ศักยภาพของยาไอวาบราดีนในการฟื้นฟูการทำงานของหัวใจในสุนัขที่มีภาวะลิ้นหัวใจไมตรัลเสื่อมแบบไม่แสดงอาการที่เกิดขึ้นตามธรรมชาติ

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POTENTIALS OF IVABRADINE TO IMPROVE CARDIAC FUNCTION IN DOGS WITH NATURALLY OCCURRING, ASYMPTOMATIC DEGENERATIVE MITRAL VALVE DISEASE

Miss Prapawadee Pirintr

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Animal Physiology
Department of Veterinary Physiology
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ประภาวดี ไพรินทร์ : ศักยภาพของยาไอวาบราดีนในการฟื้นฟูการทำงานของหัวใจในสุนัขที่มีภาวะลิ้นหัวใจไมตรัลเสื่อมแบบไม่แสดงอาการที่เกิดขึ้นตามธรรมชาติ (POTENTIALS OF IVABRADINE TO IMPROVE CARDIAC FUNCTION IN DOGS WITH NATURALLY OCCURRING, ASYMPTOMATIC DEGENERATIVE MITRAL VALVE DISEASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร.อนุศักดิ์ กิจถาวรรัตน์, 101 หน้า.

สมมุติฐานหลักของการศึกษานี้คือ ยาไอวาบราดีนขนาด 1.0 มก./กก. สามารถลดอัตราการเต้นของหัวใจ ลดปริมาณความต้องการใช้ออกซิเจนของกล้ามเนื้อหัวใจ และช่วยฟื้นฟูการทำงานของหัวใจในสุนัขที่มีภาวะลิ้นหัวใจไมตรัลเสื่อม เนื่องจากมีการลดลงของการตายของเซลล์กล้ามเนื้อหัวใจแบบอะพอพโทซิส เพื่อทดสอบสมมุติฐานดังกล่าว จึงทำการแบ่งการศึกษาออกเป็น 3 ส่วน โดยส่วนที่ 1 ได้ทำการศึกษาเพื่อหาขนาดยาที่เหมาะสม ที่สามารถลดอัตราการเต้นของหัวใจ และปริมาณความต้องการใช้ออกซิเจนของกล้ามเนื้อหัวใจที่ประเมินได้จากค่า rate-pressure product ซึ่งคำนวณได้จากอัตราการเต้นของหัวใจ และความดันซิสโตลิก โดยขนาดยาที่เหมาะสมที่ได้จากการศึกษาที่ 1 จะถูกนำไปใช้ต่อในการศึกษาส่วนที่ 2 เพื่อศึกษาผลของการให้ยาไอวาบราดีนในรูปแบบกินที่ให้อย่างต่อเนื่อง ต่อปริมาณความต้องการใช้ออกซิเจนของกล้ามเนื้อหัวใจ ความดันโลหิตร่างกาย การทำงานของหัวใจห้องล่าง คลื่นไฟฟ้าหัวใจ และความแปรปรวนในอัตราการเต้นของหัวใจ ในสุนัขที่ภาวะลิ้นหัวใจไมตรัลเสื่อมแบบไม่แสดงอาการ การศึกษาส่วนที่ 3 จะดำเนินควบคู่ไปกับการศึกษาส่วนที่ 2 โดยมีวัตถุประสงค์เพื่อประเมินสัดส่วนของโปรตีนที่เหนี่ยวนำให้เกิดการตายของเซลล์แบบอะพอพโทซิส ต่อโปรตีนที่ยับยั้งการตายของเซลล์แบบอะพอพโทซิสของกล้ามเนื้อหัวใจส่วนที่ติดกับเอ็นโดคาร์เดียม เปรียบเทียบระหว่างก่อนและหลังได้รับยาไอวาบราดีน 3 เดือน ในการศึกษาส่วนที่ 1 นำสุนัขพันธุ์บีเกิ้ลที่มีภาวะลิ้นหัวใจไมตรัลเสื่อมแบบไม่แสดงอาการ (ระยะ B2) ที่เกิดขึ้นเองโดยธรรมชาติจำนวน 7 ตัว มาติดเครื่องบันทึกคลื่นไฟฟ้าหัวใจแบบต่อเนื่องเพื่อวัดการเต้นของหัวใจเป็นเวลา 24 ชั่วโมง และวัดความดันโลหิตแบบต่อเนื่องเป็นเวลา 12 ชั่วโมง สุนัขแต่ละตัวจะถูกสุ่มเพื่อรับยาในขนาดต่าง ๆ ได้แก่ ยาหลอก หรือไอวาบราดีนในขนาด 0.5 1.0 และ 2.0 มก./กก. ผลการทดลองพบว่า ขนาดของยามีผลในการลดอัตราการเต้นของหัวใจ และปริมาณความต้องการใช้ออกซิเจนของกล้ามเนื้อหัวใจโดยขึ้นอยู่กับขนาดของยาที่ได้รับ และไม่พบผลข้างเคียงจากขนาดยาที่ใช้ ยกเว้นยาในขนาด 2 มก./กก. ที่มีผลลดความดันโลหิตซีสโตลิก และความดันโลหิตเฉลี่ย จากการทดลองสรุปได้ว่าการให้ยาไอวาบราดีในรูปแบบกินในขนาด 1.0 มก./กก. เหมาะสมที่จะนำมาใช้ในสุนัขที่มีภาวะลิ้นหัวใจไมตรัลเสื่อม โดยสามารถช่วยลดอัตราการเต้นของหัวใจ และปริมาณความต้องการใช้ออกซิเจนของกล้ามเนื้อหัวใจได้โดยไม่มีผลต่อความดันโลหิตของร่างกาย ในการศึกษาส่วนที่ 2 นำสุนัขพันธุ์บีเกิ้ลที่มีภาวะลิ้นหัวใจไมตรัลเสื่อมแบบไม่แสดงอาการ (ระยะ B2) ที่เกิดขึ้นเองโดยธรรมชาติจำนวน 4 ตัว มาติดเครื่องบันทึกคลื่นไฟฟ้าหัวใจแบบต่อเนื่อง 24 ชั่วโมง เพื่อวัดการเต้นของหัวใจ และนำไปหาค่าความแปรปรวนในอัตราการเต้นของหัวใจ ทำการวัดความดันโลหิตและอัตราการเต้นของหัวใจเพื่อนำไปคำนวณค่า rate-pressure product ทำการวัดคลื่นไฟฟ้าหัวใจและทำการวิเคราะห์การทำงานของหัวใจด้วยเครื่องเสียงสะท้อนความถี่สูง โดยสุนัขแต่ละตัวได้รับยาไอวาบราดีนในขนาด 1.0 มก./กก. วันละ 2 ครั้ง เช้า-เย็น เป็นเวลา 3 เดือน และทำการเก็บข้อมูลที่ช่วงเวลาก่อนได้รับยาและหลังการป้อนยา ที่ระยะเวลา 1 เดือน 2 เดือน และ 3 เดือน ผลการทดลองพบว่าการได้รับยาไอวาบราดีนแบบระยะยาวมีผลในการลดอัตราการเต้นของหัวใจ ความดันโลหิต และปริมาณความต้องการใช้ออกซิเจนของกล้ามเนื้อหัวใจอย่างมีนัยสำคัญ ($P < 0.05$) โดยไม่พบผลข้างเคียง ที่ระยะ 3 เดือนหลังจากที่สุนัขได้รับยาไอวาบราดีนพบว่าค่าความแปรปรวนในอัตราการเต้นของหัวใจวิเคราะห์ตามช่วงเวลาและตามความถี่เพิ่มขึ้นอย่างมีนัยสำคัญ ($P < 0.05$) ในส่วนของการวัดการทำงานของหัวใจด้วยเครื่องเสียงสะท้อนความถี่สูง แบบ speckle-tracking พบว่าที่ระยะ 2 และ 3 เดือนหลังจากที่สุนัขได้รับยา ค่า global radial strain, global circumferential strain และ fractional area change เพิ่มขึ้นอย่างมีนัยสำคัญ ($P < 0.05$) เมื่อเทียบกับช่วงก่อนได้รับยา ในการศึกษาส่วนที่ 3 ทำการวัดการไหลเวียนเลือด และการทำงานของหัวใจด้วยวิธี invasive ในช่วงก่อนที่สุนัขจะได้รับยา และ3 เดือนหลังจากที่สุนัขได้รับยา ผลการทดลองพบการได้รับยาไอวาบราดีนแบบต่อเนื่องไม่มีผลต่อการไหลเวียนเลือด และการทำงานของหัวใจ ยกเว้นค่าความสามารถในการบีบตัวของหัวใจ โดยพบว่าค่าความสามารถในการบีบตัวของหัวใจเพิ่มขึ้นอย่างมีนัยสำคัญ ($P < 0.05$) เมื่อเทียบกับช่วงก่อนได้รับยา การวิเคราะห์ชิ้นกล้ามเนื้อหัวใจส่วนที่ติดกับเอ็นโดคาร์เดียมพบว่าไอวาบราดีนสามารถลดการเกิด cardiac fibrosis และ มีแนวโน้มในการลดสัดส่วนของโปรตีนที่เหนี่ยวนำให้เกิดการตายของเซลล์แบบอะพอพโทซิสต่อโปรตีนที่ยับยั้งการตายของเซลล์แบบอะพอพโทซิสซึ่งสัมพันธ์กับการลดลงของกระบวนการการตายของเซลล์แบบอะพอพโทซิสของกล้ามเนื้อหัวใจ จากผลการทดลองทั้งหมดจึงสรุปได้ว่ายาไอวาบราดีนรูปแบบกินในขนาด 1.0 มก./กก. วันละ 2 ครั้ง เช้า-เย็น สามารถลดอัตราการเต้นของหัวใจ ปริมาณความต้องการใช้ออกซิเจนของกล้ามเนื้อหัวใจ และช่วยฟื้นฟูการทำงานของหัวใจในสุนัขที่มีภาวะลิ้นหัวใจไมตรัลเสื่อมแบบไม่แสดงอาการ ส่วนหนึ่งเนื่องจากการลดลงของการตายของเซลล์กล้ามเนื้อหัวใจแบบอะพอพโทซิส

ภาควิชา สรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สรีรวิทยาการสัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560

5875508031 : MAJOR ANIMAL PHYSIOLOGY
KEYWORDS: APOPTOSIS, DEGENERATIVE MITRAL VALVE DISEASE, DOG, HEART RATE VARIABILITY, IVABRADINE, MYOCARDIAL OXYGEN CONSUMPTION, VENTRICULAR FUNCTION

PRAPAWADEE PIRINTR: POTENTIALS OF IVABRADINE TO IMPROVE CARDIAC FUNCTION IN DOGS WITH NATURALLY OCCURRING, ASYMPTOMATIC DEGENERATIVE MITRAL VALVE DISEASE. ADVISOR: ASST. PROF. ANUSAK KIJTAWORNRAT, D.V.M., Ph.D., 101 pp.

The main hypothesis of the present study is that ivabradine (1.0 mg/kg, orally, twice daily) can reduce heart rate (HR), myocardial oxygen consumption (MVO_2) and improve cardiac function in dogs with degenerative mitral valve disease (DMVD) partly due to a reduction of cardiomyocyte apoptosis. In order to test the hypothesis, this study was divided into three parts. The first part aimed to determine the appropriate single oral dose of ivabradine for reduction of HR and MVO_2 as assessed by rate-pressure product (RPP= HR x systolic blood pressure). Once the appropriate dose was achieved, the second part was conducted to investigate the long-term effects of repeated oral dose of ivabradine on MVO_2, blood pressure (BP), ventricular function, electrocardiographic (ECG) parameters and HR variability (HRV). Simultaneously with study part 2, the study part 3 aimed to evaluate cardiomyocyte apoptosis by investigated the ratio of Bax (pro-apoptotic protein) to Bcl-2 (anti-apoptotic protein) from the endomyocardial tissues compared between before and 3 months after treatment with ivabradine. In the study part 1, seven beagles with naturally occurring DMVD stage B2 were instrumented with the Holter recorder and an oscillometric device to measure ECG and BP for 24 and 12 h, respectively, after drug administration. Each dog was randomly subjected to receive either placebo or ivabradine (0.5, 1.0 and 2.0 mg/kg). The results revealed that oral administration of ivabradine significantly decreased the HR and RPP in a dose-dependent manner without any significant adverse effects. The highest dose of 2.0 mg/kg significantly reduced systolic and mean BP. Therefore, the findings imply that a single oral administration of ivabradine at a dose of 1.0 mg/kg is suitable for dogs with asymptomatic DMVD to reduce the HR and MVO_2 without remarkable effects on BP. For the study part 2, four beagles with naturally occurring DMVD stage B2 were instrumented with a 24-h Holter recorder to measure HR and HRV, a device to acquire HR and BP to calculate RPP, ECG to measure cardiac electrical activity and an echocardiography to measure cardiac function. Dogs were given ivabradine (1.0 mg/kg twice daily, orally) for 3 months. Data were obtained at baseline and monthly after oral administration of ivabradine for 3 months (M1 = 1 month, M2 = 2 months, and M3 = 3 months). The results revealed that chronic administration of IVA significantly decreased the HR, BP, and RPP without adverse effects ($P < 0.05$). All indices of time- and frequency- domains of HRV at M3 were increased significantly when compared with baseline values ($P < 0.05$). Indices of speckle-tracking echocardiography including global radial strain, global circumferential strain, and fractional area change measured at M2 and M3 were significantly increased when compared with baseline ($P < 0.05$). In the study part 3, hemodynamic and cardiac function were assessed by invasive technique at baseline and M3. The results revealed that chronic ivabradine treatment did not affect hemodynamic and cardiac function except for the contractility index in which it was increased. The tissue biopsy from endomyocardium of dogs at before and after treatment revealed that ivabradine decreased cardiac fibrosis and tended to reduce BAX to Bcl-2 ratio which is relating to the reduction in cardiomyocyte apoptosis. All of these results suggested that ivabradine (1.0 mg/kg, orally, twice daily) reduces HR, MVO_2 and improve cardiac function in dogs with DMVD partly due to a reduction of cardiomyocyte apoptosis.

Department: Veterinary Physiology
Field of Study: Animal Physiology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....

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LIST OF ABBREVIATION

2D	2-dimension
%	percent
β	beta
℃	degree celsius
f	funny
μm	micrometer
®	registered trademark symbol
ACE	angiotensin converting enzyme
ACVIM	American College of Veterinary Internal Medicine
AF	atrial fibrillation
AI	apoptosis index
ALP	alkaline phosphatase
ALT	alanine transaminase
ANOVA	analysis of variance
ANS	autonomic nervous system
AoP	aortic pressure
Bax	Bcl-2 associated X protein; pro-apoptotic proteins expression
Bcl-2	B-cell Lymphoma 2; an anti-apoptotic proteins expression
BP	blood pressure
bpm	beat per minute
BUN	blood urea nitrogen
cAMP	cyclic adenosine monophosphate
CANS	cardiac autonomic nervous system
CBC	complete blood count
CHF	congestive heart failure
CI	contractility index
cm	centimeter
cmH_2O	centimeter of water

CO	cardiac output
CO_2	carbon dioxide
DBP	diastolic blood pressure
DMVD	degenerative mitral valve disease
DNA	deoxyribonucleic acid
dP/dt_{max}	the maximum rate of rise of the left ventricular pressure curve
dP/dt_{min}	the maximum rate of fall of the left ventricular pressure curve
$dyn.s.cm^{-5}$	units for measuring vascular resistance
ECG	electrocardiogram
ECM	extracellular matrix
EDA	end-diastolic area
EDV	end-diastolic volume
EF	ejection fraction
ESA	end-systolic area
ESV	end-systolic volume
FAC	fractional area change
FDA	Food and Drug Administration
FFT	Fast Fourier transformation
Fr	french gauge
FS	fractional shortening
GCS	global circumferential strain
GRS	global circumferential strain
h	hour
H & E	hematoxylin and eosin
HCN	hyperpolarisation-activated cyclic nucleotide-gated
HF	heart failure/ high frequency
HR	heart rate
HRV	heart rate variability
IHC	Immunohistochemistry
IVSd	interventricular septum diastole

IVSs	interventricular septum systole
I_f	funny current
IVA	ivabradine
K^+	potassium ion
K_2EDTA	dipotassium ethylenediaminetetraacetic acid
kg	kilogram
LA	left atrium
LF	low frequency
LV	left ventricle
LVIDd	left ventricular internal diameter diastole
LVIDs	left ventricular internal dimension systole
LVP	left ventricular pressure
LVPWd	left ventricular posterior wall diastole
LVPWs	left ventricular posterior wall systole
MBP	mean blood pressure
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MV E/A	mitral valve early filling/atrial filling velocities
mg	milligram
min	minute
mL	milliliter
mmHg	millimeters of mercury
M-mode	motion display of the ultrasound wave
MR	mitral regurgitation
ms	millisecond
ms^2	millisecond square
MVO_2	myocardial oxygen consumption
Na^+	sodium ion
NN	normal to normal RR intervals

NNA	the mean NN intervals
NNDrms	the square root of the mean of the squares of the differences between successive normal to normal RR intervals
NNSD	standard deviation of all normal to normal RR intervals
O_2	oxygen
PAP	pulmonary artery pressure
PAS	periodic acid-Schiff
PCD	programmed cell death
PCWP	pulmonary capillary wedge pressure
pNN50	the percentage of successive normal RR intervals exceeding 50 ms
P.O.	per os or oral administration
PQ interval	the interval from the beginning of the P wave to the beginning of the QRS complex
PRSW	preload recruitable stroke work
PSAXAP	parasternal short axis apex
PSAXB	parasternal short axis base
PSAXM	parasternal short axis middle
PSD	power spectral density
P value	the probability of obtaining a test static at least as extreme as the one that was actually observed
PVR	pulmonary vascular resistance
QRS complexes	A combination of the Q wave, R wave and S wave of an electrocardiogram
QT interval	the duration from the beginning of Q wave to the end of T wave
QTc	The corrected QT interval
RAAS	renin-angiotensin aldosterone system
RAP	right atrial pressure
rMSSD	the square root of the mean of the squares of the differences between successive normal to normal RR intervals
RPP	rate pressure product

RR intervals	the interval from the peak of the R wave to the peak of the next R wave
SA	Sinoatrial
SASD	the standard deviation of 5-minute mean RR intervals
SBP	systolic blood pressure
SDANN	the standard deviation of 5-minute mean RR intervals
SDNN	standard deviation of all normal to normal RR intervals
SDNN index	the mean of the standard deviation of all normal-to-normal RR intervals for all 5-minute segments
SEM	standard error of mean
SF	shortening fraction
SNS	sympathetic nervous system
SSDA	the mean of the standard deviation of all normal-to-normal RR intervals for all 5-minute segments
STE	speckle tacking echocardiography
SV	stroke volume
SVPC	supraventricular premature complexes
SVR	systemic vascular resistance
Tau	isovolumic relaxation time constant
TM	trade mark sign
TP	total power
VHS	Vertebral heart score
VPC	ventricular premature complexes

CHAPTER I
INTRODUCTION

Canine degenerative mitral valve disease (DMVD) is the most common acquired heart disease found in older dogs (Häggström et al., 2009). Small breed dogs are more susceptible than larger breeds and some breeds are predisposed to the DMVD especially the Cavalier King Charles Spaniel (Häggström et al., 1992). The beginning of the degenerative process results in valve thickening in which it prevents complete valve closure. As a consequence, blood flows backward into the left atrium (LA) during systole known as mitral regurgitation (MR) (Pomerance and Whitney, 1970; Kogure, 1980). Approximately 30% of dogs with DMVD and MR progress to heart failure (HF) (Borgarelli and Häggström, 2010). In small animal medicine, there are no medications that has been proved to prevent left ventricular (LV) remodeling or delayed the DMVD progression. According to the American College of Veterinary Internal Medicine (ACVIM), DMVD can be divided into four stages: asymptomatic stages A and B and symptomatic stages C and D. The most prescribed medicines when clinical signs develop are angiotensin converting enzyme (ACE) inhibitors, diuretics and pimobendan (Atkins and Häggström, 2012). While there is a consensus on pharmacological treatment of symptomatic DMVD, the management of asymptomatic DMVD is still unclear.

The stimulation of sympathetic nervous system (SNS) and the renin-angiotensin aldosterone system (RAAS) are the major compensatory mechanisms responsible for reduction in cardiac output (CO) (Ware et al., 1990). The consequences of neuro-hormonal activation are including increased blood pressure (BP) and heart rate (HR), and impaired quality of life. Elevated HR resulted in increased workload of the heart so that the heart needs more energy as well as oxygen to perform its task. A recent publication demonstrated that dogs with asymptomatic DVMD had a significantly higher HR and systemic blood pressure than age-matched control dogs (Pirintr et al., 2017). An elevated HR and systemic blood pressure contributes to increase myocardial oxygen consumption (MVO_2) (Saito et al., 1981). In addition, coronary blood flow and

myocardial perfusion are reduced while the left ventricular end diastolic pressure is increased (Cober et al., 2011). These factors may aggravate the compensation mechanism of the heart. Therefore, a reduction of the HR may be useful in managing an increased MVO_2 in dogs with asymptomatic DMVD. Moreover, the progression of DMVD leads to decrease cardiac autonomic nervous system (CANS) activity as assessed by decreased heart rate variability (HRV) that has been used as a predictor of risk for sudden death after myocardial infarction (Rasmussen et al., 2012).

The pharmacological modulation of HR is important in the treatment of this condition. In veterinary medicine, β-blockers (i.e. atenolol, carvedilol) are the most frequently used drugs for reducing HR. While they are effective BP reducer, they possess adverse effects on cardiac functions such as decreased myocardial contractility (Kijtawornrat et al., 2014). Therefore, concerns related to adverse effects of β-blockers may limit its clinical utility.

Ivabradine is a relatively novel agent that has been developed and demonstrated a pure HR reduction and reduced MVO_2 in conscious dogs (Colin et al., 2004). Ivabradine has been approved for clinical use in patients with coronary artery disease and ischemic heart disease by the European Medicines Agency since 2005 and by the United States Food and Drug Administration since 2015 but it is no veterinary-labeled for use in dogs and cats (Bucchi et al., 2007; Rosano et al., 2014; FDA, 2015b)

The compensatory mechanisms are important to survive but those are maladaptive and may injure the heart and circulatory system. If there is no intervention, HF will occurred as a result of cardiac apoptosis. The study of Becher and colleagues (2012) showed that ivabradine could improve systolic and diastolic LV functions associated with less cardiac hypertrophy, fibrosis, inflammation and cardiac apoptosis in rats with mild arterial hypertension and congestive heart failure (CHF) when compared with metoprolol treatment (Becher et al., 2012). In cats, several studies showed that ivabradine has negative chronotropic effect in healthy cats, reduced MVO_2 and safety administered at 0.3 and 0.5 mg/kg (Cober et al., 2011; Riesen et al., 2011). In healthy cats, long-term treatment with ivabradine at a dose of 0.3 mg/kg (P.O. q 12 h) for 4 weeks, demonstrated more favorable effects than atenolol

at a dose of 1.0-1.7 mg/kg (P.O. q 12 h) with regard to negative chronotropic effect, reduction of MVO_2, LV function, LA performance and clinical tolerance after (Riesen et al., 2011).

In several animal models and humans, HR reduction by ivabradine is showing possibility to prevent LV remodeling or delay the DMVD progression. However, there are no data to support the use of ivabradine in dogs. As far as we known, the therapeutic dose of ivabradine for HR reduction and cardiovascular effects of ivabradine in dogs with asymptomatic MR have not yet been studied. Furthermore, its mechanisms to improve cardiac function remain unclear.

The research questions of this study were as follow:

1) What is the appropriate oral ivabradine dose (0.5, 1.0 and 2.0 mg/kg) for reduction of HR and MVO_2 in dogs with asymptomatic DMVD stage B2?
2) What are the long-term effects of repeated oral dose of ivabradine on HR, BP, MVO_2, cardiac function, electrocardiographic (ECG) parameters, heart rate variability (HRV) and hemodynamic in dogs with asymptomatic DMVD stage B2?
3) What are the role of cardiomyocyte apoptosis assessed by changes of the percentage of cardiomyocyte apoptosis, anti-apoptotic proteins (Bcl-2) expression, pro-apoptotic proteins (Bax) expression and the ratio of Bax to Bcl-2 after long-term treatment with repeated oral dose of ivabradine in dogs with asymptomatic DMVD stage B2?

Therefore the objectives of the present study were as follow:

1) To determine the appropriate single oral dose of ivabradine for reduction of HR and MVO_2 in dogs with asymptomatic DMVD stage B2.
2) To investigate the long-term effects of repeated oral dose of appropriate ivabradine on HR, BP, MVO_2, cardiac function, ECG parameters, HRV and hemodynamic in dogs with asymptomatic DMVD stage B2.

3) To explore the role of the long-term treatment with repeated oral dose of ivabradine involving hemodynamic, cardiac function and cardiac apoptosis in dogs with asymptomatic DMVD stage B2.

The hypotheses of this study were as follow:

1) The oral dose of ivabradine at 1.0 mg/kg is appropriate to reduce HR and MVO_2 in dogs with asymptomatic DMVD stage B2.
2) The long-term treatment with appropriate repeated oral dose of ivabradine can reduce HR, BP, MVO_2 while it improves cardiac autonomic activity and function, ECG parameters and hemodynamic in dogs with asymptomatic DMVD stage B2.
3) The percentage of cardiomyocyte apoptosis, anti-apoptotic proteins (Bcl-2) expression, pro-apoptotic proteins (Bax) expression and the ratio of Bax to Bcl-2 in dogs with asymptomatic DMVD may be reduced by long-term treatment with appropriate repeated oral dose of ivabradine when compare with baseline.

The expectations of the present study were as follow:

1) In study part 1, the appropriate oral dose of ivabradine for HR reduction and improvement of MVO_2 in dogs with DMVD stage B2 was clarified and was use in the study part 2.
2) In study part 2, the long-term effect of repeated oral doses of ivabradine on HR reduction, BP, MVO_2, HRV, cardiac function, ECG parameters and hemodynamic in dogs with DMVD stage B2 were elucidated.
3) In study part 3, the role of ivabradine that improve cardiac function in dogs with DMVD were explored.

CHAPTER II
LITERATURE REVEWS

A. Canine degenerative mitral valve disease

DMVD is the most common acquired heart disease in older dogs (Häggström et al., 2009). It represents the 75% of all cardiovascular diseases; affecting primary the mitral valve, while in the 30% of the affected dogs involves both mitral and tricuspid valves (Atkins et al., 2009). The cause of canine DMVD is currently unknown, although a genetic tendency to develop the disease has been proven. In Cavalier King Charles Spaniel, a polygenic mode of inheritance is suggested by a strong inherited component, and recently two loci have been associated with DMVD (Parker and Kilroy-Glynn, 2012). In addition, C-reactive protein is high in dogs with DMVD, suggesting a possible role of systemic inflammation in the pathogenesis (Rush et al., 2006).

Clinical presentation:

The early clinical finding is a systolic heart murmur in the absence of any sign of cardiac decompensation known as pre-clinical phase. The period between the first identification of heart murmur and the onset of clinical sign is vary but generally takes several years. The first symptom is exercise intolerance, while cough and dyspnea appear in the second stage of the HF. When progressive cardiac remodeling reaches a certain point, overt HF occurs. Death due to DMVD is the most often mediated by CHF can occasionally occur.

ACVIM consensus:

Recently, the American College of Veterinary Internal Medicine (ACVIM) Specialty of Cardiology consensus panel convened to formulate guidelines for the diagnosis and treatment of chronic valvular heart disease twice, first in 2009 and second in 2012 (Atkins et al., 2009; Atkins and Häggström, 2012). The current ACVIM classification system describes 4 stages of heart disease and failure and is meant to complement the previous functional classification. Classification A is patients at high risk for developing heart disease but that currently have no identifiable structural

disorder of the heart. Classification B is asymptomatic patients that have no radiographic or echocardiographic evidence of cardiac remodeling in response to chronic valvular heart disease (B1) or asymptomatic patients that have hemodynamically significant valve regurgitation (B2). Classification C is patients with clinical signs of acute CHF, secondary to severe DMVD and Mitral regurgitation (MR), requiring hospitalization (C1) or stable enough to allow home therapy (C2). Classification D is patients with clinical signs of congestive and/or low output HF, requiring hospitalization (D1) or stable enough to allow home therapy (D2).

Diagnosis of canine DMVD:

In asymptomatic dogs, heart murmur with point of maximal intensity at left apex is the typical clinical finding. The intensity of murmur can be characterized into 6 grades (I-VI/VI) and it has been shown not to correlate with severity of the pathology of DMVD (Ljungvall et al., 2009). The diagnostic finding should be included: physical examination, thoracic radiographs (to verify the presence/absence of cardiomegaly), echocardiography (to confirm the diagnosis and evaluate heart dimension and function) and blood pressure measurement (to exclude systemic hypertension)

Treatment of canine DMVD:

Currently, there is no medical therapy has been proven to stop the progression of disease. In dogs with stage B1/B2, medical therapy is aimed to prolong or delay the onset of CHF while pharmacological therapy of dogs in stages C and D is aimed to improve quality of life. According to ACVIM consensus, no medical therapy is needed in dogs with stage B (Atkins and Häggström, 2012). Dogs with stage C of HF are benefit from given diuretics, angiotensin converting enzyme inhibitors and phosphodiesterase 3 inhibitors together with dietary sodium restriction (Atkins and Häggström, 2012). In stage D dogs, the evidence from clinical trials for different treatments in the veterinary literature is limited; therefore, the ACVIM consensus panel did reach consensus for the following pharmacological measures, in addition to oxygen supplementation, optimal nursing care, sedation, and mechanical measures, such as furosemide, pimobendan and ACE inhibitors (Atkins and Häggström, 2012).

The surgical treatments for MR include valve replacement and valve repair. However, in veterinary medicine, mitral valve surgery has been performed rarely and found only in some countries (Uechi et al., 2012). This may be due to economic and practical limitations.

B. Ivabradine

In the normal, non-diseased state, the funny current (I_f) is highly expressed in the sinoatrial (SA) node, one of the most important ionic currents for regulating the spontaneous diastolic depolarization of the SA node. The I_f current is a voltage-gated, time dependent mixed Na^+ and K^+ inward current that activated by hyperpolarization of membrane potentials and directly regulated by intracellular cyclic AMP (cAMP). The I_f current is carried by the hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels that are intermembrane proteins in the plasma membranes located in brain and heart (Luthi and McCormick, 1998). The HCN channels are encoded by four genes (HCN 1, 2, 3, 4), and HCN4 channel is the most highly expressed in SA node tissue while it has low expression in normal myocardial tissue (Michels et al., 2005). Activation of I_f current leads to increase membrane permeability to sodium and potassium causing a less negative membrane potential.

The I_f current are directly modulated by autonomic nervous system (ANS). Increased sympathetic tone leads to the formation of cAMP through the stimulation of β-receptors. The cAMP binds directly to I_f current activating the channel at a less negative potential which increases the slope of early diastolic depolarization and increases HR. On the other hand, parasympathetic stimulation causes HR reduction (Bucchi et al., 2007).

Ivabradine is a relatively novel agent that has been developed and possessed a pure HR reduction. Ivabradine is a highly selective and specific antagonist of the I_f current at the HCN4 channels (I_f-channels) in SA node tissue without affecting on other ionic currents (Bois et al., 1996). Ivabradine decreased HR by blockade of I_f-channels in

a dose-dependent manner. It binds to I_f-channels in the open state. Consequently, it displays the greatest effect when the HR is highest (Sulfi and Timmis, 2006).

Ivabradine blocks the HCN4 channels in 3 steps: 1) ivabradine diffuses through the cell membrane of the SA node, 2) when the channels are in the open state and the outward current drive, ivabradine enters the pore of HCN4 channel from the intracellular side and tightly binds at its binding site that is located within the inner cavity of HCN4 channels with van der Waals and hydrophobic interactions, and 3) when the channels are closed, the ivabradine is "trapped" in the cavity and it cannot be displaced by either inward or outward flow (Bois et al., 1996; Bucchi et al., 2013).

Ivabradine provides pure HR reduction by decreasing the rate of spontaneous diastolic depolarization through I_f current in SA node without affecting the duration of the action potential, slowing the HR, and allowing more time for blood to perfuse the myocardium by prolonging diastolic filling and coronary diastolic perfusion time (DiFrancesco and Mangoni, 1994; Thollon et al., 1994; Sulfi and Timmis, 2006; de Silva and Fox, 2009).

After oral administration, ivabradine was quickly absorbed with peak plasma concentrations achieved between 0.53 and 1.1 h. The peak effect for reduction in HR is varies between 3 and 4 h. The averaged oral bioavailability is approximately 40%. The 1st phase half-life was up to 1.39 h while the 2nd elimination phase half-life was up to 22 h. The main active metabolite is N-desmethylated. The administration of ivabradine with meal is recommended due to decrease intra-individual viability in exposure because food delayed drug absorption ~1 h and increased plasma exposure by 20-30%. The plasma protein binding of ivabradine is 70%. The cytochrome P450 3A4 at the liver and gut is the only enzyme used to metabolyze ivabradine (FDA, 2015a).

Recently, efficacy and safety of ivabradine in small animals has been conducted. Many studies of ivabradine in cat have shown that treatment with ivabradine resulted in significant reductions of HR and myocardial oxygen consumption, increased LV function, with blunted chronotropic effects of the catecholamine during

stress or exercise but did not affect systemic arterial BP (Cober et al., 2011; Riesen et al., 2012). In healthy, normal dog's heart, ivabradine primarily suppresses the SA node with only mild or no effect on other myocardial tissues (Berdeaux, 2007). Moreover, ivabradine can be used safely in aging dogs and without the risk of atrial fibrillation (AF) in dog even under the vagal stimulation (Li et al., 2015; Uemura et al., 2017).

In cat, many studies of ivabradine have been conducted and optimum dose has been performed. However, no data to support the use of ivabradine in dogs.

C. Myocardial oxygen consumption

Myocardial oxygen consumption (MVO_2) is amount of oxygen that myocardium consumed per minute. Whereas, the oxygen demand is amount of oxygen that myocardium needed. In some conditions, oxygen demand of myocardium may exceed oxygen consumption because the latter may be limited by the delivery of oxygen to the myocardium. The two terms, oxygen consumption and oxygen demand, are often used interchangeably. Heart is a highly oxidative organ and requires a high demand for oxygen and has a relatively high oxygen consumption (Cardiovascular Physiology Concepts, 2007).

Tachycardia is a major contributor to increased MVO_2. Persistent tachycardia limits diastolic coronary blood flow, decreasing myocardial perfusion, leads to an imbalance between myocardial oxygen supply and demand, may induce myocardial ischemia ultimately leading to tissue fibrosis and cardiac remodeling.

MVO_2 can be estimated by using an indirect index such as the rate pressure product (RPP) which equals to systolic blood pressure (SBP) multiply by HR. This can be useful, for example, in clinical trials to determine if a drug reduces oxygen demand. The RPP is based on the observation that MVO_2 is closely related to ventricular wall tension (Cardiovascular Physiology Concepts, 2007).

Efficacy of ivabradine to reduce HR is similar to β-blockers and calcium channel blockers, but lack of adverse effects and contraindications. Moreover, ivabradine could reduce MVO_2 in cats as assessed by the RPP (Cober et al., 2011; Riesen et al., 2012).

As a result, ivabradine may be useful in controlling unwanted tachycardia and reduce MVO_2 to balance myocardial oxygen supply and demand in dogs with asymptomatic MR to delay the DMVD progression or to prevent LV remodeling.

D. Cardiac function

The main function of the heart is to pump enough volume of blood (cardiac output; CO) into vessels in order to generate adequate blood pressure and to provide the organs with enough perfusion pressure. The heart function can be estimated from LV function. The LV function can be assessed by either non-invasive or invasive methods. The gold standard for measurement of ventricular function is the pressure-volume loops relationship (i.e. end-systolic pressure volume relationship, preload recruitable stroke work, end-diastolic pressure volume relationship). However, these methods are invasive and difficult to perform in clinical setting. Currently, the advance in ultrasound technology has made the non-invasive estimation of cardiac function possible. Echocardiography is widely used in the clinic to measure cardiac function since it can be used to diagnosis and monitor treatment responses. By using 2-dimensional (2D), M-mode the contractility of the heart can be estimated from ejection fraction (EF) and shortening fraction (SF). However, these parameters are load dependent so that the afterload and preload should be considered when using these parameters. Recently, two dimensional speckle tracking echocardiography (STE), a new imaging modality that permits measurement of myocardial deformation and velocities (i.e. strain and strain rate), has been developed (Blessberger and Binder, 2010). Strain quantifies the myocardial deformation; therefore, it measures the magnitude of myocardial fiber contraction and relaxation. In rats, it was demonstrated that preload recruitable stroke work (PRSW) correlated robustly with global circumferential strain (GCS) (Kovács et al., 2015).

E. Heart rate variability

In the healthy heart, the beat to beat (RR) intervals are not absolutely regular. Under resting conditions, the ECG of healthy individuals exhibits the variation in RR intervals. The fluctuation in RR intervals is known as HRV (Subbalakshmi et al., 2009). HRV is the result of various influences of the ANS on the HR (Stein and Kleiger, 1999). HRV reflects cardiac autonomic modulation of heart rhythm and detects early improvement of cardiac autonomic regulation (Sztajzel, 2004).

The standard measurements in the analysis of HRV consist of time- and frequency-domain analysis that obtained from the continuous ECG. Changing of the sympathetic and/or parasympathetic activity of the heart could be assessed from the time- and frequency-domain parameters of HRV (Task Force, 1996). Abnormalities of autonomic inputs to the heart result in the decreased indices of HRV.

In clinic practice, HRV has been used as a standard screening method for determination of cardiac autonomic activity (Task Force, 1996; Lewis, 2005). HRV in small animals was used to determine the clinical usefulness of cardiac event and many medical research fields. To date, several articles have been published to observe the relationships between HRV and heart diseases in dogs such as in case of MR (Häggström et al., 1996; Fujii and Wakao, 2003; Spiljak Pakkanen et al., 2012), dilated cardiomyopathy (Minors and O'Grady, 1997; Calvert and Jacobs, 2000; Calvert and Wall, 2001; Pereira et al., 2008) and myocardial infarction (Hull et al., 1990). Previous publications are also indicated that HRV is a very sensitive tool for detecting mild MR in dogs before the onset of clinical signs suggesting the benefit of using this tool to follow up the treatment (Fujii and Wakao, 2003). Moreover, the progression of DMVD assessed by decreased HRV could be used as a predictor of a risk for cardiac sudden death after myocardial infarction (Rasmussen et al., 2012).

In patients and dogs with CHF, the progression of cardiac diseases promote alterations in the cardiac autonomic function which expressed as a sympathovagal imbalance. This may result from sympathetic over activity and/or parasympathetic withdrawal. Therefore, it leads to an increase in HR and a decrease in HRV (Vanderlei

et al., 2009; Oliveira et al., 2014). In addition, the decreased HRV in CHF patients has prognostic significance for mortality (Karcz et al., 2003).

Recent clinical trials in DMVD dogs with MR have suggested that several cardiovascular drugs including enalapril and sildenafil could improve HRV and cardiac function in asymptomatic DMVD dogs (Chompoosan et al., 2014; Kijtawornrat et al., 2017; Pirintr et al., 2017). Despite these numerous clinical investigations, the effects of ivabradine treatment on HRV in DMVD dogs are not available.

F. Apoptosis

Apoptosis is the process of programmed cell death (PCD). It is generally characterized by distinct morphological characteristics of energy-dependent biochemical mechanisms (Elmore, 2007). Series of typical morphological events are morphological characteristic of apoptosis such as loss of cell volume or cell shrinkage, plasma membrane blebbing, nuclear chromatin condensation, chromatin aggregation, DNA fragmentation and membrane-bound apoptotic bodies (Kerr et al., 1972). The pathognomonics of apoptosis are nuclear chromatin condensation and nuclear fragmentation while form dense apoptotic bodies and rapid phagocytosis without induction of an inflammatory response (Dorn, 2009). The mechanisms of apoptosis are highly complex and involving an energy-dependent cascade of molecular events. To date, there are two main apoptotic pathways: the extrinsic or death receptor pathway which utilizes cell surface receptor and the intrinsic pathway involves mitochondria and cytoplasmic reticulum. The Bcl-2, anti-apoptotic, proteins play an important roles as the initiator, regulators and effectors of intrinsic pathway apoptosis while the Bax is a pro-apoptotic protein of intrinsic pathway (Gross et al., 1999). In patients with cardiac pressure overload, Bax protein expression has been found to be increased while Bcl-2 had been found to be decreased indicating apoptosis in those patients (Condorelli et al., 1999).

In HF patients with severe MR and normal sinus rhythm, mitochondrial apoptotic pathway in the atrial cardiomyocyte has been noted (Pu et al., 2009). It has

been found that LV remodeling was observed in animals and patients with asymptomatic MR which implies that apoptosis may be occurred (Zile et al., 1984; Carabello et al., 1989). Interestingly, previous study showed that ivabradine could reduce and/or prevent cardiac hypertrophy, fibrosis, inflammation and cardiac apoptosis when compared with metoprolol treatment (Becher et al., 2012). However, the exact role of ivabradine on cardiomyocyte apoptosis in dogs with MR remain uncover.

CHAPTER III
MATERIALS AND METHODS

Approvals:

This study was approved by the Institutional Animal Care and Use Committee of Chulalongkorn University Laboratory Animal Center (protocol number 1673003). All animal procedures were performed in compliance with the Regulations and Animals for Scientific Purposes Act (A.D. 2015) and followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Experimental design:

In order to test the hypotheses, this study was divided into 3 study parts. The first part aims to determine the appropriate single oral dose of ivabradine for reduction of HR and MVO_2 in dogs with asymptomatic DMVD. Once the appropriate dose was achieved, the second part was conducted to investigate the long-term effects of repeated oral dose of appropriate ivabradine on HR, BP, MVO_2, HRV, cardiac function, ECG parameters and hemodynamic in dogs with asymptomatic DMVD. At the same time of studying long-term effects of oral ivabradine, the role of cardiac apoptosis assessed by changes of the percentage of cardiomyocyte apoptosis, anti-apoptotic proteins (Bcl-2) expression, pro-apoptotic proteins (Bax) expression and the ratio of Bax to Bcl-2 after long-term treatment with repeated oral dose of ivabradine were conducted. The biopsy of endocardium of the LV anterior free wall was performed and tissues were kept for further molecular analysis.

Study part 1: The appropriate single oral dose of ivabradine for reduction of HR and MVO_2 in dogs with asymptomatic DMVD

1. Animals

Seven beagles (*Canis familiaris*) of both genders (two males, five females) were transferred from a breeding colony of the Department of Obstetric Gynecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. They were housed in a group from the time of arrival to the end of the study in a dog run maintained at a temperature between 19°C and 23°C, a relative humidity between 30% and 70%, and a 12-h:12-h dark: light cycle. All animals received commercial chow twice daily, and water was provided ad libitum in stainless steel containers. Physical examination, routine lead II ECG recording, 2D, M-mode and Doppler echocardiography, thoracic radiograph, complete blood cell count (CBC), and blood chemistry analyses were performed to evaluate the health status in all dogs, and to confirm that all dogs were in ACVIM stage B2 (i.e. dogs presented with mitral regurgitation with structural changes but no clinical sign) before beginning the experiment. None of the dogs were on any pharmacological treatment.

2. Drug administration

Ivabradine (Coralan® 7.5 mg/tablet, Les Laboratories Serveir, France) was used in this study (Figure 3-1). Previous publication had shown that oral administration of ivabradine (2 mg/kg) causes adverse effect to the eyes (European Medicines Agency, 2014). We decided to limit our maximum dose of 2 mg/kg and step downward by half of the dose. The dose selection was 0, 0.5, 1.0 and 2.0 mg/kg.

Figure 3-1 The Coralan® 7.5 mg film-coated tablet (Les Laboratories Serveir, France) supplied in a blister strip, in packs containing 56 tablets.

3. Study design and experimental protocol

Repeated and placebo-controlled studies were performed in this study. Each dog underwent four study periods, each continued for 24 h, and was randomized to receive either one dose of ivabradine (0.5, 1.0 and 2.0 mg/kg) or placebo (Table 3-1). The washout period between treatments was at least 2 days as described in a previous publication (Cober et al., 2011).

Table 3-1 Four treatments and four study periods of 7 dogs.

Treatment	Period 1	Period 2	Period 3	Period 4
Dog 1	Placebo	IVA 0.5 mg/kg	IVA 1 mg/kg	IVA 2 mg/kg
Dog 2	IVA 2 mg/kg	Placebo	IVA 0.5 mg/kg	IVA 1 mg/kg
Dog 3	IVA 1 mg/kg	IVA 2 mg/kg	Placebo	IVA 0.5 mg/kg
Dog 4	IVA 0.5 mg/kg	IVA 1 mg/kg	IVA 2 mg/kg	Placebo
Dog 5	Placebo	IVA 0.5 mg/kg	IVA 1 mg/kg	IVA 2 mg/kg
Dog 6	IVA 2 mg/kg	Placebo	IVA 0.5 mg/kg	IVA 1 mg/kg
Dog 7	IVA 1 mg/kg	IVA 2 mg/kg	Placebo	IVA 0.5 mg/kg

IVA: ivabradine

Experimental procedures were started after at least 2 h of fasting. All dogs were brought from their cages to a quiet study room and were attached to a continuous ECG recording instrument (Fukuda Denshi Co., Ltd., Tokyo, Japan). Baseline recordings of arterial blood pressure including SBP, DBP and MBP were performed prior to administration of placebo or ivabradine using an indirect blood pressure method, an oscillometric device (petMAP™, CardioCommand, Inc., Florida, USA), which has been validated and described previously (Vachon et al., 2014). Thereafter, dogs were orally given placebo (i.e. a meat ball) or ivabradine (i.e. a meat ball containing the pills). The feeding behavior of the dogs was monitored for at least 15 min afterward to ensure drug administration. The dogs were left in the room with examiners who took the BP measurement. The arterial blood pressure and pulse rate were recorded every 15 min for the first 12 h, consecutively. The clinical observation after drug administration was monitored hourly for the first 12 h as well. At 12 h after drug administration, the dogs were returned to their home cages. At 24 h after drug administration, the dogs were brought back to the study room to record the last BP, and the Holter monitor was removed. Heart rate and ectopic beats of ECG were monitored continuously for 24 hours using a Holter monitor (Figure 3-2).

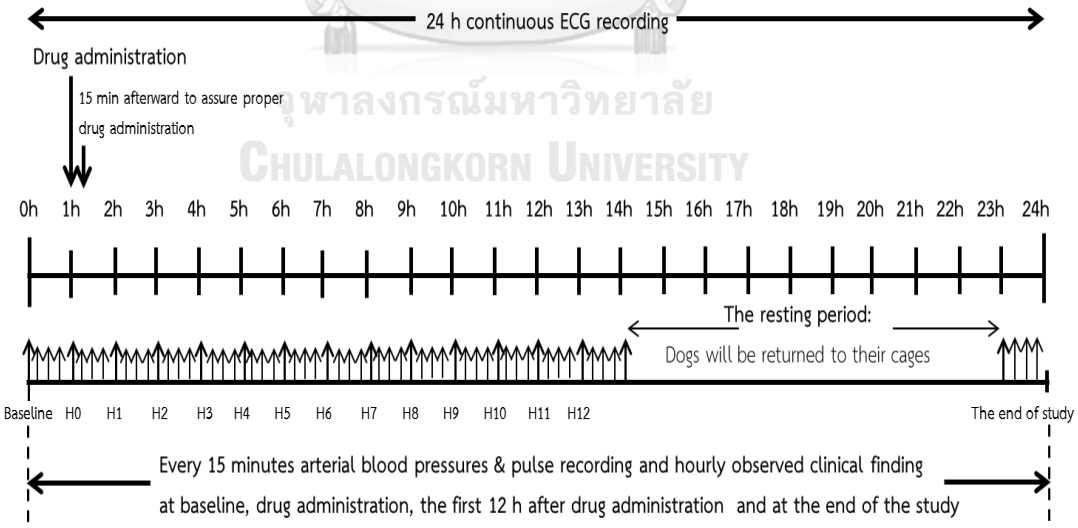


Figure 3-2 The time line and experimental procedure of the study part 1. h = hour after holter recording, H = hour after receiving placebo or ivabradine (0.5, 1.0 or 2.0 mg/kg)

4. Experimental procedures and analytical methods

4.1 Determination of hematology and chemistry profiles

At baseline and at the end of the first study, blood (2 mL) was collected from either cephalic or saphenous veins. One milliliter of blood was kept in heparinized tube and the rest was kept in K_2EDTA tube. Both tubes were submitted to the Diagnostic unit, Faculty of Veterinary Science, Chulalongkorn University for CBC, blood chemical profile analysis (i.e. blood urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase) and blood parasite determination.

4.2 Determination of hourly heart rate by Holter monitor

The Holter monitor was attached to the dogs two hours after they were fed. A standard 5-ECG electrodes were attached to the anterior chest wall of dogs with precodial leads (Figure 3-3). These leads were connected to the monitoring system (Fukuda Denshi Co., Ltd., Japan). The 24 hours of continuous ECG monitoring were performed in a quiet experimental room. The dogs were relaxed in a recumbent position either sternal or lateral and stay awake with minimal restraint and regular breathing. The 24-h continuous ECG was recorded and stored on an SD card for further analysis of hourly HR using SCM-510 Holter software (Fukuda Denshi Co., Ltd., Japan).

All QRS complexes from the 24-h ECG were automatically analyzed by the program and it was carefully manually inspected for the RR intervals by an experienced operator for calculated hourly HR.

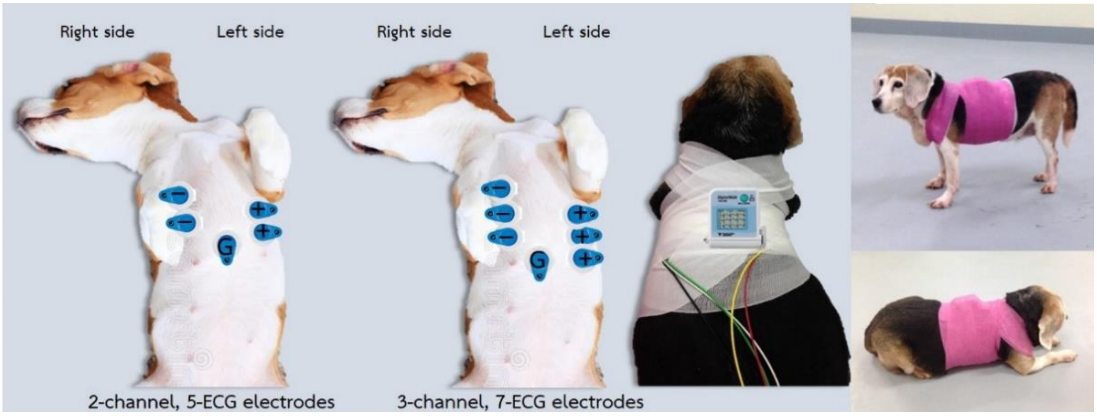


Figure 3-3 The instrumentation for the 24-h continuous ECG recording in experimental dog.

4.3 Determination of blood pressure

The hourly SBP, DBP and MBP were obtained using an oscillometric device (petMAP™, CardioCommand, Inc., Florida, USA). A pressure cuff of appropriate width (approximately 40% of the leg's circumference) was placed at forelimb upon the median artery between the elbow and the carpal pad (Figure 3-4). Every 15 min, five consecutive measurements of BP were performed and average of 3 consistent BP was used.

4.4 Determination of myocardial oxygen consumption

MVO_2 was calculated from the rate-pressure product (RPP), which is SBP multiplied by HR (Nordlander et al., 1989; Cober et Al., 2011).

Figure 3-4 The instrumentation for the indirect blood pressure method, an oscillometric device (petMAP™, CardioCommand, Inc., Florida, USA) in experimental dog.

5. Statistical analysis

All numerical data were presented as mean ± standard error of mean (SEM). Statistical analyses were performed using commercially available software. A normality test (D'Agostino-Pearson omnibus test) was performed to determine whether data were normally distributed. In each figure (Figure 4-1, 4-3 and 4-4), the adjusted baseline of each parameter was plotted against collected time points. The HR, BP (SBP, DBP, and MBP) and RPP were compared among treatment groups (placebo and ivabradine 0.5, 1.0 and 2.0 mg/kg) and time points (baseline, 1-12 h and 24 h) using two-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. In all cases, a *P* value < 0.05 was considered to be statistically significant.

Study part 2: The long-term effects of repeated oral dose of appropriate ivabradine on HR, BP, MVO_2, cardiac function, ECG parameters and HRV in dogs with asymptomatic DMVD

1. Animals

Four beagles (*Canis familiaris*) of both genders (two males, two females) were transferred from the first study. Dogs were housed as previously described in the study part 1. Physical examination, routine lead II ECG recording, 2D, M-mode and Doppler echocardiography, thoracic radiograph, complete blood count, and blood chemistry analyses were performed to evaluate the health status in all dogs, and to confirm that all dogs were in ACVIM stage B2 before beginning the experiment. None of the dogs were on any pharmacological treatment.

2. Drug administration

The dose of ivabradine (Coralan® 7.5 mg/tablet, Les Laboratories Serveir, France) used in the second study was chosen base on the study part 1. The criteria for selection were as follow: 1) no adverse reaction (i.e. induced vomiting, syncope), 2) reduced HR more than 5% from baseline but less than 20% from baseline, 3) did

not increase RPP and 4) did not induce systemic hypotension and hypertension. The drug was administered twice a day according to our result in the study part 1 and previous publications in humans and cats (Swedberg et al., 2010; Cober et al., 2011).

3. Study design and experimental protocol

A prospective cohort study of long-term effects of repeated oral dose of ivabradine was conducted in this part. The dose of ivabradine for each dog was prepared by inserted in a meat ball just before dosing. The treatment of ivabradine was started one week after baseline endomyocardial tissue biopsy in study part 3. Ivabradine was administered to all dogs twice daily by the researchers or laboratory animal technicians. Dogs were monitored for at least 15 min after dosing to ensure proper drug administration. Physical examination, blood collection for hematology and biochemistry profile (i.e. blood urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase) and thoracic radiograph were performed in all dogs at baseline and again at the end of study period (3 months). The Holter monitoring, ECG, echocardiography and BP measurements were evaluated at baseline and monthly after dosing for a total of 3 months (Figure 3-5). In addition, the clinical variables of all dogs such as appetite, behavior, activity, defecation, urination, vomiting, respiration and BP were evaluated before dosing of each day to monitor adverse reaction (e.g. low BP and bradycardia).

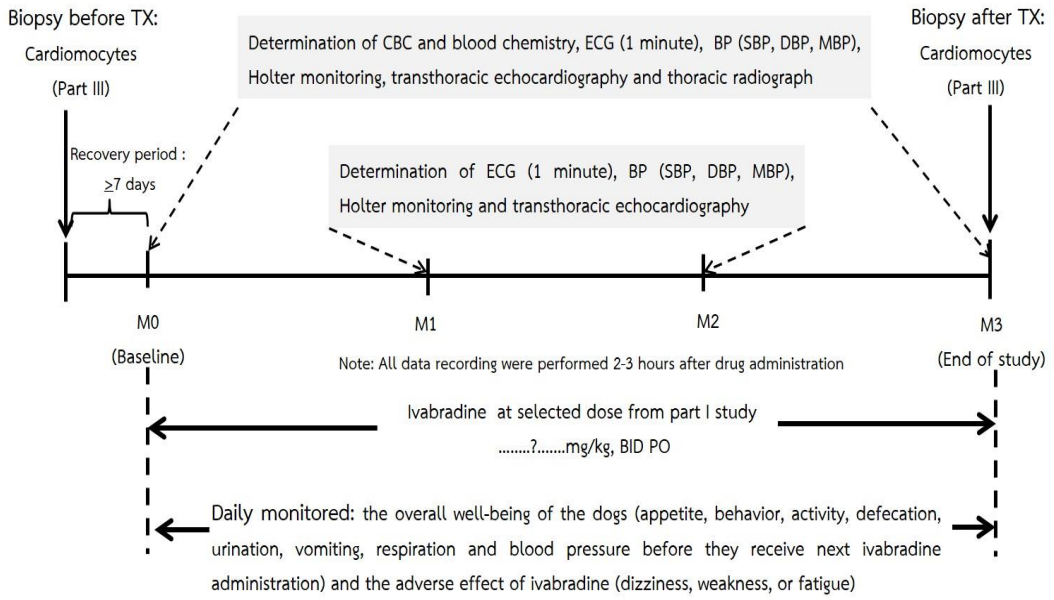


Figure 3-5 The time line and experimental procedure of the study part 2. M = month after receive ivabradine, CBC = complete blood count, BP = blood pressure, SBP=systolic blood pressure, DBP = diastolic blood pressure, MBP = mean blood pressure, ECG = electrocardiography

4. Experimental procedures and analytical methods

4.1 Determination of hematology and chemistry profiles

At baseline and at the end of the study (i.e. 3 months after repeated oral dose of ivabradine), hematology and chemistry profiles were measured as previously described in study part 1.

4.2 Determination of blood pressure

At baseline and monthly (1-3 month after repeated oral dose of ivabradine), arterial BP measurement was performed 1 hour before Holter recording. The HR, SBP, DBP and MBP were obtained using an oscillometric device (petMAP™, CardioCommand, Inc., Florida, USA). Daily BP measurement was also performed 1 hour before dosing each day. Determination of BP was measured as previously described in the study part 1.

4.3 Determination of myocardial oxygen consumption

MVO_2 was inferred from the rate-pressure product, which is calculated as SBP multiplied by HR

4.4 Determination of heart rate and heart rate variability (HRV)

The Holter monitor (Fukuda Denshi Co., Ltd., Japan) was attached to the dogs at indicated time points (baseline, M1, M2 and M3) as previously described in study part 1. The 24-hour continuous ECG was recorded and stored on an SD card for further analysis of HR and HRV using SCM-510 Holter software (Fukuda Denshi Co., Ltd., Japan). All QRS complexes from ECG were automatically analyzed by the software followed by manually inspection of the correct RR intervals by a single experienced operator. The recording was acceptable if 85% or more of raw R wave were normal beats. Signals were filtered through a Hamming window and transformed into a spectrum by fast Fourier transformation (FFT). HRV parameters were analyzed from 512 samples of consecutive RR intervals and interval to analyze was every 1 h for 24 h. In this study, the power spectrum consists of frequency bands ranging from 0-0.5 (total power, TP), low frequency (LF) band ranging from 0.041 to 0.15 Hz, and high frequency (HF) band ranging from 0.15 to 0.5 Hz (Pirintr et al., 2012). The frequency-domain (power spectral density; PSD) parameters of HRV including LF, HF, TP and the ratio of low frequency to high frequency (LF /HF ratio) (Figure 3-6 and Table 3-2). In addition, the time-domain parameters were evaluated for the mean NN intervals (NNA), standard deviation of all normal to normal RR intervals (SDNN or NNSD), the standard deviation of 5-minute mean RR intervals (SDANN or SASD), the mean of the standard deviation of all normal-to-normal RR intervals for all 5-minute segments (SDNN index or SSDA), the percentage of successive normal RR intervals exceeding 50 ms (pNN50 or total RR50%), and the square root of the mean of the squares of the differences between successive normal to normal RR intervals (rMSSD or NNDrms) (Table 3-3) (Pirintr et al., 2012).

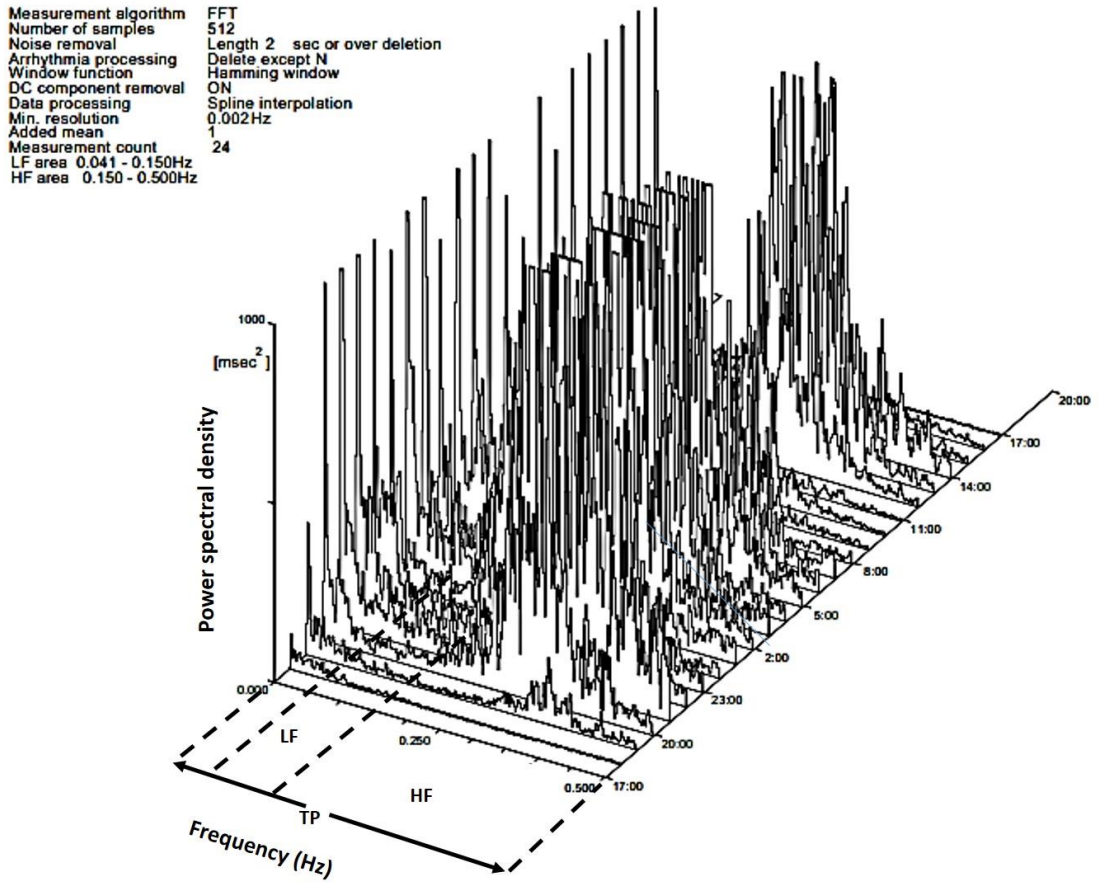


Figure 3-6 The canine power spectral components corresponding to different parts of frequency bands consisted of LF= low frequency power (0.04 to 0.15 Hz), HF= high frequency power (0.15 to 0.5 Hz), TP = total power (0 to 0.5 Hz). This graph depicted from experimental dog.

Table 3-2 Description for frequency domain parameters

Variables	Frequency band	Representation/ Description
Low frequency (LF)	0.041- 0.150 Hz	The slow changes in the heart rate, is related to baroreflexes and represents sympathetic and parasympathetic activity.
High frequency (HF)	0.150- 0.500 Hz	The quicker changes in the heart rate, is primarily due to the parasympathetic nervous system (vagal activity) during respiration.
Total power (TP)	0- 0.500 Hz	The total parasympathetic (vagal) and sympathetic activity.
Low frequency to High frequency ratio (LF/HF ratio)	-	The index of sympathetic to parasympathetic balance

Source: (Stein et al., 1994; Calvert, 1998; Sztajzel, 2004; Pirintr et al., 2012)

Table 3-3 Description for time domain parameters

Variables	Unit	Description
NNA	ms	Mean of the normal RR interval in the entire recording
SDNN	ms	SD of all normal RR intervals in the entire recording
SDNN index	ms	Mean of the SDs of all normal RR intervals for all 5-min segment in the entire recording
SDANN	ms	SD of the mean of all 5-min segments of normal RR intervals in the entire recording
RMSSD	ms	Square root of the mean of the squared differences between adjacent RR intervals in the entire recording
pNN_{50}	%	Percentage of differences between adjacent normal RR intervals that are >50 ms computed in the entire recording

Source: (Stein et al., 1994; Calvert, 1998; Sztajzel, 2004)

4.5 Determination of electrocardiograms

A body surface ECG was obtained for 1 minute from conscious dogs positioned on right lateral recumbent position. All limbs were held perpendicular to the long axis of the body. After attached electrodes onto the skin of all four limbs, using alligator clips, both unipolar (i.e. aVR, aVL, aVF) and bipolar (i.e. leads I, II and III) were recorded by using Acknowledge acquisition program version 3.8 (Biopac MP150, Santa Barbara, California, USA) and stored in hard drive for further analysis. The six consecutive cardiac cycles of Lead II ECG were measured for RR, PQ, QRS and QT intervals (Figure 3-7). The rate corrected QT interval (QTc) was calculated by using Van de Water formula (Van de Water et al., 1989). In addition, rhythm was also determined.

$$QTc = QT - 0.087 \times (RR-1000)$$

where QTc = corrected QT interval, QT = QT interval (ms), RR = RR interval (ms)

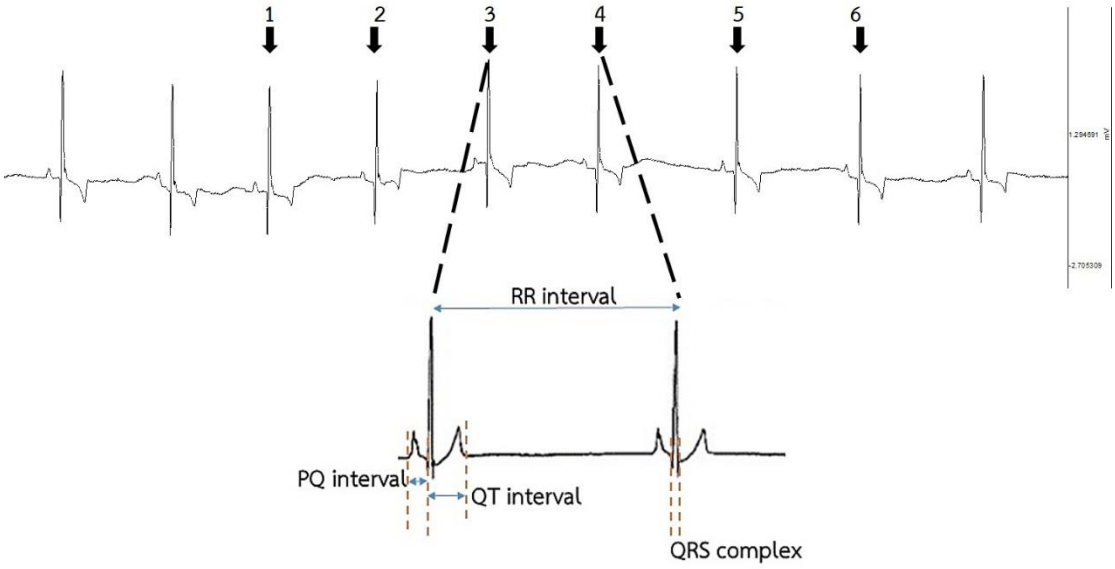


Figure 3- 7 The representative ECG tracing from experimental dog obtained from Lead II ECG demonstrating the measured intervals.

4.6 Echocardiography determination of left ventricular function

Conventional echocardiography (standard M-mode, 2D, color-flow and spectral Doppler) and speckle-tracking echocardiography (STE) were performed by a single examiner on echocardiographic machine (Mindray Medical - M9 Shenzhen Mindray Bio-Medical Electronics Co., Ltd) equipped with 2-4 MHz phased array cardiac probes (Mindray Medical - M9 Shenzhen Mindray Bio-Medical Electronics Co., Ltd). The ECG was also recorded simultaneously. All examinations were performed in conscious dogs positioned in right and left lateral recumbence. Guidelines for the American Society of Echocardiography was followed during all examinations. Pulse wave Doppler, color flow and 2D M-mode were used to evaluate flow patterns of aortic, pulmonary, and atrio-ventricular valves, cardiac function and structure and chamber dimensions. Standard M-mode dimensions (Figure 3-8) were obtained in right parasternal short axis views using 2D guidance projection at the level beneath mitral valve and measured using the leading edge-to-leading edge method (Sahn et al., 1978). The quantification of MR jet (%) was determined by using Doppler echocardiography from the left apical four-chamber view. The area of mosaic color observed during systole inside the left atrium was measured and compared with the total area of the left atrium as described previously (Chompoosan et al., 2014). In addition to conventional echocardiography, at least three cine loops of the right parasternal short-axis views of the left ventricle were obtained for STE analysis that were acquired each segment of the heart (basal, middle and apical segments; Figure 3-9). The software package (tissue tracking QA package, M9, Mindray, China) were used for calculation of global radial strain (GRS), global circumferential strain (GCS), fractional area change (FAC), end-diastolic area (EDA) and end-systolic area (ESA). Global radial strain and global circumferential strain were computed with program including parasternal short axis base (PSAXB, Figure 3-9A), parasternal short axis middle (PSAXM, Figure 3-9B) and parasternal short axis apex (PSAXAP, Figure 3-9C). The mean strain from three regional segments was used for comparison between each time point (M1, M2 and M3) and baseline.

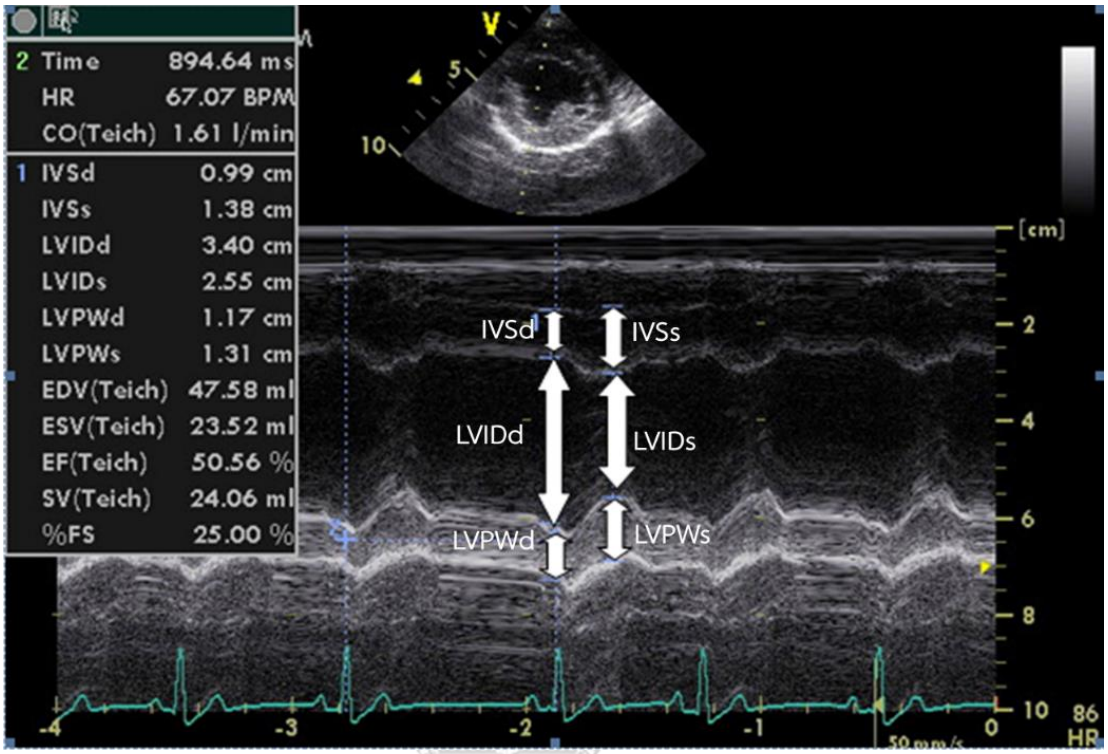


Figure 3-8 Standard 2D, M-mode echocardiogram of dog. IVSd = interventricular septum diastole, IVSs = interventricular septum systole, LVIDd = left ventricular internal diameter diastole, LVIDs = left ventricular internal dimension systole, LVPWd = left ventricular posterior wall diastole, LVPWs = left ventricular posterior wall systole, EDV = end-diastolic volume, ESV = end-systolic volume, EF = ejection fraction, SV = stroke volume, FS = fractional shortening, CO = cardiac output, HR = heart rate

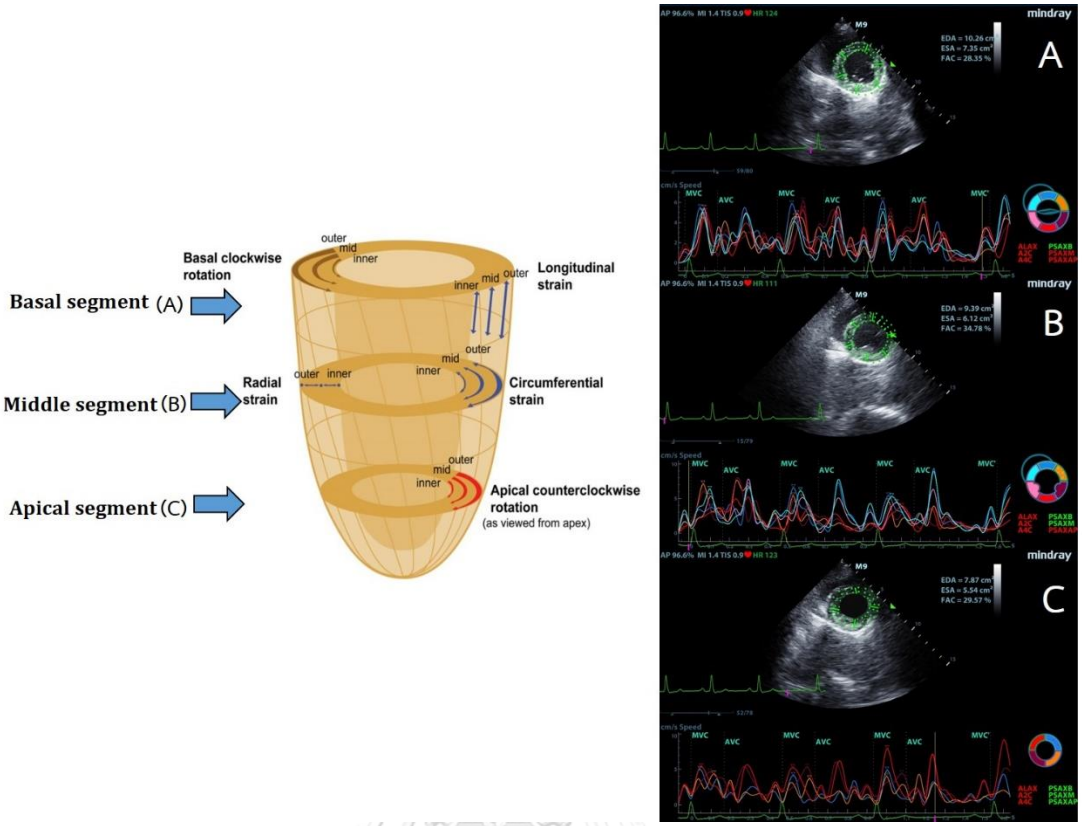


Figure 3-9 The three cross-sectional imaging of the heart (basal, middle and apical segments) and the tissue tracking QA image with circular marks placing on the myocardium from the basal (PSAXB), middle (PSAXM) and apical segments (PSAXAP) of the left ventricle that used for measurement of global radial strain and global circumferential strain, fractional area change, end-diastolic area and end-systolic area.

5. Statistical analysis

All numerical data were presented as mean ± SEM. Statistical analyses were performed using commercially available software. HR, BP (SBP, DBP, and MBP), RPP, ECG, HRV and echocardiographic parameters were compared among time points (baseline, M1, M2 and M3) using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. In all cases, a P value < 0.05 was considered to be statistically significant.

Study part 3: The role of the long-term treatment with repeated oral dose of ivabradine involving hemodynamic, cardiac function and cardiac apoptosis in dogs with asymptomatic DMVD stage B2.

1. Animals

The cardiac tissues were collected from LV free wall of all four dogs that has been used in the second study. The dogs were housed as previously described in the study part 1 and 2.

2. Study design and experimental protocol

A prospective cohort study of long-term effect of repeated oral dose of ivabradine, in DMVD stage B2 dogs, on hemodynamic and cardiac function, and cardiac structural remodeling and changes in the percentage of cardiac apoptosis, expression levels of anti-apoptotic proteins (Bcl-2) and pro-apoptotic proteins (Bax) and the ratio of Bcl-2 to Bax were studied in this part. Dogs were anesthetized twice, at baseline and at the end of the study (3 months after long-term treatment with ivabradine) to collect tissue samples of endomyocardium. The surgical procedures were started after at least 8 h of fasting period. After baseline measurement, dogs were anesthetized with propofol (6-8 mg/kg, iv) followed by isoflurane (1.5-2%). Cardiac catheterization was performed to measure LV pressure (LVP), aortic pressure (AoP), pulmonary arterial pressure (PAP), right atrial pressure (RAP) and pulmonary capillary wedge pressure (PCWP). Tissue biopsy forceps were used to sample the LV endomyocardium. Approximately 3-5 pieces of tissues were obtained per each surgery. The location for biopsy was located at the LV free wall. Dogs were recovered from anesthesia and ivabradine was given for 3 months (as previously described in part II). After 3 months of treatment, dogs were anesthetized again to obtain pressures and collect tissue samples of LV endomyocardium (Figure 3-10). After surgery, dogs were allowed to recover from anesthesia and return to the breeding colony at the end of the study.

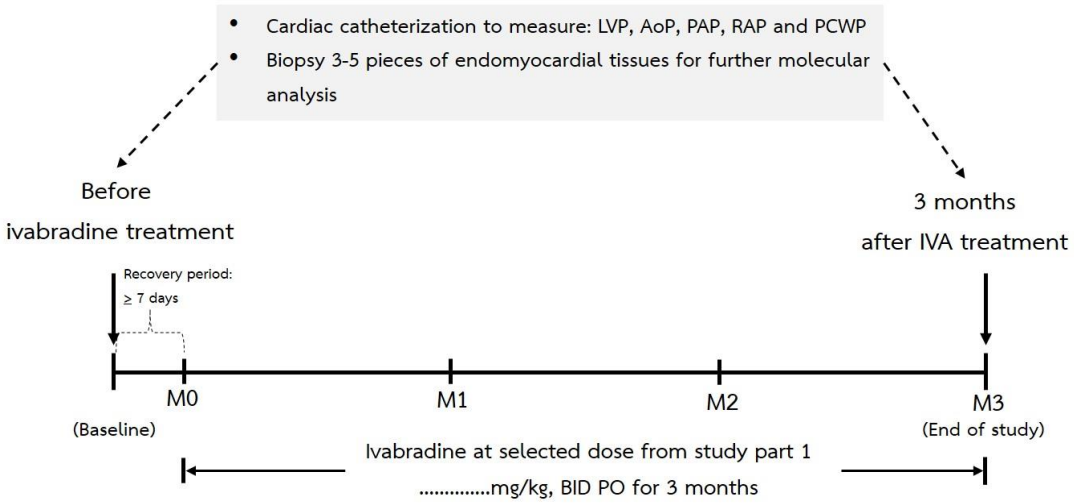


Figure 3-10 The time line and experimental procedure of the study part 3. M = month after receive ivabradine, LVP = LV pressure, (AoP) = aortic pressure, PAP = pulmonary arterial pressure, RAP = right atrial pressure and PCWP = pulmonary capillary wedge pressure

3. Anesthesia, experimental procedures and analytical methods

3.1 Anesthesia

Propofol (6-8 mg/kg) was given intravenously for induction. Orotracheal intubation was performed and ventilated mechanically with ascending-bellows, volume-cycled, pressure-regulated ventilator. The ventilator was set to deliver a tidal volume of 12-15 ml/kg (maximum allowed pressure, 20 cmH_2O) at a rate of 8 to 12 breaths per minute, sustaining the end-tidal partial pressure of CO_2 between 35 and 45 mmHg and that of O_2 greater than 80 mmHg. The endotracheal tube was connected to a circle anesthetic rebreathing circuit, and anesthesia was maintained with isoflurane in oxygen delivered by a use of vaporizer. The end-tidal inhalant concentration was maintained between 1.5-2%. Body temperature was maintained at 36.5 - 37°C by a warm water heating pump. Each animal was shaved and scrubbed at the surgical areas and prepared as aseptic technique (cervical area). Before cardiac catheterization, dogs were given tramadol 1-3 mg/kg, SC for pain relief and cefazolin 15-25 mm/kg, IV for the prevention of surgical-site infections.

3.2 Cardiac catheterization for pressure measurement and endomyocardial biopsy

The cardiac catheterization procedures were performed under fluoroscopic guidance. Figure 3-11 showed the instrumentation for this study. Dogs were placed on dorsal recumbency. The disposable ECG electrodes were attached onto right forelimb, right and left hindlimbs for standard lead II electrocardiographic measurement. The right cephalic vein was cannulated for intravenous drugs administration. The left jugular vein was located and cut down was performed over the vessel. A 6 Fr vascular sheet was inserted into the left jugular vein. The 5 Fr Swan-Ganz catheter was placed into the right atrium to measure the right atrial pressure (RAP, mmHg) and into the pulmonary trunk to measure the pulmonary artery pressure (PAP, mmHg). The balloon at the tip of the catheter was inflated for 30 second to obtain the pulmonary capillary wedge pressure (PCWP, mmHg). Then the left carotid artery was located and cut down was performed over the vessel in order to measure arterial and ventricular pressures. A 6 Fr vascular sheet was placed into the carotid artery. A mikro-tip® catheter pressure transducer (5Fr, Millar® instrument, Houston, TX, USA) was inserted into the left carotid artery via the vessel sheet and advanced to the aortic arch and left ventricle for measuring the aortic pressure (AoP, mmHg) and the left ventricular pressure (LVP, mmHg), respectively. The Swan-Ganz was connected to fluid-filled pressure sensors. Both fluid-filled pressure sensors and Millar catheter were connected to pressure amplifiers. Data were collected by using IOX acquisition program version 1.8.5 (EMKA Technologies, Falls Church, VA, USA). Dogs were stabilized for at least 15 min before all pressure measurements. After that all pressure catheters were removed.

The LV endomyocardial biopsies were obtained by a transarterial intracardiac catheter technique. Endomyocardial biopsy forceps was inserted through the left carotid artery and push against the endocardium of the left ventricular free wall to obtain 3-5 pieces of endomyocardium (size ~0.3 x 0.2 cm) (Figure 3-12). Lead II ECG was used to monitor for arrhythmias. After tissue samples, vessels were sutured with 6-0 prolene. Tissues and muscles were sutured with absorbable 3-0 suture materials. Skin was closed with sterile silk (3/0). Tramadol (1-3 mg/kg, twice a day,

P.O.) and cephazolin (15-25 mg/kg, once a day, SC or P.O.) were administered for 7 days.

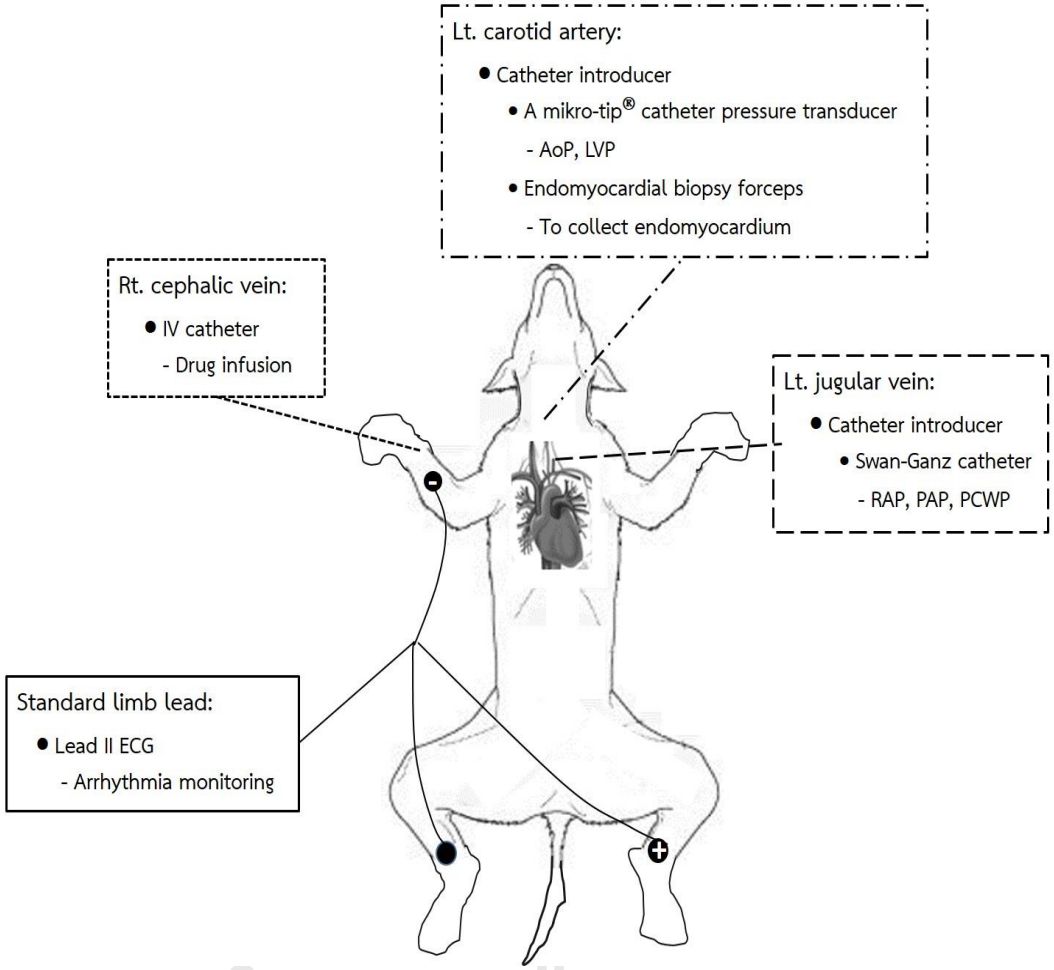


Figure 3-11 The instrumentation for the study the effects of ivabradine on hemodynamics and left ventricular functions at baseline (before treatment with ivabradine) and at the end of study (M3) of treatment with ivabradine 1 mg/kg, orally in anesthetized DMVD dogs. AoP = aortic pressure, LVP left ventricular pressure, RAP = right atrial pressure, PAP = pulmonary artery pressure, PCWP = pulmonary capillary wedge pressure, ECG = electrocardiogram

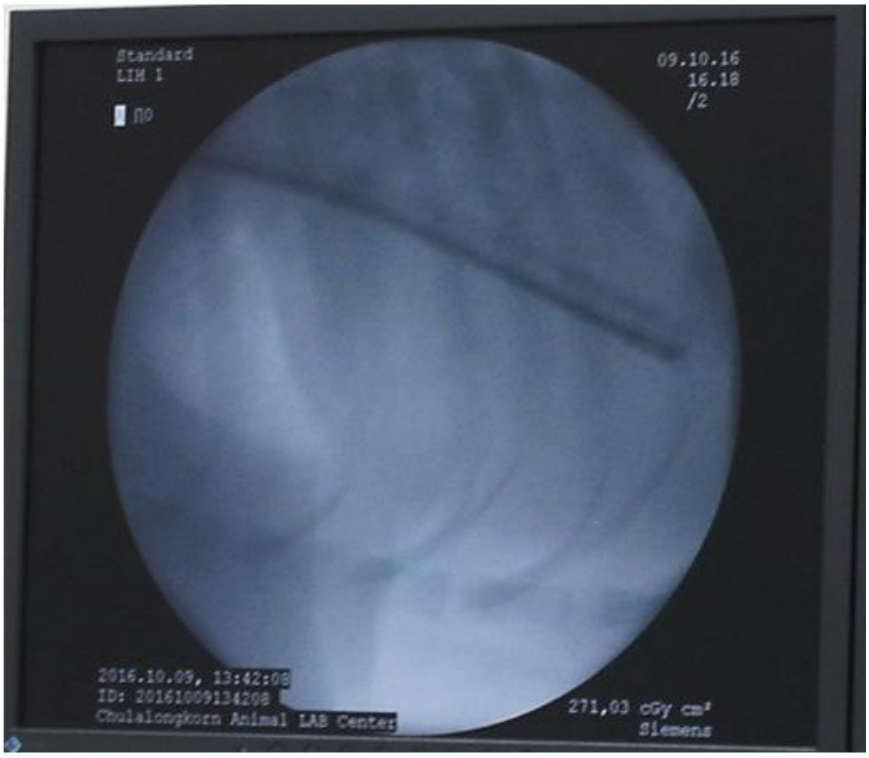


Figure 3-12 The location for endomyocardium biopsy under the guidance of fluoroscope in isoflurane- anesthetize beagle dog.

3.3 Determination of hemodynamic and cardiac function parameters

3.3.1 Mean blood pressure

Recording of aortic blood pressure (Figure 3-13) was analyzed for systolic and diastolic blood pressure by using ECG auto program. Mean blood pressure (MBP) was calculated by using the following equation:

$$MBP = DBP + 1/3\ PP \quad \text{or}$$
$$= DBP + 1/3\ (SBP - DBP)$$

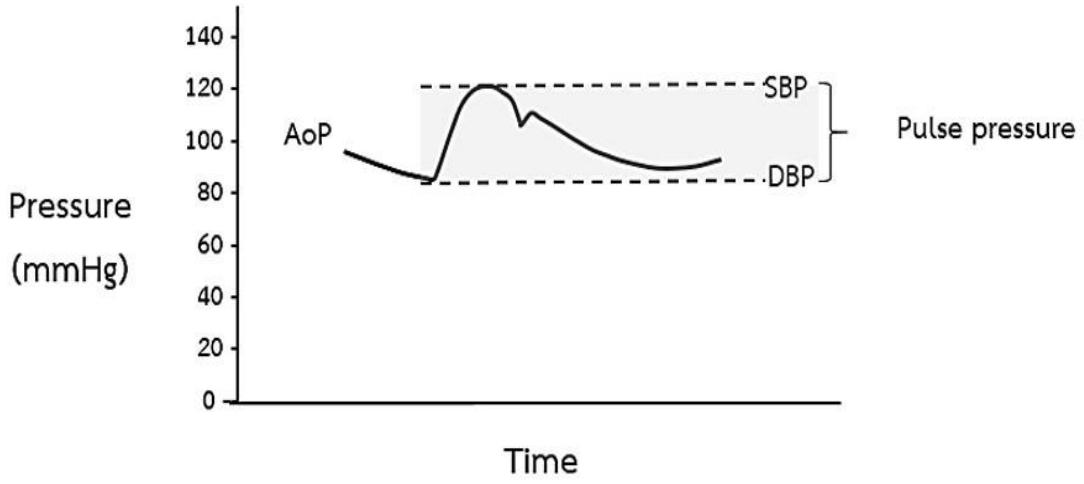


Figure 3-13 The aortic pressure (AoP) representing systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP).

3.3.2 Vascular resistances

Recording of aortic pressure, pulmonary artery pressure and pulmonary capillary wedge pressure were analyzed for determination of the vascular resistances.

Vascular resistances were determined by using Ohm's law, the the pressure gradient from the inlet (i) of the vessel to the outlet (o) (P_i - P_o) divided by the flow (Q); R = (P_i - P_o)/Q.

The systemic vascular resistance (SVR), the resistance to blood flow presented by all the vascular system excluding the pulmonary vasculature, was calculated by using the following equation:

$$SVR = 80x(mAoP - mRAP)/CO$$

Where SVR = systemic vascular resistance, mAoP = mean aortic pressure, mRAP = mean right atrial pressure, CO = cardiac output.

The pulmonary vascular resistance (PVR), the resistance in the pulmonary vasculature, was calculated as the following equation:

$$PVR = (mPAP - mPCWP)/CO$$

Where PVR = pulmonary vascular resistance, mPAP = mean pulmonary arterial pressure, mPCWP = mean pulmonary capillary wedge pressure, CO = cardiac output.

3.3.3 Left ventricular pressure

The LV pressure (LVP) was analyzed for inotropic (contractility index, CI; the maximum rate of rise of the left ventricular pressure curve, dP/dt_{max}) and lusitropic (isovolumic relaxation time constant, Tau; the maximum rate of fall of the left ventricular pressure curve, dP/dt_{min}) indices (Figure 3-14).

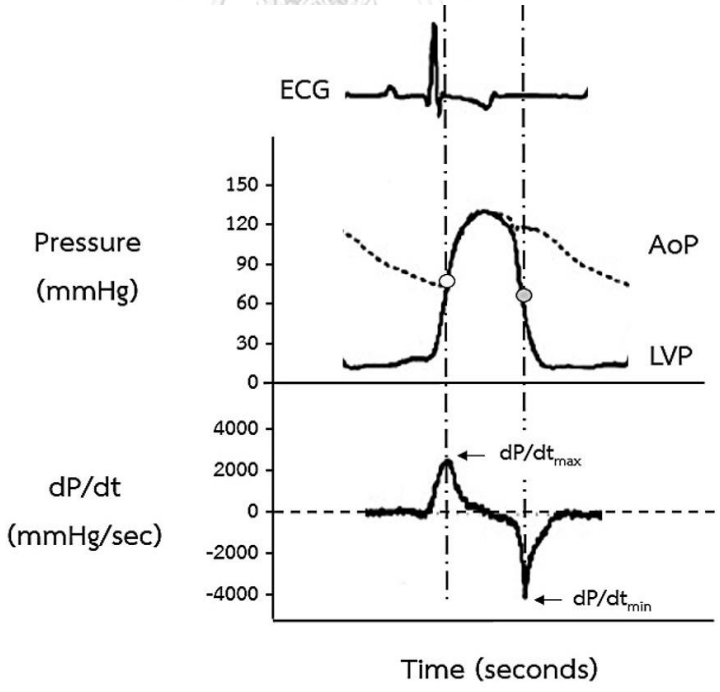


Figure 3-14 The maximum rate of rise of the left ventricular pressure curve (dP/dt_{max}) and the maximum rate of fall of the left ventricular pressure curve (dP/dt_{min}). ECG = electrocardiogram, AoP= aortic blood pressure, LVP = left ventricular pressure

3.3.4 Contractility index and relaxation time constant

Contractility index (CI) is defined as the ratio of maximal rate of rise of the left ventricular pressure over the left ventricular pressure at that point (CI = $dP/dt_{max}/P$).

Relaxation time constant (Tau) is the exponential decline of ventricular pressure during isovolumic relaxation calculated from Glantz method (Raff and Glantz, 1981).

3.4 Tissue preparation

After tissue biopsy, LV endomyocardial tissues were fixed in 10% formalin for 24 hours followed by routine histologic tissue processed, paraffin embedded and 4 μm thickness sliced. Serial sectioning of 4 μm thickness of the LV endomyocardial tissue samples were performed for further histological and immunohistochemistry (IHC) detection of LV remodeling, LV apoptosis and apoptotic protein expression.

3.5 Histological detection of left ventricular remodeling and apoptosis

The 4 μm thickness of LV endomyocardial tissue sections were stained with hematoxylin and eosin (H & E stain), Periodic acid-Schiff (PAS stain) and Masson's trichrome stain. All stained slides were identified myocardial tissue and observed morphological changes of cardiomyocytes and extracellular matrix. Histopathological finding of ventricular remodeling were included white blood cell infiltration, myocardial apoptosis and cell death, vacuolization of myocytes, reduction of myofibril, ruptured myocardial fiber, myocardial interstitial and perivascular fibrosis, fibrofatty infiltration, thin and wavy myocardial fibers and lipofuscin accumulation (Davies, 2000; Tidholm and Jönsson, 2005; Radu et al., 2012).

Morphological changes of cardiomyocyte and myocardium were examined on H&E-stained section under light microscope (Figure 3-15A-B). PAS staining and Masson's trichrome staining were used to determine extracellular matrix

(ECM) morphologic changes. PAS staining was used to detect the accumulation of glycogen, glycoprotein and proteoglycans, typically found in connective tissues (Figure 3-15C-D). Masson's trichrome staining is a three-color staining that has been used in histology for differentiating cells and their components from the surrounding connective tissue. Masson's trichrome staining was used to evaluate collagen bundle disruption and elastic fiber fragmentation.

The endomyocardial tissue sections were evaluated by randomly chosen for total 50 fields per study groups (5-10 different areas of each slide according to size of tissue section) under ×40 objective magnification (Olympus BX 40, Japan). H & E and PAS stain slides were scored 0-4 modified from the previous publication (Adali et al., 2016). The score 0 equals to no damage (no apparent), 1 = minimal damage (< 10% in apparent area), 2 = mild damage (10-30% in apparent area), 3 = moderate damage (30-60 % in apparent area) and 4 =severe damage (> 60% in apparent area).

For evaluation of cardiac fibrosis, slides were semi-quantitatively scored according to the amount and staining intensity of collagen fiber area on Masson's trichrome stain and represented by graded on a score of 0-4 modified from cardiac fibrosis scoring of the previous publication (Singh et al., 2008) (Table 3-4 and Figure 3-16).

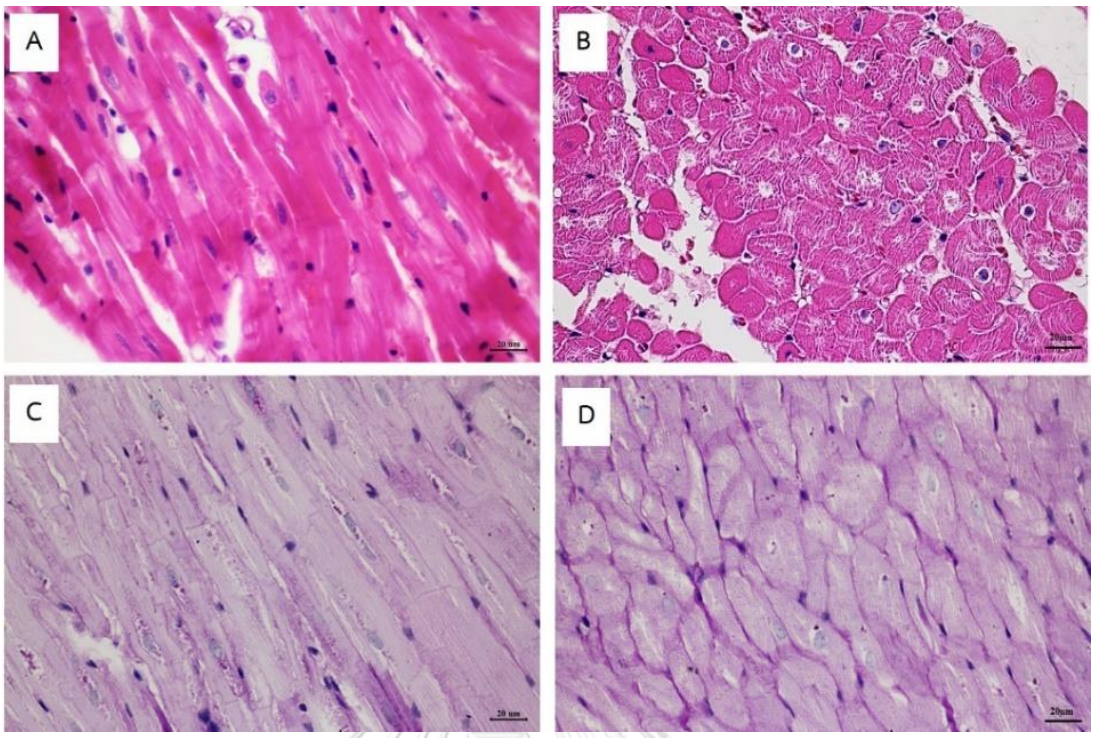


Figure 3-15 Longitudinal and cross sections of left ventricular myocardial tissue of experimental dogs stained with H & E stain (A-B) and PAS stain (C-D) (×40 objective magnification, scale bar = 20 μm).

Table 3-4 Scoring of the cardiac interstitial fibrosis on left ventricular myocardial tissue using a light microscope under ×40 objective magnification (modified from Singh et al., 2008).

Score	Quantity	Staining apparent area of collagen fiber
0	No fibrosis	No apparent collagen fiber proliferation except for small islets of fibrous tissue around the capillaries
1	Focal or minimal fibrosis	collagen fiber proliferation < 10%
2	Mild patchy fibrosis	10- 30% collagen fiber proliferation
3	Moderate diffuse fibrosis	30- 60% collagen fiber proliferation
4	abundant prominent fibrosis	> 60% collagen fiber proliferation

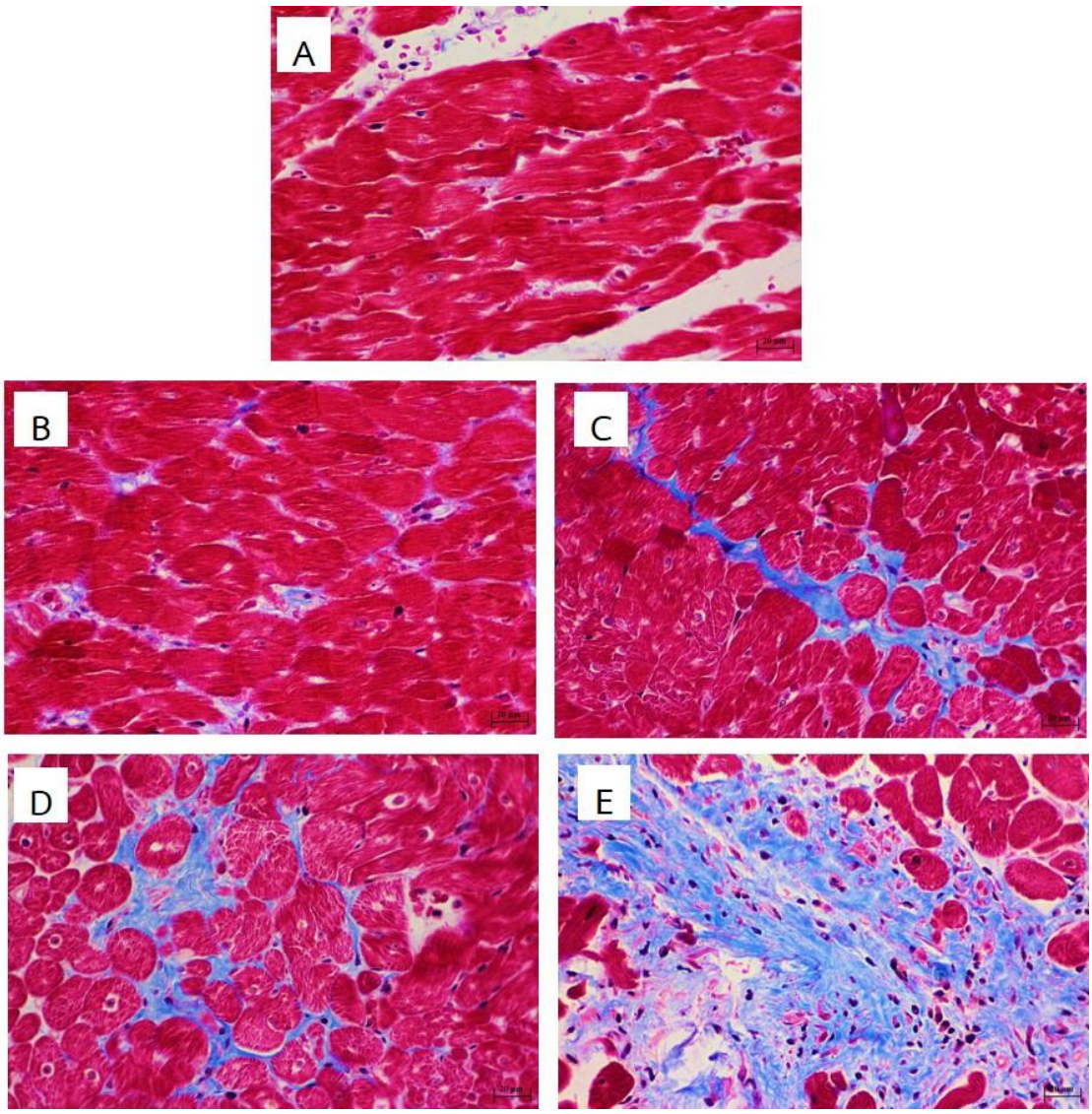


Figure 3-16 The semi-quantitation of interstitial fibrosis on left ventricular myocardial tissue. Intensity was assessed by an four-point score where (A) score of 0 indicated an absence of staining, (B) scores of 1, (C) 2, (D) 3 and (E) 4 indicated minimal, mild, moderate and abundant, respectively (×40 objective magnification, scale bar = 20 μm).

3.6 Determination of left ventricular apoptosis by histochemical, Hoechst 33342 fluorescence dye

The unstained slides with 4 μm thickness of LV endomyocardial tissue section were stained with Hoechst 33342 fluorescence dye. Hoechst 33342 fluorescence dye was used to localize and determine the expression of DNA and

chromatin fragmentation in apoptotic cells in paraffin-embedded sections. Hoechst 33342 fluorescence dye staining was modified from the previous publication (Kraiphet et al., 2017) First of all, the paraffin-embedded endomyocardial sections were incubated at 60°C for 30 min and cool down in room temperature for 3-5 min before deparaffinized in xylene series (xylene I, xylene II, and xylene III) for 20 min each, xylene plus alcohol for 2 min and rehydration in graded alcohol solutions (absolute alcohol I, absolute alcohol II, 95% alcohol, 80% alcohol and 70% alcohol for 2 min each. Next, the sections were rinsed by running water for 5 min and 1 min in distilled water (DW). To permeabilize the myocardial cell membrane, tissue sections were treated with 1% Triton X-100 diluted in phosphate-buffered saline (PBS) and incubated at room temperature for 10 min. Then, the sections were washed in PBS three times for 5 min each. After that, each tissue section was added with 70-100 µL of Hoechst 33342 fluorescence dye (working concentration 5 µg/mL diluted in PBS, Thermo Fisher Scientific) and all slides were incubated in dark chamber at room temperature for 5 min. Then, the sections were washed in PBS four time for 5 min each. Lastly, 50-100 µL of antifade-mounting media (glycerol PBS in the ratio 9:1) were added onto the tissue sections before mounted with a coverslip. Nuclei of apoptotic cells, characterized by DNA and chromatin fragmentation, were evaluated by Hoechst 33342 fluorescence dye stained, examined under fluorescent microscope (Olypus BX51-P, Japan) with blue fluorescence (excitation 352 nm and emission 461 nm) at ×40 objective magnification. The positive sites of apoptotic cardiomyocytes were small clump or condense stained with bright blue stain whereas nuclei of normal cardiomyocytes were smooth and stained with dark blue (Figure 3-17). Quantitative measure of the apoptotic cells with Hoechst 33342 fluorescence-positive nuclei were counted in 5-10 fields for each section according to size of tissue section. The apoptosis index (AI) were calculated as follow (Dirisina et al., 2011):

AI (%) = number of apoptotic cells/total number of cells ×100

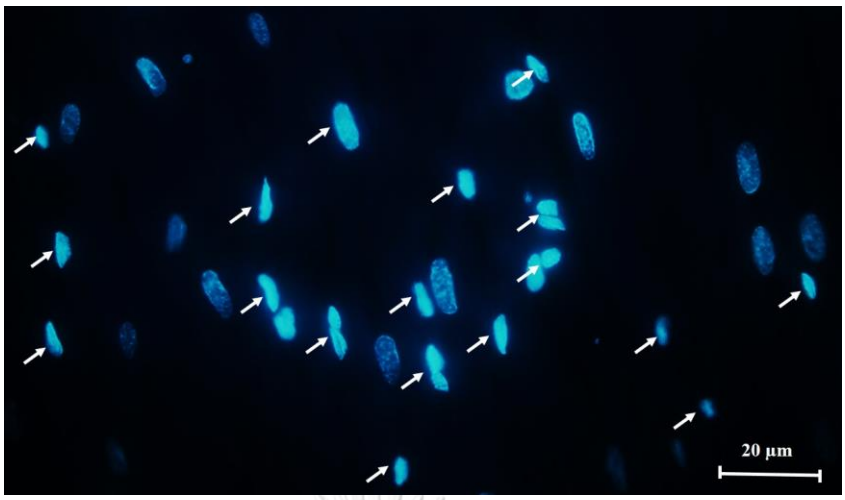


Figure 3-17 Nuclear staining of left ventricular cardiomyocytes obtained from experimental dog with asymptomatic DMVD. Bright blue stain and nuclear fragmentation (thin arrows) represented the positive sites of apoptotic cardiomyocytes, smooth nuclei and stained with dark blue represented normal cardiomyocytes (×40 objective magnification, scale bar = 20 µm).

3.7 Determination of expression of apoptotic proteins (Bcl-2 and Bax proteins) and the ratio of Bcl-2 to Bax

Immunohistochemical method (LSAB method) for Bcl-2 and Bax protein expression in paraffin-embedded sections were performed according to previous publication (Kraiphet et al., 2017). Firstly, the paraffin-embedded endomyocardial sections were deparaffinized in xylene series (xylene I, xylene II, and xylene III) for 10 min each, xylene plus alcohol for 5 min and rehydration in graded alcohol solutions (absolute alcohol I, absolute alcohol II, 95% alcohol, 80% alcohol and 70% alcohol for 5 min each. Next, the tissue sections were rinsed by running water, DW and PBS for each 5 min. To unmask antigen epitopes, tissue sections were treated with autoclave heat at 121°C for 10 min in 10 mM citrate buffer pH 6, subsequently cool down in room temperature for 20 min. Next, tissue sections were pre-incubated with 3% H_2O_2 in methanol to inactivate endogenous peroxidase for 5 minutes at room temperature. After that, the tissue sections were washed in PBS three time for 5 min each. The 1% bovine serum albumin (BSA) were used for blocked non-specific enzymes for 30 min

at 37°C and they were washed by PBS three times for 5 min each. Next, primary antibodies against for Bcl-2, monoclonal mouse anti-human Bcl-2 oncoprotein (NCL-bcl-2-486, Leica, UK) at dilution 1: 100 and Bax, Polyclonal rabbit anti-human Bax (coad no. A3533, Dako®, Denmark) at dilution 1: 200 were applied into sections. These sections were incubated with primary antibodies (Bcl-2 or Bax) overnight at 4°C in humidified chamber. After that, slides were incubated with secondary antibodies, Envision polymer (Dako®, Denmark) for 45 minutes at room temperature. Between each incubation step, slides were washed in PBS three time for 5 min each. Subsequently, visualization of peroxidase activity was performed for 5 min with 3,3'-diaminobenzidine (DAB) solution concentration 1:50 freshly prepared (DAB 20 µL in solution 980 µL). Then, the stain of tissue in the slides was checked and washed by DW and running water for 5 min. Finally, slide sections were air-dried, stained with Meyer's hematoxylin for less than 2 minutes, washed in running water, dehydrated in graded ethanol, soaked in xylene and mounted with permount solution. Negative control sections, primary antibody was replaced with universal negative control for N-series mouse antibodies. For positive control sections, Bcl-2 and Bax immunolabeling against canine lymphoma were used. Expression of Bcl-2 and Bax proteins was examined under light microscope. The positive sites of Bax were stained in brown color in the cytoplasm (Figure 3-18A), while nuclear positive and intracytoplasmic cells of Bcl-2 (Figure 3-18B), were evaluated. The apoptotic cells that expressed Bcl-2 and Bax proteins were evaluated by randomly chosen for a total of 50 fields per study groups (5-10 different areas of each slide according to size of tissue section) using a light microscope under ×40 objective magnification (Olympus BX 40, Japan) and the ratio of Bax to Bcl-2 were also calculated. Slides were semi-quantitatively scored according to the amount and staining intensity of Bax (Figure 3-19) and Bcl-2 (Figure 3-20) and represented by four-point score modified from immunohistochemical scoring of the previous publications (Numata et al., 2013; Adali et al., 2016) (Table 3-5).

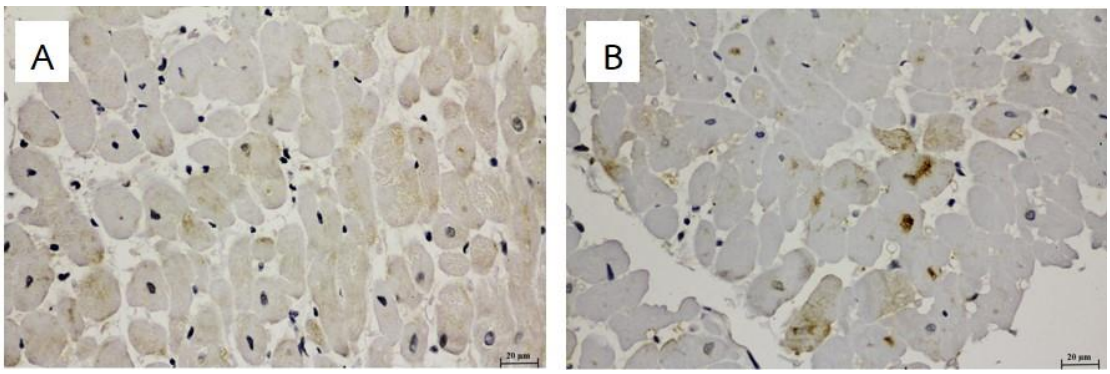


Figure 3-18 The intracytoplasmic expression of Bax and Bcl-2 proteins on left ventricular cardiomyocytes obtained from experimental dogs with asymptomatic DMVD. The positive sites of Bax were stained in brown color in cytoplasm (A), while nuclear positive and intracytoplasmic cells of Bcl-2 (B). (×40 objective magnification, scale bar = 20 μm).

Table 3-5 Four-point score of the Bax and Bcl-2 proteins expression on left ventricular cardiomyocytes using a light microscope under ×40 objective magnification (modified from Numata et al., 2013; Adalıl et al., 2016).

Score	Quantity	Amount of positive cells and staining intensity area
0	Non-staining (minimal staining)	Not see or < 10% staining intensity area
1	Weak-staining (mild-staining)	10- 30% staining intensity area
2	Median-staining (moderate-staining)	30- 60% staining intensity area
3	Strong-staining (intense-staining)	> 60% staining intensity area

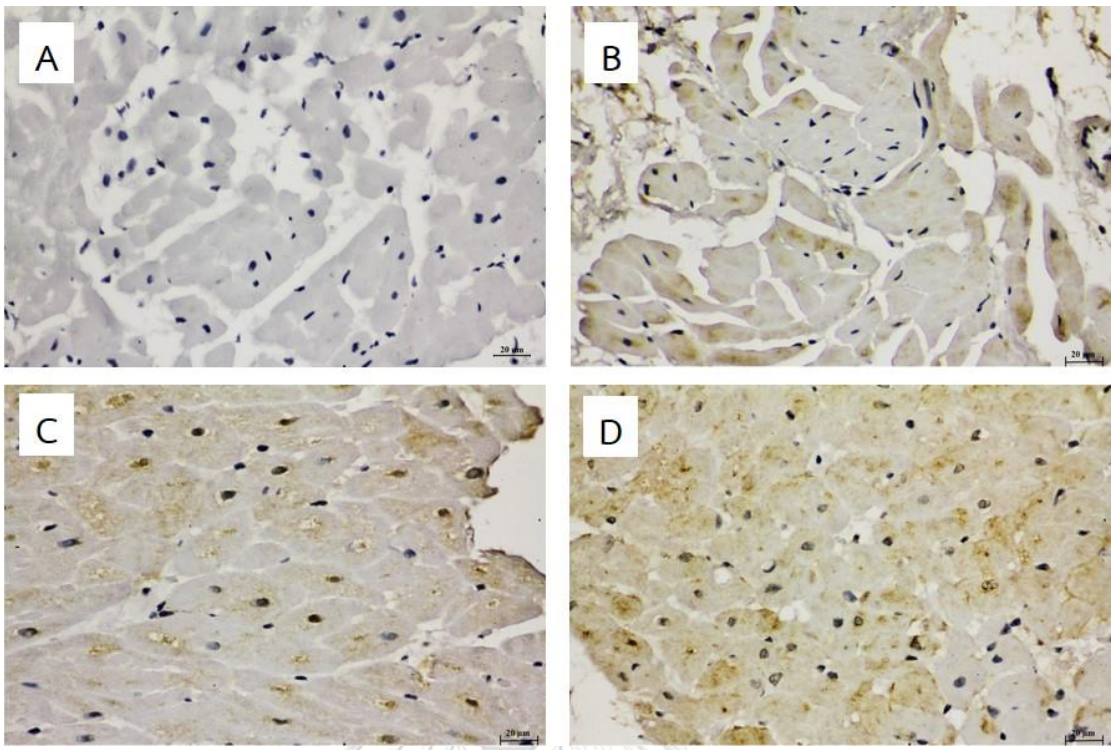


Figure 3-19 The semi-quantitation of Bax immunostaining on left ventricular cardiomyocytes using a light microscope under ×40 objective magnification. The positive sites of Bax were stained in brown color in cytoplasm. The immunostaining intensity evaluated in the current study was assessed by a four-point score where (A) score of 0 indicated an absence of staining, (B) scores of 1, (C) 2 and (D) 3 indicated mild, moderate and abundant, respectively (×40 objective magnification, scale bar = 20 μm).

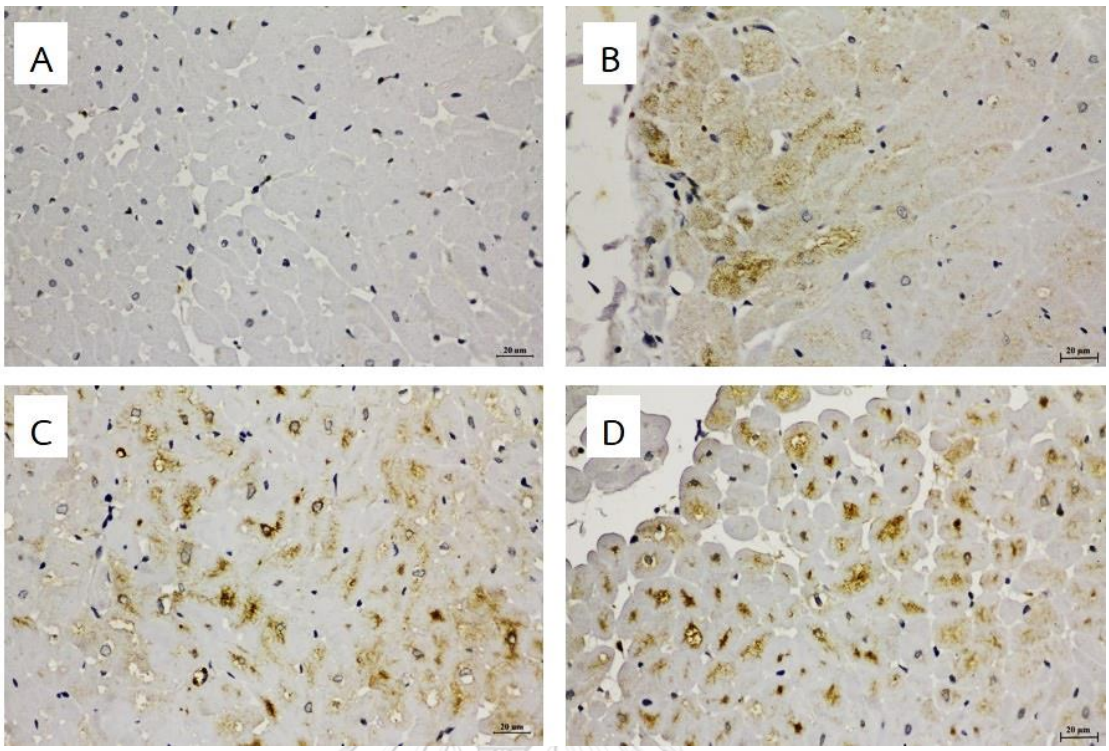


Figure 3-20 The semi-quantitation of Bcl-2 immunostaining on left ventricular cardiomyocytes using a light microscope under ×40 objective magnification. The positive sites of Bcl-2 were stained in brown color in nucleus and intracytoplasmic cells. The Immunostaining intensity evaluated in the current study was assessed by a four-point score where (A) score of 0 indicated an absence of staining, (B) scores of 1, (C) 2 and (D) 3 indicated mild, moderate and abundant, respectively (×40 objective magnification, scale bar = 20 μm).

4. Statistical analysis

Descriptive statistics were used for histological changes in H & E and special staining. All numerical data were reported as mean ± SEM. Statistical analyses were performed using commercially available software. The percentage of cardiomyocyte apoptosis or AI, Scoring of cardiac fibrosis and expression levels of anti-apoptotic proteins (Bcl-2), pro-apoptotic proteins (Bax) and the ratio of Bcl-2 to Bax between before and after 3 months treatment with ivabradine were determined by using paired *t*-test. A $P<0.05$ was considered to be statistically significant.

CHAPTER IV
RESULTS

The results of this study were organized into 3 study parts according to the materials and methods as follow:

Study part 1: The appropriate single oral dose of ivabradine for reduction of HR and MVO_2 in dogs with asymptomatic DMVD

1. General characteristics and health status of the experimental dogs

Seven beagle dogs of both genders (two males, five females) were used in this study. The general characteristics and grade of heart murmur of all seven beagle dogs are shown in Table 4-1.

Table 4-1 The general characteristics and heart murmur intensity (grade I-VI) of all 7 DMVD dogs.

Variables	Mean	Range
Age (years)	8.57 ± 0.57	6-10
Body weight (kg)	14.75 ± 2.12	9.70-26.80
Body condition score (1-5)	3.64 ± 0.36	3-5
Grade of systolic murmur (I-VI)	-	III-IV/VI

Data are presented as mean ± SEM. kg = kilogram

The complete blood cell count and blood chemical profiles of all seven dogs were within normal limits (Table 4-2). The thoracic radiograph and echocardiography revealed that all dogs were in ACVIM stage B2 (i.e. dogs presented with MR and structural changes but no clinical sign) (Table 4-3). No major adverse effects were observed in this study.

Table 4-2 The complete blood count and plasma chemical profiles at baseline and at the end of study of treatment with placebo and ivabradine (0.5, 1 and 2 mg/kg, orally) in 7 DMVD dogs.

Variables	Normal range	Baseline	End of study
Complete blood count			
Red blood cell ($x10^6$) per µL	5.1-8.5	6.08 ± 0.27	6.54 ± 0.37
Hemoglobin (g/dL)	11.0-19.0	14.51 ± 0.69	15.40 ± 0.96
Hematocrit (%)	33.0-56.0	41.56 ± 1.89	45.5 ± 2.83
MCV (fL)	60.0-76.0	67.67 ± 1.51	69.11 ± 1.18
MCH (pg)	20.0-27.0	23.69 ± 0.68	23.41 ± 0.47
MCHC (g/dL)	30.0-38.0	35.09 ± 0.23	33.86 ± 0.22
Platelets ($x10^3$) per µL	117.0-490.0	341.43 ± 52.58	350.71 ± 45.18
White blood cells ($x10^3$) per µL	6.0-17.0	11.64 ± 0.65	8.51 ± 0.89
Neutrophils (%)	52.0-81.0	72.89 ± 2.89	70.72 ± 3.15
Lymphocytes (%)	12.0-33.0	19.72 ± 2.82	21.81 ± 2.57
Monocytes (%)	2.0-13.0	5.80 ± 1.35	5.82 ± 1.43
Eosinophils (%)	0.5-10.0	1.50 ± 0.63	1.03 ± 0.28
Basophils (%)	0.0-1.3	0.08 ± 0.07	0.60 ± 0.20
Serum Chemistry			
Creatinine (mg%)	0.6-2.0	0.60 ± 0.00	0.67 ± 0.05
BUN (mg%)	7.0-30.0	12.06 ± 1.36	16.14 ± 1.90
ALT (U/L)	4.0-91.0	28.86 ± 4.85	33.14 ± 7.10
ALP (U/L)	43.0-115.0	83.29 ± 10.50	96.71 ± 10.10

Data are presented as mean ± SEM. Abbreviations: MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, BUN = blood urea nitrogen, ALT = alanine transaminase, ALP = alkaline phosphatase. *Normal ranges are from Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University.

Table 4-3 The vertebral heart score and echocardiographic parameters of all 7 dogs with DMVD.

Variables	Mean
Thoracic radiograph	
VSH	10.5 ± 0.2
Echocardiography	
LA/Ao	1.63 ± 0.05
IVSd (cm)	0.82 ± 0.08
IVSs (cm)	1.18 ± 0.07
LVIDd (cm)	3.09 ± 0.28
LVIDs (cm)	1.84 ± 0.12
LVPWd (cm)	0.80 ± 0.04
LVPWs (cm)	1.19 ± 0.04
FS (%)	3.94 ± 2.5
HR (beat per minute)	120 ± 5
EDV (ml)	40.11 ± 9.21
ESV (ml)	10.93 ± 1.82
SV (ml)	29.2 ± 7.5
CO (L)	3.62 ± 1.02
EF (%)	70.9 ± 2.7
MR Jet (%)	58.88 ± 5.18

Data are presented as mean ± SEM. Abbreviations: LA = left atrium, Ao = aorta, IVSd = interventricular septum diastole; IVSs = interventricular septum systole, LVIDd = left ventricular internal diameter diastole, LVIDs = left ventricular internal diameter systole, LVPWd = left ventricular posterior wall diastole, LVPWs = left ventricular posterior wall systole, FS = fractional shortening, HR = heart rate, EDV = end-diastolic volume, ESV = end-diastolic volume, SV = stroke volume, CO = cardiac output, EF = ejection fraction, MR Jet = mitral regurgitation jet area.

The HR, systemic blood pressure and RPP were obtained at all time points indicated in the protocol (Figure 3-1). No major adverse effects were observed in this study.

2. Effects of ivabradine on the heart rate

The baseline HR for placebo and ivabradine (0.5, 1.0 and 2.0 mg/kg) groups was 139 ± 6.4 bpm, 146 ± 4.7 bpm, 140 ± 4.2 bpm and 144 ± 5.2 bpm, respectively. Figure 4-1 shows the baselines of the adjusted HR for all groups. It was clear that ivabradine reduced HR in a dose-dependent manner. The HR of the placebo group was not different among time points. After dogs received ivabradine at 0.5 mg/kg, the HR was significantly decreased 3 h after administration (P<0.05) and remained significantly lower until 12 h after administration, however, all these time points did not differ from the placebo group evaluated at the same time points. When compared with baselines, ivabradine 1.0 mg/kg significantly reduced the HR 3 h after administration (P<0.05), while ivabradine 2.0 mg/kg significantly lowered the HR 1 h after administration (P<0.05). In both groups, the HR remained at the decreased levels until 12 h after administration. When the ivabradine groups (1.0 or 2.0 mg/kg) were compared with the placebo group at the same time points, ivabradine 1.0 mg/kg significantly reduced the HR 7 to 10 h after (P<0.05) while ivabradine 2.0 mg/kg significantly reduced the HR 3 to 12 h after administration (P<0.05).

The average HR recorded for 24 h after administration was decreased for the three doses of ivabradine. The mean 24-h HR after ivabradine administration of 1.0 mg/kg (117 ± 2.1 bpm) and 2.0 mg/kg (112 ± 1.9 bpm) was significantly lower than that of the placebo (124 ± 1.7 bpm; P<0.05) (Figure 4-2). However, no significant differences were found between the average 24-h HR after ivabradine administration (0.5 mg/kg; 123 ± 2.3 bpm) and the placebo. No dog in any groups had a minimal instantaneous HR below 60 bpm (Table 4-4).

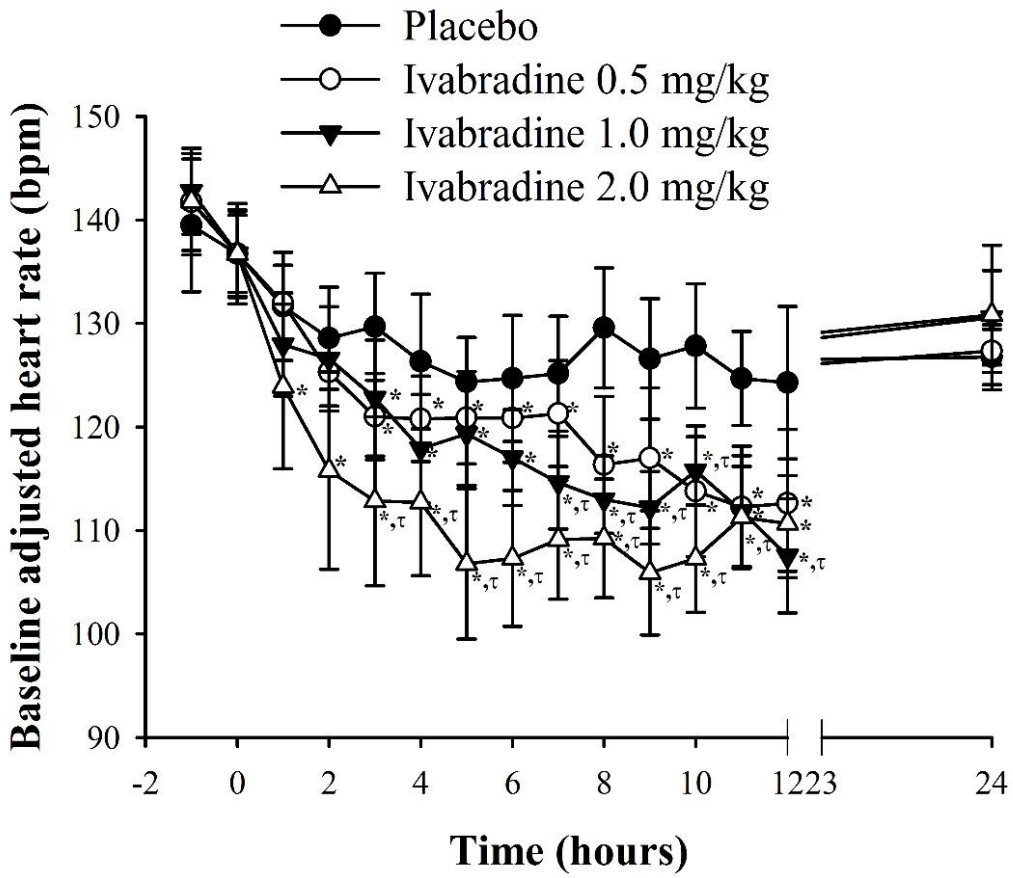


Figure 4-1 Plots of baseline adjusted heart rate against time (hours) before and after receiving placebo or ivabradine (0.5, 1.0 or 2.0 mg/kg, orally) in dogs with DMVD (N=7). *indicates *P*<0.05 when compared with baseline (0 hour) in the same group and $^{\tau}$indicates *P*<0.05 when compared with placebo group at the same time point using two-way ANOVA with repeated measures followed by Dunnett post-hoc analysis.

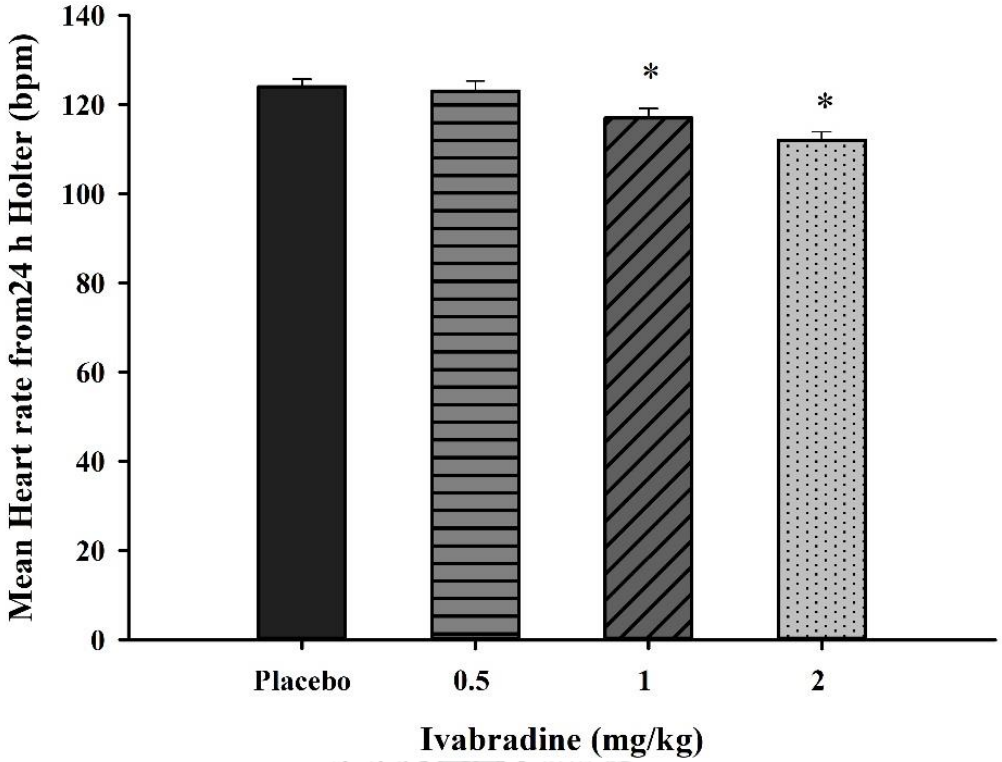


Figure 4-2 Histogram illustration of an average of 24-h HR before and after receiving placebo or ivabradine (0.5, 1.0 or 2.0 mg/kg, orally) in dogs with DMVD (N=7). *indicates $P<0.05$ when compared with placebo group using two-way ANOVA with repeated measures followed by Dunnett post-hoc analysis.

Table 4-4 The individual minimal instantaneous heart rate of all 7 dogs during 24 hour after receiving placebo and ivabradine (0.5, 1.0 and 2.0 mg/kg).

Dog number	Minimal instantaneous heart rate (bpm)			
	Placebo	Ivabradine 0.5 mg/kg	Ivabradine 1.0 mg/kg	Ivabradine 2.0 mg/kg
1	71	74	68	61
2	73	87	70	87
3	84	68	67	66
4	64	63	63	66
5	94	85	87	87
6	77	71	65	62
7	80	74	77	74

3. Effects of ivabradine on systemic blood pressure

Figure 4-3 (a-c) demonstrates the baseline adjusted SBP, MBP and DBP, respectively, for all groups. The baseline SBP of the placebo and ivabradine (0.5, 1.0 and 2.0 mg/kg) groups were 155 ± 6.6 mmHg, 154 ± 8.6 mmHg, 167 ± 5.8 mmHg and 168 ± 12.4 mmHg, respectively (Figure 4-3a). The SBP in the groups receiving ivabradine at 0.5 and 1.0 mg/kg was not altered, whereas that in the ivabradine 2.0 mg/kg group was significantly reduced at 5 and 6 h after administration when compared with its baseline or the placebo group at the same time points (P<0.05). The baseline MBP of the placebo and ivabradine (0.5, 1.0 and 2.0 mg/kg) groups were 112 ± 4.8 mmHg, 113 ± 5.5 mmHg, 119 ± 4.2 mmHg and 119 ± 11.8 mmHg, respectively (Figure 4-3b). Dogs receiving placebo and ivabradine responded similarly as shown by their SBP, but the MBP even with ivabradine (2.0 mg/kg) was not different from the baseline. The baseline DBP of the placebo and ivabradine (0.5, 1.0 and 2.0 mg/kg) groups were 91 ± 4.5 mmHg, 91 ± 3.9 mmHg, 96 ± 4.2 mmHg and 106 ± 5.8 mmHg, respectively (Figure 4-3c). There was no difference among the four groups.

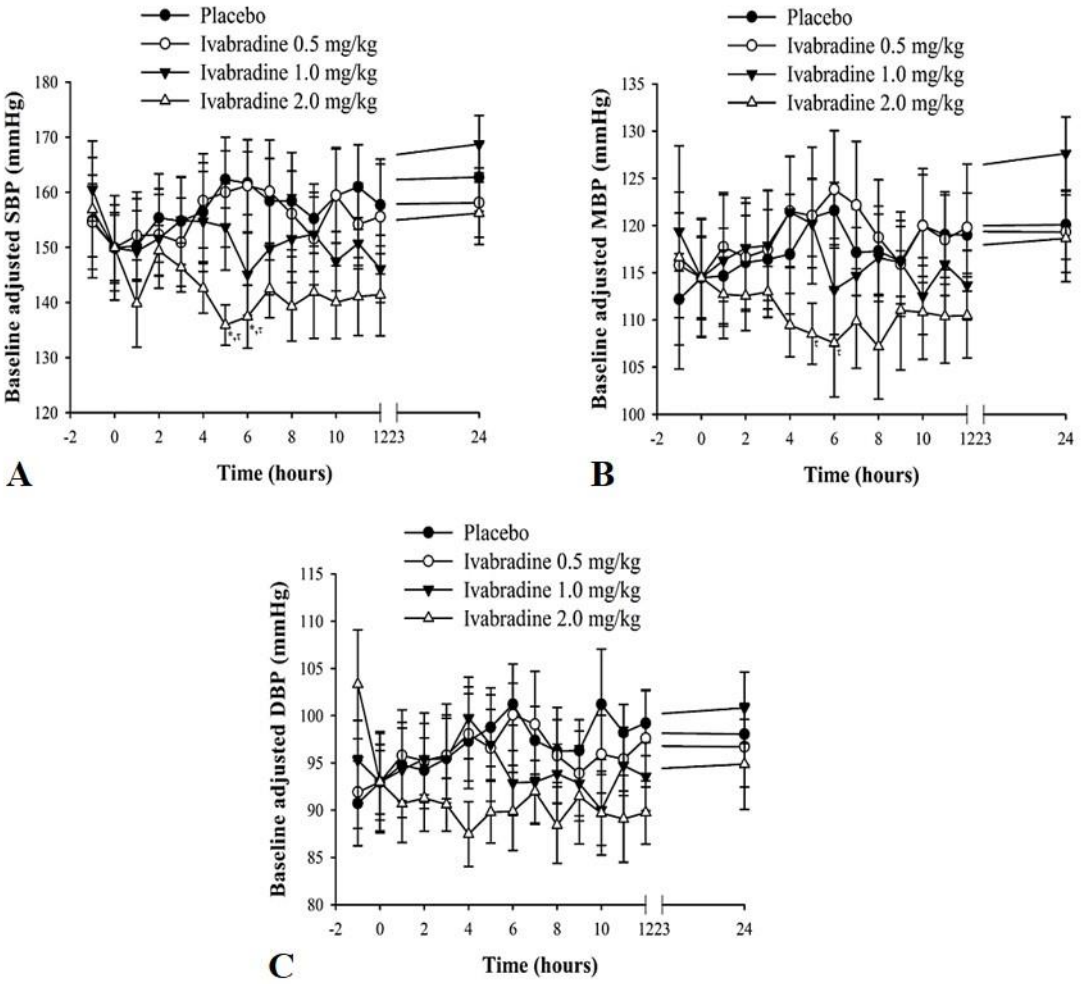


Figure 4-3 Plots of baseline adjusted systolic blood pressure (SBP; A), mean blood pressure (MBP; B) and diastolic blood pressure (DBP; C) against time (hours) before and after receiving placebo or ivabradine (0.5, 1.0 or 2.0 mg/kg, orally) in dogs with DMVD (N=7). Error bars are expressed as mean ± SEM. *indicates $P<0.05$ when compared with baseline (0 hour) in the same group and $^{\tau}$indicates $P<0.05$ when compared with placebo group at the same time point using two-way ANOVA with repeated measures followed by Dunnett post-hoc analysis.

4. Effects of ivabradine on rate pressure product

The baseline RPP of the placebo and ivabradine (0.5, 1.0 and 2.0 mg/kg) groups were 21,813±1,887 bpm.mmHg, 22,773 ± 1,715 bpm.mmHg, 23,327 ± 1,098 bpm.mmHg and 24,263 ± 1,912 bpm.mmHg, respectively. Figure 4-4 shows the baseline adjusted RPP for all groups. It can be noticed that ivabradine administration significantly reduced RPP in a dose-dependent manner ($P<0.05$). The RPP for the placebo and ivabradine (0.5 mg/kg) groups were not different between group except for the RPP at 11 h after administration in which it was significantly lowered in ivabradine group ($P<0.05$). When compared among ivabradine groups with placebo, RPP for the ivabradine (1.0 and 2.0 mg/kg) groups was significantly lowered 6 h and 5 h after administration, respectively, and the decreased levels were maintained until 12 h after administration.

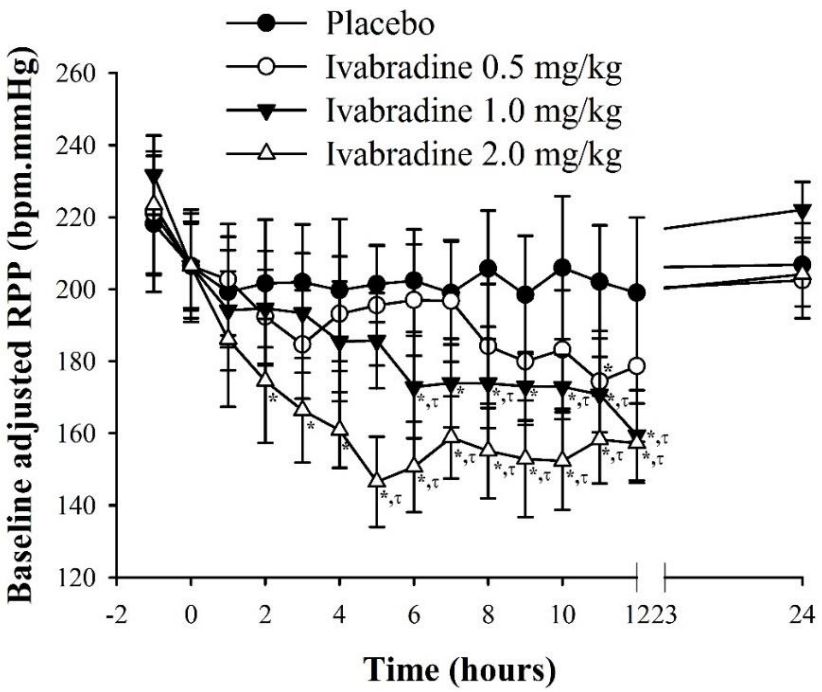
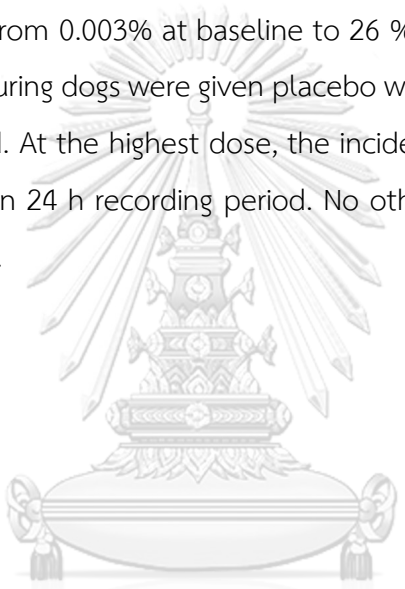


Figure 4-4 Plots of baseline adjusted rate pressure product (RPP) against time (hours) before and after receiving placebo or ivabradine (0.5, 1.0 or 2.0 mg/kg, orally) in dogs with DMVD (N=7). The scale of RPP was divided by 100. *indicates $P<0.05$ when compared with baseline (0 hour) in the same group and $^{\tau}$indicates $P<0.05$ when compared with placebo group at the same time point using two-way ANOVA with repeated measures followed by Dunnett post-hoc analysis.

5. Effects of ivabradine on 24 h electrocardiogram

Supraventricular premature complexes (SVPC) were found in approximately 0.001 % (2 beat out of 173,875 normal beats) to 0.117 % (227 beats out of 189,289 normal beats) in four out of seven dogs, both before and after receiving placebo and ivabradine. Ventricular premature complexes (VPC) were found in approximately 0.001 % (1 beat out of 192,639 normal beats) to 0.061 % (114 beats out of 187,895 normal beats) in three out of seven dogs, both before and after receiving placebo and ivabradine. One dog receiving ivabradine 2.0 mg/kg had an increase in % of VPC beyond normal limits (from 0.003% at baseline to 26 % at 2 mg/kg). The incidence of sinus pause observed during dogs were given placebo was varied from 0 to 20 episodes in 24 h recording period. At the highest dose, the incidence of sinus pause was varied from 0 to 28 episodes in 24 h recording period. No other arrhythmias were observed during the study period.



Study part 2: The long-term effects of repeated oral dose of appropriate ivabradine on HR, BP, MVO_2, cardiac function, ECG parameters and HRV in dogs with asymptomatic DMVD

1. General characteristics and health status of the experimental dogs

Four beagle dogs of both genders (two males, two females) were used in this study. The characteristics and heart murmur intensity (grade I-VI) of all four beagle dogs were showed in Table 4-5. All four dogs used in this study finished the study period of 3 months without progression of the disease to CHF.

The thoracic radiograph revealed cardiomegaly (DMVD with ACVIM stage B2) in which the both left atrial and left ventricle were enlarged (Table 4-6).

Complete blood count and blood chemistry profiles obtained during the 3-month study period did not demonstrate any clinically abnormal changes (Table 4-7).

Table 4-5 The general characteristics and murmur grade of all 4 DMVD dogs.

Variables	Mean	Range
Age (years)	7.7 ± 0.50	6.2-8.2
Body weight (kg)	12.08 ± 0.80	9.85-13.35
Body condition score (1-5)	3.63 ± 0.24	3-4
Grade of systolic murmur (I-VI)	-	III-IV

Data are presented as mean ± SEM. kg = kilogram

Table 4-6 The Vertebral heart score (VSH) at baseline and at the end of study (M3) of treatment with ivabradine 1 mg/kg, orally in 4 DMVD dogs.

Variables	Normal range*	Baseline	M3
Thoracic radiograph			
VSH	10.3 ± 0.4	10.5 ± 0.18	10.5 ± 0.30

Data are presented as mean ± SEM. *Normal ranges are from (Kraetschmer et al., 2008).

Table 4-7 The complete blood count and plasma chemical profiles at baseline and at the end of study (M3) of treatment with ivabradine 1 mg/kg, orally in 4 DMVD dogs.

Variables	Normal range*	Baseline	M3
Complete blood count			
Red blood cell ($x10^6$) per µL	5.1-8.5	6.48 ± 0.33	6.40 ± 0.42
Hemoglobin (g/dL)	11.0-19.0	15.58 ± 0.76	16.08 ± 0.78
Hematocrit (%)	33.0-56.0	45.48 ± 2.28	44.15 ± 2.43
MCV (fL)	60.0-76.0	70.20 ± 1.06	69.18 ± 1.10
MCH (pg)	20.0-27.0	24.03 ± 0.45	25.23 ± 0.62
MCHC (g/dL)	30.0-38.0	34.23 ± 0.22	36.50 ± 0.32
Platelets ($x10^3$) per µL	117.0-490.0	403.75 ± 69.36	353.25 ± 68.87
White blood cells ($x10^3$) per µL	6.0-17.0	8.92 ± 1.53	10.01 ± 0.88
Neutrophils (%)	52.0-81.0	69.80 ± 3.72	72.70 ± 3.94
Lymphocytes (%)	12.0-33.0	22.93 ± 1.59	16.28 ± 2.30
Monocytes (%)	2.0-13.0	6.20 ± 2.86	8.92 ± 1.16
Eosinophils (%)	0.5-10.0	0.38 ± 0.33	1.43 ± 0.81
Basophils (%)	0.0-1.3	0.68 ± 0.43	0.71 ± 0.32
Serum Chemistry			
Creatinine (mg%)	0.6-2.0	0.70 ± 0.09	0.83 ± 0.13
BUN (mg%)	7.0-30.0	16.63 ± 2.73	29.25 ± 5.57
ALT (U/L)	4.0-91.0	32.75 ± 7.61	43.25 ± 10.04
ALP (U/L)	43.0-115.0	112.00 ± 31.91	78.25 ± 32.83

Data are presented as mean ± SEM. Abbreviations: MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, BUN = blood urea nitrogen, ALT = alanine transaminase, ALP = alkaline phosphatase. *Normal ranges are from Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University.

2. Effect of ivabradine on BP, HR and MVO_2

The SBP, DBP and MBP at baseline were 169 ± 8.9 mmHg, 104 ± 5.9 mmHg and 128 ± 7.0 mmHg, respectively (Figure 4-5). In response to repeated oral dose of ivabradine, SBP and MBP were significantly lower at M2 (P<0.05; -9.15 % and –8.93 %, respectively) and M3 (P<0.05; -15.82 % and –15.48 %, respectively) when compared with baseline; DBP was significantly lower only at M3 (P<0.05; -14.42 %) when compared with baseline. The BP did not change at M1 when compared with baseline. The HR obtained from the oscillometric device at baseline was 152 ± 4.0 bpm and it was significantly lower at M1 (-10.91 %, P<0.05), M2 (-18.19 %, P < 0.05) and M3 (-23.30 %, P<0.05) when compared with baseline (Figure 4-6A). The MVO_2 as estimated from RPP at baseline was 25,779 bpm.mmHg and this was significantly lower at M1 (-12.96 %, P<0.05), M2 (-25.78 %, P<0.05) and M3 (-35.55 %, P<0.05) when compared with baseline (Figure 4-6B).

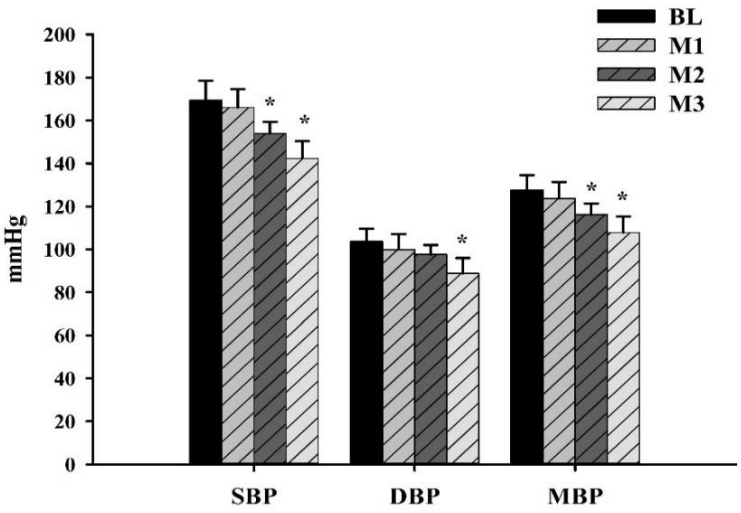


Figure 4-5 Histogram illustration of an average of systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) of dogs with DMVD (N = 4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day (M1 = 4 weeks, M2 = 8 weeks, and M3 = 12 weeks). *indicates P<0.05 when compared with baseline using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. Abbreviations: bpm = beat per minute, mmHg = millimeter of mercury

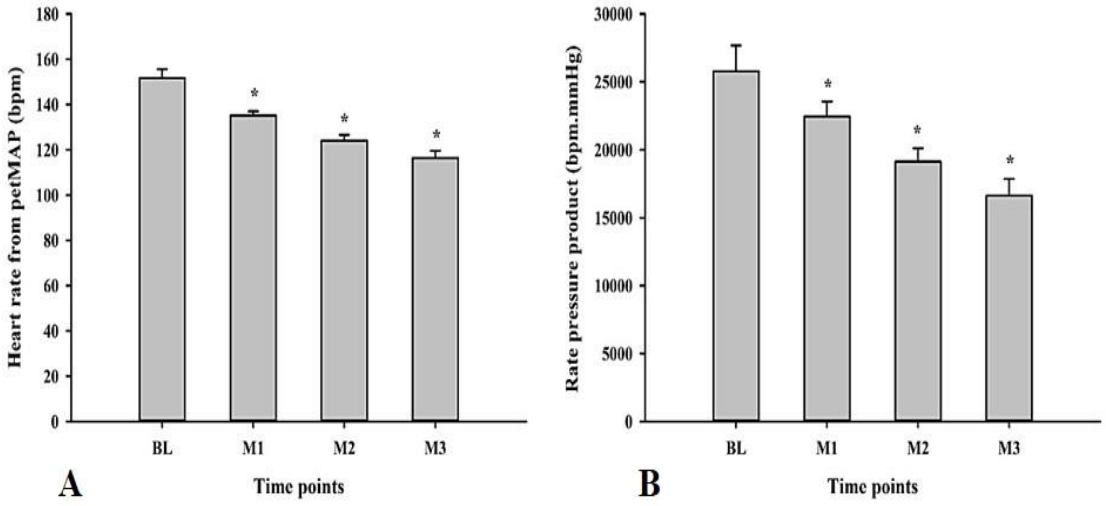


Figure 4-6 Histogram illustration of an average of heart rate (A) and rate pressure product (B) of dogs with DMVD (N = 4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day (M1 = 4 weeks, M2 = 8 weeks, and M3 = 12 weeks). *indicates $P<0.05$ when compared with baseline using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis.

3. Effect of ivabradine on ECG parameters

All 1 minute ECG tracings demonstrated no increase of either supraventricular or ventricular arrhythmias nor changes of ECG intervals and amplitudes after receiving ivabradine, except for the RR interval, which prolonged after receiving ivabradine (479.73 ± 54.64 ms to 689.50 ± 40.02 ms, $P<0.05$) (Figure 4-7).

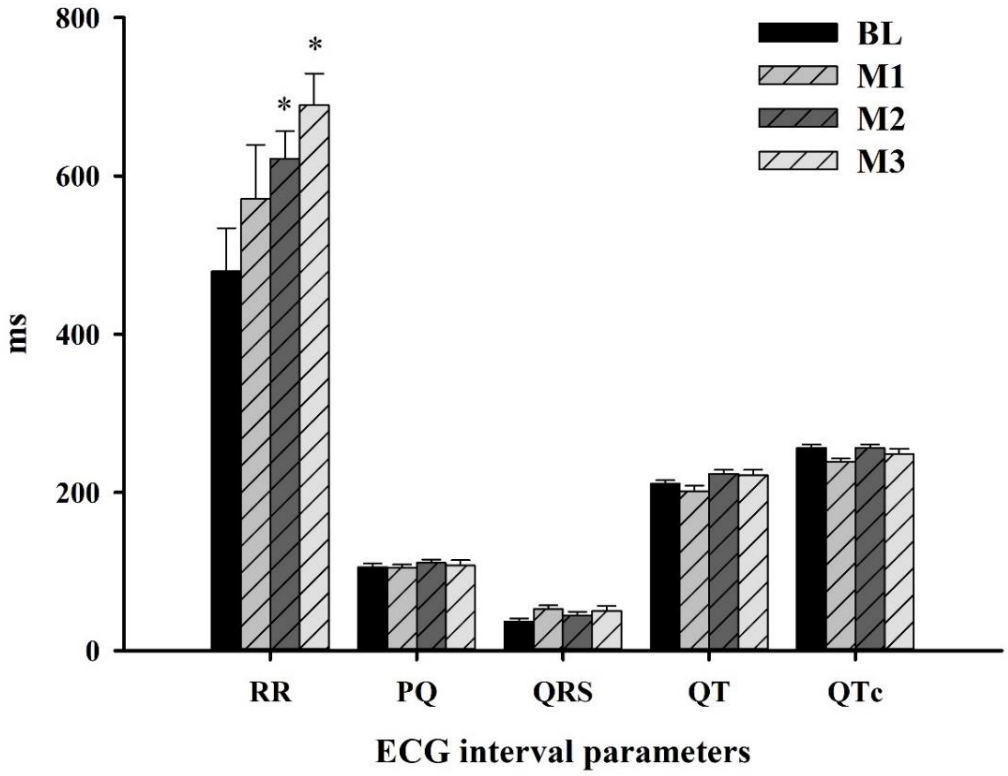


Figure 4-7 Histogram illustration of an average of ECG intervals of dogs with DMVD (N=4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day (M1 = 4 weeks, M2 = 8 weeks, and M3 = 12 weeks). *indicates *P*<0.05 when compared with baseline using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. Abbreviations: ECG = electrocardiogram, RR interval= the interval from the peak of the R wave to the peak of the next R wave, PQ interval = the interval from the beginning of the P wave to the beginning of the QRS complex; QT interval = the duration from the beginning of Q wave to the end of T wave, QTc = the QT interval corrected for heart rate by van de Water equation (Van de Water et al., 1989).

4. Effect of ivabradine on HRV

The mean HR obtained from 24 h continuous ECG recording at baseline was 133 ± 4.0 bpm and it was significantly lower at M1 (-8.3 %, *P*<0.05), M2 (-12.1 %, *P*<0.05) and M3 (-17.2 %, *P*<0.05) when compared with baseline (Figure 4-8). The maximum instantaneous HR was unaltered when compared among time points. The minimum instantaneous HR at baseline was 80 ± 4.0 bpm and it was lower at M1 (-8.1 %) and significantly lower at M2 (-11.8 %, *P*<0.05) and M3 (-15.6 %, *P*<0.05) when compared with baseline.

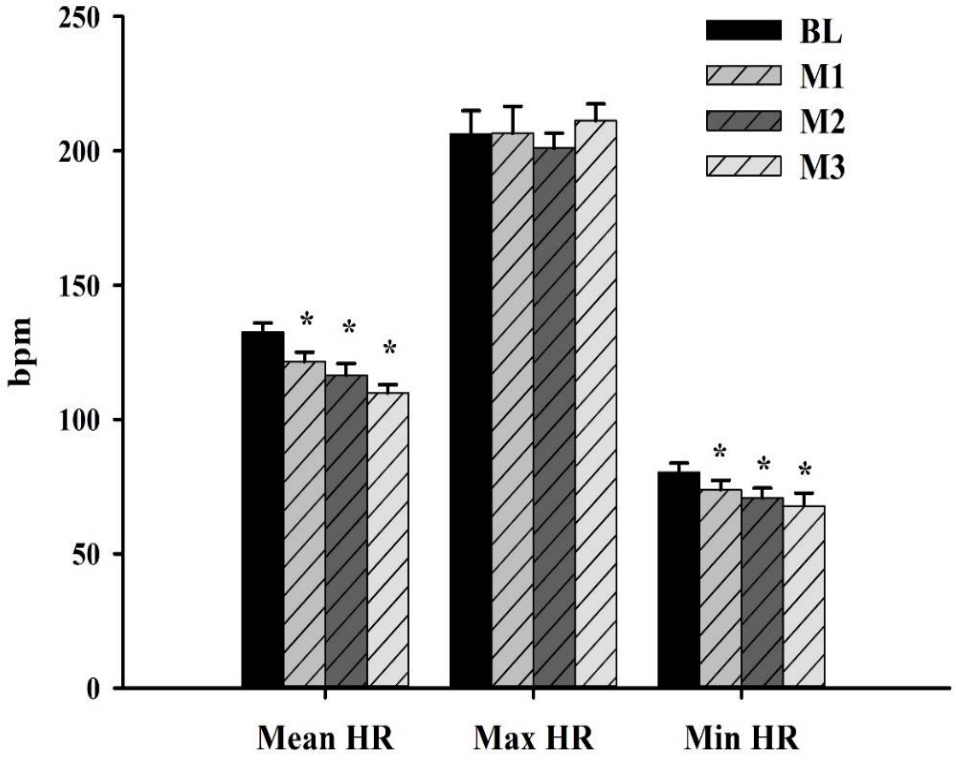


Figure 4-8 Histogram illustration of an average of 24-h Holter monitoring parameters including heart rate (mean HR), maximum instantaneous heart rate (Max HR), and minimum instantaneous HR (Min HR) of dogs with DMVD (N = 4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day (M1 = 4 weeks, M2 = 8 weeks, and M3 = 12 weeks). *indicates *P*<0.05 when compared with baseline using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. Abbreviations: bpm = beat per minute

The incidence of cardiac arrhythmia was also evaluated from Holter recording. The mean supraventricular and ventricular arrhythmias at baseline were 0.0815 % and 0.0065 %, respectively. After receiving ivabradine, one of the four dogs had higher supraventricular (from 0.26% at baseline to 1.34% at M3) and ventricular (from 0.009% at baseline to 0.015% at M3) arrhythmias while arrhythmias of the rest of the dogs did not change.

The result of time domain analysis of HRV is shown in Figure 4-9. In response to repeated oral dose of ivabradine, SDNN, SDANN, SDNN index, pNN50% and rMSSD when measured at M3 were significantly higher (54.5 %, 57.8 %, 53.8 %, 56.8 % and 77.1 %, respectively) when compared with baseline (P<0.05). After treatment with ivabradine for 2 months (M2) and 3 months (M3), the NNA value was significantly higher than the value at baseline (13.9 % and 21.9 %, respectively) (P<0.05).

The result of frequency domain (PSD, power spectral density) analysis of HRV was shown in Figure 4-10 and Figure 4-11. Dogs receiving ivabradine for 3 months had significant higher low frequency (104.7 %), high frequency (159.8 %) and total power (135.9 %) when compared with baseline (P<0.05) but no significant difference was found for the ratio of low frequency to high frequency at all time points when compared with baseline.

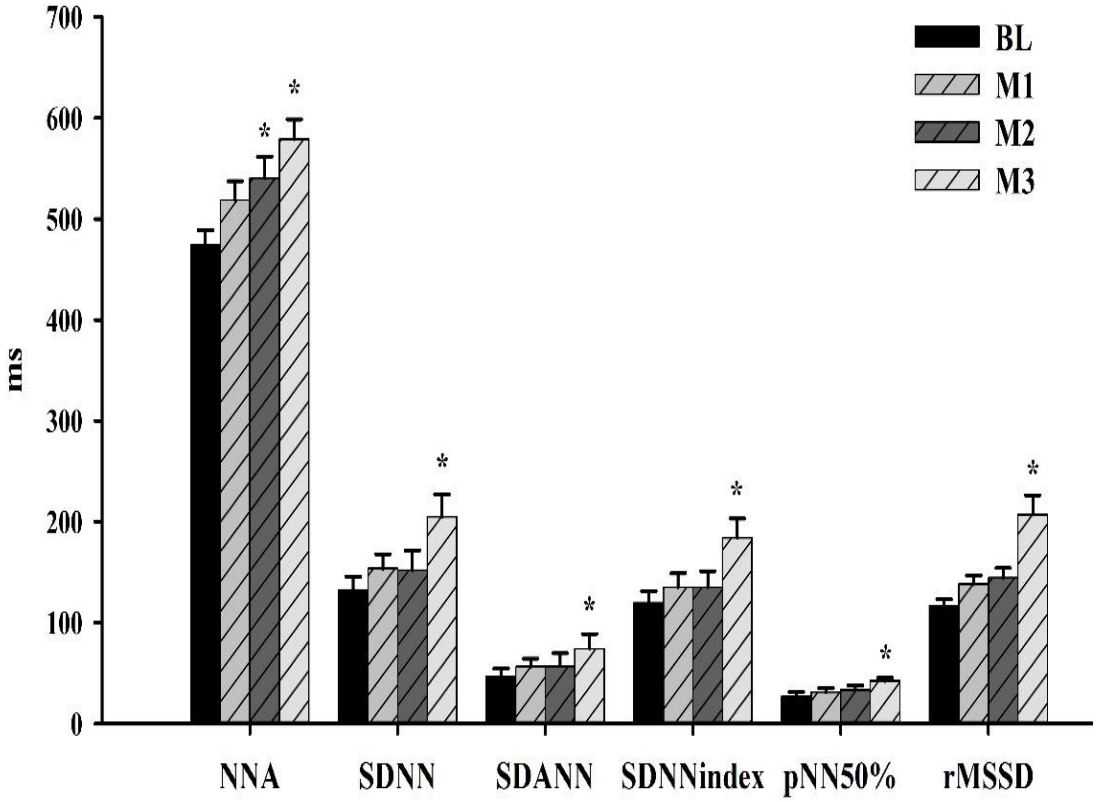


Figure 4-9 Histogram illustration of an average of time domain indices of heart rate variability including the mean NN intervals (NNA), standard deviation of all normal to normal RR intervals (SDNN), the standard deviation of 5-min mean RR intervals (SDANN), the mean of the standard deviation of all normal-to-normal RR intervals for all 5-min segments (SDNN index), the percentage of successive normal RR intervals exceeding 50 ms (pNN50) and the square root of the mean of the squares of the differences between successive normal to normal RR intervals (rMSSD) of dogs with DMVD (N = 4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day (M1 = 4 weeks, M2 = 8 weeks, and M3 = 12 weeks). *indicates $P<0.05$ when compared with baseline using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. Abbreviations: PSD = power spectral density, ms^2 = millisecond square

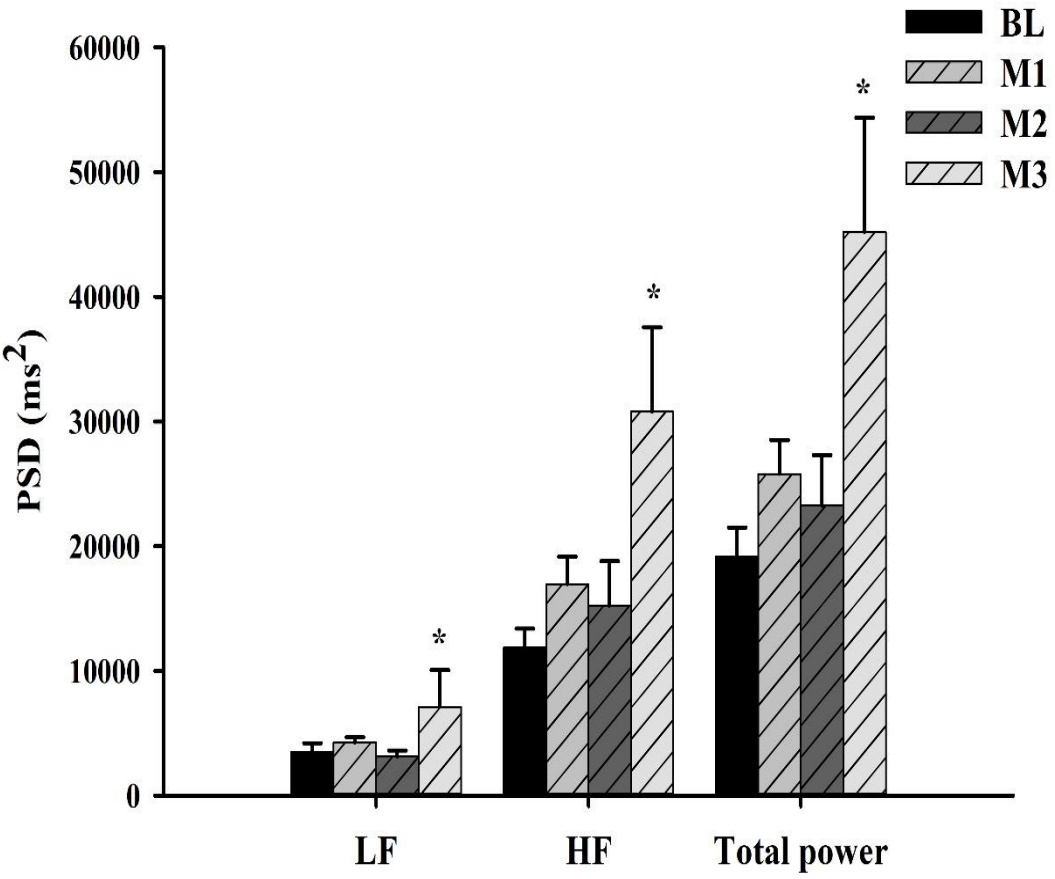


Figure 4-10 Histogram illustration of an average of frequency domain indices of heart rate variability including low frequency (LF), high frequency (HF), and total power of dogs with DMVD (N = 4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day (M1 = 4 weeks, M2 = 8 weeks, and M3 = 12 weeks). *indicates $P<0.05$ when compared with baseline using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. Abbreviations: PSD = power spectral density, ms^2 = millisecond square

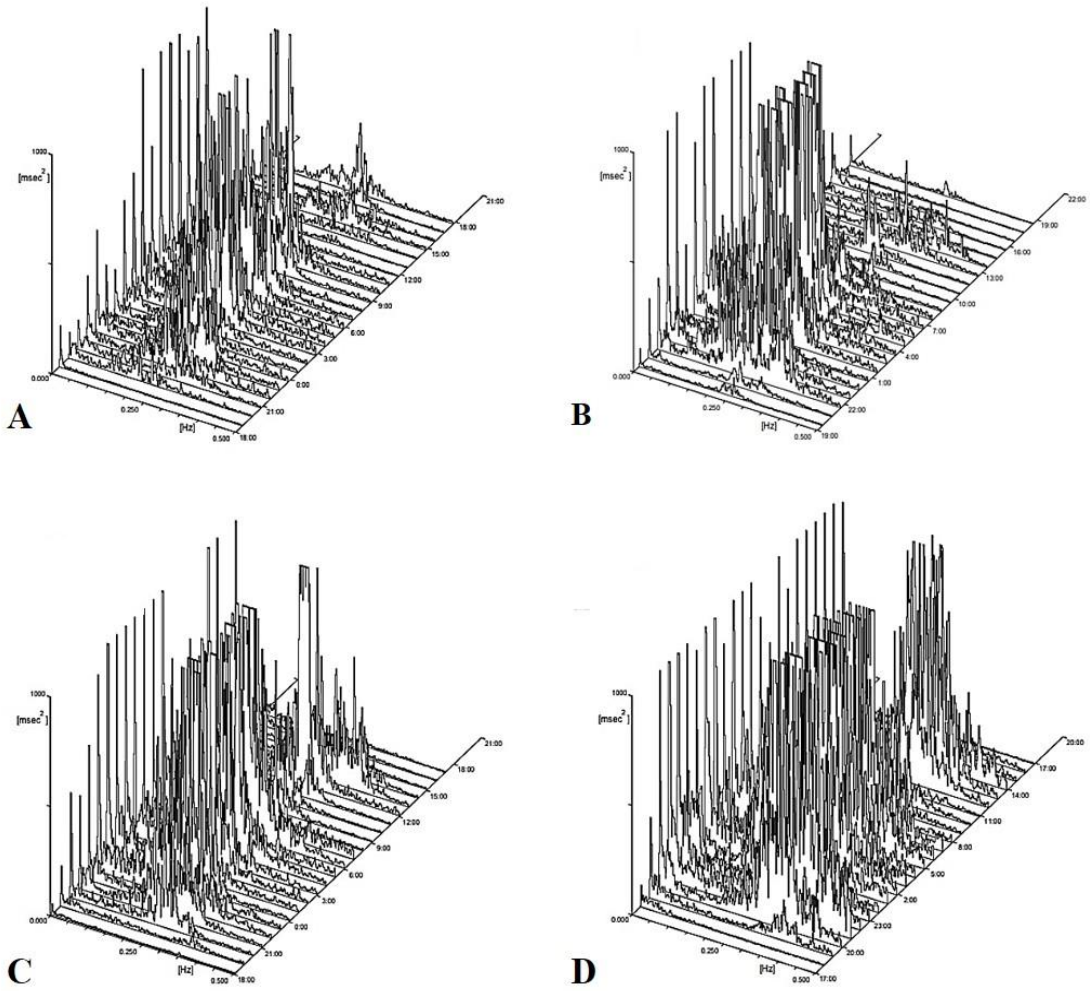


Figure 4-11 Examples of power spectrum density of one dog with naturally occurring, asymptomatic DMVD obtained at baseline (A), after treatment with ivabradine 1 mg/kg ivabradine orally twice a day for 1 month, (B) 2 months (C) and 3 months (D), respectively.

5. Effect of ivabradine on echocardiographic parameters

Conventional echocardiographic and STE data of ivabradine are summarized in Table 4-8. When comparing with baseline data, some parameters of STE changed significantly. The global radial strain, global circumferential strain and fractional area change at M2 and M3 were significantly higher than those at baseline ($P<0.05$). Other parameters obtained at any time point in the study did not achieve statistical significance when compared with baseline.

Table 4-8 Effects of ivabradine on conventional echocardiographic parameters and speckle-tracking echocardiographic parameters in dogs with naturally occurring, asymptomatic DMVD (N = 4).

Variables	Baseline	M1	M2	M3
Conventional echocardiographic parameters				
LVIDd (cm)	3 ± 0.15	3.1 ± 0.12	3 ± 0.1	3.1 ± 0.13
LVIDs (cm)	1.8 ± 0.1	1.9 ± 0.26	1.8 ± 0.24	1.9 ± 0.21
EF (%)	71.6 ± 3.56	69 ± 8.34	69.5 ± 7.81	71.6 ± 6.46
EDV (ml)	34.4 ± 3.95	38.5 ± 3.67	36.3 ± 3.03	38.5 ± 3.76
ESV (ml)	10 ± 1.37	12.1 ± 4.03	11.3 ± 3.43	11.4 ± 3.48
HR (bpm)	123 ± 6.85	120 ± 9.62	119 ± 6.52	100 ± 4.8
Jet area (%)	60.5 ± 7.57	49.3 ± 5.71	50.7 ± 7.79	49.8 ± 10.85
MV E/A	1.9 ± 0.23	1.31 ± 0.54	1.29 ± 0.17	1.12 ± 0.33
Speckle-tracking echocardiographic				
GRS (%)	17.3 ± 2.93	20.2 ± 3.8	23.3 ± 3.28*	26.1 ± 2.24*
GCS (%)	13.7 ± 1.12	15.3 ± 2.63	17.3 ± 2.19*	17.7 ± 1.80*
FAC (%)	38.3 ± 2.44	41.4 ± 5.45	46.8 ± 5.18*	49.7 ± 6.02*
EDA	7.2 ± 0.55	7.9 ± 1.39	7.4 ± 1.44	7.7 ± 1.04
ESA	4.4 ± 0.34	4.7 ± 1.12	4 ± 0.85	3.9 ± 0.9

Data are presented as mean ± SEM. *indicates $P<0.05$ when compared with baseline using one-way ANOVA with repeated measures followed by Dunnett post-hoc analysis. Abbreviations: EDA = End-diastolic area, EDV: End-diastolic volume, EF = Ejection fraction, ESA = End-systolic area, ESV= End-systolic volume, FAC = Fractional area of change, GCS = Global circumferential strain, GRS = Global radial strain, HR = Heart rate, Jet area = Mitral regurgitation jet area, LVIDd = Left ventricular internal diastole diameter, LVIDs = Left ventricular internal systole diameter, M1 = 4 weeks after treatment, M2 = 8 weeks after treatment, M3 = 12 weeks after treatment, MV E/A = The mitral valve early filling/atrial filling velocities

Study part 3: The role of the long-term treatment with repeated oral dose of ivabradine involving hemodynamic, cardiac function and cardiac apoptosis in dogs with asymptomatic DMVD stage B2.

1. Effect of ivabradine on hemodynamics

Effect of ivabradine on hemodynamics are summarized in Table 4-9. All of systemic blood pressure parameters (SBP, DBP and MBP) in four dogs receiving ivabradine for 3 months did not change when compared with baseline. In addition, pulmonary circulation parameters (i.e. PAPsys, PAPdias, mPAP, mRAP and PCWP) did not change. Both systemic and pulmonary vascular resistances were unchanged when compared with baseline.

2. Effect of ivabradine on LV mechanical function

Data of ivabradine on LV mechanical function are summarized in Table 4-10. In response to repeated oral dose of ivabradine, the percent changes of CI and Tau measured at M3 were significantly higher than those of baseline (7.57 % and 15.38 %, respectively; $P<0.05$) while other parameters (i.e. ESP, dP/dt_{max}, EDP, and dP/dt_{min}) were not different from baseline values.

Table 4-9 Effects of long-term treatment with repeated oral dose of ivabradine on hemodynamics in anesthetized dogs with naturally occurring, asymptomatic DMVD (N = 4).

Variables	Baseline	M3
Systemic blood pressure		
SBP (mmHg)	68.97 ± 4.28	92.40 ± 14.10
DBP (mmHg)	47.90 ± 2.94	68.48 ± 12.42
MBP (mmHg)	54.92 ± 3.15	76.45 ± 12.98
Pulmonic blood pressure		
PAPsys (mmHg)	28.58 ± 3.51	24.05 ± 5.77
PAPdias (mmHg)	19.43 ± 2.10	17.50 ± 1.74
mPAP (mmHg)	22.48 ± 2.55	19.04 ± 3.05
mRAP (mmHg)	7.05 ± 0.67	6.88 ± 0.98
mPCWP (mmHg)	9.26 ± 0.76	10.13 ± 0.56
Vascular resistances		
SVR (dyn.s.cm^{-5})	1,354.70 ± 205.97	1,988.13 ± 242.75
PVR (dyn.s.cm^{-5})	386.41 ± 103.21	247.93 ± 60.61

Data are presented as mean ± SEM. Abbreviations: M3 = 12 weeks after treatment, SBP = systolic blood pressure, DBP = diastolic blood pressure, MBP = mean blood pressure, PAPsys = systotic pulmonary artery pressure, PAPdias = diastolic pulmonary artery pressure, mPAP = mean pulmonary artery pressure, mRAP = mean right atrial pressure, mPCWP = mean pulmonary capillary wedge pressure, EDP = end diastolic pressure, ESP = end systolic pressure, SVR = systemic vascular resistance, PVR = pulmonary vascular resistance

Table 4-10 Effects of long-term treatment with repeated oral dose of ivabradine on LV pressure and indices of contractility and relaxation in dogs with naturally occurring, asymptomatic DMVD (N = 4).

Variables	Baseline	M3
Systolic LV function		
CI	41.60 ± 2.27	44.75 ± 2.65*
ESP (mmHg)	70.30 ± 5.51	88.98 ± 13.93
dP/dt_{max} (mmHg/sec)	1,398.75 ± 183.94	1,489.50 ± 147.43
Diastolic LV function		
EDP (mmHg)	4.58 ± 0.42	5.30 ± 0.28
Tau (ms)	16.25 ± 3.30	18.75 ± 0.18
dP/dt_{min} (mmHg/sec)	-1,402.75 ± 100.97	-1,529.00 ± 306.18

Data are presented as mean ± SEM. *indicates $P<0.05$ when compared with baseline using paired *t*-test. Abbreviations: M3 = 12 weeks after treatment, EDP = end diastolic pressure, ESP = end systolic pressure, CI = contractility index, dP/dt_{max} = the maximum rate of rise of the left ventricular pressure, Tau = isovolumic relaxation time constant, dP/dt_{min} = the maximum rate of fall of the left ventricular pressure

3. Effect of ivabradine on left ventricular structural remodeling

Cardiac structural remodeling was determined by assessment of histological changes. H & E stain, PAS stain and Masson's trichrome stain were used to identify morphological changes of cardiomyocytes and extracellular matrix. The results of histopathological examination of endomyocardial tissues of four dogs with asymptomatic DMVD were shown in Table 4-11. White blood cell infiltration, vacuolization of myocytes, reduction of myofibril, lipofuscin pigment granules, accumulation of glycogen and cardiac (interstitial, perivascular and fibrofatty) fibrosis were observed in both of timepoints (i.e. before and 3 months after treatment). Dogs receiving ivabradine for 3 months had significant lower in the percentage of

vacuolization of myocytes, glycogen accumulation, interstitial fibrosis and cardiac fibrofatty than those of baseline (65.8%, 47.6%, 57.3%, 77.8%, respectively).

Table 4-11 Effects of long-term treatment with repeated oral dose of ivabradine on histopathological parameters in dogs with naturally occurring, asymptomatic DMVD (N = 4).

Variables	Baseline	M3
H & E stain		
white blood cell infiltration	1.80 ± 0.18 (0-3)	1.60 ± 0.16 (0-3)
vacuolization of myocytes	1.17 ± 0.20 (0-3)	0.40 ± 0.16* (0-3)
reduction of myofibril	0.71 ± 0.15 (0-2)	0.68 ± 0.15 (0-2)
ruptured myocardial fiber	0.00 ± 0.00 (0)	0 ± 0.00 (0)
wavy myocardial fibers	0.00 ± 0.00 (0)	0 ± 0.00 (0)
lipofuscin accumulation	2.34 ± 0.22 (0-4)	2.20 ± 0.23 (1-4)
PAS stain		
Glycogen accumulation	2.29 ± 0.18 (0-4)	1.20 ± 0.22* (0-3)
Masson's trichrome stain		
interstitial fibrosis	2.06 ± 0.24 (0-4)	0.88 ± 0.17* (0-2)
perivascular fibrosis	0.43 ± 0.12 (0-2)	0.40 ± 0.10 (0-1)
fibrofatty	0.90 ± 0.15 (0-2)	0.20 ± 0.08* (0-1)

Data are presented as mean ± SEM and data in parenthesis represents the range of semi-quantitative score. *indicates $P<0.05$ when compared with baseline using paired *t*-test. Abbreviations: M3 = 12 weeks after treatment, H & E = hematoxylin and eosin, PAS = Periodic acid-Schiff

4. Effect of ivabradine on left ventricular apoptosis

The result of apoptotic index (AI) of LV endocardium tissue was showed in Figure 4-12. The AI of LV endocardium tissue was calculated from the number of apoptotic cells that were appeared in bright blue color within the total cells. In response to repeated oral dose of ivabradine, the percentage of AI when measured at M3 were significantly decreased (17.0 %) when compared with baseline ($P<0.05$).

5. Effect of ivabradine on expression of apoptotic proteins (Bax and Bcl-2 proteins) and the ratio of Bax to Bcl-2

The results of expression of apoptotic proteins (Bax and Bcl-2 proteins) were showed in Figure 4-13. Both Bax and Bcl-2 protein expressions observed in LV endocardium tissue of four dogs with naturally occurring, asymptomatic DMVD. Before and 3 months after treatment with ivabradine, the score of pro-apoptotic proteins (Bax) were expressed at 1.70 ± 0.10 and 1.60 ± 0.20, respectively. In response to repeated oral dose of ivabradine, anti-apoptotic proteins (Bcl-2) when measured at M3 tended to increase (from score 1.24 ± 0.14 to 1.62 ± 0.12) when compared with baseline ($P=0.06$).

An intrinsic pathway of apoptosis related with an increasing of the ratio of Bax to Bcl-2. In endocardium tissue of dogs with naturally occurring, asymptomatic DMVD at the time points before and 3 months after treatment with ivabradine, the ratio of Bax to Bcl-2 were 1.56 ± 0.43 and 1.13 ± 0.11, respectively.

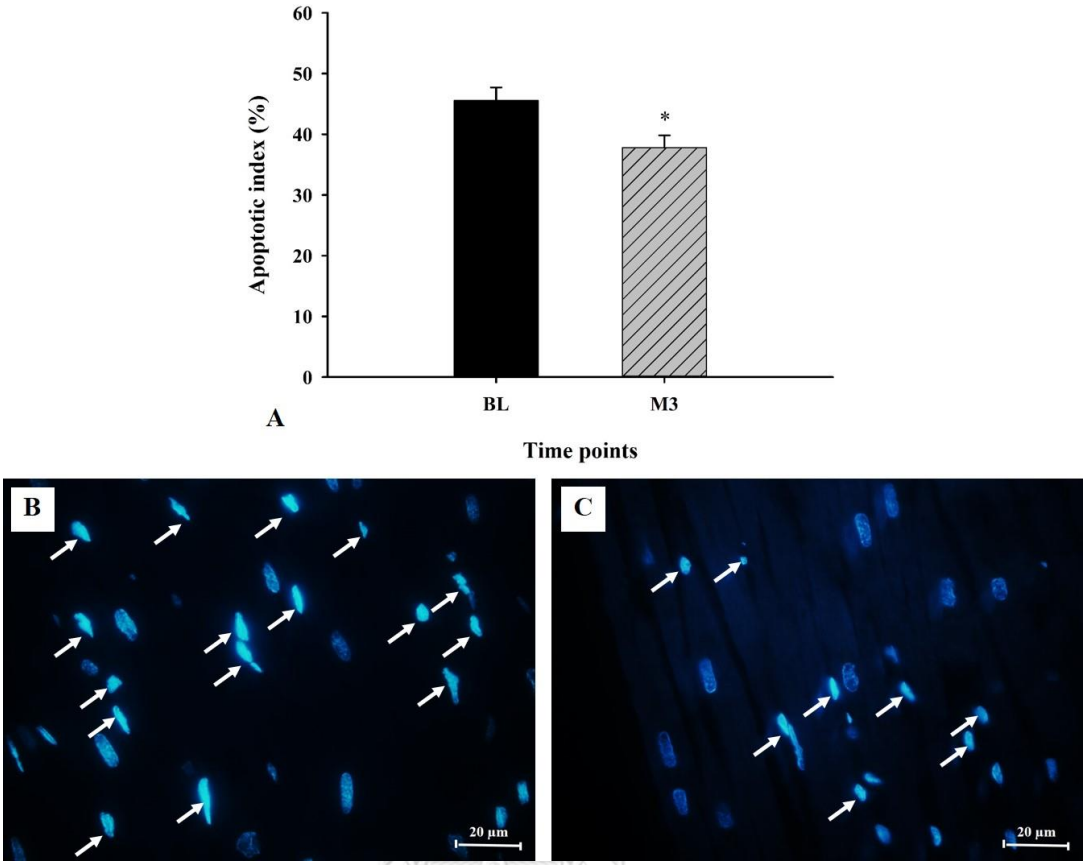


Figure 4-12 (A) Histogram illustration of semi-quantitative analysis of cardiac apoptotic cells using Hoechst 33342 fluorescence dye of dogs with DMVD (N = 4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day for 3 months (M3). Values are expressed as mean ± SEM. *indicates $P<0.05$ when compared with baseline using paired *t*-test. The nuclear staining of left ventricular cardiomyocytes obtained from experimental dogs with asymptomatic DMVD at baseline (B) and after treatment with ivabradine 1 mg/kg ivabradine orally twice a day for 3 month (C). The thin arrow pointed at the bright blue color and/or nuclear fragmentation which represented the positive sites of apoptotic cardiomyocytes. The smooth nuclei and stained with dark blue color represented normal nucleus of cardiomyocytes (×40 objective magnification, scale bar = 20 µm).

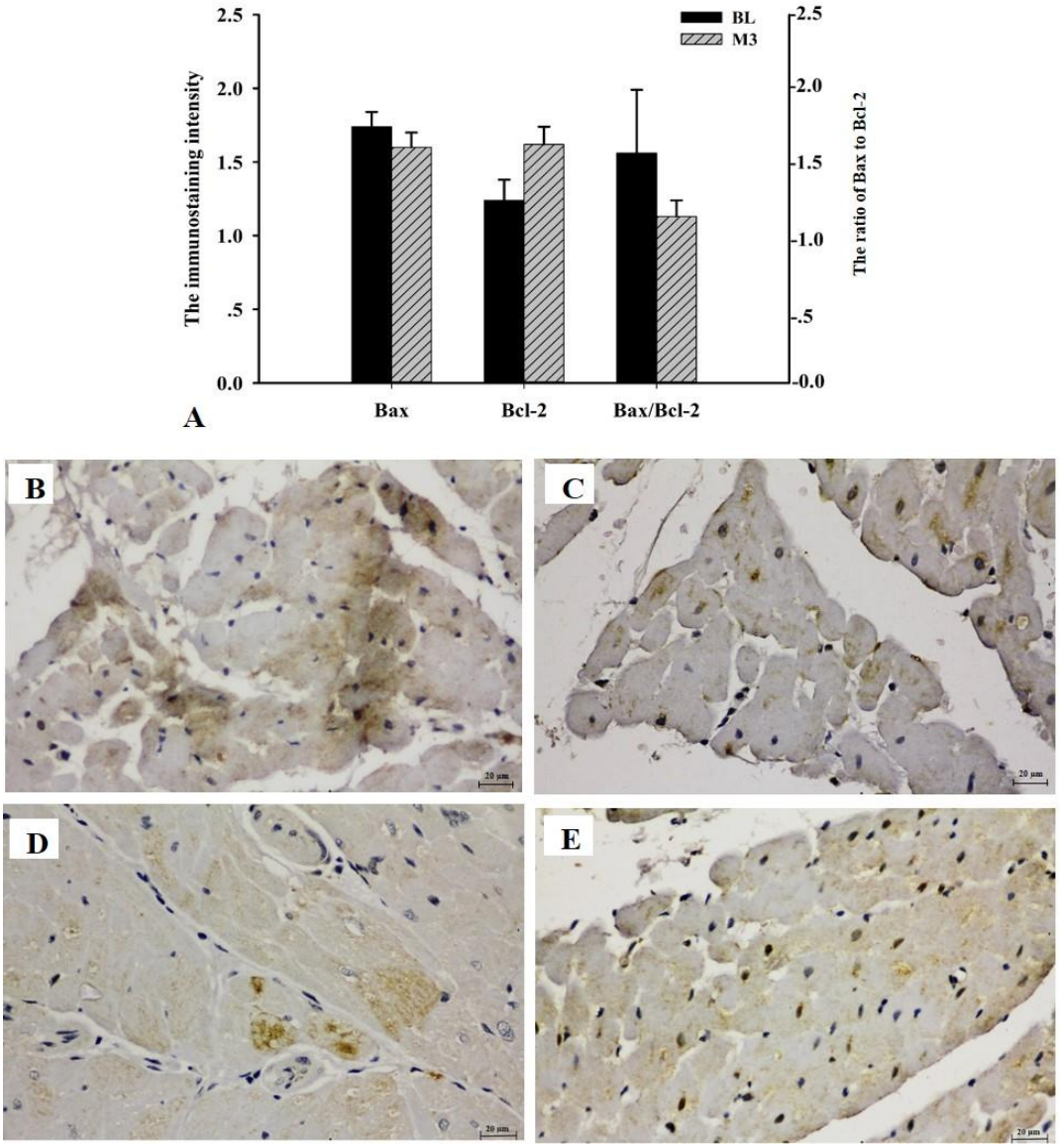


Figure 4-13 (A) Histogram illustration of semi-quantitative analysis of expressions of proapoptotic proteins (Bax), anti-apoptotic proteins (Bcl-2) and the ratio of Bax to Bcl-2 in dogs with DMVD (N = 4) obtained at baseline (BL) and after chronic administration of 1 mg/kg ivabradine orally twice a day for 3 months (M3). Values are expressed as mean ± SEM. The expressions of Bax at BL (B) and M3 (C) were also showed as well as the expression of Bcl-2 at BL (D) and M3 (E). The positive sites of Bax were stained in brown color in the cytoplasm while positive sites of Bcl-2 were stained in brown color in the nucleus and intracytoplasmic cells (×40 objective magnification, scale bar = 20 μm).

CHAPTER V
DISCUSSIONS

The discussion of this study were organized into 3 study parts as follow:

Study part 1: The appropriate single oral dose of ivabradine for reduction of HR and MVO_2 in dogs with asymptomatic DMVD

The results of this study in dogs with DMVD demonstrated that ivabradine when given orally at 1.0 mg/kg significantly decreased the HR and RPP (i.e. reduced MVO_2) without adverse effects on blood pressure. It also did not induce supraventricular and ventricular arrhythmias.

Ivabradine is a hyperpolarization-activated cyclic nucleotide (HCN) channel blocker, which acts on the SA node to reduce the HR by reducing the slope of the diastolic potential of pacemaker cells (DiFrancesco and Mangoni, 1994; Thollon et al., 1994). The slope of diastolic potential is regulated by the funny current (I_f) which is a voltage-gated, time-dependent mixed Na^+ and K^+ inward current that activated by hyperpolarization of membrane potentials and intracellular cyclic AMP (cAMP). Activation of the I_f current leads to increase membrane permeability to Na^+ and K^+ causing a less negative membrane potential which increases the slope of diastolic depolarization (phase 4 of action potential). Because it is a selective HCN blocker, most studies have indicated that ivabradine possesses neither direct negative nor positive inotropic, dromotropic nor lusitropic effects, determined by tests both in humans and animals (Gardiner et al., 1995; Simon et al., 1995; Camm and Lau, 2003; Berdeaux, 2007; Savelieva and Camm, 2008). Interestingly, a recent study in cats with hypertrophic cardiomyopathy showed that intravenous ivabradine administration possessed slightly negative inotropic and lusitropic effects (Riesen et al., 2012). The present study did not investigate those effects; therefore, it may be necessary to investigate further to elucidate the inotropic and lusitropic effects of ivabradine in dogs with DMVD.

In the present study, the highest dose of ivabradine (2.0 mg/kg) reduced the HR and blood pressure, thus lowering the RPP more than with the other two doses

(0.5 and 1.0 mg/kg). This is consistent with a previous study in which ivabradine exerted a dose-dependent effect (Cober et al., 2011). In addition, another study indicated that intravenous ivabradine administration (0.25, 0.5 and 1.0 mg/kg) demonstrated a dose-dependent reduction not only in normal conscious dogs but also in exercise-induced tachycardia dogs (Colin et al., 2004). It has been known that BP is determined by HR, stroke volume, and total peripheral resistance. Ivabradine possesses a pure funny channel blocker without any effect on cardiac contractility and vascular smooth muscle (DiFrancesco and Mangoni, 1994). Therefore, the highest dose of ivabradine (2 mg/kg) in the current study causes markedly decreased HR and leads to a falling of BP.

To the best of our knowledge, there is no pharmacokinetic study of ivabradine in dogs with DMVD. In normal dogs, the oral ivabradine administration was rapidly absorbed at roughly 40% of bioavailability. The plasma protein binding was roughly 50%-70%. The peak plasma concentration achieved 1 h after administration was approximately 1 L/kg volume of distribution at a steady state. In addition, the elimination of ivabradine mainly occurred via hepatic circulation, and its main half-life was less than 2 h (European Medicines Agency, 2005). The hemodynamics of DVMD dogs are different from healthy dogs (Oyama, 2009); therefore, further pharmacokinetic investigation of ivabradine should be conducted in various stages of DMVD dogs to ensure the proper dose.

Previous studies in normal cats with HCM indicated that ivabradine was clinically well tolerated without undesired side effects (Cober et al., 2011; Riesen et al., 2011; Blass et al., 2014). In the present study, ivabradine was also clinically well tolerated in dogs with DMVD. The oral dose of 1 mg/kg was the maximum dose used in the current study that significantly reduced the HR and RPP without lowering SBP or increasing numbers of arrhythmic beats per 24 h. In the current study, one dog showed increased numbers of VPCs after receiving ivabradine at 2.0 mg/kg. The effect of ivabradine on arrhythmia induction was unclear. The literature has suggested that the risk of atrial fibrillation (AF) when using ivabradine was about 1 in 10,000 patients. However, studies conducted in dogs with age-related AF and in dogs with vagal nerve stimulation demonstrated that ivabradine did not increase the risk of AF in those dogs (Li et al., 2015; Uemura et al., 2017). A recent study in patients with decompensated

HF indicated that ivabradine was effective in reducing the VPCs induced by low and medium doses of dobutamine infusion (Mert et al., 2017). Furthermore, studies conducted in failing heart mice and rats with acute myocardial infarction (MI) revealed that ivabradine was effective in preventing β-adrenergic stimulation-induced abnormal automaticity or partially preventing the proarrhythmic effects of MI (Kuwabara et al., 2013; Mackiewicz et al., 2014).

In humans, several clinical trials have demonstrated that there was a benefit of HR reduction in cardiovascular disease because the clinical outcomes were improved (Fox et al., 2008; Swedberg et al., 2010). The HR is an independent factor for cardiovascular diseases and is mainly related to the MVO_2 (Colin et al., 2004; Cooney et al., 2010). In the present study, MVO_2 was decreased in response to oral ivabradine administration in a dose-dependent manner along with HR reduction. This finding agreed with previous studies in resting and exercising dogs, in which the reduction of the HR by ivabradine led to decreased MVO_2 and to an increased diastolic time interval (Colin et al., 2004). MVO_2 decreased by ivabradine improved the balance between oxygen demand and supply during cardiovascular diseases whereas increased diastolic time improved myocardial calcium cycling (Lechat, 1998). Both mechanisms may be beneficial to dogs with DMVD.

In conclusion, the current study indicated that ivabradine can be safely used in dogs with DMVD at 1.0 mg/kg, P.O. A long-term treatment should be performed to ensure the safety. In addition, further study in symptomatic DMVD dogs should be conducted to ensure our findings.

Study limitations: This study did not measure BP in detail from the 12^{th} to 24^{th} h after ivabradine administration. The lack of the data on BP during that period may not confound with the outcome of this study because the BP trend in Figure 4-3 began to stabilize from the 5^{th} h after dosing.

Study part 2: The long-term effects of repeated oral dose of appropriate ivabradine on MVO_2, BP, ventricular function, ECG parameters and HRV in dogs with asymptomatic DMVD

The first goal of this study was to assess the chronic effects of ivabradine on alteration of cardiac ANS activity in stage B2 DMVD dogs. ANS activity was estimated by evaluation of time- and frequency-domain parameters of HRV, because ANS activity is difficult to evaluate directly. Abnormal ANS input to the heart resulting in the decreased of HRV parameters (Stein et al., 1994). The time-domain parameters consist of NNA, SDNN, SDANN, SDNNindex, rMSSD and pNN50. The NNA is a mean of RR interval in the entire recording, increased NNA related to the decreased of HR (a reciprocal of RR interval). The SDNN reflects changes in sympathetic and parasympathetic activities whereas the SDANN and SDNNindex reflect sympathetic and parasympathetic activities that are attributable to baroreceptor reflex (Calvert, 1998; Sztajzel, 2004). In this study, increased SDNN indicates the increase of sympathetic and parasympathetic activities while increased SDANN and SDNNindex suggest the increase of baroreflex activity. In addition, SDNN, SDANN and SDNNindex have been shown to be associated with LF (Calvert, 1998). The pNN50 and rMSSD are associated with HF in which these indices are attributed to the effect of vagal activity (Calvert, 1998). In the present study, increased pNN50, rMSSD, and HF suggest the increase of vagal activity. The Total power (TP) is the sum of all spectral frequency bands in which it was increased in the present study; therefore, the overall cardiac autonomic regulation is improved (Stein et al., 1994; Task Force, 1996; Calvert, 1998; Sztajzel, 2004). The LF/ HF is the index of sympathetic and parasympathetic balance (i.e. sympatho-vagal balance). In the current study, the LF/ HF did not different among time-points after treatment (M1, M2, and M3) and baseline while other HRV parameters are augmented. This discrepancy may support by Billman (2013) in which it was demonstrate that the LF/HF ratio does not accurately measure cardiac sympatho-vagal balance since the ratio is rested upon several interrelated assumptions.

The present study demonstrated that asymptomatic DMVD dogs have impaired cardiac ANS activities suggested by low values of both time- and frequency- domain parameters at baseline (i.e. SDNN, SDANN, SDNN index, pNN50%, rMSSD, low frequency, high frequency and total power). This finding is consistent with previous studies of DMVD dogs (Fujii and Wakao, 2003; Rasmussen et al., 2012; Pirintr et al., 2017). In the present study, HRV increased gradually and a statistically significant increase was recorded at M3 after ivabradine treatment. To our knowledge, this is the first study showing the benefit of ivabradine to improve HRV in DMVD dogs. Similar findings have been reported previously in patients with nonischemic dilated cardiomyopathy (Kurtuglu et al., 2014).

Because the impairment of sympathovagal balance (i.e. sympathetic over activation and/or parasympathetic withdrawal) is a hallmark of CHF, a drug that can improve HRV might be beneficial in clinical use. In addition, a lower HRV has been shown to be associated with poor cardiovascular outcome (Billman, 2011). Therefore, the increase in HRV after receiving ivabradine in the present study suggest the potential use of ivabradine in asymptomatic DMVD. The current study did not investigate the possible mechanisms responsible for the improvement of HRV by ivabradine; however, evidence from several studies suggests that it may be due to the lower HR (i.e. increase diastolic filling time) together with reverse remodeling (Kurtuglu et al., 2014; Sabbah et al., 2014).

A lower in NNA (the reciprocal of a higher HR) is related to severity of DMVD; therefore, NNA can be used for evaluation of autonomic dysfunction (Rasmussen et al., 2012). In the present study, NNA was significantly higher after treatment with ivabradine. This result is similar to previous studies observed in DMVD and normal dogs (Colin et al., 2004) because ivabradine is a pure funny channel blocker that affects spontaneous diastolic depolarization at the SA node (DiFrancesco and Camm, 2004). Several clinical trials in humans have demonstrated that ivabradine can lower HR and improve of cardiac function, and improve quality of life without negative inotropism (Fox et al., 2009; Swedberg et al., 2010).

The current study also showed that the minimum instantaneous HR decreased whereas the maximum instantaneous HR was unchanged after treatment with

ivabradine (Figure 4-8). This change in HR did not induce bradycardia or any other arrhythmia. This is consistent with the finding of the current study that most of the dogs showed unchanged of the incidence of supraventricular and ventricular arrhythmias after receiving ivabradine. The effect of ivabradine on arrhythmia induction was unclear. Studies conducted in dogs with age-related AF and in dogs with vagal nerve stimulation showed that ivabradine did not increase the risk of AF in those dogs (Li et al., 2015; Uemura et al., 2017).

In addition to lowering HR, the current study in asymptomatic DMVD dogs demonstrated that systemic BP is decreased in response to chronic administration of ivabradine (Figure 4-5). The finding is contrast to our previous study in a similar group of dogs in which a single oral administration of 1 mg/kg ivabradine did not alter systemic BP (Pirintr et al., 2018). A decrease in BP may be caused by lower HR, because BP is determined by HR, stroke volume and total peripheral resistance. Furthermore, MVO_2 estimated by RPP is reduced which helps to reduce the workload of the heart and improve cardiac efficiency (Suga, 1979)

The second goal of this study was to assess the chronic effects of ivabradine on cardiac function in stage B2 DMVD dogs investigated by both conventional and STE. The STE is a newly established system that can be used for characterization and quantification of myocardial deformation (Bansal and Kasliwal, 2013). The indices obtained from STE are unlike those obtained from tissue Doppler and conventional echocardiography because they are angle-independent and less load-dependent. Myocardial deformation, also known as strain imaging, is reported as the percent change of length of the myocardial segment during a given period; thus, it reflects both systolic and diastolic functions of the heart (Bansal and Kasliwal, 2013). The global radial strain measures myocardial thickening, whereas the global circumferential strain measures myocardial shortening; these are both obtained from the short-axis view. A recent study by Kovacs and colleagues (Kovács et al., 2015) demonstrated that STE indices correlate with pressure-volume loop-derived contractility indices. The current study demonstrated that ivabradine improved several STE indices (global radial strain, global circumferential strain, and fractional area change) at M3 when compared with baseline, suggesting that both left ventricular systolic and diastolic function are

improved by ivabradine. However, significant changes of conventional echocardiographic parameters seen at M3 were not detected. This may be due to the lack of sensitivity of conventional echocardiography to detect subtle changes in response to chronic ivabradine treatment in DMVD dogs. The high sensitivity of STE has been supported by several investigators showing that the STE is more sensitive early changes in pathological condition of the heart than conventional echocardiography (Bauer et al., 2011; Chu et al., 2015; An et al., 2016). In addition, similar results showing ivabradine improves systolic and diastolic function have been observed in CHF dogs induced by microsphere embolization (Sabbah et al., 2014). That study suggested that ivabradine improves heart function by prevention of progressive left ventricular remodeling suggested by improvement of myocardial calcium handling, reduction plasma biomarkers, and lowering apoptotic process.

In conclusion, both the time- and frequency-domain of HRV indices were low in DMVD dogs, suggesting parasympathetic withdrawal and/or sympathetic activation. Dogs with mild DMVD receiving long-term treatment with ivabradine (1 mg/kg, twice daily, orally) had increased cardiac autonomic modulation, as suggested by both time- and frequency-domain indices. Long-term administration of ivabradine lowered HR and BP without increasing cardiac arrhythmia in DMVD dogs. In addition, a decrease in MVO_2 suggested the improvement of cardiac efficiency. Therefore, ivabradine may be useful for maintaining normal ANS activity in dogs with asymptomatic DMVD. Furthermore, long-term treatment with ivabradine improves systolic and diastolic function of the left ventricle, suggesting that using ivabradine in a clinical setting is beneficial.

Study limitations: There are a couple limitations in the present study; therefore, the result must be interpreted with caution. First, the study was performed in a small sample size due to the availability of subjects. The lack of statistically significant differences in some parameters between ivabradine and baseline may be influenced by this small sample size. However, the small sample size did not affect the overall outcome of the study. In addition, the study supports the use of the 3R concept (i.e. replacement, reduction, and refinement) by using fewer animals to achieve the objectives of the study. Second, the plasma concentrations of ivabradine in DMVD dogs were not performed. The data of plasma ivabradine concentration may

provide more detail of the relationship between drug concentration and its effect; however, it is not a major goal of the study. The pharmacokinetic of ivabradine in DMVD dogs has not been investigated; therefore, further investigation of ivabradine should be conducted in various stages of DMVD dogs.

Study part 3: The role of the long-term treatment with repeated oral dose of ivabradine involving hemodynamic, cardiac function and cardiac apoptosis in dogs with asymptomatic DMVD stage B2.

In this study, the objectives were to: 1) assess the chronic effects of ivabradine on hemodynamic and cardiac function by using invasive technique (i.e. cardiac catheterization for pressure measurement) and 2) evaluate the chronic effects of ivabradine on cardiac structural remodeling and apoptosis using both histopathological and immunohistochemical detections in DMVD stage B2 dogs. The main findings in this part are: 1) chronic administration of ivabradine did not alter hemodynamics and most of cardiac function parameters except for the contractility index in which it was elevated significantly when compared with baseline and 2) chronic administration of ivabradine improves structural remodeling suggested by reduction of myocardial vacuolization, glycogen accumulation, interstitial fibrosis and fibrofatty, and reduces myocyte apoptotic process (i.e. reduction in AI).

In the present study, the hemodynamic and ventricular function at baseline were not severely impaired. This is consistent with previous reports (Chetboul and Tissier, 2012; Zois et al., 2014) in which the hemodynamic and ventricular function in dogs with mitral valve regurgitation due to DMVD stage B2 are preserved until the advanced symptomatic disease has developed (Kittleson et al., 1984). This is because of compensatory mechanism that has developed to compensate for reduction of cardiac output (Dal-Bianco et al., 2014). After dogs were given repeated oral dose of ivabradine, none of the hemodynamic and ventricular function parameters has changed except for contractility index. In this study, the CI of dogs receiving ivabradine increases 7.57% from baseline. This result is in agreement with previous report in heart

failure dogs induced by intracoronary microembolization (Sabbah et al., 2014). In that study, the EF was reduced when dogs receiving microsphere embolization indicated congestive heart failure; however, treatment with ivabradine improves the systolic and diastolic functions. The authors suggested that the calcium homeostasis and ATPase activity may play important role on the improvement of those functions. Moreover, the result of increased CI in this part is also supported the rise of global circumferential and radial strain of dogs after treatment with ivabradine in part 2. Interestingly, the present study did not observe any change in diastolic function after treatment with ivabradine. Previous studies demonstrated that mice and dogs presented with HF have increased diastolic time interval and ventricular relaxation indicated by dP/dt_{min} after treatment with chronic ivabradine (Becher et al., 2012; Sabbah et al., 2014). The disagreement between our study and previous studies may be due to the different stages of heart disease. In dogs with DMVD stage B2, the cardiac function is reserved while in HF experimental animals the cardiac function is impaired; therefore, the effects of ivabradine appear obviously (Norman et al., 2011).

In addition, MR also be responsible for varying degree of structural remodeling of the myocardium demonstrated from the findings of changes of the histopathological lesions observed at baseline (i.e. vacuolization of cardiomyocytes, glycogen accumulation, interstitial fibrosis, and fibrofatty). This is a hallmark of cardiac adaptation to volume overload (Melenovsky, 2013). After chronic treatment with ivabradine, those lesions were decreased significantly. This is consistent with the findings in HF mice treated with ivabradine in which the cardiac fibrosis was reduced significantly after treatment (Becher et al., 2012). The authors suggested that reduction in cardiac fibrosis may result from the reduction in afterload and improvement of arterial-ventricular coupling after ivabradine administration (Becher et al., 2012).

In the present study, Hoechst fluorescence dye was used to stain the condense chromatin of apoptotic cell showed as bright blue color which represents the fragmentation of late stage of cell death (Kraiphet et al., 2017). The results were showed as %AI in which it was reduced by 17% from baseline when observed after treatment with ivabradine. This is in agreement with recent publication in murine model of myocarditis in which ivabradine administration for one month significantly

attenuates the process of apoptosis when compared with control group (Li-Sha et al., 2018).

It has been known that the anti-apoptotic protein, Bcl-2, prevents apoptosis by preventing the reduction of mitochondrial membrane potential while the pro-apoptotic protein, Bax, is an important element for cellular induced apoptosis. The migration and translocation of Bax increases mitochondrial membrane permeability that will activate caspases and apoptosis (Webster, 2012). In the present study both Bcl-2 and Bax were determined in which the Bcl-2 tended to increase while Bax tended to decrease. However, those changes did not reach statistical significance. This due to a limited number of sample for each dogs that can be obtained. In general concept, these two proteins possess opposite function to regulate program cell death (Czabotar et al., 2014). The ratio of Bax to Bcl-2 calculated in the present study also shows a trend to reduce after treatment with ivabradine which indicated the improvement of cell apoptosis. This result is also in agreement with the result of reduction in histopathological lesions observed by H&E and special staining (Mason's trichrome and PAS).

In conclusion, this study demonstrated that ivabradine improves systolic function without changes in hemodynamic and diastolic function in dogs with DMVD stage B2. The study also showed that ivabradine decreases the cardiac structural remodeling and the cellular apoptosis suggested from histopathological findings, AI, Bcl-2 and Bax proteins. These results indicate that ivabradine could attenuate the deterioration of ventricular function partly by reduction of cardiac apoptosis suggested from the reductions of the Bax to Bcl-2 ratio and cardiac fibrosis.

Study limitations: In this study, the authors are aware that the number of animals enrolled in the study is too small which could limit the extrapolation of result. However, several essential parameters are statistically significant when compared with the baseline which ensures that the study is valid. Another potential limitation is the number of samples collected from each dogs. In the current study, only 3-5 pieces of endomyocardium were obtained from each dog which limits the measurement of some parameters from histopathological study.

CHAPTER VI

SUMMARY

Degenerative mitral valve disease (DMVD) is the most common acquired cardiac disease in small aging dogs leading to impaired cardiac autonomic activity and functions. Dogs with asymptomatic DVMD usually have a significantly higher HR and systemic BP than healthy dogs. An elevated HR and systemic BP contributes to increase MVO_2. In addition, coronary blood flow and myocardial perfusion are reduced while the LV end diastolic pressure is increased. These factors may aggravate the compensation mechanism of the heart disease. Therefore, a reduction of the HR may be useful in managing an increased MVO_2 in dogs with asymptomatic DMVD. Ivabradine has been approved for clinical use in patients with coronary artery disease and ischemic heart disease by the European Medicines Agency since 2005 and by the United States Food and Drug Administration since 2015 but it is no veterinary-labeled for use in dogs and cats. In several animal models and humans, HR reduction by ivabradine is showing possibility to prevent LV remodeling or delay the DMVD progression. However, there are no data to support the use of ivabradine in dogs. To the author's knowledge, the therapeutic dose of ivabradine for HR reduction and cardiovascular effects of ivabradine in dogs with asymptomatic DMVD have not been studied. Furthermore, its mechanisms to improve cardiac function remain unclear. The overall objective of the present study was to investigate the potential of ivabradine to improve cardiac autonomic activity and functions in dogs with naturally occurring, asymptomatic DMVD partly due to a reduction of cardiomyocyte apoptosis.

Firstly, the appropriate single oral dose of ivabradine for reduction of HR and MVO_2 as assessed by RPP (RPP= HR x systolic blood pressure) were determined in seven beagles with naturally occurring DMVD stage B2. All animals were instrumented by the Holter recorder and an oscillometric device to measure ECG and BP for 24 and 12 h, respectively. Each dog was randomly subjected to receive either placebo or ivabradine (0.5, 1.0 and 2.0 mg/kg, orally). The results revealed that oral administration of ivabradine significantly decreased the HR and RPP in a dose-dependent manner

without adverse effects. The highest dose of 2.0 mg/kg significantly reduced SBP and MBP. Therefore, the findings demonstrated that a single oral ivabradine administration at a dose of 1.0 mg/kg is suitable for dogs with asymptomatic DMVD to reduce the HR and MVO_2 without marked effects on BP and other adverse effects. It also did not induce arrhythmias that observed by Holter monitoring.

Next, the study part 2 and part 3 were designed to investigate the long-term effects of repeated oral dose of ivabradine 1.0 mg/kg twice daily for 3 months in four beagles with naturally occurring DMVD stage B2. For the study part 2, HR, MVO_2, BP, ventricular function, ECG parameters and HRV were determined. In addition, the clinical variables of all dogs such as appetite, behavior, activity, defecation, urination, vomiting, respiration and BP were evaluated before dosing of each day to monitor adverse reaction (e.g. low BP and bradycardia). Moreover, hematology and chemistry profiles were measured at baseline and at the end of the study to determine the side effect of drug on renal and hepatic function. Simultaneously with study part 2, the study part 3 was designed to investigate the hemodynamic and cardiac function using invasive technique and evaluate cardiac apoptosis from the endomyocardial tissues that were biopsied at baseline and 3 months after treatment with ivabradine. The long-term treatment of oral dose of ivabradine was started one week after baseline endomyocardial tissue biopsy in study part 3. For the study part 2 and part 3, the results revealed that chronic administration of ivabradine significantly decreased the HR, systemic BP, and MVO_2 without adverse effects ($P < 0.05$). All indices of time- and frequency- domain of HRV, using to measure of cardiac ANS activity, at M3 were increased significantly when compared with baseline values ($P < 0.05$). Indices of speckle-tracking echocardiography, using to estimate the cardiac systolic and diastolic function, including global radial strain, global circumferential strain, and fractional area change measured at M2 and M3 were significantly increased when compared with baseline ($P < 0.05$). The findings of this study imply that long-term treatment with ivabradine at a dose of 1.0 mg/kg twice daily in dogs with asymptomatic DMVD improves cardiac ANS activity and LV function. All of systemic BP parameters, pulmonary circulation parameters, systemic and pulmonary vascular resistances

except for cardiac contractility in dogs receiving ivabradine for 3 months did not change when compared with baseline. In addition, dogs receiving ivabradine for 3 months had significant lower in the percentage of vacuolization of myocytes, glycogen accumulation, interstitial fibrosis and cardiac fibrofatty than those of baseline (65.8%, 47.6%, 57.3%, 77.8%, respectively). Moreover, the percentage of apoptotic index when measured at M3 were significantly decreased (17.0 %) when compared with baseline (P<0.05). Both Bax and Bcl-2 protein expressions were observed in LV endocardium tissue of dogs with naturally occurring, asymptomatic DMVD. Before and 3 months after treatment with ivabradine, the score of Bax were expressed at 1.70 ± 0.10 and 1.60 ± 0.20, respectively. In response to repeated oral dose of ivabradine, Bcl-2 when measured at M3 tended to increase (from score 1.24 ± 0.14 to 1.62 ± 0.12) when compared with baseline (P=0.06). In endomyocardial tissue of dogs with naturally occurring, asymptomatic DMVD at the time points before and 3 months after treatment with ivabradine, the ratio of Bax to Bcl-2 were 1.56 ± 0.43 and 1.13 ± 0.11, respectively. The study part 3 demonstrated that ivabradine improves systolic function without changes in hemodynamic and diastolic function in dogs with DMVD stage B2. The study also showed that ivabradine decreases the cardiac structural remodeling and the cellular apoptosis suggested from histopathological findings and apoptotic index. These results indicate that ivabradine could attenuate the deterioration of ventricular function partly by reduction of cardiac apoptosis and cardiac fibrosis.

Beneficial to veterinary practitioners

The novel findings in all of these studies demonstrated that the long-term effects of repeated oral dose of ivabradine at a dose of 1.0 mg/kg twice daily for 3 months are save and suitable for use in dogs with asymptomatic DMVD to reduce the HR, systemic BP, MVO_2, and improve cardiac autonomic activity and functions without any cardiovascular adverse reaction (e.g. bradycardia, increasing cardiac arrhythmia, alter hemodynamic) or other effects on renal and hepatic function. The possible mechanism responsible for this result partly due to a reduction of cardiomyocyte apoptosis. Therefore, HR reduction by ivabradine may be useful for maintaining

normal cardiac autonomic activity in dogs with asymptomatic DMVD. Furthermore, long-term treatment with ivabradine are showing possibility to prevent LV remodeling or delay the DMVD progression, improves systolic and diastolic function of the LV, suggesting that using ivabradine in a clinical setting is beneficial.

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