THE STUDY OF CHANGES IN GENE REGULATION OF SEROTONIN SIGNALING PATHWAY AND CIRCULATING SEROTONIN CONCENTRATION IN DOGS WITH PULMONARY HYPERTENSION DUE TO DEGENERATIVE MITRAL VALVE DISEASE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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น.ส.ณัฐวรรณ ตั้งมหากุล

จุฬาลงกรณ์มหาวิทยาลัย
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ณัฐวรรณ ตั้งมหากุล : การศึกษาการเปลี่ยนแปลงของการควบคุมยีนในวิถีของซีโรโทนินและความเข้มข้นของซีโรโทนินในสุนัขที่มีภาวะความดันปอดสูงเนื่องจากโรคลิ้นหัวใจไมทรัลเสื่อม. (THE STUDY OF CHANGES IN GENE REGULATION OF SEROTONIN SIGNALING PATHWAY AND CIRCULATING SEROTONIN CONCENTRATION IN DOGS WITH PULMONARY HYPERTENSION DUE TO DEGENERATIVE MITRAL VALVE DISEASE) อ.ที่ปรึกษาหลัก : รศ. สพ.ญ. ดร.สิริลักษณ์ สุรเชษฐพงษ์, อ.ที่ปรึกษาร่วม : รศ. สพ.ญ. ดร.สมพร เตชะงามสุวรรณ

ภาวะความดันปอดสูง คือ การเพิ่มขึ้นอย่างผิดปกติของความดันในหลอดเลือดภายในปอด ในสุนัขภาวะความดันปอดสูงพบได้มากในโรคลิ้นหัวใจไมทรัลเสื่อม โดยเฉพาะอย่างยิ่งในสุนัขพันธุ์เล็ก ซีโรโทนินเป็นไบโอจีนิกเอมีนที่มีบทบาทต่อกระบวนการทางสรีรวิทยาและความผิดปกติของหลายๆอวัยวะ โดยเป็นสารที่มีผลต่อหลอดเลือดซึ่งเป็นหนึ่งในตัวกลางที่อาจทำให้เกิดภาวะความดันปอดสูง มีการศึกษาถึงความเกี่ยวข้องของซีโรโทนินทั้งในระดับเฉพาะที่และในระบบไหลเวียนโลหิตต่อพยาธิกำเนิดของภาวะความดันปอดสูงทั้งในคนและในสัตว์ โดยในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาวิถีของซีโรโทนินภายในปอดและหลอดเลือดพัลโมนารีอาร์เทอรีในปอด ตลอดจนศึกษาแหล่งกำเนิดและความแตกต่างของซีโรโทนินในกระแสเลือด ระหว่างสุนัขปกติ สุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม และสุนัขที่มีภาวะความดันปอดสูงเนื่องจากโรคลิ้นหัวใจไมทรัลเสื่อม โดยเก็บตัวอย่างเนื้อเยื่อปอดและหลอดเลือดพัลโมนารีอาร์เทอรี จากสุนัขพันธุ์เล็ก 14 ตัว แบ่งเป็น กลุ่มควบคุม 4 ตัว กลุ่มโรคลิ้นหัวใจไมทรัลเสื่อม 5 ตัว และกลุ่มโรคลิ้นหัวใจไมทรัลเสื่อมร่วมกับภาวะความดันปอดสูง 5 ตัว ใช้ปฏิกิริยาลูกโซ่พอลิเมอเรสแบบเรียลไทม์แบบย้อนกลับ (qRT-PCR) และเวสเทิร์น บลอท (Western blot) ในการศึกษาแสดงออกของยีนและโปรตีนเกี่ยวข้องกับวิถีของซีโรโทนิน วัดความเข้มข้นของซีโรโทนินภายในเกล็ดเลือดและพลาสมาในตัวอย่างเลือดทั้งหมดของสุนัข 62 ตัว แบ่งเป็นกลุ่มควบคุม 22 ตัว กลุ่มโรคลิ้นหัวใจไมทรัลเสื่อม 20 ตัว และกลุ่มโรคลิ้นหัวใจไมทรัลเสื่อมร่วมกับภาวะความดันปอดสูง 20 ตัว เตรียมเลือดและวัดด้วยวิธีอีไลซา (ELISA) การแสดงออกของโปรตีน TPH-1, SERT, 5-HTR2A, ERK1/2 and pERK1/2 มีแนวโน้มเพิ่มขึ้นในเนื้อเยื่อหลอดเลือดพัลโมนารีอาร์เทอรีของสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อมทั้งที่มีและไม่มีภาวะความดันปอดสูง ในขณะที่การแสดงออกของยีนและโปรตีนในเนื้อเยื่อปอดนั้นมีความแปรผันไปหลากหลายรูปแบบ ความเข้มข้นของซีโรโทนินในเกล็ดเลือดของสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อมที่มีความน่าจะเป็นของภาวะความดันปอดสูงมาก (35.82 [2.69 - 126.35] นาโนกรัม/เกล็ดเลือด 1 พันล้านเซลล์) ลดลงเมื่อเปรียบเทียบกับสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม (325.99 [96.84 - 407.66] นาโนกรัม/เกล็ดเลือด 1 พันล้านเซลล์) (p = 0.008) ความเข้มข้นของซีโรโทนินภายในพลาสมาระหว่างกลุ่มสุนัขไม่มีความแตกต่าง ผลการศึกษาครั้งนี้แสดงให้เห็นถึงแนวโน้มการเพิ่มขึ้นของโปรตีนในวิถีของซีโรโทนินในหลอดเลือดพัลโมนารีอาร์เทอรีในสุนัขที่มีและไม่มีภาวะความดันปอดสูงแสดงถึงผลเฉพาะที่ของซีโรโทนินในหลอดเลือดพัลโมนารีอาร์เทอรีในสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อมที่มีและไม่มีภาวะความดันปอดสูง ในกระแสเลือดระดับความน่าจะเป็นภาวะความดันปอดสูงในสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อมมีความสัมพันธ์กับการลดลงของความเข้มข้นของซีโรโทนินในเกล็ดเลือด ควรศึกษาเพิ่มเติมถึงบทบาทของซีโรโทนินในพัลโมนารีอาร์เทอรีและซีโรโทนินในเกล็ดเลือดที่เกี่ยวข้องกับผลเฉพาะที่และในกระแสเลือดของวิถีซีโรโทนินต่อการเกิดภาวะความดันปอดสูงโดยธรรมชาติในสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม

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Nattawan Tangmahakul : THE STUDY OF CHANGES IN GENE REGULATION OF SEROTONIN SIGNALING PATHWAY AND CIRCULATING SEROTONIN CONCENTRATION IN DOGS WITH PULMONARY HYPERTENSION DUE TO DEGENERATIVE MITRAL VALVE DISEASE. Advisor: Assoc. Prof. Sirilak Surachetpong, D.V.M., M.S., Ph.D., D.T.B.V.M. Co-advisor: Assoc. Prof. SOMPORN TECHANGAMSUWAN, D.V.M., M.S., Ph.D., D.T.B.V.P.

Pulmonary hypertension (PH) is an abnormal increase in pulmonary vascular pressure. In dogs, PH is commonly found secondary to degenerative mitral valve disease (DMVD), especially in small breed dogs. Serotonin, a biogenic amine playing an essential role in both physiology and abnormalities of several organs, is a vasoactive substance that has been one of the suspicious mediators for the development of PH. Both local and circulating effects of serotonin have been investigated to discover the involvement of serotonin and the pathogenesis of PH in both humans and animals. The present study aimed to investigate the local serotonin signaling in lung and pulmonary arteries (PA) and the source and differences of serotonin in platelets and plasma of dogs with PH secondary to DMVD compared to healthy dogs and DMVD dogs without PH. The lung and PA tissues of fourteen small-breed dogs were collected and divided into the control (n = 4), DMVD (n = 5) and DMVD+PH (n = 5) groups. The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot were used to assess the expression of genes and proteins associating with the serotonin signaling pathway. To measure the platelet and plasma serotonin concentrations, whole blood was collected from sixty-two small-breed dogs divided into the control (n = 22), DMVD (n = 20) and DMVD+PH (n = 20) groups. The blood samples were prepared and measured by enzyme-linked immunosorbent assay (ELISA). The tendency of upregulated TPH-1, SERT, 5-HTR2A, ERK1/2 and pERK1/2 protein in PA tissues were found in DMVD dogs with and without PH, whereas the gene and protein expression in lung tissues was varied. The concentration of platelet serotonin of dogs with DMVD and high probability of PH (35.82 [2.69 - 126.35] ng/10^9 platelets) was decreased compared to DMVD dogs without PH (325.99 [96.84 - 407.66] ng/10^9 platelets) (p = 0.008). The concentration of plasma serotonin did not differ among all groups. These findings revealed that proteins related to the serotonin signaling pathway increased in dogs affected with DMVD with and without PH suggesting the local effect of serotonin in PA in dogs affected with DMVD with and without PH. In circulation, the degree of PH probability of dogs with PH secondary to DMVD is correlated with a decrease in platelet serotonin concentration. Roles of serotonin in PA and platelet serotonin should be investigated to elucidate the involvement of the local and systemic effect of the serotonin signaling pathway in dogs with naturally occurring PH due to DMVD.

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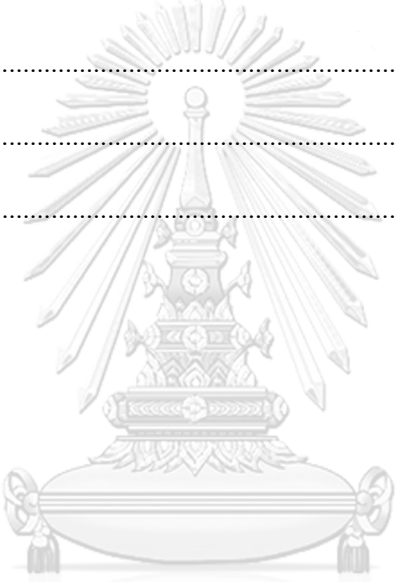

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ABBREVIATION

DMVD: degenerative mitral valve disease

DPG: diastolic pressure gradient

ELISA: enzyme-linked immunosorbent assay

ERK1/2: extracellular signal-regulated kinase ½

htr2a: serotonin receptor 2A (gene)

htr1b: serotonin receptor 1B (gene)

LA: left atrium

LA/Ao: the ratio of LA and aorta

LHD: left heart disease

LV: left ventricle

LVIDd: left ventricular internal diameter at end diastole

mPAP: mean pulmonary arterial pressure

MPV: mean platelet volume

PA: pulmonary artery

PAECs: pulmonary artery endothelial cells

PAH: pulmonary arterial hypertension

PAP: pulmonary arterial pressure

PASMCs: pulmonary artery smooth muscle cells

PAWP: pulmonary arterial wedge pressure

pERK1/2: phosphorylated extracellular signal-regulated kinase 1/2

PH: pulmonary hypertension

PPHN: persistent pulmonary hypertension in newborns

PPP: platelet poor plasma

PRP: platelet rich plasma

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PVR: pulmonary vascular resistance

qRT-PCR: real-time reverse transcription polymerase chain reaction

RV: right ventricle

SERT: serotonin transporter (protein)

slc6a4: serotonin transporter (gene)

TPH-1: tryptophanhydroxylase-1 (protein)

tph1: tryptophanhydroxylase-1 (gene)

TR: tricuspid regurgitation

5-HT: serotonin

5-HTR2A: serotonin receptor 2A (protein)

5-HTR1B: serotonin receptor 1B (protein)

5-HTR2B: serotonin receptor 2B (protein)

CHAPTER I
INTRODUCTION

Importance and rationale

Pulmonary hypertension (PH) is an abnormal elevation of pulmonary vascular pressure caused by multiple disorders increasing the pulmonary arterial pressure (PAP) greater than 25 mmHg at rest and 30 mmHg at doing activities (MacLean and Dempsie, 2009; Hoeper et al., 2013). However, the 6th World Symposium on Pulmonary Hypertension (WSPH) redefined that the mean PAP (mPAP) of patients with PH is greater than 20 mmHg with concern about pulmonary arterial wedge pressure (PAWP) and pulmonary vascular resistance (PVR) to differentiate the pre- and post-capillary PH (Simonneau et al., 2019). In the veterinary field, the American College of Veterinary Internal Medicine (ACVIM) also defined PH by using PAP, hemodynamic and pathological changes assessed by echocardiography (Reinero et al., 2020). The common clinical signs in dogs with PH is not specific to the disease and include exercise intolerance, coughing, changes in breathing sound and pattern, respiratory distress, lethargy, syncope and cyanosis (Johnson et al., 1999; Kellum and Stepien, 2007; Reinero et al., 2020). In dogs, PH ordinarily occurs secondary to degenerative mitral valve disease (DMVD) commonly presented in elder small breed dogs (Pyle et al., 2004). In humans, the incidence of PH was increased over time. Several studies in 1986-2009 reported that the prevalence of pulmonary arterial hypertension (PAH) in the United Kingdom, Ireland, France, Spain, and the United States of America was between 0.9-26 cases per million populations with shortening survival time in older patients (McGoon et al., 2013). The studies in 2004-2015 reported that the prevalence of PH in dogs with DMVD was between 13.94-53.33% (Borgarelli et al., 2004; Serres et al., 2006; Guglielmini et al., 2010; Schober et al., 2010; Borgarelli et al., 2015). Dogs with more severity of DMVD were prone to develop PH. The study of Borgarelli et al. (2015) presented that 39.15% of dogs with stage B2 and C DMVD had PH. Also, two-third of PH dogs were in stage C DMVD. Moreover, the median survival time of dogs with PH

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(456 days) was shorter than DMVD dogs without PH (758 days). Similarly, the study of Serres et al. (2006) reported that Doppler echocardiography–derived evidence of pulmonary arterial hypertension was frequently developed in the higher stage of DMVD dogs.

Serotonin involves the physiology, development, and abnormalities of several organs in both humans and animals (Nebigil and Maroteaux, 2001). The medical profession has been interested in the serotonin hypothesis since increased evidence of PH in women who were medicated with anorectic drugs such as aminorex, fenfluramines and chlorphentermine. The incidence of PH in Switzerland and Germany during the aminorex exposure period was 5-fold increased and the incidence in France and Belgium during the dexfenfluramine exposure period was increased by 20% (Kramer and Lane, 1998). These medicines are serotonin transporter substrates that increase serotonin efflux leading to promote the elevation of extracellular serotonin concentration (Rothman et al., 1999). Although brain tissue was the expected target for these anorectic drugs, Ramamoorthy et al. (1993) discovered that serotonin transporter (SERT) expression in human lung tissues was greater than that in the brain. This evidence implied that SERT in lung tissues might be a predominant route for serotonin action. Interestingly, the previous publications reported that circulating serotonin is associated with PH in humans (Herve et al., 1995; Kereveur et al., 2000; Kirillova et al., 2009). Furthermore, using antidepressant, selective serotonin reuptake inhibitors (SSRIs), in late-gestation women may increase the risk of persistent pulmonary hypertension in newborns (PPHN) (Chambers et al., 2006). Also, the previous studies in ovine and mouse models reported that serotonin involved in the development of PPHN in the fetus by an increase in PVR, increase in serotonin production from the pulmonary artery endothelial cells (PAECs) and increase in serotonin receptor and SERT in pulmonary artery smooth muscle cells (PASMCs) (Delaney et al., 2011; Delaney et al., 2013; Delaney et al., 2018). Besides, several previous studies reported an increase in circulating serotonin levels and the occurrence of PH in patients with abnormalities of platelet, the storages of circulating serotonin,

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such as platelet storage pool deficiency (Herve et al., 1990; Herve et al., 1995), collagen vascular disease (Hoeper, 2002), and Raynaud's phenomenon (Halpern et al., 1960; Marasini et al., 1988).

Nowadays, medical innovations extend the life span and improve the quality of pets' life leading to an increase in age-related diseases including DMVD, which can induce PH. Most of the studies of PH in dogs focused on diagnosis and treatment strategies. The studies in humans and animal models revealed strong evidence indicating that serotonin may mediate PH. Several previous studies in laboratory animal models and cell culture revealed that inhibition of the effectors in the serotonin signaling pathway, such as SERT, serotonin receptor 2A (5-HTR2A), Rho-associated protein kinase (ROCK) and extracellular signal-regulated kinase 1/2 (ERK1/2), may inhibit and/or reverse the pathological changes of the pulmonary artery (PA) with PH condition (Hironaka et al., 2003; Abe et al., 2004; Guignabert et al., 2005; Liu et al., 2013a; Liu et al., 2013b; Zhang et al., 2015). However, the data of gene regulation as well as local and systemic effects of serotonin pathway induced naturally occurring PH in dogs with DMVD was scarce. As an increased prevalence of PH in dogs is found similarly to humans, and the present treatment (e.g., vasodilator reducing pulmonary arterial pressure) may not eliminate the certain cause of PH. An investigation of the cause of this condition, such as targeted gene expression and regulation in serotonin signaling pathway, may lead to targeted therapy for direct inhibition of the cause and progression of PH in dogs and may provide an early detection of abnormal gene regulation to prevent the disease prior to an abnormal change in structure and function of pulmonary arteries, and clinical sign appearance.

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Objectives of the study

1. To investigate the local serotonin signaling in lung tissue and pulmonary arteries of PH dogs due to DMVD compared to healthy dogs and DMVD dogs without PH by determining the expression and regulation of targeted genes and proteins in the serotonin signaling pathway including

tryptophanhydroxylase-1 (TPH-1), serotonin receptor 2A (5-HTR2A), and serotonin transporter (SERT), and the downstream effectors, extracellular signal-regulated kinase ½ (ERK1/2), and phosphorylated ERK1/2 (pERK1/2).

2. To assess the source and difference of serotonin in circulation by measurement of serotonin concentration in circulating plasma and platelets of dogs with naturally occurring PH due to DMVD compared to DMVD dogs without PH and healthy dogs.

Hypothesis

1. Genes of TPH-1 (*tph1*), 5-HTR2A (*htr2a*) and SERT (*slc6a4*) and TPH-1, 5-HTR2A, SERT, ERK1/2 and pERK1/2 protein expressions are upregulated in lung and pulmonary artery of dogs with PH due to DMVD compared to those without PH and healthy dogs.

2. Serotonin concentrations in circulating plasma and platelets are increased in dogs with PH due to DMVD and significantly greater than the concentrations in healthy dogs.

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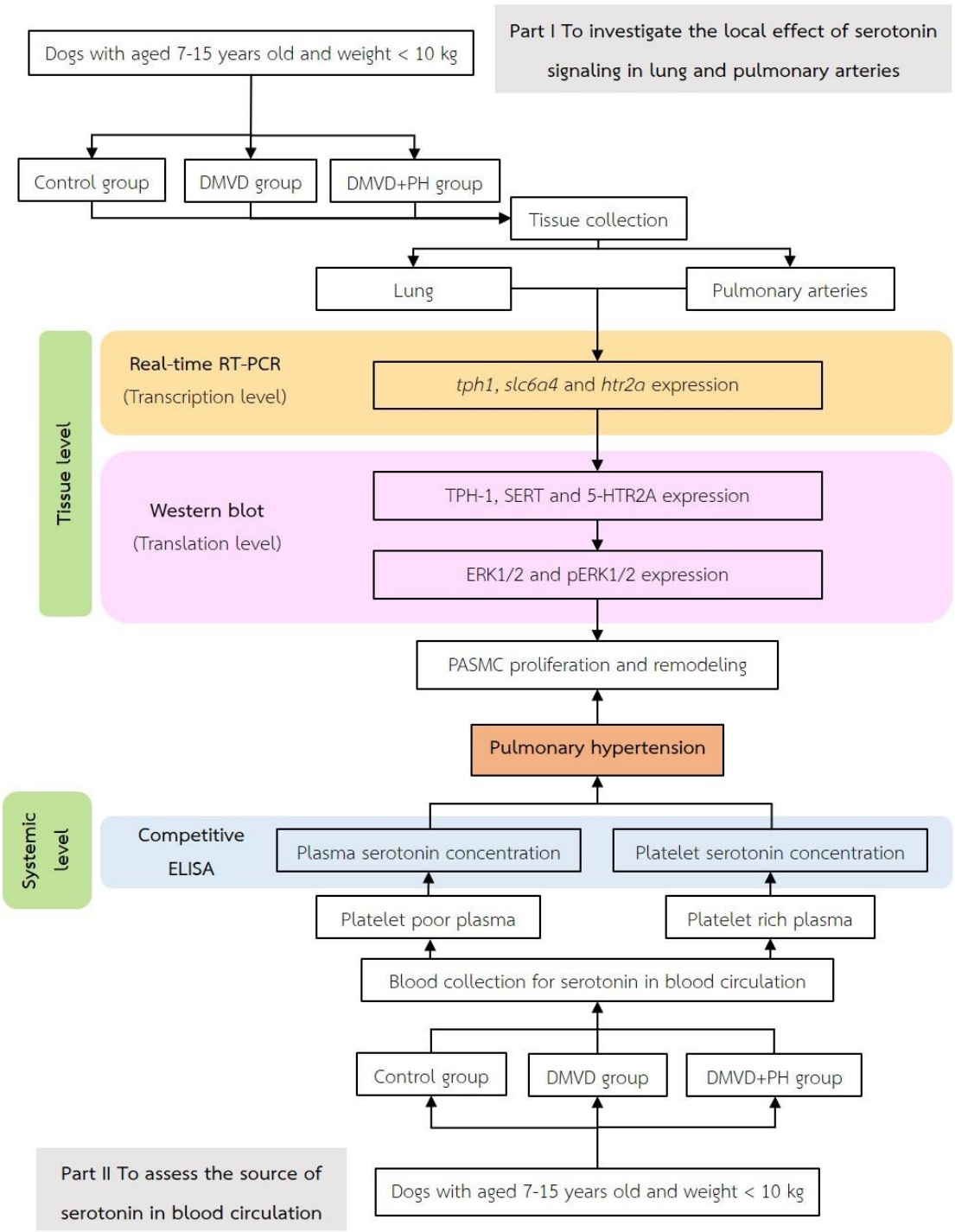
Conceptual Framework



Advantages of Study

This study presents the expression of genes and proteins related to the serotonin signaling pathway in the lung and PA, and the serotonin concentrations in the circulation of dogs with PH secondary to DMVD. The changes in gene and protein expressions of lung and PA tissues showed in the present study provide the location (lung and/or PA) affected by serotonin. Moreover, the alteration of platelet and plasma serotonin concentrations provide the involvement of circulating serotonin in PH due to DMVD of dogs.

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CHAPTER II
LITERATURE REVIEWS

Definition of pulmonary hypertension

Pulmonary hypertension (PH) is an abnormal increase in pulmonary vascular pressure. Since the first World Symposium on Pulmonary Hypertension (WSPH) in 1973, PH was usually defined as the mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg at rest diagnosed by right heart catheterization (Hatano et al., 1975). Pulmonary blood flow, pulmonary vascular resistance (PVR) and pulmonary venous pressure influence PAP (Kellihan and Stepien, 2012; Reinero et al., 2020). However, using the mPAP cut-off value of ≥ 25 mmHg as a single measurement to diagnose PH is not enough to identify the clinical condition or the pathological process because increased PAP can result from several different causes, such as elevated cardiac output, left-to-right cardiac shunt, increased pulmonary arterial wedge pressure (PAWP) in left heart disease (LHD), hyperviscosity and pulmonary vascular disease (PVD) (Simonneau et al., 2019). Therefore, PH can be classified as pre-capillary PH (pulmonary arterial hypertension [PAH] or active PH) and post-capillary PH (pulmonary venous hypertension [PVH] or passive PH) (Kellihan and Stepien, 2012) using mPAP with PVR, PAWP, and diastolic pressure gradient (DPG) (Simonneau et al., 2019; Reinero et al., 2020).

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Classification of PH

The consensus statement guidelines of the American College of Veterinary Internal Medicine (ACVIM) classified PH in dogs into 6 categories and 1 sub-category following the etiology, clinical signs, hemodynamic attributes, pathophysiology, and treatment (Reinero et al., 2020). The classification of PH shows in Table 1, and PH secondary to DMVD is categorized as group 2b1a.

Table 1 Classification of canine pulmonary hypertension (Reinero et al., 2020)

1. Pulmonary arterial hypertension (PAH)
1a. Idiopathic (IPAH)
1b. Heritable
1c. Drugs and toxins induced
1d. Associated with (APAH)
1d1. Congenital cardiac shunts
1d2. Pulmonary vasculitis
1d3. Pulmonary vascular amyloid deposition
1'. Pulmonary veno-occlusive disease (PVOD) or pulmonary capillary hemangiomatosis (PCH)
2. Pulmonary hypertension due to left heart disease
2a. Left ventricular dysfunction
2a1. Canine dilated cardiomyopathy
2a2. Myocarditis
2b. Valvular disease
2b1. Acquired
2b1a. Myxomatous mitral valve disease
2b1b. Valvular endocarditis
2c1. Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
2c1a. Mitral valve dysplasia
2c2a. Mitral valve stenosis
2c3a. Aortic stenosis
3. Pulmonary hypertension secondary to respiratory disease, hypoxia, or both
3a. Chronic obstructive airway disorders
3a1. Tracheal or mainstem bronchial collapse
3a2. bronchomalacia
3b. Primary pulmonary parenchymal disease
3b1. Interstitial lung disease
3b1a. Fibrotic lung disease
3b1b. Cryptogenic organizing pneumonia/secondary organizing pneumonia
3b1c. Pulmonary alveolar proteinosis
3b1d. Unclassified interstitial lung disease

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Table 1 Classification of canine pulmonary hypertension (Reinero et al., 2020) (continued)

3b1e. Eosinophilic pneumonia/eosinophilic bronchopneumopathy
3b2. Infectious pneumonia: Pneumocystis; Ehrlichia
3b3. Diffuse pulmonary neoplasia
3c. Obstructive sleep apnea/sleep disordered breathing
3d. Chronic exposure to high altitude
3e. Developmental lung disease
3f. Miscellaneous: bronchiolar disorders; bronchiectasis; emphysema; pneumonectomy
4. Pulmonary emboli/thrombi/thromboemboli (PE/PT/PTE)
4a. Acute PE/PT/PTE (Massive PE/PT/PTE with right ventricular (RV) dysfunction or submassive PE/PT/PTE without RV dysfunction)
4b. Chronic PE/PT/PTE
5. Parasitic disease (*Dirofilaria* or *Angiostrongylus* infection)
6. Pulmonary hypertension with multifactorial or unclear mechanisms
6a. Disorders having clear evidence of 2 or more underlying group 1-5 pathologies contributing to PH
6b. Masses compressing the pulmonary arteries (e.g., neoplasia, fungal granuloma, etc.
6c. Other disorders with unclear mechanisms

Pre-capillary PH is an increase in PAP or pre-capillary resistance, which is caused by an increase in pulmonary blood flow or PVR without increasing left atrial (LA) pressure (Reinero et al., 2020). On the other hand, post-capillary PH is an elevation of pulmonary arterial pressure secondary to a passive increase in LA pressure, which increases right ventricular (RV) load and further elevates the systolic RV pressures (Atkinson et al., 2009; Kellihan et al., 2015; Reinero et al., 2020). Isolated postcapillary PH (Ipost-PH) can be progressed to combined postcapillary and precapillary PH (C-PH) by chronic progressive of LHD and postcapillary PH leading to vasoconstriction of pulmonary arteries (PA) and PVD resulting in increased PVR (Kellihan and Stepien, 2012; Guazzi and Naeije, 2017).

Pathogenesis and pathological changes of pulmonary arteries in pulmonary hypertension secondary to degenerative mitral valve disease

PH is a complication of the left-sided heart disease including DMVD (Pyle et al., 2004). Chronic increased PAWP may occur in DMVD dogs with congestive heart failure (Falk et al., 2006). Chronic mitral regurgitation causes an elevation of LA pressure and passive increase in the pulmonary venous pressure leading to pulmonary venous congestion, capillary disturbance, and pulmonary edema. These abnormalities can cause vasoconstriction, remodeling and dysfunction of PA, which induce PH. PH finally generates right ventricular overload leading to the dilated chamber and abnormal contractility (Delgado et al., 2005; Patel et al., 2014).

Imbalance of vasoconstriction and vasodilation is found in the contracted and remodeled pulmonary arteries with PH. The evidence of pulmonary arterial vasoconstriction and remodeling including the proliferation and thickening of smooth muscle layer in tunica media of pulmonary arteries has been found in human and animal models with PH, such as rats and mice. (Quinlan et al., 2000; Delgado et al., 2005). Moreover, medial hypertrophy of muscular pulmonary arteries can be found in human patients with chronic heart failure without PH (Delgado et al., 2005). The histopathological remodeling of large pulmonary arteries of PH patients includes an increased thickness of vascular wall due to smooth muscle cell proliferation and hypertrophy. In severe PH patients, medium to small pulmonary arteries form the plexiform lesions, a concentric hypertrophy of PAECs in mesh pattern with multichannel formation occluding the lumen of the arteries and worsening the blood flow toward the post-obstructive areas. The capillaries are dilated and thickened with leakage of red blood cells to alveoli through the ruptured basement membrane (Delgado, 2010). The plexiform lesions develop in distal to branch points or the bifurcation of pulmonary arteries with or without concentric-obliterative lesions, an onion-like lesions proximal to plexiform lesion. The plexiform lesions may terminate as aneurysmal-like dilatation lesions or end with disappearance of the vessels. Neovascularization of small thinned-wall dilated vessels may present adjacent to the

plexiform vessels. The inflammatory cytokines and growth factors may affect the development of plexiform lesions. (Tuder et al., 1994; Cool et al., 1999)

Diagnosis of canine pulmonary hypertension

The clinical signs of PH are lethargy, coughing, exercise intolerance, changed breathing sound and pattern, respiratory distress, heart murmur at the tricuspid area, syncope, and cyanosis which are similar clinical signs of other cardiac or respiratory diseases (Johnson et al., 1999; Serres et al., 2006; Kellum and Stepien, 2007; Kellihan and Stepien, 2012; Kellihan et al., 2015). Physical examination for dogs with DMVD and PH commonly reveals cardiac murmurs, respiratory crackles, and cyanosis. In severe cases, signs of right-sided heart failure such as ascites and jugular pulse are found. Radiographic findings show either normal lung and cardiac silhouette or the abnormalities of the heart and lungs such as cardiomegaly, respiratory infiltration, and abnormal vascular patterns (Johnson et al., 1999; Pyle et al., 2004; Kellum and Stepien, 2007). The gold standard method for diagnosis of PH in human is a direct measurement for hemodynamic parameters including mPAP, PAWP and PVR by the right heart catheterization; however, this method is invasive for dogs (Simonneau et al., 2019). Therefore, the diagnostic procedure for PH in dogs is an indirect method which is the Doppler echocardiographic measurement for peak tricuspid regurgitation (TR) velocity and the calculation of the estimated PAP using simplified Bernoulli equation (pressure gradient [PG] = 4 x TR velocity^2) (Reinero et al., 2020). Following the ACVIM consensus statement guidelines, the severity of PH is classified into low, intermediate and high probability of PH depending on peak TR velocity and the number of different anatomic sites of echocardiographic signs of PH in ventricles, pulmonary artery, and right atrium and caudal vena cava (Reinero et al., 2020). (Table 2 and 3) Moreover, the echocardiographic evidences of left heart disease and the definite LA enlargement were the additional concern for PH secondary to left heart disease in dogs including DMVD (Reinero et al., 2020).

Table 2 Echocardiographic probability of PH in dogs (Reinero et al., 2020)

Peak tricuspid regurgitation velocity (m/s)	Number of different anatomic sites of echocardiographic signs of PH*	Probability of PH
≤3.0 or not measurable	0 or 1	Low
≤3.0 or not measurable	2	Intermediate
3.0 to 3.4	0 or 1	Intermediate
>3.4	0	Intermediate
≤3.0 or not measurable	3	High
3.0 to 3.4	≥2	High
>3.4	≥1	High

* See Table 3

Table 3 Anatomic sites of echocardiographic signs of PH (Reinero et al., 2020)

Anatomic sites 1: Ventricles	Anatomic sites 2: Pulmonary artery	Anatomic sites 3: Right atrium and caudal vena cava
Flatting of the interventricular septum (especially systolic flattening)	Pulmonary artery enlargement (PA/Ao >1.0)	Right atrial enlargement
Underfilling or decreased size of the left ventricle[a]	Peak early diastolic PR velocity >2.5 m/s	Enlargement of caudal vena cava
RV hypertrophy (wall thickening, chamber dilation, or both)	RPAD index <30%	
RV systolic dysfunction	RV outflow Doppler acceleration time (<52-58 ms) or acceleration time to ejection time ratio (<0.30) Systolic notching of the Doppler RV outflow profile (caution: false positives are possible)	

PR = pulmonary regurgitation; RPAD = right pulmonary artery distensibility; RV = right ventricular.

[a] Not applicable for dogs with group 2 PH due to the confounding effects of LV remodeling secondary to LHD.

Serotonin and pulmonary hypertension

Serotonin or 5-hydroxytryptamine (5-HT) is one of the mediators that may cause PH. Typically, serotonin is commonly found in the intestinal wall, platelets and CNS (Nebigil et al., 2000). Serotonin is divided into central and peripheral pools. The central pool of serotonin is a neurotransmitter in the central nervous system (CNS) synthesized by tryptophan hydroxylase-2 (TPH-2), the rate-limiting enzyme, in the raphe nuclei located on the midline of the brainstem (Declerck and Boone, 2016). Serotonin plays a crucial role in the cognition and development of the nervous system as well as the abnormalities of the serotonergic system including temperament and behavior disorders (Moiseiwitsch et al., 2001; Fukumoto et al., 2005; Kaneko et al., 2008; De-Miguel et al., 2015). The peripheral pool of serotonin is a biogenic amine synthesized by TPH-1, which is mainly produced in the enterochromaffin cells in the intestine (Berger et al., 2009). Serotonin plays an essential role in physiology, development of organs in embryo and abnormalities of several organs of human and animals (Lauder, 1993; Nebigil et al., 2000; Nebigil et al., 2001; Nebigil and Maroteaux, 2001; Fukumoto et al., 2005). Ninety-five percentage of serotonin is synthesized in enterochromaffin cells of the intestine, and the other 5% is produced by the brain and lung (Adnot et al., 2013). Synthesis of serotonin uses the two-step enzymatic pathway. Firstly, TPH-1 is used for the hydroxylation of tryptophan and forming 5-hydroxytryptophan (5-HTP), then amino acid decarboxylase (AADC) converts 5-HTP into serotonin (Linder et al., 2007). After releasing to the blood stream, 99% of serotonin is uptaken by platelets and stored in an inactive form in the dense (delta) granules in platelet cytosol by vesicular monoamine transporter 2 (VMAT2) mediating serotonin uptake from platelet cytosol to dense granules (Tranzer et al., 1966; White, 1969; Bentfeld-Barker and Bainton, 1982; Adnot et al., 2013). Uptake of serotonin to platelet cytosol mediates via serotonin receptors and serotonin transporters (SERT) resulting in low plasma serotonin concentration (Adnot et al., 2013). Serotonin metabolism mainly occurs in the liver; however, the metabolism also occurs in the lung (Egermayer et al., 1999). In the lung, serotonin enters pulmonary vascular endothelial cells by passing

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via serotonin receptors and SERT on the plasma membrane. In the endothelial cells, serotonin is metabolized by monoamine oxidase (MAO), which converts serotonin into 5-hydroxyindoleacetic acid (5-HIAA), which then are excreted via urine (Gillis and Pitt, 1982).

Evidence of serotonin involving PH have been discovered since the incidence of PH was increased in women using anorectic drug including aminorex, fenfluramine and chlorphenteramine which are SERT substrates. These drug molecules exchange for endogenous serotonin leading to an increase in serotonin efflux. An elevation of extracellular serotonin concentration results in an increased risk of PH (Rothman et al., 1999). The involvement of platelet serotonin and PH was found in many studies in both humans and dogs. Aggregated platelets from human patients with primary PH-induced the increased platelet serotonin release simultaneously with decreased platelet serotonin and increased plasma serotonin concentrations (Herve et al., 1995). In addition, serotonin release from canine aggregating platelets can induce vasospasm of PA (McGoon and Vanhoutte, 1984). Moreover, platelet serotonin may involve in the pathogenesis of DMVD in dogs since increased platelet serotonin concentration was found with elevated serotonin levels in the mitral valve and myocardial tissues of the left ventricle (Cremer et al., 2014).

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In lung tissues, serotonin synthesis originates via tryptophan hydroxylase-1 (TPH-1), a rate-limiting enzyme, in pulmonary artery endothelial cells (PAECs) (Gairhe et al., 2012). After the synthesis, serotonin is then transferred to pulmonary artery smooth muscle cells (PASMCs) in paracrine fashion through serotonin receptors and SERT on the plasma membrane and then activate downstream effector signaling pathways such as extracellular signal-regulated kinase (ERK) 1/ERK2, mitogen-activated protein kinase (MAPK) and Rho/Rho kinase (ROCK) (Lee et al., 1999; Lee et al., 2001; Liu et al., 2004). The downstream signaling stimulates contraction, proliferation, and differentiation of the smooth muscle cells of pulmonary arteries (MacLean and Dempsie, 2009) resulting in vasoconstriction and remodeling following by the development of PH. Gairhe et al. (2012) revealed that serotonin passing through

another route, myoendothelial gap junction, on PASMC membrane activates latent transforming growth factor β (TGF-β) and TGF-β synthesis. Active TGF-β combines with TGF-β receptors and triggers phosphorylate SMAD 2 signaling cascade stimulating smooth muscle cell differentiation (Figure 1). Moreover, serotonin passing through serotonin receptor 1B (5-HTR1B) and SERT in human PASMC culture, and 5-HTR2A in lung tissues of rats inhibits apoptosis of PASMCs via increasing the stimulation of phosphorylated ERK1/2 and pyruvate dehydrogenase kinase (PDK) reducing the release of apoptosis-inducing factors (Liu et al., 2013a; Liu et al., 2013b) (Figure 2). Chen et al. (2014) reported that serotonin activating 5-HTR2A in the adventitial cell culture of rat main pulmonary arteries promotes the downstream TGF-β1/Smad3 pathway to mediate proliferation and migration of adventitial fibroblast, and phenotypic alteration of adventitial fibroblast to myofibroblast (Figure 3). These changes result in adventitial fibrosis and remodeling of pulmonary arteries. Eddahibi et al. (2006) revealed that in humans with PH, overexpression of TPH-1 in PAECs caused an increase in serotonin synthesis. In addition, SERT overexpression on PASMCs in PH patients caused an excess of serotonin entering to PASMCs and induced smooth muscle cell proliferation resulting in thickening of the wall and decreasing the lumen capacity.

The objectives of treatment of PH are to decrease PAP by reducing the left atrial pressure, improving left ventricular function and inducing pulmonary arterial vasodilation (Kellihan and Stepien, 2012). Common medication for therapy of PH is selective phosphodiesterase-5 (PDE-5) inhibitors such as sildenafil, vardenafil and tadalafil. PDE-5 inhibitors promote the nitric oxide pathway to relax the muscular layer of pulmonary arteries and induce vasodilation. Moreover, PDE-5 inhibitors inhibit PDE-5 in PASMCs to stop the conversion of cyclic guanosine monophosphate (cGMP); appropriate levels of cGMP promote the signaling pathway which inhibit PASMCs hyperplasia and decrease intracellular calcium concentration resulting in smooth muscle relaxation and a decrease in PAP (Wharton et al., 2005; Hori et al., 2014). Even though PDE-5 inhibitors are an effective treatment to improve the clinical signs of PH, the discovery of the specific target therapy for inhibition of the mechanism and

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progression of PH may directly inhibit the pathogenic changes with less or without adverse effects of other organs. The previous studies in induced PH animal models and PASMC culture revealed that inhibition of 5-HTR2A (Hironaka et al., 2003; Liu et al., 2013a; Zhang et al., 2015) and SERT (Guignabert et al., 2005; Liu et al., 2013b) may inhibit and/or reverse the pathological change of PH by decreasing of downstream effector activation including ERK1/2. The studies of Liu et al. (2013a) and Liu et al. (2013b) also mentioned that inhibition of ERK1/2 may decrease proliferation and increase apoptosis of PASMCs. These effects may result in the reduction of pulmonary arterial wall thickening leading to a decrease in PAP. TPH-1 inhibition, as well as SERT, 5-HTR2A and ERK1/2 inhibition, in PAECs may reduce peripheral serotonin inducing PH (Cianchetta et al., 2010). Therefore, researching and understanding of etiology, pathogenesis, and pathophysiology of PH, such as the serotonin signaling pathway, are essential to developing the specific target therapy for PH in the future.

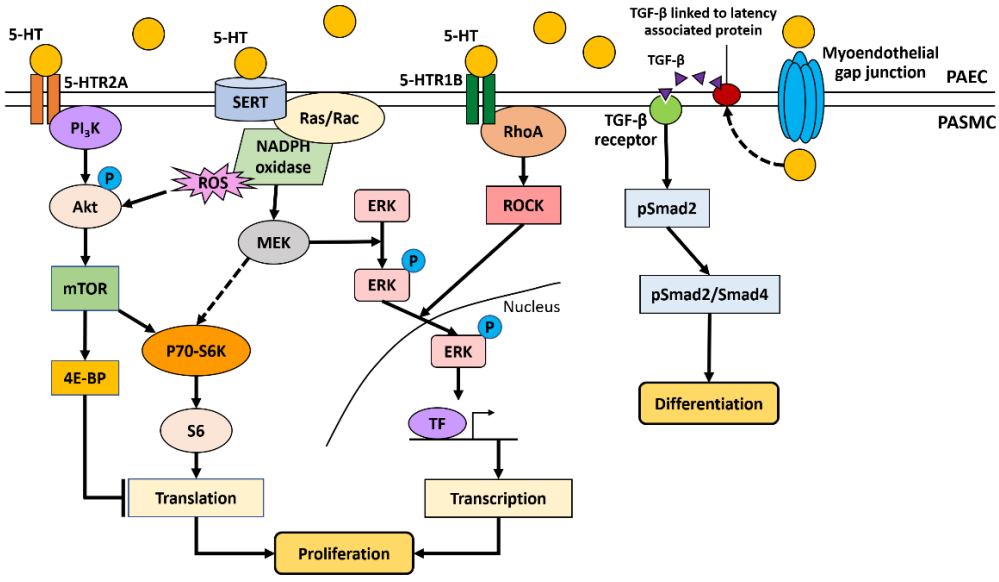

Figure 1 Diagram of serotonin signaling pathway in pulmonary artery smooth muscle cells (PASMCs) indicating the cooperation of several signaling pathway mediating the PASMC proliferation and differentiation (modified from Liu et al. (2004) and Gairhe et al. (2012))
Abbreviation: ERK1/2 = extracellular signal-regulated kinase 1/2, pERK1/2 = phosphorylated extracellular signal-regulated kinase 1/2, SERT = serotonin transporter, TF = transcription factor, 5-HT = serotonin, 5-HTR2A = serotonin receptor 2A

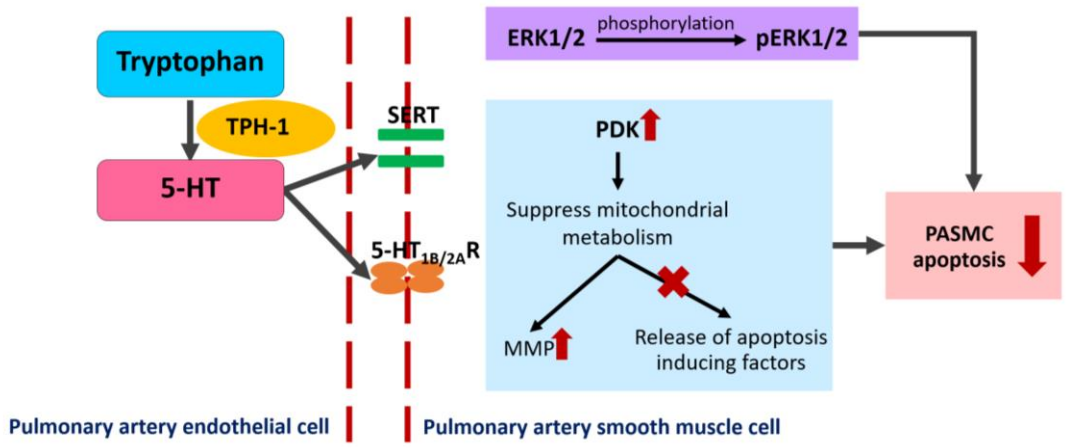

Figure 2 Diagram of serotonin signaling pathway inhibiting PASMC apoptosis via SERT and serotonin receptor (5-HTR1B in human and 5-HTR2A in animal models)

Abbreviation: ERK1/2 = extracellular signal-regulated kinase 1/2, PDK = pyruvate dehydrogenase kinase, pERK1/2 = phosphorylated extracellular signal-regulated kinase 1/2, TPH-1 = tryptophan hydroxylase-1, 5-HT = serotonin, $5\text{-HT}_{1B/2A}R$ = serotonin receptor 1B/2A

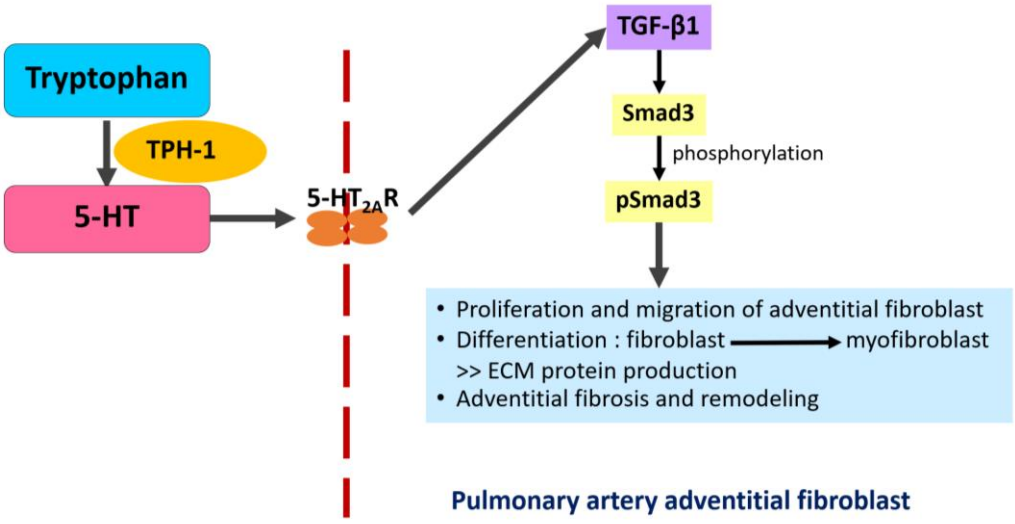

Figure 3 Diagram of serotonin signaling pathway inducing the proliferation, migration, and phenotypic alteration of adventitial fibroblast to myofibroblast

Abbreviation: Smad3 = Mothers against decapentaplegic homolog 3, pSmad3 = phosphorylated Mothers against decapentaplegic homolog 3, TGF-β1 = transforming growth factor β1, TPH-1 = tryptophan hydroxylase-1, 5-HT = serotonin, 5-HTR2A = serotonin receptor 2A

CHAPTER III
MATERIALS AND METHODS

Part I To investigate the local effect of serotonin signaling in lung tissues

The expression of the targeted genes in serotonin signaling pathway including TPH-1, serotonin receptor, SERT and the downstream effector, ERK1/2 and pERK1/2, was evaluated in lung tissues of dogs with PH due to DMVD comparing to DMVD dogs without PH and healthy dogs.

1.1. Animals

Small breed dogs aged between 7-15 years old, weight lesser than 10 kilograms were recruited in this study. The dogs were patients of Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University and Prasu Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University.

1.2. Inclusion and exclusion

The dogs enrolled to this part of the study were the patients naturally died by the causes following the inclusion criteria. Dogs were divided into 3 categories including the healthy group (as the control group), the DMVD group, and the DMVD+PH group.

1.2.1. The control group was composed of four dogs with normal cardiovascular and respiratory structure and function that naturally died by causes not involving cardiovascular and respiratory abnormalities.

1.2.2. The DMVD group was composed of five dogs naturally died with DMVD stage C or D. Dogs had no history of PH and other concurrent cardiovascular and primary respiratory diseases. The dogs with DMVD was enrolled to the study using the information in the medical records which DMVD was diagnosed following the guideline of the American College of Veterinary Medicine (ACVIM) (Keene et

al., 2019). The echocardiographic data showed mitral valve thickening and regurgitation with left atrial (LA) and ventricular (LV) enlargement indicating by the ratio of LA and aorta (LA/Ao) diameter in the right parasternal short-axis view in early diastole > 1.6, and the normalized left ventricular internal diameter at end diastole (LVIDd) > 1.7 cm/kg.

1.2.3. The DMVD+PH group was composed of five dogs naturally died by PH due to DMVD stage C or D. Dogs had to have no other concurrent cardiovascular and primary respiratory diseases which can be causes of PH. The intermediate probability of PH was identified by peak tricuspid regurgitation (TR) velocity $\leq$3 m/s with 2 anatomic sites of echocardiographic signs of PH or more than 3 m/s with or without 1 anatomic site of echocardiographic signs of PH. The high probability of PH was characterized by TR $\leq$3 m/s with 3 anatomic sites of echocardiographic signs of PH, TR 3-3.4 m/s with $\geq$2 anatomic sites, and >3.4 m/s with >1 anatomic sites of echocardiographic signs of PH (Reinero et al., 2020).

The exclusion criteria included dogs with pregnancy, infectious diseases, other systemic diseases inducing pulmonary hypertension and cardiovascular diseases (e.g. primary respiratory disease and chronic kidney disease), and other cardiovascular diseases (e.g. heartworm infestation, congenital heart diseases and other acquired heart diseases). The inclusion and exclusion criteria are concluded in Figure 4.

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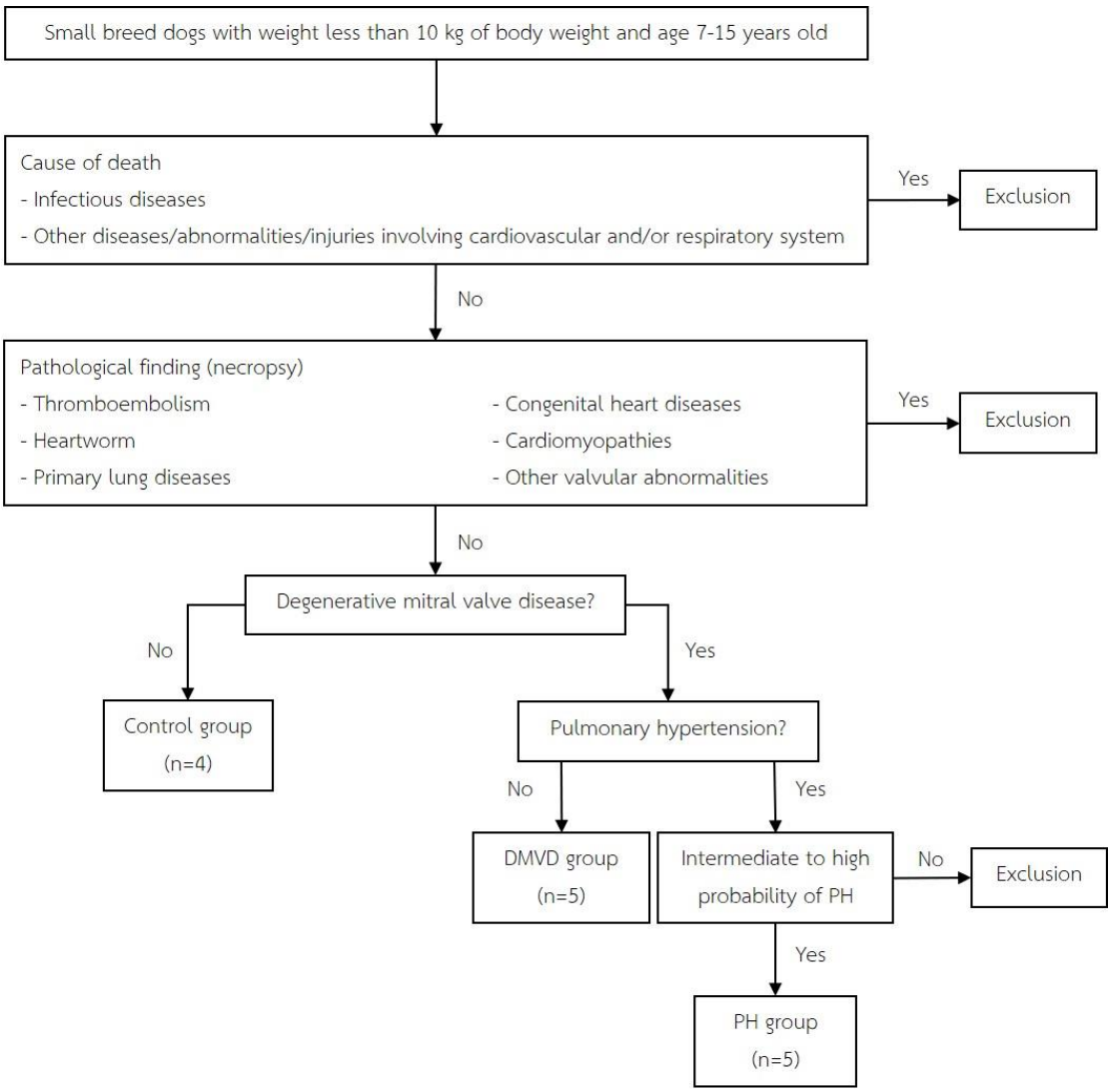

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Figure 4 Inclusion and exclusion criteria for investigating the local effect of serotonin signaling in lung and PA tissue

1.3. Lung and pulmonary artery tissues collection

Lung tissue and branched of pulmonary arteries (PA) were collected by necropsy. The samples of dogs in the control group were from 4 dogs that died without pathological lesions of cardiovascular and respiratory system. Likewise, lung and PA tissues from the DMVD group (n=5) and the PH group (n=5) were collected. The samples were stored at -80 ˚C before processing for the total RNA extraction.

1.4. Investigation of genes associated in serotonin signaling pathway by real-time reverse transcription polymerase chain reaction (qRT-PCR)

1.4.1. Total RNA extraction

Thirty mg of lung and branched PA tissues stored at -80 °C were cut into powder-liked pieces by using sterile surgical blade on dry ice to preserve the RNA in tissues. The tissues were then extracted for total RNA using NucleoSpin RNA kit (Macherey-Nagel, USA). The homogenized tissue was added by 350 µl of lysis buffer (Buffer RA1) and 3.5 µl of β-mercaptoethanol, and vigorously mixed with a Vortex mixer. The lysate was then transferred to filtrate through the filter column and centrifuged for 1 minute at 11000 g. The filter was then discarded. The homogenized lysate was added by 350 µl of 70% ethanol and mixed by pipetting up and down. The prepared lysate was then loaded to the RNA-binding column and centrifuged for 30 seconds at 11000 g. The RNA was bound to the silica membrane of the column. The collecting tube was discarded and placed with the new one. To desalt the silica membrane, 350 µl of membrane desalting buffer was added and centrifuged for 1 minute at 11000 g. The contaminated genomic DNA was then digested. The DNase reaction mixture was prepared by mixing 10 µl of reconstituted rDNase to 90 µl of reaction buffer for rDNase. Ninety-five µl of DNase reaction mixture was applied onto the silica membrane of the column and incubated for 15 minutes at room temperature. After incubation, the column was added with 200 µl of the first wash buffer (Buffer RAW2) and centrifuged for 30 seconds at 11000 g. After that, the column was moved to a new collection tube. The second wash buffer (Buffer RA3) (600 µl) was applied to the column and centrifuged for 30 seconds at 11000 g. The flowthrough in collection tube was discarded. The membrane was then washed by 250 µl of Buffer RA3 and centrifuged for 2 minutes at 11000 g to dry the membrane completely. Finally, the RNA was eluted by adding 60 µl of RNase-free water and centrifuged for 1 minutes at 11000 g. The total RNA concentration was quantified and qualified by NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, USA).

1.4.2. DNase treatment

DNase treatment by TURBO DNA-*free*™ Kit (Invitrogen, USA) was done to remove the remaining DNA contamination from RNA preparation. Sixty µl of the RNA was added with 6 µl of 10X TURBO DNase Buffer and 1 µl of TURBO DNase and mixed gently. The RNA was incubated at 37 °C for 20-30 minutes. After incubation, 6 µl of DNase Inactivation Reagent was added and incubated at room temperature for 5 minutes with mixing 2-3 times during the incubation period. The treated RNA was then centrifuged at 10000 g for 1 minute 30 seconds. The supernatant was transferred to a new tube and measured for the RNA concentration.

1.4.3. Reverse transcription (RT)

The treated RNA was converted into complementary DNA (cDNA) by using the Omniscript RT kit (Qiagen, Germany). In brief, the total volume of cDNA was 20 µl which divided into 12 µl of 100 ng RNA and 8 µl of the master mix. Both RNA and all master mix solutions were thawed in ice. The 8-µl master mix which composed of 10X Buffer RT (2 µl), dNTP Mix (2 µl), random primers (1 µl) (Random primers; Promega, USA), RNase inhibitor (1 µl) (RNaseOUT™ Recombinant Ribonuclease Inhibitor; Invitrogen™, USA), reverse transcriptase (1 µl) and RNase free water (1 µl) was prepared. All RNA samples were diluted with nuclease-free water for the similar concentration of 5 ng/µl at total reaction volume of 20 µl. The diluted RNA was incubated at 65 °C for 5 minutes to denature the secondary structure. The template RNA (12 µl) was then added with the prepared master mix (8 µl), mixed carefully, and incubated at 37 °C for 60 minutes. The obtained cDNA was finally stored at -20 °C.

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1.4.4. Real-time polymerase chain reaction (qPCR)

To investigate the change in regulation of the genes associating in serotonin signaling pathway, qPCR was performed to assess the relative quantitation of *tph1*, *slc6a4* and *htr2a* expression.

The primers of *tph1*, *slc6a4* and *htr2a* were designed by using the online program, Primer 3 Plus, which are shown in Table 4. The internal reference (housekeeping) genes for normalization of the gene expression were chosen from the most stable genes in the previous studies. The reference gene for tissue was ribosomal protein L32 (*RPL32*) (Peters et al., 2007) and for PA tissue was ribosomal protein S19 (*RPS19*) (Tangmahakul et al., 2019).

The conventional PCR and gel electrophoresis was performed to replicate and identify. The conventional PCR condition included initial denaturation at 95 °C for 5 minutes followed by 35 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute and final extension at 72 °C for 5 minutes.

The specific PCR products of *tph1*, *slc6a4* and *htr2a* were separated on 1.5% agarose gel and visualized the specific band by ethidium bromide. The 100 bp DNA ladder (ONE MARK 100 DNA ladder, Bio-Helix, Taiwan) was used for band-size estimation. The specific bands of PCR products were cut off the gel and weighed. The bands were purified by Wizard® SV Gel and PCR Clean-up System (Promega, USA). In brief, the cut bands were added by the membrane binding solution with 10 µl of the solution/10 mg of gel slice and mixed by a Vortex mixer. The gel slices were incubated at 50-65 °C for 10 minutes and mixed with a Vortex mixer a few minutes to promote the dissolution. The dissolved gels were briefly centrifuged at room temperature and then transferred to the membrane column. After adding the dissolved gel, the column was incubated for 1 minute at room temperature, then centrifuged at 16000 g for 1 minute. The flowthrough was discarded. The column was added by 700 µl of membrane wash solution and

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centrifuged at 16000 g for 1 minute. Then, the membrane column was secondly washed by adding 500 µl of membrane wash solution and centrifuged at 16000 g for 5 minutes. The purified PCR products were eluted by applying 50 µl of nuclease-free water on the membrane, incubating at room temperature for 1 minute and centrifuging at 16000 g for 1 minute. The purified PCR products were then sequenced by Sanger sequencing (Macrogen, Korea) to confirm the specific PCR product sequences of the target genes. After confirming of the PCR product, all primers were used to analyze the targeted gene expression by relative qRT-PCR. The qRT-PCR was operated in Roter-Gene Q (Qiagen, Germany). The reaction components included 10 µl of SYBR green dye (KAPA SYBR Green Fast qPCR kit Mastermix (2X) Universal, Kapabiosystems, USA), forward and reverse primers (5 µM of primers for targeted genes and 10 µM of primers for reference genes), cDNA template, and PCR-grade water up to 20 µl. The thermal cycling conditions of all genes were present in Table 5. The melting curves were analyzed at the temperature between 65 °C-95 °C with increasing rate of 1 °C per second. The PCR efficiency was analyzed by plotting the standard curve of 2-fold serial dilution of the standard (20 to 1.25 ng/µl). The acceptable PCR efficiency is between 80 and 110% (Rocha et al., 2016). The qRT-PCR were done in triplicate. The relative quantitation of gene expression was measured by the comparative C_T method ($\Delta\Delta C_T$ method) from the formula showed below (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

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$$\Delta C_T = C_{T\ \text{target gene}} - C_{T\ \text{reference gene}}$$

$$\Delta\Delta C_T = \Delta C_{T\ \text{disease group}} - \Delta C_{T\ \text{control group}}$$

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

Table 4 Primers for polymerase chain reaction of genes associating serotonin signaling

Genes	Accession no.	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)	Tm (°C)	Reference
tph1	NM_001197191.1	CTGTGGAGTTTGGTCTCTGTAAG	TGTGATGAGACACTCCTGTTTG	158	82.77	This study
slc6a4	NM_001110771.1	GGCTGAGATGAGGAACGAAG	TTGGACCAGATGTGTGGAAA	222	84.43	This study
htr2a	NM_001005869.1	TCTTTCAGCTTCCTCCCTCA	TCCTCGTTGCAGGACTCTTT	227	84.70	This study
RPS19	XM_005616513.3	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	95	80.69	(Brinkhof et al., 2006)
RPL32	XM_022406256.1	TGGTTACAGGAGCAACAAGAAA	GCACATCAGCAGCACTTCA	100	81.54	(Peters et al., 2007)

Table 5 The qRT-PCR conditions of the targeted and reference genes

	Cycles	Reference genes		*tph1*		*slc6a4*		*htr2a*	
		Temperature	Duration	Temperature	Duration	Temperature	Duration	Temperature	Duration
Initial denaturation	1	95°C	5 min	95°C	3 min	95°C	3 min	95°C	3 min
Denaturation	40	95°C	3 sec	95°C	3 sec	95°C	3 sec	95°C	3 sec
Annealing		60°C	25 sec	63°C	25 sec	63°C	25 sec	63°C	30 sec
Extension				72°C	30 sec	72°C	30 sec	72°C	30 sec

1.5. Investigation of the downstream effectors of serotonin signaling pathway by Western blot

The samples of lungs and branched pulmonary artery tissues stored at -80 °C were powdered by using mortar and pestle with liquid nitrogen. The powdered tissues were lysed and homogenized with 1 ml/300 mg tissue of cold RIPA buffer (consisting of 25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) (Pierce® RIPA Lysis and Extraction Buffer, Thermo Fisher Scientific, USA) added by protease inhibitors (composing of 1 mM AEBSF, 800 nM aprotinin, 50 µM bestatin, 15 µM E64, 20 µM leupeptin and 10 µM pepstatin A) (Halt™ Protease Inhibitor Cocktail, EDTA Free, Thermo Fisher Scientific, USA) with 1/100 volume of total volume and phosphatase inhibitors (1 mM Sodium orthovanadate, Sigma-Aldrich, Germany). The lysate was then centrifuged at 15,000 g for 30 minutes at 4°C. The supernatant was collected and stored at -80°C until processing.

To determine the protein concentration, the supernatants were quantified by Bradford protein assay using colorimetric assay dye (composing of 50-100% phosphoric acid and 10-20% methanol) (Bio-Rad Protein Assay Dye Reagent concentrate, Bio-Rad®, USA). The protein assay dye was filtrated by filter papers with 90 mm of pore diameter. The filtrated dye was diluting by distilled water in the ratio of dye: water = 1:4. The standard curve of protein concentration was plotted by using bovine serum albumin (BSA) (Albumin, Bovine Serum, Low Heavy Metals PROTEIN, Merck, Germany). The standard BSA was prepared by serial dilution at the concentration of 2.0, 1.5, 1.0, 0.75, 0.5, 0.25, 0.125 and 0 µg/µl as blank. The protein concentration was measured in duplicate. Twenty µl of BSA standards and samples were transferred to the new tube and mixed by 1 ml of the diluted protein assay dye. The optical density (OD) was measured by a spectrophotometer at 595 nm. The standard curve was plotted using OD as y-axis against the protein concentration as x-axis in the linear equation in Microsoft Excel program (Microsoft, USA). The protein concentrations of the samples were calculated following the standard curve. The protein samples were diluted by bidistilled water and mixed by the sodium dodecyl sulfate (SDS) sample buffer

(consisting of 65.8 mM Tris HCl pH 6.8, 26.3% (w/v) glycerol, 2.1% SDS and 0.01% bromophenol blue) (2x Laemmli Sample Buffer, Bio-Rad®, USA) and β-mercaptoethanol. Preparation of the samples used 1 part of the diluted protein sample with 1 part of the SDS sample buffer. Beta-mercaptoethanol was added with 1/10 volume of the total volume. The protein samples were heated at 95°C for 5 minutes except the samples for investigation of SERT were heated at 85°C for 2 minutes.

The prepared protein samples contained 50 µg of total protein per well of SDS/polyacrylamide gel. The protein samples were separated by running on 10% SDS/polyacrylamide gel using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separating by molecular weight, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane (Immun-blot PVDF membrane, Bio-Rad®, USA) by wet transfer (Mini Trans-Blot Electrophoresis Transfer Cell, Bio-Rad®, USA). Then, the proteins on the membrane were blocked of non-specific protein binding to decrease the background in the final products and eliminate the false positives by applying TBS-T buffer (consisting of 40 mM Tris HCl pH 7.7, 200 mM NaCl and 0.1% Tween 20) containing 2.5% BSA for 30 minutes for SERT and 1 hour for 5-HTR2A and α-Tubulin, and 5% BSA for 1 hour for TPH-1, ERK1/2 and pERK1/2. The membranes were then washed three times with TBS-T. The membranes were incubated overnight at 4°C with primary monoclonal antibodies diluted with TBS-T containing 1% non-fat milk including 1:500 mouse anti-TPH-1 antibody (Monoclonal Anti-Tryptophan Hydroxylase antibody produced in mouse clone WH-3, ascites fluid (T0678), Sigma-Aldrich, Germany), 1:1000 mouse anti-SERT antibody (SERT Mouse Monoclonal (AB-N40), Advanced Target Solutions, USA), 1:1000 mouse anti-5-HTR2A antibody (Anti-SR-2A Antibody (A-4): sc-166775, Santa Cruz Biotechnology, USA), 1:1000 rabbit-anti ERK1/2 antibody (Phospho Plus p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody Duet (No. 4370S + 4695S), Cell Signaling Technology, USA), and 1:1000 mouse anti-α-Tubulin antibody (alpha Tubulin Monoclonal Antibody (DM1A), Invitrogen™, USA). Detection of pERK1/2 expression, the membranes were incubated overnight at

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4°C with 1:1000 rabbit-anti pERK1/2 monoclonal antibody (Phospho Plus p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody Duet (No. 4370S + 4695S), Cell Signaling Technology, USA) diluted with TBS-T containing 1% BSA. After incubation, the membranes were washed three times with TBS-T and incubated with horseradish peroxidase conjugated anti-rabbit/mouse secondary antibody (EnVision Detection Systems, Peroxidase/DAB, Rabbit/Mouse, Agilent Technologies, USA) diluted by TBS-T buffer with EnVision : TBS-T of 1:3. The duration of secondary antibody incubation for detection of TPH-1, SERT, ERK1/2 and pERK1/2 was 1 hour and those of 5-HTR2A and **α**-Tubulin was 2 hours. The membranes were then washed three times by TBS-T and incubated with 3,3'-Diaminobenzidine (DAB) diluted with its buffer in ratio of 1:100. The incubation duration for TPH-1, 5-HTR2A, ERK1/2 and pERK1/2 detection were 2 minutes and those for SERT and **α**-Tubulin were 4 minutes. The reaction of DAB was stopped by soaking the membrane in bidistilled water. Finally, the membranes were captured as pictures and the intensity of the targeted and reference protein bands were quantified by ImageJ software (ImageJ bundled with 64-bit Java 1.8.0_172, NIH, USA). The Western blot procedures of all protein detections are summarized in Table 6.

1.6. Statistical analyses

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The statistical analyses of gene expression from qPCR were executed by the the comparative C_T method (**ΔΔ**C_T method) (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The C_T values of *tph1*, *slc6a4*, *htr2a*, *RPS19*, and *RPL32* of all groups were examined for fold changes of the disease groups comparing to the control group and reported as median (interquartile range). The statistical difference among the groups was analyzed by Kruskal-Wallis test with Dunn's post-hoc test in the computer-based program, SPSS version 22 (IBM, USA). The significant difference at $p<0.05$ was considered.

To analyze the protein expression, the intensity of the targeted protein bands was normalized by the intensity of the reference protein bands, **α**-tubulin. The

quantified intensity of the targeted and reference protein bands from Western blot were reported as mean ± SEM of the protein band intensity. The normalized targeted protein band intensity of the disease groups was compared to those of the control group using one-way ANOVA following with Bonferroni post hoc test in the computer-based program, SPSS version 22 (IBM, USA). A $p<0.05$ was considered as the significant difference. The experimental design for investigating the local effect of serotonin signaling in lung tissues is concluded in Figure 5.

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Table 6 Summary of the Western blot procedure

Step	TPH-1	SERT	5-HTR2A	ERK1/2	pERK1/2	αTubulin
1. Tissue lysis and protein extraction	RIPA lysis buffer + Halt™ Protease inhibitor cocktail + Na_3VO_4 Centrifuge 15,000 g, 4°C 30 min					
2. Total protein amount/well	50 µg					
- protein preparation	95°C, 5 min	85°C, 2 min	95°C, 5 min			
3. Gel electrophoresis	180 Volts, 1 hour (Bio-Rad)					
% separating gel	10%					
4. Transfer-blotting method	Wet transfer 0.25 A, 3 hours (on ice)					
5. Blocking						
- Blocking agent	5% BSA	2.5% BSA	2.5% BSA	5% BSA	5% BSA	2.5% BSA
- Duration	1 hour	30 min	1 hour	1 hour	1 hour	1 hour
- Washing with TBS-T	10 min x 3	10 min x 3	10 min x 3	10 min x 3	5 min x 1	10 min x 3
6. Primary antibody						
- Dilution	1:500	1:1000	1:1000	1:1000	1:1000	1:1000
- Diluent (diluted with TBS-T)	1% non-fat milk	1% non-fat milk	1% non-fat milk	1% non-fat milk	1% BSA	1% non-fat milk
- Incubation period	4°C overnight					
- Washing with TBS-T	10 min x 3					
7. Secondary antibody						
- Dilution	EnVision 1:3 (diluted with TBS-T)					
- Incubation period	1 hour	1 hour	2 hours	1 hour	1 hour	2 hours
- Washing with TBS-T	10 min x 3					
8. Detection						
- Dilution	DAB 1:100					
- Duration	2 min	4 min	2 min	2 min	2 min	4 min

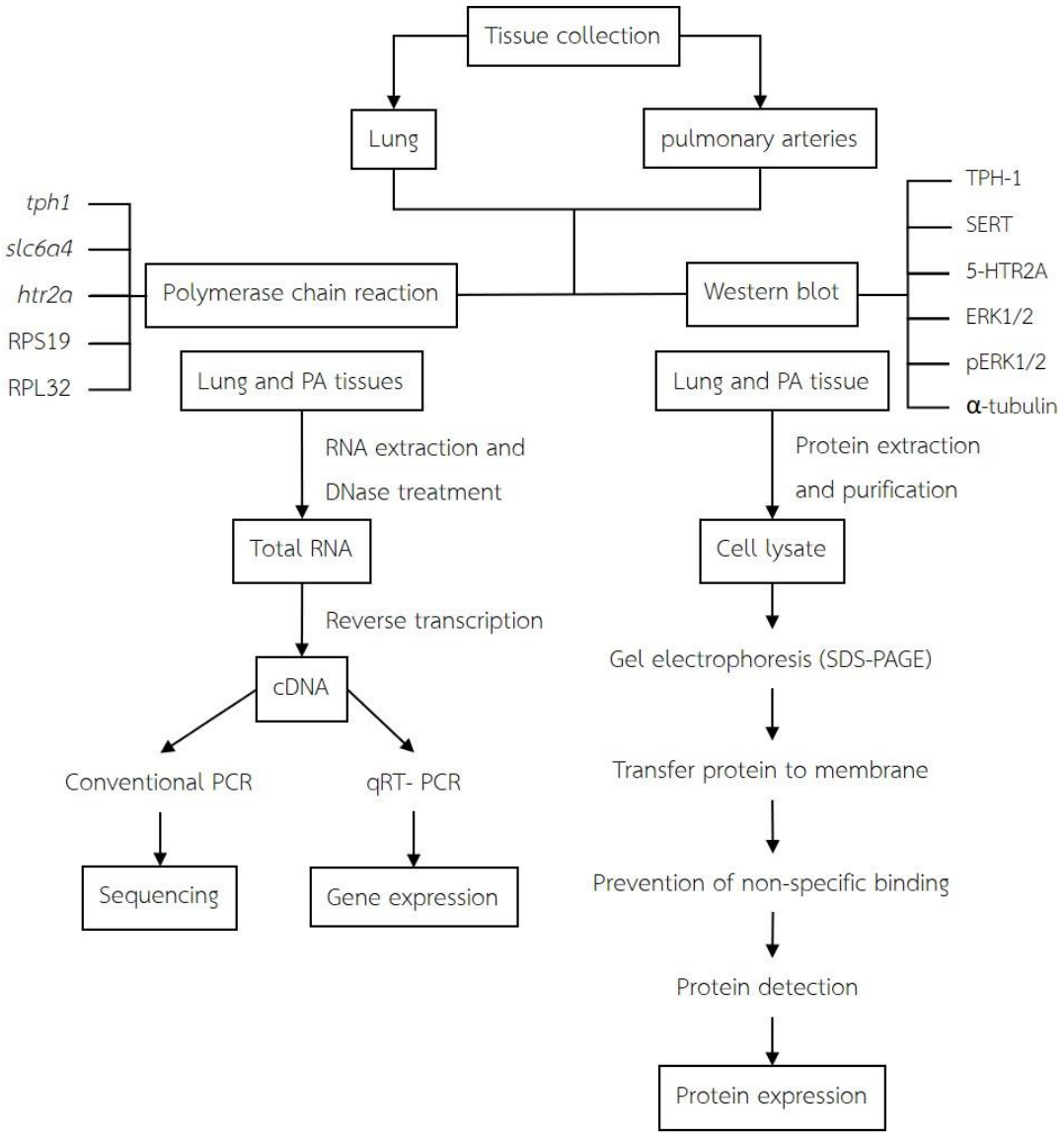

Figure 5 Experimental design for investigating the local effect of serotonin signaling in lung and PA tissue

Part II To assess the source of serotonin in blood circulation

The serotonin concentration in circulating platelet and plasma was measured to reveal the source of serotonin dogs affected with PH secondary to DMVD compared to DMVD and healthy dogs.

2.1. Animals

The methods used in the present study was approved by the Institutional Animal Care and Committee, Faculty of Veterinary science, Chulalongkorn University (number 1831053). Small breed dogs, aged between 7-15 years old and weight lesser than 10 kilograms, were included in this study. All dogs were patients of Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University.

2.2. Inclusion and exclusion

The history taking and clinical evaluation were operated for enrollment dogs to the study. The history of home care and environment, feeding, annual vaccination and parasite prevention of all dogs were recorded. Physical examinations including observation for hydration status, mucous membrane, measurement of vital signs (temperature, capillary refill time, heart rate, respiratory rate, and blood pressure), and heart and lung auscultation were evaluated. One ml of blood was collected for analysis of routine complete blood counts (red blood cell, white blood cell and platelet count and morphology), blood parasite, blood chemical profiles (serum glutamate-pyruvate transaminase, alkaline phosphatase, blood urea nitrogen, creatinine, total protein, and albumin), and results of a rapid test kit for blood parasite and heartworm antigen detection (SNAP i.d. ® 2.0 Protein Detection System, Merck Millipore, USA). The mean platelet volume (MPV) was also measured. Thoracic radiographic examination was assessed for evaluating hemodynamic changes. Electrocardiography was evaluated for detecting of cardiac arrhythmia. Echocardiography was assessed for evaluating of the cardiac structure and function as

well as the surrounding vasculature for diagnosis and staging the severity of DMVD and PH.

The inclusion criteria following clinical evaluation, radiographic and echocardiographic finding were used to divide the enrolled dogs into 3 categories.

2.2.1 The control group was composed of 22 healthy dogs with normal cardiovascular and respiratory structure and function without an evidence of internal organ abnormalities revealed by clinical evaluation, radiographic and echocardiographic findings.

2.2.2 The DMVD group was composed of 20 dogs that have DMVD stage C without PH and other concurrent cardiovascular and primary respiratory diseases confirmed by physical examination, radiography, and echocardiography. The DMVD stage C was diagnosed following the guideline of ACVIM (Keene et al., 2019). The clinical signs of dogs with DMVD stage C including cough, tachypnea, weight loss and respiratory distress were found with murmur at the left apical area on thoracic auscultation. The radiographic finding presented the left-sided heart failure or cardiogenic pulmonary edema. A 4-12 MHz phased array transducer-ultrasound machine (M9, Mindray, China) was used for echocardiography by an experienced cardiologist. The echocardiographic findings of canine DMVD stage C revealed mitral valve thickening and regurgitation. The LA and LV were enlarged which were characterized by LA/Ao in the right parasternal short-axis view in early diastole $\geq$ 1.6, and the normalized LVIDd $\geq$ 1.7 cm/kg.

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2.2.3 The DMVD+PH group was composed of 20 dogs with DMVD stage C and intermediate to high probability of PH caused by DMVD. All dogs in this group did not have other concurrent cardiovascular and primary respiratory diseases which can induce PH. The intermediate probability of PH was identified by the peak TR velocity $\leq$ 3 m/s with 2 anatomic sites of echocardiographic signs of PH or more than 3 m/s with or without 1 anatomic site of echocardiographic signs of PH. The high probability of PH was characterized by TR $\leq$3 m/s with 3 anatomic

sites of echocardiographic signs of PH, TR 3-3.4 m/s with ≥2 anatomic sites, and >3.4 m/s with ≥1 anatomic sites of echocardiographic signs of PH.

The exclusion criteria included dogs with pregnancy, infectious diseases, other systemic diseases inducing pulmonary hypertension and cardiovascular diseases (e.g. primary respiratory disease, and chronic kidney disease), and other cardiovascular diseases (e.g. heartworm infestation, congenital heart diseases and other acquired heart diseases). The inclusion and exclusion criteria are concluded in Figure 6.

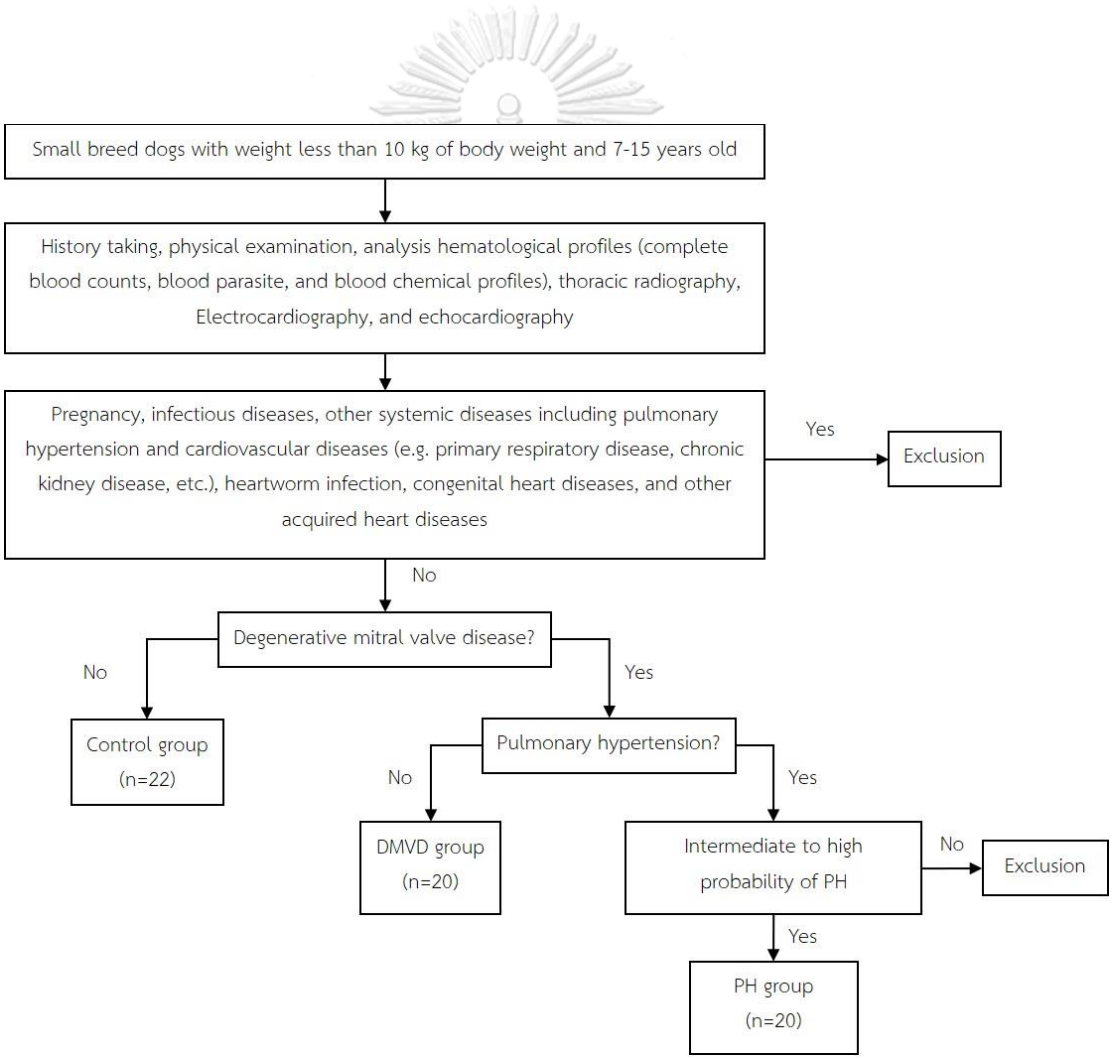

Figure 6 Inclusion criteria for investigating the source of serotonin in blood circulation

2.3. Blood sample collection and preparation for measurement of plasma and platelet serotonin concentration

The feeding history of all dogs was considered. Dogs fed with food composing of serotonin, such as tomatoes, pineapple, bananas, plums and kiwi fruits, were rejected (Feldman and Lee, 1985). All enrolled dogs were fasted for at least 8 hours and collected 5 ml of blood on the day of clinical examination. Direct venipuncture was done from cephalic or lateral saphenous vein with a 21-gauge needle. The blood samples were then transferred to 5-ml evacuated EDTA-containing tubes. One ml of blood samples was divided for rapid test of blood parasite and heart worm antigen and sent to the hematology unit to analyze the routine hematology and blood chemistry profile. The remaining four ml of blood samples was then processed for measurement of platelet and plasma serotonin concentration. The whole blood was centrifuged at 200 g for 10 minutes to separate platelet-rich plasma (PRP) which was the supernatant. To prepare platelet sample, 200 µl of PRP was transferred to the new tube with 800 µl of physiological saline. Then, the diluted PRP was centrifuged at 4500 g for 10 minutes at 4°C and the supernatant was discarded. The sediments were added with 200 µl of distilled water and mixed on a Vortex mixer. The platelet samples were stored at -20°C until assay. To analyze the platelet serotonin concentration, the frozen platelet samples were thawed and centrifuged at 10000 g for 2 minutes and collected the supernatant for measurement. The remaining PRP samples were centrifuged at 1500 g for 20 minutes and aspirated the supernatant which was platelet-poor plasma (PPP) for measurement of plasma serotonin concentration. The PPP samples were then stored at -20°C until assay. The procedures of blood sample preparation are concluded in Figure 7 (Mangklabruks and Surachetpong, 2014).

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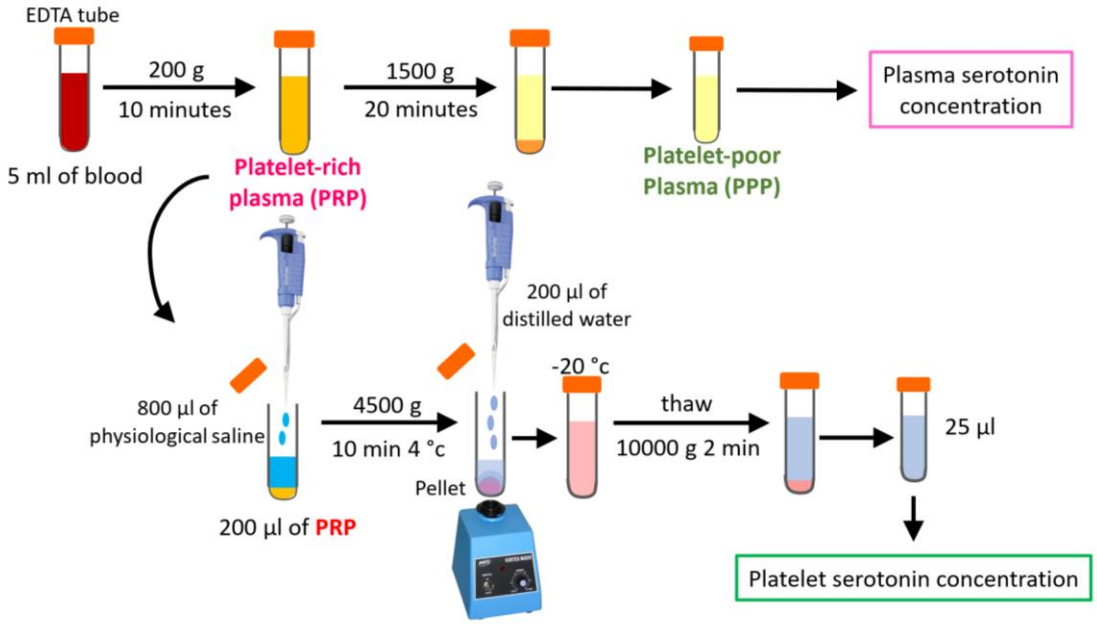

Figure 7 Sample preparation for investigating the source of serotonin in blood circulation

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2.4. Serotonin concentration measurement

The competitive enzyme-linked immunosorbent assay (ELISA) was used to evaluate platelet and plasma serotonin concentration. The Serotonin ELISA (IBL International GMBH, Germany) test and the Serotonin High Sensitive ELISA test (IBL International GMBH, Germany) were used for analysis of platelet and plasma serotonin concentrations, respectively.

2.4.1. Platelet serotonin concentration measurement

2.4.1.1. Sample preparation

The materials supplied by the kit were prepared by following the instructions before starting the sample preparation. In brief, the assay buffer (15 ml) and wash buffer (15 ml) were diluted by adding bidistilled water (135 ml and

285 ml, respectively). The diluted assay buffer and wash buffer were stored at 2-8°C for 2 and 4 weeks, respectively. The lyophilized controls of the kit were added with 0.5 ml of bidistilled water, and standed for 15 minutes and mixed gently to avoid foaming formation. The enzyme conjugate (60 µl) was diluted by 6 ml of the diluted assay buffer. The prepared controls were aliquoted and stored at ≤20°C until expired date.

Acylation was performed to prepare the samples for ELISA. All platelet samples and the assay controls (20 µl) were thawed and diluted with the diluted assay buffer (100 µl) in glass tubes and mixed on a Vortex mixer. Acylation reagent (25 µl) were added to each tube of the samples and assay controls, and immediately mixed by a Vortex mixer. All tubes were then covered and incubated for 15 minutes at 37°C in a water bath. After that, 2 ml of the diluted assay buffer was added to the glass tubes and mixed. All samples and assay controls were centrifuged at 1500 g for 10 minutes. The supernatant was then transferred to the microtiter plate. The sample preparation provided 107-fold dilution of the platelet samples.

2.4.1.2. ELISA procedure

The assay standards, acylated controls and acylated samples (50 µl) were pipetted into each well of the microtiter plate in duplicate following the pipetting scheme. The serotonin biotin (50 µl) was added to each well. The serotonin antiserum (50 µl) was then pipetted to each well. The plate was covered by adhesive foil, shaken carefully and incubated for 16-20 hours at 2-8°C. After incubation, the incubation solution in the wells was discarded and washed with diluted wash buffer (250 µl) for 3 times. Then, 150 µl of fresh-prepared enzyme conjugate was pipetted into each well and incubated for 60 minutes at room temperature (18-25°C) on the orbital shaker (500 rpm). After that, the incubation solution in each well was discarded and washed with diluted wash buffer (250 µl) for 3 times. Two hundred microliters of PNPP substrate solution were added into each well and the plate was incubated for 30 minutes at room temperature on

the orbital shaker (500 rpm). The PNPP stop solution (50 µl) was then pipetted into each well to stop the reaction of the substrate. The microtiter plate was gently mixed. The optical density (OD) of the reaction was measured by a photometer (Epoch 2 microplate reader, BioTex, USA) at 405 nm within 60 minutes after pipetting the stop solution.

2.4.1.3. Calculation of the results

The OD of the standards were plotted against their concentration by using the linear OD as y-axis and logarithmic concentration as x-axis. The automate method using to plot the standard curve and calculate the concentration was 4 Parameter Logistics. The equation used for calculation are shown below.

$$y = d + \frac{a-d}{1+\left(\frac{x}{c}\right)^{b}}$$

Then

$$x = c\left(\frac{a-d}{y-d} - 1\right)^{\frac{1}{b}}$$

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y = OD of the reaction

x = the concentration of platelet serotonin (ng/ml)

a = the minimum value that can be acquired by this assay

d = the maximum value that can be acquired by this assay

c = the point on the halfway of the curve between a and d

b = the slope factor (Hill slope)

The concentrations obtained from the equation were in ng/ml. As a result of sample dilution in sample preparation step, all platelet concentrations calculated from this equation were multiplied by 107. The assay suggested that the serotonin concentration was referred to 10^9 platelets, therefore, the platelet count was used for calculation the platelet serotonin concentration in ng/10^9 platelets. In brief, the equation for this calculation is

$$y = \left(\frac{x}{a}\right)(10)^6$$

y = platelet serotonin concentration (ng/10^9 platelets)

x = calculated serotonin concentration in ng/ml

a = platelet count (platelets/µl)

2.4.2. Plasma serotonin concentration measurement

2.4.2.1. Sample preparation

The materials supplied by the kit were prepared by following the instructions before starting the sample preparation. In brief, the assay buffer (50 ml) was added with 0.1% ascorbic acid (0.5 ml) to prevent the degradation of serotonin during the process. The prepared assay buffer was aliquoted and stored at -20°C until the expired date. The standards were diluted by the prepared assay buffer following Table 7.

Table 7 Standard dilution for the Serotonin High Sensitive ELISA test

Standard	Concentration (ng/ml)	Assay buffer volume (µl)	Standard volume
Std 6	5	990	10 µl of concentrated standard (500 ng/ml)
Std 5	1	800	200 µl of Std 6
Std 4	0.335	933	67 µl of Std 6
Std 3	0.1	980	20 µl of Std 6
Std 2	0.0335	993	6.7 µl of Std 6
Std 1	0	1000	-

The controls (10 µl) were diluted by 5 ml of the prepared assay buffer. Lyophilized acylation buffer was added by 4 ml of distilled water and added with 200 µl of concentrated acylation buffer, then mixed gently on a roll mixer for 30 minutes. The prepared acylation buffer was stored at -20°C until the expired date. The acylation reagent was prepared immediately before use by dissolving the supplied lyophilized acylation reagent with 2.5 ml of the solvent and shaking for 5 minutes on the orbital shaker. The prepared acylation reagent was discarded immediately after use. The wash buffer concentrate (25X) was diluted by 500 ml of distilled water. The diluted wash buffer was stored at 2-8°C up to 4 weeks. Acylation of the standards, assay controls and samples were done on the supplied acylation plate before the ELISA testing. The prepared acylation buffer (25 µl) was pipetted into each well of the acylation plate. Then, the standards, controls and samples (20 µl) were added into each well and mixed for 10 seconds. After that, the prepared acylation reagent (10 µl) was added into each well and the plate immediately incubated for 60 minutes on orbital shaker with avoiding the direct sunlight approach. After incubation, the deactivator (25 µl) was added. The acylation plate was covered with adhesive foil and incubated for 3 hours at room temperature on an orbital shaker avoiding the direct sunlight approach. After finishing incubation with the deactivator, 50 µl of the acylated standards, assay

controls and samples were pipetted and added into the microtiter plate for ELISA. The sample preparation provided 4-fold dilution of PPP samples.

2.4.2.2. ELISA procedure

The acylated standards, assay controls and samples were added into each well of the microtiter plate in duplicate following the pipetting scheme. The plate was then covered by adhesive foil and incubate for 15-20 hours at 2-8°C. After incubation, the incubation solution in each well was discarded and washed with diluted wash buffer (250 µl) for 4 times. The enzyme conjugate (100 µl) was then added into each well. The plate was covered and incubated for 60 minutes at room temperature on an orbital shaker (500 rpm). After that, the incubation solution was discarded, and the plate was washed by the diluted wash buffer (250 µl) for 4 times. The TMB substrate solution (100 µl) was pipetted into each well and incubated for 30 minutes at room temperature on the orbital shaker (500 rpm). The TMB stop solution (100 µl) was added and briefly mixed to each well to stop the reaction. The OD of the reaction in each well were measured by a spectrophotometer (Epoch 2 microplate reader, BioTex, USA) at 450 nm within 15 minutes after adding the stop solution.

2.4.2.3. Calculation of the results

The plasma serotonin concentration was analyzed by the similar method as the platelet serotonin concentration and using the similar equation to calculate the serotonin concentration in ng/ml. The concentration was multiplied by 4.

2.4.3. In-house validation of the assay

Intra-assay precision, inter-assay precision and spike-recovery assessment were analyzed to validate the ELISA kits. Intra-assay precision was analyzed by calculation for coefficient of variability (CV) from duplicate mean and standard deviation of the samples and reported as %CV. The acceptable intra-assay precision was less than 10%. Inter-assay precision was assessed by calculation for %CV of the similar set of samples between ELISA plates. The acceptable inter-

assay precision was less than 15%. Spike-recovery was calculated from the sets of a known-amount analyte in different concentrations. The spike-recovery was calculated from the observed concentration dividing by the expected concentration and converted to percentage of recovery. The acceptable spike-recovery was between 80-120%.

2.5. Statistical analyses

The statistical analyses were executed by the computer-based program, SPSS version 22 (IBM, USA). The continuous data were analyzed for the data distribution using Shapiro-Wilk test. The continuous data among 3 groups with normal distribution including vital sign measurement and VHS were analyzed by one-way ANOVA. The homogeneity of variance of data was tested by Levene's test. The data with equal variance was then analyzed with the Bonferroni post-hoc test whereas the data with unequal variance was then analyzed with Tamhane test. The data were presented as mean $\pm$ standard deviation (SD). The continuous data among 3 groups with non-normal distribution including the plasma and platelet serotonin concentrations, age, body weight, hematology and blood chemistry profiles, and echocardiographic data were analyzed by the Kruskal-Wallis test and Dunn's post hoc test. Comparisons between 2 groups of those variables were analyzed by the Mann-Whitney U test. The data were shown as median and interquartile range (IQR). The correlations between the circulating serotonin concentrations and other variables including body weight, age, PAP, platelet count, and MPV were investigated by the Spearman correlation. The association between the circulating serotonin concentrations and gender and breed of dogs were analyzed by the multiple regression analysis. A $p<0.05$ was considered as the significant difference of all tests.

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CHAPTER IV
RESULTS

Part I To investigate the local effect of serotonin signaling in lung tissues

Fourteen dogs were enrolled to the study and categorized into 3 groups, the control (n=4), DMVD (n=5) and DMVD+PH (n=5) groups. The control group composed of 3 males (2 intact males and 1 castrated male) and 1 sterile female. Breeds of dogs included 2 Shih Tzu, 1 Cocker and 1 mixed breed dogs. The DMVD group consisted of 1 male and 4 females (1 intact female and 3 sterile females). Breeds of dogs were 3 Poodles, 1 Shih Tzu and 1 Pomeranian. The DMVD+PH group comprised 3 males (2 intact males and 1 castrated male) and 2 females (1 intact female and 1 sterile female). Breed of dogs included 2 Chihuahuas, 2 Poodles and 1 Pomeranian. The clinical diagnosis and pathologic finding on necropsy revealed the causes of death of dogs. In the control group, the causes of death were acquired porto-systemic shunt due to post-operation of peritoneal-pericardial diaphragmatic hernia (1), acquire hydrocephalus (1), and cancers (2) (gastric signet ring cell carcinoma and abdominal neuroendocrine tumor) which were not associated to cardiorespiratory diseases. In the DMVD group, the cause of death was heart failure due to DMVD stage C or D without any infectious or systemic diseases associated with other cardiorespiratory diseases. In the DMVD+PH group, the cause of death was heart failure due to DMVD stage C or D and PH without any infectious or systemic diseases associated with other cardiorespiratory diseases. The characterization of all dogs is presented in Table 8. There was no significant difference of age and body weight among groups representing the age and weight matched of the enrolled dogs.

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Table 8 The characterization of the enrolled dogs in the study

		Control group (n = 4)	DMVD group (n = 5)	DMVD+PH group (n = 5)	*p* value
N (%)		4 (28.57)	5 (35.71)	5 (35.71)	-
Age (years)		8 (7.75-10.00)	15 (14.00-15.00)	14 (13.00-15.00)	0.283
Gender	**Male**	3 (M = 2; Mc = 1)	1 (M)	3 (M = 2; Mc = 1)	-
	Female	1 (Fs)	4 (F = 1; Fs = 3)	2 (F = 1; Fs = 1)	
Weight (kg)		5.60 (5.28-6.93)	3.92 (3.70-4.14)	4.30 (2.80-6.90)	0.320

F = intact female; Fs = sterile female; M = intact male; Mc = castrated male

The data is presented as median and interquartile range.

The *p*-value was analyzed by Kruskal-Wallis test.

The echocardiographic findings from the medical records of dogs in the DMVD and DMVD+PH groups showed no significant difference between the DMVD and DMVD+PH groups except the left ventricular internal diameter at end diastole (LVIDd), the left ventricular internal diameter at end systole (LVIDs) and the fractional shortening (FS). The LVIDd of the DMVD group was greater than those of the DMVD+PH group (2.84 $\pm$ 0.87 cm/kg vs 1.66 $\pm$ 0.62 cm/kg, p = 0.038). The LVIDs of the DMVD group was greater than those of the DMVD+PH group (1.59 $\pm$ 0.50 cm/kg vs 0.72 $\pm$ 0.30 cm/kg, p = 0.010). The FS of the DMVD group was less than those of the DMVD+PH group (42.14 $\pm$ 5.82% vs 55.16 $\pm$ 7.91%, p = 0.018). The echocardiographic data are presented in Table 9. In the DMVD+PH group, all dogs had the peak TR velocity greater than 3.4 m/s. Two dogs had intermediate probability of PH without anatomic site of echocardiographic signs of PH. Three dogs had high probability of PH with 1 and 2 anatomic sites of echocardiographic signs of PH. Dog with 1 anatomic site of echocardiographic signs of PH had PA/Ao more than 1.0. Dog with 2 anatomic sites of echocardiographic signs of PH had decreased size of the left ventricle and right atrial and ventricular enlargement.

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Table 9 Echocardiographic data of dogs in DMVD and DMVD+PH groups

	DMVD (n = 5)	DMVD+PH (n = 5)	*p* value
LA index (cm/kg)	1.67 ± 0.28	1.83 ± 0.72	0.642
Ao index (cm/kg)	1.01 ± 0.42	1.02 ± 0.28	0.952
LA/Ao	1.80 ± 0.56	1.75 ± 0.38	0.862
IVSd index (cm/kg)	0.59 ± 0.13	0.56 ± 0.14	0.663
LVIDd index (cm/kg)	2.84 ± 0.87	1.66 ± 0.62	0.038
LVPWd index (cm/kg)	0.55 ± 0.24	0.48 ± 0.17	0.621
IVSs index (cm/kg)	0.92 ± 0.30	0.84 ± 0.29	0.707
LVIDs index (cm/kg)	1.59 ± 0.50	0.72 ± 0.30	0.010
LVPWs index (cm/kg)	0.85 ± 0.33	0.71 ± 0.10	0.407
%FS	42.14 ± 5.82	55.16 ± 7.91	0.018
PAP (mmHg)	-	72.03 ± 25.96	-

Abbreviation: Ao = aorta; FS = fractional shortening; IVSd = interventricular septal thickness at end diastole; IVSd = interventricular septal thickness at end systole; LA = left atrium; LA/Ao = left atrium to aorta ratio; LVIDd = left ventricular internal diameter at end diastole; LVIDs = left ventricular internal diameter at end systole; LVPWd = left ventricular posterior wall thickness at end diastole; LVPWs = left ventricular posterior wall thickness at end systole; PAP = pulmonary arterial pressure

The data are expressed as mean ± standard deviation

The *p*-values represent the significant difference among 2 groups by independent t-test.

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Investigation of genes associated in serotonin signaling pathway by real-time reverse transcription polymerase chain reaction (qRT-PCR)

The relative expression of genes associated in the serotonin signaling pathway, *tph1*, *slc6a4* and *htr2a*, were found in all lung and PA samples. The mean and standard error of mean (SEM) of the relative gene expressions normalized to the reference genes are presented in Table 10. Comparison of the relative gene expression among three groups did not show the significant difference. However, the trends of changes in the relative gene expression were found and revealed as the heatmaps in Figure 8. The overall pattern of the gene expression levels of lung tissues (Figure 8A) was different from those of PA tissues (Figure 8B).

In lung tissues, the relative *tph1* expression of dogs among three groups were similar. The relative *slc6a4* expression of dogs was upregulated in the control group and had a trend to downregulate in the DMVD and DMVD+PH group. The relative *htr2a* expression was varied in the control group while the tendency of downregulation was found in the DMVD group. The *htr2a* was tended to upregulate in the DMVD+PH group.

In PA tissues, the relative *tph1* expression in PA was similar among three groups. 2 out of 5 dogs in the DMVD group had an upregulation of the relative *tph1* expression. The *tph1* expression of 1 dog in the DMVD+PH group was not detected. The relative *slc6a4* expression of the control and DMVD+PH groups were similar, except an obvious upregulation in 1 DMVD dogs with PH. The relative expression of *slc6a4* in the DMVD group was found only in 2 out of 5 dogs which was upregulated. The relative *htr2a* expression in PA tissues in the control group was varied. The downregulation of *htr2a* expression was found in the DMVD group while the tendency of upregulation was discovered in the DMVD+PH group. The *htr2a* expression was not detected in 1 dog in the control group.

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Table 10 Relative gene expression of lung and PA tissue

Gene	Control (n=4)	DMVD (n=5)	DMVD+PH (n=5)	*p* value
Lung (normalized to RPL32)				
tph1	1.99 (0.18-5.54)	0.95 (0.67-2.36)	0.51 (0.45-0.72)	0.680
slc6a4	1.78 (1.05-2.47)	0.62 (0.40-1.20)	0.15 (0.12-0.24)	0.081
htr2a	0.92 (0.77-1.24)	0.28 (0.26-0.54)	0.91 (0.83-0.93)	0.154
Pulmonary artery (normalized to RPS19)				
tph1	0.81 (0.24-5.03)	7.81 (5.74-18.50)	4.16 (2.19-6.27)	0.117
slc6a4	1.98 (0.33-4.48)	N/A[ŧ]	0.92 (0.34-0.99)	1.000*
htr2a	2.31 (1.22-2.76)	0.35 (0.21-0.52)	1.02 (0.63-3.34)	0.267

Abbreviation: *tph1* = tryptophan hydroxylase 1 gene; *slc6a4* = solute carrier family 6 member 4 (serotonin transporter gene); *htr2a* = 5-hydroxytryptamine receptor 2A gene

The data are expressed as median (interquartile range). The *p* value was analyzed by Kruskal-Wallis test.

* The *p*-value of *slc6a4* expression between control and DMVD+PH group was analyzed by Mann-Whitney U test.

[ŧ] The *slc6a4* expression of PA tissue could not be analyzed because the gene expression was found in only 2 samples.

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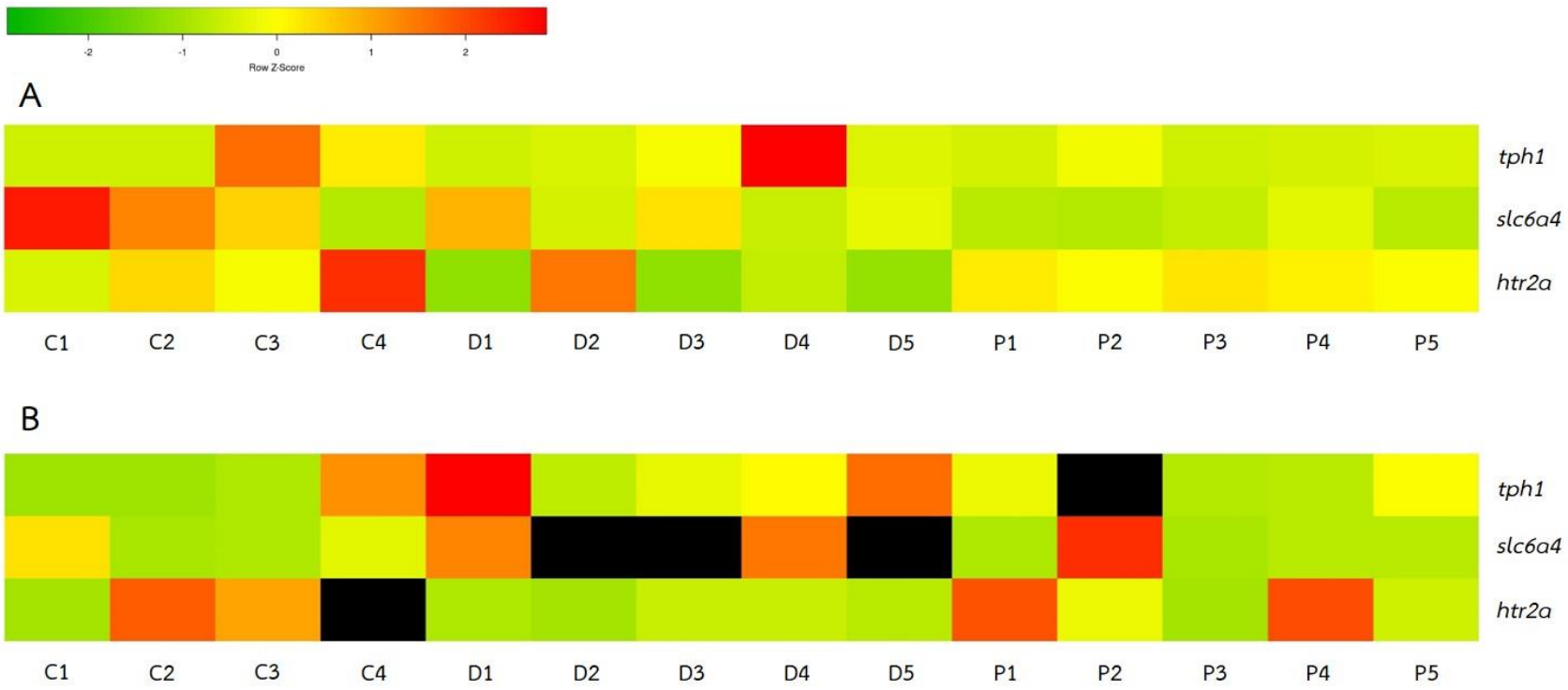

Figure 8 Heatmaps reveal the relative gene expression associating the serotonin signaling pathway. A, The relative gene expression normalized to RPL32 in lung tissue of all dogs. B, The relative gene expression normalized to RPS19 in PA tissue of all dogs. The gene upregulation represents in red, and the gene downregulation represents in green. The missing data are represented in black. Genes are shown as row and samples as column. C = dogs in control group, D = dogs in DMVD group and P = dogs in DMVD+PH group.

Investigation of the downstream effectors of serotonin signaling pathway by Western blot

The relative protein expressions of TPH-1, SERT, 5-HTR2A, ERK1/2 and pERK1/2 were found in both lung and PA tissues of all dogs. Comparison of all protein expressions among groups in both lung and PA tissues revealed no statistical difference (Table 11). However, the tendencies of relative protein expressions were found. The relative expression patterns of proteins associated with the serotonin signaling pathway are illustrated as the heatmaps in Figure 9. The overall protein expression pattern of the lung tissues (Figure 9A) was different from those of the PA tissues (Figure 9B) even in the same dog.

In lung tissue, the relative expression of TPH-1 and 5-HTR2A among groups were similar. Both up and downregulation of TPH-1 and 5-HTR2A were found in all three groups. The relative SERT expression was varied in the control group while downregulation was found in the DMVD group and upregulation was discovered in the DMVD+PH group. The expression of ERK1/2 and pERK1/2 were varied in all groups and did not different among groups.

In PA tissues, the relative expression of TPH-1, SERT, 5-HTR2A, ERK1/2 and pERK1/2 were upregulated in the DMVD and DMVD+PH groups while they were downregulated in the control group.

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Table 11 The relative protein expression normalized to α-Tubulin associated with the serotonin signaling pathway in lung and PA tissue

Proteins	Control (n=4)	DMVD (n=5)	DMVD+PH (n=5)	*p* value
Lung				
TPH-1	2.33 (1.14-3.52)	1.59 (0.84-3.59)	1.56 (1.37-4.96)	0.362
SERT	1.14 (0.40-2.10)	0.46 (0.21-0.63)	1.49 (0.27-1.52)	0.373
5-HTR2A	4.40 (1.62-7.90)	2.59 (1.96-6.60)	3.15 (2.42-5.75)	0.656
ERK1/2	1.74 (1.03-3.35)	1.41 (1.21-4.97)	2.16 (1.42-3.98)	0.680
pERK1/2	0.72 (0.46-1.15)	1.02 (0.39-1.27)	0.21 (0.18-1.21)	0.284
Pulmonary artery				
TPH-1	0.65 (0.60-0.78)	0.81 (0.41-2.07)	1.85 (1.08-2.33)	0.401
SERT	0.23 (0.20-0.28)	0.14 (0.14-0.47)	0.40 (0.13-0.70)	0.761
5-HTR2A	0.68 (0.60-0.77)	0.99 (0.75-1.63)	1.58 (1.39-2.43)	0.350
ERK1/2	0.86 (0.79-0.94)	0.81 (0.78-2.19)	1.04 (0.66-1.96)	0.831
pERK1/2	0.61 (0.43-0.78)	0.45 (0.29-1.05)	0.56 (0.21-0.85)	0.990

Abbreviation: TPH-1 = tryptophan hydroxylase-1; SERT = serotonin transporter; 5-HTR2A = serotonin receptor 2A; ERK1/2 = extracellular signal-regulated kinase 1/2; pERK1/2 = phosphorylated extracellular signal-regulated kinase 1/2

The data are expressed as median (interquartile range). The *p* value was analyzed by Kruskal-Wallis test.

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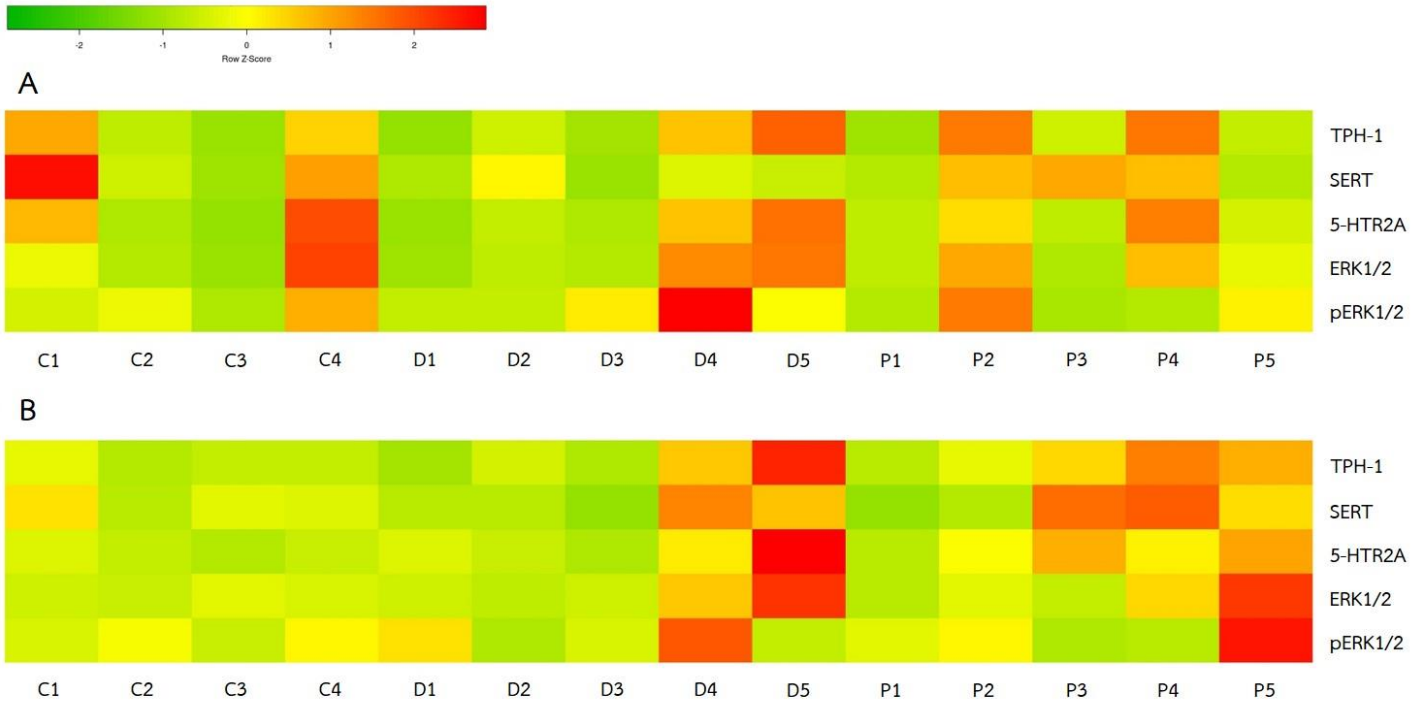

Figure 9 Heatmaps reveal the relative protein expression associating with the serotonin signaling pathway normalized to **α**-Tubulin. A, The relative protein expression in lung tissues of all dogs. B, The relative protein expression in PA tissues of all dogs. The upregulated proteins represent in red, and the downregulated proteins represent in green. Proteins are shown as row and samples as column C = dogs in control group, D = dogs in DMVD group and P = dogs in DMVD+PH group.

Part II To assess the source of serotonin in blood circulation

Sixty-two dogs were included in the study. The dogs were divided into 3 groups, the control, DMVD and DMVD+PH groups. The control group composed of 22 dogs which were 8 males (1 intact and 7 castrated males) and 14 sterile females. Breeds of dogs were 8 Shih Tzus, 5 Chihuahuas, 5 Yorkshire Terriers, 3 mixed breed dogs, and 1 Dachshund. The median (interquartile range (IQR)) of the age of the control group was 8.00 (7.00 - 9.75) years old and the body weight was 4.65 (2.87 – 6.00) kg. The DMVD group consisted of 20 dogs which were 15 males (9 intact and 6 castrated males) and 5 sterile females. Breeds of dogs were 5 Pomeranians, 5 Chihuahuas, 4 Poodles, 1 Shih Tzu, 1 Yorkshire Terrier, 1 Chinese Crested Hairless Dog, 1 Beagle, 1 Finnish Spitz, and 1 mixed breed dog. The median (IQR) of the age was 12.00 (11.00 - 13.25) years old and the body weight was 5.16 (3.95 – 6.51) kg. The DMVD+PH group comprised 20 dogs which were 9 males (4 intact and 5 castrated males) and 11 females (3 intact and 8 sterile females). Breeds of dogs were 6 Poodles, 5 Chihuahuas, 4 mixed breed dogs, 2 Shih Tzus, 2 Miniature Pinschers and 1 Jack Russel Terrier. The median (IQR) of the age was 12.00 (10.00 - 14.00) years old and the body weight was 5.22 (4.47 - 6.16) kg. The median age of dogs in the control group was significant difference from other 2 groups ($p < 0.0001$). There was no significant difference of the body weight between groups. The signalment of all groups are presented in Table 12.

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Table 12 Summary of the signalment of control, DMVD and DMVD+PH group

	Control (n = 22)	DMVD (n = 20)	DMVD+PH (n = 20)	*p*-value
Gender (male/female)	8/14	15/5	9/11	-
Age (years)	8.00 (7.00-9.75)	12.00 (11.00-13.25) [a]	12.00 (10.00-14.00) [a]	<0.0001
Weight (kg)	4.65 (2.87-6.00)	5.16 (3.95-6.51)	5.22 (4.47-6.16)	0.191

The data are expressed as median (interquartile range).

*The *p*-values represent the significant difference among 3 groups by the Kruskal-Wallis test.

[a] indicates significant difference compared to the control group at $p < 0.05$.

The clinical signs of dogs in the DMVD and DMVD+PH groups from history taking were coughing (16/20 and 17/20), exercise intolerance (9/20 and 9/20), respiratory distress (4/20 and 6/20) and syncope (1/20 and 3/20). The physical examination of DMVD and DMVD+PH groups revealed systolic murmur (20/20 and 20/20), increased lung sound (9/20 and 15/20), lung crackles (4/20 and 4/20), pale pink mucous membrane (1/20 and 5/20), and ascites (0/20 and 4/20). The physical examination revealed the difference of heart rate among groups ($p = 0.019$) which the heart rate of dogs in the DMVD+PH group was greater than the control group ($p = 0.016$). The respiratory rate of the DMVD+PH group was more than the DMVD group ($p = 0.026$). No significant difference of systolic blood pressure, diastolic blood pressure and mean arterial pressure was found between groups (Table 13). The clinical findings from history and physical examination between male and female dogs were not different.

Analysis of hematology and blood chemistry profiles showed that RBC, Hb, Hct, platelet count, MPV, WBC, SGPT, creatinine, total protein and albumin of all dogs were in the normal ranges. ALP and BUN of some dogs in all groups was more than the upper limit of normal range. Dogs in the DMVD and DMVD+PH groups had higher ALP, BUN and creatinine than dogs in the control group ($p < 0.05$) and dogs in the DMVD group had higher total protein level than the control and DMVD+PH groups ($p = 0.014$).

However, the creatinine and total protein levels of all enrolled dogs were in the normal range. The age and body weight of the dogs in this study were positively correlated with ALP (p = 0.013 and p = 0.005), creatinine (p < 0.001 and p = 0.047) and total protein (p = 0.014 and p = 0.006). Moreover, there was positive correlation between BUN and the age of the dogs in this study (p = 0.001). The results of hematology and blood chemistry profiles are shown in Table 14.

Table 13 Summary of the vital signs and vertebral heart score of control, DMVD and DMVD+PH groups

Parameter	Control (n = 22)	DMVD (n = 20)	DMVD+PH (n = 20)	*p*-value*
Heart rate (beats/minute)	122.91 ± 27.13	132.40 ± 28.58	148.05 ± 28.98 [a]	0.019
Respiratory rate (breath/minute)	41.26 ± 14.55	35.15 ± 11.07 [b]	51.38 ± 20.05 [b]	0.027
Systolic blood pressure (mmHg)	149.14 ± 23.65	139.75 ± 20.09	138.50 ± 19.06	0.208
Diastolic blood pressure (mmHg)	100.86 ± 24.43	93.28 ± 16.86	97.32 ± 15.84	0.487
Mean arterial pressure (mmHg)	111.95 ± 21.44	104.65 ± 16.56	108.43 ± 16.30	0.485
Vertebral heart score	9.58 ± 0.39	11.24 ± 0.92 [a]	11.87 ± 1.05 [a]	<0.001

The data are expressed as mean ± standard deviation

*The *p*-values represent the significant difference among 3 groups by the one-way ANOVA. Bonferroni post-hoc test was used to analyze the difference between 2 groups including heart rate, respiratory rate, systolic blood pressure, diastolic blood pressure and mean arterial pressure. The Tamhane test was used as the post-hoc test for vertebral heart score.

[a] indicates significant difference compared to the control group at p < 0.05.

[b] indicates significant difference between the DMVD and the DMVD+PH group at p < 0.05.

Table 14 Summary of hematology and blood chemistry profiles of all dogs

Parameter	Normal range	Control group	DMVD group	DMVD+PH group	*p*-value*
RBC (10^6 cell/μl)	5.10 – 8.50	6.51 (6.06 – 7.26)	7.14 (6.31 – 8.06) [b]	6.29 (5.71 – 6.71) [b]	0.037
Hb (g/dL)	11.00-19.00	15.65 (14.95 – 17.08)	16.15 (13.93 – 17.38)	15.15 (13.58 – 16.15)	0.177
Hct (%)	33.00 – 56.00	42.00 (39.30 – 45.00)	42.70 (38.90 – 46.85)	40.80 (36.68 – 43.65)	0.332
Platelet count (ng/10^9 platelets)	117.00 – 490.00	268.50 (223.00 - 323.75)	303.00 (252.50 – 400.50)	299.50 (245.50 – 367.25)	0.267
MPV (fL)	8.00 – 14.10	10.00 (8.90-10.50)	9.50 (9.10-11.05)	10.20 (9.55-11.25)	0.621
WBC (cells/μl)	6,000.00 – 17,000.00	7,875.00 (6,920.00 – 9,832.50)	7,840.00 (7,077.50 – 9,900.00)	10,405.00 (8,267.50 – 11,617.50)	0.073
SGPT (U/L)	4.00 – 91.00	47.00 (37.00 – 55.50)	61.50 (44.75 – 81.75)	48.50 (33.00 – 70.50)	0.143
ALP (U/L)	3.00 – 61.00	39.50 (19.50 - 82.00)	108.00 (43.25 – 175.00) [a]	67.00 (51.75 – 110.25) [a]	0.026
BUN (mg/dL)	7.00 – 30.00	20.75 (15.95 – 23.35)	25.45 (23.50 – 32.78) [a]	33.85 (25.20 – 41.68) [a]	<0.0001
Creatinine (mg/dL)	0.60 – 2.00	0.75 (0.60 – 0.80)	0.90 (0.78 – 1.23) [a]	1.05 (0.88 – 1.13) [a]	0.001
Total protein (g/dL)	5.80 – 8.80	6.30 (5.78 – 6.85)	6.90 (6.60 – 7.25) [a,b]	6.25 (5.80 – 6.85) [b]	0.014
Albumin (g/dL)	2.60 – 4.30	3.35 (3.10 – 3.60)	3.40 (2.75 – 3.73)	3.05 (2.7 – 3.43)	0.149

Abbreviation: ALP = alkaline phosphatase; BUN = blood urea nitrogen; Hb = hemoglobin; Hct = hematocrit; RBC = red blood cell; SGPT = serum glutamate-pyruvate transaminase; WBC = white blood cell

*The *p*-values represent the significant difference among 3 groups by the Kruskal-Wallis test.

[a] indicates significant difference compared to the control group at $p < 0.05$.

[b] indicates significant difference between the DMVD and the DMVD+PH group at $p < 0.05$.

The radiographic examination showed normal appearance of heart and lung in the control group. The radiographic findings of dogs in the DMVD and DMVD+PH groups included LA enlargement (17/20 and 19/20), LV enlargement (12/20 and 17/20), pulmonary edema (3/20 and 7/20), and pulmonary vein distention (2/20 and 3/20). Moreover, PA distention (3/20) and right heart enlargement (7/20) were found in the DMVD+PH group. Vertebral heart score (VHS) of dogs in the DMVD and DMVD+PH groups were greater than control group ($p < 0.001$) (Table 13).

The echocardiographic findings revealed greater LA, LA/Ao, LVIDd and %fractional shortening (%FS) in the DMVD and DMVD+PH groups compared to the control group ($p < 0.05$). The DMVD group had larger Ao and LVIDs compared to the control group ($p < 0.05$). The Ao index in the DMVD group was larger than those of the DMVD+PH group (Table 15). In the DMVD+PH group, DMVD dogs with an intermediate probability of PH (n=11) had a lower peak TR velocity and estimated PAP than those with a high probability of PH (n = 9) ($p = 0.004$) (Table 16). The examples of the echocardiographic findings of all three groups are shown in Figure 10.

No difference of both platelet and plasma serotonin concentration between 3 groups was found by using the Kruskal-Wallis test (Figure 11A and 11B). However, comparison of platelet serotonin concentration between normal, DMVD, DMVD with intermediate probability of PH (DMVD + intermediate probability of PH) and DMVD with high probability of PH (DMVD + high probability of PH) represented statistically significant difference ($p = 0.014$). Analysis with Dunn's post hoc test exhibited lower platelet serotonin concentration in DMVD with high probability of PH compared to the DMVD group ($p = 0.008$) (Table 16 and Figure 12). There was no significant difference in plasma serotonin concentration between all four groups ($p = 0.175$) (Table 16). The platelet serotonin concentration of male dogs was not significantly different from those of female dogs (194.99 [96.08-373.64] ng/10^9 platelets vs. 226.37 [80.29-405.16] ng/10^9 platelets, $p = 0.789$). Moreover, the plasma serotonin concentration between male and female dogs was not significantly different (2.14 [1.14-4.53] ng/ml vs. 1.89 [0.57-4.90] ng/ml, $p = 0.828$, respectively). A weak negative correlation between platelet and

plasma serotonin levels was found (r = -0.253, p = 0.049) (Figure 13A). MPV was weakly negatively correlated with platelet serotonin concentration and platelet count (r = -0.297, p = 0.022 and r = -0.345, p = 0.007, respectively) (Figure 13B and 13C). Body weight and age of dogs were weakly negatively correlated with plasma serotonin level (r = -0.353, p = 0.005 and r = -0.281, p = 0.028, respectively) (Figure 13D and 13E). No association among both circulating serotonin concentrations and sex and breed of dogs. In the DMVD+PH group, no correlation between the estimate PAP and both circulating serotonin concentration was found.

In-house ELISA validation showed that the intra-assay precision of the serotonin ELISA and the serotonin high sensitive ELISA tests were 6.56% and 4.89%, respectively, and the inter-assay precision were 6.54% and 9.16%, respectively. Moreover, the spike-recovery assessment was 104.19% and 101.00%, respectively.

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Table 15 Summary of echocardiographic data, platelet and plasma serotonin concentrations, platelet counts, and mean platelet volume of control, DMVD and DMVD+PH group

Parameter	Control (n = 22)	DMVD (n = 20)	DMVD+PH (n = 20)	*p*-value
LA (cm/kg)	1.00 (0.93-1.08)	2.23 (1.91-2.59) [a]	1.69 (1.40-2.50) [a]	<0.0001
Ao (cm/kg)	0.78 (0.69-0.88)	1.18 (0.94-1.28) [a, b]	0.81 (0.67-1.13) [b]	0.007
LA/Ao	1.27 (1.56-1.40)	1.82 (1.58-2.34) [a]	2.19 (2.06-2.24) [a]	<0.0001
IVSd (cm/kg)	0.45 (0.40-0.48)	0.47 (0.40-0.50)	0.45 (0.40-0.54)	0.725
LVIDd (cm/kg)	1.27 (1.21-1.39)	1.86 (1.73-1.96) [a]	1.80 (1.75-1.98) [a]	<0.0001
LVPWd (cm/kg)	0.37 (0.33-0.42)	0.37 (0.34-0.43)	0.40 (0.35-0.43)	0.886
IVSs (cm/kg)	0.59 (0.51-0.63)	0.57 (0.52-0.64)	0.63 (0.57-0.73)	0.137
LVIDs (cm/kg)	0.75 (0.72-0.88)	1.02 (0.87-1.74) [a]	0.77 (0.68-1.09)	0.009
LVPWs (cm/kg)	0.64 (0.56-0.68)	0.66 (0.61-0.74)	0.70(0.58-0.79)	0.226
%FS	38.52 (35.53-43.78)	44.15 (38.47-47.61) [a]	50.65 (43.34-56.37) [a]	0.001
PAP (mmHg)	-	-	52.88 (44.66-67.16)	-
Platelet serotonin ($ng/10^9$ platelets)	179.73 (102.37-352.24)	325.99 (96.84-407.66)	135.11 (21.21-312.22)	0.081
Plasma serotonin (ng/ml)	2.92 (1.76-7.50)	1.23 (0.27-4.23)	1.75 (1.19-2.72)	0.920
Platelet counts ($x\ 10^3$ cells/ml)	268.50 (223.00-323.75)	303.00 (252.50-400.50)	299.50 (245.50-367.25)	0.267
MPV (fL)	10.00 (8.90-10.50)	9.50 (9.10-11.05)	10.20 (9.55-11.25)	0.621

Abbreviation: Ao = aorta; FS = fractional shortening; IVSd = interventricular septal thickness at end diastole; IVSd = interventricular septal thickness at end systole; LA = left atrium; LA/Ao = left atrium to aorta ratio; LVIDd = left ventricular internal diameter at end diastole; LVIDs = left ventricular internal diameter at end systole; LVPWd = left ventricular posterior wall thickness at end diastole; LVPWs = left ventricular posterior wall thickness at end systole; MPV = mean platelet volume; PAP = pulmonary arterial pressure

The data are expressed as median (interquartile range).

The *p*-values represent the significant difference among 3 groups by the Kruskal-Wallis test.

[a] indicates significant difference compared to the control group at $p < 0.05$.

[b] indicates significant difference between the DMVD and the DMVD+PH group at $p < 0.05$.

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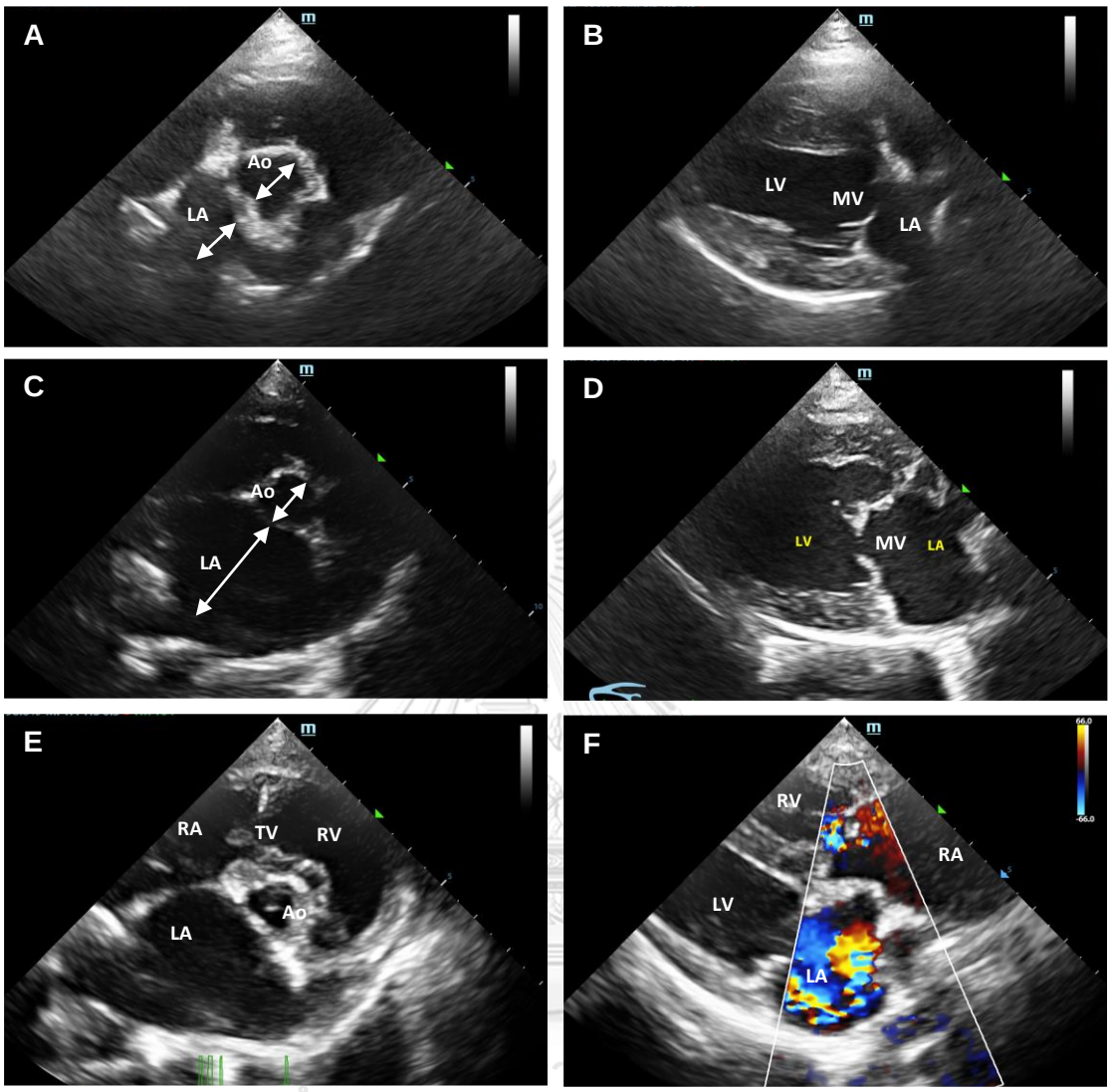

Figure 10 The examples of the two-dimensional echocardiographic findings of dogs in this study. A, The right parasternal short-axis view of a dog in the control group. The white arrows show the dimension of left atrium (LA) and aorta (Ao). The LA/Ao ratio is 1 which reflects the normal size of LA. B, The right parasternal long axis 4 chamber view of a dog in the control group illustrates the thin and regular mitral valve (MV) leaflets with a normal size of left atrium and ventricle (LV). C, The right parasternal short-axis view of DMVD dog shows an enlarged LA with LA/Ao ≥ 1.6 (LA/Ao = 2.23). D, The right parasternal long axis 4 chamber view of a dog with DMVD reveals the enlarged LA and LV with thickening and irregular surface of the mitral valve. E, The right parasternal short-axis view of a DMVD dog with PH presents the enlarged LA with LA/Ao ≥ 1.6 (LA/Ao = 2.25), tricuspid valve (TV) leaflet thickening with irregular surface. F, Color-flow Doppler echocardiography of right parasternal long axis 4 chamber view of a dog with DMVD and PH illustrates all four-chamber enlargement with mitral and tricuspid valve thickening and regurgitation.

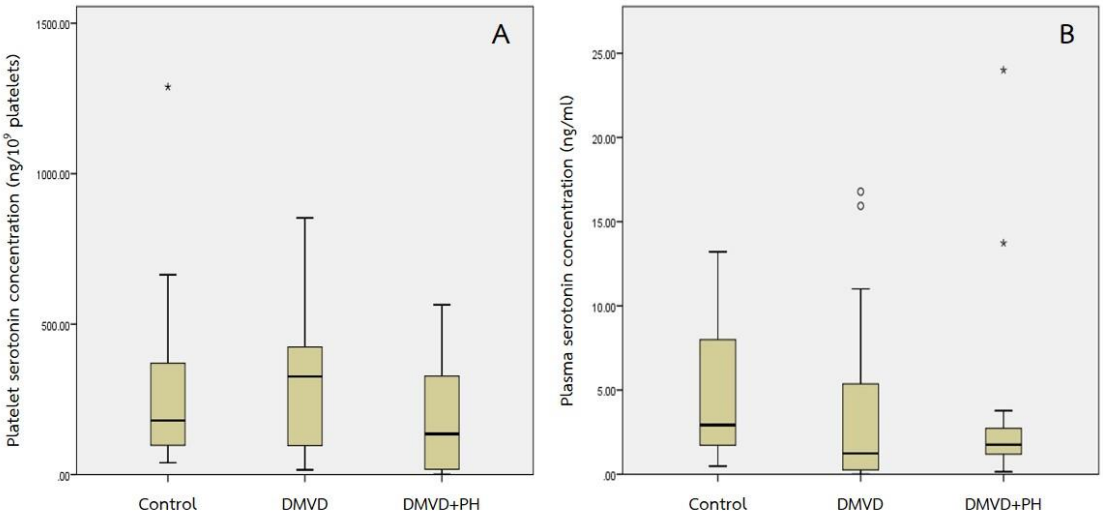

Figure 11 Box plot of platelet and plasma serotonin of the control (n = 22), DMVD (n = 20) and DMVD+PH groups (n = 20). There was no statistically significant difference among the groups in both platelet and plasma serotonin concentration. A, Platelet serotonin concentrations. B, Plasma serotonin concentration. Median values of platelet and plasma serotonin levels are represented as horizontal lines. The ends of the box are the 25th and 75th quartiles. The ends of the whiskers reveal the minimum and maximum of all the data. Data not included between the whiskers are plotted as an outlier with a circle and an asterisk.

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Table 16 Platelet and plasma serotonin concentration of the control, DMVD, DMVD + intermediate probability of PH and DMVD + high probability of PH groups, and peak TR velocity and estimated PAP of DMVD with intermediate probability of PH and DMVD with high probability of PH group. Data are showed as median (interquartile range).

	Control (n = 22)	DMVD (n = 20)	DMVD + intermediate probability of PH (n = 11)	DMVD + high probability of PH (n = 9)	*p*-value
Platelet serotonin concentration (ng/10^9 platelets	179.73 (102.37 - 352.24)	325.99 (96.84 - 407.66)[a]	291.11 (106.69 - 400.84)	35.82 (2.69 - 126.35)[a]	0.014
Plasma serotonin concentration (ng/ml)	2.92 (1.76 - 7.50)	1.23 (0.27 - 4.23)	1.50 (1.22 - 1.93)	2.37 (0.57 - 3.49)	0.175
Peak TR velocity (m/s)	-	-	3.40 (3.29 – 3.62)	4.12 (3.76 – 4.59)	0.004
PAP (mmHg)	-	-	46.19 (43.42 – 52.43)	67.90 (56.55 – 84.31)	0.004

Abbreviation: PAP = pulmonary arterial pressure; TR = tricuspid regurgitation

The *p*-values reveal the significant difference among groups. The platelet and plasma serotonin concentrations were analyzed by the Kruskal-Wallis test. The peak TR velocity and PAP were analysed by Mann-Whitney U test.

[a] indicates significant difference between DMVD without PH and DMVD with high probability of PH groups analyzed by Dunn's post hoc test at $p < 0.05$.

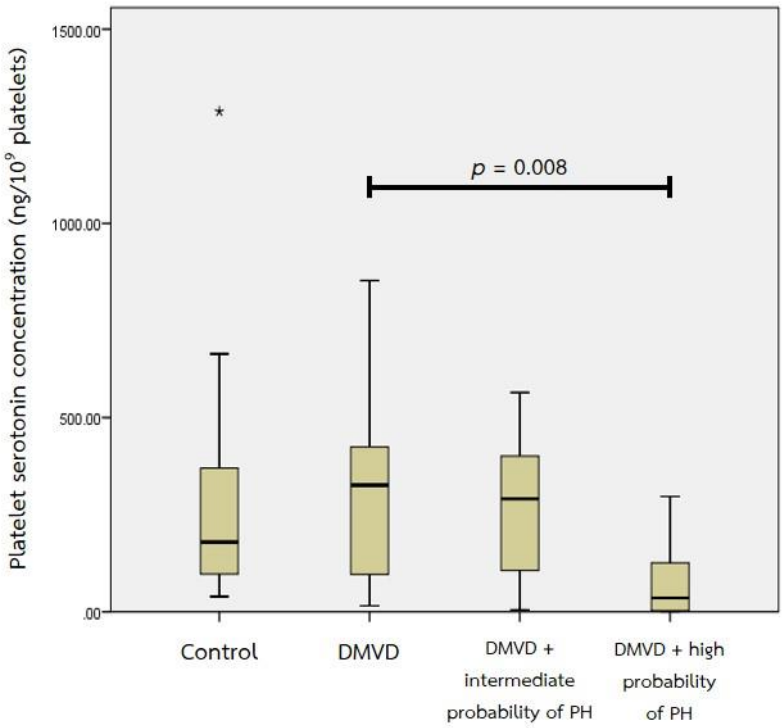

Figure 12 Box plot of platelet serotonin concentration of dogs in the control (n = 22), DMVD (n = 20), DMVD+PH which the dogs in DMVD+PH group were divided into intermediate (n = 11) and high probability of PH (n = 9). The platelet serotonin concentration of DMVD dogs with high probability of PH was significantly lower than those of DMVD dogs without PH (p = 0.008). Median values of platelet serotonin levels are represented as horizontal lines. The ends of the box are the 25th and 75th quartiles. The ends of the whiskers reveal the minimum and maximum of all the data. Data not included between the whiskers are plotted as an outlier with an asterisk.

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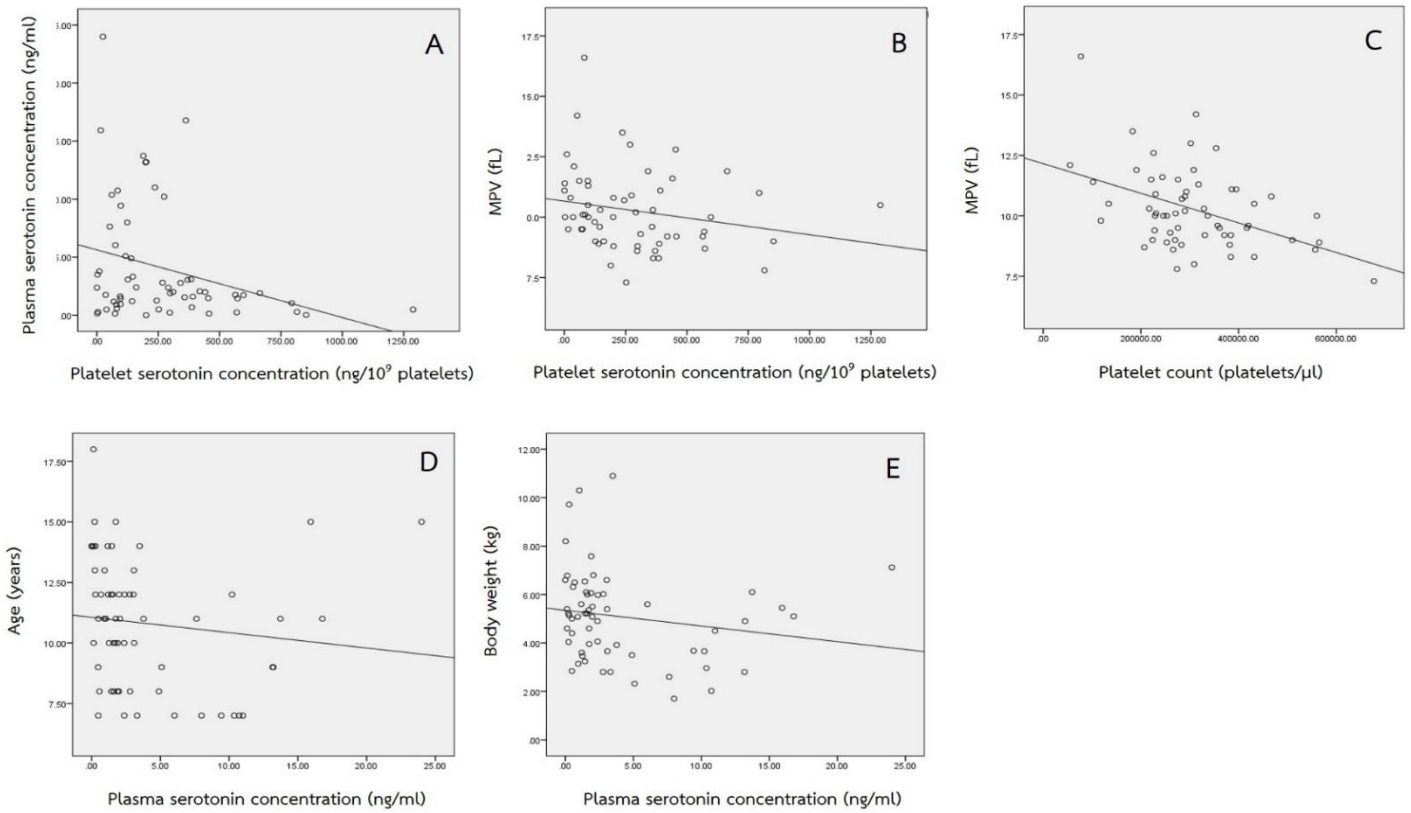

Figure 13 The weak negative correlation revealed in the present study. A, Weak negative correlation between platelet and plasma serotonin concentration (r = -0.253, p = 0.049). B, Weak negative correlation between platelet serotonin concentration and MPV (r = -0.297, p = 0.022). C, Weak negative correlation between platelet count and MPV (r = -0.345, p = 0.007). D, Weak negative correlation between plasma serotonin concentration and age (r = -0.353, p = 0.005). E, Weak negative correlation between plasma serotonin concentration and body weight (r = -0.281, p = 0.028).

CHAPTER V
DISCUSSION

Part I The local effect of serotonin signaling in lung tissues

Discussion

In human and other animal models, the involvement of serotonin signaling pathway in PH has been proven. The present study tried to investigate the association between the serotonin signaling pathway in the lung and PA tissues and PH secondary to DMVD in dogs. This study did not discover a significant difference in the relative gene and protein expression involving the serotonin signaling pathway in both canine lung and PA tissues among the control dogs and DMVD dogs with and without PH. However, trends of the gene and protein expressions in each group were found and the patterns of gene and protein expression in lung tissues were unlike those in PA tissues.

TPH-1 is the rate-limiting enzyme for serotonin synthesis which is also found in PAECs of lungs. The expression of *tph1* in the lung tissues of human patients with idiopathic PAH was increased together with elevated serotonin synthesis (Eddahibi et al., 2006). However, the relative gene and protein expressions of TPH-1 in lung tissues in this study were not different among three groups. This finding is similar to the study in hypoxic induced PH mice which *tph1* expression in the lung tissues did not change during exposure to hypoxia, however, this expression was measurable even in a small amounts (Abid et al., 2012). The previous research of Aiello et al. (2017) in rat lung tissues with induction of the different PH phenotype, idiopathic PAH and hypoxic PH, revealed the upregulated *tph1* with increased serotonin levels and increased mast cell numbers in lung tissues of the idiopathic PAH model while the *tph1* expression, serotonin levels and the mast cell numbers in the lung tissues of hypoxic PH model was not changed. Inhibition or knockout of TPH-1 improved the pulmonary hemodynamic parameters and decreased pulmonary vascular remodeling (Morecroft

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et al., 2007; Aiello et al., 2017). These evidences suggested that TPH-1 involves in serotonin induced PH and the difference in *tph1* expression of the two models was resulted from intrapulmonary synthesis of serotonin and the inflammatory response leading to increase in mast cell accumulation and degranulation providing elevated serotonin release to the pulmonary vasculature of the idiopathic PAH model but not in hypoxic PH model (Aiello et al., 2017). The study of Izikki et al. (2007) found that the gene polymorphism of *tph2* may affect the severity of hypoxic induced PH in *tph1* knockout mice. Additionally, hypoxic condition may accelerate the turnover of serotonin and cause dysregulated serotonin synthesis in the mice lung tissues which was intimately linked to the phenotype of PH. Taken together, the TPH-1 expression can be found in the lung tissues with PH, but the level of expression may be affected by several factors associating PH including the different forms of PH, gene polymorphism of TPH-2, and the inflammatory response occurring in the lung with PH.

SERT mediates the transportation of serotonin across the cells including passing to the pulmonary vascular endothelial cells for metabolism (Gillis and Pitt, 1982; Ni and Watts, 2006). In the present study, the relative expressions of *slc6a4* and SERT in lung tissues of dogs with DMVD were downregulated. This finding was similar to the previous studies reported the downregulated *slc6a4* in rat induced mitral valvulopathy by serotonin injection (Elangbam et al., 2008), as well as, the decreased SERT expression in canine myxomatous mitral valve (Disatian and Orton, 2009). Therefore, the changes of the gene and protein expression of SERT in lungs may be similar to the expression in mitral valve in dogs with DMVD. In lung tissues of DMVD dogs with PH in the present study, the upregulated SERT was found. Hypoxia in lung induces an increased protein expression of SERT in lung tissues of mice and SERT is the key role of PA muscularization (Eddahibi et al., 2000). Inhibition of SERT in hypoxic induced PH mice models exhibited less right ventricular hypertrophy and PA muscularization (Marcos et al., 2003). SERT has mitogenic effect on PASMC. In transgenic mice with overexpressed SERT, PA remodeling and upregulated gene and protein expression of SERT in lung tissues were found with increased serotonin uptake to the lung suggesting

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that overexpressed SERT induced development of PH (Guignabert et al., 2006). Conversely, the study of Morecroft et al. (2005) showed that rats had the decreased relative *slc6a4* expression and increased serotonin mediated vasoconstriction after hypoxic condition induced PH. The expression and function of SERT are affected by other effectors in the serotonin signaling pathway. The study in neuronal cell culture exhibited that serotonin receptor 2B controls SERT function by mediating the phosphorylation of SERT (Lamiay et al., 2006). Delaney et al. (2018) revealed that *slc6a4* expression was increased in fetal mice induced PPHN without changes in *htr2a* and 5-HTR2A expression and inhibition of 5-HTR2A activity decreased the expression of *slc6a4* and *htr2b*. Therefore, it is possible that a change in serotonin receptors may also influence the expression of SERT in lung tissues of DMVD dogs with and without DMVD.

Serotonin receptors compose of seven families, serotonin receptor 1-7, with 14 different structures and 5-HTR2A activation causes vasoconstriction in many arteries including PA of non-human species (MacLean and Dempsie, 2009). Study of changes in 5-HTR2A may indicate the involvement of serotonin and PH in DMVD dogs. However, the relative gene and protein of 5-HTR2A expression in the present study did not differ between groups. The variation of 5-HTR2A expression in the lung tissue was found in several previous studies. Investigation of the lung tissue of rat with PA remodeling found the upregulated 5-HTR2A gene and protein expression together with the increased SERT protein expression (Hofmann et al., 2014). Besides, the inhibition of 5-HTR2A of induced PAH rats attenuated PAH and prolonged the survival time as well as reduced the inflammation in the lung tissue (Hironaka et al., 2003). The studies in induced-PPHN ovine fetus, increased 5-HTR2A protein expression were found in both lung tissue and PASMC culture (Delaney et al., 2013) and inhibition of 5-HTR2A improved the pulmonary hemodynamic effects of the induced-PPHN ovine fetus (Delaney et al., 2011). In controversy, the study of Delaney et al. (2018) revealed that the gene and protein expressions of 5-HTR2A in the lung tissue of induced-PH fatal mouse model were not differ from the normal mice whereas an increased *htr2b* and

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slc6a4 expressions, increased TPH-1 expression and decreased pERK1/2 expression were found. Moreover, improvement of the hemodynamic parameters and pathological changes of the pulmonary vessels in induced-PH fatal mouse model were found with decreased *htr1b* and *slc6a4* expression and increased pERK1/2 and pArk expression (Delaney et al., 2018). Nevertheless, the study of other serotonin receptors in shunt-induced PH piglet model indicated that the gene expression of 5-HTR1B was predominately upregulated while the gene expression of 5-HTR2B, 5-HTR1D, 5-HTR4 and SERT were not changed. Treatment of idiopathic PAH and chronic hypoxic PH rat model by the inhibitors with high affinity for 5-HTR2A, 5-HTR2B and 5-HTR7 and moderate affinity for SERT exhibited improvement of pulmonary hemodynamic parameters with attenuation of pulmonary vascular remodeling (Bhat et al., 2017b; Bhat et al., 2017a; Bhat et al., 2018). These evidences suggested that 5-HTR2A cooperatively work with other serotonin receptors and SERT to mediate remodeled PA, and the function and expression of serotonin receptors and SERT in the lung tissue may varied between species and the various forms of PH.

ERK1/2 and pERK1/2 are the downstream effectors in MAPK/ERK pathway mediated by serotonin passing through SERT and activating Ras protein and NADPH oxidase leading to production and shift of reactive oxygen species (ROS), superoxide (O_2^-) (Lee et al., 1999; Liu et al., 2004). Conversion of the superoxide into hydrogen peroxide activates mitrogen-activated protein kinase kinase (MEK) which MEK then induces the phosphorylation of ERK1/2 in the cytosol of PASMC (Lee et al., 2001; Liu et al., 2004). Rho kinase activates the nuclear translocation of pERK1/2 which subsequently stimulates the nuclear transcription factors and ameliorates PASMC proliferation (Liu et al., 2004). ERK1/2 and pERK1/2 are also involved in apoptosis of PASMC which serotonin induced 5-HTR2A activates the phosphorylation of ERK1/2 participating in decreasing of PASMC apoptosis in induced PH rat model (Liu et al., 2013a). Taken together, serotonin inducing changes in ERK1/2 and pERK1/2 expression reflect the involvement of serotonin signaling pathway in PASMC proliferation and PA remodeling. The present study revealed the variation of ERK1/2 and pERK1/2

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expression in all groups without the trend of different expression among the groups. This finding may be due to the variation of the expression of the upstream effectors.

In PA tissues, although, the relative gene expressions among three groups were similar and individually varied, the relative protein expressions of all five proteins were upregulated in DMVD dogs with and without PH. These finding suggested that serotonin signaling activation was increased in dogs with naturally occurring DMVD with and without PH. Increased medial thickness of PA was reported in dogs with naturally occurring DMVD with congestive heart failure suggesting that pathological changes of PA can occurred in DMVD dogs without PH and serotonin signaling is one of the pathway mediating the thickening and abnormalities of pulmonary vascular walls (Falk et al., 2006; Sakarin et al., 2020). Moreover, upregulation of the relative protein expressions in DMVD and DMVD+PH groups in this study is similar to the previous study which the upregulation of these proteins was found in DMVD dogs with and without PH on immunohistochemistry (Sakarin et al., 2020). These evidences suggest that changes of serotonin signaling pathway may be occurred in PASMCs of dogs with DMVD and pulmonary edema prior to the occurrence of PH.

The previous studies in human and animal models also used PA to investigate the involvement of serotonin pathway in PH in addition to the lung. In this study, the trend of TPH-1 protein expression was upregulated in DMVD dogs with and without PH whether the relative *tph1* expression of all groups was similar. The finding of upregulated TPH-1 protein expression in DMVD dogs without PH is similar to the study of Disatian et al. (2010) which found an increase in TPH-1 expression in the mitral valve of DMVD dogs and human without PH. Therefore, upregulation of TPH-1 may be simultaneously found in mitral valve and PA of dogs with DMVD. The upregulated TPH-1 found in DMVD dogs with PH in this study was supported by the studies in both human and animal models. Increase in the mRNA expression of TPH-1 was revealed in both whole-lung homogenates and PAEC culture together with the gene upregulation of SERT in both whole-lung homogenates and PASMC culture from patients with idiopathic PAH (Eddahibi et al., 2006). Inhibition of TPH-1 in PAH-induced rat model

resulted in the decreases in TPH-1 and SERT protein expression, and inflammatory cytokine in lung tissues (Bai et al., 2014). Also, in chronic hypoxic PH study, the upregulated *tph1* was discovered in both rat lung and PA, and human PAECs, therefore, hypoxic can induce TPH-1 activity and subsequently increases pERK1/2 expression (Morecroft et al., 2012; De Raaf et al., 2015).

The present study found that the relative SERT expression of the DMVD and DMVD+PH groups was upregulated even though the *slc6a4* expression was not clearly different. The previous studies showed that SERT causes PA remodeling by inducing PASMC proliferation and medial thickening of PA in various conditions representing different forms of PH. Guignabert et al. (2005) reported upregulated SERT expression in PA of the PAH-induced rat model and inhibition of SERT reversed the medial thickening of PA. In hypoxic induced PH rat model, a mitogenic effect of serotonin inducing muscularization of PASMCs and medial thickening of PA was depended on SERT activity since SERT gene expression was upregulated in chronic hypoxic PH and inhibition of SERT prevented the proliferation of PASMCs (Eddahibi et al., 1999; De Raaf et al., 2015). However, SERT did not affect the remodeling of PAECs which is also the hallmark of PH (De Raaf et al., 2015). Therefore, it is possible that hypoxic condition from pulmonary edema of DMVD dogs may induce PH. Nevertheless, the difference of the relative *slc6a4* expression was found in the various groups of PH. The mRNA expressions of SERT in PASMCs from patients with primary PH and pulmonary veno-occlusive disease (PVOD) were increased while those from patients with secondary PH resulting from various diseases was not changed compared to the control patients (Marcos et al., 2004). This variation of SERT expression may affected by the gene polymorphisms. In human, *slc6a4* gene polymorphism is found in the region of promotor which changes the transcription level. The *slc6a4* genotype composed of 2 alleles of L allele and S allele with a 44-bp insertion or deletion (Lesch et al., 1993). The L allele provides a higher *slc6a4* transcription level than S allele. The *slc6a4* gene polymorphism affects the *slc6a4* expression, the serotonin uptake and PASMC proliferation which were higher in individuals with homozygous L variants (LL),

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intermediate in individuals with heterozygous LS variants (LS) and lower in individuals with homozygous S variants (SS) (Eddahibi et al., 2001). Therefore, LL genotype affects the genetic susceptibility to severe PH in primary PH, PVOD, secondary PH and chronic obstructive pulmonary disease inducing PH (Eddahibi et al., 2003; Marcos et al., 2004). From these suggestions, gene polymorphism of SERT may affect the variation of the relative *slc6a4* expression and severity of PH.

The variation of gene expression was also found in the PA tissues. The previous study of Marcos et al. (2004) exhibited that the mRNA expressions of 5-HTR2A, 5-HTR1B and 5-HTR2B in PASMCs from patients with primary PH, PVOD and secondary PH from various diseases were not different from the controls. Besides, De Raaf et al. (2015) found that in rat induced PH by chronic hypoxia had a trend of *tph1* upregulation whereas the expression of *htr1b* and *htr2a* did not differ from the normoxic condition. However, the study in rat PA indicated that 5-HTR2A activation inhibits voltage-gated K^+ channels on PASMC leading to increase in Ca^{2+} concentration and mediation of PA vasoconstriction (Cogolludo et al., 2006). Additionally, 5-HTR2A of induced PAH rat model, and 5-HTR1B and SERT of induced PAH human PASMC culture reduce the apoptosis of PASMC via pyruvate dehydrogenase kinase (PDK) and pERK1/2 pathway (Liu et al., 2013a; Liu et al., 2013b). Therefore, 5-HTR2A may play an essential role in development of PH. The pulmonary artery adventitial fibroblasts (PAFs) driven by 5-HTR2A activation were proliferated and differentiated resulting in fibrosis and remodeling of cultured rat PAFs incubated in serotonin via TGF-β1/Smad3 pathway (Chen et al., 2014) and chronic hypoxic rat PAFs via p38 MAPK pathway (Welsh et al., 2004). Similarly to the lung tissue, other serotonin receptors are also involved in mediation of serotonin signaling pathway in PH such as 5-HTR1B (Keegan et al., 2001; Rondelet et al., 2003) and 5-HTR2B (Launay et al., 2002). The variation of serotonin receptor gene expression may also be occurred with the species and forms of PH similarly to the expression in the lung tissues but this study mentioned that gene and protein expressions of 5-HTR2A were upregulated in dogs with PH. Taken together, it could be suggested that SERT may be the major mediator for serotonin induced PASMC

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proliferation (Eddahibi et al., 2001) while 5-HTR2A increases PA vasoconstriction and reduces PASMC apoptosis, therefore, medial remodeling of PA is a key role of PA remodeling in PH.

The increase in pERK1/2 expression was found in PA of hypoxic PH in rats and human (Morecroft et al., 2012; De Raaf et al., 2015; Jiang et al., 2017) The present study revealed the upregulation of ERK1/2 and pERK1/2 in PA of DMVD dogs with and without PH. This finding was supported by the previous study using immunohistochemistry of Sakarin et al. (2020) which found the increased protein expression of TPH-1, SERT, 5-HTR2A and ERK1/2 in PA of DMVD dogs with and without PH, and increased pERK1/2 expression in dogs with PH secondary to DMVD. Upregulated ERK1/2 and pERK1/2 illustrated serotonin inducing the increased PASMC proliferation via SERT and the decreased PASMC apoptosis via 5-HTR2A (Lee et al., 1999; Lee et al., 2001; Liu et al., 2004; Liu et al., 2013a). Altogether, serotonin may play an essential role for induction of PH secondary to DMVD in dogs and the evidence of serotonin activated PA remodeling may be found prior to the occurrence of PH in DMVD dogs.

The previous studies showed the upregulated TPH-1 and pERK1/2 in both rat lung and PA, and human PAECs with chronic hypoxia PH (Morecroft et al., 2012; De Raaf et al., 2015). However, the results of the present study showed the different patterns of relative gene and protein expressions among lung and PA. The gene and protein expressions in lung tissues was varied whereas the trend of protein expression was found in PA tissues. Therefore, in my opinion, PA tissues are the better samples to represent the serotonin signaling pathway associating PH secondary to DMVD in dogs.

Serotonin induced PH is also associated with other pathways including 5-HTR2A activating phosphatidylinositol 3-kinase/serine-threonine protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway that mediates mitogenic response or proliferation of PASMCs (Liu and Fanburg, 2006). Moreover, the pathogenesis of PH can be affected by several mediators or signaling pathways such as endothelin pathway

(Kim et al., 2000; Moraes et al., 2000; Ray et al., 2008), prostanoid pathway (Kellihan and Stepien, 2012), nitric oxide pathway (Cooper et al., 1996; Moraes et al., 2000), natriuretic peptides (Yamamoto et al., 2004; Kellihan and Stepien, 2012) or inflammatory process (Rabinovitch, 2012; Kherbeck et al., 2013).

Limitation of the study

The limitation of this study was small sample size which may cause the variation of the relative expression of genes and proteins related to serotonin signaling pathway. Further studies should perform with larger sample size.

Conclusion

The present study revealed the tendency of the upregulated mediators and downstream effectors associated in serotonin signaling pathway in PA of DMVD dogs with and without PH. With cooperative working of the serotonin related mediators, serotonin may play an important role in PH of dogs with DMVD. Variation of gene and protein expression of the mediators in serotonin pathway may be caused by the activation of other associating mediators (e.g., other serotonin receptors), genetic polymorphism, inflammatory response, and the forms of PH. The further study should investigate other serotonin receptors to elucidate the involvement of serotonin signaling pathway in PA of dogs with PH.

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Part II The source of serotonin in blood circulation

Discussion

Analysis of the circulating serotonin concentration revealed lesser platelet serotonin concentration in DMVD dogs with high probability of PH compared to dogs in the DMVD group. There was no significant difference of the plasma serotonin levels between 3 groups. Nevertheless, a trend of elevating platelet serotonin level of dogs in DMVD group was found. This finding was consistent with a previous study in elder small-breed dogs (Mangklabruks and Surachetpong, 2014). On the other hand, Arndt et al. (2009) revealed the increasing serum serotonin concentration in small-breed DMVD dogs compared with normal large-breed dogs.

In the normal condition, dense granules in platelets cytosol are the storage of serotonin (Tranzer et al., 1966; Bentfeld-Barker and Bainton, 1982). Serum serotonin level is likely to be an approximate total of plasma and platelet serotonin levels (Leon et al., 2012), thus using plasma serotonin to investigate circulating serotonin without platelet activation may be better. Herve et al. (1995) reported that during in vitro platelet aggregation, platelets increased the serotonin release resulting in a decrease in platelet serotonin concentration and an increase in plasma serotonin concentration. This finding supported the negative correlation among platelet and plasma serotonin concentration that was found in the present study. In dogs with DMVD, increased plasma and serum serotonin suggests an association between DMVD and circulating serotonin, nevertheless, serotonin has not been proved as the cause or effect of DMVD.

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The difference in breeds had an influence on the serum serotonin concentration in healthy dogs. Cavalier King Charles Spaniel (CKCS) was reported the higher plasma and serum serotonin level compared to other small-breed dogs in both healthy and DMVD condition (Cremer et al., 2015; Hoglund et al., 2018). Therefore, the design of this study concerned about a breed-matched study. No correlation between the circulating serotonin levels and breed in this study suggested that interbreed variation of platelet and plasma serotonin concentration was minimal.

Gender was found that can affected the level of serum serotonin. The study of Hoglund et al. (2018) revealed that male dogs had lower serum serotonin concentration than female dogs. The study in human showed that women have a greater incidence of PH than men (White et al., 2011). The involvement of estrogen in the regulation of the serotonin pathway mediators was found in animal models and human patients suffered from pulmonary arterial hypertension (White et al., 2011). The present study did not furnish the sex-matched design between groups. Most of dogs in DMVD group were males whereas females were more than males in control and DMVD+PH groups. However, there was no difference of platelet and plasma serotonin concentrations between male and female dogs in this study. Moreover, no correlation between gender and circulating serotonin concentration was found. Therefore, sex was not the profound effect on circulating serotonin concentrations in this study.

In this study, dogs in the control group were younger than dogs in DMVD and DMVD+PH groups. In addition, negative correlation was found between age of dogs and plasma serotonin concentration. The previous study of Arndt et al. (2009) also reported that the age was negatively correlated with serum serotonin concentration. These findings advise an intense effect of aging on circulating serotonin concentrations.

Negative correlation between body weights of dogs and plasma serotonin concentration was report by Park et al. (2015). However, the present study had body weight-matched design for all groups. Accordingly, the body weights of dogs had a minimal effect on circulating serotonin concentrations.

The plasma serotonin levels among dogs in the control and DMVD+PH groups appeared no difference. This finding was resembled to the previous study in human which the plasma serotonin concentrations of patients with PH secondary to chronic thromboembolism and pulmonary arterial hypertension did not differ from those of controls (Ulrich et al., 2011). Dogs in DMVD groups did not have a difference in plasma serotonin level comparing to dogs in DMVD+PH groups. However, the platelet serotonin concentration of dogs with DMVD and high probability of PH was decreased

compared to those of DMVD dogs without PH. This finding suggested that the platelet serotonin concentration can be decreased in dogs with a high probability of PH secondary to DMVD whereas the platelet serotonin level of the DMVD dogs without PH was similar to those with intermediate probability of PH. The study of Ulrich et al. (2011) advised that in human patients with PH, a decrease in platelet serotonin concentration may be secondary to an elevated serotonin release from platelets to PAECs and PASMCs.

The present study revealed that MPV was negatively correlated with platelet serotonin concentration and platelet count. This finding is supported by the previous studies of MPV (Bancroft et al., 2000; Dastjerdi et al., 2006). MPV indicates the production of platelets which decreased MPV reflects a small size of platelets resulting from an excessive platelet production. On the other hand, increased MPV reflects a large size of platelets resulting from an inadequate platelet production. In human patients with chronic obstructive pulmonary disease (COPD), the previous study showed that MPV of the COPD patients with PH was higher than the COPD patients without PH. Moreover, the positive correlation between MPV and PAP was found which can be suggested an association among MPV and severity of PH in human patients (Mohamed et al., 2019). However, there is no difference of MPV between groups in this study. This result can be implied that the platelet production of normal dogs, and DMVD dogs with and without PH was similar.

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The results of hematology and blood chemistry profiles showed that dogs with DMVD with and without PH had a significant increase in ALP, BUN and creatinine compared to normal dogs. Some dogs in these two groups had ALP and BUN levels over the upper limit of the normal values while all dogs in this study had normal creatinine level. Age was positively correlated with both ALP and BUN, and the body weight was positively correlated with ALP. However, the body weights among groups of dogs in this study were not significantly different, therefore, the body weights may not be a profound effect of change in ALP levels. On the other hand, dogs in the control group had younger than dogs in DMVD and DMVD+PH groups, thus age

provided the profound effect on both ALP and BUN levels. Moreover, in patients with congestive heart failure due to DMVD and DMVD+PH. Diuretics was used to treat the dogs with congestive heart failure. DMVD dogs in this study had a long-term treatment with furosemide which can cause diuretic resistance and impaired renal function (Keene et al., 2019). Therefore, BUN and creatinine levels of dogs in DMVD and DMVD+PH group were higher than those of the control group.

Limitation of the study

Small sample size was the first limitation of the present study. The small sample size may influence the statistical significance of the data. The further studies should be concerned with the greater number of samples. The duration of sample collection was another limitation found in this study. The blood samples of all enrolled dogs could not be collected concurrently. However, prevention of serotonin degradation was done following the manufacturing recommendation by preserving the blood samples up to a year at -80˚C with avoidance of repeated freeze-thaw cycles.

Conclusion

The present study exhibited the association between a decrease in platelet serotonin concentration and the degree of PH probability. The plasma serotonin concentration among the control and DMVD dogs with or without PH did not differ. Clarification of the role of platelet serotonin involving in PH secondary to canine DMVD should be executed in the further studies.

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APPENDIX

Publications

1. Selection of the Appropriate Reference Genes for Relative Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) in Canine Pulmonary Arteries

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Abstract

A normalisation of targeted genes using the appropriate reference genes is necessary for reliable quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments. This study aimed to investigate the appropriate reference genes and the numbers of the genes required for qRT-PCR in canine pulmonary arteries. Pulmonary arteries were collected from twenty-three dogs at necropsy. Candidate reference genes including ribosomal protein L32 (RPL32), ribosomal protein L13a (RPL13A), ribosomal protein S18 (RPS18), TATA box binding protein (TBP), beta-2-microglobulin (B2M), ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from pulmonary arteries were examined by qRT-PCR and analysed using four major algorithms, including geNorm, NormFinder, the comparative delta-CT method, and RefFinder. The optimal number of genes was analysed using geNorm. The ranking of the appropriate reference genes from all algorithms was evaluated by RefFinder. The appropriate reference genes for the normalisation of the targeted gene in canine pulmonary arteries are RPS19 and RPL32.

2. Assessment of Platelet and Plasma Serotonin in Canine Pulmonary Hypertension Secondary to Degenerative Mitral Valve Disease.

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Abstract

Background: Pulmonary hypertension (PH) is a common complication of degenerative mitral valve disease (DMVD), the most common cardiovascular disease in dogs. Serotonin has been suspected to play a role in the pathogenesis of PH, so this study aimed to investigate the differences in platelet and plasma serotonin between normal, DMVD and DMVD with PH (DMVD+PH) dogs.

Materials and Methods: Sixty-two small-breed dogs were enrolled to the study and divided into the normal (n = 22), DMVD (n = 20), and DMVD+PH (n = 20) groups. The platelet and plasma serotonin concentrations were measured by the competitive ELISA.

Results: The Kruskal–Wallis revealed the difference among the four groups of normal (179.73 [102.37–352.24] ng/10^9 platelets), DMVD (325.99 [96.84–407.66] ng/10^9 platelets), DMVD with intermediate probability of PH (291.11 [106.69–400.84] ng/10^9 platelets) and DMVD with high probability of PH (35.82 [2.69–126.35] ng/10^9 platelets) (p = 0.014). The Dunn's post-hoc test showed a decrease in the platelet serotonin concentration of the DMVD dogs with high probability of PH compared to the DMVD group (p = 0.008). The plasma serotonin concentration was not different between normal, DMVD, and DMVD+PH dogs.

Conclusion: In conclusion, a decrease in platelet serotonin concentration, which is associated with a degree of PH probability was found in DMVD dogs with PH. Further studies investigating roles of platelet serotonin in PH secondary to DMVD should be performed.

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Supplementary data for Chapter IV Part I

Table S1 Signalment of enrolled dogs.

ID	Name	Age (years)	Breed	Gender	Weight (kg)
Control group					
C01	ปุ่ยปุย	8	Shih Tzu	M	5.20
C02	Twonie	8	Cocker	Fs	5.90
C03	เขียวหวาน	7	Mixed	M	10.00
C04	Brownie	16	Shih Tzu	Mc	5.30
DMVD group					
D07	CD	10	Poodle	Fs	3.70
D10	หนุงหนิง	15	Poodle	Fs	6.60
D11	Sushi	15	Shih Tzu	M	4.14
D12	Toffy	16	Poodle	F	3.58
D13	Snow	14	Pomeranian	Fs	3.92
DMVD+PH group					
P03	วุ้นเส้น	13	Poodle	M	4.30
P05	โมเม	16	Chihuahua	Fs	1.80
P06	อิคิว	14	Poodle	M	10.0
P10	Euro	12	Pomeranian	Mc	2.80
P11	หมีห่า	15	Chihuahua	F	6.90

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Table S2 Echocardiographic data of dogs in DMVD and DMVD+PH groups.

ID	LA (cm/kg)	Ao (cm/kg)	LA/Ao	IVSd (cm/kg)	LVIDd (cm/kg)	LVPWd (cm/kg)	IVSs (cm/kg)	LVIDs (cm/kg)	LVPWs (cm/kg)	%FS	PAP (mmHg)
DMVD group											
D07	1.68	1.11	1.51	0.63	2.88	0.56	0.97	1.41	0.72	51.09	-
D10	1.88	1.69	1.11	0.80	3.87	0.94	1.22	2.02	1.41	43.42	-
D11	1.98	0.77	2.60	0.51	2.44	0.30	0.48	1.48	0.57	37.60	-
D12	1.30	0.65	2.00	0.55	3.38	0.53	1.14	2.15	0.88	36.39	-
D13	1.50	0.83	1.80	0.48	1.62	0.41	0.77	0.91	0.66	42.20	-
DMVD+PH group											
P03	1.79	1.25	1.43	0.64	1.52	0.34	0.64	0.47	0.68	67.68	112.78
P05	2.38	1.15	2.07	0.63	2.50	0.36	1.06	1.09	0.69	56.00	62.88
P06	0.73	0.58	1.28	0.33	0.82	0.50	0.63	0.41	0.58	47.93	53.29
P10	1.71	0.93	1.84	0.52	1.53	0.44	0.65	0.67	0.79	55.38	81.70
P11	2.56	1.21	2.13	0.66	1.92	0.76	1.24	0.98	0.82	48.81	49.51

Supplementary data for Chapter IV Part II

Table S3 Signalment of dogs in the control groups.

ID	Name	Age (years)	Breed	Gender	Weight (kg)
C01B	Pocky	9	Yorkshire Terrier	Fs	2.32
C02B	Brownie	9	Yorkshire Terrier	Fs	2.84
C03B	Sumo	7	Yorkshire Terrier	Mc	2.96
C04B	ยูตะ	7	Chihuahua	Mc	3.68
C05B	Lucky	7	Yorkshire Terrier	Mc	2.80
C06B	จั่นเจา	8	Shih Tzu	Mc	6.00
C07B	ซูโม่	12	Mixed (Poodle + Pug)	Mc	6.60
C08B	กาละแมร์	11	Shih Tzu	Fs	6.80
C09B	ปุ๊กปิ๊ก	9	Shih Tzu	Fs	4.90
C10B	ปุ้มปุ้ย	10	Shih Tzu	Mc	7.58
C11B	หวานเย็น	12	Shih Tzu	Fs	5.98
C12B	นีน่า	7	Shih Tzu	Fs	5.60
C13B	มีมี่	11	Shih Tzu	Fs	4.40
C14B	ใยไหม	8	Mixed (Lhasa Apso + Yorkshire Terrier)	Fs	3.50
C15B	ชัมเก้	7	Chihuahua	Fs	2.02
C16B	โมเช่	7	Chihuahua	M	1.70
C17B	ฮุชชู่	8	Chihuahua	Fs	3.24
C18B	คันโช่	8	Chihuahua	Mc	6.02
C19B	เบ็นเท็น	8	Shih Tzu	Fs	6.06
C20B	น้อย	9	Yorkshire Terrier	Fs	2.80
C21B	ถั่วแดง	10	Dachshund	Fs	5.36
C22B	ดุ๊กดิ๊ก	7	Mixed	Fs	5.00

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Table S4 Signalment of dogs in the DMVD groups.

ID	Name	Age (years)	Breed	Gender	Weight (kg)
D01B	ข้าวหอม	11	Chihuahua	Fs	5.08
D02B	ไข่หวาน	14	Poodle	Mc	6.60
D03B	ไข่ขาว	13	Poodle	M	4.04
D04B	โมจิ	12	Beagle	Fs	9.72
D05B	Edward	10	Chihuahua	Mc	3.66
D06B	สี่	11	Shih Tzu	Mc	10.00
D07B	โกโก้	14	Poodle	M	5.40
D08B	ฮัตสึ	12	Pomeranian	M	3.66
D09B	ตาหวาน	12	Poodle	Fs	5.50
D10B	ซูกัส	18	Mixed	Fs	4.60
D11B	บุ้งกี๋	12	Pomeranian	M	6.50
D12B	Kitty	13	Chihuahua	Mc	3.14
D13B	ลูกหมู	7	Chihuahua	M	4.50
D14B	ซูชิ	10	Pomeranian	M	5.22
D15B	ยูโร	12	Pomeranian	M	2.80
D16B	Teddy	12	Pomeranian	M	6.54
D17B	โป้งเหน่ง	11	Chihuahua	Mc	2.60
D18B	ไวท์	14	Finnish Spitz	M	8.20
D19B	ฮิกกี้	15	Yorkshire Terrier	Mc	5.45
D20B	โบวี่	11	Chinese Crested Hairless Dog	Fs	5.10

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Table S5 Signalment of dogs in the DMVD+PH groups.

ID	Name	Age (years)	Breed	Gender	Weight (kg)
P01B	เหมา	14	Mixed	Mc	10.00
P02B	โดโด้	15	Poodle	M	4.60
P03B	คาซึ	10	Shih Tzu	F	4.90
P04B	จีจี๊	15	Poodle	Fs	7.12
P05B	Roxy	11	Chihuahua	Fs	3.96
P06B	เปรี้ยว	11	Chihuahua	F	3.92
P07B	จีโน่	14	Shih Tzu	Mc	5.60
P08B	เบน	14	Miniature Pinscher	M	5.14
P09B	หมูตุ๋น	13	Poodle	Mc	5.40
P10B	มะยม	12	Chihuahua	M	3.60
P11B	Bobby	12	Poodle	Fs	6.10
P12B	Cartoon	8	Mixed	F	6.32
P13B	ข้าวหอม	8	Poodle	Fs	5.08
P14B	ทองแดง	14	Miniature Pinscher	Fs	5.22
P15B	โมจิ	13	Jack Russel Terrier	Fs	7.20
P16B	Baby	10	Poodle	Fs	6.78
P17B	บ้องแบ๊ว	15	Mixed	Fs	5.22
P18B	ทองคำ	10	Chihuahua	Mc	3.46
P19B	โตโต้	11	Mixed	M	6.10
P20B	หมั่นโถว	7	Chihuahua	Mc	4.06

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Table S6 The vital signs and vertebral heart score of the control group

ID	Heart rate (bpm)	Respiratory rate (bpm)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Mean arterial pressure (mmHg)	Vertebral heart score
C01B	108	44	138	91	99	10
C02B	92	28	102	65	76	9.2
C03B	108	36	122	74	85	9.2
C04B	176	48	165	136	140	9.5
C05B	104	40	157	127	133	10.5
C06B	104	N/A	158	99	111	9.6
C07B	160	36	144	81	99	9.5
C08B	156	36	150	115	123	9.2
C09B	92	32	166	87	108	9.3
C10B	128	N/A	146	90	106	9.5
C11B	120	36	171	117	132	N/A
C12B	132	24	152	116	122	10
C13B	108	20	196	132	138	10.2
C14B	160	60	162	145	149	9.7
C15B	80	36	120	82	92	9
C16B	168	40	132	100	109	9.2
C17B	136	60	143	111	119	9.6
C18B	88	60	121	77	87	N/A
C19B	112	76	160	100	113	9.5
C20B	124	48	195	125	142	9.8
C21B	124	24	160	50	76	9.2
C22B	124	N/A	121	99	104	9.8

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Table S7 The vital signs and vertebral heart score of the DMVD group

ID	Heart rate (bpm)	Respiratory rate (bpm)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Mean arterial pressure (mmHg)	Vertebral heart score
D01B	102	33	99	73	81	10.7
D02B	140	32	151	109	121	12
D03B	100	28	133	105	111	12.3
D04B	104	48	147	97	110	12.3
D05B	168	N/A	155	122	130	12
D06B	172	N/A	130	N/A	N/A	10.9
D07B	84	N/A	159	96	110	9.8
D08B	164	28	130	79	93	10.8
D09B	144	24	145	98	104	10.9
D10B	164	64	154	114	122	11.8
D11B	170	N/A	120	N/A	N/A	11.5
D12B	112	32	127	69	86	10.9
D13B	108	40	126	78	91	10.7
D14B	100	N/A	125	93	N/A	11
D15B	116	28	163	87	107	10.4
D16B	140	N/A	163	117	126	12.5
D17B	128	24	153	87	97	9.3
D18B	120	N/A	178	109	124	10.6
D19B	144	40	128	73	86	12.8
D20B	168	36	109	73	80	11.6

จุฬาลงกรณ์มหาวิทยาลัย

Table S8 The vital signs and vertebral heart score of the DMVD+PH group

ID	Heart rate (bpm)	Respiratory rate (bpm)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Mean arterial pressure (mmHg)	Vertebral heart score
P01B	132	60	128	82	N/A	9.8
P02B	150	92	126	98	N/A	12.8
P03B	173	N/A	135	92	N/A	12
P04B	156	N/A	151	90	N/A	10.3
P05B	154	32	127	90	99	12
P06B	200	80	167	113	124	13
P07B	150	48	148	121	N/A	9.8
P08B	120	32	112	82	88	11.3
P09B	172	24	120	N/A	N/A	12.6
P10B	144	54	140	81	99	11.5
P11B	190	52	111	90	94	12.5
P12B	184	64	142	111	121	11
P13B	128	44	142	105	118	12.4
P14B	156	N/A	161	131	136	12.8
P15B	144	32	121	85	94	11.5
P16B	124	52	135	97	104	13.1
P17B	104	N/A	186	115	134	11.6
P18B	140	56	129	68	86	13.5
P19B	80	76	131	105	112	12.2
P20B	160	24	158	93	109	11.6

จุฬาลงกรณ์มหาวิทยาลัย

Table S9 Echocardiographic data of dogs in control group

ID	LA (cm/kg)	Ao (cm/kg)	LA/Ao	IVSd (cm/kg)	LVIDd (cm/kg)	LVPWd (cm/kg)	IVSs (cm/kg)	LVIDs (cm/kg)	LVPWs (cm/kg)	%FS	PAP (mmHg)
C01B	1.04	0.69	1.52	0.47	1.25	0.38	0.67	0.88	0.47	28.05	-
C02B	1.04	0.83	1.27	0.62	1.21	0.51	0.64	0.91	0.69	23.35	-
C03B	1.03	0.72	1.43	0.52	1.38	0.43	0.67	0.73	0.72	45.79	-
C04B	0.98	0.79	1.25	0.40	1.45	0.31	0.61	0.90	0.52	36.15	-
C05B	0.86	0.68	1.27	0.25	1.18	0.28	0.55	0.72	0.58	37.50	-
C06B	0.98	0.72	1.37	0.51	1.30	0.37	0.53	0.92	0.53	26.82	-
C07B	0.70	0.64	1.12	0.45	1.31	0.29	0.50	0.57	0.76	54.59	-
C08B	0.87	0.62	1.41	0.49	1.62	0.38	0.61	1.01	0.56	35.44	-
C09B	0.76	0.51	1.48	0.43	1.15	0.42	0.54	0.62	0.73	44.38	-
C10B	0.96	0.76	1.28	0.59	1.44	0.48	0.63	0.80	0.68	41.98	-
C11B	1.06	0.87	1.23	0.45	1.43	0.33	0.52	0.80	0.68	41.43	-
C12B	0.98	0.88	1.12	0.46	1.26	0.37	0.43	0.86	0.57	29.33	-
C13B	0.93	0.66	1.41	0.48	1.27	0.29	0.49	0.91	0.56	26.24	-
C14B	0.94	0.76	1.25	0.41	1.43	0.29	0.58	0.74	0.65	46.60	-
C15B	1.01	0.76	1.33	0.47	1.37	0.38	0.63	0.84	0.58	37.87	-
C16B	0.84	0.83	1.02	0.45	1.19	0.45	0.62	0.74	0.62	37.41	-
C17B	1.08	1.23	0.88	0.53	1.17	0.49	0.66	0.69	0.68	39.17	-
C18B	1.43	1.03	1.39	0.33	1.21	0.33	0.47	0.74	0.45	40.00	-
C19B	1.37	1.37	1.00	0.28	0.98	0.53	0.63	0.48	0.74	49.48	-
C20B	1.26	0.86	1.47	0.41	1.39	0.41	0.6	0.74	0.68	46.06	-
C21B	1.79	1.59	1.13	0.39	1.22	0.35	0.48	0.75	0.66	35.82	-
C22B	1.52	1.11	1.38	0.37	1.26	0.33	0.46	0.72	0.54	40.48	-

Table S10 Echocardiographic data of dogs in DMVD group

ID	LA (cm/kg)	Ao (cm/kg)	LA/Ao	IVSd (cm/kg)	LVIDd (cm/kg)	LVPWd (cm/kg)	IVSs (cm/kg)	LVIDs (cm/kg)	LVPWs (cm/kg)	%FS	PAP (mmHg)
D01B	1.21	0.60	2.02	0.40	1.80	0.32	0.52	1.14	0.46	34.14	-
D02B	1.34	0.77	1.75	0.46	1.90	0.38	0.55	1.01	0.68	44.64	-
D03B	1.81	0.66	2.75	0.40	2.07	0.35	0.61	1.06	0.70	46.93	-
D04B	3.56	1.53	2.33	0.50	1.76	0.46	0.61	1.06	0.58	36.67	-
D05B	2.10	1.23	1.70	0.48	1.93	0.30	0.51	0.94	0.60	49.64	-
D06B	3.06	1.43	2.14	0.46	2.05	0.37	0.55	1.11	0.61	43.21	-
D07B	2.49	1.90	1.31	0.38	1.84	0.28	0.46	0.88	0.78	50.28	-
D08B	1.92	1.24	1.55	0.66	1.28	0.43	0.62	0.66	0.65	46.85	-
D09B	2.15	1.22	1.76	0.49	1.87	0.51	0.68	1.13	0.51	37.33	-
D10B	2.23	1.17	1.91	0.50	1.94	0.36	0.78	0.76	0.80	59.55	-
D11B	2.61	1.68	1.55	0.43	1.52	0.43	0.39	0.82	0.82	43.75	-
D12B	2.22	1.18	1.87	0.63	1.77	0.47	0.73	1.06	0.69	38.89	-
D13B	1.96	1.23	1.59	0.48	1.78	0.43	0.56	1.04	0.6	39.74	-
D14B	1.86	1.18	1.58	0.40	1.64	0.33	0.56	0.97	0.63	38.51	-
D15B	2.33	0.87	2.66	0.46	1.49	0.40	0.51	0.90	0.63	38.33	-
D16B	2.58	0.95	2.71	0.39	2.02	0.37	0.57	1.06	0.67	45.29	-
D17B	1.45	0.92	1.58	0.5	1.63	0.49	0.83	0.66	0.88	58.62	-
D18B	2.25	1.40	1.60	0.47	1.91	0.37	0.49	1.15	0.65	37.14	-
D19B	3.04	1.10	2.77	0.53	2.17	0.28	0.88	0.75	0.87	64.00	-
D20B	2.74	1.16	2.37	0.37	2.26	0.39	0.63	1.21	0.72	44.55	-

Table S11 Echocardiographic data of dogs in DMVD+PH group

ID	LA (cm/kg)	Ao (cm/kg)	LA/Ao	IVSd (cm/kg)	LVIDd (cm/kg)	LVPWd (cm/kg)	IVSs (cm/kg)	LVIDs (cm/kg)	LVPWs (cm/kg)	%FS	Peak TR velocity (m/s)	PAP (mmHg)
P01B	0.71	0.46	1.54	0.40	0.82	0.41	0.49	0.44	0.49	43.37	4.03	64.96
P02B	1.65	0.76	2.19	0.54	1.81	0.46	0.57	1.02	0.59	41.55	3.76	56.55
P03B	1.72	0.78	2.23	0.43	1.87	0.34	0.72	1.13	0.30	37.06	4.12	67.90
P04B	1.37	0.69	2.01	0.40	1.71	0.32	0.51	0.73	0.72	55.74	3.83	58.68
P05B	1.90	0.62	3.10	0.53	2.00	0.48	0.85	0.94	0.78	51.64	3.72	55.35
P06B	1.63	0.53	3.10	0.48	1.78	0.35	0.62	0.67	0.82	61.25	3.55	50.41
P07B	1.47	0.91	1.63	0.49	1.77	0.43	0.60	1.07	0.70	37.19	4.09	66.91
P08B	1.25	0.56	2.24	0.41	1.80	0.35	0.78	0.72	0.70	58.30	3.28	43.03
P09B	1.41	0.68	2.09	0.56	1.36	0.45	0.59	0.64	0.94	50.61	4.48	80.28
P10B	1.87	0.84	2.24	0.33	2.29	0.40	0.64	1.19	0.73	46.75	3.30	43.56
P11B	1.54	0.72	2.14	0.45	2.16	0.36	0.56	1.18	0.56	43.44	3.51	49.28
P12B	0.85	0.55	1.55	0.25	0.85	0.28	0.35	0.40	0.48	50.69	5.69	129.50
P13B	2.99	1.44	2.08	0.4	2.40	0.41	0.67	1.45	0.57	37.28	3.52	49.51
P14B	2.48	1.13	2.19	0.34	1.76	0.33	0.54	0.76	0.73	55.1	3.40	46.19
P15B	3.39	1.64	2.07	1.44	1.89	0.43	0.76	0.75	0.85	58.38	3.20	40.87
P16B	1.29	1.63	0.79	0.74	1.35	0.45	0.70	0.43	0.88	66.67	4.591	84.31
P17B	2.55	1.13	2.25	0.46	1.76	0.37	0.65	0.78	0.61	53.96	5.36	115.06
P18B	2.93	1.14	2.58	0.54	1.97	0.36	1.00	0.68	0.81	64.67	3.33	44.23
P19B	3.70	1.66	2.23	0.42	2.36	0.39	0.60	1.25	0.59	45.16	3.35	44.80
P20B	2.19	0.95	2.29	0.54	1.88	0.41	0.76	1.04	0.62	43.23	3.29	43.28

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