EFFECTS OF MODERATE - INTENSITY EXERCISE TRAINING ON CARDIAC ANGIOGENIC CAPACITY AND FIBROSIS IN MIDDLE-AGED AND AGED RATS

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ฐิฒิภรณ์ เมฆรุ่งเรืองวงศ์ : ผลของการฝึกออกกำลังกายระดับปานกลางต่อความสามารถในการสร้างหลอดเลือดใหม่และการเกิดพังผืดในหัวใจของหนูกลางวัยและหนูแก่. (EFFECTS OF MODERATE - INTENSITY EXERCISE TRAINING ON CARDIAC ANGIOGENIC CAPACITY AND FIBROSIS IN MIDDLE-AGED AND AGED RATS) อ.ที่ปรึกษาหลัก : ศ. ดร.สุทธิลักษณ์ ปทุมราช, อ.ที่ปรึกษาร่วม : ดร.ชีพสุมน วิบูลย์วรกุล

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลการฝึกออกกำลังกายต่อความสามารถในการสร้างหลอดเลือด และการเกิดพังผืด ที่เกี่ยวข้องกับ พี เอเคที 1 อี นอส เอ็ม ดี เอ็ม 2 พี 53 วีอีจีเอฟ คลัสเตอร์ของความแตกต่าง 31 และ ที จี เอฟ บีตา 1 ในหัวใจหนูกลางวัยและหนูแก่ หนูเพศผู้ สายพันธ์วิสตาร์ ถูกแบ่งออกเป็นห้ากลุ่ม: กลุ่ม 1 หนูเด็ก อายุ 4 เดือน กลุ่ม 2 หนูกลางวัยที่ไม่ได้ออกกำลังกาย อายุ 14 เดือน กลุ่ม 3 หนูแก่ที่ไม่ได้ออกกำลังกาย อายุ 22 เดือน กลุ่ม 4 หนูกลางวัยที่ออกกำลังกาย อายุ 14 เดือน และกลุ่ม 5 หนูแก่ที่ออกกำลังกาย อายุ 22 เดือน ในกลุ่มหนูที่ไม่ได้ออกกำลังกายหนูจะถูกแช่น้ำเป็นรายตัวเป็นเวลา 30 นาที ต่อ วัน 5 วัน ต่อสัปดาห์เป็นเวลา 8 สัปดาห์ในถังรูปทรงกระบอกที่เต็มไปด้วยน้ำ ความลึก 5 ซม. อุณหภูมิ 33-36 องศาเซลเซียส ในกลุ่มหนูที่ออกกำลังกาย หนูจะว่ายน้ำเป็นรายตัว นาน 60 นาที ต่อวัน 5 วัน ต่อ สัปดาห์เป็นเวลา 8 สัปดาห์ในถังรูปทรงกระบอกที่เต็มไปด้วยน้ำ ความลึก 50-55 ซม. อุณหภูมิ 33-36 องศาเซลเซียส หลังจาก 8 สัปดาห์ของระยะเวลาออกกำลังกายหนูจะพักเป็นเวลา 24 ชั่วโมงก่อนการทดลอง หนูถูกวัดความดันโลหิตและประเมินการแสดงออกของ พี 53 เอ็ม ดี เอ็ม 2 คลัสเตอร์ของความแตกต่าง 31 และการเกิดพังผืด โดยใช้วิธีอิมมูโนฮีสโตเคมี และการย้อมสีพิเศษ ตามลำดับ. หัวใจที่ถูกบดจะถูกใช้สำหรับทดสอบมาลอนไดดีไฮด์ (เอ็ม ดี เอ) โดยใช้วิธีการทำปฏิกริยากับกรดไทโอบาร์บิทูริก (ทีบาร์) และระดับของ พี เอเคที 1, อี นอส, วีอีจีเอฟ และ ที จี เอฟ บีตา 1 โดยใช้วิธี อีไลซ่าเทคนิค ผลการศึกษาพบว่ากลุ่มหนูแก่มีความดันโลหิตสูงกว่าหนูเด็ก และ หนูกลางวัยอย่างมีนัยสำคัญ กลุ่มหนูแก่ที่ออกกำลังกายมีค่าความดันโลหิตลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มหนูแก่ที่ไม่ได้ออกกำลังกาย กลุ่มหนูกลางวัยที่ออกกำลังกาย แสดงค่าความดันโลหิตที่ต่ำกว่ากลุ่มหนูกลางวัยที่ไม่ได้ออกกำลังกายแต่ยังไม่มีนัยสำคัญ ระดับของ พี เอเคที 1 อี นอส วีอีจีเอฟ และ คลัสเตอร์ของความแตกต่าง 31 เพิ่มขึ้นอย่างมีนัยสำคัญในกลุ่มออกกำลังกายทั้งหมดเมื่อเปรียบเทียบกับหนูกลางวัยและหนูแก่ที่ไม่ได้ออกกำลังกำลังกาย ระดับ เอ็ม ดี เอ็ม ทรู ในกลุ่มหนูเด็กเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับหนูกลางวัยและหนูแก่ที่ไม่ได้ออกกำลังกาย และมีเพียงหนูแก่ที่ออกกำลังกายเท่านั้นที่ เอ็ม ดี เอ็ม 2 เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มหนูแก่ที่ไม่ได้ออกกำลังกาย อย่างไรก็ตามไม่มีความแตกต่างอย่างมีนัยสำคัญของ พี 53 ทั้ง 5 กลุ่ม การสะสมของคอลลาเจน และ ระดับของ ที จี เอฟ บีตา 1เพิ่มขึ้นอย่างมีนัยสำคัญในกลุ่มหนูแก่ที่ไม่ได้ออกกำลังกายเมื่อเทียบกับหนูเด็กและการออกกำลังกายสามารถลดผลเหล่านี้ได้ นอกจากนี้ ระดับของ เอ็ม ดี เอ ของกลุ่มหนูกลางวัย และหนูแก่ที่ออกกำลังกายลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มไม่ได้ออกกำลังกายที่ควบคุมอายุเท่ากัน ดังนั้นผลลัพธ์เหล่านี้บ่งบอกว่า การฝึกออกกำลังกายแบบฝึกฝนที่ใช้ในการศึกษานี้สามารถป้องกันการลดลงของความสามารถในการสร้างหลอดเลือด และการเพิ่มขึ้นของพังผืด และผลการศึกษานี้มีความเกี่ยวข้องกับผลของความเครียดออกซิเดชัน พี เอเคที 1 อี นอส เอ็ม ดี เอ็ม 2 พี 53 วีอีจีเอฟ คลัสเตอร์ของความแตกต่าง 31 และ ที จี เอฟ บีตา 1

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Titiporn Mekrungruangwong : EFFECTS OF MODERATE - INTENSITY EXERCISE TRAINING ON CARDIAC ANGIOGENIC CAPACITY AND FIBROSIS IN MIDDLE-AGED AND AGED RATS. Advisor: Prof. SUTHILUK PATUMRAJ, Ph.D. Co-advisor: SHEEPSUMON VIBOOLVORAKUL, Ph.D.

This study aimed to investigate the protective effects of exercise training against age-induced the reduction of cardiac angiogenic capacity associated with p-Akt1, eNOS, Mdm2, p53, VEGF, and CD31 in rat hearts. Additionally, age-induced cardiac fibrosis association with TGF-β1 was also observed. Male Wistar rats were divided into five groups: Sedentary - young group (SE-Young, aged 4 months), Sedentary - middle-aged group (SE-Mid-Age, 14 months), Sedentary – aged group (SE-Age, aged 22 months), Exercise-trained middle-aged group (ET-Mid-Age, aged 14 months), and Exercise-trained -aged group (ET-Age, aged 22 months). In the SE- groups, rats were immersed individually for 30 minutes/day, 5 days/week for 8 weeks in cylindrical tanks filled with water; depth of 5 cm, the temperature at 33-36 °C. In ET groups, rats swam individually for 60 minutes/day, 5 days/week for 8 weeks in cylindrical tanks filled with water; depth of 50-55 cm, the temperature at 33-36 °C. After 8 weeks of exercise training, the rats were rest for 24 hours before the experiment. The rats were measured blood pressure and assessed the expression of p53, Mdm2, CD31, collagen accumulation by using immunohistochemistry and Masson's Trichrome Staining, respectively. Heart homogenates were used for the assay of malondialdehyde levels (MDA) by using the thiobarbituric acid reactive substances assay (TBARS) and the levels of p-Akt1, eNOS, VEGF and TGF-β1 using enzyme-linked immunosorbent assay (ELISA). Our results showed that the SE-Age group had significantly high blood pressure when compared to the SE-Young and SE-Mid-Age. The ET-Age group showed significantly decreased blood pressure when compared to SE-Age. The ET-Mid-Age group showed a lower blood pressure than in the SE-Mid-Age group, but not yet significant. The levels of p-Akt1, eNOS, VEGF, and CD31 were significantly increased in all ET groups when compared with SE–Mid-Age and SE-Age. Mdm2 levels were significantly increased in SE-Young when compared to SE-Mid-Age and SE-Age. Only Mdm2 levels in the ET-Age had a significant increase when compared to SE-Age, while no significant difference of p53 in all groups. The collagen accumulation and TGF-β1 levels were significantly increased in SE-Age when compared to SE-Young and exercise training could reduce these. Furthermore, tissue MDA in ET-Mid-Age and ET-Age rats were significantly reduced when compared to SE-Mid-Age and SE-Age control. Therefore, these results implied that the exercise training program used in this study prevented age-induced the reduction of angiogenic capacity and fibrosis, and these results were associated with its effects on oxidative stress, p-Akt, eNOS, Mdm2, p53, VEGF, CD31, and TGF-β1.

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LIST OF ABBREVIATIONS

CD31	=	cluster of differentiation 31
eNOS	=	endothelial nitric oxide synthase
ET	=	exercise-training
MDA	=	malondialdehyde
Mdm2	=	murine double minute 2
phosphor-Akt1	=	AKT 1 phosphorylation on serine 473
P53	=	tumor suppressor p53
ROS	=	reactive oxygen species
SE	=	sedentary
TGF-β 1	=	transforming growth factor beta 1
VEGF	=	vascular endothelial growth factor

CHAPTER I

INTRODUCTION

1.1 Background and rationale

From the reports of Department of Economic and Social Affairs, United Nations in 2015, they showed that the number of older persons — those aged 60 years or over — in the world will increase from 901 million (12%) in 2015 to 2.1 billion (22%) in 2050 [1]. In Thailand, the number of aging populations has grown up rapidly and tends to increase continuously in the next decades. From the previous report, they demonstrated that the percentage of Thai population, aged 60 or over will increase from 13.2% in 2010 to 32.1% in 2040[2]. The rapid growth of the aging population may affect a *socio*-economic status as well as health issues[2]. In 2010, it was reported that cardiovascular disease was a leading cause of death in people aged 65 and older both in men and women [3].

In aging group, morphological and structural changes of the heart would lead to functional impairment, impaired myocardial contraction, and relaxation, resulting in cardiovascular morbidity and mortality[4]. Left ventricular hypertrophy and fibrosis in the aging cardiac lead to diastolic dysfunction[4]. Moreover, clinical and experimental studies suggested that cardiac capillary rarefaction found in chronic cardiac hypertrophy induced tissue hypoxia, cell death, and fibrosis [5, 6]. It contributes to the progression from compensated hypertrophy to contractile dysfunction and heart failure[5-7]. Fibrosis suppress systolic function through disruption of myocardial excitation-contraction coupling[8]. Moreover, cardiac fibrosis induced organ structure distortion[9, 10], myocardial stiffness, electrical heterogeneity, resulting in cardiac dysfunction and sudden death[11]. Angiogenesis is the formation of new capillaries from pre-existing blood vessels by sprouting of differentiated endothelial cells [12]. In aging process, the reduction of angiogenic capacity of many organs is common, with the cardiovascular system being particularly affected[13].

Angiogenic capacity is the maximum ability of the endothelial cells to form new blood vessels. Angiogenic capacity appears to described by the dynamic balance

between the expression of pro-and antiangiogenic factors and the responsiveness of inducers of angiogenesis[14]. The impaired angiogenic capacity of aged endothelial cells compromises the less clinical response to interventions which aimed to stimulated capillarization in the ischemic myocardial infarction in elderly people[15, 16]. Moreover, the cardiac function has been shown to depend on the angiogenic capacity of the cardiac vessels[13]. The previous report showed that the venular capillary portion and total capillary density of myocardium in aged animals were less than that in the young animals, which might be related to angiogenic capacity reduction[17]. It is thought that the age-associated decline in the ability to form new blood vessels in the myocardium impairs both cardiac repair processes and adaptation to changes in myocardial oxygen supply and demand in the elderly. However, the molecular mechanism involved in the regulation of cardiac angiogenesis, particularly, the reduction of angiogenic capacity associated with cardiac fibrosis in aging is not well understood.

P53 plays a critical role in the progression of heart failure. The elevation of p53 was observed in myocardial biopsies of patients with heart disease [18] and found to associate with disease severity[19]. Moreover, the regulation of genes that control biological processes such as differentiation, migration or angiogenesis is regulated by p53 [20]. P53 plays as anti-angiogenesis by transcriptionally activating the α (II) collagen prolyl-4hydroxylase gene, resulting in the antiangiogenic fragments of collagen type 4 and 18 release[21] Therefore, the inhibition of p53 may be beneficial for capillary formation. During chronic pressure-overload, excessive p53 leads to blood vessel rarefaction and fibrosis in ECs[9]. These findings suggested that cardiac capillary rarefaction and fibrosis might be involved by p53 activation.

Murine double minute 2 (Mdm2) is a nuclear phosphoprotein that has been shown a negative regulator of the tumor suppressor p53. Several reports showed that Mdm2 expression is correlated with an increased level of the VEGF which may facilitate vascularization as well as intravasation and seeding of tumor cells in distant places[22]. However, the study of Mdm2 and p53 in non-tumor cells, especially in the aging heart, has not been elucidated.

The free radical hypothesis remains a good explanation for the mechanism of aging[23]. The elevation of reactive oxygen species (ROS) production and reduction of

antioxidant capacity are associated with the aging process by the oxidative modification of different macromolecules such as lipid, protein, and DNA[23]. ROS also influences the processes of on cell growth, survival, and development[24]. Age-dependent impairment of mitochondrial function associated with increased production of ROS. Moreover, it has been reported that sedentary older human showed NRF2 Keap1 dysfunction, but an active life style increased NRF2 function and thereby maintains redox homeostasis in skeletal muscle of older human[25]. Impairment of mitochondrial function has been wildly documented in heart failure in both human patients and mouse models[4]. In addition, a significant increase in superoxide radical production was seen in mitochondria prepared from aging rat hearts[4, 26]. Direct evidence for the critical role of mitochondrial ROS in cardiac aging was revealed the overexpression of mitochondrial-targeted catalase (mCAT) reduced cardiomyocyte hypertrophy, diminished cardiac fibrosis and attenuated diastolic dysfunction[27]. These data suggested an important role for ROS in the pathogenesis of aging-associated fibrotic cardiac remodeling. Moreover, the previous study showed that aged induced endothelial cells (ECs) dysfunction appears to involve ROS increment in the face of unchanged or reduce antioxidant defense [28]. In aging humans, the bioavailability of nitric oxide (NO) was reduced in parallel with endothelium-derived ROS elevation [29].

The loss of NO bioavailability in the endothelium is one of the main characteristics of ECs dysfunction, leading to reduced vasodilation as shown by decreased vascular smooth muscle cell (VSMC) relaxation [30]. Endothelial nitric oxide synthase (eNOS) is the major enzyme responsible for NO synthesis in the vasculature [31]. The reduction of NO bioavailability is largely due to an excessive reduction in eNOS and phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) in aged blood vessels [31, 32]. LeBlanc AJ *et al* [33] showed that impaired endothelium-dependent dilation in coronary arterioles of the aged rats was associated with PI3K/Akt signaling dysregulation. Moreover, Iemitsu M *et al*[17] found that mRNA and protein of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR), phosphorylation Akt and eNOS, eNOS protein and capillary density in cardiomyocyte were reduced with advance aged.

Cardiac fibrosis is the abnormal accumulation of extracellular matrix in the heart. Progressive fibrosis may contribute to the development of diastolic heart failure in elderly patients[34]. Age-associated fibrosis and architectural remodeling in the heart are primarily an imbalance of collagen degradation, collagen synthesis, and proliferation of fibroblasts[10]. TGF-β plays critical roles in aging-associated cardiac fibrosis by inducing myofibroblast transdifferentiation, and by enhancing matrix protein synthesis by cardiac fibroblasts. Moreover, TGF-β may exert potent matrix –preserving action by suppressing the activity of MMPs together with inducing synthesis of protease inhibitors, such as plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs)[4]. The study of Kwak et al (2011) showed that age-induced collagen accumulation and fibrosis through a signaling pathway that down-regulated MMPs level by increasing of TIMP1 and TGF- β1 activities [10], indicated an important role of TGF- β1 within signaling cascades of cardiac aging and fibrosis. Together, these data revealed the pathway that responsible for aging-induced cardiac fibrosis is related to TGF- β1. Moreover, oxidative stress may regulate the quantity and quality of the extracellular matrix by modulating both collagen synthesis and metabolism[35].

Exercise training (ET) is well established as a preventive and therapeutic approach for cardiovascular diseases with several cardiovascular benefits[36]. In response to moderate exercise training, nuclear factor-erythroid 2 (NRF2) protein was significantly increased in the heart of old mice, indicating that moderate training-induced stability of Nrf2. Consequently, the protein levels of majors targets of NRF2 such as glutathione reductase (GSR), heme oxygenase 1 (HO1), glutathione peroxidases (GPX1) were increased[37]. Corresponding to Gounder SS *et al*[38] showed that moderate exercise training promoted antioxidant levels such as catalase (CAT), GSR through the improved transcriptional activity of Nrf2 in the age-associated myocardial oxidative stress. Furthermore, Hassan AF and Kamal MM[39] showed that exercise training - induced increases of capillary density may turn into a beneficial adaptation as the capillary network increases oxygen supply and energy substances for the aging heart. It is hypothesized that exercise training can decrease oxidative damage in the aging cardiovascular system through the induction of antioxidant enzyme and increasing capillary growth[38]. The potential of exercise training on p53 expression was observed

in several studies [40, 41]. Ziaaldini MM *et al*[40] showed that p53 expression and ROS levels were increased in the skeletal muscle of aged rat and endurance training could lower p53 expression. Al-Jarrah M *et al* [41] examined the level of inducible nitric oxide synthase (iNOS) and p53 expression in the cardiac muscle of type I diabetic rats and non-diabetic rats after treadmill running 4 weeks. They found that the expression of the p53 levels in the diabetic control group increased when compared to inactive control rats. The level of p53 expression, but not iNOS level, was reduced by exercise training. Roudier E *et al*[42] showed that Mdm2 expression level was an important factor for exercise-induced skeletal muscle angiogenesis. It was found that the level of a human double minute2 protein, Hdm2 protein, did not differ between young and aging skeletal muscle. However, exercise training caused a dramatic increment of this protein by 38% in skeletal muscle of young subjects and 81% of the senior subjects[43]. These results confirmed the important role of Mdm2 on angiogenesis, probably, on angiogenic capacity.

The coronary vascular adaptations by exercise regulation lead to increased coronary blood flow and transport reserves, resulting in structural change in the size and number of the blood vessel as well as alterations in systemic, neurohumoral, local vascular control and capillary density[44]. Iemitsu M *et al*[17] demonstrated that the downregulation of the VEGF angiogenic signaling cascade is restored by exercise training in the heart of old rats.

Kwark HB *et al*[10] examined the potential effects of exercise training on cardiac fibrosis in the aging rat. They found that exercise training reduced transforming growth factor-beta (TGF-β), an upstream regulator of tissue inhibitor of matrix metalloproteinase (TIMP), resulting in a dysregulation of matrix metalloproteinases (MMPs) and a reduction of cardiac fibrosis. Moreover, Liao PH *et al*[45] also showed that aging-induced cardiac fibrosis primarily occurred through the fibroblast growth factor 2 (FGF2), urokinase plasminogen activator (uPA) and MMP 2 pathway. Exercise training demonstrated beneficial effects by reducing uPA and MMP2 expression.

It is quite clear that the alteration of the angiogenetic pathway is age-dependent[46]. However, in the aging heart, the relationship between exercise training and angiogenesis in together with roles of p53, Mdm2, and VEGF, is still not well

understood. Moreover, in the middle-aged population, it seems to be a lack of knowledge to describe the relationship between angiogenic capacity / cardiac fibrosis and the consequences of exercise training. Wright KJ *et al*[47] found that exercise training in late middle-age subjects could attenuate cardiac fibrosis through the reduction of advanced glycation end products (AGEs) accumulation and subsequent less collagen cross-linking. Therefore, in the present study, we aim to investigate the protective effects of exercise training against age-induced the reduction of cardiac angiogenic capacity and the risk of cardiac fibrosis in middle-aged and aged rats. In addition, this study also assessed the effects of exercise training on the multiple factors, p53, Mdm2, VEGF, p-Akt, eNOS, TGF- β1, that involved cardiac angiogenic capacity and cardiac fibrosis in both middle-aged and aged rats.

1.2 Research questions

1. Can exercise training prevent age-induced changes in cardiac angiogenic capacity and fibrosis in middle-aged and aged rats?
2. Whether exercise training has any effects on p53, Mdm2, VEGF, p-Akt, eNOS, TGF- β1 in middle-aged and aged rat hearts?

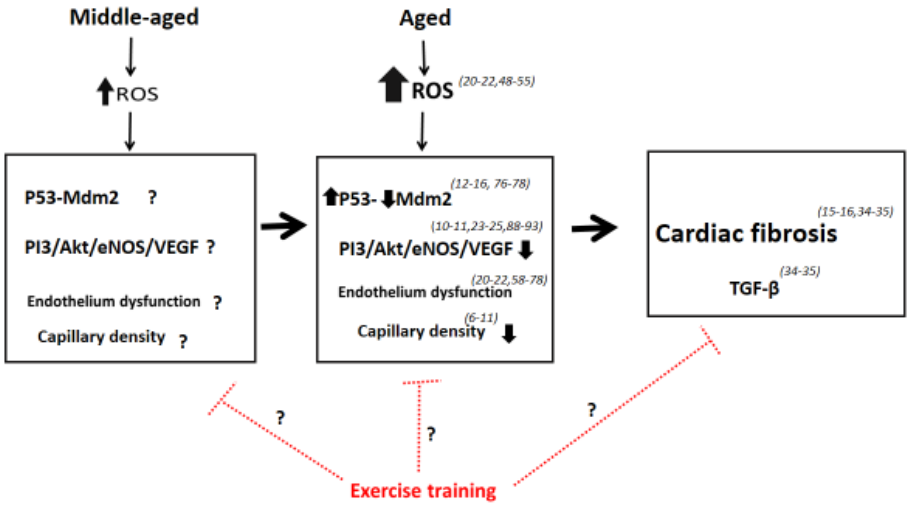
1.3 Research objectives

1. To investigate the effects of exercise training on age-induced changes in cardiac angiogenic capacity and fibrosis in middle-aged and aged rats.
2. To investigate the effects of exercise training on p53, Mdm2, VEGF, p-Akt, eNOS, TGF- β1 in middle-aged and aged rat hearts.

1.4 Hypothesis

Exercise training can prevent age-induced decline cardiac angiogenic capacity and fibrosis, which is associated with improvement of endothelial function and increasing angiogenic capacity in middle-aged and aged rats.

1.5 Conceptual framework



CHAPTER II

LITERATURE REVIEWS

The impairment of angiogenesis with aging is related to several deficits in cell functions[48]. Several reports showed that aging had a negative impact on angiogenesis in humans and animals. Moreover, clinical and experimental studies suggested that the reduction of cardiac capillary density induced tissue hypoxia, cell death, and fibrosis[5, 6].

2.1 Cardiac fibrosis

The excessive of extracellular matrix (ECM) proteins, which included collagen, fibronectins, laminins, and proteoglycan, of cardiac interstitial spaces is a hallmark of cardiac fibrosis. The most abundant myocardial ECM fibrillar proteins are collagen. The collagen-based cardiac matrix network not only functioned as a scaffold for the cellular components but also played a role in the contractile force transmission. Myocardial collagen type I, which is about 85% of total myocardial collagen, is concomitant with thick fibers that transmit tensile strength. In contrast, collagen type III phenotype (11%) that associates with thin fiber and maintain the elasticity of the matric network[34]. Synthesis of both collagen types is dramatically increased in the remodeling fibrotic heart. In hypertensive cardiac fibrosis and myocardial infarction models, collagen type I is more intense and prolonged upregulation than collagen type III. However, in ischemic cardiomyopathy patient, type I to type III ratio was decreased, indicated that patterns of collagen isoform expression in the fibrotic heart may rely on the contextual factors[34]. The significant of fibrosis interrupted the organization of myocardial excitation-contraction coupling, resulting in cardiac dysfunction[11]. Cardiac fibrosis induced organ structure distortion which is often found in heart failure patients[9] and the aged populations[10]. Moreover, interstitial collagen accumulation induced myocardial stiffness and electrical heterogeneity, resulting in blood pressure dysfunction and sudden death[8].

TGF-β has been implicated as a critical regulator to tissue fibrosis in several organs including cardiac fibrosis as evidenced by the overexpression of TGF-β in

myocardium hypertrophy in animal and human heart failure. TGF-β can induce the expression of the ECM component such as fibrillary collagen, collagen, proteoglycan, fibronectin, and fibroblast proliferation. The elevation of TGF-β is associated with the severity of fibrosis in pressure overload of human hearts[11]. In the latent complex form, TGF-β has bounded to dimeric of latency-associated protein (LAP) and latent TGF-β-binding protein (LTBP). Oxidants have two pathway to activate TGF-β latent form to mature form, directly by LAP oxidation and indirectly through activation of MMPs, which finally cleave LAP to release active TGF-β, indicating that redox imbalance can activate latent TGF-β and also induces TGF-β expression[49]. Both aging and fibrosis are linked with redox imbalance and oxidative stress[50]. TGF-β levels were increased with advance aged[10, 45]. Kwark HB *et al*[10] investigated the level of TGF-β in the young-rat (3 months) and old-rat (31 months) hearts. They found that TGF-β levels in the left ventricle were increased 95% in old sedentary rats compared with the young sedentary group. Interestingly, TGF-β is a potent stimulator of TIMP 1 and collagen accumulation. Furthermore, the study of Liao PH *et al*[45] in hearts of 3- month, 12- month and 18- month old rats indicated that TGF-β1, the ligan in TGF dependent fibrosis pathway, was up-regulated in the sedentary aged (12- and 18 –month old) as well as connective tissue growth factor (CTGF) overexpression when compared to the sedentary young group. Importantly, CTGF expression in the fibrosis pathway up regulated and led to the fibroblast proliferation after translocating to the cell nucleus.

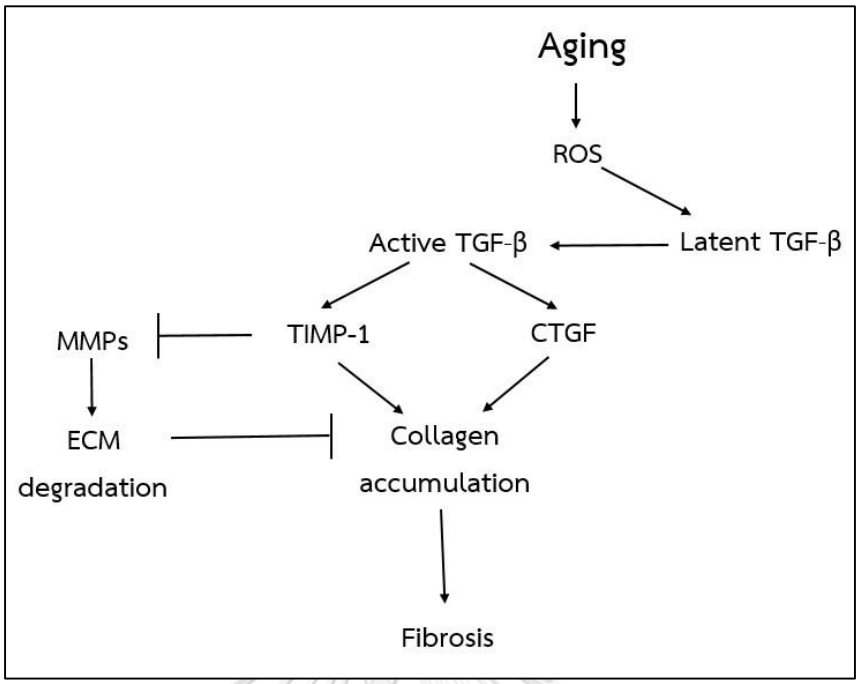


Figure 1 Aging-induced heart fibrosis via TGF-β dependent pathway
In an aging heart, oxidative stress can activate latent TGF-β to active TGF-β. After that, TGF-β activates TIMP, which suppresses ECM degradation and induces CTGF, leading to promotes collagen accumulation and fibrosis. (Modified from Kwark HB et al. (2011) [10] and Liao PH et al. (2015) [45], Liu RM et al. (2015) [49])

2.2. Hypertension induced cardiac hypertrophy

Persistent hypertension negatively affects both myocardial structure and function by serving as a substrate for the induction of pathological hypertrophy. The pathophysiology is initiated by pressure overload resulting in left ventricle concentric hypertrophy for normalizes systolic wall stress, and then the cardiac function is preserved in the face of an enhanced hemodynamic load. This process is referred to as compensatory hypertrophy [51, 52]. The mechanical stretch activates intracellular signaling cascades, resulting in gene expression and protein syntheses such as actin, myosin, which organize in the sarcomere that characteristically increases cardiomyocyte area and width[51, 53]. Compensatory hypertrophy is frequently demonstrated with the alteration of myocardial systolic and diastolic function,

including increased LV chamber stiffness and fibrosis. The compensatory response is irreversible followed by an alteration to heart failure and progressive contractile dysfunction[51]. The regulation of compensated hypertrophy is associated with the activation of endocrine, paracrine, and autocrine growth factors. These factors trigger the growth of cardiomyocyte hypertrophy mediated through G-protein coupled ligand-receptor, which regulated Ca2+ release in relation to the enhanced afterload and developed cardiac hypertrophy[51].

2.3. Angiogenic capacity

The angiogenic capacity plays a vital role in the aging process and has been related in cardiac diseases, including cardiac hypertrophy and heart failure[13]. Angiogenic capacity is the ability of the endothelial cell, at its maximum, in order to form new blood vessels. Partialy, it is simply explained by dynamic balance between the expression of pro- and anti-angiogenic factors, and appeared to include the responsiveness of inducers of angiogenesis significantly[14]. Aging significantly impairs angiogenic capacity [17, 46, 54], weakening their responsiveness to angiogenic stimulation. It is believed that the angiogenesis capacity in the aged myocardium impairs both cardiac repair processes and adaptation to oxygen supply and demand changes. In aged endothelial cells, the damaged angiogenic capacity concessions the less clinical response to interventions which aimed to stimulated capillarization in the ischemic myocardial infarction in elderly people[15, 16]. Importantly, the majority of preclinical trials promoting therapeutic angiogenesis are conducted in young animals, which do not recapitulate age-associated endothelial dysfunction and unresponsiveness of aged endothelial cells to inducers of angiogenesis. The discrepancy could explain the failure to translate promising experimental results into a clinical benefits[16]. Therefore, interventions which improve endothelial function in aging appear to have beneficial effects on endothelial angiogenic capacity and /or microvascular capillary density[14]. During hypertrophy development, capillary endothelial cells and cardiac fibroblasts dynamically underwent a phenotypic change to support myocardium contraction[55, 56]. The hypertrophic heart required more oxygen demand and led to increasing

myocardial angiogenesis to overcome the hypoxic state and to preserve cardiac function[7]. The reduced capillary in pathologic hypertrophy[7, 57, 58] indicating that the number of the capillary is regulated by the myocardium and that capillary rarefaction might cause myocardial hypoxia and contractile dysfunction[7]. Akt is a serine-threonine protein kinase that is related to hypertrophy of cardiomyocytes. Short term Akt1 activation up-regulated VEGF expression, resulting in physiological hypertrophy. In contrast, long term Akt1- activation-induced pathological hypertrophy[7]. Shiojima I et al[6] have studied, using cardiac-specific inducible Akt1 transgenic mice, which long -term Akt activation reduced VEGF-A, Ang-2, CD31, and impaired coronary angiogenesis and contractile dysfunction in the myocardium. Moreover, blockage of VEGF signaling induces capillary rarefaction and early transition to heart failure. The reduction of angiogenic capacity could explain at least in part of the mechanism of LVH to cardiac failure in the elderly[59]. The previous report showed that the venular capillary portion and total capillary density of myocardium in aged animals were less than that in the young animals. Moreover, both the arteriolar and intermediate capillary portion was increased by aging. These results suggested that the pathological cardiac disease was related to total capillary density reduction and the proportion of venular and arteriolar capillaries alteration[17]. Impaired angiogenic capacity in the heart might alter the aging-induced cardiac dysfunction because the myocardial contraction depends on energy substance and oxygen from blood supply [17]. Edelberg JM *et al.*[15] demonstrated that myocardial infarction in the elderly is associated with less favorable clinical outcomes. They investigated myocardial infarction after pretreatment with PDGF-AB increased von Willebrand factor (vWF) expression and minimized the extent of myocardial infarction area in aging hearts when compared to vehicle-aged control. The mechanism of this alteration was believed to be involved in the suppression of angiogenic capacity in the aging heart. Moreover, Maizel J *et al*.[13] examined the cardiac function of 30-40 weeks endothelial Sirtuin-deleted ($Sirt1^{endo-/-}$) mice by using echocardiography and IHC assay and found diastolic dysfunction (DD) and capillary density reduction. Sirtuin 1 inhibitor administration reduced mRNA VEGFR expression in ECs. Ex-vivo, the VEGF response in capillary sprouting from aortic explants were reduced in the $Sirt1^{endo-/-}$ mice.

The importance of sirtuin 1 in coronary endothelial cells is related to a reduction of VEGFR expression, compromising the angiogenesis.

2.3.1 Factors involved in age reduced angiogenic capacity

2.3.1.1 Oxidative stress

The free radical remains a good explanation for the aging mechanism. The imbalance between ROS and antioxidants are closely associated with the aging process [23]. In the living cells, mitochondria, endoplasmic reticulum, peroxisomes, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, NOX complex, are predominant endogenous oxidant sources that produced ROS *via* multiple pathways [60, 61]. During this process, NADH and FADH released electrons and transferred to molecular oxygen by using the four enzymatic complexes ensuring phosphorylation of ADP into ATP. Finally, oxygen molecules reduce to water however, these processes cannot completely reduce. It produces a superoxide anion (O_2^-). Superoxide dismutase (SOD) can react with O_2^- and give rise to hydrogen peroxide (H_2O_2). The catalase (CAT), glutathione peroxidase (GPXs) converts H_2O_2 into water. H_2O_2 can react with redox-active metals to generate the hydroxyl radical (OH^-) through the Fenton/Haber-Weiss reaction. OH^- production can damage protein, lipid, carbohydrate, and DNA. Apart from that, several exogenous oxidant sources such as radiation, heavy metals, particularly smoking have a critical role in oxidative stress. ROS interaction with DNA leads to DNA damage, resulting in activating p53 expression. Elevation of p53 activity upregulates Bcl2 family proteins that Bax causes to release cytochrome c from mitochondria. These processes initiate the apoptosis pathway. Free radicals are clarified into two groups that (i) ROS such as O_2^-, OH^- H_2O_2 and (ii) reactive nitrogen species (RNS) such as nitric oxide radical (NO^-) nitrogen dioxide radical (NO_2^-)[60, 61]. Elevated ROS production leads to attenuate capillary growth in aging[16]. Several pieces of evidence showed that the increment of oxidative stress contributes to vascular endothelial dysfunction with aging. The mechanism contributing to aged induced endothelial dysfunction appears to involve ROS increment in the face of unchanged or reduce antioxidant defense[28]. Isolation of the endothelial cell and inhibition of eNOS in the aorta of aged rats have been shown to

decrease ROS generation[62]. In aging humans, the bioavailability of nitric oxide (NO) was reduced in parallel with endothelium-derived ROS elevation[29].

ROS generation from mitochondria are elevated in the aging animals that indicate mitochondria dysfunction from multiple causes including age-related accumulation of mitochondrial mutations[63]. Hyperglycemia induced ROS dependent alteration of the mitochondrial network resembling early signs of vascular aging[64]. Furthermore, ECs of middle-age diabetic subjects showed a premature derangement of organelle structures, which related to reduced flow-mediated vasodilation of the brachial artery[65]. O_2^- can inhibit angiogenesis *via* multiple mechanisms, including acting as a NO scavenger and inhibiting eNOS activity. Therefore, exaggerated O_2^- in aged endothelium deteriorated both endothelium-dependent vasodilation and collateral vessel formation[16]. In addition, the sources of ROS in aged endothelium include excessive stimulation of NAD(P)H oxidase, xanthine oxidase (XO) and uncoupled eNOS. Therefore, the balancing between ROS and antioxidants might prevent endothelial dysfunction from oxidative stress[66]. Antioxidants are substances that play a major role in preventing the formation and in scavenging of free radicals. Nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor has been shown the master regulator of cellular redox homeostasis and restored antioxidant levels[67]. A previous study reported that NRF2 activity such as glutathione synthesizing enzyme, glutamate-cysteine ligase decreased with age, referring that the cell's glutathione pool can be down-regulated significantly by an age-related decline in NRF2 function [68]. Moreover, NRF2 also regulate gene-associated with the thioredoxin-based antioxidant system (TXN1, TXNRD1), suggesting that NRF2 modulates the proteins redox state[69]. Therefore, NRF2 dysfunction is also a key factor of aging and age-related diseases. The relationship between ROS and angiogenic capacity was studied in several models[70, 71] and found that excessive mitochondrial-derived oxidative stress could reduce angiogenesis[71]. Wei T et al[70] showed that overexpression of Sirtuin 3 (SIRT3) in cardiac microvascular endothelial cells (CMVECs) reduced ROS generation and mitophagy caused in AngII treatment. Moreover, SIRT3 activated FOXO3-dependent

antioxidants, catalase and manganese superoxide dismutase (MnSOD), and inhibited Ras activation, downstream mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and PI3K/Akt, resulting in reduced cardiac hypertrophy [72]. Coenzyme Q10 reduced Ang II-induced mitochondrial ROS and improved angiogenesis in CMVEC. Therefore, these results suggested that reducing mitochondrial oxidative stress might improve CMVECs angiogenic capacity[70]. In Ang II infusion mice, SIRT3 deficiency reduces microvasculature which involved in the cardiac mitochondrial dysfunction and enhanced collagen I and collagen III expressions, resulting in cardiac fibrosis[70].

Taken together, these data indicated that ROS accumulation in endothelial cells impaired angiogenesis by suppressing angiogenic capacity. And impaired angiogenesis resulted in myocardial microvascular rarefaction that could contribute to the progression of heart diseases including cardiac fibrosis [6, 7, 70].

2.3.1.2. Endothelial cells (ECs) dysfunction

ECs play a role in the arterial tone, permeability, angiogenesis, blood clotting and inflammatory process[73]. ECs dysfunction is a prominent factor of vascular disease, which caused the alteration of metabolic and gene expression, leading to reduced angiogenic capacity and NO and increased pro-inflammatory and prothrombotic mediators expression [74]. The characteristics of ECs are flattened and enlarged in the elderly. In advanced aged, ECs with polyploid nuclei increased permeability, the arrangement, and integrity of cytoskeletal alteration, and increased cell cycle inhibitor expression[75]. Moreover, ECs dysfunction and increasing pro-inflammatory state that found in advanced age could induce vascular disease including atherosclerosis, hypertension, and stroke[74].

The loss of NO bioavailability in the endothelium is one of the main characteristics of ECs dysfunction, leading to reduced vasodilation as shown by decreased vascular smooth muscle cell (VSMC) relaxation[30]. Moreover, it was shown that vasodilatory responses were altered in aged vasculature as well as vascular

stiffness. Impaired vasorelaxation induces hypertension and is predictive of future adverse cardiovascular disease. In addition, the reduction of endothelial NO stimulates the proliferation and migration of VSMC, results in the stenosis of the vessel[74].

In senescent ECs, it was found that there were reduced eNOS expression[76-78], impaired angiogenic and repair capability[79] and altered metabolism, resulting in ECs dysfunction and vascular disease[80, 81]. The eNOS enzyme, encoded by the nitric oxide synthase 3 (NOS3) gene, is the major producer of NO in ECs. Several studies have shown that NOS3/eNOS knockout mice induce hypertension, thrombosis, stroke and atherosclerosis [82-84]. Furthermore, ROS, the superoxide anion, react with NO producing peroxynitrite could reduce NO bioavailability[85]. Excessive production of ROS increased prothrombotic and pro-inflammatory mediators expression leading to EC dysfunction [86, 87]. Moreover, augment ROS and loss of NO promote cellular senescence[87-89]. EC senescence or aged EC causes ECs dysfunction and induces vascular disease[80, 81]. Therefore, factors that negatively affect eNOS activity or augment ROS production may promote endothelial dysfunction. Moreover, stresses including hyperglycemia, turbulent flow, smoking increase ROS production in ECs during aging, resulting in DNA damage and p53 activation [90]. The endothelial senescence which is characterized by the p53 activation and induction of its downstream target p21CIP1/WAF1 can be induced by turbulent blood flow via a p53 dependent pathway. However, treatment with a SIRT1 activator can block this[90]. In the spontaneously hypertensive rat model, endothelial senescence was partially blocked by a SIRT1 activator which was related to a reduction to p53 acetylation and increased NOS3 transcription [91]. SIRT1 plays a critical role in the regulation of p53 and in the control of cellular senescence which was found in several tissues including the endothelium[92].

Cluster of differentiation 31 (CD31) also known as platelet endothelial cell adhesion molecule (PECAM)-1 is highly expressed on the surface of endothelial cells in both large and small vessel endothelium including in the capillary networks. CD31 and well established for the monitoring of vessel density and involved in the formation of new vessels.[93] Although, CD31 is a usually use as an EC marker to detect angiogenesis, its expression in other cells may express different forms of CD31 with different functions. Each domain of CD31 plays distinctive roles in the development of

vascular and other inflammatory diseases. Such as, CD31 loses its intercellular junction expression pattern after ECs are exposed to inflammatory cytokines.[94] Therefore, CD31 also expressed on platelet, inflammatory cells, and endothelial cells. The functions of CD31 are related to endothelial mechanosensor and vascular permeability [95].

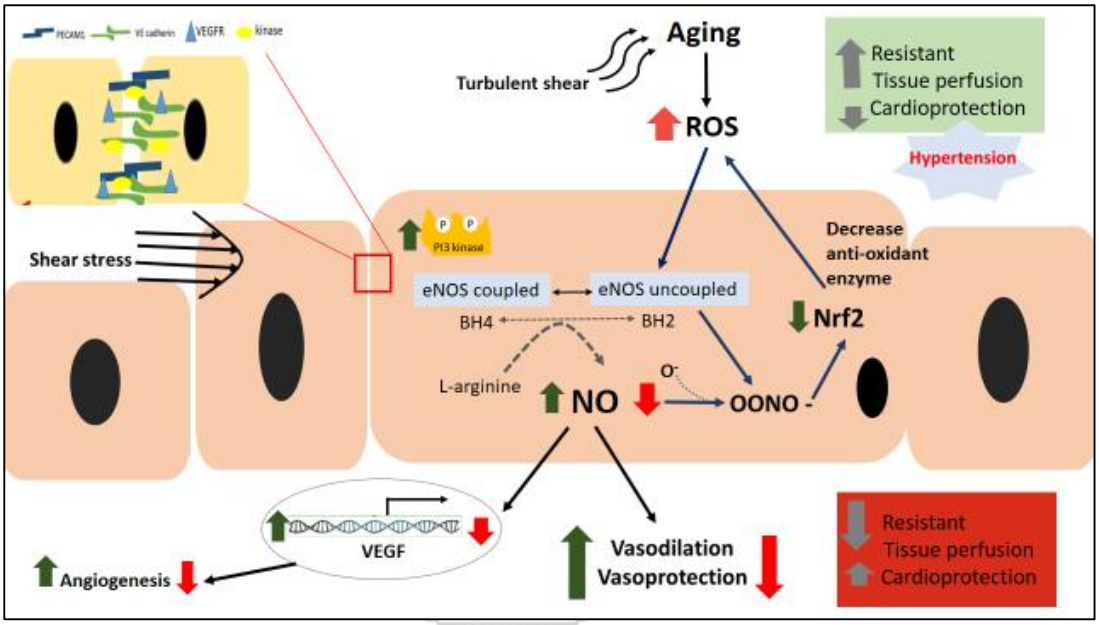


Figure 2 Age-induced impaired NO bioavailability in endothelial cells

Shear stress induces PI3/AKT and eNOS activity via mechanotransduction pathway, leading to increase NO and enhance vasoprotection and angiogenesis. In age endothelial cells, ROS production and turbulent flow increase induce the forming of BH2 which promotes eNOS uncoupling, increasing superoxide, and decreasing NO. $OONO^-$ which generated from the reaction between NO and O^- reduces NRf2 that regulates antioxidant enzyme, resulting in enhances oxidative stress (Modified from Ungvari Z et al (2018)[14]).

At adherence junction, the mechanosensory complex in cells is composed of vascular endothelial (VE)-cadherin, PECAM-1, and vascular endothelial growth factor receptor (VEGFR) 2 were capable of fluid shear stress detection[96]. Shear stress is a laminar flow that we found in the normal vascular wall and increase when exercise. Shear stress-induced mechanotransduction leads to activates Akt and eNOS activity. This enzyme generates NO from L-arginine. NO regulates EC survival, vasodilation, angiogenesis, and antioxidant properties. In aged endothelial cells, ROS, and turbulent

shear increase, resulting in decreased bioavailability of NO by forming peroxynitrite. eNOS can convert to uncoupled status when NOS substrate, L-arginine, or the cofactor tetrahydrobiopterin (BH_4) is not present. Under normal physiological conditions, electron flows from the reductase domain of one subunit to the oxygenase domain of the other subunit, while in eNOS uncoupled status, they are changed to molecular oxygen instead of L-arginine, leading to the production of superoxide anions instead of NO[97]. Peroxynitrite ($OONO^-$) which generated from the reaction between NO and superoxide (O^-) reduces NRF2-dependent antioxidant defense pathway worsens vascular oxidative stress endothelial dysfunction. Moreover, lack of NO causes vasodilator dysfunction. Impaired NO bioavailability is an important component of hypertension in aging [14, 96].

2.3.1.3. Tumor suppressor protein p53

During chronic cardiac pressure-overload, the p53 enlargement in ECs related to blood vessel reduction and fibrosis [9]. Gogiraju R et al [9] found that endothelial p53 deletion prevented the capillary rarefaction in heart failure mice. Furthermore, endothelial p53 deletion is related to reduced apoptotic death of cardiac endothelial, but also non-endothelial cells. Moreover, endothelial p53 deletion had beneficially to change the factors involved in ECM production and turnover and to control the mesenchymal differentiation in cardiac endothelial. From the results, endothelial p53 deletion reduced the development of LV dilation and systolic dysfunction present in End.WT-p53 mice and improved survival. Thus, these data supported the importance of ECs during pressure overload-induced cardiac remodeling and suggested a direct link between endothelial p53 expression, cardiac angiogenesis, and fibrosis.

In ECs, there is evidence supporting that the NOS3 promoter exhibits a functional p53 binding site[98], however, the directional regulation of p53 to inhibit the NOS3 promoter has not been found. However, p53 was demonstrated to indirectly suppress NOS3 transcription by inhibiting the KLF2 expression, a direct transactivator of the NOS3 gene in human umbilical vein endothelial cells (HUVECs)[99]. Furthermore, Overexpression of p53 in ECs inhibited Akt phosphorylation, leading to reduced eNOS activity in mice [100].

The p53 tumor suppressor protein has been proved to play a critical role in cellular responses to oxidative stress. Transgenic Arf/p53 exhibits the elevation of antioxidant activity and the reduction of endogenous oxidative stress levels, correlating with an increased life span [101]. At a low level of oxidative stress, p53 showed antioxidant activities and promote cellular survival. P53 target genes such as sestrin, glutathione peroxidase (GPX), and aldehyde dehydrogenase (ALDH) are involved in reducing ROS. P53 decreased the intracellular level of ROS by regulation of cellular metabolism. P53 exhibited glycolysis and promoted NADPH production lead to a decreased ROS level. Moreover, p53 can inhibit phosphoglycerate mutase (PGM) expression, resulting in less pyruvate required for oxidative respiration in mitochondria and thus reduced ROS production [23, 102].

At a high level of oxidative stress, p53 shows pro-oxidative activity to induce cellular apoptosis. P53 can induce p53 inducible gene3 (PIG3) and proline oxidase gene, resulting in increased oxidative stress. P53 also induces the p53 up-regulated modulator of apoptosis (PUMA) and BAX expression, which induce apoptosis through the releases of cytochrome c from mitochondria. Moreover, p53 can inhibit the antioxidant genes expression such as superoxide dismutase2 (SOD2) and nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2), resulting to elevated oxidative stress to induce apoptosis[23]. Interestingly, p53 also induced MnSOD and GPx elevation, but not catalase, increases ROS and apoptosis[103].

2.3.1.4. Murine double minute 2 (Mdm2)

Mdm2 is a nuclear phosphoprotein that has been shown a negative regulator of the tumor suppressor p53. Mdm2 function is an E3 ubiquitin ligase which together with the transcriptional co-activator p300, mediates the ubiquitination and proteasome-dependent degradation of the p53 tumor suppressor protein and other growth regulatory protein. Moreover, Mdm2 inhibits the interaction between p53 and transcriptional apparatus, mediates translocation of p53 to the cytoplasm, thereby removing it from its active site, and recruits the histone deacetylases 1 (HDAC1) to deacetylase key lysine residues in the COOH terminus of p53 thus making them available for ubiquitination[104]. VGFR2 which is receptor upstream of Mdm2 relays its

signal to downstream kinase including Phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/Akt) and Mitrogen-activated protein kinase (MAPKs). Both of PI3K/Akt and MAPKs pathway could phosphorylation Mdm2 on serine residues 166 and 186[105]. Cancer cell studies showed the regulation of VEGF by Mdm2 in vitro studies [22, 106]. Skinner HD *et al*[106] exhibited that Mdm2 interacted with HIF-1 to activate VEGF transcription in response to hypoxia and growth factor. On the other hand, Zhou S *et al*[22] demonstrated that Mdm2 bound directly to the VEGF 3' UTR and regulated VEGF translation. On the other hand, insulin-like growth factor receptor 1 (IGFR1) and β-adrenergic receptors (β–AR) are important for cellular processes in the heart such as hypertrophy, contractility, and cell survival. IGF1-IGFR1 interaction induced Mdm2 phosphorylation mediated through the Akt and MAPK pathway which led to Mdm2 nuclear localization and enhance its stability and activity. β–AR also activated both Akt and MAPK pathways. The administration of isoproterenol, a β adrenergic agonist, was reported to increase Ataxia telangiectasis mutated (ATM) kinase which is a kinase upstream of Mdm2[107]. Mdm2 can regulate the β –AR signal in cardiomyocytes independently p53 which involved G-protein coupled receptor kinase 2 (GRK2) and β-arrestin2. GRK2 and β-arrestin2 play a critical role in the desensitization of β –AR signaling by inhibiting G-protein coupling subsequently reduce contractility. In cardiomyocyte, Mdm2 can bind and ubiquitinate both GRK2 and β-arrestin2[105]. Jean-Charles P.Y *et al*[108] reported that the polyubiquitination of GRK2 was reduced in Mdm2/p53/KO heart mice, resulting in the impairment of heart contractility. Since the delivery of the Mdm2 gene can improve cardiac contractility, suggesting that the p53-independent physiological role of Mdm2 is required for proper signaling of the β –AR in cardiomyocyte. Furthermore, Roudier E *et al*[42] showed that Mdm2 expression level was an important factor for exercise-induced skeletal muscle angiogenesis. After exercise training, VEGF-A expression and capillary to fiber ratio were significantly increased in wild-type animals, whereas Mdm2 hypomorphic did not show these changes.

2.3.1.5. Phosphoinositide-3-kinase–protein kinase B/Akt (PI3/Akt), endothelial nitric oxide synthase (eNOS) vascular endothelial growth factor (VEGF)

ECs are mainly characterized by a progressive reduction of the bioavailability of NO. This process related to an increase in ROS and nitrogen species (RNS) production [30]. The balance between NO production via nitric oxide synthase (NOS) and NO degradation primarily by superoxide anion means NO bioavailability [109]. eNOS is the major enzyme responsible for NO synthesis in the vasculature [31]. The reduction of NO bioavailability is largely due to an excessive reduction in eNOS[31, 32]. Interestingly, eNOS dysfunction related to ROS production had played a key role in microvascular dysfunction and loss of ventricular contractility in aging[32]. Redox imbalance in aging endothelial cells can increase the uncoupling of eNOS function[32]. Phosphorylation of serine residue 1177 (p-eNOS^{ser1177}) by the PI3K/Akt pathway plays an important role in eNOS activation and NO production [31]. LeBlanc *et al*.[33] showed that impaired endothelium-dependent dilation in coronary arterioles of aged rats was associated with PI3K/Akt signaling dysregulation. Recently, the study of Trott DW *et al*.[110] showed that the impairment of PI3K/Akt/NO signaling led to endothelial dysfunction in old arteries, this event associated with the p-eNOS^{ser1177}

Pathological and physiologic angiogenesis is regulated by VEGF, which a potential angiogenic factor. The proliferation, sprouting, migration and tube formation of ECs are influenced by VEGF[111]. VEGF family is a group of glycoproteins consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (placental growth factor). There are interacted with a family of 3 receptor tyrosine kinase: VEGFR1 (VEGF receptor 1), VEGFR2 and VEGFR3 (Figure 3). VEGF-A binds to both of VEGFR1 and VEGFR2. VEGF-B and PlGF bind to VEGFR1. While, VEGFC and VEGFD bind to both of VEGFR2 and VEGFR3[112]. VEGFA plays an important role during organ remodeling and diseases that involves blood vessels such as in angiogenesis[111]. The VEGFRs are found on wide variety of cell types. VEGFR1, also called Flt-1 (fms-like tyrosine kinase 1), is found on vascular endothelial cells, hematopoietic stem cells, monocytes, and macrophages. VEGFR2, also called KDR (kinase insert domain) or Flk-1 (fetal liver kinase 1),

is expressed on vascular and lymphatic endothelial cells. VEGFR3 (also called Flt-4) is restricted to lymphatic endothelial cells[112].

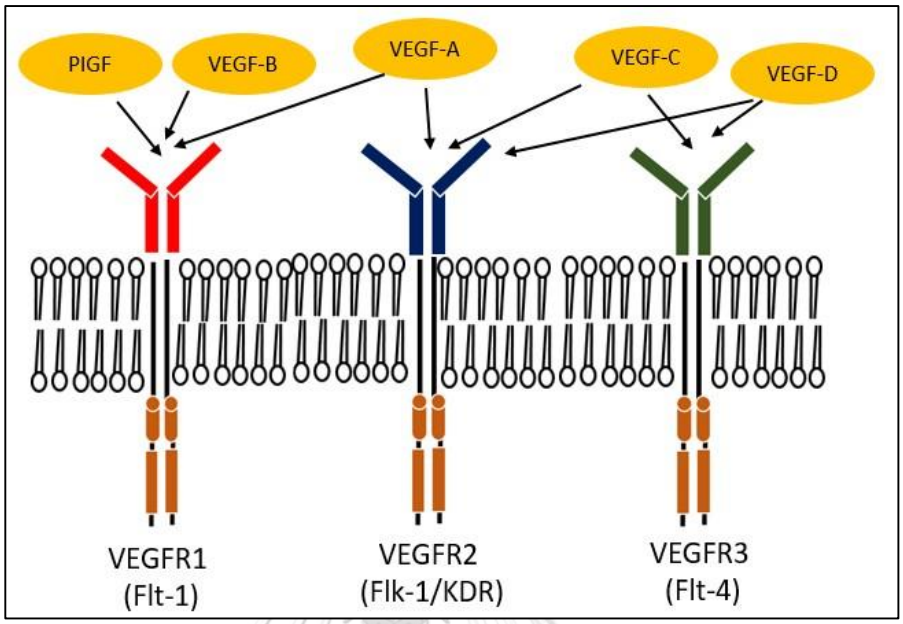


Figure 3 VEGF ligands and receptors.
VEGFR1 binds to PlGF, VEGFA, and VEGFB. VEGFR2 bind to VEGFA, VEGFC, and VEGFD, whereas VEGFR3 binds VEGFC and VEGFD (Modified from Pandey AK (2018)[112]).

The expression of VEGFR 2 is down-regulated in ECs of the adult vessel[111]. Importantly, VEGF and VEGFR are decreased in aged animals [113] especially its also reduced in cardiac myocyte[15]. The mRNA and protein expressions of VEGF, VEGFR1 and VEGFR2 were significantly decreased in the heart of aged rats [17]. VEGF causes vasodilation through the induction of the eNOS and subsequently increases in NO production. Although VEGF acts mostly on endothelial cells, it also binds VEGF receptors on hematopoietic stem cells (HSCs), monocytes, osteoblasts, and neurons. VEGF activates recruitment of inflammatory cell and enhances the protease expression that has been implicated in pericellular matrix degradation in angiogenesis[111]. The regulation of VEGF production is involved in several factors including cytokines, growth factors, and tumor suppressor factors [114]. Hypoxia stimulates VEGF gene expression *via* hypoxia-inducible factor (HIF)[115]. Under the hypoxic conditions,

the aryl hydrocarbon nuclear translocator (ARNT) binds hypoxia-responsive elements (HREs), resulting in transcriptional genes induction, including genes involves in angiogenesis such as VEGF genes[111]. On the other hand, several studies showed the involvement of the PI3K/Akt pathway contributed to the cytoprotection of endothelial cells by VEGF [116, 117]. P-eNOS^{ser1177}, which rendering enzyme activity calcium-independent, is directly activated by PI3K/Akt[31, 118]. On ligand binding, VEGFR2 dimerizes and activates an activity of tyrosine kinase receptor, leaded to an intracellular domains autophosphorylation. This activation initiates variety of signaling pathways. With signaling downstream, one major pathway is an activation of PI3K /Akt signaling. An activation of eNOS, a product of PI3K/Akt phosphorylation, increases NO level. In addition, VEGF dimerized VEGFR activates PLC**γ** (phospholipase C gamma), which then converts phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). After that, IP3 triggers the release of intracellular calcium while DAG mediates the activation of protein kinase C (PKC). The activation of PKC activates phospholipase A2 (PLA2) via mitogen-activated protein kinases (MAPKs) increases prostacyclin (PGI2) level. Both PGI2 and NO mediate many biological consequences within the VEGF signaling cascade; including enhanced vascular permeability, vasorelaxation, and endothelial survival (Figure 4)[112]. However, the VEGFA/VEGFR2 signaling exhibits difference in the process of arterial–venous endothelial cell differentiation[119].

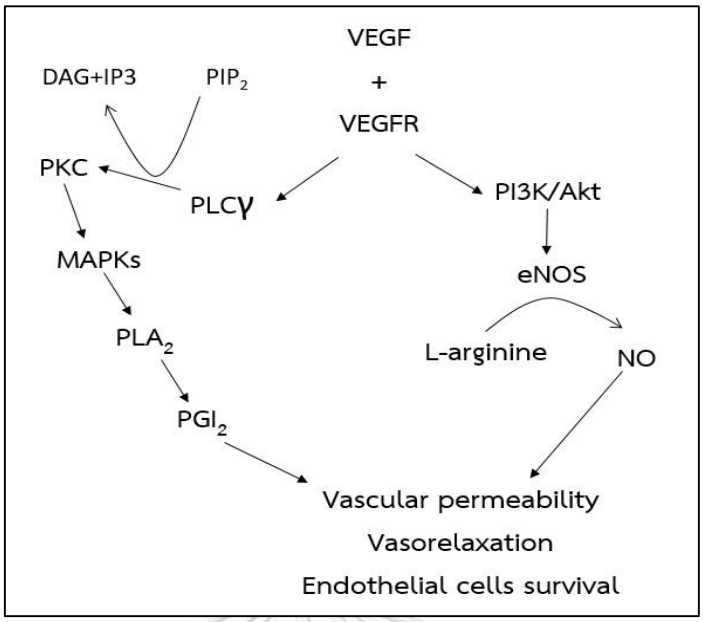


Figure 4 Intracellular signaling pathway for VEGFA.

VEGF-VEGFR dimerization activates PI3K/Akt, which then induces conversion of L-arginine to NO by eNOS activation. In parallel, PLCγ activated by VEGFR converts PIP2 into IP3 and DAG. Then, DAG triggers PKC, leading to increases of PGI2 via MAPKs and PLA2. All these changes contribute to in vascular permeability, vasorelaxation, and EC survival. (Modified from Pandey AK et al. 2018[112])

2.4. Impact of exercise training induce beneficial effects in the heart

Regular physical activity has health benefits for cardiovascular disease, diabetes, hypertension. In aged animals, the impairment of angiogenic responses especially, VEGF was found in many tissues including heart, skeletal muscle, and brain[120]. The impaired angiogenesis during aging-induced several pathological complications and delayed wound healing process [121]. Several reports showed that exercise improved angiogenesis in various young and aged tissues. However, the effect of exercise to induce angiogenesis was different from organ to organ. Coronary artery disease (CAD) is related to insufficient angiogenesis and physical inactivity [122, 123]. In aged subjects, physical activity improved the angiogenesis. Therefore, physical activity programs were necessary for old people who are at high risk of CAD due to impaired angiogenesis[120].

Gounder SS *et al* [38]showed that moderate exercise training promoted myocardial antioxidant levels such as catalase (CAT) Glutathione reductase (GSR) in the aging mouse. Furthermore, Hassan AF and Kamal MM[39]showed that exercise training induced increases of capillary density may turn into a beneficial adaptation as the capillary network increases oxygen supply and energy substances for the aging heart. It is hypothesized that exercise training can decrease oxidative damage in the aging cardiovascular system through the induction of antioxidant enzyme and increasing capillary growth [38].

The potential of exercise training on p53 expression was observed in several studies [40, 41]. Ziaaldini MM *et al*.[40] investigated the effect of endurance training on treadmill 6 weeks in skeletal muscles of young rats (three months) and old rats (eight months) and found that p53 expression and ROS levels were increased in the skeletal muscles of old rats and endurance training could lower p 53 expression in both old and young rats. Al-Jarrah *et al* [41] examined the level of iNOS and p53 expression in the cardiac muscle of type I diabetic rats and non-diabetic rats after treadmill running 4 weeks. They found that p53 expression in the diabetic control group increased when compared to inactive control rats. However, the level of p53 expression, but iNOS expression level, was attenuated by exercise training suggesting that the reduction of p53 might be associated with the reduction of ROS.

Mdm2 could be considered as a critical regulator of angiogenesis such as the growth of blood capillaries under exercise situations. Roudier E *et al*.[42] showed that Mdm2 expression level was an important factor in maintenance capillaries for exercise-induced skeletal muscle angiogenesis. They used transgenic mice (40% Mdm2 expression) and found that after exercise VEGF-A expression and capillary to fiber ratio were significantly increase in wild-type animals, but Mdm2 hypomorphic did not show these changes. In the human study, it was found that the level of a human double minute2 protein, Hdm2 protein, did not differ between young and aging skeletal muscle. However, exercise training caused a dramatic increment of this protein by 38% in skeletal muscle of young subjects and 81% of the senior subjects[43]. These results confirmed the important role of Mdm2 on angiogenesis, probably, on angiogenic capacity.

Rossman MJ *et al.*[124] demonstrated that aerobic exercise prevented EC senescence induced- vascular endothelial dysfunction in aging healthy humans. They evaluated the level of senescence marker such as p53, p21, and p16 in ECs obtained from antecubital veins of human aging with or without exercise training. Their results indicated the reduction of p53, p21, and p16 expression seem to appear only in ECs of exercise aging, but not in sedentary aging subjects. Moreover, they also found that EC proteins, p53, p21, and p16, were inversed correlation with vascular endothelial function, as measured by brachial artery flow-mediated dilation. Therefore, they suggested that exercise training could prevent the expression of senescence and impaired vascular endothelial function in sedentary aging. Taking together, the previous reports also showed the reduction of oxidative and inflammatory signaling in ECs obtained from older aerobic exercise-trained when compare to older sedentary [125, 126]

Exercise training increased capillary mediated to be triggered by local mechanical and angiogenic growth factors[120]. Several studies demonstrated that exercise training induced capillary growth as evidenced by the elevation of mRNA VEGF expression in both humans and animals [127, 128]. Both skeletal muscle in humans and animals, exercise training induced the expression of VEGF genes and VEGF protein [129, 130]. However, the up-regulation of VEGF genes depended on the intensity of exercise and the level of hypoxia [120]. Iemitsu *et al.*[17] revealed that exercise training improved the age-induced downregulation of mRNA and VEGF protein and VEGFR in the heart. Moreover, TGF-β1, b-FGF, angiopoietin, PlGF and hepatocyte growth factor-induced angiogenesis in the heart by exercise training regulation [17, 131].

Exercise training induces physiologic hypertrophy, which increases by approximately 7% in cell length[132]. Aerobic exercise induces stretch of cardiomyocyte growth by increased plasma volume while activation of growth signals like insulin-like growth factors[133, 134]. These changes may be a good correlation between left ventricular function during diastole and aerobic fitness[135]. A previous study showed that chronic exercise training increases hypertrophy at both the organ and cellular level in laboratory animals[136]. Moreover, cardiac hypertrophy is distributed across several walls of the left ventricular myocardium (anterior, posterior, and septal wall). Previous reports showed that swimming training increased weight and internal diastolic

diameter of left ventricular in the spontaneously hypertensive rats[137]. Swimming training also increased cardiomyocyte cross-sectional area, reduced apoptosis, and normalized calcineurin without any significant changes in the Akt pathway. Consequently, the reduction of fibrosis and improvement of vascularization[137].

In ECs, eNOS and NO appeared associated with the positive effects of exercise [138, 139]. Importantly, eNOS activation induced by exercise was specific in tissue manner. In the heart, exercise up-regulated eNOS-PSer1177 expression and down-regulated eNOS-PThr495 expression without changing of total eNOS. However, exercise increased the total eNOS and decreased the eNOS-PThr495 expression in the skeletal muscle [140]. During exercise, the eNOS activation may be involved in several signaling pathways including Akt, protein kinase A (PKA) and/or AMPK. Akt signaling blockage decreased eNOS-PSer1177 expression in exercise mice when compared with the control group, however, phosphorylated CREB (PKA signaling) or AMPK expression did not change [141]. Furthermore, β3-adrenergic receptors (β3-AR) has been proved to play an important role in regulating eNOS –PSer1177 and maintaining the basal level of myocardial eNOS during exercise[140, 142]. Therefore, the correlation of β3-AR, eNOS, and Akt by exercise regulation future in myocardial injury treatment.

The sedentary lifestyle appeared to accelerate heart aging and regular exercise especially, endurance exercise improved cardiac function both of the young and senior populations[10]. Exercise training improved maximal cardiovascular work capacity by elevated stroke volume and cardiac output [143]. Exercise training in aging reduced the accumulation of connective tissue including collagen content [10, 144]. These results are consistent with the result of other studies [10, 45]. Kwark HB *et al* [10]. investigated the potential effects of 12- week exercise training on cardiac fibrosis in young and aging rats. They found that exercise training reduced TGF-β, an upstream regulator of TIMP, resulting in a dysregulation of MMPs and a reduction of cardiac fibrosis.

CHAPTER III

MATERIALS AND METHODS

3.1 Animals

Male Wistar rats (aged 8 weeks) were purchased from National Laboratory Animal Center (Salaya, Mahidol University, Thailand). The animals were allowed to rest for at least one week before being used in the experiment. They were housed 3-4 rats per cage, in the animal room with the controlled temperature at 20±2°C and 12:12 h light: dark cycle (on 7.00 a.m. off 7.00 p.m.) with free access to food and water *ad libitum*. The experiment protocols were conducted in accordance with the guidelines for experimental animals by the National Research Council of Thailand, and authorized by the Committee of Animal Care and Use (CU-ACUP), Faculty of Medicine, Chulalongkorn University (NO.023/2561).

Sample size determination: To determine the sample size for each group, we selected G power program to analysis[145]. We calculated the number of rats per group by consulting with statistician and referred to the article of our college who performed the similar study, Viboolvorakul S, Patumraj S[146]. And the following values were used to calculate the effective sample size from the equation.

$$n = 2 \times \frac{(Z_{(1-\alpha/2)} - Z_{\beta})^2}{d^2} \qquad [1]$$

When α value 0.05, $Z_{(1-\alpha/2)} = 1.96$

Power (1-β value) 0.99, $Z_{\beta} = -2.326$

Effect size (d^2) 0.82

From the results, the total sample size is 45 animals, so each group is 9. The result of G power window was shown in appendix A. However, , from our pilot study, the finding of heart weight changes appeared to become significant difference even though the sample size was only at 6, therefore, we decided to reduce the number of each group equal to 8 along with the Ethical Committee allowance. (NO.023/2561).

3.2 Experimental protocols

To determine the effects of selected exercise training program used in this study, how it did on physiological changes. Rats were measured body weight, heart weight, resting blood pressure, resting heart rate.

Male Wistar rats were randomly divided into 5 groups, [147, 148] as indicated below:-

Group 1: Sedentary – young group (SE-Young, aged 4 months) (n=8). They were subjected to the same swim environment as the trained-aged animals, except they remained freely in their cages

Group 2: Sedentary- middle-aged group (SE-Mid-Age, aged 14 months) (n=8). They were immersed individually for 30 minutes/day, 5 days/week for 8 weeks in cylindrical tanks filled with water: depth of 5 cm, temperature at 33-36 °C.

Group 3: Exercise trained – middle-aged group (Ex-Mid-Age, aged 14 months) (n=8). They swam individually for 60 minutes/day, 5 days/week for 8 weeks in cylindrical tanks filled with water: depth of 50-55 cm, temperature at 33-36 °C.

Group 4: Sedentary- aged group (SE-Age, aged 22 months) (n=8). They were immersed individually for 30 minutes/day, 5 days/week for 8 weeks in cylindrical tanks filled with water: depth of 5 cm, temperature at 33-36 °C.

Group 5: Exercise trained – aged group (Ex-Age, aged 22 months) (n=8). They swam individually for 60 minutes/day, 5 days/week for 8 weeks in cylindrical tanks filled with water: depth of 50-55 cm, temperature at 33-36 °C.

NOTE: The age of experimental rats in each group was design according to the comparison age between human and rat as shown in Table 1[149]

Table 1 Rat's age versus human's age.

Rat's age (months)	Human's age (years)
6	18
12	30
18	45
24	60
30	75
36	90
42	105
45	113
48	120

Rats were subjected to measure body weight every Monday for 8 weeks starting from the first day of experiment. The results were represented by % change of body weight according to the formula below.

$$\% \text{ change of body weight} = \frac{\text{final body weight} - \text{initial body weigh}}{\text{initial body weight}} \times 100 \quad [2]$$

After 8 weeks of exercise training program, the rats rested for 24 hours. Then, rats were anesthetized with pentobarbital sodium (60 mg/kg per body weight, intraperitoneally). After that, rats were tracheotomized and canulated a catheter into the carotid artery for measurement resting blood pressure and heart rate by using a pressure transducer (Statham, USA) connected to a polygraph system (Nihon Koden, Japan). At the end of experiment, rat hearts were perfused with 250 ml ice-cold phosphate-buffered saline (PBS) containing heparin (25 UI/ml) and excised it. Heart tissues were rinsed in ice-cold PBS to remove contaminating blood. The hearts were weighed for determining the ratio of heart weight and final body weight. Then, the hearts were cut for 2 millimeters (mm) at the mid-ventricular level. The rat heart sections were fixed in 10% neutral buffered formalin (250 ml.) for 24 hours. After

fixation process, the heart tissues were embedded into paraffin wax blocks. The rest heart tissues were stored at -80 ^{o}C for the determination of phospho-Akt, eNOS, VEGF, TGF-β and, MDA. All surgical procedures were performed by aseptic techniques.

According to resting blood pressure, systolic blood pressure (SBP), diastolic blood pressure (DBP) were measured. From graph recording (Figure 5), 1 column equaled 1 second. The duration was 10 seconds. In this current study, the blood pressure data were collected by 3 time points, which were the 3rd second (1st time point), the 6th second (2nd time point) and the 9th second (3rd time point). The blood pressure data of both SBP and DBP in 3 time were represented in average value. Heart rate was represented in beats per minute Furthermore, mean arterial blood pressure (MAP) was calculated by (2xDBP) +SBP before sacrificing rats.

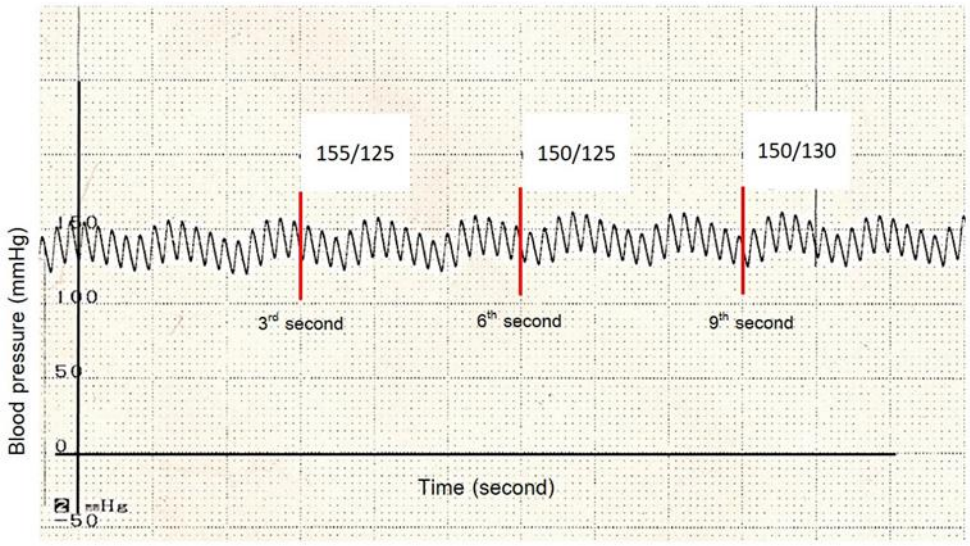


Figure 5 Measurement of resting blood pressure of SBP and DBP from a common carotid artery with pressure transducer (Statham, USA) which connected to polygraph system (Nihon Koden, Japan) at the the 3rd second (1st time point), the 6th second (2nd time point) and the 9th second (3rd time point).

3.2.1 Moderate intensity exercised training program

The program of swimming training was described and modified from the method of Iemitsu M *et al*. [17] and Viboolvorakul S *et al*.[146]. In the exercise group,

rats were transported to an exercise training room. They swam in cylindrical tanks (65 cm height with 50 cm diameter) containing water maintained the temperature at 33-36 ^{o}C. The cylindrical tank contained water at depth of 50-55 cm. Rats were exercised once a day, during 9.00-12.00 a.m., 5 days per week. The protocol for exercise shows that rat swam for 15 minutes for the first 2 days for adaptation, and then the swimming duration was gradually increased by one-week period from 15 minutes to 60 minutes. After that, the trained-group continued to swim 60 minutes per day for 7 weeks as we showed in Figure 6 and Table 2 [17, 146]. From the exercise training program, the rats swam continuously without the addition of tail weight loading. They were performed at a metabolic rate 2-3 METs with a VO_2 ranging from 46-63 ml-min-kg^{-1} body weight^{-1}. Because of the maximum oxygen uptake (VO_{2max}) of normal rat ranges from 85-100 ml-min-kg-1 body weight-1. Therefore, the selected swimming exercise program was equivalent to the moderate-intensity with the value of VO_{2max} was about 45% - 65%.[148, 150]

$$VO_2 \text{ max} = HR_{max} \times SV_{max} \times a\text{-}vO_2 \text{ difference}_{max} \quad [3]$$

After each training session, rats were dried with a towel and a hairdryer and then returned to their cages. The SE-Young animals were subjected to the same training room, remained in their cages during training hour and handled daily. In SE-Mid-Age and SE-Age groups, rats were transported to the exercise training room, and they were individually immersed in cylindrical tanks containing water at a depth of 5 cm which the water controlled at the same temperature as the exercise groups. Rats were placed in the cylindrical tanks for 30 minutes per day, 5 days per week for 8 weeks. Rats were placed for 15 minutes for the first 2 days, and then the immersed duration was gradually increased by one-week period from 15 to 30 minutes. After 8 weeks, all groups were rested at least 24 hours. As we showed in Figure 7.

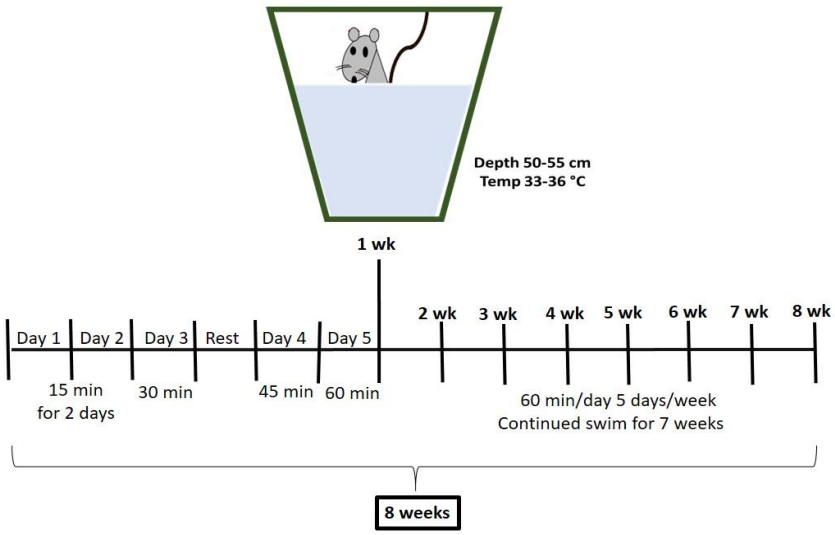


Figure 6 Exercise training program

(Modified from the method of Iemitsu M et al., (2006)[17] and Viboolvorakul S et al., (2014)[146]).

Table 2 Exercise training program

	Day	Duration time (minutes)
1st week	1	15
	2	15
	3	30
	4	rest
	5	45
	6	60
	7	rest
2nd-8th week	1	60
	2	60
	3	60
	4	rest
	5	60
	6	60
	7	rest

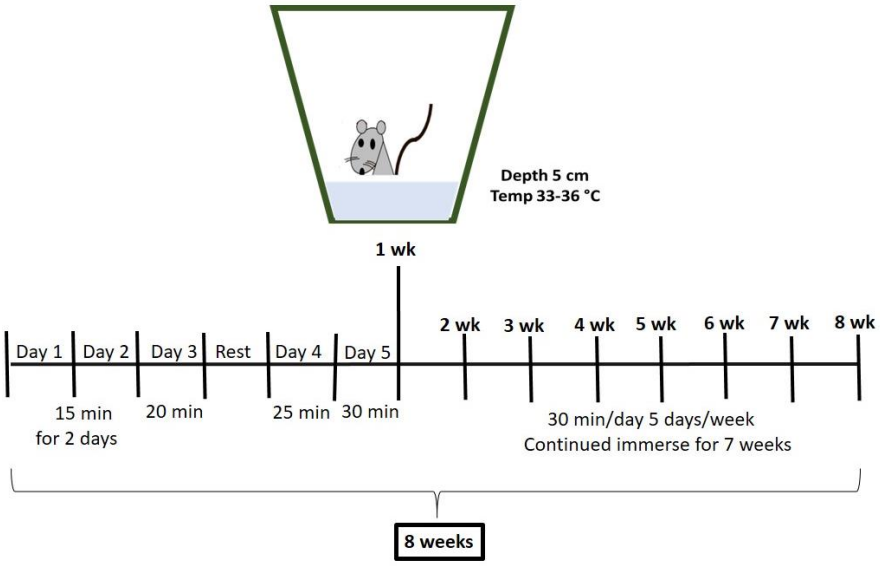


Figure 7 Immerse program
(Modified from the method of Iemitsu M et al., (2006)[17] and Viboolvorakul S et al., (2014)[146]).

3.2.2. Methods for evaluation cardiac pathology

From each heart, two midventricular slices (at the level of the papillary muscles) were processed and embedded in paraffin, because they are most representative of the scale of LV remodeling and the extent of the transmural scar[151] and LV mid-wall fibrosis also associated with an increased risk of heart failure hospitalizations, ventricular arrhythmias and cardiac death[152]. We showed the process in Figure 8

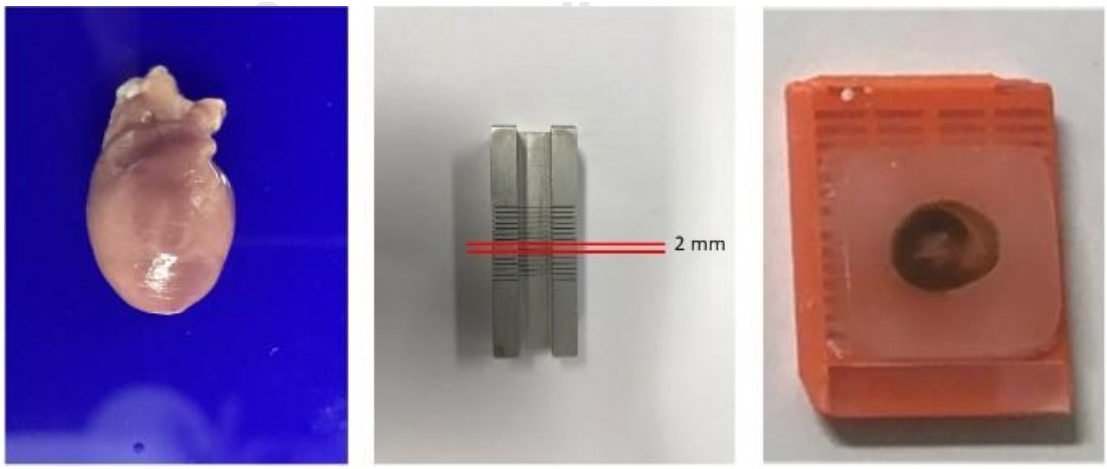


Figure 8 The process for preparing to paraffin-embedded tissue

3.2.2.1. Masson's trichrome stain for collagen accumulation

To evaluate the collagen content in the hearts, Masson's trichrome staining was used in this study. In this technique, cardiomyocytes, collagen fibers, and nuclei were stained a bright red, blue and black, respectively. Briefly, 3 μm thickness heart paraffin sections were deparaffinized by soaking in xylene and rehydrated following re-fix in Bouin's solution to improve staining quality. After washing, the sections were stained in Weigert's iron hematoxylin, washed, and stained with 1% Biebrich scarlet-acid fuchsin solution. After washing and differentiation in 2.5% phosphomolybdic-phosphotungstic acid solution, the sections were transferred to 2.5% alanine blue solution and differentiated in 1% acetic solution, dehydrated, then cleared in xylene, mounted with coverslip. The results were visualized using a Nikon light microscope. The positive area was evaluated at 400X magnification in four areas of the heart (anterior, posterior, lateral and septum wall), three pictures per area (Figure 9). Sections in 8 rats/group were analyzed for the percentage of collagen area by using Image-Pro Plus 6.0 program [10] as showed in Figure 10. These results were confirmed by blind assessment as showed in appendix B1

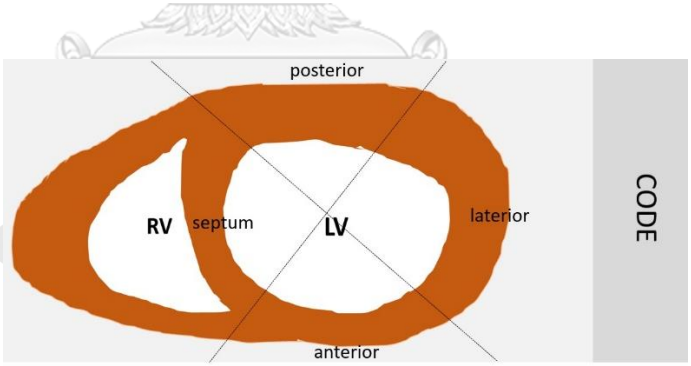


Figure 9 The collagen accumulation were assessed under a light microscope at 400X magnification in four areas (anterior, posterior, lateral, septum)

The results were represented by the percentage of positive-blue staining pixels according to the formula below.

$$\% \text{ of collagen accumulation} = \frac{\text{Number of pixels with the blue color X 100}}{\text{Total number of pixels in the study frame}} \quad [4]$$

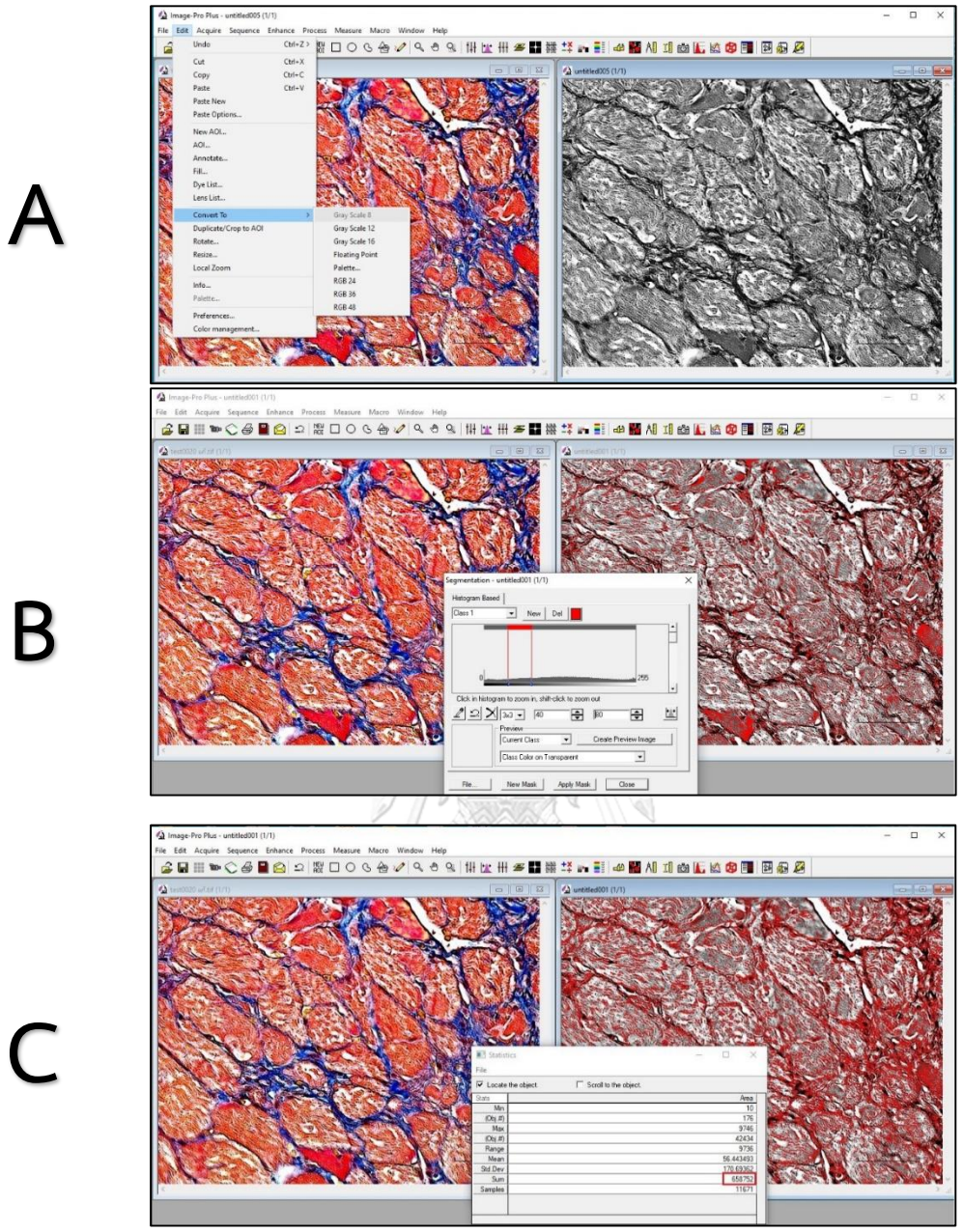


Figure 10 The analysis of collagen content with sum of density method by using Image analysis program software (Image-Pro Plus 6.0). (A) To calibrate the total positive-blue staining pixels, we need to converte to grey scale and (B) To select the threshold range and (C) To measure the density of staining.

3.2.2.2. Immunohistochemistry (IHC) evaluation for p53 Mdm2 and CD31

The expression of p53, Mdm2, and CD31 was examined by using IHC. According to Mdm2, the 3 µm thickness heart paraffin sections were treated with target retrieve

solution buffer pH 6 (DAKO, USA) in a microwave oven for 13 minutes for antigen retrieval after deparaffinized. Then paraffin sections were incubated with 3.0% H_2O_2 for a block of endogenous peroxidase activity. After that, the sections were blocked with non-specific protein blocking reagent, followed with mouse monoclonal against Mdm2 at a dilution 1:50 (33-7100, Thermo Fisher, USA) overnight at 4℃. The next day, after the sections were washed extensively with wash buffer, sections were incubated with horseradish peroxidase (HRP) polymer-conjugate anti-mouse (DAKO, USA) for 30 minutes at room temperature. After the sections were washed, 3,3'-Diaminobenzidine (DAB) substrates were applied. Finally, the sections were counterstained with hematoxylin, dehydrated, mounted with coverslip. The immunoreactivities were assessed under a light microscope at 400X magnification in four areas of the heart (anterior wall, posterior wall, lateral wall, and septum wall), three pictures per area similar to Figure 9. The number of positive areas were analyzed by using Image-Pro Plus 6.0 program as showed in Figure 11. These results were confirmed by blind assessment as showed in appendix B2

To determine the level of p53 and CD31 staining, antigens were retrieved in Dako EnVision FLEX Target Retrieval Solution, high pH (pH 9.0), using Dako PT Link for 20 min at 97°C according to the manufacturer's instructions (Dako, Glostrup, Denmark). The IHC for p53 and CD31 were performed using a rabbit polyclonal against p53 at a dilution of 1:250 (FL-393, Santa Cruz Biotechnology, CA) and rabbit polyclonal against CD31 at a dilution 1:100 (MA5-13188, Thermo Fisher, USA) for 30 min at room temperature and the Dako Envision FLEX system was used for visualization. The immunoreactivities were assessed under a light microscope at 400X magnification in four areas of the heart (anterior wall, posterior wall, lateral wall, and septum wall), three pictures per area similar to Figure 9. The number of positive areas were analyzed by using Image-Pro Plus 6.0 program as showed in Figure 11. These results were confirmed by blind assessment as showed in appendix B3.

The results were represented by the percentage of positive-brown staining pixels according to the formula below.

$$\%\ \text{of p53/Mdm2/CD31 staining} = \frac{\text{Number of pixels with the brown color}}{\text{Total number of pixels in the study frame}} \times 100 \quad [5]$$

A

B

C

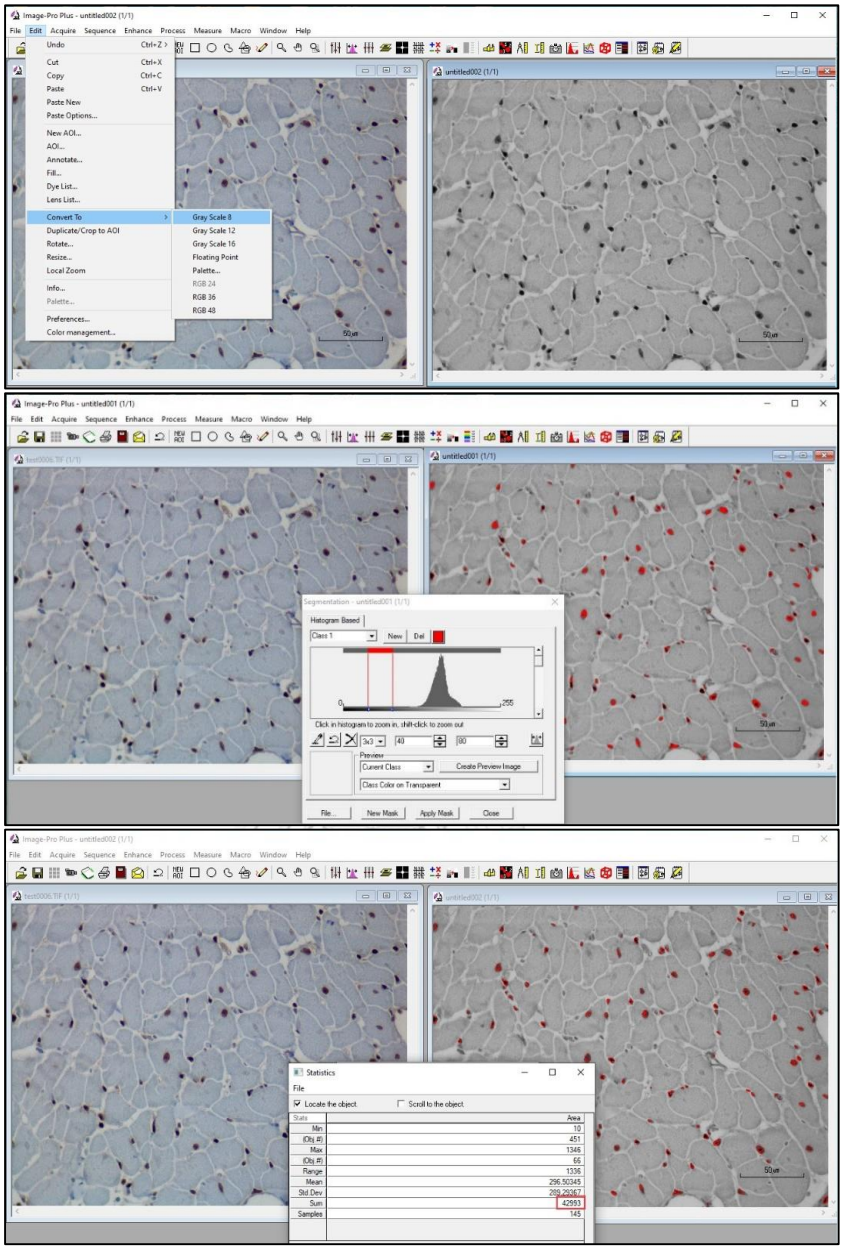


Figure 11 The analysis of the p53, Mdm2 and CD31 with sum of density method by using Image analysis program software (Image-Pro Plus 6.0). (A) To calibrate the total positive-brown staining pixels, we need to convert to grey scale and (B) To select the threshold range and (C) To measure the density of staining.

3.2.3 Homogenization of heart tissue

Perfused heart tissues were homogenized with a Dounce homogenizer in 1000 µl ice-cold RIPA lysis buffer containing protease inhibitor cocktails and phosphatase

inhibitor cocktails (Sigma Chemical Co, USA). Tissue homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. After that, the supernatants were collected, aliquoted and stored at -80 °C for further experiments. The protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Pierce, USA), according to manufacturer's instruction.

3.2.3.1 Enzyme-linked immunosorbent assay (ELISA) for determining phospho-Akt, eNOS, VEGF and TGF-β protein expression

Phospho-Akt 1 protein levels were determined by a DuoSet IC ELISA kit (DYC2289C-2, R&D system, USA). This technique was performed under the manufacture's protocol. For plate preparation, the phospho-Akt 1 antibody was diluted at concentration 4 µg/ml in PBS and pre-coated microplate with 100 µL per well of the diluted phosphor-Akt1 antibody, then plates were sealed and incubated overnight at room temperature. After washing 3 times, the plates were blocked with blocking buffer to each well, incubated at room temperature for 1.30 hours and washed before using. After plate preparation, samples or standard solution were added in IC diluent #3 (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 1 M urea in PBS, pH 7.2-7.4) to normalized and incubated 2 hours at room temperature. The plates were aspirated and washed 3 times. After that, the detection antibody was diluted at concentration 100 µg/ml in IC diluent #1(1% BSA in PBS, pH 7.2-7.4) and added 100 µL of the diluent detection antibody to each well and incubated 2 hours at room temperature. After washing, Streptavidin-HRP was diluted in IC diluent #1 and added 100µL of the diluted Streptavidin-HRP to each well and incubated 20 minutes at room temperature. Avoid direct exposure to intense light. The plates were washed again and 100 µL of substrate solution (1:1 mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine)) were added to each well and incubated 20 minutes at room temperature in the darkroom. The stop solution was added to each well and mixed. The plates were measured at a wavelength of 450 nm. The differences in absorbance were calculated and the sample values were determined from a standard curve which expressed as pg/mg protein. The standard curve of p-Akt1 was showed in appendix C

eNOS protein levels were determined by a DuoSet IC ELISA kit (DY950-05, R&D system, USA). This technique was performed under the manufacture's protocol. For plate preparation, the eNOS antibody was diluted at concentration 1 µg/ml in PBS and pre-coated microplate with 100 µL per well of the diluted eNOS antibody, then plates were sealed and incubated overnight at room temperature. After that, the plates were aspirated and washed 3 times. The plates were blocked with blocking buffer to each well, incubated at room temperature for 1.30 hours and washed before using. After plate preparation, the samples or standard solution were added to the wells, followed by the diluent detection antibody. After incubation 2 hours, the plates were aspirated and washed 3 times with wash buffer to remove unbound material. After that, 100 ul of diluted Streptavidin-HRP was added to each well and incubated 20 minutes at room temperature. Avoid direct exposure to intense light. The plates were washed again and 100 µL of substrate solution (1:1 mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine)) were added to each well and incubated 20 minutes at room temperature in the darkroom. The stop solution was added to each well and mixed. The plates were measured at a wavelength of 450 nm. The differences in absorbance were calculated and the sample values were determined from a standard curve which expressed as pg/mg protein. The standard curve of eNOS was showed in appendix D

VEGF protein levels were determined by a quantikine ELISA kit (MMV00, R&D Systems, USA). According to the manufacture's protocol. Briefly, the samples or standard solution were added in 96- well plate that pre-coated with a polyclonal antibody specific for VEGF. The plates were incubated for 2 hours and washed 3 times with wash buffer. After that, streptavidin-HRP was added to each well, incubated for 2 hours and washed 3 times as before. After that, the TMB substrate solution was added to each well and incubated for 20 minutes in the dark. The stop solution was added to each well. The plates were measured at a wavelength of 450 nm. The differences in absorbance were calculated and the sample values were determined from a standard curve which expressed as pg/mg protein. The standard curve of VEGF was showed in appendix E.

TGF-β1 protein levels were determined by a Duoset ELISA kit (DY1679-05, R&D Systems, USA). This technique was performed under the manufacture's protocol. For plate preparation, TGF-β1 antibody was diluted at concentration 4 μg/ml in PBS and pre-coated microplate with 100 μL per well of the diluted TGF-β1 antibody, then plates were sealed and incubated overnight at room temperature. After that, the plates were aspirated and washed 3 times. The plates were blocked with blocking buffer to each well, incubated at room temperature for 1.30 hours, washed before using. From the manufacture's protocol, the samples were prepared by added 1N HCl and 1N NaOH for neutralizing before starting the test procedure. After plate and sample preparation, the samples or standard solution were added to the wells, followed by the diluent detection antibody. After incubation 2 hours, the plates were aspirated and washed 3 times with wash buffer to remove unbound material. After that, 100 ul of diluted Streptavidin-HRP was added to each well and incubated 20 minutes at room temperature. Avoid direct exposure to intense light. The plates were washed again and 100 μL of substrate solution (1:1 mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine)) were added to each well and incubated 20 minutes at room temperature in the darkroom. The stop solution was added to each well and mixed. The plates were measured at a wavelength of 450 nm. The differences in absorbance were calculated and the sample values were determined from a standard curve which expressed as pg/mg protein. The standard curve of TGF-β1 was showed in appendix F

3.2.3.2 Lipid peroxidation

To evaluate the malondialdehyde levels (MDA), the thiobarbituric acid reactive substances assay (TBARS) was used in this study. The TBARS assay was performed according to the procedure described by the kit (Cayman, USA). Briefly, the samples or MDA standards were added into the separated tube followed by incubation with sodium dodecyl sulfate (SDS) solution. After that, the mixtures were incubated with TBA reagent at 95 ^{o}C for 60 minutes. After 60 minutes, the tubes were immediately placed in an ice bath to stop the reaction and incubated 10 minutes. After that, the mixtures were centrifuged at 1600 xg for 10 minutes at 4 ^{o}C and incubated at room temperature for 30 minutes. The MDA concentrations were measured at a wavelength

of 540 nm. The differences in absorbance were calculated and MDA concentrations were determined from a standard curve which expressed as μM/mg protein. The standard curve of MDA was showed in appendix G.

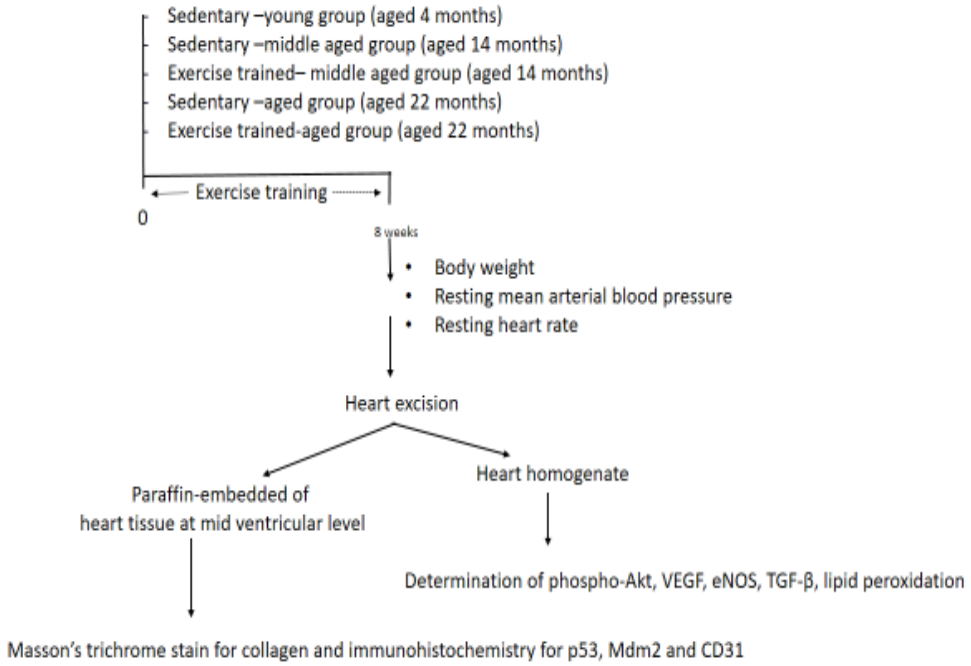


Figure 12 Schematic diagram showing the protocol for study effects of moderate-intensity exercise training on cardiac angiogenic capacity and fibrosis in middle-aged and aged rats

3.3. Statistical analysis

Data were represented as means ± standard error of means (SEM). Comparisons between groups were statistically assessed by one-way analysis of variance (ANOVA), and the differences between means were evaluated by Least significant difference (LSD) test. P-values ≤ 0.05 were statistically significant. Data were analyzed by SPSS 22 for Windows (SPSS Inc., USA)

3.4. Ethical consideration

The experimental protocols were conducted in accordance with the guidelines for experimental animals by National research Council of Thailand, and authorized by the Committee of animal care, Faculty of Medicine, Chulalongkorn University. (MED-ACUC number 023/2561)

CHAPTER IV

RESULTS

4.1 Body weight, heart weight and blood pressure changes

Rat Body weights (BW) were measured every week for 8 weeks and then analyzed by using the equation [2] as shown above. In Table 3 the percentage changes of BW of rats in each different group showed that % BW in ET-Mid-Age and the ET-Age groups significantly decreased than those in sedentary control ($p<0.001$). Heart weight per body weight (HW/BW) of the SE-Age group significantly increased than those in SE-Young and SE-Mid-Age group, and there was no significant difference between exercise training and non-ET in both middle-aged and aged rats. There were significantly increased in systolic blood pressure, diastolic blood pressure and mean arterial blood pressure in SE-Mid-Age and SE-Age. And exercise training seems to express the beneficial effect of decreasing hypertension in ET-Age when compared to SE-Age group ($p<0.001$)

There were no significant differences in heart rate among the groups of SE-Young, SE-Mid-Age, and SE-Age. And only in the ET-Age group significantly decreased than those in SE-Age ($p<0.01$)

Table 3 Percentage change of body weight, heart weight per body weight (HW/BW) and hemodynamic parameters in SE-Young, SE-Mid-Age, ET–Mid-Age, SE-Age and ET- age rats.

	SE-Young (n=8)	SE-Mid-Age (n=8)	ET–Mid-Age (n=8)	SE–Age (n=8)	ET–Age (n=8)
% Change of body weight	17.03±0.92	-5.08±1.37***	-7.83±0.90	-6.78±1.25***	-12.30±0.89***###
Heart weight / Body weight	2.69±0.04	2.60±0.07	2.63±0.06	3.22±0.11***	3.23±0.10
Systolic blood pressure, mmHg	114.16±2.00	124.63±1.09***	122.54±1.80	138.75±0.88***	116.66±1.09###
Diastolic blood pressure, mmHg	90.21±2.21	105.50±2.66***	107.92±4.20	115.42±2.45***	92.88±2.07###
Mean arterial blood pressure, mmHg	98.20±2.00	111.80±1.89***	112.58±3.04	123.18±1.86***	100.79±1.69###
Heart rate, beats/min	358±9.21	348±9.96	349±8.33	355±16.26	305±14.27##

Values are means±SE.

SE-Young = sedentary - young group, SE-Mid-Age = sedentary - middle-aged sham group, ET-Mid-Age = exercise trained – middle-aged group, SE-Age = sedentary - aged sham group and ET-Age = exercise trained – aged group.

* = p<0.05, ** = p<0.01, *** = p<0.001 compared to the SE-young

= p<0.05, ## = p<0.01, ### = p<0.001 compared to the indicated groups without exercise training

4.2 Exercise training effects on lipid peroxidation

Malondialdehyde levels (MDA), the indicator of oxidative stress, in the heart homogenate of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET –Mid-Age rats, and ET-Age rats were showed in Figure 13.

The present data of heart-MDA level supports the idea that oxidative stress significantly increased with increasing age. MDA levels were significantly increased in the SE-Mid-Age (2.32±0.08 μM/mg protein) group and SE-Age group (2.58±0.13 μM/mg protein)

when compared to SE-Young group (1.71±.0.05 μM/mg protein) ($p<0.01$). However, the MDA levels significantly decreased in the ET–Mid-Age group (2.00±0.05 μM/mg protein) and in the ET –Age group (2.23±0.09 μM/mg protein) ($p<0.05$).

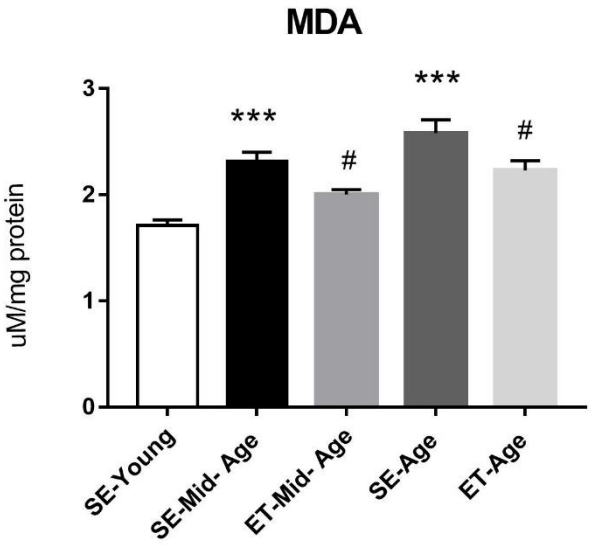


Figure 13 The level of MDA in hearts of SE-Young (n=8), SE-Mid-Age (n=8), ET-Mid-Age (n=8), SE-Age (n=8) and ET-Age (n=8).

Data are expressed as means±SE

** = $p<0.01$, *** = $p<0.001$ compared to the SE-Young

= $p<0.05$ compared to the indicated groups without exercise training

4.3 Exercise training effects on cardiac angiogenic capacity.

4.3.1 VEGF levels

In this study, we determined the protein expression of vascular growth factor levels (VEGF) in the heart homogenate of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET –Mid-Age rats, and ET-Age rats in order to indicate the cardiac angiogenic capacity of each group as showed in Figure 14.

The VEGF levels in hearts were significantly decreased in the SE-Mid-Age group (52.48±2.09 pg/mg protein) and SE-Age group (74.30±3.75 pg/mg protein) when compared to SE-Young group (90.31±5.37 pg/mg protein) ($P<0.001$). However, the VEGF levels significantly increased in the ET-Mid-Age group (62.84±2.16 pg/mg protein) when

compared to SE-Mid-Age group (p<0.05). And VEGF levels significantly increased in the ET-Age group (85.07±3.62 pg/mg protein) when compared to SE-Age group (p<0.05).

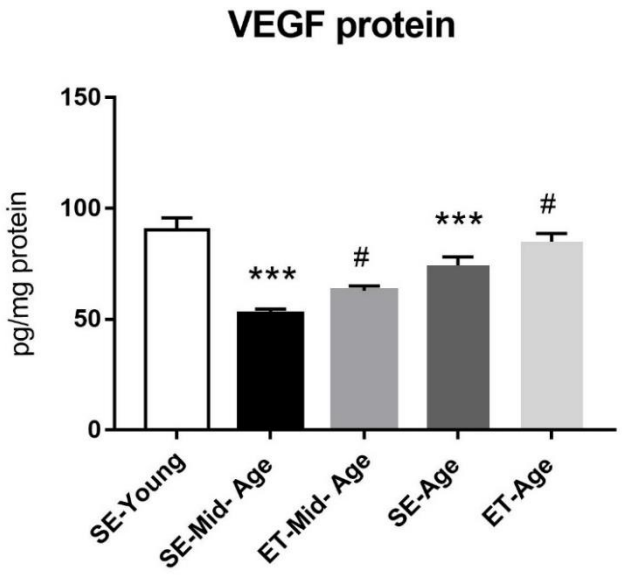


Figure 14 Expression of VEGF protein in hearts of SE-Young (n=8), SE-Mid-Age (n=8), ET–Mid-Age (n=8), SE–Age (n=8) and ET–Age (n=8).

Data are expressed as means±SE

*** = p<0.001 compared to the SE-Young

= p<0.05, ## = p<0.001 compared to the indicated groups without exercise training

4.3.2 Phosphor-Akt 1 levels

In this study, we determined the protein expression of phosphor-Akt 1 in the heart homogenate of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET–Mid-Age rats, and ET-Age rats in order to indicate the cardiac angiogenic capacity of each group as showed in Figure 15

The phosphor-Akt1 levels in hearts were significantly decreased in the SE-Mid-Aged group (3,424.71±173.92 pg/mg protein) and SE-Age rats (3,939.53±219.24 pg/mg protein) when compared to SE–Young group (4,809.04±220.74 pg/mg protein) (p<0.001 and p<0.01, respectively). However, the phosphor-Akt1 levels significantly increased in the ET-Mid-Age group (4,527.64±139.26 pg/mg protein) when compared to SE-Mid-Age group (p<0.01). And the phosphor-Akt1 levels significantly increased in the ET-Age group (4,959.61±268.10 pg/mg protein) when compared to SE-Age group (p<0.01).

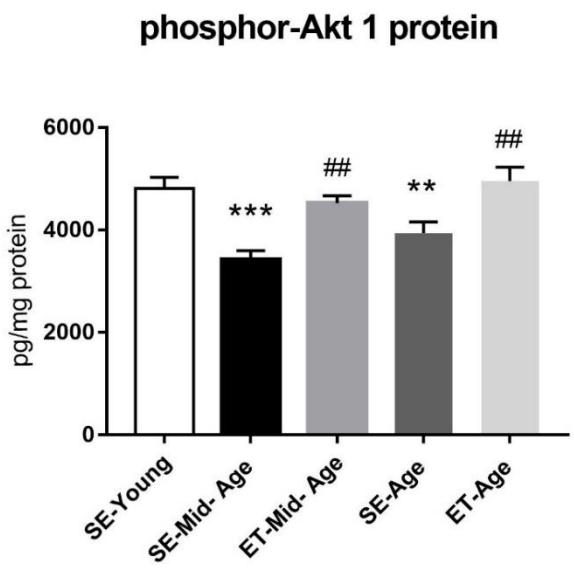


Figure 15 Expression of phosphor Akt1 protein in hearts of SE-Young (n=8), SE–Mid-Age (n=8), ET–Mid-Age (n=8), SE–Age (n=8) and ET–Age (n=8).

Data are expressed as means±SE

** = p<0.01, *** = p<0.001 compared to the SE-Young

= p<0.01 compared to the indicated groups without exercise training

4.3.3 eNOS levels

In this study, we determined the protein expression of eNOS in the heart homogenate of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET –Mid-Age rats, and ET-Age rats in order to indicate the cardiac angiogenic capacity of each group as showed in Figure 16.

The eNOS levels in hearts were significantly decreased in the SE-Mid-Age group (644.27±43.50 pg/mg protein) and SE-Age rats (746.22±49.09 pg/mg protein) when compare to SE–Young group (916.93±31.66 pg/mg protein) (p<0.01 and p<0.05, respectively). However, eNOS levels significantly increased in the ET-Mid-Age group (1080.94±69.42 pg/mg protein) when compared to SE-Mid-Age group (p<0.001). And eNOS levels significantly increased in the ET-Age group (1036.59±61.68 pg/mg protein) when compared to SE-Age group (p<0.001).

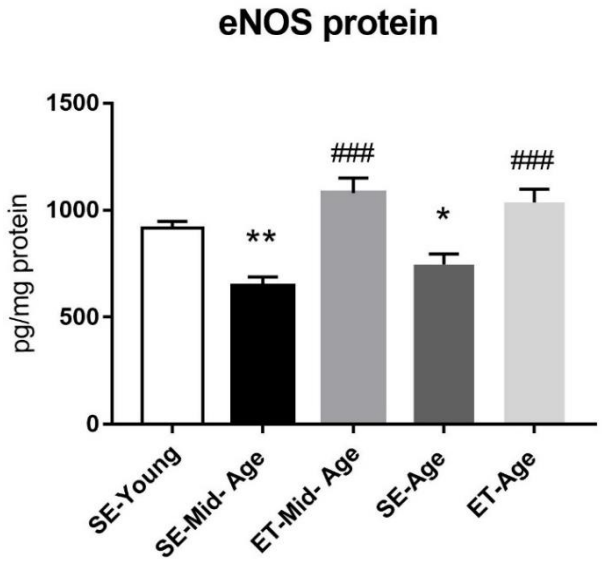


Figure 16 Expression of eNOS protein in hearts of SE-Young (n=8), SE–Mid-Age (n=8), ET– Mid-Age (n=8), SE–Age (n=8) and ET–Age (n=8).

Data are expressed as means±SE

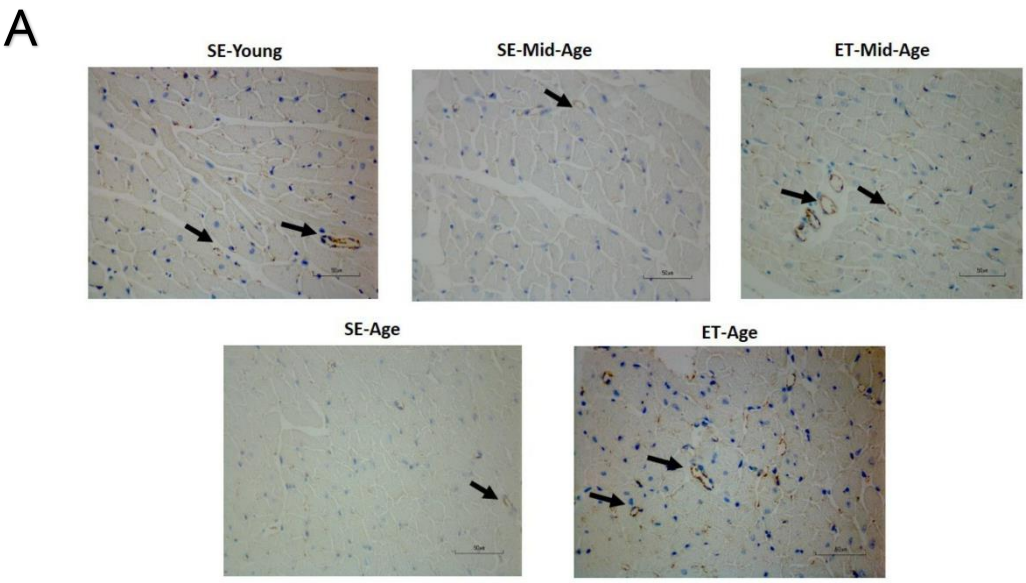
** = $p<0.01$, * = $p<0.05$ compared to the SE-Young

= $p<0.001$ compared to the indicated groups without exercise training

4.3.4 CD31 expression

In this study, we determined the protein expression of CD31 in the heart section of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET–Mid-Age rats, and ET-Age rats in order to indicate the cardiac angiogenic capacity of each group as showed in Figure 17

The expression of CD31 was detected by immunohistochemistry staining. According to Figure 17A, the black arrows pointed at the positive CD31 which was stained in brown color in each group. As in Figure17B, the results were represented by the percentage of CD31 staining. The percentage of positive CD31 in the hearts significantly decreased in SE-Mid-Age group (0.21±0.01 % positive area) and SE-Age group (0.20±0.03 % positive area) when compare to SE-young group (0.47±0.03 % positive area) ($p<0.001$). However, CD31 expression significantly increased in the ET-Mid-Age group (0.33±0.03 % positive area) when compared to SE-Mid-Age ($p<0.01$). And CD31 expression significantly increased in the ET-Age group (0.34±0.02 % positive area) when compared to SE-Age group ($p<0.01$)



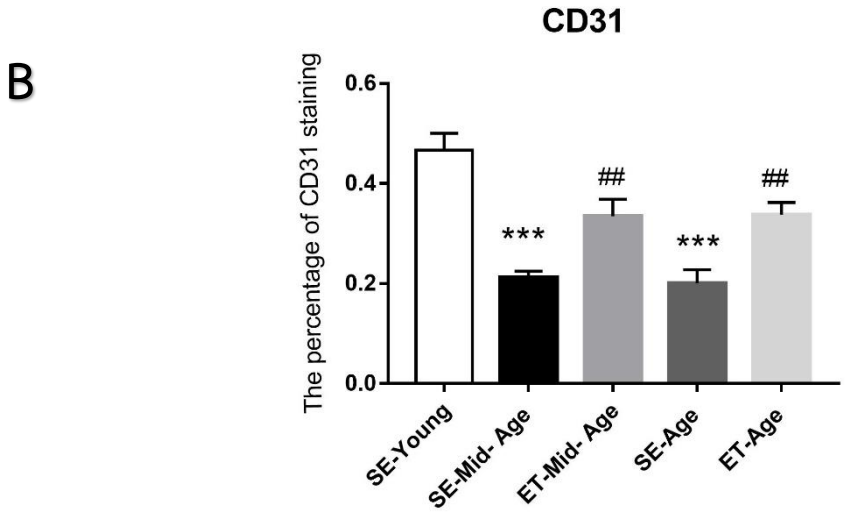


Figure 17 Effect of exercise training on CD31 expression (A) using an immunohistochemistry staining and observed under a light microscope with 400X magnification as indicated by black arrows. The scale bar is 50 µm. (B) The percentage of CD31 staining in the hearts of SE-Young (n=8), SE–Mid-Age (n=8), ET– Mid-Age (n=8), SE–Age (n=8) and ET–Age (n=8).

Data are expressed as means±SE

*** = $p<0.001$ compared to the SE-Young

= $p<0.01$ compared to the indicated groups without exercise training

4.3.5 Mdm2 expression

In this study, we determined the protein expression of Mdm2 in the heart section of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET–Mid-Age rats, and ET-Age rats in order to indicate the cardiac angiogenic capacity of each group as showed in Figure 18

The expression of Mdm2 was detected by immunohistochemistry staining. According to Figure 18A, the black arrows pointed at the positive Mdm2 which was stained in brown color in each group. As in Figure 18B, the results were represented by the percentage of Mdm2 staining. The percentage of positive Mdm2 in the hearts significantly decreased in the SE-Mid-Age group (0.91±0.07 % positive area) and SE-Age group (0.81±0.03 % positive area) when compared to the SE-Young group (1.48±0.23 % positive area) ($p<0.05$ and $p<0.01$, respectively). Interestingly, Mdm2 expression significantly increased in the ET-Age group (1.33±0.14 % positive area) when compared to SE-Age group ($p<0.05$). However, there was no significant for Mdm2 expression in the ET-Mid-Age group (1.10±0.05 % positive area) when compared with SE-Mid-Age group.

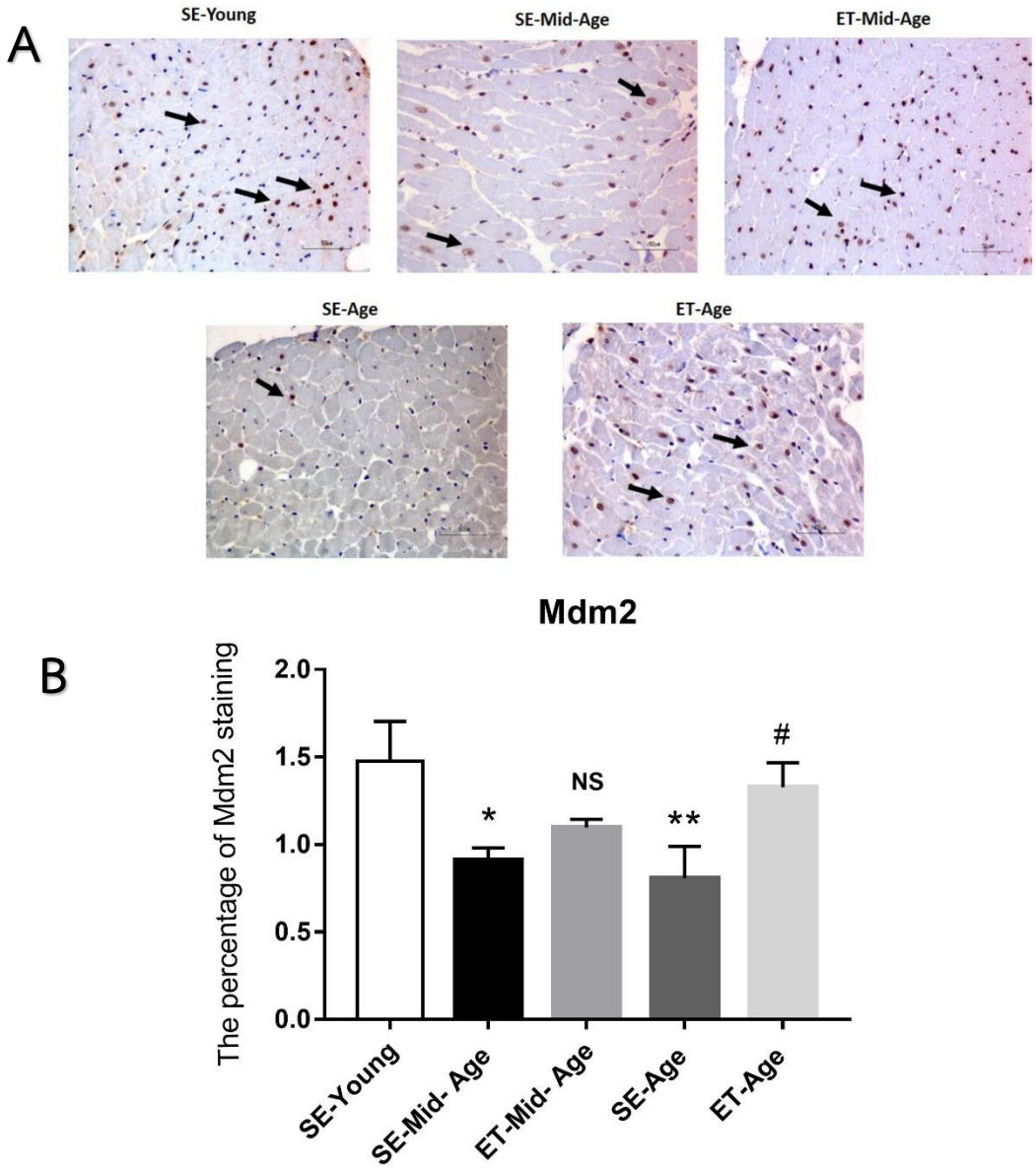


Figure 18 Effect of exercise training on Mdm2 expression (A) using an immunohistochemistry staining and observed under a light microscope with 400X magnification as indicated by black arrows. The scale bar is 50 µm. (B) The percentage of of MDM2 staining in hearts of SE-Young (n=7), SE–Mid-Age (n=8), ET–Mid-Age (n=8), SE–Age (n=8) and ET–Age (n=8). Data are expressed as means±SE

** = $p<0.01$, * = $p<0.05$ compared to the SE-Young

= $p<0.05$ compared to the indicated groups without exercise training

4.3.6 P53 expression

P53 expression in the heart section of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET–Mid-Age rats, and ET-Age rats were showed in Figure 19.

The expression of p53 was detected by immunohistochemistry staining. According to Figure 19A, the black arrows pointed at the positive p53 which was stained in brown color in each group. As in Figure 19B, the results were represented by the percentage of p53 staining. The percentage of positive p53 in the hearts was no significant difference in the SE-Mid-Age group (0.0012±0.0005 % positive area) and SE-Age group (0.0014±0.0004 % positive area) when compared to SE-Young group (0.0004±0.0001 % positive area). Moreover, there were no significant for p53 expression in both of ET-Mid-Age groups (0.0004±0.0003 % positive area) and in the ET-Age groups (0.0006±0.0004 % positive area) when compared to SE-Mid-Age group and SE-Age group, respectively.

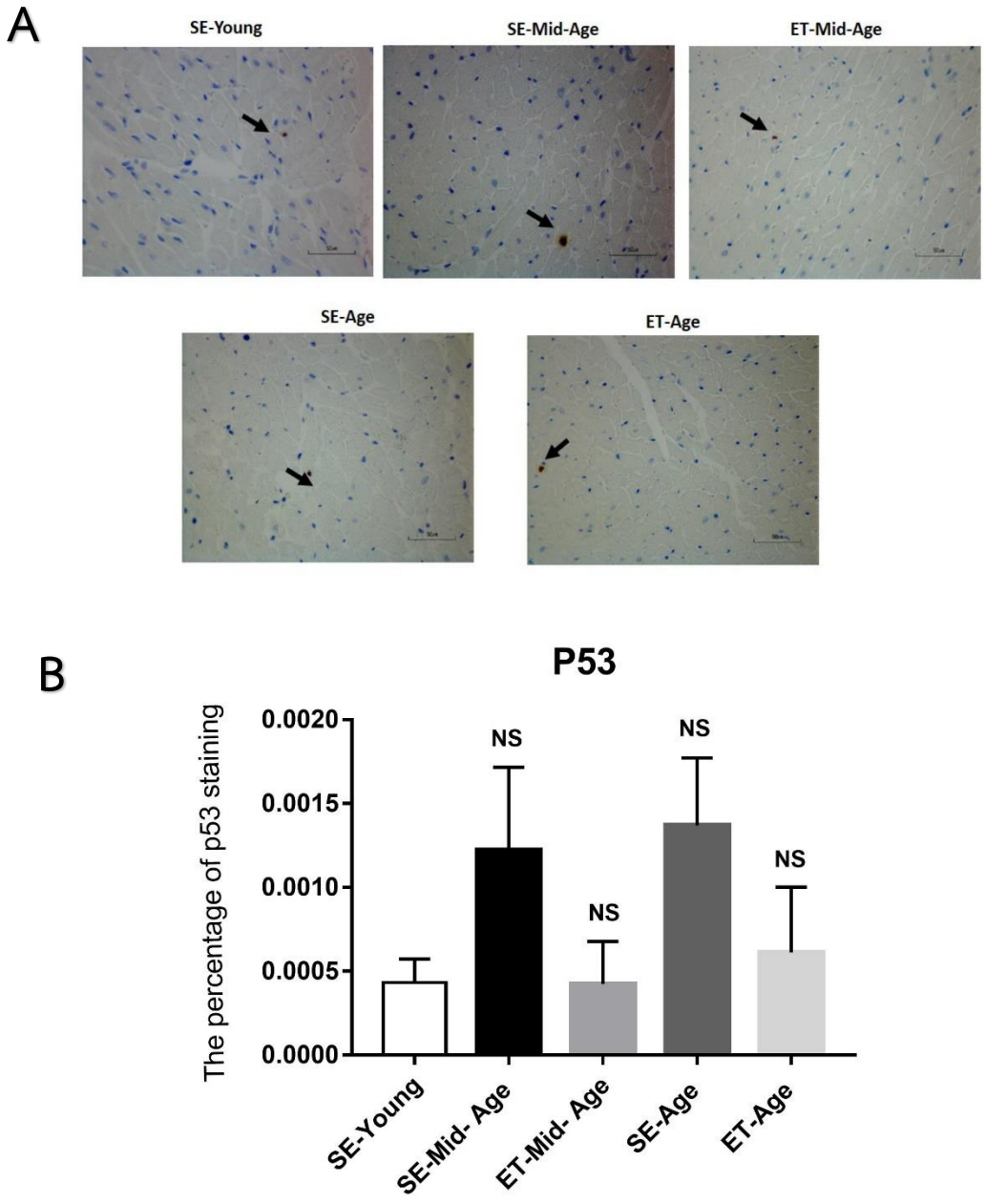


Figure 19 Effect of exercise training on p53 expression (A) using an immunohistochemistry staining and observed under a light microscope with 400X magnification as indicated by black arrows. The scale bar is 50 µm. (B) The percentage of p53 staining in heart of SE-Young (n=7), SE–Mid-Age (n=8), ET– Mid-Age (n=8), SE–Age (n=7) and ET–Age (n=8).

Data are expressed as means±SE.

4.4 Exercise training effects on cardiac fibrosis

Collagen accumulation and TGF-β1 levels in the heart section and heart homogenate of the SE-Young rats, the SE-Mid-Age rats, the SE-Age rats, ET–Mid-Age rats, and ET-Age rats were showed in Figure20 and Figure21, respectively.

Masson'trichrome staining was used to evaluate collagen accumulation. According to Figure 20A, the black arrows pointed at the collagen fiber which was stained in blue color in each group. As in Figure 20B, the results were represented by the percentage of collagen accumulation. The percentage of collagen accumulation in hearts significantly increased in the SE-Age group (6.63±0.77 % positive area) when compared to SE-Young group (2.00±0.09 % positive area) ($p<0.001$). Interestingly, collagen accumulation significantly decreased in the ET-Age group (3.32±0.31 % positive area) when compared with SE-Age group ($p<0.001$). And there was no significant in the ET-Mid-Age group (2.83±0.16 % positive area) when compared to SE-Mid-Age group. (2.77±0.20 % positive area).

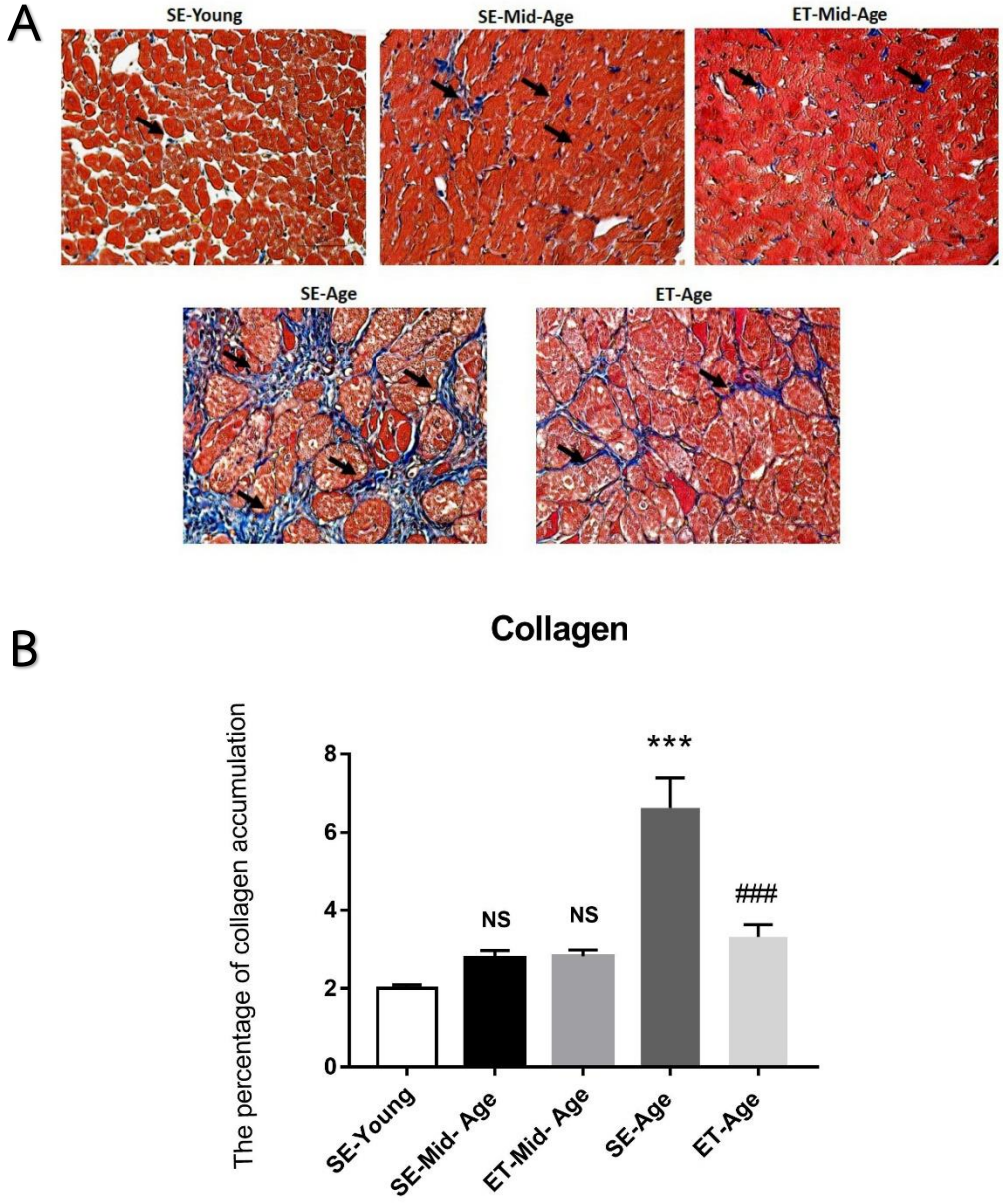


Figure 20 Effect of exercise training on collagen accumulation (A) using a Masson trichrome staining and observed under a light microscope with 400X magnification as indicated by black arrows. The scale bar is 50 µm. (B) The percentage of collagen accumulation in the hearts of SE-Young (n=8), SE-Mid-Age (n=8), ET–Mid-Age (n=8), SE–Age (n=8) and ET–Age (n=8).

Data are expressed as means±SE

*** = P<0.001 compared to the SE-Young

= P<0.001 compared to the indicated groups without exercise training

We also investigated the profibrotic mediator, TGF-β1. The TGF-β1 levels in hearts were significantly increased in the heart of the SE-Age group (26.41±3.80 pg/mg protein) when compared to SE-Young group (12.47±0.97 pg/mg protein) ($p<0.001$). Interestingly, TGF-β1 levels significantly decreased in the ET-Age group (14.28±1.90 pg/mg protein) when compared to SE-Age group ($p<0.001$). And there was no significant in the ET-Mid-Age group (7.89±0.43 pg/mg protein) when compared to SE-Mid-Age group (11.89±1.32 pg/mg protein) (Figure 21).

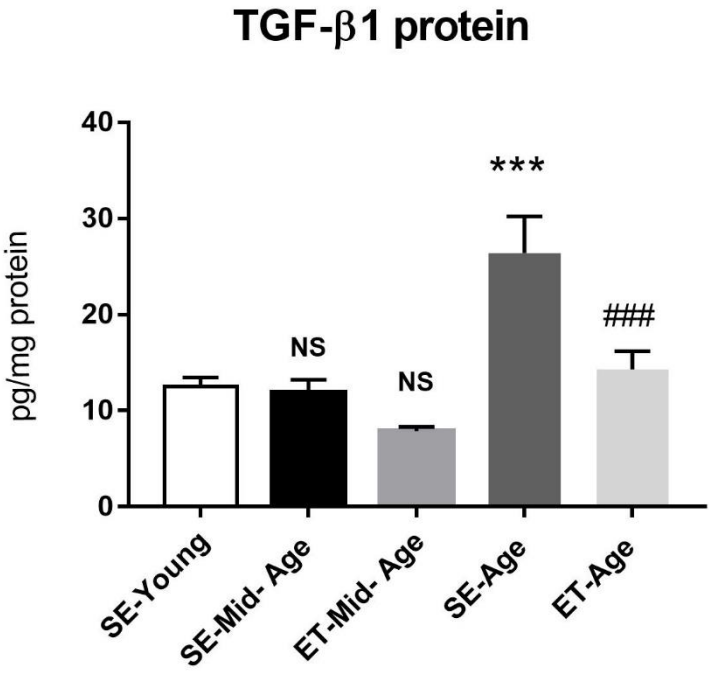


Figure 21 Expression of TGF-β1 in the hearts of SE-Young (n=8), SE–Mid-Age (n=8), ET–Mid-Age (n=8), SE-Age (n=8) and ET–Age (n=8). Data are expressed as means±S

*** = P<0.001 compared to the SE-Young

= P<0.001 compared to the indicated groups without exercise training

CHAPTER V

DISCUSSIONS

Age-induced changes of body weight and heart weight and effects of exercise training

Aging was associated with increases in total adiposity both in humans and rodents[153]. The aging process causes several changes in body composition, without often concomitant change in body weight and body mass index (BMI). Generally, the increase of body fat percentage and decrease of lean mass and bone mineral density happen at individual age. In the abdominal region, which is related to cardiovascular disease and diabetes, the elevation in fat mass (FM) has distribution more specifically[154]. Furthermore, the alteration in energy balance is associated to the alteration in body composition, such as a positive energy balance leads to weight gain while negative balance leads to weigh loss[154]. Basal metabolic rate (BMR), as refers as the metabolic rate during rest, reduces with advancing age and is attributed to alteration in muscle and organ weights[155]. From the Payne and Dugdale model[156] showed the four compartment, which separated into different tissue metabolic types, was developed to simulate BMR. The fast lean consisted of the metabolically active organ, including heart, brain and the slow lean tissue refers to muscle (Figure 22).

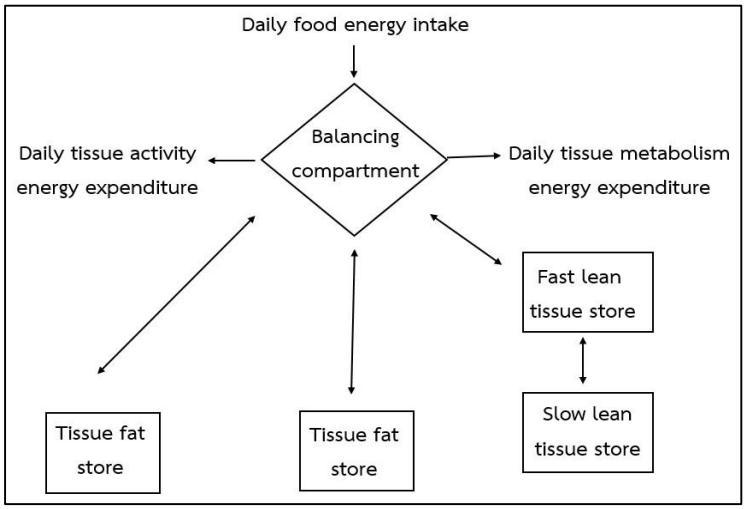


Figure 22 Payne and Dugdale model for energy regulation (Modified from Henry J (2000)[155])

Although the muscle mass is the largest component which make up about 40% of body weight, there is represented BMR only 20%. While major organs including heart, brain, which make up about 4% of body weight, have represent approximately 80% of BMR. Therefore, the increment of heart size with aging may influence the observed increase in BMR[155]. Corresponding to our results showed that HW/BW in SE-Age and ET-Age were significantly increased than SE-Young and SE-Mid-Age groups, suggesting that the increment of heart activity in aging induce heart size enlargement.

Moreover, a sedentary life-style tend to have a major risk for weight gain[157]. Exercise training reduced body mass in aging rat mediated through enhancing fat oxidation[158]. Current study, we found exercise training reduced the percentage change of body weight between the SE-Age (-6.78±1.25%) and ET-Age (-12.30±0.89 %) ($p<0.001$), suggesting that swimming training program has a beneficial effect in aging. However, exercise training did not change the % change of body weight between the SE-Mid-Age (-5.08±1.37 %) and the ET-Mid-Age (-7.83±0.90) (Table 3). These results may be associated with the old age has a less metabolic rate, leading to the accumulation of fat mass while middle age has a greater metabolic rate than age group. Consequently, the fat mass in aging larger than in middle age, resulting in exercise effects has clearly in the age group.

Persistent hypertension changes the structure and compromises the function of the cardiac muscle. The pathophysiology is initiated by pressure overload resulting in left ventricle concentric hypertrophy for normalizes systolic wall stress, and then the cardiac function is preserved. This adaptation leads to a remodeling of structural cardiac muscle, including cardiomyocyte loss and cardiac hypertrophy. The compensatory response is irreversible followed by an alteration to heart failure and progressive contractile dysfunction [52, 159]. Our results demonstrated that heart weight per body weight (HW/BW) in SE-Age and ET-Age were significantly increased than SE-Young and SE-Mid-Age groups, suggesting that age-induced cardiac hypertrophy occurred. As we know that "cardiac hypertrophy" is the abnormal enlargement, or thickening, of the heart muscle, resulting from increases in cardiomyocyte size and changes in other heart muscle components, such as

extracellular matrix. As mention above, the age-induced persistent hypertension changes the structure and compromises the function of the cardiac muscle. Exercise training induces physiologic hypertrophy, which increase by approximately 7% in cell length [(132)]. Lengthening of cardiomyocytes induced cavity of left ventricular enlargement and increased wall thickness. These changes are a good correlation between diastole function and aerobic fitness[(135)]. In animals laboratory, chronic exercise training increases hypertrophy at both the organ and cellular level[(136)]. Moreover, cardiac hypertrophy is distributed across the walls of the left ventricular myocardium. Consistent with the previous study had shown swimming exercise increased left ventricular weight and left ventricular internal diastolic diameter in the spontaneously hypertensive rats[(137)]. Our results also showed a significant increase in the HW/BW of the trained-aged group as a result of the swimming training program.

Moreover, we also observed the angiogenic capacity was significantly decreased in SE-Mid-Age and SE-Age rats when compared to SE–Young rats. Interestingly, there was a tendency of VEGF, in the SE-Age group higher than in the SE-Mid-Age group ($p<0.05$), although VEGF should decrease with increasing age[(17)]. It was suggested that the augmentation of angiogenesis is only observed in the early stages of cardiac hypertrophy, whereas continues exposure to hypoxia was shown to leads to progressive hypertrophy without additional angiogenesis.[(160)]. Therefore, cardiac hypertrophy requires more oxygen to dissolve hypoxic situations and to maintain cardiac contractile dysfunction by increase myocardial angiogenesis. These responses are related to cardiac myocyte and amount of microvasculature[(7)]. On the other hand, augmentation of cardiac VEGF-A expression has been found in ischemic cardiomyopathy patients[(161, 162)]. Furthermore, serum VEGF-A levels were significantly increased in dilated cardiomyopathy patients[(163)]. Hypoxia is one of the potent stimulators for VEGF expression. Under the hypoxic condition, HIF-1α activates inducible nitric oxide synthase (iNOS) which is the downstream genes of VEGF in cardiomyocytes and endothelial cells, resulting in an increase in angiogenesis and cardioprotection[(164, 165)]. Therefore, it could be assumed that the upregulation of

VEGF-A as an adaptive mechanism to improve vascularization and contractility of the failing heart muscle before the degeneration of the aging heart [166].

Our results also showed that there was no significant difference of HW/BW between SE-Young, SE-Mid-Age, and ET-Mid-Age, although angiogenic capacity, especially CD31 levels were reduced in SE-Mid-Age when compared to SE-Young, suggesting that the Mid-Age is early aged could worsen endothelial cells and reduce angiogenic capacity without hypertrophy. However, there was a tendency of exercise training to reduce blood pressure in this group (Table 3). Moreover, the level of angiogenic capacity also increased after exercise training. Therefore, exercise training in middle-aged should be recommended.

Age-induced oxidative stress and effects of exercise training

Our findings also demonstrated that lipid peroxidation, MDA, was significantly increased with increasing age (Figure 13). MDA levels were significantly increased in SE-Age and SE-Mid-Age than in SE-Young ($p<0.001$). Similarly, the previous study demonstrated that the elevation of ROS and impairment of antioxidants were found in the aging hearts[38]. Luceri C et al (2017) reported that the increment of ROS during aging occurs already in middle age rats of 15 months, resulting in an increased amount of protein carbonyls in plasma[167]. It was also suggested that oxidative stress could be consequently caused by decreased peripheral vascular compliance and augmented afterload leads to increase oxygen consumption, and energy deficits[45, 146, 168]. Oxidative stress is a term that is an imbalance between oxidant and antioxidant due to enhancing of production free radical or reducing of antioxidant. In a normal cell, the antioxidant defense mechanism can eliminate them by using an antioxidant enzyme. However, the dysregulation of the antioxidant defense system or excessive generation of free radicals leads to damage biomolecules such as carbohydrate, lipid, protein, and nucleic acid. Lipid is a basic element of the membrane that selectively lets ions and organic molecules pass and thus to control the movement of substances in and out of cells. Moreover, cell membranes are in the involvement of several cellular processes such as cell signaling, and cell adhesion [169]. Therefore, the dysfunction of the cell membrane leads to significant effect.

Although acute exercise in aging showed the elevation of ROS level, exercise training reduced lipid peroxidation[170]. Exercise training increased capacity for antioxidant defense, including increased free radical scavenging enzyme and mitochondrial oxidative enzymes, in both young and old rats[171]. In response to moderate exercise training, nuclear NRF2 protein was significantly increase in the heart of both young and old mice, suggesting that exercise training can stabilize NRF2[37]. This process was related to the reduction of VEGF /PI3K/Akt and the NRF2-dependent antioxidant system in aging [38, 172]. Likewise, our study showed that the level of MDA was significantly decreased after exercise training ($p<0.05$), indicating the decrease in oxidative damage. This MDA result are consistent with the previous study[173]. We demonstrated that exercise training effects on the positive correlation between MDA levels and tissue capillary vascularity in aging animals[173]. This may suggest that the alterations of cardiac angiogenic capacity in the middle-aged and aged animals by exercise training partly related oxidative stress reduction in the hearts.

Age-induced hypertension and effects of exercise training

The present study demonstrated that aging rats tended to developing hypertension as shown in Table 3. Our results showed that there was a significant difference in SBP, DBP, and MAP between SE-Young, SE-Mid-Age and SE-Age. As shown in the previous studies they indicated the prevalence of hypertension increase with age. Moreover, hypertension-associated cardiovascular disease was found in the aged than in the young [168, 174, 175]. Several studies have described the pathophysiology of this age-induced hypertension lies in that age-induced endothelial dysfunction and subsequent impaired NO bioavailability, thus promoting the pathogenesis of hypertension and cardiovascular disease. ROS is the leading factor of aging-associated NO bioavailability impairment and endothelial dysfunction [14]. The augmentation of ROS induces uncouple the eNOS-catalyzed reduction of molecular oxygen from the oxidation of L-arginine, resulting in the paradoxical production of the superoxide anion instead of NO[14, 97]. Peroxynitrite is cytotoxic and may cause oxidative damage to proteins, lipids, and DNA [176]. Recently, it has been indicated that $ONOO^-$ have deleterious effects on the activity and function of prostacyclin synthase[177] and

eNOS[178]. Several evidences support the role of these pathways in the development and the progression of arterial hypertension[97, 179]

It is well known that endothelium-derived NO plays a significant the protective effects of the cardiovascular system by regulating cell division and survival, platelet aggregation, inflammatory cell adhesion, pro-inflammatory signaling pathways, mitochondrial function and cellular energy metabolism[14, 180]. An age-associated increase in central arterial wall stiffness, which characterization of pulse wave velocity increment, is causing factor of extracellular matrix (ECM) remodeling, including enhanced collagen content and crosslinking elastin fragmentation, and vascular calcification[175, 180]. In this study, SBP, DBP, and MAP significantly up-regulated in the SE-Age group when compared to the SE-Young group ($p<0.001$). The positive staining of CD31 in the SE-Age groups was significantly lower when compared to the SE-Young group ($p<0.001$), suggesting that the age-induced worsen of endothelial cells which related to abnormal of the coronary arteriolar wall.

The present study also showed SBP, DBP and MAP significantly increased in the SE-Mid-Age group when compared to the SE-Young group ($p<0.001$). The positive staining CD31 in the SE-Mid-Age groups was significantly lower when compared to the SE-Young group ($p<0.001$), suggesting that the alteration of arterial blood pressure was linked to endothelial cells as well as in SE-Age group. Similarly, a previous study, hypertension was found in the middle-aged and young populations[181]. Because of these sub-populations, insulin resistance and overproduction of adipokines impair endothelial and heart function resulting in accelerated cardiovascular aging[181].

Our results also showed that there was significant effect of exercise training to prevent developing hypertension in the ET-Age group ($p<0.001$). Moderate exercise training reduced oxidative damage in aging and this provides a protective mechanism in the cardiovascular system through the induction of antioxidant enzymes such as catalase (CAT), Glutathione reductase (GSR) and increasing capillary growth[38]. Moreover, exercise training increased shear stress that restored NO bioavailability and reduced blood pressure by increasing arterial distensibility, arterial compliance and decrease sympathetic tone [182]. As well as in our study, exercise training could reduce

MDA, increase eNOS protein expression, resulting in the improvement of NO bioavailability and balancing of vasodilator and vasoconstrictor.

Age-induced the reduction of angiogenic capacity and effects of exercise training

Because of our results of angiogenic capacity have many parameters, therefore we would like to summarize by table below-:

	SE-Young	SE-Mid-Age	ET-Mid-Age	SE-Age	ET-Age
VEGF (pg/mg)	90.31±.5.37	52.48±2.09*** ⬇	62.84±2.16# ⇧	74.30±3.75*** ⬇	85.07±3.62# ⇧
p-AKT (pg/mg)	4809.04±.220.74	3424.71±173.92*** ⬇	4527.64±139.26## ⇧	3939.53±219.24*** ⬇	4959.61±268.10## ⇧
eNOS (pg/mg)	916.93±.31.66	644.27±43.50** ⬇	1080.94±69.42### ⇧	746.22±49.09* ⬇	1036.59±61.68### ⇧
% CD31	0.47±0.03	0.21±0.01*** ⬇	0.33±0.03## ⇧	0.20±0.03*** ⬇	0.34±0.02## ⇧
% MDM2	1.48±0.23	0.91±0.07* ⬇	0.81±0.03	1.10±0.05** ⬇	1.33±0.14# ⇧
% P53	0.0004±0.0001	0.0012±0.0005	0.0004±0.0003	0.0014±0.0004	0.0006±0.0004

* = P<0.05, ** = P<0.01, *** = P<0.001 compared to the SE-young

= P<0.05, ## = P<0.01, ### = P<0.001 compared to the indicated groups without exercise training

An age-induced downregulation of cardiac angiogenesis capacity as described by VEGF, eNOS, p-Akt, Mdm2, and CD31 in SE-Mid-Age and SE-Age groups were indicated in our results (Figure 14-18). PI3K/Akt/eNOS signaling was found to decrease in the hearts of SE-Mid-Age and SE-Age rats compared to SE-Young rats. The decline of PI3K/Akt signaling was also observed in the brain[96, 183] and skeletal of aging animals[184]. In hepatocytes of the aging rat, the impairment of Akt phosphorylation was remediated by alpha-lipoic acid through PI3K, PTEN, and PP2A[185]. Aging is associated with endothelial dysfunction and NO bioavailability impairment, these factors contribute to angiogenic processes decrease and microvascular rarefaction[186]. The previous study showed that the impairment PI3K/Akt/NO signaling related to endothelial dysfunction in arteries skeletal muscle of aged animals[110] and uncoupling eNOS increment associated with ROS production was observed in ECs of aging[32]. The loss of NO

bioavailability characterized ECs dysfunction leads to impairment of VSMCs[30]. The NO bioavailability impairment in aging is probably exacerbated by dysregulation of eNOS expression,[76, 187]reducing the availability of tetrahydrobiopterin[188] and/or decreasing intracellular L-arginine availability[189]. These factors are contributed to the impairment of vascular permeability and angiogenesis process. Moreover, ECs dysfunction also increases the incidence of atherosclerosis, hypertension, and stroke[74]. The underlying mechanisms of age-associated endothelial dysfunction involve increased oxidative stress and alterations in molecular pathways impacting common aging processes[14].

An augmentation of NAD(P)H oxidase (NOX) activity and mitochondrial oxidative stress are related to endothelial dysfunction in age coronary arteries[187]. Previous studies revealed that up-regulation of endothelial NOX and mitochondria-derived ROS in the aging vasculature may involve the accumulation of pro-inflammatory mediators[190] and/or increased renin-angiotensin activity[191], dysregulation of the electron transport chain[192], and impairment of antioxidant enzymes[193]. CD31 expression, also known as PECAM-1 was widely used as a way to understand its distribution[194]. Our results demonstrated the positive staining CD31 in the SE-Mid-Age and SE-Age groups was significantly lower when compared to the SE-Young group. Moreover, MDA levels were increased in SE-Age and SE-Mid-Age than in SE-Young, suggesting that age-induced worse endothelial cells are corresponding with ROS production. This result consistency with other studies that showed ROS-induced eNOS impairment in vascular age by promoting ECs dysfunction in both aged adults[195] and older laboratory animals[187].

VEGF is a main proangiogenic factor that is up-regulated in response to hypoxia to restore tissue vascularization. VEGF is also critically involved in the formation of the blood vessel in physiologic and pathologic process [196]. Age-induced the reduction of VEGF, VEGFR, and eNOS in the cerebral vessel[146] and in cardiac myocyte[15]. Similarly, our results demonstrated that VEGF levels in the SE-Mid-Age and SE-Age groups were significantly decreased compared to the SE-Young group. The previous report showed that VEGF-mediated signaling was essential for ECs survival in some tracheal capillaries. Because the ECs should begin to undergo apoptosis soon after the onset of VEGF inhibitor treatment[197]. Furthermore, eNOS plays a pivotal role

in VEGF-induced angiogenesis and vascular permeability[198]. A previous study showed that VEGF induces NO production mediated through eNOS phosphorylation at serine 1177 residual by Akt[31]. This phosphorylation is essential for VEGF-induced endothelial cell migration[31]. In our study, the VEGF levels seem to increase in SE-Age than those in SE-Mid-Age, even though the percentage of CD31 staining still declined. It assumed that the dysfunction responsiveness of VEGF inducer contributes to the age-related impairment of endothelial angiogenic capacity. Likewise, the previous study reported that in primary microvascular endothelial cells derived from aged rodents, there was a decreased angiogenic response to exogenous VEGF administration, indicating that endothelial cells turn into resistant to inducers of angiogenesis with aged rodents[14]. Moreover, the results of this study were somewhat consistent with the previous study. They demonstrated that aging impairs the intrinsic angiogenic capacity of cerebromicrovascular endothelial cells (CMVECs), indicating by the disruption of autocrine pituitary adenylate cyclase-activating polypeptide (PACAP) signaling.[199]

Regular exercise training especially, endurance exercise improves cardiac function in both young and senior subjects. The beneficial effects of exercise training in angiogenesis were observed in several studies[39, 44]. Hassan AF and Kamal MM[39] showed that exercise training-induced increases of capillary density that lead to increases in oxygen supply and energy substances for the aging heart. Exercise training also induces coronary vascular adaptations which lead to structural alterations in the size and number of the blood vessels[44] Moreover, Iemitsu *et al*[17] showed exercise training restored the age-caused downregulation of VEGF, eNOS, and p-Akt protein in the hearts. Our study demonstrated that exercise training restored the reduction of p-Akt1, eNOS, and Mdm2 in the rat hearts, and these results are in line with the change of VEGF and CD31 levels. Exercise training did not only enhance VEGF and downstream signaling through Akt and eNOS pathway for cardiac angiogenesis processes but also preserved endothelial cells, indicated by the percentages of positive CD31 in both exercise training groups were significantly increased compared with sedentary aged control. The mechanosensory complex at endothelial cell-cell junctions which were composed of PECAM-1 and VEGFR was capable of fluid shear stress detection[96]. Shear stress-induced mechano-transduction leads to activate PI3/AkT/eNOS activity.

eNOS activates NO from L-arginine and BH4 cofactor, which then regulates vasodilation and angiogenesis process[14]. Shear-stress is defined as blood flow that has vector paralleled to the long axis of the blood vessel wall[200]. Moderate exercise can induce shear stress which is one of the most important mechanisms to improve vascular function via PI3/Akt/eNOS activity and NO synthesis[14]. Our study, swimming training showed beneficial effects for improving vascular function associated with the enhancement of molecular mechanisms as indicated by PI3/Akt/eNOS activity induced shear stress.

Mdm2 is a negative regulator of p53 and required for capillary maintenance in exercise stimulation[42]. The present study showed the percentage of Mdm2 significantly decreased in the SE-Age group when compared to the SE-Young group ($p<0.01$) and the angiogenic capacity in the SE- Age groups was significantly lower when compared to the SE-Young group ($p<0.05$). Mdm2 has several p53 independent effects; for example, Mdm2 has a number of p53 independent effects. For example, Mdm2 has p53 independent transcription factor-like effects in nuclear factor-kappa beta (NF-ƙB) activation, and Mdm2 can promote tissue inflammation and has adverse implications in experimental autoimmune disorder [201]. Moreover, capillary staining of muscle sections from $Mdm2^{puro/\Delta 7-9}$ sedentary mice with wild-type or knockout background for p53 showed that the depletion in Mdm2 resulted in 20% capillary regression independently of p53 status[42]. Although in our study also showed no significant difference of p53 between SE-Young and SE-Age group, it should be investigated the ratio both of phosphor-Mdm2 and p53 per total Mdm2 /or p53 in heart tissue in the future.

Our results showed the Mdm2 levels in aging rats following exercise training have shown a significantly increased in its expression and correlated with angiogenic capacity in heart tissue compared to the SE-Age group ($p<0.05$) indicating exercise-induced Mdm2 expression plays an important role in cardiac angiogenesis even to the slight non-significant reduction of p53 expression. This significant increase in Mdm2 versus non-significant reduction in p53 may suggest that exercise training may also interfere with the increment of Mdm2 through other pathways that ultimately improved the cardiac angiogenesis in aging rats. This result showed the same trend

with previous study of Emilia *et al*[42] showing the mechanism responsible for capillary regression in Mdm2$^{puro/\Delta 7\text{-}9}$ mice is p53 independent. Moreover, they revealed the compensatory mechanism in the transgenic animal model that the decreased capillarization in Mdm2$^{puro/\Delta 7\text{-}9}$ mice lead to hypoxia, which then causes stabilization of HIF-1α and increases VEGF-A. Furthermore, Mdm2$^{puro/\Delta 7\text{-}9}$ also increased antiangiogenic TSP-1and FOXO1. Thus, Mdm2 is a critical regulator of skeletal muscle angioadaptation, exerting various and complex roles on both sides of the angioadaptive balance. However, we cannot exclude the possibility of cardiac degeneration in the aging rat that was insufficient to change in the molecular of p53 mechanism. Unlike the increment of p53 in cardiovascular disease[18, 19]. Pathological hypertrophy enhanced degradation of HIF-1α through p53-mediated recruitment of the Mdm2, resulting downstream angiogenic events[202]. The metabolic or energy stress can induce Mdm2 phosphorylation on residue serine 166[203] to increase stability of Mdm2 protein[204]. Furthermore, shear stress could induce Mdm2 in ECs of skeletal muscle[205]. They reported that Mdm2 phosphorylation on Ser166 that is increased by shear stress and VEGF-A induced EC migration via binds Fox O1 and lead to decrease p27, TSP-1 and Sprouty-2[205, 206] Therefore, we suggested that several exercise-induced stimuli, such as metabolic, mechanical or hemodynamic stresses could induce Mdm2 expression and Mdm2 bind to other binding partners, except p53 might be important to induce cardiac angiogenesis. However, our study showed no significant change in Mdm2 levels after exercise training in ET-Mid-Age group (Figure 18), they also did not show significant change of p53 (Figure 19). Although, the percentage of Mdm2 in SE-Mid-Age was significantly reduced in SE-Mid-Age when compared to SE-Young ($p<0.05$). Therefore, neither enhance Mdm2 or decreased p53 expression can explain the increment of angiogenic capacity observed in ET-Mid-Age. However, the mechanism of Mdm2 and p53 should be investigated in the future.

Age-induced cardiac fibrosis and effects of exercise training

Cardiac fibrosis and remodeling are related to the reduction of collagen degradation, an increment of collagen synthesis, and proliferation of fibroblasts. Aging alters the connective tissue geometry due to extent collagen fibrils diameter,

and reduction of collagenous myocyte sheaths linearity [10]. Our results demonstrated the percentage of collagen accumulation was significantly increased in SE-Age than in SE-Young ($p<0.001$). However, exercise training increases the linearity of the collagen network by reducing the internal work of the aging heart leading to reduce metabolic and blood flow requirements, reducing fibrosis, alleviating arrhythmias, suppressing excess apoptosis and leading to improve cardiac function[10]. In this study, we found exercise training decreased or reversed collagen accumulation as evidenced by Masson's trichrome staining. Moreover, exercise training improved the age-induced increased of collagen accumulation in the heart, especially in aged hearts, and these corresponded to the changes in the TGF-β1. These results are consistent with the result of other studies. The previous study showed that exercise training in aging can decrease connective tissue accumulation including collagen content [90, 144]. Twelve weeks of exercise training reduced TGF-β, an upstream regulator of TIMP, resulting in a dysregulation of MMPs and a reduction of cardiac fibrosis [10]. Moreover, swimming training demonstrated beneficial effects by reducing uPA and MMP2 expression and led to reduced cardiac fibrosis[45].

Cardiac fibroblasts are involved in cardiac homeostasis by contributing to extracellular matrix (ECM) synthesis deposition. In addition, fibroblasts are significantly in heart healing after MI and in pathogenesis of heart fibrosis[207]. Activated fibroblasts are differentiate into contractile myofibroblasts in response to TGF-β. The fibroblast's phenotype alters and its ability to mature into a functional myofibroblast determine the efficiency of healing after MI, which is compromised in the aging heart[208]. Overpopulation of myofibroblasts interrupts myocyte coupling and conduction process leading to cardiacmyocyte stiffness[209] and uncontrolled fibrosis[210-212] which cause pathological ventricular remodeling, hypertrophy, arrhythmia, and even heart failure[213]. Our results showed collagen accumulation and cardiac hypertrophy which occurred only in SE-Age but not in SE-Mid-Age and exercise training can reduce collagen accumulation in ET-Age, suggesting that SE-Mid-Age had not shown the aging phenotype and the exercise training should be recommended.

Active TGF-β modulated gene expression and fibroblast phenotype can reduce matrix degradation and increasing collagen and fibronectin synthesis, thus

enhancing ECM deposition in the myocardium infarction[214]. TGF-β-induced CTGF may enhance hypertrophy of cardiomyocyte in the cardiac remodeling, while promoting fibrosis through interaction with TGF-β[215]. TGF-β also collaborates with the renin-angiotensin system (RAS) to encourage fibroblast proliferation, cardiomyocyte hypertrophy, and ECM protein expression in cardiac remodeling[160, 216]. Moreover, TGF-β plays a pivotal role in the hypertrophic and dilative ventricular remodeling pathogenesis after cardiomyocyte stimulating and interstitial fibrosis-inducing[160, 214]. Our results showed cardiac hypertrophy and collagen accumulation in aging rats and these results correlated to TGF-β levels, suggesting that TGF-β may partly related to hypertrophy and fibrosis. It has been shown that TGF-β can increase ROS production and suppress the antioxidant system leading to imbalanced redox[217]. The redox imbalance brings TGF-β an important role in fibrosis pathogenesis. ROS exerts its function through mediating TGF-β-induced profibrotic effects. TGF-β mediated fibrosis mechanisms include activation of resident fibroblast, stimulation of apoptosis in epithelial and endothelial cells, induction of epithelial-or endothelial-mesenchyme transition, production of ECM matrix proteins, and suppressing ECM degradation[49]. As a latent complex with its prodomain, TGF-β is stored in the extracellular matrix. Activation of TGF-β requires ROS to cleave LAP and release active TGF-β [49]. As mention above, exercise training can reduce ROS, suggesting exercise training may reduce TGF-β via ROS reduction (Figure 23).

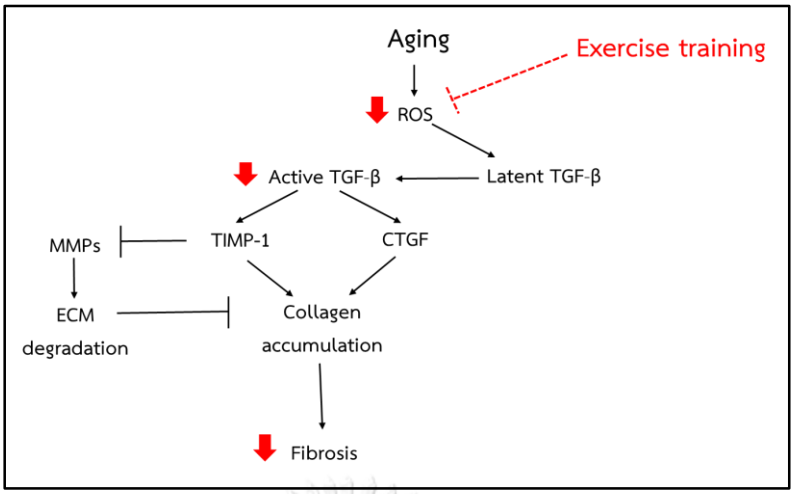


Figure 23 Exercise training reduced TGF-β via ROS reduction, leading to declined cardiac fibrosis

Our study also showed the positive correlation between MDA and TGF-β (r = 0.423, p = 0.007), indicating that ROS and TGF-β might be a significant mediator of exercise training protection against fibrosis as result of aging (Figure 24). Therefore, the potency of exercise training in diminishing TGF-β1 protein expression has important clinical relevance.

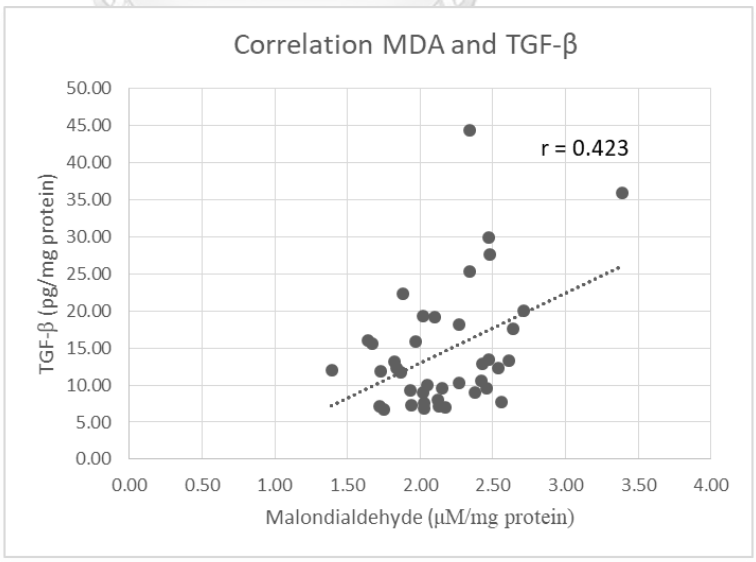


Figure 24 The correlation between MDA and TGF-β (r = 0.0423, p value = 0.007)

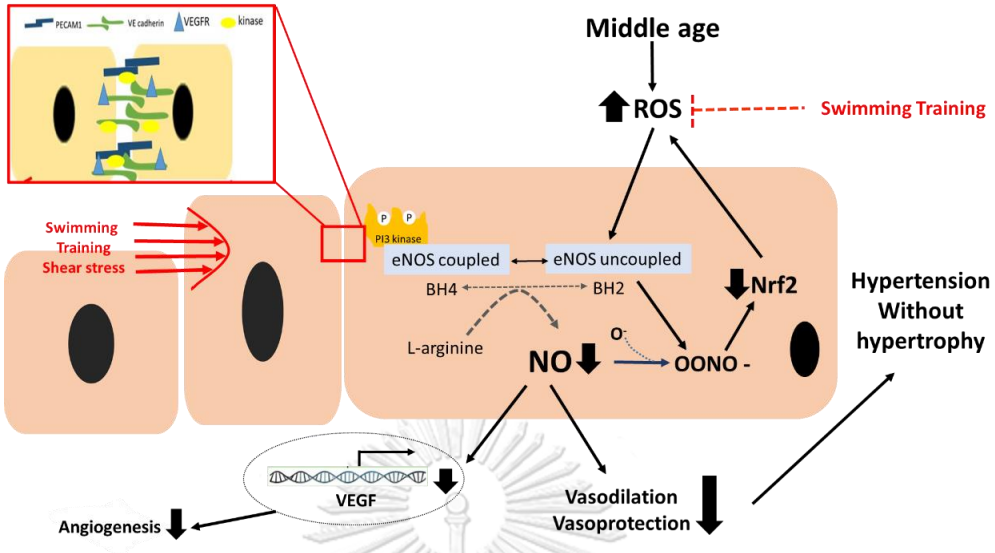


Figure 25 The proposed mechanism in middle-aged

The proposed mechanism in middle-aged from the present study is that in middle-aged, antioxidant defense impairs with increasing age. ROS may induce endothelium dysfunction and downstream angiogenic capacity events. ROS increment induces eNOS uncoupled and generates $OONO^-$ via the reaction of NO and oxygen, followed by enhances oxidative stress. At the same time, the reduction of NO induces hypertension without hypertrophy and decreases angiogenesis. Exercise training reduces ROS by an increment of antioxidant capacity and upstream angiogenic cascade. Exercise training prevents endothelium dysfunction by induced mechanotransduction via increase PI3/Akt/eNOS activity, followed by the enhancement of NO and its downstream increasing VEGF and restores antioxidant defense.

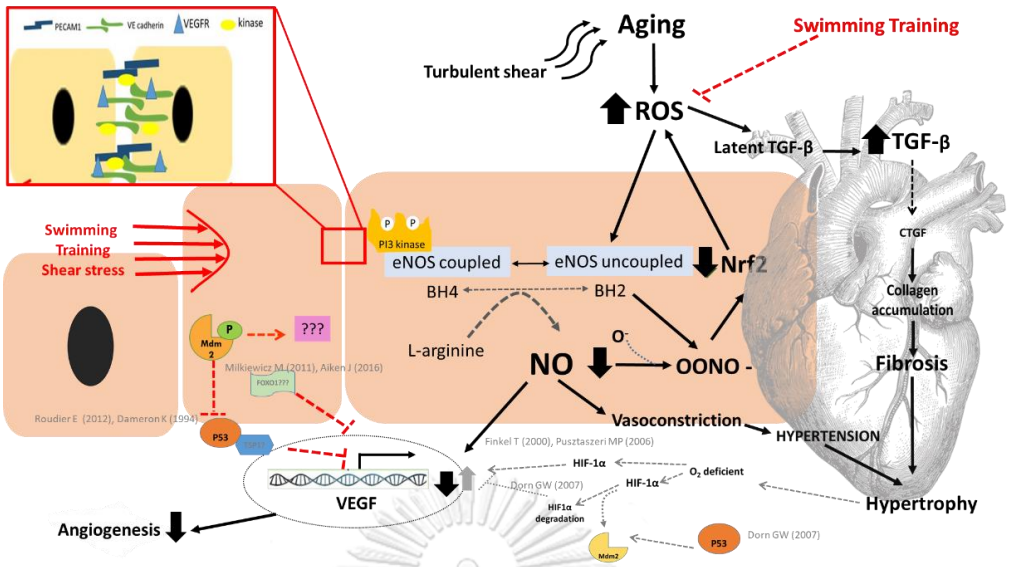


Figure 26 The proposed mechanism in aging.

The proposed mechanism in aging from the present study is that the age-impaired antioxidant defense could increase ROS, resulting in endothelium dysfunction and downstream angiogenic capacity events. ROS increment induces eNOS uncoupled and generates OONO- via the reaction of NO and oxygen, followed by enhances oxidative stress. At the same time, the reduction of NO induces hypertension and decreases angiogenesis. ROS also induces TGF-β1, resulting enhances collagen accumulation and consequent cardiac fibrosis. Cardiac hypertrophy is induced by increased hypertension and associated with TGF-β1 mediated through Connective Tissue Growth Factor (CTGF) expression. In the early stage, core hypoxia of hypertrophied myocytes induces expression of HIF-1, resulting in increases in VEGF levels. However, Mdm2 interaction with HIF-1 leads to HIF-1 ubiquitination and degradation. Exercise training prevents endothelium by the restored antioxidant defense and its downstream angiogenic cascade. Exercise training-induced mechanotransduction via increase PI3/Akt/eNOS activity, followed by the enhancement of NO and its downstream increasing VEGF and vasodilation. Exercise training increases the phosphorylation of Mdm2 on serine 166. By the expression of Mdm2 phosphorylation restrains the expression of FOX-O1 and stimulates angiogenesis. The resulting decrease p53 can favor angiogenesis by limiting angiostatic gene expression, such as Thrombospondin-1 (TSP-1). Exercise training also reduces TGF-β1 activation via the reduction of ROS, leading to alleviated cardiac fibrosis.

CHAPTER VI

CONCLUSIONS

The present study aimed to define whether exercise training could prevent age-induced the reduction of cardiac angiogenic capacity and fibrosis in middle-aged and aged rats or not. To understand the mechanism of exercise training effects, MDA, p-Akt, eNOS, p53, Mdm2, VEGF, CD31 and TGF- β1 were also examined:-

The data from the present study could be summarized as follow: -

1. The systolic blood pressure, diastolic blood pressure and mean arterial blood pressure increase with increasing age. The significant reduction of blood pressure was demonstrated after 8 weeks swimming exercise in the age group
2. Age-induced the reduction of cardiac angiogenic capacity is associated with increased oxidative stress, as described by malondialdehyde (MDA) levels elevation.
3. Age-related to the reduction of CD31 expression, the endothelial cell indicator, suggested that there was a degeneration of the endothelial cells. However, swimming exercise training could improve it.
4. Age-associated the reduction of VEGF, p-Akt1, eNOS protein expression was observed, however, they could be improved by the swimming exercise training
5. The level of Mdm2 was reduced by aging. The significant increased Mdm2 was observed only in the ET-Age group
6. P53 protein expression was trend to increase with increasing age
7. Swimming exercise training could prevent age-induced cardiac fibrosis and appeared to correspond with the alteration of TGF β1.

In conclusion, these findings suggested that exercise training had benefited in the aging heart. Exercise training reduced oxidative stress in the aging heart rats as demonstrated by the reduction of MDA levels. Exercise training also could increase the CD31 expression, as to represent endothelial cells marker. Moreover, age-induced cardiac microvascular changes were involved in the downregulation of VEGF, p-Akt1, eNOS, Mdm2 expression but p53 was no change. Furthermore, exercise training can attenuate the myocardial fibrosis in the aged group through the reduction of TGF β1. Thus, we propose that exercise training could prevent age-induced the reduction of angiogenic capacity and fibrosis are associated with the change in the oxidant-antioxidant balance.

The limitation of the present study

The limitation of this study is there was no aged rat available in a commercial. The researcher needs to take care of all rat for several months from 4-6 week – old to 22-month-old) therefore, it took a lot of time to do this research with hard-working

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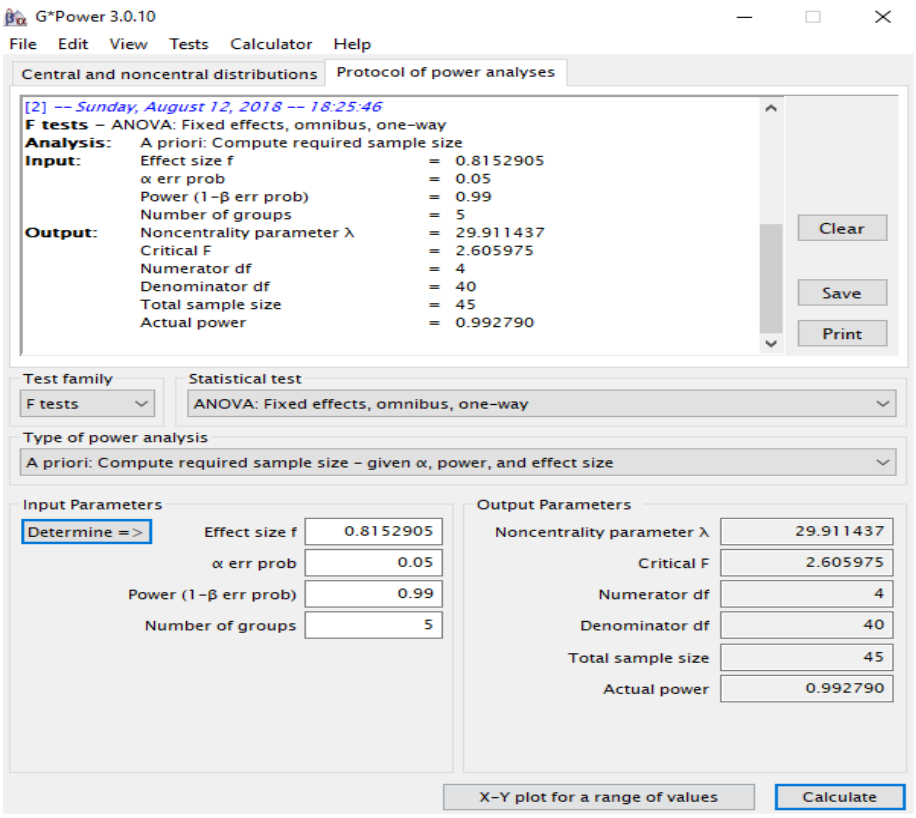
APPENDIX

APPENDIX A: G power program

We calculated the number of rats per group (from consulted with statistician) perform as Viboolvorakul S, Patumraj S[146]. In this research, the results showed Mean±SEM. Therefore, we must calculate SEM to SD by using SD = SEM * √n. From the calculation, the results showed the systolic blood pressure of sedentary- young (n=5) was 121±13.30, sedentary –aged (n=8) was 144.58±14.34, immersed-aged (n=5) was 144.83±7.67 and trained-aged rat (n=7) was 130.42±10.85. Therefore, Mean SD (11.82), α value (0.05), power (1-β value = 0.99) and effect size (0.82) were used for sample size calculation. From the results, the total sample size is 45 animals, so each group is 9.



APPENDIX B Blind' results

APPENDIX B1. In blind assessment, the result of collagen accumulation

The result of collagen accumulation in this study was confirmed by blind assessment as showed below: -

	SE-Young (n=5)	SE-Mid-Age (n=5)	ET-Mid-Age (n=5)	SE-Age (n=5)	ET-Age (n=5)
Mean	5.77	7.22	5.99	14.46	8.00
SE	0.57	0.68	0.60	1.21***	1.08###

*** = P<0.001 compared to the SE-Young

= P<0.001 compared to the indicated groups without exercise training

APPENDIX B2. In blind assessment, the result of Mdm2

The result of Mdm2 expression in this study was confirmed by blind assessment as showed below: -

	SE-Young (n=5)	SE-Mid-Age (n=5)	ET-Mid-Age (n=5)	SE-Age (n=5)	ET-Age (n=5)
Mean	3.69×10^{-3}	1.61×10^{-3}	1.73×10^{-3}	1.77×10^{-3}	3.27×10^{-3}
SE	1.00×10^{-3}	0.44×10^{-3}	0.47×10^{-3}	0.66×10^{-3}	0.92×10^{-3}

No significant

APPENDIX B3. In blind assessment, the result of CD31

The result of CD31 expression in this study was confirmed by blind assessment as showed below: -

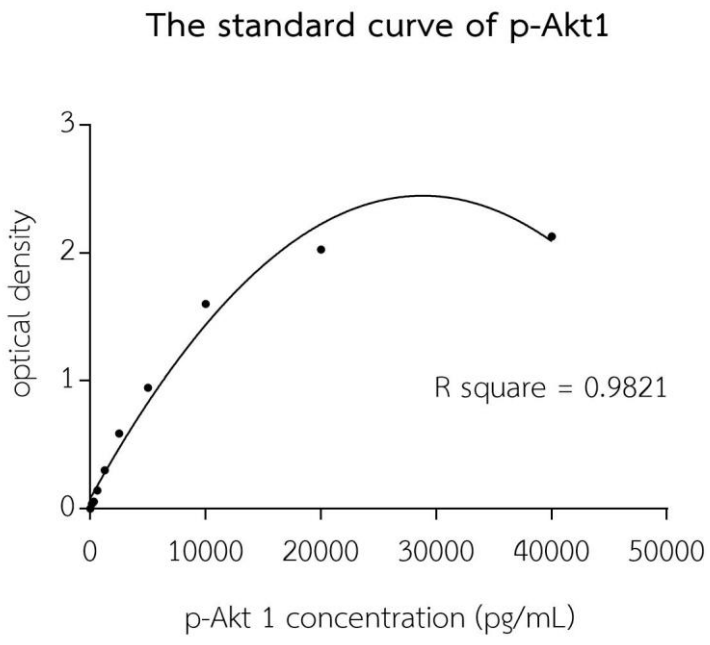
	SE-Young (n=5)	SE-Mid-Age (n=5)	ET-Mid-Age (n=5)	SE-Age (n=5)	ET-Age (n=5)
Mean	1.09×10^{-3}	0.042×10^{-3}	0.94×10^{-3}	0.40×10^{-3}	0.88×10^{-3}
SE	0.08×10^{-3}	$0.06\times10^{-3***}$	$0.06\times10^{-3###}$	$0.05\times10^{-3***}$	$0.08\times10^{-3###}$

*** = P<0.001 compared to the SE-Young

= P<0.0 1 compared to the indicated groups without exercise training

APPENDIX C: The standard curve of p-Akt1 protein

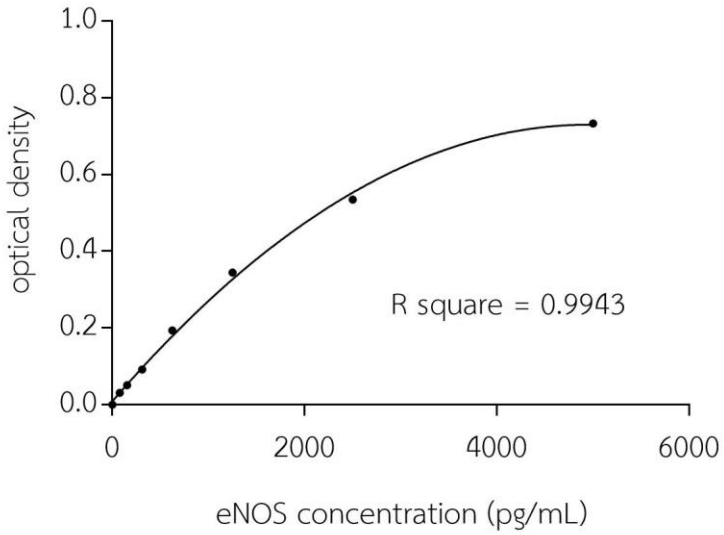
p-Akt1	Optical density				
(pg/mL)	I	II	Average	Corrected I	Corrected II
40000	2.419	2.437	2.428	2.120	2.136
20000	2.324	2.328	2.326	2.025	2.027
10000	1.930	1.872	1.901	1.631	1.571
5000	1.249	1.244	1.2465	0.950	0.943
2500	0.896	0.883	0.8895	0.597	0.582
1250	0.599	0.604	0.6015	0.300	0.303
625	0.434	0.452	0.443	0.135	0.151
312.5	0.342	0.373	0.3575	0.043	0.072
156.25	0.328	0.347	0.3375	0.029	0.046
0	0.299	0.301	0.3	0.000	0.000



APPENDIX D: The standard curve of eNOS protein

eNOS (pg/mL)	Optical density				
	I	II	Average	Corrected I	Corrected II
5000	0.868	0.833	0.8505	0.760	0.706
2500	0.658	0.646	0.652	0.550	0.519
1250	0.478	0.446	0.462	0.370	0.319
625	0.297	0.325	0.311	0.189	0.198
313	0.210	0.209	0.2095	0.102	0.082
156	0.169	0.168	0.1685	0.061	0.041
78.1	0.146	0.151	0.1485	0.038	0.024
0	0.108	0.127	0.1175	0.000	0.000

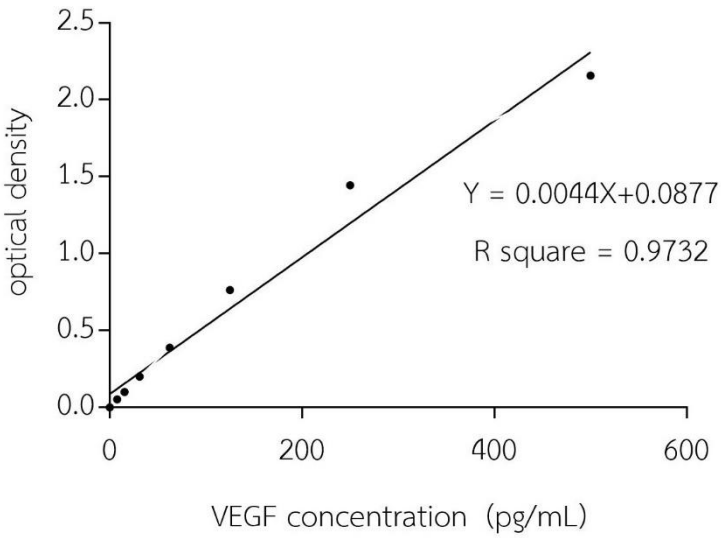
The standard curve of eNOS



APPENDIX E: The standard curve of VEGF protein

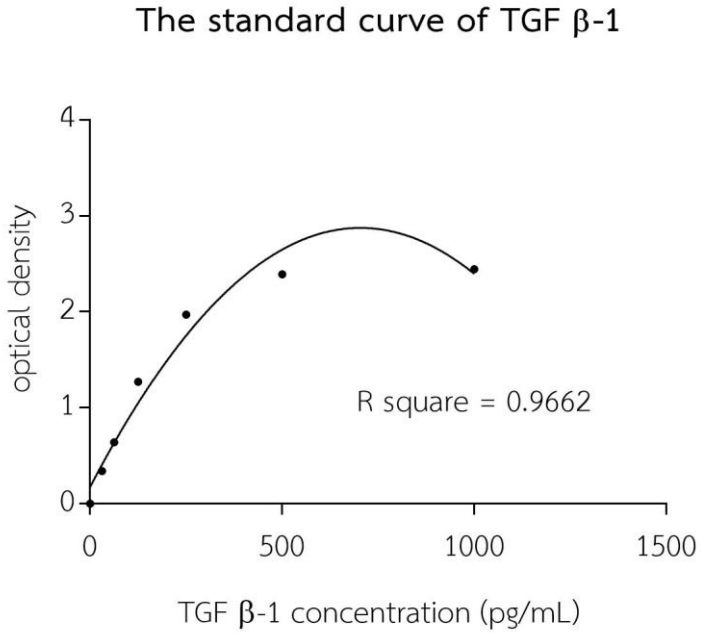
VEGF	Optical density			
(pg/ml)	I	II	Average	Corrected
500	2.247	2.235	2.241	2.157
250	1.503	1.553	1.528	1.444
125	0.85	0.843	0.8465	0.7625
62.5	0.485	0.459	0.472	0.388
31.3	0.29	0.282	0.286	0.202
15.6	0.189	0.183	0.186	0.102
7.8	0.141	0.133	0.137	0.053
0	0.085	0.083	0.084	0

The standard curve of VEGF



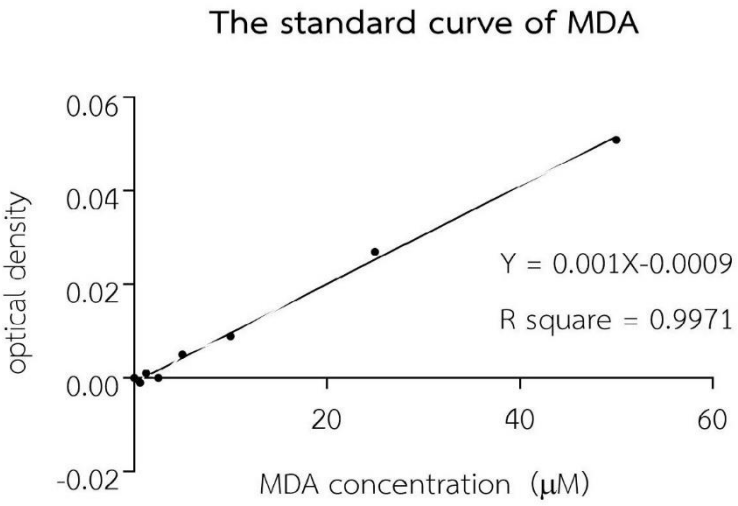
APPENDIX F: The standard curve of TGF - β1 protein

TGF-β1 (pg/mL)	Optical density				
	I	II	Average	Corrected I	Corrected II
2000	2.463	2.462	2.4625	2.444	2.437
1000	2.466	2.468	2.467	2.447	2.443
500	2.41	2.417	2.4135	2.391	2.392
250	1.986	2.004	1.995	1.967	1.979
125	1.314	1.273	1.2935	1.295	1.248
62.5	0.655	0.671	0.663	0.636	0.646
31.3	0.363	0.364	0.3635	0.344	0.339
0	0.019	0.025	0.022	0	0



APPENDIX G: The standard curve of MDA level

MDA	Optical density			
(μM)	I	II	Average	Corrected
50	0.083	0.085	0.084	0.051
25	0.059	0.061	0.060	0.027
10	0.043	0.042	0.043	0.009
5	0.039	0.038	0.039	0.005
2.5	0.034	0.033	0.034	0.000
1.25	0.035	0.033	0.034	0.001
0.625	0.033	0.033	0.033	-0.001
0	0.034	0.033	0.034	0.000



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