in case of stage B2. In stage B, the treatment benefits are still controversial. Pouchelon et al., 2008 stated that angiotensin-converting enzyme (ACE) inhibitors can prolong asymptomatic period in dogs with DMVD at stage B. Nonetheless, some veterinary researchers and practitioners still question about the benefits of ACE-inhibitors in the treatment of asymptomatic DMVD. Therefore, the treatment protocol for stage B dogs currently depends on the veterinarian judgment. Stage C is the symptomatic DMVD, the conventional treatment should be given to dogs at this stage. ACE-inhibitors reduce the neurohormonal activation as well as decrease preload and total peripheral resistance. As the result, ACE inhibitor would help to increase cardiac output. Next, diuretic, mostly furosemide, is given in order to reduce edema and fluid retention. Lastly, pimobendan is given to improve cardiac contraction in case that has systolic dysfunction. Spironolactone or/and digoxin could be given as needed. Dogs with stage D are refractory to the standard treatment. Other additional drugs such as spironolactone, hydrochlorotiazide, digoxin, cough suppressants and bronchodilators should be administered (Atkins et al., 2009).

The prognosis of DMVD is good to fair if a veterinarian can manage the disease well enough. Most of dogs with DMVD would die with other causes rather than the disease itself (Haggstrom et al., 2005). Dogs with DMVD can live long and have a good quality of life. Asymptomatic dogs may live for 2 years or more before heart failure development. 60% of dogs with ISACHA class I heart failure could survive more than 70 months after the diagnosis. The left sided congestive heart failure is the most concern for the disease. Significant prognostic factors are syncope, and high heart rate, and increased LA/Ao ratio that would reduce the survival rate (Borgarelli et al., 2008).

Serotonin

Serotonin or 5-hydroxytyptamine (5HT) is the monoamine neurotransmitter which is mostly produced by enterochromaffin cells in the gastrointestinal tract (90%) (Ruddell et al., 2008). The serotonin synthesis begins with the essential amino acid L-tryptophan, which is rich in bananas, tomatoes, turkeys, and milk. Then, L-tryptophan is reacted with enzyme tryptophan hydroxylase, forming of 5-hydroxy-L-tryptophan. Next, enzyme decarboxylase removes carbon dioxide from 5-hydroxy-L-tryptophan, and forming 5-hydroxytryptamine or serotonin (Vu, 2003). After serotonin production, it is released from enterochromaffin cells and exported around the body. In the circulation, serotonin is rapidly taken up by the platelet within its dense granule (Meyer et al., 1982), so platelets are the major source of circulating serotonin. Many stimuli trigger serotonin releasing from platelets. Serotonin outside the platelets binds with its receptor or is taken up by serotonin transmembrane transporter (SERT), leading to its function or metabolism.

Liver is the important organ for the serotonin metabolism. At the liver, serotonin metabolism is taken place. Serotonin is catabolized into the breakdown products such as 5-hydroxyindoleacetaldehyde (5-HIAA) and 5-hydroxyindole acetic acid, and is excreted from the body via urine (Ruddell et al., 2008).

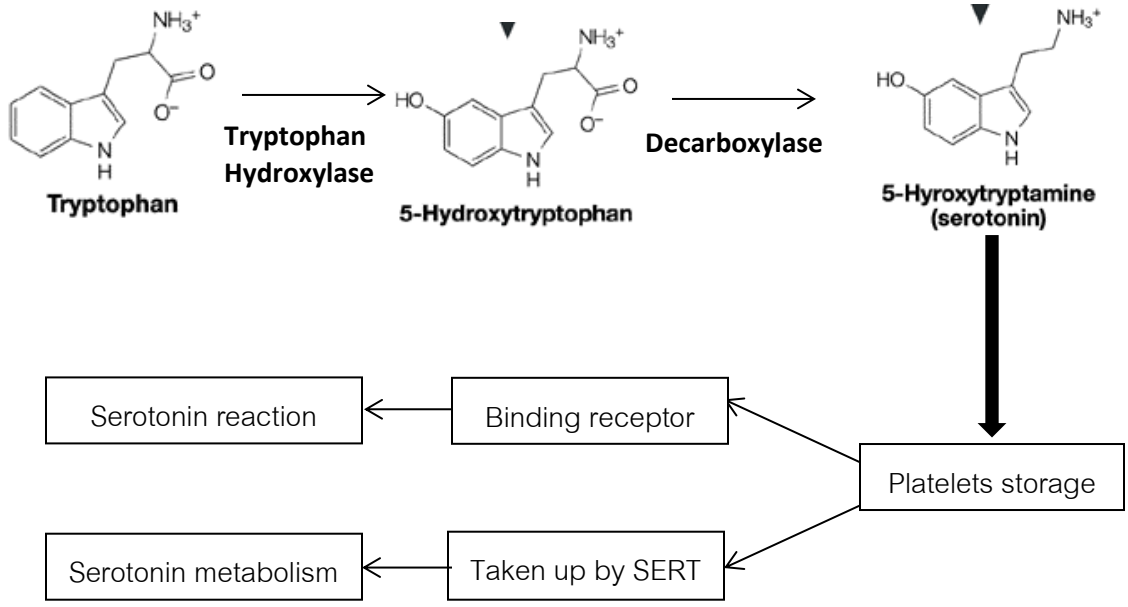

Fig 1: The synthesis of serotonin

(Modified from http://scienceblogs.com/clock/2008/11/26/tryptophan)

The minority serotonin is synthesized by serotonergic neuron in the central nervous system (CNS) (Morti and Jantos, 2008). Although it is only small amount of serotonin, these neurons are widely spread and early developed in the brain. Serotonin is expressed in both immature human and rodent brain, so does its receptors, enzymes and transporters. The serotonin system in CNS begins to reduce by the early adulthood. In CNS, serotonin plays important roles in mood, appetite, metabolism, behavior, learning, sleep, and anxiety (Whitaker-Azmitia, 2001).

In addition to its function in CNS, serotonin is able to mediate vascular contraction and relaxation, gastrointestinal motility, cell proliferation and platelet aggregation. Endothelial damage, platelet aggregation, and serotonin receptor agonist can induce serotonin releasing from platelets (Jonnakuty and Gragnoli, 2008). Bleeding causes serotonin release leading to vasoconstriction.

Serotonin in cardiovascular disease

Outside CNS, serotonin is produced from tryptophan by the rate-limiting step in the enterochromaffin cells. Once serotonin is released, it is taken up by the platelets via serotonin transmembrane transporter (5HT-reuptake transporter or SERT). Serotonin will be released again by triggering of many stimuli such as endothelial damage, platelet aggregation, and serotonin receptor (5HT-R) agonist. Both 5HT-R and SERT are rich in the cardiovascular system (CVS) (Oyama and Chittur, 2006; Disatian and Orton, 2009).

SERT is found along the pulmonary and coronary endothelium as well as the valvular tissues in the mature rodent. SERT acts as the local serotonin clearance in the CVS. After serotonin is free, SERT takes serotonin up into the cells and metabolizes into 5-hydroxyindole acetic acid, which is excreted out of the body via urine. Its functions are regulation of local vascular tone and local serotonin clearance. Serotonin mediates its function through serotonin receptor (5HT-R). There are at least 5 family of 5HT-R that is found in CVS. So, CVS is rich of the ability to bind and react to serotonin. The actual function of SERT and serotonin in valvular tissues is unknown; however, since it is rich in CVS, it is possible that SERT and serotonin may have roles in maintaining the healthy heart function (Oyama and Chittur, 2006).

The ideal model of serotonin mediating vulvular disease in human is carcinoid tumor. Carcinoid tumor is the neoplasia of enterochromafin cells which are 70% found along the gastrointestinal tracts such as intestine, colon, and liver. The tumors secrete large amount of serotonin into the circulation resulting in “carcinoid syndromes” in the patients (Fox and Khattar, 2004). The clinical signs include abdominal pain, cutaneous erythema, and diarrhea. Forty percent of the patients develop pulmonary and tricuspid valve diseases. The morphology of right-sided vulvular changes is grossly thickening and opaque. From histopathology, valvular interstitial cell (VIC) is proliferation and differentiation. The glycosaminoglycan (GAG) accumulates together with collagen fibers within valve tissues.

This pathology of valves in carcinoid patients, even not a 100 percent match, it is very close to the valvular changes in canine DMVD (Simula et al., 2002).

In addition to carcinoid tumor, people who use serotonergic drugs also relate to the development of valve degeneration. For example, human who use an anorectic drug, fenfluramine-phenteramine, have a risk factor of 2.2 and 1.6 for the incidence of aortic regurgitation and mitral regurgitation, respectively. Patients with Parkinson using ergot derivatives such as pergolide and with migraine headaches using methysergid for a long period produce the lesion on both tricuspid and mitral valves (Sachdev et al., 2002; Antonini and Poewe, 2007)

The experiments studying serotonin mediating valvular diseases have been done in experimental animals. Rats injected with the exogenous serotonin substance everyday developed valvulopathy and VIC activation (Gustafsson et al., 2005). This process could be inhibited by the injection of serotonin blocker (Droogmans et al., 2007). Moreover, SERT knock-out mice also developed the valvulopathy and myocardial fibrosis secondary to the decrease of serotonin clearance (Mekontso-Dessap et al., 2006)

Serotonin in dogs

Serotonin function in dogs is similar with other higher mammals. It is a neurotransmitter agent in CNS that plays important roles about mood, appetite, sleep, and behavior. Most of the serotonin researches in dogs are related with the aggressive behavior. Low serotonin concentration in cerebrospinal fluid and circulation is associated with the low thresholds for the aggression (Cakiroglu et al., 2007; Leon et al., 2012).

There is one case report about carcinoid tumors in dogs. The researchers reported the serum serotonin concentration in dogs with carcinoid tumor was increased when compared to other four healthy dogs. (Sako et al., 2003).

Serotonin in canine degenerative mitral valve disease

The actual etiology of DMVD is still unclear. The initial stimuli could be the mechanical stimuli such as the blood regurgitation resulting in endothelial damage. Valvular degeneration can also be induced together with chemical stimuli such as serotonin (Orton et al., 2012).

Serotonin signaling mechanisms are believed to play an important role in naturally occurring DMVD in dogs. Several studies including transcriptional, immunohistochemical, cell culture and protein studies suggest that serotonin signaling may involve in canine DMVD (Oyama and Levy, 2010). Serotonin binds to serotonin receptors and activates mitogen-activated pathway resulting in differentiating of valvular interstitial cells and remodeling of extracellular matrix in valve tissues. The affected valve and chordae tendineae are thickened. Glycosaminoglycans deposit in the extracellular matrix and cause dysfunction of mitral valve leaflets.

Many previous studies suggest a possible involvement of serotonin signaling in canine DMVD (Figure 2) as follows,

- The mRNA and protein expression of serotonin receptors increased in mitral valves of DMVD dogs (Oyama and Chittur, 2006; Disatian and Orton, 2009).

- An increase of tryptophan hydroxylase 1, a rate limiting enzyme involved in serotonin production, in the affected valves from DMVD dogs indicates an increase of local production of serotonin (Disatian and Orton, 2009).

- Serotonin could increase production of extracellular matrix and collagen in cultured canine mitral valve interstitial cells. Blockage of serotonin by adding ketanserin, a 5HT-2A receptor blocker could prevent the extracellular matrix production (Connolly et al., 2009) suggesting a role of serotonin receptors in extracellular matrix remodeling.

- Serotonin transmembrane transporter (SERT), a key structure required for serotonin uptake and metabolism is down-regulated in myxomatous valves (Disatian and Orton, 2009). Taken together, these findings suggest a decrease of serotonin metabolism and an increase of serotonin availability to bind with serotonin receptors which may lead to an increase in downstream serotonin signaling.

- Another potential source of serotonin signaling is serotonin in circulation. A previous study found an increased serum serotonin concentration in dogs with DMVD compared to control dogs. Serum serotonin was increased by approximately 50% in dogs with DMVD as well as small breed dogs predisposed to DMVD compared with healthy large breed control dogs. This study indicated that the dogs with naturally occurring DMVD as well as non-DMVD Cavalier King Charles Spaniels have increased serum serotonin concentration. The increased serum serotonin concentration may be another source of increased serotonin signaling (Arndt et al., 2009).

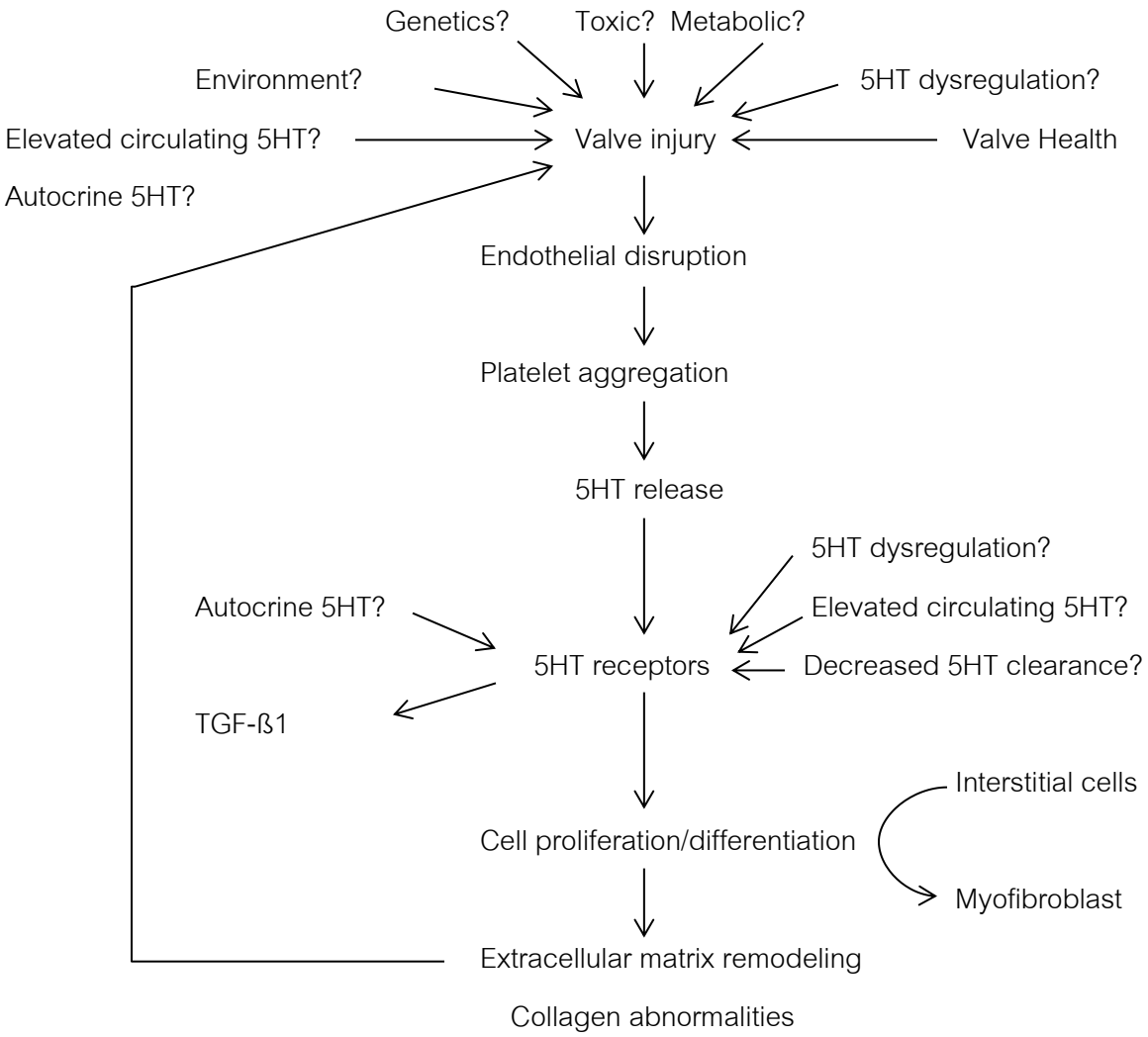

Fig. 2 The diagram of hypothesis involving 5-hydroxytryptamine (5HT) or serotonin signaling as a potential contributor to canine DMVD
(Modified from Oyama and Levy, 2010)

Based on the previous study by Arndt et al. (2009), serum serotonin concentration has been found increased in dogs affected with DMVD compared to normal dogs. However, more than 98 % of the circulating serotonin is located in platelets and release during blood

clotting. Therefore, serum serotonin could exaggerate the amount of serotonin in the circulation. To assess the real value of circulating serotonin in dogs, plasma serotonin should be measured. Simultaneously, platelet serotonin should be evaluated as it is the major source of serotonin in the circulation. Since serum, plasma, and platelet serotonin concentrations of dogs with DMVD have not been published yet, it is important to evaluate these three parameters of circulating serotonin in order to understand the relationship between serotonin signaling and DMVD in dogs.

CHAPTER III
MATERIALS AND METHODS

1. Animals

This study obtained the ethical approval from Chulalongkorn University Animal Care and Use Committee. Forty-three dogs were presented to Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University. After consent form was signed by the owner, all dogs were performed examination procedures including owner interview, physical examination, blood collection for hematology and blood chemistry, thoracic radiography and echocardiography. Since food that is rich with tryptophan has effects on level of circulating serotonin, dogs were fasted 4 - 6 hours before blood collection. Moreover, dogs receiving medication that could alter serotonin concentration (such as fluoxetine) were excluded from the experiment. Dogs were divided into 2 groups.

Group1. Healthy control dogs

Control group consisted of 20 clinically healthy small breed dogs less than ten kilograms and older than six years, presenting with normal physical examination, no evidence of heart murmurs, normal blood results, no cardiovascular, or clinical evidence of other diseases. All dogs in this group had normal echocardiographic profiles including normal valve appearance without evidence of mitral regurgitation, normal cardiac chamber dimension and wall thickness as well as normal cardiac function.

Group 2: DMVD group

DMVD group consisted of 23 dogs with DMVD that were small breed dogs less than 10 kilograms, and older than 6 years. Dogs presented either with or without clinical signs of DMVD classified in stage B, C and D according to table 1. All dogs were newly diagnosed for DMVD and never been received any cardiovascular medicine. DMVD was confirmed by

echocardiography. On echocardiography, all dogs in this group were found mitral valve thickening and mitral regurgitation. Clinically diseased dogs were given the medicine at the day of the DMVD diagnosis. Dogs with other significant abnormalities such as renal disease or skin disease were excluded from the study.

2. Clinical Procedures

Every dog was performed all the procedures including owner interview, complete physical examination, thoracic radiography, blood collection for complete blood count (CBC) and blood chemistry, and echocardiogram.

2.1 The owner of each dog was interviewed by the questionnaire including past and current clinical signs, pet diet, and past medical history.

2.2 Complete physical examination was carried out and recorded in the physical examination form.

2.3 Thoracic radiography was performed and interpreted by the veterinarians of imaging unit in Chulalongkorn Small Animal Hospital. Two radiographic views, dorsoventral and left lateral views, were required.

2.4 CBC and blood chemistry including blood urea nitrogen, creatinine, alanine aminotransferase and alkaline phosphatase as well as the evidence of blood parasite infection were evaluated by Chulalongkorn Veterinary Laboratory. The normal values referred to Textbook of Veterinary Internal Medicine (Ettinger and Fledman, 2010).

2.5 Echocardiography was performed by a Vivid 5 echocardiograph (GE-medical). All dogs were un-sedated. The right parasternal four-chamber view was used to determine valvular changes including valve thickening or (Fig. 3) prolapse as well as the rupture of chordea tendinea. M-mode echocardiography was performed to measure parameters including: interventricular septal thickness at end-diastole

(IVSd), interventricular septal thickness at end-systole (IVSs), left ventricular internal dimension at end-diastole (LVIDd), left ventricular internal dimension at end-systole (LVIDs), left ventricular posterior wall thickness at end-diastole (LVPWd), left ventricular posterior wall thickness at end-systole (LVPWs), left atrial dimension (LAD), aortic root dimension (AOD), left atrial to aortic root ratio (LAD/AOD). Fractional shortening (FS) was calculated. Color flow Doppler was performed to estimate the degree of mitral regurgitation including mild, moderate, and severe (Fig.4) (Fuentes, 2008).

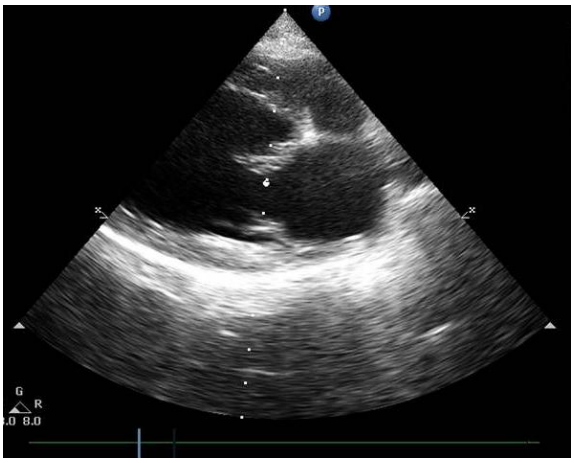

Fig. 3 The picture from 2 dimensional echocardiography of thickened mitral valve leaflets (Surachetapong, unpublished data)

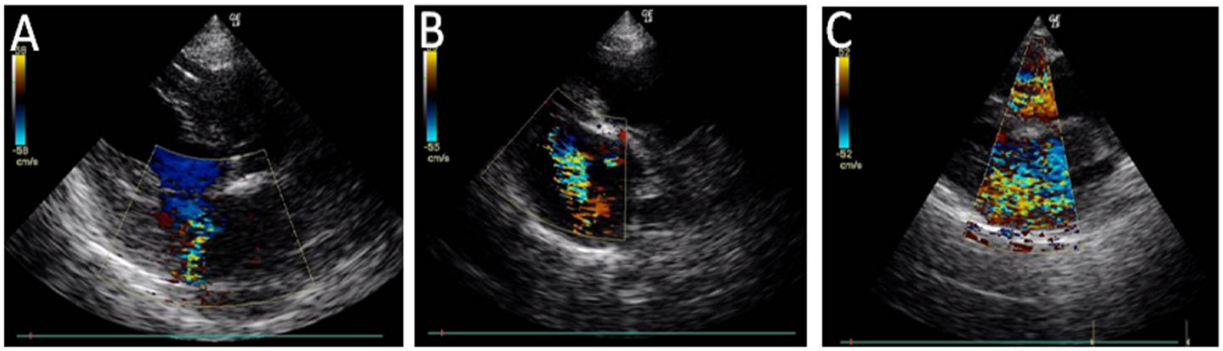

Fig. 4 The picture from color flow Doppler echocardiography of mitral regurgitation at different severity of DMVD. Fig. 4A mild degree, jet size <15%; Fig. 4B moderate degree, jet size 15 -50%; Fig. 4C severe degree, jet size > 50%
(Surachetapong, unpublished data)

3. Sample collection and preparation:

All dogs were fasted 4 – 6 hours before blood collection. Five milliliters of blood was collected by venipuncture from cephalic or saphenous vein. Blood was contained in the EDTA tube and plain tube.

Serum sample preparation: Clotted blood samples were set at room temperature for 30 minutes and then centrifuged at 1,500g for 5 minutes for serum separation. Serum was transferred and kept in aliquots at -20°C.

Plasma sample preparation: Within 6 hours in room temperature, whole blood was centrifuged at 200 g for 10 minutes for platelet-rich plasma (PRP). PRP was centrifuged at 1,500 g for 20 minutes for platelet-poor plasma (PPP). The platelet-poor plasma was used as the plasma sample. All samples were kept in -20°C.

Platelet sample preparation: The platelet sample was obtained by adding 800 μl of physiological saline to 200 μl of PRP (containing between 350,000 – 500,000 platelets/μl) and centrifugation (4,500 x g, 10 minutes at 4°C). Then, the supernatant was discarded. 200 μl of distilled water was added to the pellet and mixed thoroughly on a vortex mixer. The suspension was stored frozen at < -20°C. The frozen sample was thaw before the serotonin measurement. After thawing, the frozen sample was centrifuged at 10,000 x g for 2 minutes at room temperature. 25 μl of the supernatant was used as the platelet sample for serotonin measurement (Immuno-biological laboratories, Inc. 2009[a]).

Whole blood sample was smeared and stained with Wright-Giemsa stain. Under light microscope, any platelet morphology abnormalities such as megaplatelets were recorded. The direct platelet count was done by using the Neubauer counting chamber for estimating the number of platelets in each PRP samples in order to calculate the content of serotonin in referred platelets (10^9 platelets). All of these procedures were done under the same condition at the Faculty of Veterinary Science, Chulalongkorn University.

4. Measurement of serotonin concentrations:

All serum, plasma, and platelet samples were stored at -20°C before batch serotonin measurement. Serum and platelet serotonin concentrations were measured by the Serotonin ELISA® test (produced by Immuno-Biological Laboratories,Inc., Minneapolis, Minnesota, USA). While plasma serotonin concentrations were measured by Serotonin (Research) ELISA® test (produced by Immuno-Biological Laboratories,Inc., Minneapolis, Minnesota, USA). Serum and plasma serotonin concentrations were quantitatively analyzed and shown as ng/ml, whereas the quantitation of platelets serotonin concentration was shown in ng/10^9 platelets. All procedures were done according to the test kit direction.

According to the serotonin ELISA test kit and the serotonin (research) ELISA test kit 2009, the antigen is bound to the solid phase of the microtiter plate. The acylated standard, controls, samples and the solid phase bound analyte compete for fixed number of antiserum binding sites. After the system is equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by anti-rabbit IgG-peroxidase conjugate using tetrametyhl benzidine (TMB) as a substrate. The reaction is monitored at 450 nm by photometer. All samples are assayed in duplicate. Quantitative of unknown samples is achieved by comparing their absorbance with reference curve prepared with known standard concentrations (Immuno-biological laboratories, Inc., 2009[b]). The 4-parameters logistics regression model was plotted from the

standard solutions A-F, and this non-linear curve was used as the standard curve. The unknown samples absorbance was calculated by the ReaderFit® program for the serotonin concentration. Plasma and serum serotonin concentrations were read directly from the standard curve. While the platelet serotonin concentrations had to refer to 10^9 platelets, the sample concentrations were analyzed again for the serotonin concentration in referred platelets (10^9 platelets) using this equation;

$$\text{Serotonin concentration}/10^9 \text{ platelets} = \frac{\text{Sample serotonin} \times 10^9}{\text{Number of platelets in PRP} \times 10^9}$$

5. Statistical analysis:

Statistical analysis was performed by the computer-based software, SPSS program. The data was tested for the normality using the histogram plot. The data of signalment, CBC and blood chemistry parameters, and echocardiogenic index were presented as mean ± standard error of mean (SEM). The means of these data were analyzed by independent T-test. The data of circulating serotonin concentrations were non-parametric and were presented as median and 25^{th} and 75^{th} percentile of data. The medians of serum, plasma, and platelets serotonin concentrations between normal dogs and dogs with DMVD were analyzed by Mann-Whitney U test. The correlations between serotonin concentrations and age, platelets count, as well as echocardiographic indices were analyzed by Spearman's rank correlation. P-value of less than 0.05 was considered significant.

CHAPTER IV

RESULTS

Part I General information

1.1 Signalment and Owner interviewer

43 dogs were participated in this study. Normal group consisted of 20 dogs including ten males and ten females with 7 Poodles, 7 Shih Tzus, 3 Chihuahuas, and 1 each of Pomeranian, Jack Russell, and mixed breed. DMVD group consisted of 23 dogs including eleven males and twelve females with 16 Poodles, 3 Shih Tzus, and 1 each of Spritz, Chihuahua, Yorkshire terrier, and mixed breed. None of these dogs were received cardiovascular medicines or continuously on other medicines at least one month before the study.

The mean age of dogs between DMVD group (10.7 ± 0.5 years) was significantly older than the normal group (8.8 ± 0.3 years) ($p < 0.01$). The mean weight of dogs between normal group (6.0 ± 0.4 kilograms) and DMVD group (6.4± 0.6 kilograms) was not significantly different ($p > 0.05$) (table2).

Table 2: Signalment of the normal dogs and dogs with DMVD (Mean ± SEM)

Parameters	N	Male	Female	Age	Weight
Normal	20	10	10	8.8 ± 0.3	6.0 ± 0.4
DMVD	23	11	12	10.7 ± 0.5**	6.4 ± 0.6

** represent statistically significance at $p < 0.01$ when compared the age of the normal and DMVD group.

From the owner interview, seven in 23 dogs of DMVD group (30%) were reported the clinical signs of DMVD. The clinical signs observed from those dogs included; coughing (7/7), exercise intolerance (7/7), and syncope (1/7). 30 in 43 dogs of both groups (70%) were fed with commercial dog diet combined with homemade diet, while the other 13 dogs were fed only with commercial dog diet.

From the physical examination, the abnormalities signs observed in dogs with DMVD at the first day of disease diagnosis included: systolic heart murmur (23/23; 100%), increased lung sound (10/23; 43.4%), pale-pink mucous membrane (12/23; 52.1%), cardiac cachexia (3/23; 13%), and cataract (5/23, 21.7%).

1.2 Complete blood count and blood chemistry

The CBCs data of normal and DMVD group is shown as mean ± SEM and presented in Table 3. There was no significant difference in RBCs between the clinical normal dogs and dogs with DMVD as well as in hematocrits between the clinical normal dogs the dogs with DMVD ($p > 0.05$). Moreover, WBCs in dogs with DMVD were not significantly different from normal dogs, but neutrophils in dogs with DMVD were significantly higher than the normal dogs ($p < 0.05$). The means of lymphocytes, basophils, and monocytes are shown in the Table 3. There were also no significant differences in lymphocytes, basophils and monocytes numbers between the clinically normal and the DMVD dogs. Lastly, there was no significant difference in platelets count between normal dogs and dogs with DMVD. All means ± SEM of CBC parameters were within the normal value limit (Table 3).

Table 3. The means CBC of the clinically normal dogs and the dogs with degenerative mitral valve disease (mean ± SEM)

Parameter	Unit	Normal value#	Normal (n=20)	DMVD (n=23)	*p*-value
RBC	$\times 10^6$ cells/ml	5.2 - 8.06	6.37 ± 0.15	5.99 ± 0.25	0.110
Hematocrit	%	29.8 - 57.5	45.6 ± 1.07	42.43 ± 1.64	0.063
WBC	cells/ml	5,400 -15,300	9,623 ± 855	12,900 ± 1,790	0.057
Neutrophil	cells/ml	3,000-12,000	6,129 ± 618	8,827 ± 1,156	0.027*
Lymphocyte	cell/ml	530-4,800	2,600 ± 505	2,585 ± 470	0.491
Eosinophil	cell/ml	0-1,900	258.4 ± 58.85	725 ± 264	0.056
Monocyte	cells/ml	100-1,800	624.45 ± 64.35	798 ± 126	0.124
Basophil	cells/ml	< 100	0	29.47 ± 22.06	0.110
Platelets	$\times 10^3$ cells/ml	160 - 525	303 ± 34.72	316 ± 29.20	0.387

* indicate significant difference at $p < 0.05$ when compared the means of neutrophil between normal and DMVD group.

Normal reference value from Textbook of Veterinary Internal Medicine (Ettinger and Fledman, 2010).

RBC = Red blood cell; PCV = Pack cell volume; WBC = White blood cell.

The mean blood chemistry profiles of both groups are showed in Table 4. There were no significant differences in plasma ALT, ALP, BUN, and creatinine between clinical normal dogs and dogs with DMVD. The mean of plasma ALT, BUN, and creatinine in both groups were within the normal limit, while mean ALP in both groups were higher than the normal value. But the value was not higher than 3 times of reference value indicating clinically insignificance. In addition, none of these dogs had shown signs of liver disease.

Table 4. Blood chemistry of the clinically normal dogs and dogs with degenerative mitral valve disease (mean ± SEM)

Parameter	Unit	Normal value	Normal (n=20)	DMVD (n=23)	*p*-value
ALT	IU/L	4 - 91	49.25 ± 6.26	69.61 ± 11.93	0.079
ALP	IU/L	3 - 60	124.45 ± 43.52	95.90 ± 16.16	0.268
BUN	mg/dl	7 - 26	19.33 ± 2.23	25.04 ± 2.70	0.061
Creatinine	mg/dl	0.6 – 1.4	0.94 ± 0.04	1.02 ± 0.07	0.194

Normal reference value from Textbook of Veterinary Internal Medicine (Ettinger and Fledman, 2010).

ALT = Alanine amino transferase; ALP = Alkaline phosphatase; BUN = Blood urea nitrogen.

From the platelets count, three of 43 dogs (6.9%) were thrombocytopenia. No magaplatelets or any abnormalities were found from blood morphology under the light microscope.

1.3 Thoracic radiography

Twelve of 23 dogs with DMVD (52.1%) were diagnosed with cardiomegaly and left atrial enlargement. Three of 12 dogs reported for cardiac enlargement, also had pulmonary edema from the radiographic finding. Radiographs of normal dogs did not show any abnormalities for the thoracic radiography.

1.4 Echocardiography

As expected, the left atrium of dogs with DMVD was significantly larger than the normal dogs. Moreover, the ratio of left atrium to aortic root of dogs with DMVD was

significantly larger than normal dogs ($p<0.01$). The left ventricular internal dimension index at end systole and end diastole was significantly larger in dogs with DMVD than in control dogs. While the percentage of fractional shortening in the clinically normal dogs was lower than in dogs with DMVD. Other echocardiographic indices including aortic root dimension, interventricular septal thickness at end-systole, left ventricular posterior wall thickness at end-systole, interventricular septal thickness at end-diastole and LVPWd, left ventricular posterior wall thickness at end-diastole were not statistically different between normal and DMVD dogs (Table 5). From the degree of mitral regurgitation (MR), there were 8 dogs with mild MR, 6 dogs with moderate MR, and 9 dogs with severe MR.

Table 5: Echocardiographic data of normal and DMVD group (Mean ± SEM)

Parameter	Normal (n = 20)	DMVD (n = 23)	*p*-value
Ao index	12.82 ± 0.54	13.48 ± 0.50	0.1931
LA index	16.33 ± 0.47	20.80 ± 1.09	0.0004**
LA/Ao	1.22 ± 0.07	1.55 ± 0.04	0.0004**
IVSs index	9.74 ± 0.54	10.37 ± 0.42	0.1861
LVIDs index	13.82 ± 0.82	16.08 ± 0.63	0.0199*
LVPWs index	8.94 ± 0.37	9.93 ± 0.46	0.0540
IVSd index	7.43 ± 0.36	7.08 ± 0.35	0.2525
LVIDd index	21.54 ± 1.21	26.13 ± 0.64	0.0013**
LVPWd index	7.06 ± 0.35	6.90 ± 0.36	0.3856
%FS	34.65 ± 1.33	38.73 ± 1.82	0.0362*

*indicate significant difference at $p < 0.05$ when compared the means of LVIDs index and percentage of FS between normal and DMVD group.

**indicate significant difference at $p < 0.01$ when compared the means of LA index, LA/Ao, and LVIDd index between normal and DMVD group.

DMVD, degenerative mitral valve disease; Ao, aortic root dimension; LA, left atrial dimension ; LA/Ao, left atrial to aortic root ratio; IVSs, interventricular septal thickness at end-systole; LVIDs left ventricular end-systolic dimension; LVPWs, left ventricular posterior wall thickness at end-systole; IVSd, interventricular septal thickness at end-diastole; LVIDd, left ventricular end-diastolic dimension; LVPWd, left ventricular posterior wall thickness at end-diastole; FS, fractional shortening

Part II Circulating serotonin concentration

The serum, plasma, and platelets serotonin concentrations were non-parametric data. The serum and plasma serotonin concentrations were expressed as ng/ml, whereas the platelets serotonin concentrations were expressed as ng/10^9 platelets. The median of serum, plasma, and platelets concentration were shown in the Table 6.

Table 6: The median of serum, plasma, and platelet serotonin concentrations of normal and DMVD group (Median (25^{th} and 75^{th} percentile)).

Parameter	Unit	Normal (n=20)	DMVD (n=23)	*p*-value
Serum	ng/ml	219.14 (158.29-404.34)	84.66 (37.84-176.56)	0.005**
Plasma	ng/ml	4.29 (2.31-8.21)	3.70 (2.17-5.98)	0.268
Platelets	ng/10^9 platelets	128.61 (78.67-276.64)	176.66 (75.43-445.77)	0.457

**indicate significant difference at $p < 0.01$ when compared the medians of serum serotonin concentration between normal and DMVD group.

2.1 Serum serotonin concentration

The range of serum serotonin concentration in dogs with DMVD was 16.06 – 453.58 ng/ml and in normal dog was 35.05 – 631.63 ng/ml. The median of the data in dogs with DMVD was significantly lower than normal dogs (p <0.01).

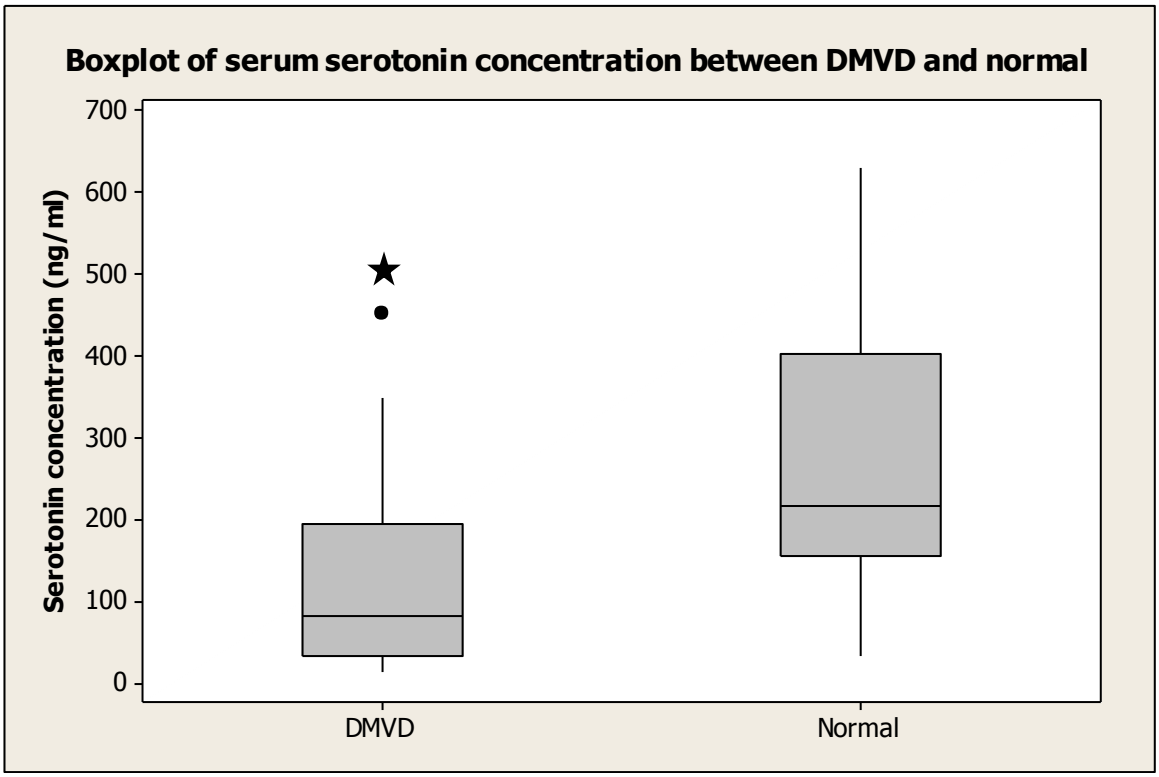

Fig 5. Boxplot of serum serotonin concentration in dogs with DMVD and normal control dogs. The line within the box represents the median value; the limits of the box represent the 25th and 75th percentile value. The outlier value (>1.5 interquartiles) are shown as dot.
* p < 0.01 when compared between the median between DMVD and normal groups

There was no significant correlation between serum serotonin concentration and age, the ratio of left atrium and aortic root, left ventricular internal dimension index at end

systole, left ventricular internal dimension index at end diastole, fractional shortening nor platelets count in the entire population. (Table 7)

Table 7: The correlations between serum serotonin concentration and other parameters

Parameters	r	P-value
Serum 5HT and age	-0.073	0.650
Serum 5HT and LA/Ao	-0.128	0.424
Serum 5HT and LVIDs index	-0.058	0.720
Serum 5HT and LVIDd index	-0.018	0.913
Serum 5HT and FS	-0.000	1.000
Serum 5HT and platelet count	0.091	0.571

Serum 5HT = serum serotonin concentration, r = Spearman's rank correlation, LA/Ao = the ratio of left atrium and aortic root, LVIDs = left ventricular internal dimension index at end systole, LVIDd = left ventricular internal dimension index at end diastole, FS = fractional shortening.

2.2 Plasma serotonin concentration

The range of plasma serotonin concentration in dogs with DMVD was 0.03 – 23.44 ng/ml and normal dogs was 0.49 – 12.10 ng/ml. The median of plasma serotonin concentration in dogs with DMVD was not significantly different from normal dogs.

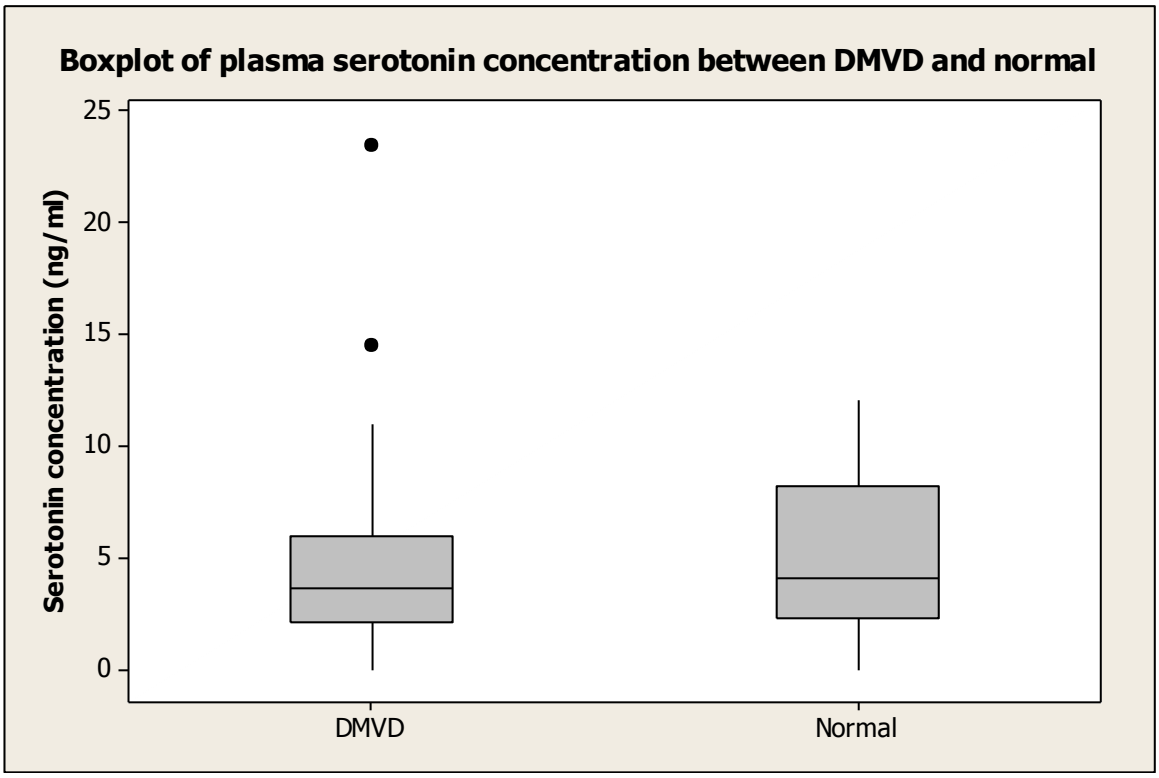

Fig 6. Boxplot of plasma serotonin concentration in dogs with DMVD and normal control dogs. The line within the box represents the median value; the limits of the box represent the 25th and 75th percentile value. The outlier value (>1.5 interquartiles) are shown as dots.

There was no significant correlation between plasma serotonin concentration and age, the ratio of left atrium and aortic root, left ventricular internal dimension index at end systole, left ventricular internal dimension index at end diastole, fractional shortening, nor platelets count in the entire population (Table 8).

Table 8: The correlation between plasma serotonin concentrations and other parameters

Parameters	r	P-value
Plasma 5HT and age	-0.123	0.433
Plasma 5HT and LA/Ao	0.120	0.444
Plasma 5HT and LVIDs index	-0.131	0.403
Plasma 5HT and LVIDd index	-0.182	0.242
Plasma 5HT and FS	0.030	0.849
Plasma 5HT and platelet count	-0.092	0.557

Plasma 5HT = plasma serotonin concentration, r = Spearman's rank correlation, LA/Ao = the ratio of left atrium and aortic root, LVIDs = left ventricular internal dimension index at end systole, LVIDd = left ventricular internal dimension index at end diastole, FS = fractional shortening.

2.3 Platelet serotonin concentration

The range of platelet serotonin concentration in dogs with DMVD was 38.25 – 1,657.79 ng/10^9 platelets and in normal dogs was 33.85 – 1,101.34 ng/10^9 platelets. The median of platelet serotonin concentration in dogs with DMVD was not significantly different from the normal dogs.

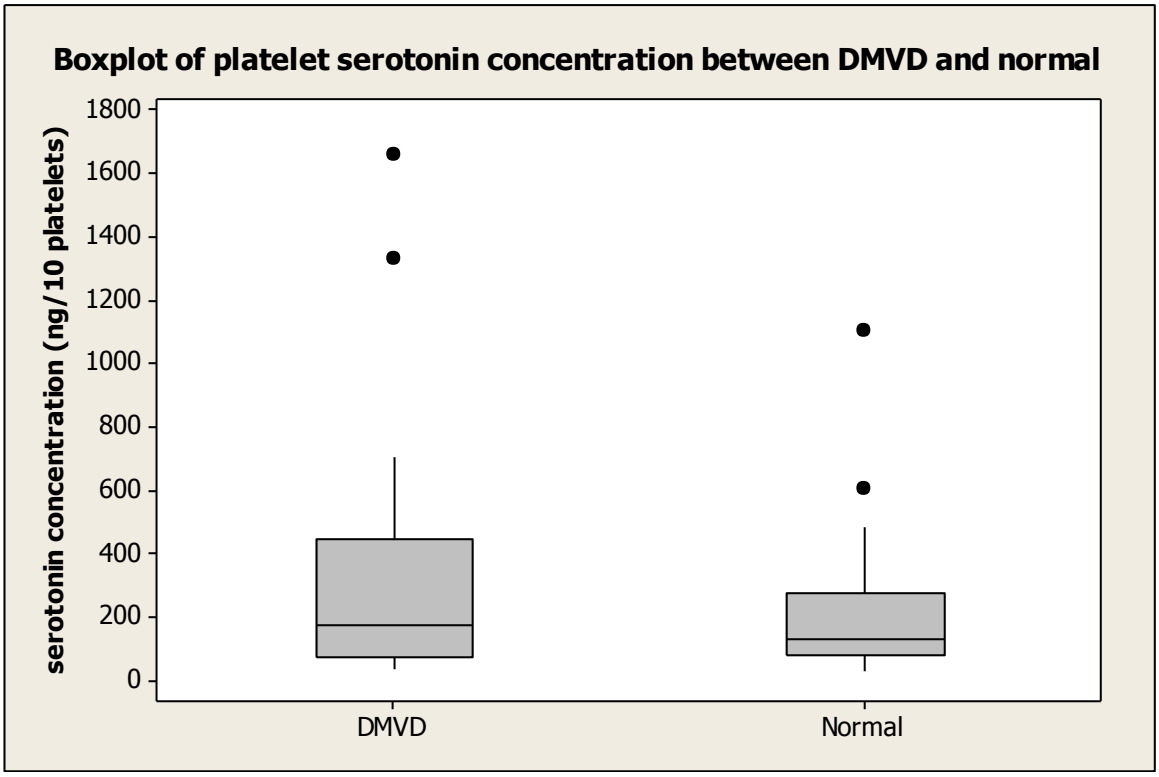

Fig 7. Boxplot of serotonin concentration in 10^9 platelets in dogs with DMVD and normal control dogs. The line within the box represents the median value; the limits of the box represent the 25th and 75th percentile value. The outlier value (>1.5 interquartiles) are shown as dots.

There was no significant correlation between platelets serotonin concentration and age, the ratio of left atrium and aortic root, left ventricular internal dimension index at end systole, left ventricular internal dimension index at end diastole, fractional shortening, nor platelets count in the entire population (Table 9).

Table 9: The correlation between platelet serotonin concentrations and other parameters

Parameters	r	P-value
Platelet 5HT and age	-0.044	0.781
Platelet 5HT and LA/Ao	0.177	0.262
Platelet 5HT and LVIDs index	-0.064	0.689
Platelet 5HT and LVIDd index	-0.074	0.643
Platelet 5HT and FS	0.089	0.576
Platelet 5HT and platelet count	-0.055	0.728

Platelet 5HT = platelet serotonin concentration, r = Spearman's rank correlation, LA/Ao = the ratio of left atrium and aorta root, LVIDs = left ventricular internal dimension index at end systole, LVIDd = left ventricular internal dimension index at end diastole, FS = fractional shortening.

There was no correlation between serum and plasma, serum and platelet, and plasma and platelet serotonin concentrations in the entire study (Table 10).

Table 10: The correlation among circulating, serum, plasma, and platelet, serotonin concentrations.

Parameters	r	P-value
Serum and plasma 5HT	-0.003	0.984
Serum and platelet 5HT	0.009	0.958
Plasma and platelet 5HT	0.210	0.193

5HT = serotonin, r = Spearman's rank correlation

CHAPTER V

DISCUSSIONS

Part I Signalment

43 client-owned dogs were enrolled in this study including 23 dogs with DMVD (11 male and 12 male) and 20 clinically normal dogs (10 male and 10 female). Although only dogs older than 6 years were included in this study, the mean age of dogs with DMVD (10.7 ± 0.5 years) was significantly higher than the clinically normal dogs (8.8 ± 0.3 years). This study agrees with a previous study, Arndt et al. study in 2009 the mean age of dogs with DMVD (8.5 years) were significantly older than predisposing (3.0 years) and large breed control dogs (6 years). However, the age significant difference in this study was not clinically significant difference because all dogs were old dog (older than 6 years), which categorized in the same age range (young, middle, and old). The reason that only dogs older than 6 years were included is to reduce the age affecting serotonin level. DMVD is commonly found in elderly population and it progresses with age. 30% of dogs that are 13 years or older have clinically detected of the disease (Abbott, 2008). Moreover, 58% of dogs older than 9 years have found pathologic changes of mitral valve leaflets from the postmortem report, and this number is increasing to 90% reported from the dogs older than 13 years (Whitney, 1974). The real etiology of DMVD related with aging is still unclear; however, the old hypothesis is that mitral valve leaflets are progressively injured from the shear stress for a long period of time. Therefore, the valve is repaired, changed, formed some nodular and loss its function eventually (Pommerance, 1966). However, not all old dogs would develop the pathologic changes and some younger dogs could develop these changes very early. So, there might be other factors that would relate to the occurring of DMVD.

The breeds of dog in this study including Poodles, Shih Tzus, Chihuahuas, Pomeranian, Jack Russell, Spritz, Yorkshire terrier, and mixed breed. Most dogs in the present study were Poodle and Shih Tzu, which are different from the other studies from the US or Europe where Cavalier King Charles Spaniel (CKCS) and Dachshund are the main breed population studying about DMVD. This might be because Poodle and Shih Tzu are popular breeds in Thailand while CKCS and Dachshund are not popular. The breed variation does not really affect the result of this study because all of them are small breeds and predisposing to the disease. Some researchers suggested that breeds such as CKCS and Dachshund should not be included and compared with other breeds in the study or research involving DMVD because CKCS and Dachshund develop DMVD in the early age which is different from the other small breeds that develop DVMD quite late in their life (Olsen et al., 1999 and Swenson et al., 2009).

The gender of dogs in this study was approximately 50/50 percent of male and female. Abbott (2008) noted that the male dog is slightly more prone to the disease than female. Haggstrom et al. (2004) stated that male dogs have developed the disease faster than female dogs. However, we did not find any difference between males and females developing DMVD in this study.

Part II History and physical examination

From the study records, the clinical signs of DMVD dogs in this study from the owner report and the physical examination included coughing, exercise intolerance, syncope, systolic heart murmur at the left heart apex, and increased lung sound. Non-productive cough is the most clinical sign that has found in these dogs with DMVD. Coughing in DMVD is usually caused by elevated of main stem bronchus resulting from left atrial enlargement. This cough happens after exercise, excitement or at night, which should

be carefully differentiated from other causes of coughing from respiratory problems (Disatian, 2010).

According to ACVIM consensus in 2009, 23 dogs with DMVD in this study were divided into stage B1 (3/23 dogs), stage B2 (13/23 dogs) and stage C (7/23 dogs). About 70% of experimental dogs have DMVD without showing any clinical signs of the disease (stage B); moreover, 80% of dogs with stage B DMVD have developed cardiac remodeling (stage B2). Dogs with DMVD in stage B were detected murmur heart sound at the mitral valve area from the physical examination on the day of normal routine health check, or the annual vaccination. This finding indicated that the truly prevalence of DMVD could be much greater in the dog population if all of the aging small breed dogs are screened. At this moment, the owners often bring their dogs in case that they have already shown the abnormal signs. Some dogs with DMVD have already developed congestive heart failure on the presenting day at the animal hospital and some dogs have diseased condition that is too late for the treatment. Therefore, it is important for aging dogs that is older than 7 years to have an annual health check. At least one time per year, the old dogs should be checked with fully physical examination, complete blood count and blood chemistry profile, and thoracic radiography in order to screen for the elderly disease such as heart disease and kidney disease and, most importantly, for the effective disease control and monitoring.

Part III Complete blood count and blood chemistry

The means of all CBCs data from normal dogs and dogs with DMVD in this study were in the normal value limit. However, neutrophils in the DMVD group are significantly higher than the normal group, which might indicate that dogs with DMVD have been undergone the stress condition or inflammatory processes in the body. The inflammation of

DMVD could come from the respiratory inflammation such as tracheitis and bronchitis (Abbott, 2008). However, the means of neutrophils in both groups are in the normal limit.

The mean value of blood chemistry, BUN, creatinine, ALT and ALP, in both groups are not significant difference. For ALP, the values of this liver enzyme in both groups were higher than the normal limit, which were about 2-fold of the normal value. ALP or alkaline phosphatase is the enzyme of the bile duct lining cells in the liver. Elevated ALP could indicate the congestion of biliary bile duct due to the bile duct obstruction. ALP can come from, liver, bone tissue and intestine. Although the values of ALP in both groups are elevated, none of these experimental dogs have shown the signs of liver disease such as icterus. ALP has high sensitivity but low specificity; therefore, elevated ALP alone is not enough to state the problems. Bone ALP could be elevated in old dogs with osteolytic disorder. Moreover, the value was not three times higher than the reference value indicating the clinical insignificance (Alvarez and Whittemore, 2009).

Part IV Radiography and Echocardiography

The radiographic diagnosis for DMVD indicates cardiomegaly, left atrial enlargement, and pulmonary edema. However, only 50% of dogs with DMVD in this study had the abnormal radiographs. Most of the abnormal radiographs were cardiomegaly and left atrial enlargement as the result of cardiac remodeling from the mitral regurgitation. As the result, radiography is not appropriate for the DMVD diagnosis; nevertheless, it is the helpful diagnostic tool especially when pulmonary edema occurs in the heart failure patient. Therefore, all dogs with stage B or higher should be taken the baseline thoracic radiograph at the time that murmur heart sound was detected (Atkins et al., 2009)

The echocardiography is the gold standard diagnostic tool for DMVD. It is very useful for the diagnosis of early DMVD and can differentiate DMVD from other diseases

(Haggstrom et al., 2004). It becomes a routinely used diagnostic method for the veterinary cardiologist because 2D and M-mode echocardiogram provides a useful information of the heart such as the valve abnormality, the heart shape, and the cardiac chamber size. Furthermore, echocardiogram can estimate the systolic function of the heart. From this study, all of dogs with DMVD had the abnormal mitral valve leaflets, which were thickening than normal, “club-like” appearance, and sometimes prolapsed. The several views of mitral valve should be seen in order to examine the lesion of entire valves (Kvart and Haggstrom, 2000). The ratio of left atrium to aorta root, the left ventricular internal dimension index at end systole, and end diastole in dogs with DMVD were larger than normal dogs, which agreed with Arndt et al. study in 2009. Left atrial enlargement or cardiac remodeling is the result from the prolonged blood regurgitation into the left atrium. The percentage of fractional shortening in the clinically normal dogs (34.65 ± 1.33%) was lower than in dogs with DMVD (38.73 ± 1.82%), which is also similar with Arndt et al. study. The more severe lesion of the mitral valve is the more severe MR occurs, consequencing in volume overloading in left atrium. Prolong volume overloading leads to cardiac remodeling in left atrium and eccentric hypertrophy or left ventricular chamber enlargement. According to the Frank starling law, the increased stretch of ventricular wall causes the increasing of cardiac contraction (Klabunde, 2010). As the result, the echocardiographic indices such as fractional shortening that are greater than normal could be found in dogs with moderate to severe MR. The decreasing or normal number of fractional shortening in moderate to severe MR indicates the poor cardiac contractility (Haggstrom et al., 2004). The Doppler color flow displays the severity of mitral regurgitation. In this study, there were 8 dogs with mild MR, 6 dogs with moderate MR, and 9 dogs with severe MR, which is subjectively categorized from the percentage of MR or jet size into left atrium (mild; <15%, Moderate 15-50%, Severe >50%). The small MR is obviously separated from moderate and severe MR. However, moderate and severe MR is difficult to separate from each other. The proximal isovelocity

surface area (PISA) is sometimes used and more quantitative value for the DMVD severity diagnosis (Doiguchi and Takahachi, 2000; al Muzzi et al., 2003; Kittleson and Brown, 2003).

Part V Circulating serotonin concentrations in dogs with DMVD

All dogs were fasted 4-6 hours before the blood collection, and no drug administration at least 1 month prior to the study. So, the serotonin level in this study would not be affected from other factors, especially from food and drugs.

1. Plasma serotonin concentration

The median of plasma serotonin concentration in dogs with DMVD (3.70; 2.17 – 5.98 ng/ml) was not significantly different from normal dogs (4.29; 2.31 – 8.21 ng/ml). The roles of serotonin induced valvular changes have been well understood in human and experimental animals, but still unclear in canine DMVD. In human who has carcinoid tumor, the large amount of serotonin is secreted from tumor into the circulation increasing the chance of having vulvulopathy in the patients (Gustafsson et al, 2008). The valve that would likely effect were the right sided heart valves including tricuspid and pulmonic valves, not mitral valve, because of some serotonin metabolism by cells in the lungs (Gustafsson et al., 2005). Moreover, patients who received serotonergic drugs or the combination drug of fenfluramine-phenteramine have increased the risk of having lesions at mitral and tricuspid valves (Connolly et al., 1997; Rothman et al., 2000; Antonini and Poewe, 2007). Another study from Gustafsson et al. (2005) showed that the myxomaotus like lesions could induce in rats by the daily injection with serotonin. All above studies suggests that overwhelmed serotonin in the circulation can induce the valve abnormality. However, in normal situation, serotonin in the circulation or in plasma is very low due to the rapid up taken from the platelets and metabolized through the lung and liver cells (Orton et al., 2012). The pathology of valvulopathy in humans and rats are similar to canine DMVD; however, there

are actually not identical. In both human and rats, the serotonin induced myxomatous valve degeneration are described as fibrous plaques, thickening leaflets and chordea tendinea, and GAG deposition in extracellular matrix while canine DMVD do not develop large fibrous plaques on the valve surface (Donnelly, 2008; Oyama and Levy, 2010; Orton et al., 2012). Together with our study, we did not find the significant difference between plasma serotonin concentration in dogs with DMVD and normal dogs. As the result, this suggests that plasma serotonin is unlikely to be the source of serotonin signaling in canine DMVD. In this case, the roles of local serotonin mediating canine DMVD are suggested, which will be explained in the last section of this chapter.

2. Platelets serotonin concentration

The median of platelet serotonin concentration in dogs with DMVD (176.66; 75.43 – 445.77 ng/10^9 platelets) was not significantly different from the normal dogs (128.61; 78.67 – 276.64 ng/10^9 platelets). After serotonin synthesis in enterochromaffin cells in the guts, it is released into the circulation and rapidly up taken by platelets. Thus, major serotonin amount in the circulation is stored in the platelets (Kema et al., 2000). Serotonin will be released from the platelets when platelets aggregate. Our study is the first study investigated the amount of serotonin in platelets in dogs with DMVD. The amount of serotonin in platelets is the largest compared with in plasma and serum. From our platelet serotonin results, there was also no significant difference between dogs with DMVD and normal dogs. This results support the local serotonin mechanism on canine DMVD as well. Nevertheless, dogs with DMVD tended to, but not significantly, have higher platelet serotonin level than normal dogs. This might suggest the hypothesis of platelets aggregation releasing serotonin on the damaged mitral valve (Yabanoglu et al., 2009) and mediating local serotonin signaling. Corcoran et al. (2004) have published the photograph of mitral valve leaflet surface of dogs with DMVD taken by scanning electron microscopy. The affected mitral valve surface have lost of the endothelial cells, accumulated with inflammatory cells and shown small amount of

platelets aggregation (Stein et al., 1989; Corcoran et al., 2004). This platelet aggregation could release serotonin into the local valve area and possibly initiating the local serotonin mechanism of canine DMVD. Even through the platelet aggregation is hardly seen from scanning electron microscope, this hypothesis is likely to be accepted. The loss of platelet aggregation on the mitral valve surface might be the consequence of the slide preparation procedure leading to a washout of some platelets. So, platelet might be the source bringing serotonin into the valve and be the source of initial chemical stimuli for the local serotonin signaling in canine DMVD.

Many studies reported platelet dysfunction in dogs with DMVD including increasing platelet aggregation, decreasing platelet lifespan, and platelet response. CKCS, the predisposing breed of DMVD is mostly used in the studies. Both higher and lower in platelet aggregation as well as other platelet abnormalities were found in CKCS with DMVD (Tanaka and Yamane, 2000; Olsen et al., 2001; Tanaka et al., 2002; Tarnow et al, 2003; Cowan et al., 2004; Tarnow et al, 2004; Nielsen et al., 2007). Moesgaard et al. (2009) reported the platelet dysfunction in Dachshund with DMVD. As a result, in CKCS and, maybe, Dachshund, the pathogenesis of DMVD might involve with the abnormal platelet dysfunction which might occur as the consequence of the blood regurgitation around the mitral area (Haggstrom et al., 2004; Moesgaard et al., 2009). The platelet function in other breeds has not been studied yet.

3. Serum serotonin concentration

The median of serum serotonin concentration in dogs with DMVD (84.66; 37.84 – 176.56 ng/ml) was significantly lower than normal dogs (219.14; 158.29 – 404.34 ng/ml) (p <0.01). From Arndt et al. study (2009), they reported the significant increasing of serum serotonin concentration in dogs with DMVD compared with normal dogs. However, their study has many limitations. First of all, the dog breeds did not match between DMVD and normal group. In their study, they selected healthy large breed dogs as a control, while

dogs with DMVD mostly are small breed dogs (Ware, 2003). Thus, the small breeds should be used as a model in the study of DMVD. Secondly, the age did not match among three experimental groups. Even though there was no relationship between age and serum serotonin concentration, the dogs with DMVD that were significant older than other groups of normal control dogs might not appropriate for the evaluation of the study. Thirdly, if the result was compared only in small breed dogs, the DMVD group (765.5 ng/ml) and predisposing small breed control group (774.9 ng/ml), the results would not be significantly different.

Our study had controlled and matched the breed and age of dogs between two experimental groups. The result of this study showed the significant lower serum serotonin concentration in dogs with DMVD compared to normal dogs. Ljungvall et al. (2012) studied about serum serotonin concentration and DMVD severity association and reported the significant lower serum serotonin concentration in dogs with severe DMVD compared with dogs predisposing to the disease. The predisposing dogs in Ljungvall study were the small breed dogs that have no evidence of the DMVD from the echocardiogram. These findings suggest that the level of serum serotonin is dependent on disease severity. Arndt et al (2009) study and this study did not separate and concentrate in disease severity among dogs with DMVD, so the majority of dogs with DMVD could be whether in mild or severe stage afterward. On another word, the severity of disease in each dog could affect the overall serum serotonin in the whole group of dogs with DMVD. Therefore, the results from Arnt et al (2009) and this study are possibly different and incomparable.

However, serum might not be a good sample representing circulating serotonin in the body. Under the normal circumstance, in the body, the major serotonin in circulation is stored in the platelet granules (Meyer et al., 1982). The serotonin is very low in the plasma. Many factors induce platelets release serotonin such as endothelial damage, platelet aggregation, and serotonin receptor agonist administration (Jonnakuty and Gragnoli, 2008).

During sample collection, blood was collected and waited for the clotting process in order to obtain serum. During the clotting phase, serotonin would be released from the platelet and would exaggerate the actual amount of serotonin in the body. Moreover, the quantity of platelet releasing serotonin when blood clotting is unclear, so the serum serotonin concentration would not be a correct value reflecting circulating serotonin (Orton et al., 2012). Indeed, plasma serotonin concentration might be a better sample for the study of circulating serotonin.

Lastly, this study supports the roles of local serotonin signaling in the occurring of canine DMVD. Orton et al. (2012) proposed the hypothesis of serotonin mediating canine DMVD shown below (Fig 8).

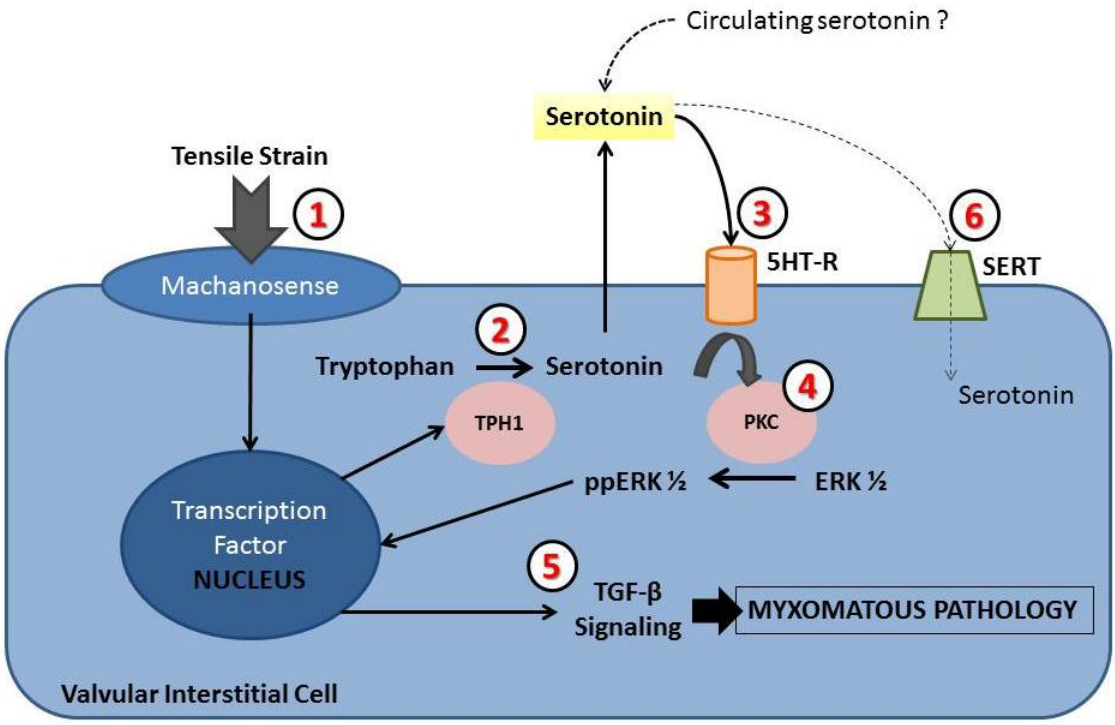

Fig. 8: The local serotonin hypothesis mediating canine degenerative mitral disease (DMVD) (Modified from Orton et al., 2012)

1. Tensile strain on the mitral valve firstly stimulate through the machanosense, resulting in the increasing of tryptophan hydroxylase 1 (TPH1), the rate limiting serotonin synthesis enzyme.
2. Increased TPH1 locally synthesize serotonin from tryptophan. Serotonin is released from the valvular interstitial cells.
3. Increased local serotonin and, maybe, together with circulating serotonin not in plasma but from platelets bind with serotonin receptors type B2 on the heart valve. Serotonin receptor type B2 has been found increased in the mitral valve of dogs with DMVD (Oyama and Chittur, 2006; Disatian and Orton, 2009; Scruggs et al., 2010)
4. After the binding with serotonin receptor, protein kinase C (PKC) is activated leading to phosphorylation of ERK ½ to the phosphorylated ERK (ppERK ½). Disatian and Orton (2009) reported the increased ppERK ½ without the change of total ERK, confirming the activation of serotonin signaling in the mitral valve tissues.
5. ppERK ½ induce the transforming growth factor ß (TGF-ß) signaling. The TGF-ß signaling leads myxomatous pathology including cell proliferation, ECM degradation, and GAG synthesis.
6. The downregulation of serotonin transporter (SERT) in the affected mitral valve decreases serotonin uptake and metabolism causing more serotonin available to react to their receptors.

The limitation of this study is use of the single laboratory serotonin measurement. As the result, the quantity of serotonin concentration has no reference value from the other standard tests. However this study was not decide to measure the standard value of serotonin in the circulation, but to compare the circulating serotonin concentration between two experimental groups; DMVD and normal groups of dogs. So, the single serotonin test of serotonin ELISA test kit would be sufficient for the results and discussion. Another limitation is a small sample size. Total 43 dogs were included in this study, which are fewer than other

serotonin studies in DMVD dogs. Arndt et al. (2009) had 86 dogs enrolled in their research, and Ljungvall et al. (2012) had 120 dogs in total. However, all dogs in our study are matched with age, breed, and size, which are the ultimate model for the comparison. Furthermore, dogs were fasted at least 4-6 hours before sample collection and no other drugs administered 1 month prior to the study. So the other factors disturbing the level of circulation serotonin concentration was minimal. Most importantly, the power of the test had reached 80% (G*Power test) when sample size was 20 dogs in each group. Normally, the power of the experiment should be 80% to 90% in order to detect the difference of the effect when there is present. Therefore, the sample size of this study is adequate to explain the difference between two experimental groups if there is the alteration.

In conclusion, in our study, dogs with naturally occurring DMVD did not have the plasma and platelet serotonin concentration different from the age and size matched control dogs. Even though the difference had found in serum, the actual and reliable serotonin amount in the body that could initially act the serotonin mechanism in DMVD is from the serotonin in plasma. Moreover, serum serotonin level has many factors affecting its concentration such as the unpredictable releasing serotonin from platelet during blood clotting. So, plasma and platelet serotonin is more trustable and represented for circulating serotonin in this case. Therefore, the present study showed that plasma serotonin might not be the major source of serotonin signaling in canine DMVD, but serotonin in platelets is still questionable for being the initial chemical stimuli of this mechanism. Lastly, the local serotonin signaling mediating DMVD is supported by the results of this study. From many updated knowledge, the etiology of canine DMVD is more clear, but more evidence about the initial stimuli of this mechanism should be investigated. The further study about the local serotonin blockage or other serotonin pathways mediating DMVD is needed in order to prevent and prolong disease progression.

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APPENDICES

Appendix A Signalment in the clinically normal dogs.

Name	Breed	Sex	Age (year)	Weight (kg)
มูมู่(1)	Poodle	F	10	10.9
ปิงปิง	Poodle	F	9	8
พริตตี้	Mixed	F	7	5.42
เศรษฐี	Shin Tzu	M	10	8
เคน	Shin Tzu	M	8	6
คาเน	Poodle	M	13	3.4
นางฟ้า	Pomeranian	F	10	4
ยูโร	Poodle	M	8	3.4
แจ็คกี้	Jack Russell	F	8	5.78
ซีดาน	Chihuahua	M	9	4.5
ดีดี้ (2)	Poodle	F	7	5.42
มูมู่ (2)	Shin Tzu	M	7	5.5
มีมี่	Shin Tzu	F	8	7.8
น้ำตาล	Poodle	M	9	5.4
ถุงเงิน	Shin Tzu	F	10	5.5
จ๊อกกี้	Chihuahua	M	8	5
โคล่า (2)	Shin Tzu	F	11	6
ไจแอนท์ (1)	Shin Tzu	M	10	7.6
เจนนี่	Chihuahua	F	8	4
โคล่า (1)	Poodle	M	7	7.6

Appendix B Signalment in the dogs with degenerative mitral valve disease.

Name	Breed	Sex	Age (year)	Weight (kg)
โรเตอร์	Poodle	M	15	4.5
ดีดี้(1)	Poodle	F	8	6
ผักกาด	Shin Tzu	F	7	6.8
หยอง	Poodle	F	7	6.4
เบน	Poodle	M	7	4
หาว	Spitz	F	10	8.6
ฟันฟัน	Shin Tzu	M	10	4.8
ฟลุ๊ค	Poodle	M	14	3
นิก	Chihuahua	F	12	2
กะทิ	Poodle	F	12	5
อ้องเต้	Poodle	M	10	6
กีกี้(1)	Poodle	M	10	8
ริชชี่	Yorkshire	M	9	3.2
เต้าฮวย	Poodle	M	10	3.7
ไจแอนท์(2)	Poodle	M	8	5
โกจิ	Poodle	F	12	5.5
โชกุน	Poodle	M	13	12
ปังปอนด์	Shin Tzu	M	12	7
ชาแตน	Poodle	F	11	6.8
กุ๊งกิ๊ง	Mixed	F	14	10
perty	Poodle	M	12	14
ไมโล	Poodle	F	11	10
กีกี้ (2)	Poodle	F	15	4.6

Appendix C. Echocardiographic indices in the clinically normal dogs.

Dog	Name	AO	LA	LA/Ao	IVSs	IVIDs	LVPWs	IVSd	IVIDd	LVPWd	FS
1	มูมู่(เก่า)	16.8	18.9	0.89	12.8	16.9	10.7	10.6	24.8	8.6	31.71
2	ปิงปิง	18.5	19.3	1	12.7	17.4	11	10.3	27.4	10.4	36
3	พริตตี้	14.4	16.8	1.16	10.4	16.8	7.6	7.6	20.7	6.5	18.85
4	เศรษฐี	13.4	16.2	1.2	17	15.1	10	10.2	24	9.2	37
5	เคน	12.3	16	0.76	9.2	13.8	9.4	7.7	20	7.7	31
6	คาเน	16.1	13.5	1.2	6.6	14.9	6.7	7	21.1	5.2	29.3
7	นางฟ้า	11.5	16.6	1.4	11.4	12.2	9.7	8	22.2	6.8	45.04
8	ยูโร	9.5	18.5	1.4	9.9	11.9	10.7	6.6	23.4	6.3	49.1
9	แจ็คกี้	12.8	17.2	1.3	10.8	15.3	9.2	8.3	24.7	6.6	38.17
10	ซีดาน	11.3	15.2	1.35	9.2	10.7	10	5.1	20.5	6.2	47.79
11	ดีดี้	11.9	18.6	1.5	7.7	13.1	8.9	5.7	21.3	6.6	38
12	มูมู่	13.2	16.6	1.26	9.1	14.9	8.2	6.7	21	6.7	28.93
13	มีมี่	14.6	16.6	1.1	9.5	8.3	8.3	7.5	18.7	5.7	31.4
14	น้ำตาล	12.4	15.6	1.2	10	10.9	9.5	4.6	18.3	9.3	40
15	ถุงเงิน	10.6	15.1	1.4	8.1	12.3	7.2	7.9	18	4.3	31
16	จ๊อกกี้	9.1	11.5	1.27	4.8	9.4	4.3	6.3	15.1	6.6	37.59
17	โคล่า	10.9	16.1	1.4	8.8	18.2	9.2	7.5	22.2	8.2	18.32

Appendix C. Echocardiographic indices in the clinically normal dogs. (cont.)

Dog	Name	AO	LA	LA/Ao	IVSs	IVIDs	LVPWs	IVSd	IVIDd	LVPWd	FS
18	ไจแอนท์	13.1	15.8	1.2	9.4	14.9	10.4	7.9	21.7	7.7	31.3
19	เจนนี่	10.3	12.7	1.2	8.3	12	7.4	5.7	20.6	4.5	41.76
20	โคล่า(เก่า)	13.8	19.8	1.4	9.2	17.4	10.4	7.4	25.1	8.1	30.8

Ao, aortic root dimension; LA, left atrial dimension ; LA/Ao, left atrial to aortic root ratio; IVSs, interventricular septal thickness at end-systole; LVIDs left ventricular end-systolic dimension; LVPWs, left ventricular posterior wall thickness at end-systole; IVSd, interventricular septal thickness at end-diastole; LVIDd, left ventricular end-diastolic dimension; LVPWd, left ventricular posterior wall thickness at end-diastole; FS, fractional shortening

Appendix D. Echocardiographic indices in the dogs with degenerative mitral valve disease.

Dog	Name	AO	LA	LA/Ao	IVSs	IVIDs	LVPWs	IVSd	IVIDd	LVPWd	FS	MR
1	โรเตอร์	10	17.2	1.72	10.1	16.6	6.6	5.5	22.5	5.5	46.22	Severe
2	ดีดี้(1)	11.6	18.6	1.6	9.4	13.4	9.4	7.5	23.4	6.8	42.58	Moderate
3	ผักกาด	14.3	18.8	1.31	11.4	14.5	7.8	8.4	23.5	5.3	38.46	Mild
4	หยอง	11.8	19.2	1.63	8.8	15.2	9.2	6	25.2	6.5	39.52	Moderate
5	เบน	10	17	1.7	10.1	18.7	6.6	5.9	29.2	5.4	35	Moderate-Severe
6	หาว	15.3	23.5	1.53	10.9	19.5	10.9	5.8	33.1	7.9	41.06	Moderate
7	ฟันฟัน	13.3	18.1	1.36	13.9	10.7	10.8	8.8	19.7	8.8	45.64	Moderate
8	ฟลุ๊ค	10.9	23.4	2.14	7.9	8.7	8.7	5.3	28.1	5.8	35.57	Severe
9	นิก	11.4	18.5	1.36	7.6	9.7	6.8	4.5	19.8	3.5	50.86	Moderate-severe
10	กะทิ	15.5	18.3	1.18	12.6	14.9	12.6	10.4	22.1	8.5	32.46	Mild
11	ฮ้องเต้	12.3	23	1.88	7.7	19.4	10.8	6.8	28.9	5.5	32.68	Moderate-severe
12	กีกี้(1)	13.2	18	1.36	9	17.5	9	7.3	25	7.1	29.93	Mild
13	ริชชี่	12.7	16.6	1.31	7.3	12.9	9.8	5.7	22.8	4.8	43.35	Mild
14	เต้าฮวย	10	20.4	2.05	10	11.9	8.3	5.6	23.1	5.3	48.37	Moderate-severe
15	ไจแอนท์(2)	16	22.8	1.43	7.2	20.6	11.3	5.7	33	5.8	37	Moderate-severe
16	โกจิ	12.3	17.22	1.4	12.6	15.2	12	10	25.2	9	39.5	Moderate
17	โชกุน	18.7	23.6	1.2	12.8	16.6	12.8	9.2	24.3	10.8	31	Mild

Appendix D Echocardiographic indices in the dogs with degenerative mitral valve disease. (cont.)

Dog	Name	AO	LA	LA/Ao	IVSs	IVIDs	LVPWs	IVSd	IVIDd	LVPWd	FS	MR
18	ปังปอนด์	13	18.3	1.38	11.3	21	8.3	7.7	15.8	7.1	25	Mild
19	ชาแตน	13.6	26.2	1.93	12.1	18.5	10.6	6.8	27.5	8.3	32.88	Severe
20	กุ๊งกิ๊ง	18.5	23.5	1.27	10.4	19.1	14.9	8.1	34.2	7.2	44	Mild
21	perty	16	22.8	1.42	13.6	18.5	12.3	10	31.5	8.9	37.13	Moderate
22	ไมโล	15.8	40.2	2.54	11.9	24.5	11.7	5.5	42.1	9.2	41.7	Severe
23	กีกี้(2)	13.9	13.2	0.95	10	12.3	7.4	6.5	21.1	5.9	41	Mild

Ao, aortic root dimension; LA, left atrial dimension ; LA/Ao, left atrial to aortic root ratio; IVSs, interventricular septal thickness at end-systole; LVIDs left ventricular end-systolic dimension; LVPWs, left ventricular posterior wall thickness at end-systole; IVSd, interventricular septal thickness at end-diastole; LVIDd, left ventricular end-diastolic dimension; LVPWd, left ventricular posterior wall thickness at end-diastole; FS, fractional shortening; MR, Degree of mitral regurgitation.

Appendix E: The subgroup analysis of serum serotonin concentrations

Table 11: Subgroup analysis among disease severity, no MR, mild MR and moderate to severe MR, of serum serotonin concentrations (Median (25^{th} and 75^{th} percentile)

Disease severity	Number	Median serotonin concentrations (ng/ml)
No MR	20	219.14 (158.29-404.33)*
Mild MR	8	88.23 (31.92-158.15)
Moderate to severe MR	15	69.89 (35.87-227.74)

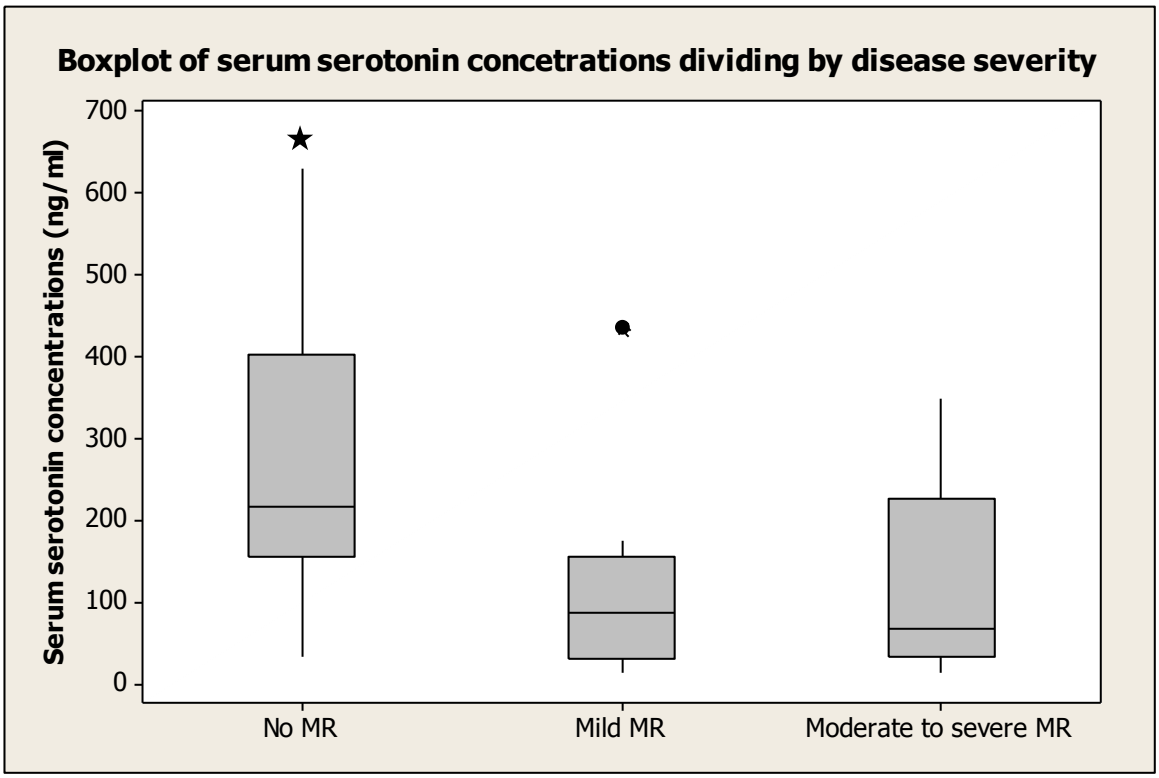

Fig 9. Boxplot of serotonin concentrations dividing by disease severity. The line within the box represents the median value; the limits of the box represent the 25^{th} and 75^{th} percentile value. The outlier value (>1.5 interquartiles) are shown as dots. MR = mitral regurgitation. * represent statistically significance at $p < 0.05$ when compared serum serotonin concentration of no MR group and mild, moderate to severe MR.

Appendix F: The owner questionnaires

Owner's name…….. HN:..... RN:…….

Patient's Name…….. Species……...Breed…… Sex……

Date of Birth/Age…… Weight…… Examination Date ……...

Clinician……..……Consultant……..

MEDICAL HISTORY

• สัตว์ป่วยมีอาการไอหรือไม่ ในกรณีที่มีมักเป็นช่วงใด บ่อยหรือไม่	☐ มี ☐ ไม่มี
• ความอยากอาหารเป็นอย่างไร • มีการเปลี่ยนแปลงของน้ำหนักอย่างไร	☐ ลดลง ☐ เพิ่มขึ้น ☐ ปกติ ☐ ไม่ทราบ ☐ ลดลง ☐ เพิ่มขึ้น ☐ ปกติ ☐ ไม่ทราบ
• มีอาการหายใจลำบาก (dyspnea) หรือไม่	☐ มี ☐ ไม่มี
• มีอาการเหนื่อยง่ายหรือไม่	☐ มี ☐ ไม่มี
• มีอาการเป็นลมหรือไม่	☐ มี ☐ ไม่มี
• มีอาการชักหรือไม่	☐ มี ☐ ไม่มี
• มีการป้องกันพยาธิหนอนหัวใจหรือไม่	☐ มี ☐ ไม่มี
• มีอาการทานน้ำเยอะกว่าปกติหรือไม่	☐ มี ☐ ไม่มี
• มีอาการปัสสาวะมาก หรือบ่อยกว่าปกติหรือไม่	☐ มี ☐ ไม่มี
• มีอาการอาเจียนหรือไม่	☐ มี ☐ ไม่มี
• มีอาการถ่ายเหลว หรือท้องเสียหรือไม่	☐ มี ☐ ไม่มี
• มีขี้ตา หรือมีน้ำมูกหรือไม่	☐ มี ☐ ไม่มี
• มีอาการจามมากกว่าปกติหรือไม่	☐ มี ☐ ไม่มี
• เจ้าของเลี้ยงสัตว์เลี้ยงมานานเท่าใด	
• เจ้าของเลี้ยงสัตว์ด้วยอาหารชนิดใด	

Appendix F: The owner questionnaires (cont.)

Owner's name…….. HN:..... RN:……

Patient's Name…….. Species……...Breed…….. Sex……

Date of Birth/Age…… Weight…… Examination Date ……...

Clinician……..……Consultant……..

MEDICAL HISTORY

• เคยได้รับการรักษาจากการเจ็บป่วยที่สำคัญหรือไม่ ในกรณีที่เคย เป็นการเจ็บป่วยประเภทใด และเมื่อไร
• เลี้ยงในบ้าน หรือนอกบ้าน
• สัตว์เลี้ยงเคยเดินทางออกนอกประเทศ หรือพักฟื้นที่โรงพยาบาล หรืออาบน้ำตัดขนที่ร้านหรือไม่ ในกรณีที่เคย ที่ไหน
• มีอาการเหนื่อยมากกว่าปกติหรือไม่ ☐ มี ☐ ไม่มี
• ในระยะนี้สัตว์เลี้ยงได้รับยาหรือไม่ ในกรณีที่เคย ยาชนิดใด ปริมาณเท่าไร
• เคยมีอาการแพ้ยาหรือไม่ ☐ มี ☐ ไม่มี ในกรณีที่เคย ชนิดใด
• พบพฤติกรรมเปลี่ยนแปลงในสัตว์เลี้ยงหรือไม่ ☐ มี ☐ ไม่มี ในกรณีที่มีเป็นอย่างไร
• ปัญหาหลักที่เจ้าของนำสัตว์เลี้ยงมาโรงพยาบาลคือ

BIOGRAPHY

Miss Tanawan Mangklabruks was born on May 19, 1981 in Chiang Mai, Thailand. She finished her high school education from Satit Chiang Mai University Demonstration School, Chiang Mai, and graduated with Doctor of Veterinary Medicine from the Faculty of Veterinary Medicine, Chiang Mai University in 2005. After that, she had worked as the general practitioner in Small Animal Teaching Hospital, Chiang Mai University for 3 years. After left from work, she spent one year in the US studying English, traveling and exploring life. Then, she decided to continue her education in veterinary medicine in Chulalongkorn University. Cardiology and internal medicine are her field of interests.