ผลของการฝึกออกกำลังกายต่อการเปลี่ยนแปลงของหลอดเลือดขนาดเล็กในสมองของหนูแก่: บทบาทของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทส

นางสาวชีพสุมน วิบูลย์วรกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECTS OF EXERCISE TRAINING ON MICROVASCULAR CHANGES IN AGING RAT BRAIN: ROLES OF VEGF AND ENOS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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Field of Study	Physiology
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ชีพสุมน วิบูลย์วรกุล : ผลของการฝึกออกกำลังกายต่อการเปลี่ยนแปลงของหลอดเลือดขนาดเล็กในสมองของหนูแก่: บทบาทของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทส. (EFFECTS OF EXERCISE TRAINING ON MICROVASCULAR CHANGES IN AGING RAT BRAIN: ROLES OF VEGF AND ENOS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. สุทธิลักษณ์ ปทุมราช, 90 หน้า.

ปัจจุบันมีรายงานว่าประชาการผู้สูงอายุมีความเสี่ยงต่อการเกิดโรคหลอดเลือดสมองเพิ่มมากขึ้น การลดลงของการไหลเวียนเลือดและการสูญเสียหลอดเลือดฝอยที่สมองเป็นปัจจัยสำคัญที่ส่งผลต่อการขาดเลือดไปเลี้ยงที่สมองในวัยชราได้ การเปลี่ยนแปลงของหลอดเลือดที่สมองในวัยชรามีความสัมพันธ์กับการลดลงของระดับวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทส การออกกำลังกายอย่างสม่ำเสมอสามารถเพิ่มการไหลเวียนเลือดและกระตุ้นการสร้างหลอดเลือดฝอยที่สมองในวัยชราได้ อย่างไรก็ตามกลไกที่อธิบายผลของการออกกำลังกายต่อการเปลี่ยนแปลงของหลอดเลือดที่สมองในวัยชรายังไม่ทราบแน่ชัด การศึกษาวิจัยนี้มีจุดประสงค์เพื่อศึกษาผลของการออกกำลังกายต่อการเปลี่ยนแปลงของหลอดเลือดและระดับวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทสที่สมองของหนูแก่ ในการทดลองใช้หนูขาวเพศผู้สายพันธุ์วิสตาร์ โดยแบ่งออกเป็น 4 กลุ่ม คือ 1) กลุ่มหนูวัยเจริญพันธุ์ 2) กลุ่มหนูแก่ไม่ได้ออกกำลังกาย 3) กลุ่มหนูแก่ได้รับการแช่น้ำ 4) กลุ่มหนูแก่ได้รับการฝึกออกกำลังกายด้วยการว่ายน้ำ 5 วันต่อสัปดาห์ เป็นเวลา 8 สัปดาห์ เราทำการศึกษาการเปลี่ยนแปลงของหลอดเลือดที่สมอง โดย 1) วัดการไหลเวียนของเลือดที่สมอง 2) ฉีดสารเรืองแสงและส่องตรวจดูด้วยกล้องจุลทรรศน์แบบส่องกราดด้วยแสงเลเซอร์และบันทึกภาพหลอดเลือดสมองที่เรืองแสงเพื่อนำไปวิเคราะห์หาปริมาณหลอดเลือดฝอย นอกจากนี้เราได้ทำการศึกษากลไลของการออกกำลังกายต่อการกระตุ้นการไหลเวียนเลือดและสร้างหลอดเลือดฝอยที่สมองของหนูแก่ โดยตรวจหาปริมาณวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทสในหลอดเลือดขนาดเล็กที่สมอง โดยวิธีการวัดปฏิกริยาแอนติบอดี-แอนติเจน ผลการวิจัยพบว่าการออกกำลังกายทำให้มีการเปลี่ยนแปลงทารสรีรวิทยาของหนูแก่ที่ดีขึ้นอย่างมีนัยสำคัญทางสถิติ คือ น้ำหนักตัว, ความดันเลือดลดลง, ระดับไขมันในเลือด และระดับไขมันเปอร์ออกไซด์ในเลือดลดลงเมื่อเทียบกับหนูแก่ไม่ได้ออกกำลังกาย นอกจากนี้ยังพบว่าการออกกำลังกายกระตุ้นการไหลเวียนเลือดและสร้างหลอดเลือดฝอยที่สมองของหนูแก่ที่ลดลงได้อย่างมีนัยสำคัญทางสถิติ อีกทั้งยังพบว่าการออกกำลังกายกระตุ้นการสร้างวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทสในหลอดเลือดขนาดเล็กที่สมองของหนูแก่อย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังพบความสัมพันธ์เชิงเส้นระหว่างปริมาณหลอดเลือดฝอยและปริมาณวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทส และความสัมพันธ์เชิงเส้นระหว่างปริมาณหลอดเลือดฝอยและระดับไขมันเปอร์ออกไซด์ ผลการวิจัยสรุปได้ว่าการออกกำลังกายสามารถกระตุ้นการไหลเวียนเลือดและสร้างหลอดเลือดฝอยที่สมองของหนูแก่ได้ โดยกลไกของการเปลี่ยนแปลงของหลอดเลือดที่สมองภายใต้อิทธิพลของออกกำลังกายส่วนหนึ่งผ่านการกระตุ้นการสร้างวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ และเอนโดทีเลียลไนตริกออกไซด์ซินเทส และการกระตุ้นการกำจัดอนุมูลอิสระ

สาขาวิชา สรีรวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: EXERCISE / AGING / CEREBRAL MICROVASCULATURE / VEGF / ENOS

SHEEPSUMON VIBOOLVORAKUL: EFFECTS OF EXERCISE TRAINING ON MICROVASCULAR CHANGES IN AGING RAT BRAIN: ROLES OF VEGF AND ENOS. ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D., 90 pp.

Aging has been reported to increase risk of cerebrovascular diseases. Reduction of basal regional blood flow and capillary loss in the brain contribute tissue perfusion insufficiency with advancing age. Microvascular deterioration in aged brain appears to be related to downregulation of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS), which both are key modulators related age-induced microvascular changes. Regular physical exercise is well known to have beneficial effect to brain health, including promoted blood flow, augmented angiogenesis and enhanced vascularization, in aging individuals. However, the underlying mechanisms are largely unknown. The present study was set to investigate effect of exercise training on age-induced cerebral microvascular alterations with modulation of VEGF and eNOS expressions. Male Wistar rats were divided into four groups: sedentary-young (4-6 months), sedentary-aged (21-22 months), immersed-aged (21-22 months) and trained-aged (21-22 months). Exercise program included swimming training 5 days/week for 8 weeks. Physiological characteristics including body weight, mean arterial blood pressure, plasma lipid profile and plasma malondialdehyde (MDA) level were determined. To investigation of microvascular networks of the brain, *in situ* study was performed for determining regional cerebral blood flow perfusion (rCBF) using a laser Doppler flowmeter and for visualizing the vasculature via cranial window, using a laser scanning confocal fluorescent microscope. Fluorescent images of the vasculature were recorded and off-line analyzed for capillary vascularity (CV) by using image analysis software. To examine possible underlying mechanism of exercise training on ameliorating cerebral vascular deterioration, VEGF and eNOS protein level in isolated brain microvessels were determined using immunoassay technique. We found that age induced significant alteration of these physiological characteristics in old rats when compared to those in young group. However, those parameters were significantly improved in trained-aged rats. In addition, CV and rCBF significantly reduced in non-exercise old rats when compared to those in young rats, however, in trained-aged rats, CV amd rCBF significantly elevated when compared to those in old rats without training. Further, age induced significant downregulation of VEGF and eNOS expression in aged rats when compared to those in young rats. In addition, exercise significantly upregulated VEGF and eNOS level in trained-aged rats when compared to those in non-exercise old rats. Moreover, the linear correlations between CV and either VEGF or eNOS levels were observed as well as a linear relationship between CV and plasma MDA level. Therefore, it implied that antioxidant effects of exercise training could protect brain microvascular and blood perfusion against aging, particularly associated with its actions on VEGF and eNOS expressions.

Field of Study Physiology Student's Signature .....

Academic Year 2011 Advisor's Signature .....

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LIST OF ABBREVIATIONS

BBB	blood brain barrier
BCA	bicinchoninic acid
CBF	cerebral blood flow
CV	capillary vascularity
ELISA	enzyme link immunosorbant assay
eNOS	endothelial nitric oxide synthase
FITC	fluorescein isothiocyanate
Flk-1	Fetal liver kinase-1
Flt-1	fms-like tyrosyl kinase-1
HDL	high density lipoprotein
iNOS	inducible nitric oxide synthase
KDR	Kinase Domain-contain Receptor
LDL	low density lipoprotein
MAP	mean arterial blood pressure
MDA	malondialdehyde
MnSOD	mitochondrial superoxide dismutase
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
PCO_2	partial pressure of carbon dioxide
$PaCO_2$	partial pressure of carbon dioxide in arterial blood
PI3K	phosphatidylinositol-3 kinase
rCBF	regional cerebral blood flow
ROI	region of interest
ROS	reactive oxygen species
TG	triglyceride
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2

CHAPTER I
INTRODUCTION

The number of elderly in the world is growing rapidly. According to the United Nations Report in 2007, since 1950 the proportion of older persons has been rising steadily, passing from 8 percent in 1950 to 11 percent in 2007, and is expected to reach 22 percent in 2050. Thailand also experienced a rapid and extensive growth, starting in the late 1960s. The Thailand's National Economic Social and Development Board Report in 2007 indicated that Thai aging population will continue at a rapid pace between 2000 and 2030 with persons aged 60 and older expected to constitute one fourth of the population by the end of that period. The rapid growth of global aging population has profound implications for many aspects of human health.

Cerebrovascular abnomalities is the one of leading cause of death in Thailand. The Bureau of Policy and Strategy, Ministry of Public Health, reported that the cerebrovascular disease is the most important leading cause of death in elderly persons (2008). According to Thailand Health Profile Report, mortality rate of cerebrovascular disease in aged persons was increased from 54.9 per 100,000 populations in 1996 to 110.9 per 100,000 populations in 2006.

It is well accepted that structural and functional alterations in cerebral vasculature are of pivotal importance in the pathogenesis of a variety of diseases affecting the brain (Gavins, Yilmaz et al., 2007), including stroke, subarachnoid hemorrhage, and neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases). Evidence from recent studies suggests that cerebrovascular dysfunction is a key contributor to age-related brain pathogenesis (Iadecola, Park et al., 2009). Moreover, aging is associated with a reduction in basal cerebral blood flow (CBF) and a dysfunction of mechanisms regulating cerebral circulation (Kalaria 2009). Regional perfusion rate and flow velocity decline were found in aged individuals (Farkas and Luiten 2001). These alterations reduce cerebral perfusion and increase susceptibility of the brain to vascular insufficiency and ischemic injury.

The microvasculature density in the brain is a necessary component of several fundamental aspects of cerebrovascular function. Microvascular density is an important determinant of the capacity for blood flow through a region of brain tissue (Sonntag, Eckman et al., 2007). In aging, brain regions exhibit marked capillary loss and increase in microvascular tortuosity, leading to increase resistance to flow and reduce tissue perfusion (Kalaria 2009). The mechanism responsible for suppression of capillary in aging appears to be related to a reduced expression of vascular endothelial growth factor (VEGF), known to be a potent angiogenic growth factor *in viv*o (Reed and Edelberg 2004).

VEGF signaling pathway is considered a major stimulating factor in both physiological and pathological angiogenesis (Shibuya and Claesson-Welsh 2006). VEGF receptor 2 (VEGFR2: KDR in human, Flk-1 in rodents) has an important role in VEGF-mediated endothelial cell proliferation, migration, and differentiation, suggesting that VEGFR2 is the major signal transducer for angiogenesis (Shibuya 2006). Extensive work has been shown age-impaired angiogenesis associated with VEGF decline in several tissues (Rivard, Fabre et al., 1999, Sadoun and Reed 2003, Wagatsuma 2006, Hoenig, Bianchi et al., 2008). In aging brain, it was also demonstrated the reduction of VEGF expression in microvessels (Hoehn, Harik et al., 2002, Ding, Li et al., 2006, Villar-Cheda, Sousa-Ribeiro et al., 2009).

Oxidative stress is increased in aging, which is associated with increases in oxidatively damaged proteins, lipids, and DNA, contribute to attenuate blood vessel growth in aging (Lahteenvuo and Rosenzweig 2012). Overall reduction in NO bioavailability seen in aged blood vessels is largely due to a markedly reduction in endothelial nitric oxide synthase (eNOS) activity (Collins and Tzima 2011). eNOS plays a role in regulation of CBF and vascular tone under normal and pathological conditions (Atochin and Huang 2011). Moreover, eNOS acts as a central integrator of signal pathways related to aging and angiogenesis (Lahteenvuo and Rosenzweig 2012). Age-related change in NO pathway was also found in the brain (Siles, Martinez-Lara et al., 2002), however, less information of age-mediated altering eNOS expression in cerebral microvessels was found. The most potent activators of eNOS is fluid shear stress on the endothelial layer of blood vessels (Malek, Alper et al., 1999).

Previous studies have shown that the reduction in NO availability in older vessels is partially in response to low shear stress (Kang, Reyes et al., 2009). It is plausible that age-related reduction of blood flow resulting in diminished shear stress to tissues could be contributed to impair angiogenesis, partly involved in eNOS modulation.

Physical exercise has been shown to reduce risk of cerebrovascular and cardiovascular events (Cotman, Berchtold et al., 2007). Accumulating evidence suggests that regular exercise training induced brain vascularization (Churchill, Galvez et al., 2002), augmented angiogenesis (Kleim, Cooper et al., 2002, Swain, Harris et al., 2003) and increased basal CBF (Swain, Harris et al., 2003, Ainslie, Cotter et al., 2008). Increased brain vascularization, and hence blood flow, might prove to be an effective strategy to minimize or delay cerebrovascular events with age. Studies have demonstrated that exercise training ameliorated impaired angiogenesis and reduced VEGF level in the brain with advancing age (Ding, Li et al., 2006, Latimer, Searcy et al., 2011). However, the mechanisms underlying the exercise-induced improvement of vascular deterioration in the aged brain are largely unknown. Chronic aerobic exercise has been suggested to induce shear stress for endothelial adaptations in vasculatures perfusing noncontractive tissues, including brain (Padilla, Simmons et al., 2011). As aforementioned, shear stress is a potent physiological stimulus for NO release via upregulation of eNOS. Together, it is interesting to investigate role of exercise training on age-induced alteration of cerebral microcirculation in the brain with modulation of VEGF and eNOS. Therefore, the goal of the present study was set to investigate the effects of exercise training on microvascular changes in aging rat brain, and the possible mechanism of exercise training on VEGF, eNOS expression in brain microvessels was examined.

Research question

Could exercise training ameliorate age-induced cerebral microvascular deterioration associated with upregulation of VEGF and eNOS?

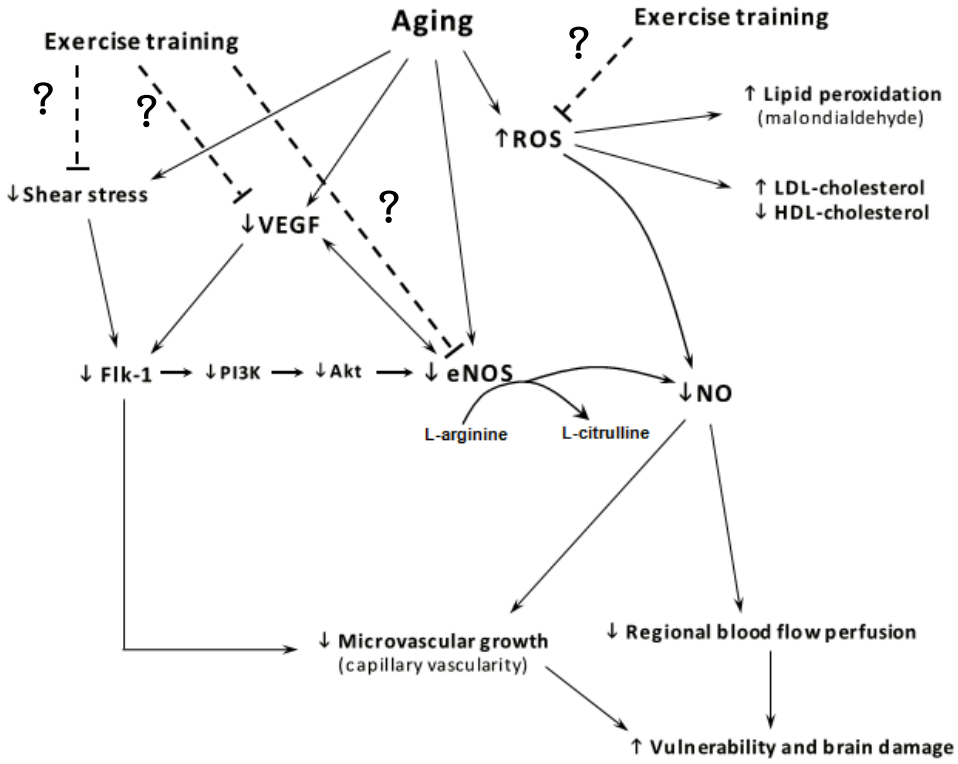
Research objective

1. To examine the effect of exercise training on cerebral microvascular deterioration in aging rat brain.
2. To investigate the possible mechanisms underlying the beneficial effects of exercise traininig on modulation of VEGF and eNOS expressions in brain microvessels.

Hypothesis

Exercise training can attenuate age-induced cerebral microvascular deterioration, partly related to VEGF and eNOS activation.

Conceptual framework



CHAPTER II
LITERATURE REVIEW

Cerebral microvasculature and blood brain barrier

Network of cerebral vessels and capillary function in the brain inherently differs from that of arteries. Generally, arteries regulate blood pressure while capillaries maintain the blood brain barrier (BBB) and sustain continuous nutrient, electrolyte and waste product trafficking between neural tissue and blood is reflected in the microvascular anatomy. Capillary densities apparently correlate with the activity and nutrient demand of the particular region (Farkas and Luiten 2001). The phenomenon that metabolically active brain regions are more heavily vascularized than less active zones is supported by the observation that capillary density appears to be most pronounced in area rich in synapses, followed by cell body population and finally neural fiber bundles.

The cerebral capillaries display a typical ultrastructure crucial to execute BBB function, particularly forming the neurovascular unit composed of endothelial cells, pericytes and astrocytes. The capillary endothelial cells form one layer around the capillary lumen and create tight junctions where they are opposed to each other. The tight junction seal the space between the meeting endothelial surfaces and are considered as morphological basis for the BBB gaining their full functional integrity with the maturation of the animal (Kniesel and Wolburg 2000). Capillary endothelial cells are characterized by a relatively high number of mitochondria, which can provide the energy requirement for the specific BBB transport.

Regulation of cerebral blood flow

The brain receives probably the most constant blood supply of all body organs maintained by a very finely tuned regulation of cerebral blood flow (CBF). Physiological fluctuations in the cerebral perfusion pressure are normally compensated by the cerebrovascular autoregulation to sustain an optimal, unterrupted CBF. An intact autoregulation is capable of keeping the CBF independent of perfusion pressure provided the perfusion pressure range approximately between 60 and 150 mmHg (Paulson, Strandgaard et al., 1990). Below or above the given values, the autoregulatory mechanisms become uncoupled from the perfusion pressure and lose accurate control of CBF. The dynamics maintenance of CBF is achieved by changes in vascular resistance, which can be controlled by local chemical factors, endothelial factors and neurotransmitters (Farkas and Luiten 2001).

Vascular endothelium plays a pivotal role in CBF regulation because an important group of vasoactive biochemical compounds are released by and act on the endothelial cells. Nitric oxide (NO) is concerned to be the most important factor in the CBF regulation. Vascular dilation mediated by NO is well-described phenomenon. NO relaxed vascular smooth muscle and increases regional CBF in response to shear stress to the endothelium or stimulation by acetylcholine, bradykinin or other biochemical compounds (Arnal, Dinh-Xuan et al., 1999). The mechanical and chemical stimuli can increase the cystolic calcium concentration and the association of the calcium/calmodulin complex to NO synthase (NOS) in the endothelial cells which in turn modulates NO production (Atochin and Huang 2011).

Cerebrovascular alterations during aging

It is well known that aging leads to a degeneration of the vascular system. In cerebral vasculature, decreasing cerebral blood flow and structural integrity of microvessels are representative degenerative features of the vascular system of the aging brain (Farkas and Luiten 2001). Several mechanisms regulate flow into and through each capillary bed, including the density of precapillary arterioles and capillaries, the structure of the vessels, and the reactivity of the arterioles (Sonntag, Eckman et al., 2007).

At the level of the cerebral microvessels, both the capillary density of distinct brain region and the ultrastructure of the capillary walls are prone to age-related (Farkas and Luiten 2001). The aged brain has been reported to exhibit marked capillary loss (Sonntag, Lynch et al., 1997, Brown, Moody et al., 2007, Shao, Li et al., 2010, Murugesan, Demarest et al., 2012) and increase in microvascular tortuosity (Kalaria 2009), changes that increase resistance to flow (Stoquart-ElSankari, Baledent et al., 2007) and reduce tissue perfusion (Iadecola, Park et al., 2009, Kalaria 2009). Age-related alterations in the structure and function of the cerebral microvasculature can interrupt delivery of oxygen and energy substrates need to the brain activities, leading to increase vulnerability of susceptible regions to ischemic hypoxic brain damage (Iadecola, Park et al., 2009). Appropriate microvascular networks (including pre-capillary arterioles and capillaries) supplying the brain has an important requirement for cerebrovascular function (Sonntag, Eckman et al., 2007). Alteration in microvascular density has a potential to result in inadequate blood flow through the brain (Ingraham, Forbes et al., 2008). In aging rats, the density of microvessels on the cortical surface was significantly lower when compared to the young adults (Sonntag, Lynch et al., 1997), suggesting that vascular density may be an important contributing factor to decrease in cerebral blood flow. Moreover, mechanism for age-induced deterioration of structure and function of brain vaculature is appeared to involve in reduction of angiogenic growth factor.

Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis in both physiological and pathological conditions (Shibuya and Claesson-Welsh 2006). VEGF was initially recognized as a factor which increased vascular permeablilty, it is now apparent that this protein regulates multiple biological functions in endothelial cells, including endothelial cell survival, proliferation and migration (Zachary 2003). The biology actions of VEGF are mediated via its receptors which located on endothelial cell membranes. Three main receptors have been identified: VEGF receptor 1 (VEGFR1: Flt-1, fms-like tyrosyl kinase-1), VEGFR2 (KDR, Kinase Domain-containg Receptor for human/Flk-1, Fetal liver kinase-1 for rodents) and VEGFR3 (Flt-4, fms-like tyrosyl kinase-4). These receptors belong to the superfamily of receptor tyrosine kinases. Most biologically relevant VEGF signaling in endothelial cells is mediated via VEGFR2 (Shibuya 2006).

VEGFR2 is the predominant mediator of VEGF-stimulated endothelial cell migration, proliferation, survival, and enhanced vascular permeability (Roskoski 2007). Although VEGFR2 has lower affinity for VEGF than VEGFR1, VEGFR2 exhibits robust protein-tyrosine kinase activity in response to it ligands. VEGF induces the dimerization of VEGFR2 that lead to receptor autophosphorylation and activation.

VEGF activates an angiogenic signaling cascade, which promotes angiogenesis in association with activation of phosphatidylinositol-3 kinase (PI3K)/Akt downstream (Shibuya and Claesson-Welsh 2006, Jiang and Liu 2008). In cultured endothelial cells, VEGF-induced angiogenesis was blocked by PI3K inhibitors and by overexpression of a dominant-negative form of Akt (Gerber, McMurtrey et al., 1998, Ilan, Mahooti et al., 1998). The PI3K/Akt-dependent signaling pathway in VEGF activated endothelial cells regulated by NO synthase (NOS) (Govers and Rabelink 2001). It has been shown that inhibiting NOS reduces the VEGF-induced cell proliferation and migration (Uhlmann, Friedrichs et al., 2001). Conversely, eNOS overexpression results in elevated VEGF-dependent angiogenesis in ischemic model (Namba, Koike et al., 2003).

NOS catalyzes the oxidation of L-arginine to L-citrulline and NO. There are three forms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS is constitutively expressed by vascular endothelial cells, and generate NO by calcium-dependent activity. NO produced by eNOS mediates a variety of physiologic functions including angiogenesis, regulation vascular tone, platelet aggregation, vascular permeability and leukocyte-endothelial interaction (Duda, Fukumura et al., 2004). Shear stress is an important stimulus for NO production by eNOS in endothelial cell in normal blood vessels (Murohara, Asahara et al., 1998).

An age-associated enhanced production of superoxide (the initial oxygen free radical generated by mitochondria) in the vasculature, mainly derived from the endothelium, occurs and is the basis for the *'oxidative-stress theory'* of vascular aging (van der Loo, Schildknecht et al., 2009). Age-associated mitochondrial dysfunction implies increased generation of superoxide and peroxynitrite by misdirection of electrons from the respiratory chain into reactive oxygen species (ROS) production due to a decline of mitochondrial function (van der Loo, Labugger et al., 2000), such as inactivation of aconitase, reduced ATP synthesis and enhanced permeability transition pore opening, leading to apoptosis. This initiates a 'vicious cycle' of increased (nuclear and mitochondrial) DNA damage, leading again to more ROS generation, entailing further mitochondrial DNA damage (Finkel and Holbrook 2000). Superoxide inhibits capillary growth through multiple mechanisms, including scavenging NO and inhibiting eNOS activity (Giacco and Brownlee 2010). During aging, endothelium-dependent vasodilation declines, this reflects an impairment of NO bioavailability (McCarty 2000, Csiszar, Ungvari et al., 2002). The overall reduction in NO bioavailability seen in aged blood vessels is largely due to a reduction of eNOS activity (Collins and Tzima 2011). Mayhan and colleagues have first demonstrated that dilator responses of cerebral arterioles to acetylcholine, bradykinin, ADP, and serotonin were impaired in aged rats as compared with adult rats (Mayhan, Faraci et al., 1990). Several studies suggest that impairment in NO production could be associated with damage and alteration of different brain functions during aging (McCann, Licinio et al., 1998, Yamada and Nabeshima 1998, Calabrese,

Bates et al., 2000). Recently, the lower level of NO production in the brain of aging rats could be related to the reduction of NO synthase (NOS) mRNA, protein and activity in the aging process (Canuelo, Siles et al., 2007). Oxidant stress may contribute to capillary rarefaction by inducing endothelial cell apoptosis and/or reducing NO needed for vascular budding and suppression of vascular endothelial growth factor (Feihl, Liaudet et al. 2006).

Age-related impaired angiogenesis has been suggested that mediated by VEGF. Mitogenic effect of VEGF has been shown on human microvascular endothelial cells in culture (Rosenstein, Mani et al., 1998), as well as, prevents cultured microvascular endothelial cells from entering replicative senescence (Watanabe, Lee et al. 1997). Age-induced impairment of cerebral angiogenesis appears to be related to reduction of VEGF expression (Rivard, Berthou-Soulie et al., 2000, Hoehn, Harik et al., 2002, Gao, Shen et al., 2009).

Many studies demonstrated that increased oxidative stress contributes to the impaired angiogenesis of endothelial cells (Cai 2005, Benndorf, Schwedhelm et al., 2008, Teng, Eis et al., 2009). Hydrogen peroxide was shown to decrease endothelial cell migration and endothelial tube formation and in vivo angiogenesis (Cai 2005). Isoprostanes, endogenously formed end products of lipid peroxidation, were inhibited not only VEGF-mediated endothelial cell migration but also vascular tube formation (Benndorf, Schwedhelm et al., 2008). This study suggested that isoprostanes may activate the thromboxane receptor, initiating robust and persistent activation of RhoA, which results in inhibition of endothelial cell migration and vascular tube formation. Recent study demonstrated impairment of angiogenesis in pulmonary artery endothelial cells associated with increased superoxide production by a NADPH oxidase-dependent mechanism, suggested that the superoxide could be scavenged by NO (an important mediator of angiogenesis) at a diffusion-limited rate, contribute to decreased NO bioavailability, resulting in impairment of angiogenesis (Teng, Eis et al., 2009). These data implicate a potential link between oxidative stress and impaired angiogenesis.

Impact of exercise training on aging vasculature

Regular physical activity has a tremendous impact on health. It leads to a variety of functional changes such as a reduction of heart rate at rest and blood pressure, an increase of myocardial oxygen uptake and several other adaptations including improved organ blood flow (Kojda and Hambrecht 2005). These alterations can be largely explained by a variety of vascular and cardiac molecular mechanisms that provide a protective environment in the cardiovascular system, particularly in the aging population, and may diminish total and cardiovascular morbidity and mortality. Its beneficial effects, however, extend to the cerebral vasculature as well.

Among a wide range of other fundamental structural and metabolic changes such as neurogenesis and enhanced synaptic plasticity, exercise promotes extensive vascular changes and adaptive mechanisms in the central nervous system, such as the induction of vascular antioxidant pathways, capillary growth, and improvement of cerebral perfusion (Churchill, Galvez et al., 2002).

Whereas acute bouts of exercise promote oxidative stress, long-term exercise elicits antioxidant and anti-atherogenic effects, presumably by arterial remodeling, plaque stabilization and functional adaptations such as increased endothelium-dependent vasodilation (Hambrecht, Walther et al., 2004). Numerous data suggest that activation of redox-sensitive signaling pathways in response to non-exhaustive exercise is induced by transient oxidative stress that in turn stimulates the longer lasting expression of antioxidant enzymes (Kojda and Hambrecht 2005). Thus, transient oxidative stress occurring during each bout of exercise may actually play an important role in the beneficial vascular response to long-term regular exercise.

In aging humans, it has been shown that chronic aerobic exercise improves regional CBF in various relevant brain structures in response to cognitive tasks along with better task performance (Colcombe, Kramer et al., 2004). Forced walking caused regional CBF enhancement particularly in the hippocampus as a key structure for memory formation and processing in both young and aged rats as a result of increased extracellular acetylcholine (ACh) release and thus NO upregulation (Uchida, Suzuki et al., 2006).

Exercise is known to increase eNOS gene expression and endothelium dependent generation of NO in humans and a wide variety of animal models. Numerous vasoprotective actions of endothelial NO appear reasonable to assume that activation of the vascular NO/cGMP pathway by induction of eNOS expression and activity is an important mechanism mediating beneficial effects of exercise on the vasculature (Kojda and Hambrecht 2005). Protein expression of eNOS is subject to extensive regulation, e.g., by lysophosphatidylcholine, cGMP-analogs, hormones, growth factors, lipoproteins, protein kinase C inhibitors and cytokines and physical shear stress is among the most important regulating factors (Li, Wallerath et al., 2002). It is well known that vasoprotection conveyed by eNOS activation extends to the cerebral vasculature as well. Recently, it has been shown that constitutive eNOS activation in mutant mice (transgenic mice expressing only a phosphomimetic form of eNOS) exhibited greater vascular reactivity, develop less severe strokes, and have improved cerebral blood flow in a middle cerebral artery occlusion model than mice expressing an unphosphorylatable form (Atochin, Wang et al., 2007). It is thus very likely that exercise-induced eNOS activation enhances vascular plasticity in the long-term and thereby adds to the general beneficial effects on the age-related cerebrovascular pathogenesis.

Exercise training profoundly changes the morphology of vasculature, including increases the number (angiogenesis) and diameter (arteriogenesis) of arterial blood vessels. The exercise-induced angiogenesis is associated with functional changes and improved organ blood flow (Prior, Yang et al., 2004). Improving CBF by counteracting the age-induced dysregulation of angiogenesis may be just another aspect of the beneficial vascular effects specifically in elderly. It has been shown that exercise-induced angiogenesis is neuroprotective, involving growth factors (Ding, Li et al., 2004). VEGF appears to be an important element mediated the effects of exercise in brain angiogenesis. Recently, exercise training has been shown to promote angiogenesis associated with an increase in brain VEGF mRNA and protein (Ding, Li et al., 2006). Activation of endothelial cells including increased generation of NO is also involved in the process of arteriogenesis in response to exercise training (Prior, Yang et al., 2004).

The mechanisms underlying vascular remodeling in response to exercise training are not fully understood. However, shear stress is a primary signal for exercise training-induced endothelial adaptations beyond the active muscle tissues, including brain (Padilla, Simmons et al., 2011). The alteration of physical forces on blood vessels especially shear stress is the important mechanotransduction mechanism in exercise-induced angiogenesis (Kojda and Hambrecht 2005). Endothelial cells respond to shear stress by changing their morphology, function, and gene expression indicates that endothelial cells recognize shear stress as a signal and transmit it into the cell. Laminar blood flow generate shear stress, frictional dragging force acting on the endothelium is the most potent physiological stimulus for NO production by activating eNOS in endothelial cells leading to angiogenesis (Boo and Jo 2003). Fluid shear stress-stimulated VEGFR2 recruits PI3K and hence leads to activation of Akt and eNOS (Jin, Ueba et al., 2003). It is known that physiological shear stress plays an important role in protecting the development of atherosclerosis mainly via NO-dependent mechanism.

As aforementioned, VEGFR2 plays a significant role in angiogenesis as well as endothelium-dependent vasodilation, and it is capable to activated by both VEGF and shear stress. Thus, an alternative mechanism is that shear stress induces a ligand-independent phosphorylation of VEGFR2 in endothelial cells. The mechanical forces trigger dimerization of VEGFR2 monomers by affecting their spatial distribution in the cell membrane, or they may activate the receptors by changing their conformation and promoting the binding of tyrosine kinases that are capable of phosphorylating the receptors. Phosphorylation of the tyrosine kinase receptors leads to activation of various protein kinases, including extracellular signal-regulated kinase (ERK), PI3K and Akt, which result in eNOS activation (Jin, Ueba et al., 2003).

In many vascular beds flow-induced dilation is known to mediated predominantly by production of NO from eNOS, cerebral vascular beds is also reported that flow-induced cerebral vasodilation in vivo involves production of NO (Paravicini, Miller et al., 2006). Recently, shear stress has been reported to induce VEGF expression in endothelial cells (dela Paz, Walshe et al., 2012). The induction of

VEGF expression by shear stress suggested a potential autocrine-juxtacrine signaling pathway.

CHAPTER III
MATERIALS AND METHODS

Animals

Male Wistar rats (8 week olds) were obtained from the National Laboratory Animal Center (Nakornpathom, Thailand). They were housed in groups of four per cage under 12:12 hour light-dark cycle in a temperature- and humidity-controlled room until used. All rats were allowed free access to normal chow and tap water *ad libitum*. This study was approved by Ethics Committee on Care and Use of Laboratory Animals, Faculty of Medicine, Chulalongkorn University. The present study was conducted in accordance with the guidelines for laboratory animals established by the National Research Council of Thailand, 1999.

Experimental protocol

Protocol 1. To determine the effects of exercise training on physiological characteristics in aging rat.

The objective of this experiment was to examine the effects of exercise training on physiological adaptations, including body weight, resting mean arterial blood pressure, lipid profile and lipid peroxide level, in aging rats.

Rats were randomly divided into 4 groups as follows:

Group 1: sedentary-young (aged 4-6 months), as age-control group, rats were subjected to the same swim environment as the trained-aged animals, except they remained in their cages.

Group 2: sedentary-aged (aged 21-22 months), as activity-control group, rats were subjected to the same swimming room as the trained-aged animals, except they remained in their cages.

Group 3: immersed-aged group (aged 21-22 months), as sham group, rats were placed individually in cylindrical tanks filled with water to a depth of 5 cm for 30 minutes/day, 5 days/week for 8 weeks.

Group 4: trained-aged group (aged 21-22 months), rats were individually swum in cylindrical tanks 1 hour/day, 5 days/week for 8 weeks.

It is noted that age of experimental rats in each group is designed according to the comparison age between human and rat (Table A).

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

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

On the day of experiment, overnight-fasted rats were subjected to measured body weight, and then were anesthetized with pentobarbital sodium (60 mg/kg body weight, intraperitonealy). Afterward, rats were tracheotomized and were allowed to breathe spontaneously throughout the experiment. A catheter was placed into the right carotid artery for measurement of arterial blood pressure using a pressure transducer (Statham, USA) connected to a polygraph system (Nihon Koden, Japan). At the end of experiment, blood was collected from abdominal aorta for determining lipid profile and lipid peroxide levels by colorimetric assay technique.

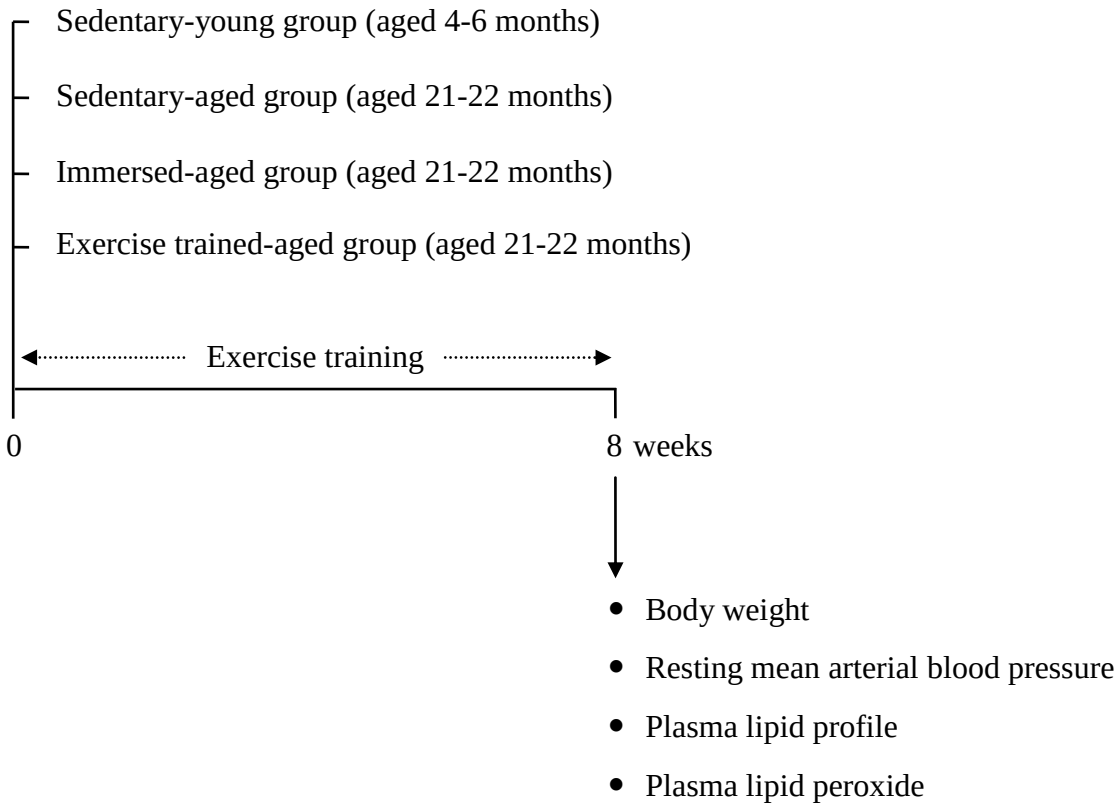


Figure A Schematic diagram showing the protocol for study the effects of exercise training on physiological characteristics in aging rat.

Protocol 2. To determine the effects of exercise training on microvascular changes in aging rat brain.

The objective of this experiment was to study the effect of exercise training on microvascular hemodynamic parameters, including brain capillary vascularity and regional cerebral blood flow, in aging rats.

Rat groups were prepared as decribed in protocol 1. Overnight-fasted rat was anesthetized with pentobarbital sodium (60 mg/kg body weight, intraperitonealy). Body temperature was maintained at 37°C by a homeothermic blanket (Comella′, Spain). The rat was tracheotomized and was mechanically ventilated with room air with supplemental oxygen by an animal ventilator (Kent Scientific, USA). A catheter was placed into the right femoral artery to obtain a blood sample for measurement of

blood gas with a portable blood gas analyzer (iSTAT Abbott Point of Care Inc., USA). The arterial blood gases were monitored throughout the experiment, and the blood gas values were recorded and maintained stable within normal ranges (pH: 7.35-7.45, PO_2: 80-120 mmHg, PCO_2: 35-45 mmHg). Another catheter was placed in the left femoral vein for injection of fluorescent tracer, supplement anesthesia and fluid replacement.

The animal was placed in a homemade stereotaxic apparatus. When the animal head was fixed in the stereotaxic frame, the skull was exposed via a midline incision. Using a dissecting microscope (Olympus, Tokyo) and a low-speed drill (World Precision Instrument Inc., USA), a 3 mm-diameter circular cranial window was performed over the left parietal cortex (2 mm posterior to left coronal suture and 2 mm lateral to the linea temporalis). To prevent overheating of the cerebral cortex during drilling, the skull was cooled with periodic application of room temperature saline. The dura mater was carefully removed using a micro-scissors (PMS GmbH, Germany). A custom-made stainless metal ring (8 mm diameter) was then sealed over the cranial window using dental cement (GC Dental Products Corp., Japan). The cerebral cortex was suffused with artificial cerebral spinal fluid (NaCl 118 mM, KCl 4 mM, NaH_2PO_4 1.2 mM, D-glucose 5 mM, $CaCl_2$ 1.5 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM).

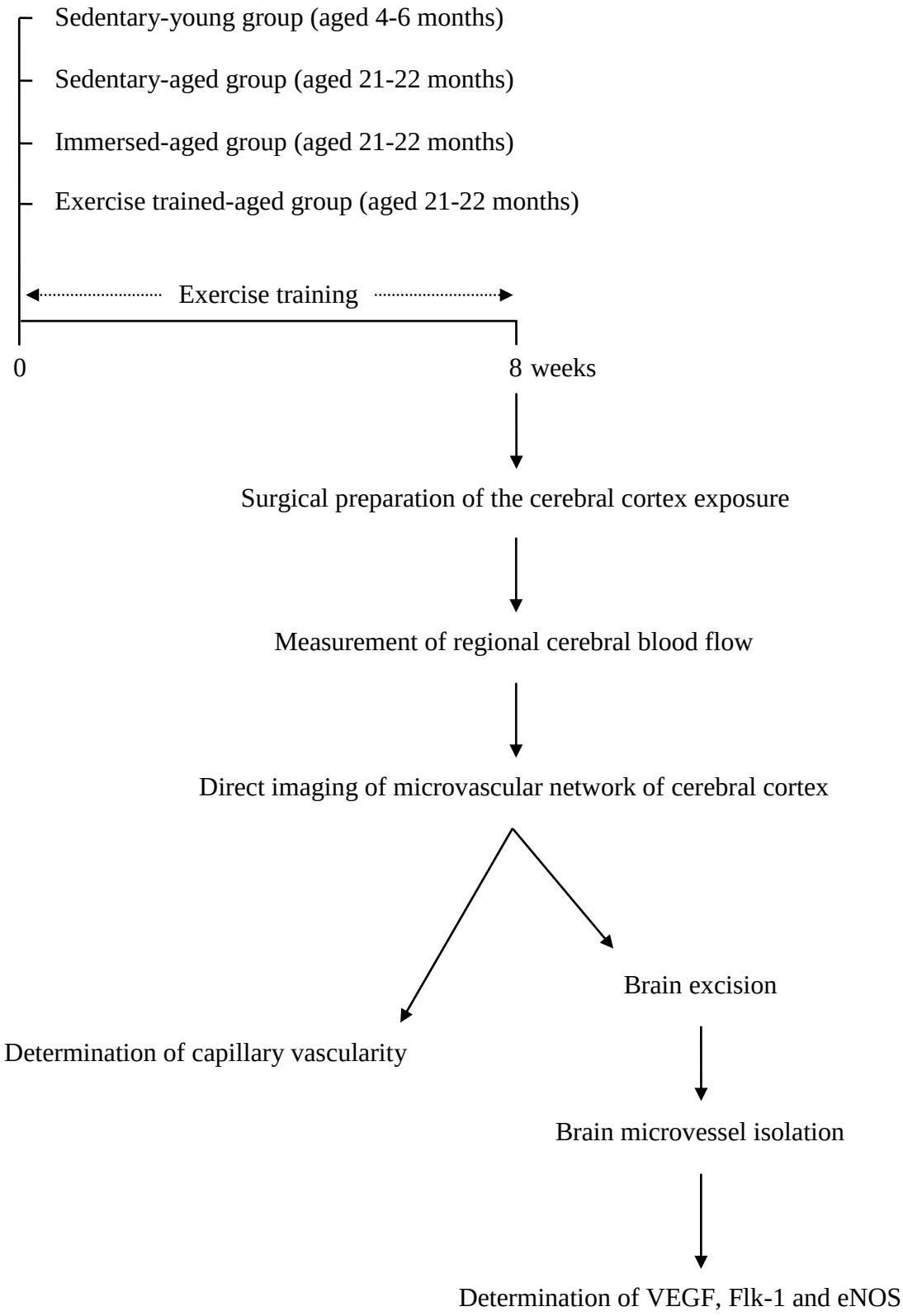


Figure B Schematic diagram showing the protocol for study the effects of exercise training on microvascular changes in aging rat brain.

After surgical preparation of the cerebral cortex exposure, measurement of regional cerebral blood flow was performed using a laser Doppler perfusion monitoring unit (PeriFlux System 5000, Perimed AB, Sweden). Arterial blood gas was sampled before and after direct imaging of cerebral microvascular network. To visualize microvessels, 0.2 ml of fluorescein isothiocyanate-dextran (50 mg/ml; molecular weight 250 kDa) was injected intravenously. The microvascular network was imaged using a laser scanning confocal microscopy system (Eclipse C1 Plus, Nikon, Japan), including an upright fluorescence microscope equipped with epi-illuminator for fluorescence provided by a high pressure mercury lamp (Ushio Inc., Japan) using an appropriate filter for FITC. The light source of using the confocal microscope was an argon-ion laser whose wavelength was 488 nm. The cranial window was explored with 10x objective lens (Plan Fluor 10x dry, Nikon, Japan). The cerebrovascular network was recorded by a computer-base frame grabber (EZ-C1, Nikon, Japan) with controlled gain, offset and exposure time. The visualization and recording of microvascular network was performed within 5 minutes. The collected fluorescent images were off-line analyzed for the capillary vascularity using GLOBAL Lab Image/2 software (Data Translation, Inc., USA).

After the imaging, a cannula was inserted into the apex of the left ventricle to allow perfusion of the brain with 200-ml ice-cold phosphate buffer saline (PBS) containing heparin (25 UI/ml). The brain was then removed and stored at -80°C until isolation of cerebral microvessels. The isolated brain microvessels were used for determining of VEGF, Flk-1 and eNOS levels by immunoassay technique.

Methods

1. Exercise training program

The present swimming exercise protocol is a nonimpact endurance exercise with moderate intensity, modified from method of Eksakulkla et al. (Eksakulkla, Suksom et al., 2009) and Iemitsu et al. (Iemitsu, Maeda et al., 2006). Each day, animals were transported to an exercise training room, and individually swam in cylindrical tanks with a diameter and height of 50 and 65 cm, respectively, in water at a depth of 50-55 cm. Rats were exercised once per day, between 2.00-4.00 p.m., 5

day/week. The animals swam for 15 min/day for the first 2 days, and then the swimming time was gradually increased by 1-wk periods from 15 to 60 min/day. Thereafter, the trained-aged group continued swam for 7 week. Therefore, the trained-aged group received 8 wk of swim training. To minimize stress associated with cold or hot water exposure, water temperature was monitored at 33-36°C. At the end of each training session, rats were dried with towel and hair-dryer. Sedentary-young and sedentary-aged animals were subjected to the same training room, remained in their cages during training hour and handled daily. Immersed-aged animals were placed individually in cylindrical tanks filled with water to a depth of 5 cm which the water was controlled same temperature as the trained-aged animals. The immersed-aged rats were placed in the swimming tanks for 30 minutes/day, 5 days/week for 8 weeks. After 8-week swimming training, immersed-aged and trained-aged animals were rested for at least 24 hour before they conducted to the experiment.

Table B Exercise training program

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

2. Measurement of regional cerebral blood flow

Regional cerebral blood flow (rCBF) was measured by a laser Doppler perfusion monitoring with laser Doppler probe (PeriFlux System 5000, Perimed AB, Sweden) interfaced to a labtop equipped with the PeriSoft data-acquisition software (Perimed Systems, Inc., Sweden). Laser Doppler flowmetry is a noninvasive method of determining tissue perfusion and is now finding widespread use (Matsuura, Fujita et al., 1999). It uses the principle that laser light backscattered from tissue is spectrally broadened by Doppler shifts produced by moving red blood cells. For each measurement, the laser Doppler probe was placed perpendicularly to the cortical surface and was avoided placing over area with large vessels. The rCBF data of each animal was obtained from 3 cortical regions and it was expressed in arbitrary "perfusion units".

3. Determination of capillary vascularity

The recorded fluorescent images were analyzed for capillary vascularity using GLOBAL Lab Image/2 software (Data Translation, Inc., USA) (Yoysungnoen, Wirachwong et al., 2005). The images were acquired by using the same camera settings (gain, offset, and exposure time). The RGB images were converted into binary images in which vascular pixels and perivascular pixels were discriminable based on grayscale intensity. The capillary vascularity was analyzed in area of 100x100 μm rectangular region of interest (ROI). Each image was selected by 5 ROI and each ROI was placed to cover microvessels (<10 μm in diameter).

The software calculated the percentage of capillary vascularity using the following equation:

$$\text{Capillary vasculairty (\%)} = \frac{\text{Number of pixels located within vessels of each ROI}}{\text{Total number of pixels in each ROI}} \text{X100}$$

The determination of capillary vascularity was performed by 5 ROI/image, 3 images/animal and 5-8 animals/group. To avoid bias measurements, a blinded person independently performed assesses the capillary vascularity.

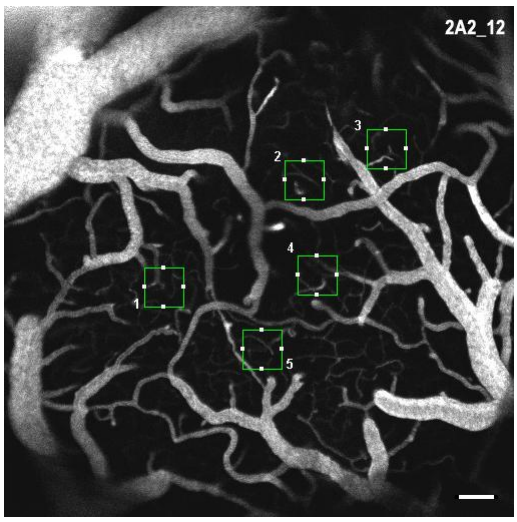


Figure C Grey-scale fluorescent image of microvasculature in the cortical surface. Five rectangular boxes were located to cover area of microvessel (diameter < 10 μm). Scale bar = 100 μm.

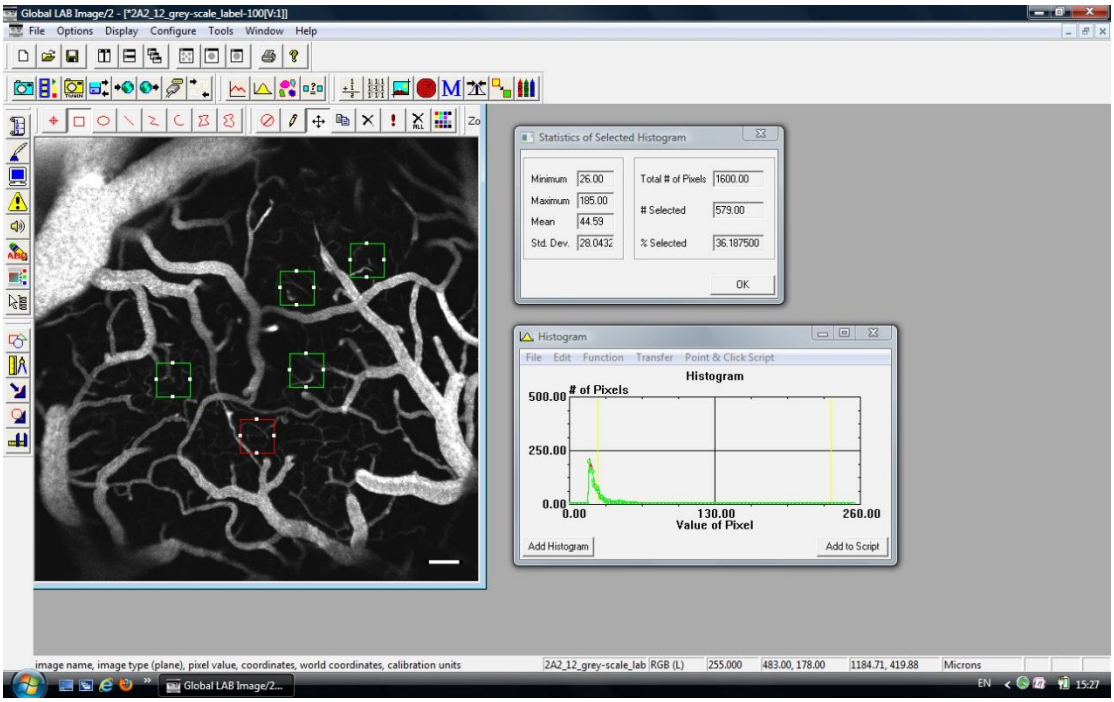


Figure D GLOBAL Lab Image/2 software shows the analysis outcome of capillary vascularity (CV) for red rectangular ROI. The total number of pixels within the ROI is 1,600.00 and the number of pixels within the capillaries is 579.00, and thus CV is estimated as 36.19%.

4. Isolation of brain microvessel

Brain microvessels were isolated according to the method of Yamakawa et al. (Yamakawa, Jezova et al., 2003). The brain was freed of cerebellum and brain stem and was rinsed in ice-cold PBS. The chopped tissue was homogenized in 3 ml of ice-cold isotonic sucrose buffer (0.32 mol/L sucrose, 3 mmol/L HEPES, pH 7.4) using a Potter-Elvehjem glass-teflon homogenizer (Glas-Col, USA) at 3,000 rpm, 10 strokes at 4°C. Homogenate was centrifuged at 1,000 xg for 10 minutes at 4°C. Supernatant was discarded, and resuspended the pellet in 3 ml of ice-cold sucrose buffer, followed by centrifugation at 1,000 xg for 10 minutes at 4°C. The pellet was suspended the sediment in ice-cold sucrose buffer, the suspension was centrifuged twice at 100 xg for 10 minutes at 4°C. The supernatants of the last two centrifugations were pooled and centrifuged at 200 xg for 2 minutes at 4°C. The pellet was washed twice with ice-cold sucrose buffer and once with ice-cold PBS + 0.1% bovine serum albumin (BSA) at 200 xg for 2 minutes at 4°C in each step. The pellet was resuspended in 1 ml of ice-cold PBS + 0.1% BSA, a separated aliquaot was evaluated the purity of microvesssel preparation. The suspension was centrifuged at 14,000 xg for 2 minutes at 4°C. The final pellet was stored at -80°C.

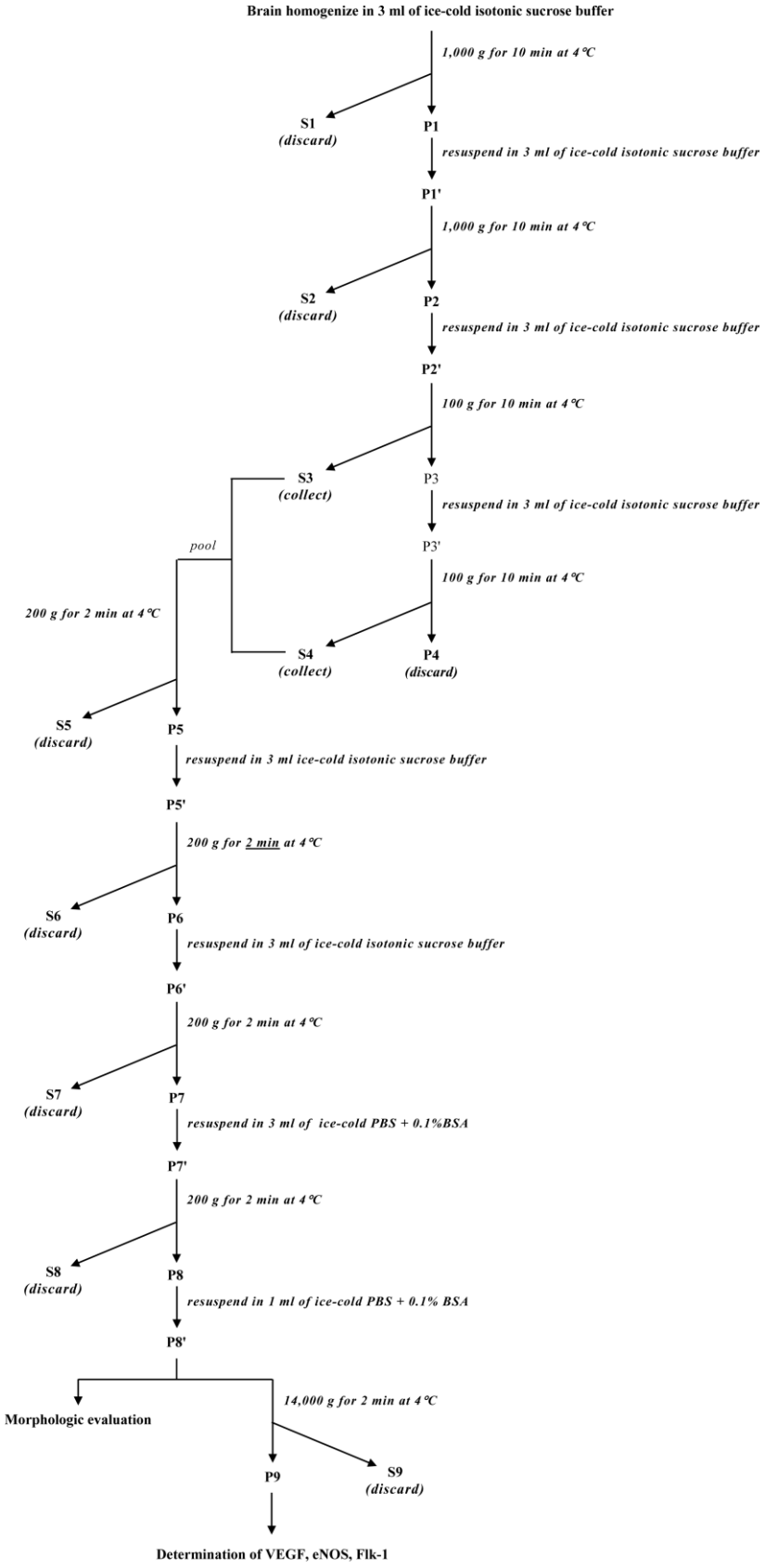


Figure E Schemic diagram showing the method for isolation of brain microvessels.

5. Preparation of plasma

After the abdominal wall was exposed, 4 ml of blood was withdrawn from abdominal aorta using a heparinized syringe. Plasma was obtained by centrifugation of blood at 3,000 rpm for 15 minutes at 4 °C. Plasma aliquot was used to measured lipid profile level by Bangkok RIA Laboratory (Thailand), and lipid peroxide concentration by a commercial colorimetric assay kit (no.10009055, Cayman Chemical, USA).

6. Sample preparation for VEGF, Flk-1 and eNOS immunoassay

Isolated brain microvessels were homogenized in 500 μl of ice-cold RIPA buffer containing protease inhibitor cocktail (Sigma, USA). The homogenate was centrifuged at 1,000 xg for 5 minutes at 4°C. The supernatant as a postnuclear fraction were aliquot and stored at -80°C. Protein concentration of postnuclear supernatant was determined using bicinchoninic acid (BCA) assay kit (no.23252, Pierece, USA). The supernatant aliquots were used for quantification of VEGF, Flk-1 and eNOS protein contents by commercial immunoassay kits (R&D system, USA).

7. Morphologic evaluation of isolated brain microvessels

To assess the purity of microvessel preparation, the brain microvessel fractions were smeared on glass slides. The dried smear slides were fixed by 95% ethanol and stained with hematoxalin. The smeared brain microvessels were observed by a light microscope (SMZ800, Nikon, Japan). The microvessel preparations were free of contamination by other cell fragments.

8. Immunoassay for VEGF, Flk-1 and eNOS

VEGF levels were quantified by a sandwich enzyme immunoassay technique using an ELISA kit (MMV00, R&D Systems, USA). The standard solution or the samples were added in a 96-well plate that was pre-coated with a polyclonal antibody specific for VEGF. The samples were incubated for 2 hours, and after washing the plate a substrate solution was added to the wells. The enzyme reaction yields a blue

product that turns yellow when the stop solution was added. The intensity of the color measured is in proportion to the amount of VEGF bound in the initial step. The sample values are then read off the standard curve.

Flk-1 expression was measured using a solid-phase sandwich ELISA kit (MVR200B, R&D systems, USA). Samples were pipetted into the wells and any Flk-1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for Flk-1 were added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded blue products that turn yellow when the stop solution was added. The intensity of the color measured was in proportion to the amount of Flk-1 bound in the initial step. The sample values were then read off the standard curve.

eNOS protein was quantitated using a commercially available solid-phase sandwich ELISA kits (DEN00, R&D systems, USA). According to the manufacturer, the assay was employ the quantitative enzyme immunoassay technique in which a monoclonal antibody specific for eNOS which was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any eNOS present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for eNOS was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of eNOS bound in the initial step. The color development was stopped after 10 min and the intensity of the color was measured. The eNOS concentration of each sample was calculated from a standard curve.

9. Statistical analysis

Results are expressed as the means±standard error of mean (SEM). The significant differences between groups were determined by using one way analysis of variance (one-way ANOVA), and the difference in pairs of means were evaluated by a least significant difference (LSD) test. The difference was statistically significant if

the statistical probability (p-value) was less than 0.05. Data were analyzed by SPSS 16.0 for Windows (SPSS Inc., USA).

CHAPTER IV
RESULTS

Effect of exercise training on physiological adaptations in aging rat

Body weight and mean arterial blood pressure

Body weight, mean arterial blood pressure, systolic blood pressure and diastolic blood pressure for sedentary-young, sedentary-aged, immersed-aged and trained-aged groups were summarized in Table 1.

Figure 1 shows body weight significantly increased in sedentary-aged (675.50±19.36 g), immersed-aged (686.80±16.87 g) and trained-aged (591.50±14.68 g) rats compared to sedentary-young rats (474.80±10.17 g, $P<0.05$). However, the body weight of trained-aged group was significantly lower when compared to sedentary-aged group ($P<0.05$).

As shown in Figure 2A, systolic blood pressure of both sedentary-aged (144.58±5.07 mmHg) and immersed-aged (144.83±3.43 mmHg) rats were significantly higher compared to sedentary-young (121.00±5.95 mmHg) rats ($P<0.05$). However, there were no significant difference between either trained-aged (130.42±4.10 mmHg) and sedentary-young rats ($P=0.106$) or trained-aged and sedentary-aged ($P=0.109$). Figure 2B demonstrated diastolic blood pressure significantly increased in sedentary-aged (122.71±5.03 mmHg), immersed-aged (123.17±2.64 mmHg) and trained-aged (106.90±4.48 mmHg) rats compared to sedentary-young (88.17±4.48 mmHg) rats ($P<0.05$). However, the diastolic blood pressure of trained-aged group was significantly lower when compared to sedentary-aged group ($P<0.05$). Figure 2C, mean arterial blood pressure (MAP) significantly higher in the sedentary-aged (130.00±5.00 mmHg), immersed-aged (130.39±2.69 mmHg) and trained-aged (115.79±4.30 mmHg) group compared to the sedentary-young (99.11±6.04 mmHg) group ($P<0.05$). However, the trained-aged rats were significantly reduced in MAP compared to the sedentary-aged rats ($P<0.05$).

Table 1 Body weight, mean arterial blood pressure, systolic blood pressure and diastolic blood pressure of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed- aged (IM-Aged) and trained-aged (ET-Aged) rats.

	SE-Young	SE-Aged	IM-Aged	ET-Aged
Body weight, g	474.80±10.17 (5)	675.50±19.36* (8)	686.80±16.87* (5)	591.50±14.68*,# (8)
Mean arterial blood pressure, mmHg	99.11±6.04 (5)	130.00±5.00* (8)	130.39±2.69* (5)	115.79±4.30*,# (7)
Systolic blood pressure, mmHg	121.00±5.95 (5)	144.58±5.07* (8)	144.83±3.43* (5)	130.42±4.10 (7)
Diastolic blood pressure, mmHg	88.17±6.41 (5)	122.71±5.03* (8)	123.176±2.64* (5)	106.90±4.48*,# (7)

Values are expressed as means ± SEM and number of rats in parentheses.

*$P<0.05$; significant differences from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

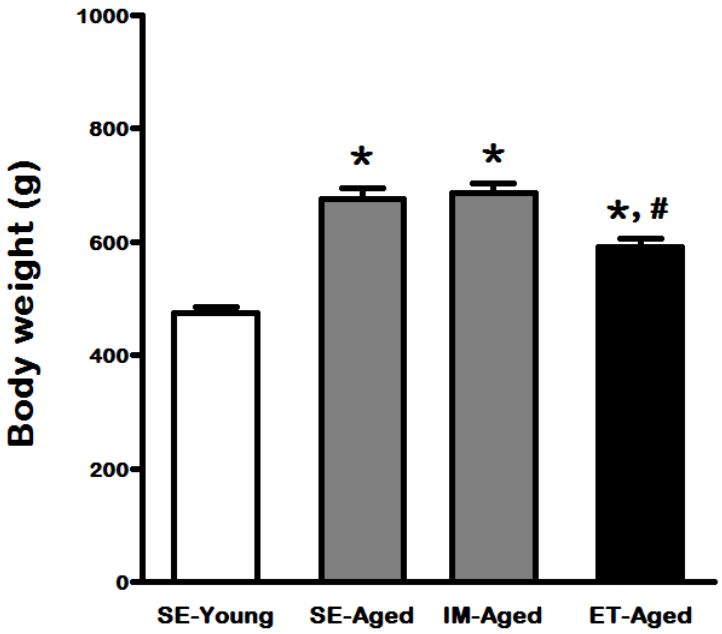


Figure 1 Effect of exercise training on body weight in sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

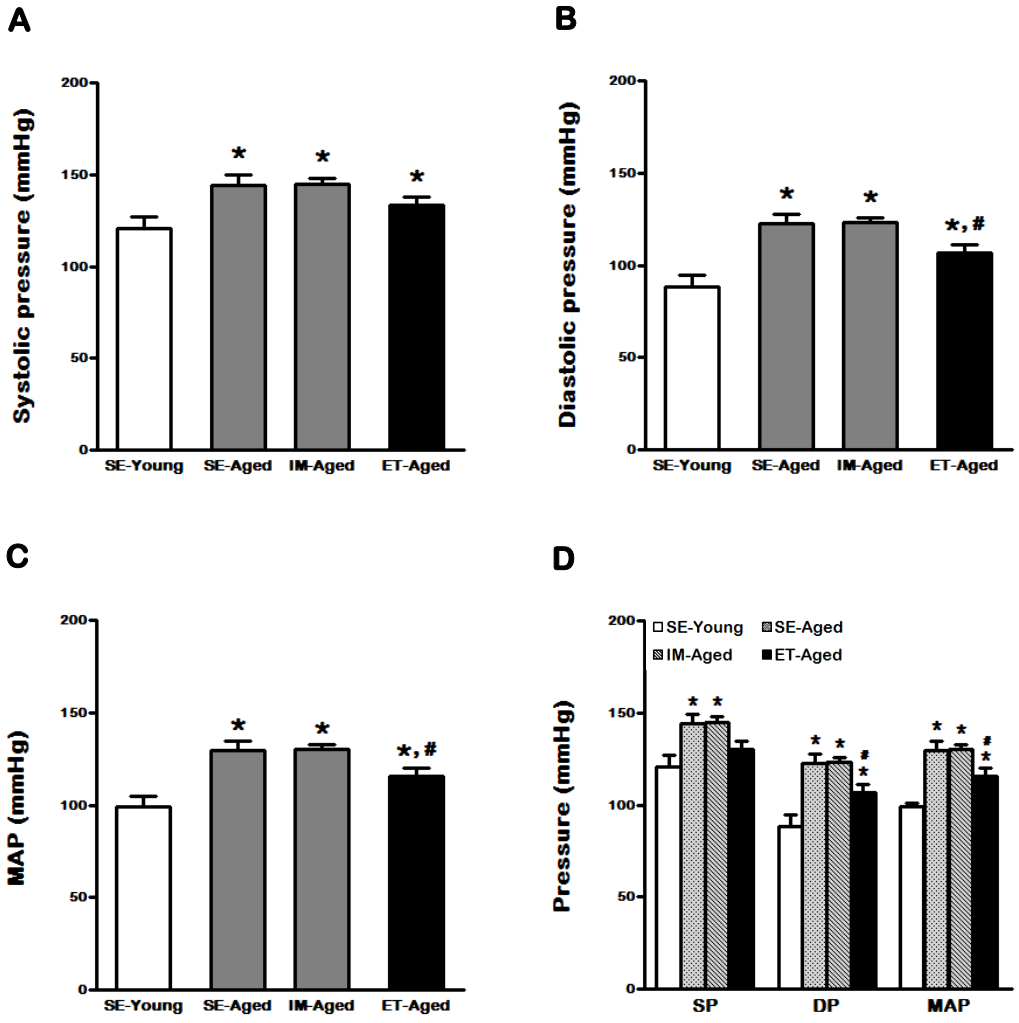


Figure 2 Effect of exercise training on (A) systolic pressure (SP), (B) diastolic pressure (DP) and (D) mean arterial pressure (MAP) in sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

**P*<*0.05*; significantly different from sedentary-young group.

#*P*<*0.05*; significantly different from sedentary-aged group.

Lipid profile

Plasma cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol levels of sedentary-young, sedentary-aged and trained-aged were summarized in Table 2.

As shown in Figure 3A and 3D, plasma cholesterol and LDL-cholesterol level of both sedentary-aged (197.71±12.59 mg/dL) and trained-aged (149.00±13.35 mg/dL) rats were significantly higher compared to the sedentary-young rats (50.67±9.67 mg/dL, $P<0.05$). Figure 3B demonstrated the significantly increase in plasma triglyceride of sedentary-aged rats (197.00±26.51 mg/dL) when compared to those of sedentary-young rats (43.67±14.34 mg/dL, $P<0.05$). However, although the triglyceride level of trained-aged rats (134.62±27.43 mg/dL) had a tendency to increase when compared with sedentary-young group, no statistical significance was observed ($P=0.104$). Figure 3C showed HDL-cholesterol level was significantly higher in both sedentary-aged (79.57±3.75 mg/dL) and trained-aged (93.13±5.49 mg/dL) groups compared to the sedentary-young group (43.00±6.11 mg/dL, $P<0.05$), however, there was no significant difference between the sedentary-aged and trained-aged groups ($P=0.061$). It is note that data of plasma lipid profile for the immersed-aged group was left out from the presenting table and figure due to high variability and lack of data with small sample size.

Lipid peroxide level

Plasma lipid peroxide level of sedentary-young, sedentary-aged, immersed-aged and trained-aged was summarized in Table 3 and Figure 4.

As shown in Figure 3 and Table 3, plasma lipid peroxide level in both sedentary-aged (11.93±2.28 μmol/L) and immersed-aged (12.08±0.94 μmol/L) groups were significantly higher when compared to those in sedentary-young group (1.84±0.46 μmol/L, $P<0.05$). In trained-aged group, plasma lipid peroxide level (5.32±1.60 μmol/L) was significantly decreased when compared to the sedentary-aged group ($P<0.05$), however, the plasma lipid peroxide level of trained-age group was not significant difference when compared to the sedentary-young group ($P=0.153$).

Table 2 Plasma cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol levesl of sedentary-young (SE-Young), sedentary-aged (SE-aged) and trained-aged (ET-Aged) rats.

	SE-Young	SE-Aged	ET-Aged
Cholesterol, mg/dL	50.67±9.67 (3)	197.71±12.59* (7)	149.00±13.35*,# (16)
Triglyceride, mg/dL	43.67±14.34 (3)	197.00±26.51* (7)	134.62±27.43# (8)
HDL-cholesterol, mg/dL	43.00±6.11 (3)	79.57±3.75* (7)	93.13±5.49* (8)
LDL-cholesterol, mg/dL	4.00±1.53 (3)	35.43±6.18* (7)	21.38±2.31*,# (8)

Values are expressed as means ± SEM and number of rats in parentheses.

*$P<0.05$; significant differences from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

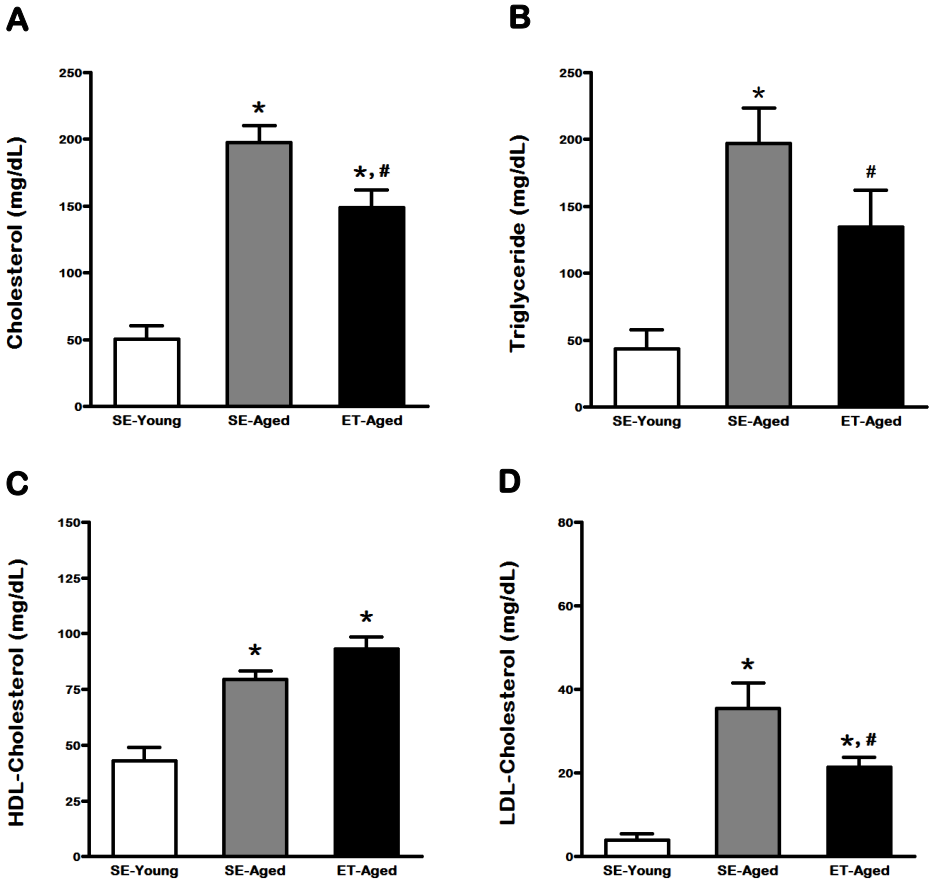


Figure 3 Effect of exercise training on cholesterol (A), triglyceride (B), HDL-cholesterol (C) and LDL-cholesterol (D) levels in sedentary-young (SE-Young), sedentary-aged (SE-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

$^{\#}P<0.05$; significantly different from sedentary-aged group.

Table 3 Plasma malondialdehyde level of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

	SE-Young	SE-Aged	IM-Aged	ET-Aged
Plasma malondialdehyde, nmol/mL	1.84±0.46 (5)	11.93±2.28* (6)	12.08±0.94* (5)	5.32±1.60# (5)

Values are expressed as means ± SEM and number of rats in parentheses.

*$P<0.05$; significant differences from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

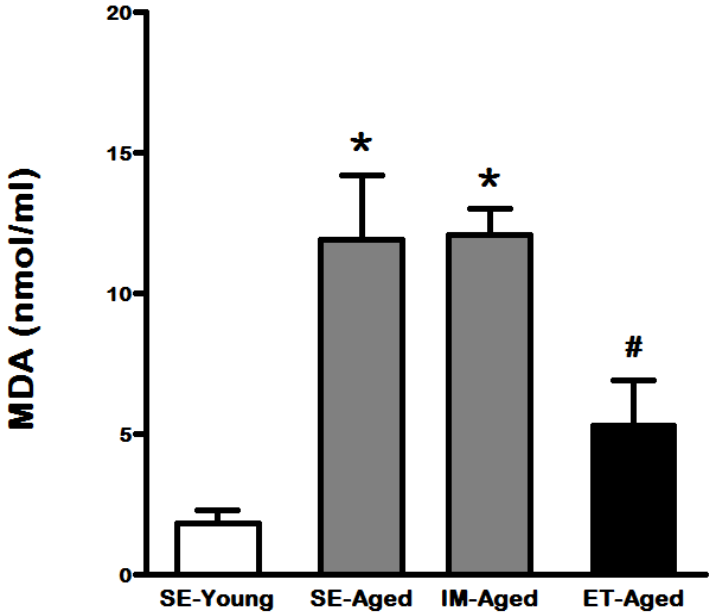


Figure 4 Effect of exercise training on plasma malondialdehyde level (MDA) in sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

$^{\#}P<0.05$; significantly different from sedentary-aged group.

Effect of exercise training on microvascular changes in aging rat brain

Regional cerebral blood flow and brain capillary vascularity

During the experiment (protocol II), the physical condition of the rats was maintained broadly unchanged. Blood gas measurement before and after experiment was summarized in Table 4. None of the differences of the blood gas values before and after experiments are statistically significant.

Regional cerebral blood flow (rCBF) and capillary vascularity of sedentary-young, sedentary-aged, immersed-aged and trained-aged groups were summarized in Table 4.

Figure 5 shows the effect of exercise training on rCBF in aging rats. The rCBF was significantly diminished ($P<0.05$) in sedentary-aged (134.99±14.74 PU), immersed-aged (131.09±17.53 PU) and also trained-aged (255.83±13.17 PU) groups when compared to those in sedentary-young group (334.27±35.00 PU). However, the rCBF in the trained-aged group was significantly higher than of that in the sedentary-aged group ($P<0.05$).

Table 4 Arterial pH, partial pressure of oxygen (PaO_2) and partial pressure of carbon dioxide ($PaCO_2$) in sedentary-young (SE-Young), sedentary-aged (SE-aged), immersed-aged and trained-aged (ET-Aged) rats before and after experiment

	SE-Young	SE-Aged	IM-Aged	ET-Aged
pH				
before	7.45±0.02	7.40±0.01	7.41±0.01	7.39±0.03
after	7.43±0.02	7.40±0.02	7.37±0.01	7.41±0.02
PaO_2, mmHg				
before	91.0±5.5	81.7±1.9	89.3±1.9	87.3±4.4
after	96.7±1.9	88.5±3.5	87.0±3.7	87.3±0.3
$PaCO_2$, mmHg				
before	37.5±2.9	41.7±4.5	45.2±1.1	43.1±2.6
after	41.6±3.1	42.2±2.6	44.5±3.6	38.4±2.4

Values are expressed as means ± SEM.

By variance analysis (ANOVA), arterial pH, PaO_2 and $PaCO_2$ were not significant differences in all groups.

Table 5 Reginal cerebral blood flow (rCBF) and capillary vascularity of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

	SE-Young	SE-Aged	IM-Aged	ET-Aged
rCBF, PU	334.26±35.00 (5)	134.99±14.74* (7)	131.09±17.53* (5)	255.83±13.17*,# (7)
% Capillary vascularity	39.25±2.18 (5)	15.85±1.25* (8)	16.46±1.59* (5)	29.81±1.64*,# (7)

Values are expressed as means ± SEM and number of rats in parentheses.

*$P<0.05$; significant differences from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

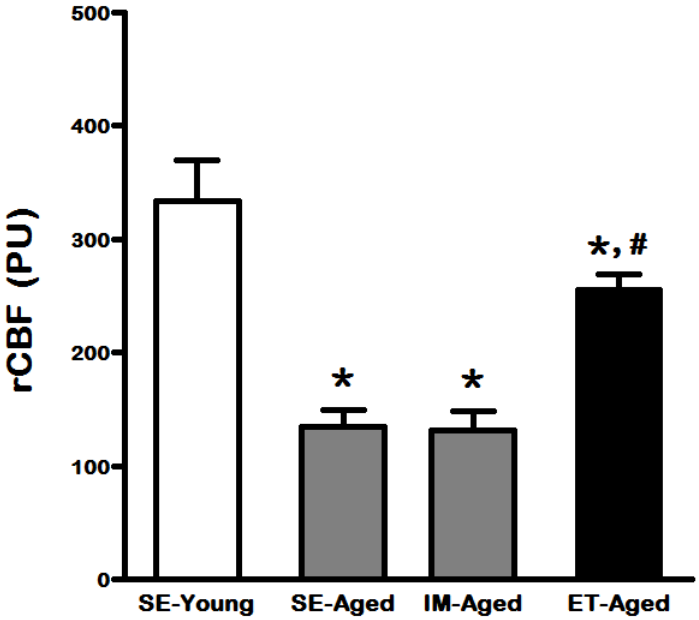


Figure 5 Effect of exercise training on reginal cerebral blood flow (rCBF) in sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

$^{\#}P<0.05$; significantly different from sedentary-aged group.

Figure 6 shows the examples of microvascular network visualized by FITC-dextran under a laser-scanning fluorescence confocal microscope with 10x objective lens from sedentary-young, sedentary-aged, immersed-aged and trained-aged groups. In sedentary-young group, microvascular network exhibited rich of capillary with structurally intact architecture (Figure 6A). In contrast, the capillary network in both aged rats without exercise shows markedly alteration with reduced density of capillaries and had striking shape abnormality (Figure 6B, 6C) when compared to the sedentary-young rats. However, in aged rats with exercise training, capillaries appeared to become greater in density and size (Figure 6D), developed by exercise training.

Figure 7 illustrates the effect of exercise training on brain capillary vascularity in aging rats. The capillary vascularity was significantly reduced ($P<0.05$) in sedentary-aged (15.85±1.25 %), immersed-aged (16.46±1.59 %) and trained-aged (29.81±1.64 %) group when compared with those in sedentary-young group (39.25±2.18 %). As similar to alteration of the rCBF, the capillary vascularity of trained-aged group was significantly greater when compared to that of sedentary-aged group ($P<0.05$).

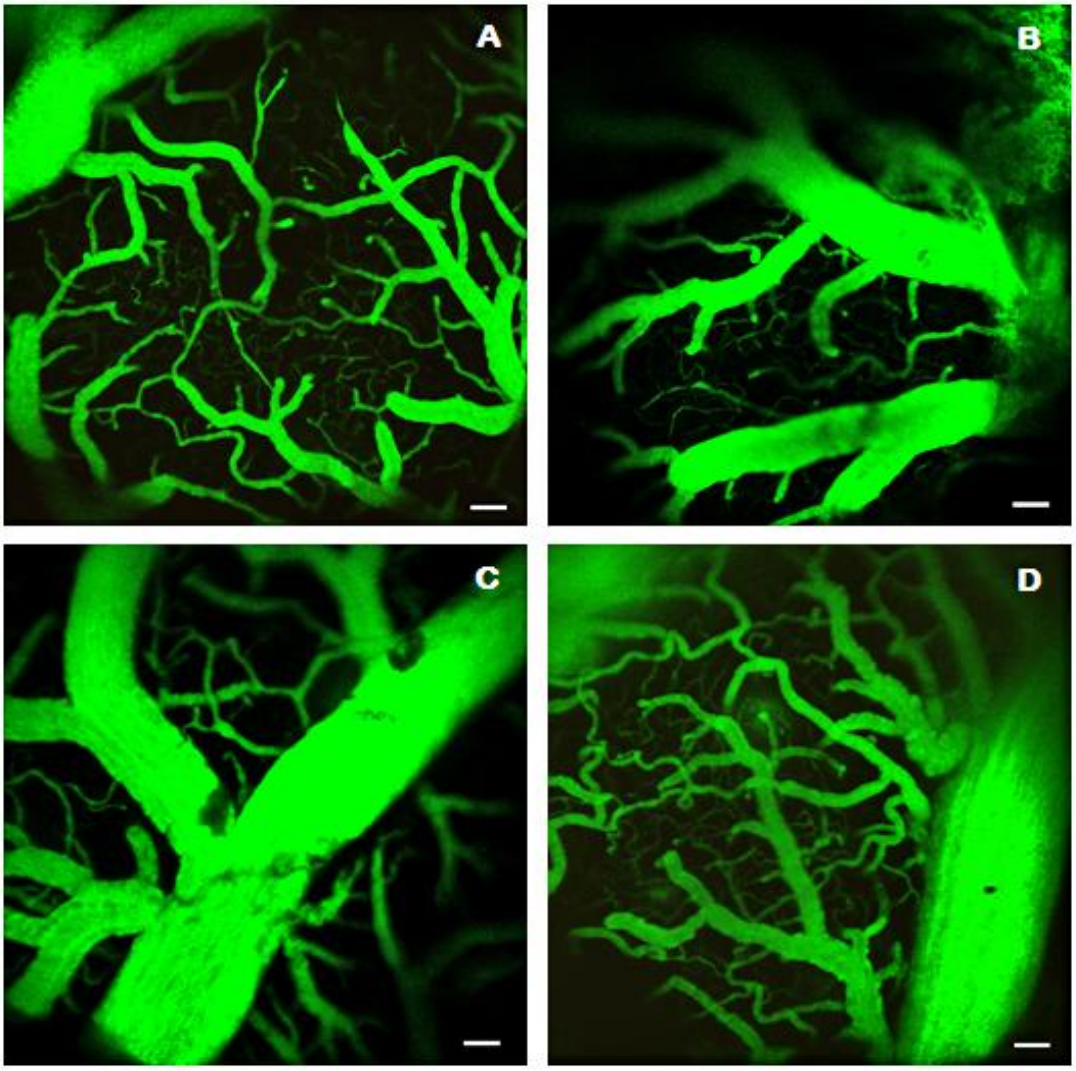


Figure 6 Example of microvascular network visualized by a laser-scanning fluorescence confocal microscope, using FITC-dextran as fluorescence tracer, via a cranial window with 10x objective lens from sedentary-young (A), sedentary-aged (B), immersed-aged (C) and trained-aged (D) groups. Scale bar = 100 μm.

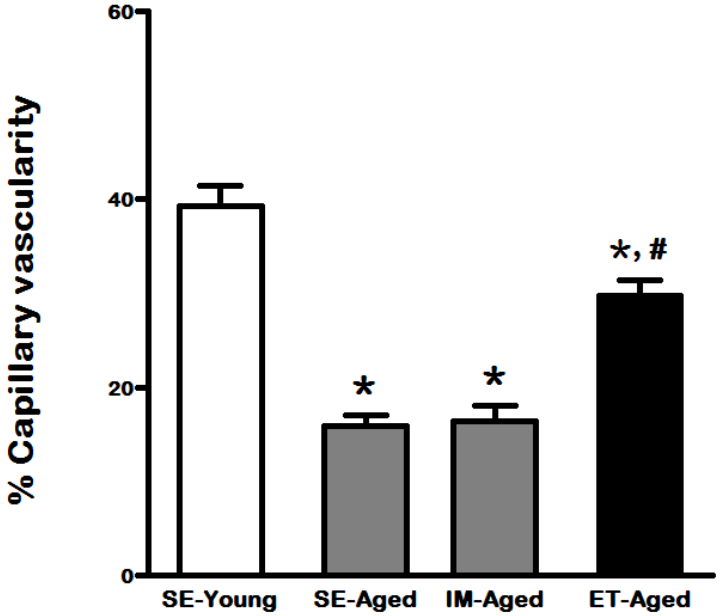


Figure 7 Effect of exercise training on capillary vascularity of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

The preparation of isolation brain microvessel in this study yielded almost exclusively microvessels (Figure 8). The isolated microvessels revealed intact tubular structures. The microvessel preparations were free of adherent debris and structural damage. On the basis of the revealing outcome, the isolation procedure was judged satisfactory.

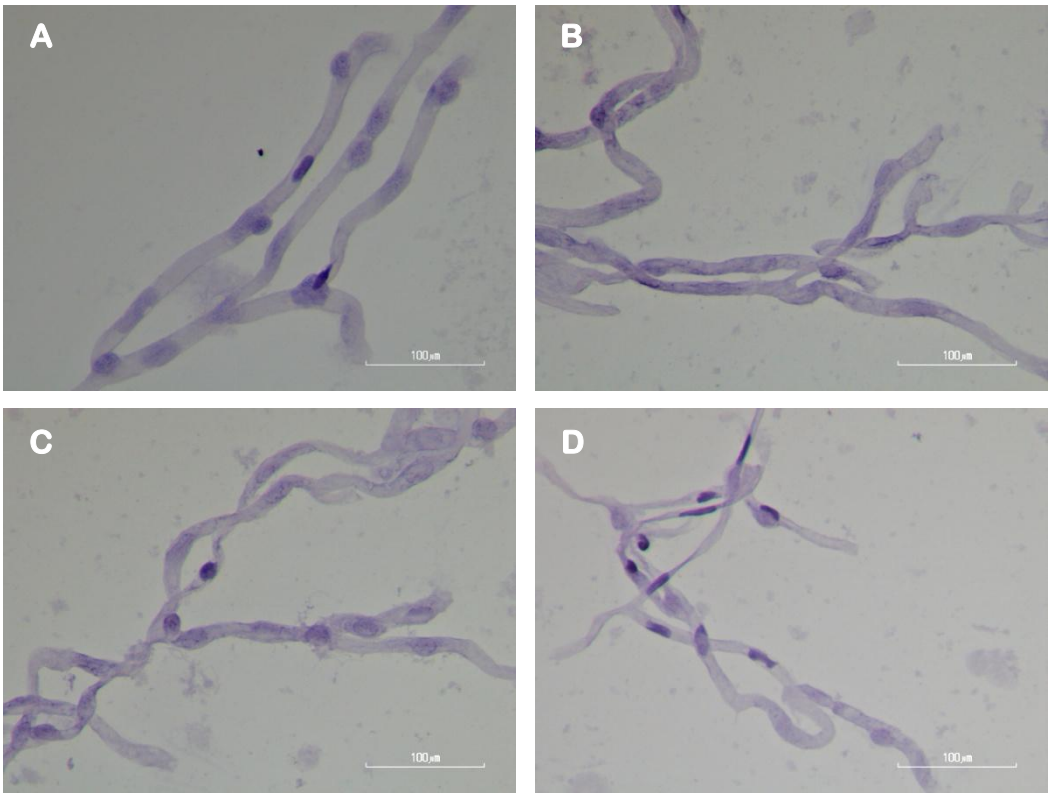


Figure 8 Example of isolated brain microvessels stained with haematoxylin from sedentary-young (A), sedentary-aged (B), immersed-aged (C) and trained-aged (D), observed under a light microscope with 40x magnification.

VEGF, Flk-1 and eNOS

VEGF, Flk-1 and eNOS levels in brain microvessels of sedentary-young, sedentary-aged, immersed-aged and trained-aged groups were summarized in Table 5.

Figure 9 shows the effect of exercise training on brain microvascular VEGF level in aging rats. The VEGF level was significantly lower ($P<0.05$) in sedentary-aged (20.51±1.75 pg/mg protein) and immersed-aged (19.07±1.27 pg/mg protein) groups compared with those in sedentary-young group (32.27±0.93 pg/mg protein). Swimming training caused significant increment ($P<0.05$) of VEGF level in trained-aged (28.35±1.53 pg/mg protein) group when compared to that of sedentary-aged group. Additionally, there was no significant difference in VEGF level when compared between sedentary-young and trained-aged groups ($P=0.075$).

Figure 10 represents the effect of exercise training on brain microvascular Flk-1 level in aging rats. The Flk-1 level of immersed-aged group (0.014±0.003 pg/mg protein) and sedentary-aged group (0.0950±0.01345 pg/mg protein) were significantly lower ($P<0.05$) than those of sedentary-young group (0.076±0.021 pg/mg protein). Besides, the Flk-1 level in trained-aged group (0.064±0.011 pg/mg protein) was not significantly different from both sedentary-young ($P=0.9880$) and sedentary-aged ($P=0.1980$) groups.

Figure 11 illustrates the effect of exercise training on brain microvascular eNOS level in aging rats. Both sedentary-aged and immersed-aged groups have a significantly lower ($P<0.05$) eNOS level (575.62±70.14 and 459.94±98.02 pg/mg protein, respectively) when compared to sedentary-young group (994.39±88.49 pg/mg protein). Regular swimming caused significantly elevated ($P<0.05$) of eNOS level in trained-aged group (926.75±65.08 pg/mg protein) when compared with that of sedentary-aged group. In addition, the eNOS level of trained-aged group was not significantly different ($P=0.5480$) from sedentary-young group.

Table 6 Vascular endothelial growth factor (VEGF), VEGF receptor 2 (Flk-1) and endothelial nitric oxide synthase (eNOS) level in brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and and trained-aged (ET-Aged) rats.

	SE-Young	SE-Aged	IM-Aged	ET-Aged
VEGF, pg/mg protein	32.27±0.93 (5)	20.51±1.75* (6)	19.07±1.27* (5)	28.35±1.53# (7)
Flk-1, pg/mg protein	0.0950±0.01345 (3)	0.0375±0.00790* (5)	0.0142±0.00324* (3)	0.0642±0.01089 (6)
eNOS, pg/mg protein	994.39±88.49 (5)	575.62±70.14* (5)	459.94±98.02* (4)	926.75±65.08# (5)

Values are expressed as means ± SEM and number of rats in parentheses.

*$P<0.05$; significant differences from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

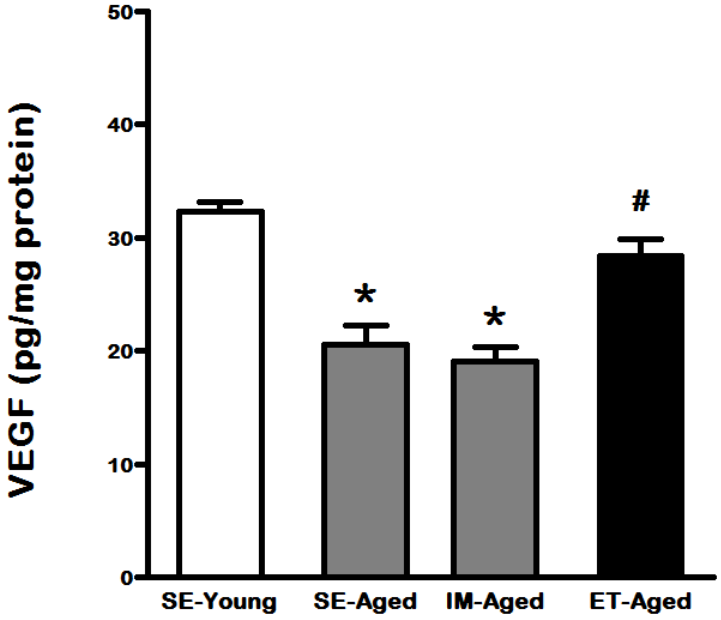


Figure 9 Effect of exercise training on vascular endothelial growth factor (VEGF) in brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

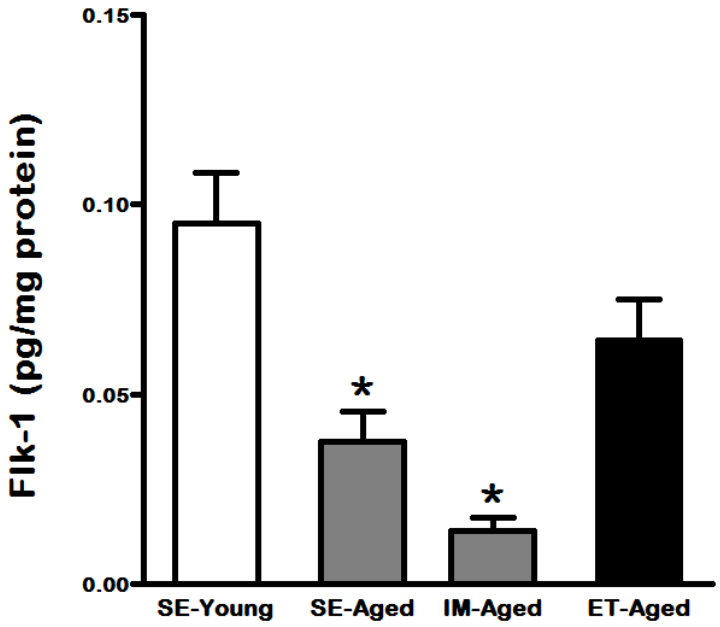


Figure 10 Effect of exercise training on vascular endothelial growth factor receptor 2 (Flk-1) in brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

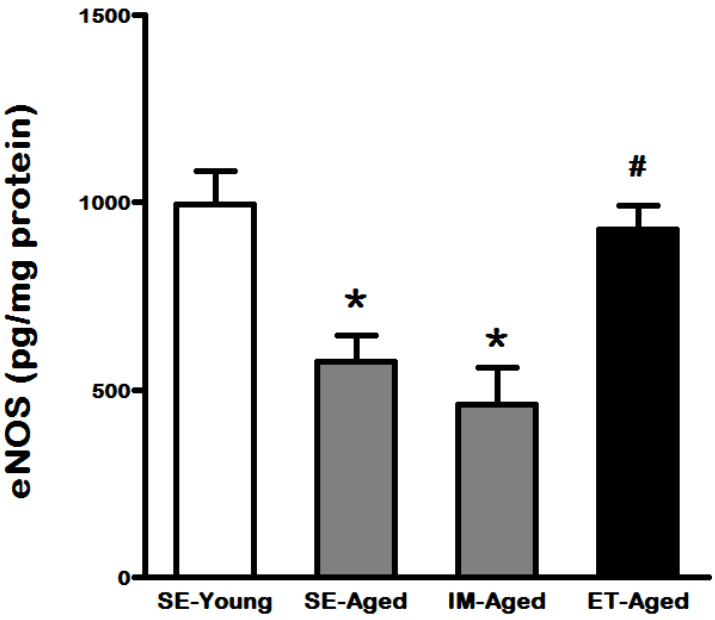


Figure 11 Effect of exercise training on endothelial nitric oxide synthase (eNOS) in brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged) rats.

*$P<0.05$; significantly different from sedentary-young group.

#$P<0.05$; significantly different from sedentary-aged group.

To further study the underlying mechanism of effect of exercise training on microvascular changes in aging rat brain, relationships between either the capillary vascularity and oxidative stress or the capillary vascularity and key angiogenic proteins were examined.

Data of the capillary vascularity and MDA level from sedentary-young, sedentary-aged, immersed-aged and trained-aged groups were collected and plotted in Figure 12. A linear relationship existed between the capillary vascularity and MDA level with a correlation coefficient, r = -0.8921 ($P<0.05$). The linear regression equation was expressed as y =-1.9368x + 40.999.

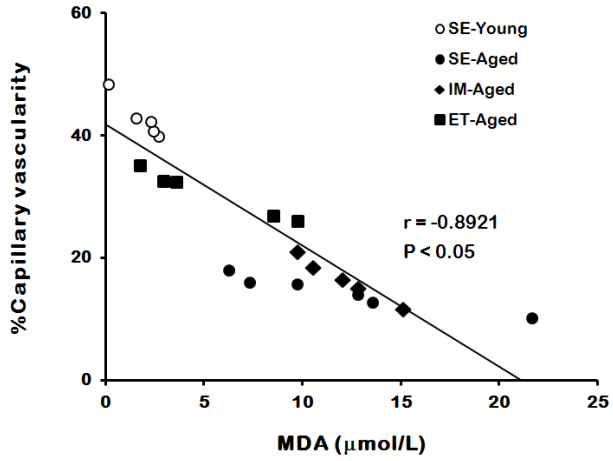


Figure 12 Plotting of data of MDA level (x) and brain capillary vascularity (y) from sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged).

Data of the capillary vascularity, VEGF and eNOS level from sedentary-young, sedentary-aged, immersed-aged and trained-aged groups were collected and plotted in Figure 13. A linear relationship shows between the capillary vascularity and VEGF protein level with a correlation coefficient, r = 0.9353. The linear regression equation was expressed as y = 0.4933x + 12.244. Likewise, A linear relationship shows between the capillary vascularity and eNOS protein level with a correlation coefficient, r = 0.8600. The linear regression equation was expressed as y = 0.0391x – 3.3039.

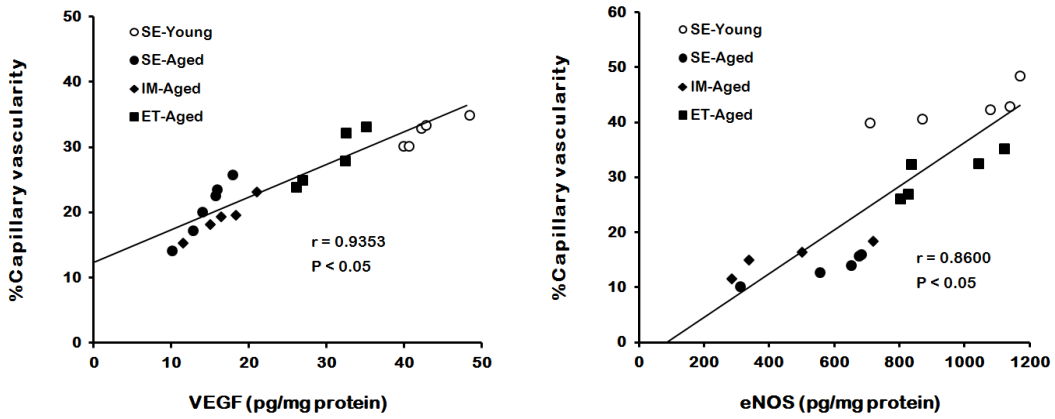


Figure 13 Relationships between percentage of brain capillary vascularity and either VEGF level or eNOS level for sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged).

Data of VEGF and eNOS level from sedentary-young, sedentary-aged, immersed-aged and trained-aged groups were collected and plotted in Figure 14. A linear relationship shows between VEGF level and eNOS level with a correlation coefficient, r = 0.8023. The linear regression equation was expressed as y = 0.0282x + 7.7287.

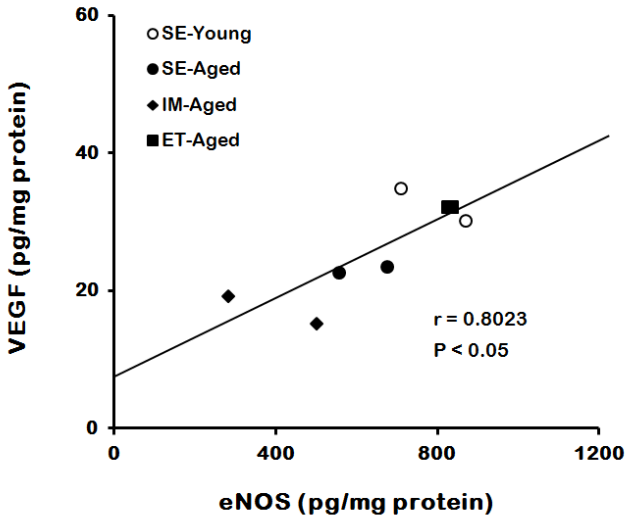


Figure 14 Relationship between VEGF level and eNOS level for sedentary-young (SE-Young), sedentary-aged (SE-Aged), immersed-aged (IM-Aged) and trained-aged (ET-Aged).

CHAPTER V
DISCUSSION

In the present study, performing *in vivo* experiment, revealed the protective effects of moderate exercise training on brain microvascular changes in advancing age. Deterioration of capillary vascularity as well as regional blood perfusion was found in aging brain. Up-regulation of VEGF and eNOS, key angiogenic proteins, may play, in part, a role in the protective effect of exercise training in amelioration of brain microvascular perfusion during aging. Moreover, the result also indicated that the moderate exercise training program could attenuate the decreases in brain capillary vascularity and in blood perfusion with significant associated to the reduction of exacerbating age-induced oxidative stress. Therefore, from these findings of the present study, it implied that ***the protective effect of exercise training on up-regulations of VEGF and eNOS expression represent as considerable factors contributing to the possible underlying mechanism against age-induced alteration of brain microvessels.***

Effects of exercise training on physiological adaptations during aging

The present study showed age induced alteration in physiological characteristics, including body weight, MAP, plasma lipid profile and oxidative stress (**Table 1-Table 3**). These deleterious alterations could be ameliorated by 8-week swimming exercise training. These results confirmed the physiological effectiveness of the swimming training protocol used in the present study.

In the present study, it was found that both sedentary-aged rats and immersed-aged rats showed significantly higher body weight when compared to sedentary-young rats. Greater body weight in old rats occurred due to alteration in body fat content and particular body fat distribution, and reduction of skeletal muscle mass and strength, which are commonly physiological changes found in the elderly (Elmadfa and Meyer 2008). It is well established that exercise training can reduce body mass in the old age, which primarily caused by the reduction of fat mass,

because of capacity of fat oxidation enhanced by endurance exercise (Clavel, Farout et al., 2002). In the current study, our results also showed the decline of body mass in trained-aged group significantly ($P<0.05$) by the swimming training program.

It is well documented that high blood pressure is widely characterized with advancing age. Stiff and rigid arterial wall of older persons are commonly found and this further compounded by hypertension and atherosclerosis. Morphologic alterations in vasculature associated with aging, including lipid and collagen deposit lead to decreased distensibility of peripheral arterial system. Elastin content is also decreased whereas collagen level is increased in elder arteries (Pugh and Wei 2001). These changes can result in less laminar intraluminal flow and increase susceptibility to lipid deposition. Present data showed hypertension also occurred in sedentary-aged rats and immersed-aged rats (**Figure 2**). Most hypertensive elderly have elevation of systolic pressure rather than diastolic pressure, because of central aorta stiffness with aging (Yung, Laher et al., 2009). When the aorta is less able to stretch and accommodates even a normal stroke volume with a higher systolic pressure. Our results confirmed about high systolic blood pressure observed in aging groups. Several mechanisms involved in hypertension in the elderly such as endothelial dysfunction (loss of vasomotor regulation), elevation of oxidative stress, inflammation, cellular apoptosis, and increased concentration of active metabolites (increased myogenic constriction) (Mateos-Caceres, Zamorano-Leon et al., 2012). However, in present study, MAP was significantly lower in trained-aged rats when compare to both sedentary-aged rats and immersed-aged rats. This suggests that age-induced hypertension could be ameliorated by regular moderate exercise training program used in this study. Much evidence affirmed aerobic endurance training could lower high blood pressure in associated to abolishing the imbalance between endothelium-derived relaxing and constricting factors (Donato, Lesniewski et al., 2005, Fagard and Cornelissen 2007, Otsuki, Maeda et al., 2007, Eksakulkla, Suksom et al., 2009). Emerging evidence suggests that aerobic exercise improves endothelial function and reduces blood pressure in hypertensive elderly through the release of endothelium-derived relaxing factors such as NO which is stimulated mainly by the rise in shear stress occurring during exercise (Cornelissen and Fagard 2005). Furthermore, age-related decreased arterial compliance and increased in sympathetic tone have been reported to improve

by exercise; it increases arterial elasticity and decreases sympathetic tone, resulting in decreased blood pressure (Yung, Laher et al., 2009).

It is widely accepted that abnormal levels of lipids and/or lipoproteins in blood are commonly found in elderly, increased the relative risk of cardiovascular diseases. The results of present study also showed that both sedentary-aged rats and immersed-aged rats have dyslipidemic profile (**Table 2**, **Figure 3**). Total cholesterol in the blood increases with age secondary to increasing low density lipoprotein (LDL) cholesterol. The increment of LDL cholesterol is primarily due to a decrease in lipid metabolism which is secondary to either a decrease in the number of functioning LDL receptors in the hepatic and extra-hepatic cells or the result of alterations in the function of LDL cholesterol receptor with age. Despite blood LDL cholesterol level is elevated with advancing age, high density lipoprotein (HDL) cholesterol is lowered as a negative risk factor for cardiovascular events in elderly. However, the current data showed that swimming training could attenuate the dislipidemia in aging rats. Our findings confirmed that physical exercise can give the beneficial effects on dyslipidemic profile which related to its antioxidant properties (Wang, Lin et al., 2000).

In Burneiko's study, it demonstrated that the reduction of HDL/TG ratio has been shown to associate with LDL cholesterol and becomes less susceptible to oxidation (Burneiko, Diniz et al., 2006). Thus, it can be plausible that a beneficial effect of physical exercise on lipid profile is antioxidant-mediated. Moreover, it is well known that endurance training can enhance HDL cholesterol level in the blood (Tambalis, Panagiotakos et al., 2009). The primary reason for the elevation in HDL cholesterol is an increase in lipoprotein lipase activity in response to exercise. Lipoprotein lipase accelerates the breakdown of triglycerides, resulting in a transfer of cholesterol and other substances to the HDL cholesterol (Stefanick, Mackey et al., 1998).

Lipid peroxidation is a major form of cellular and tissue oxidative stress. Malondialdehye (MDA) is a product of lipid peroxidation, which is widely used as an oxidative stress biomarker (Valenzuela 1991, Lykkesfeldt 2007, Burlakova, Zhizhina et al., 2010). In addition, accumulation of lipid peroxide products plays a major role in

age-related diseases (Negre-Salvayre, Auge et al., 2010). The present data demonstrated that plasma MDA level was significantly elevated in both immersed-aged and sedentary-aged rats when compared to those in sedentary-young rats (**Table 3**, **Figure 4**). High lipid with aged is a main target of oxidative attack leading to form lipid peroxidation products. Based on free radical theory of aging, various oxygen species oxidized lipids to give a diverse array of peroxide products (Yin, Xu et al., 2011). Lipid peroxidation induced disturbance of fine structures, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes, modifies LDL cholesterol to pro-atherogenic and pro-inflammatory forms, and generates potentially toxic products (Greenberg, Li et al., 2008). Although a single bout of exercise in aging has been shown to enhance level of ROS, chronic exercise training has been resulted in lower levels of lipid peroxidation (Goto, Radak et al., 2004). Endurance exercise is well known to enhance antioxidant defense capacity, including enhanced activity of free radical scavenging enzymes (e.g. SOD, glutathione peroxidase, catalase) and mitochondrial oxidative enzymes (e.g. citrate synthase, NADH oxidase) , in both young and old rats (Ji 2001, Goto, Radak et al., 2004). The present swimming training with moderate intensity also showed a lower plasma MDA level in trained-aged rats compared to sedentary-aged rats.

The present study elicits that ***8-week swimming exercise training protocol exhibits favorable physiological adaptations to the trained-aged rats, including lower body weight and resting MAP as well as attenuated dyslipidemia and oxidative stress.*** Therefore, it can be concluded that the adequate endurance training is achieved by the present swimming exercise program.

Effect of exercise training on microvascular changes in aging rat brain

The present study describes the direct application performing *in situ* investigation of cerebral microcirculation through the cranial window. The current method based on the combined use of fluorescent intravascular tracers and laser-scanning confocal fluorescence microscopy, developing for real-time *in vivo* study of cerebral microcirculation in rats. This method can be enable on-line dynamically visualize brain parenchymal microvessels. The confocal effect increased the

resolution on in-depth measurements and permits several optical sections to be studied *in vivo* brain parenchyma. Thus, the main advantage of laser-scanning confocal fluorescence microscopy is that it makes possible the high-quality visualization of microvascular network from surface through intraparenchymal at a depth of 100-200 μm. For short period of *in situ* exploration, the brain tissue was not damaged by the laser illumination, and the physiological status was preserved (stable arterial blood gas).

The present data provides clear evidence on the effect of exercise training on alteration of microvessels related to key angiogenic-mediated proteins, VEGF and eNOS, in aging rat brain. Lower brain capillary vascularity and regional blood perfusion as well as downregulation of VEGF and eNOS, exhibited with age, however, physical activity attenuated these unfavourable results associated with its antioxidant and angiogenic properties (**Table 5-6**, **Figure 7**, **9-11**).

Reduction of cerebral blood flow has been found with advancing age. Using sensitive neuroimaging methods, it demonstrated aging related regression in global and regional measurement of CBF, cerebral metabolic rate of oxygen, glucose oxidation, cerebral blood volume and CSF flow (Stoquart-ElSankari, Baledent et al., 2007). The present study confirmed by demonstrating markedly lower regional cerebral blood flow in the old groups (**Figure 5**). The study of de la Torre and colleagues revealed that cerebral neurovascular dysfunction in relation to bioavailability of NO formed by eNOS and nNOS isoforms (de la Torre, Pappas et al., 2003). Impaired vasodilation response of cerebral arteriole during aging (Mayhan, Faraci et al., 1990) may develop cerebral hypoperfusion, which related to depletion of cerebrovascular reserve, leading to increase susceptibility of the brain to vascular insufficiency (Iadecola, Park et al., 2009). Moreover, aging related changes in the systemic circulation and degenerative changes in the extra-cerebral resistance arteries may shift the lower and upper limits of the auto-regulatory plateau to cause cerebral hypoperfusion (Kalaria 2009). The declining CBF and energy metabolism of aging brain appear to have well-described morphological correlates. At the level of the cerebral microvessels, both the capillary density of distinct brain regions and the ultrastructure of the capillary walls are prone to age-related alterations (Farkas and

Luiten 2001). A number of several groups reported the reduction of cerebral capillary density both in humans (Buee, Hof et al., 1994, Brown, Moody et al., 2007) and experimental animals (Sonntag, Lynch et al., 1997, Villena, Vidal et al., 2003, Shao, Li et al., 2010, Murugesan, Demarest et al., 2012). The current data also showed that the aging animal groups had a significantly lower brain capillary vascularity, as an index of capillary density, compared with the young group (**Figure 7**).

The present study demonstrated that preparation of isolation brain microvessel yielded tubular vascular structure with diameters ranging from capillaries to small arterioles (Figure 8). Moreover, the preparation of microvessels was free of contamination by other cells. The revealing outcome of brain microvessel isolation procedure is similar to the other study (Yamakawa, Jezova et al., 2003, Maguin Gate, Lartaud et al., 2011). Therefore, the microvessel fractions are appropriated for determination of VEGF, Flk-1 and eNOS expression.

Age-related brain microvascular rarefaction has the potential to result in inadequate blood flow to the brain. Mechanism for age-related changes in the brain microvasculature is appeared to, at least in part, correlated with declined angiogenic-mediated growth factors. Vascular endothelial growth factor (VEGF) is a widely potential angiogenic-regulating protein for peripheral and central vascular systems. In the presence of VEGF, angiogenesis occurs, but in the absence of VEGF, the capillaries undergo apoptotic regression (Dore-Duffy and LaManna 2007). There appears to be an age-related decline in the capacity for cerebral angiogenesis with reducing VEGF expression (Rivard, Berthou-Soulie et al., 2000). Adeno-associated viral vector expressing VEGF was shown to attenuate cerebral vascular regression in the aging mouse brain (Gao, Shen et al., 2009). A number of investigators reported VEGF downregulation of brain tissues corresponding to reduction of brain capillary density. The present study, isolated brain microvessels were used to examine angiogenic-mediated protein expression providing accuracy result corresponding to alterations of brain microvessels, significantly lower of VEGF expression was found in the aging rats.

In vitro and *in vivo* experiments have demonstrated that eNOS enhances endothelial cell migration, proliferation, and differentiation (Dimmeler, Dernbach et al., 2000, Park, Hong et al., 2002, Bach, Sadoun et al., 2005). It has been shown that blocking eNOS reduces the VEGF-induced cell proliferation and migration (Uhlmann, Friedrichs et al., 2001). The study of Reed and colleagues reported defects in the activation of eNOS result in a defect in NO production, contributes to impaired angiogenesis in aging (Bach, Sadoun et al., 2005). In the brain vasculature, eNOS has been recently reported to play a role in maintaining pre-existing collateral density in adulthood (Dai and Faber 2010). The result of present study also confirmed that brain microvascular eNOS expression was significantly diminished in the aging rats when compared with those in young ones (**Figure 11**). The downregulation of eNOS in brain microvessels is similar to the recent report of Pachter's lab, which revealed a weaker response of eNOS in aged brain neurovascular unit tissues (Murugesan, Demarest et al., 2012).

The angiogenic action of VEGF is mainly mediated by VEGF receptor 2 (KDR/Flk-1) which is primarily expressed in the endothelial cells. Activation of the tyrosine kinase receptor Flk-1 in angiogenesis has been shown associated with activation of eNOS in endothelial cells (Jin, Ueba et al., 2003). In aging, the impairment of Flk-1 has been demonstrated the reduction of NO-mediated vasodilation in coronary arterioles (LeBlanc, Shipley et al., 2008). The data of our study (**Figure 10**) showed that expression of brain microvessel Flk-1 was significantly lower in aging rats when compared to those in young rats. This result is similar to Sun's Lab, which reported the reduction of Flk-1 expression in the cerebral vessels with advancing age, using fluorescence immunostaining technique (Yang, Zhang et al., 2003). They also demonstrated that the distribution of Flk-1 is found in neuron more than found in vessel. This corresponded to the current data that small amount of Flk-1 in isolated brain microvessels in all groups of animal. The less expression of Flk-1 in brain microvessels can reflect that the other VEGF receptors, like VEGF receptor 1 (Flt-1), may involved in regulation of angiogensis (Shibuya and Claesson-Welsh 2006).

As aforementioned, brain aging is characterized by decreases in vascularity and endothelial function. It is well known that regular exercise is associated with reduced cerebrovascular events (Cotman, Berchtold et al., 2007). Exercise exerts direct protective effects on the cerebral circulation, it exhibited increased resting cerebral blood flow in the ischemic lesion (Gertz, Priller et al., 2006). The protective effects of physical activity were abolished in animals treated with NOS inhibitors. Similarly, Endres and colleagues demonstrated that exercise training let to an elevation of resting cerebral blood flow and a reduction of cerebral infarct size in wild-type, but not $eNOS^{-/-}$ (Endres, Gertz et al., 2003). A large cross-section study provided evidence in humans that resting blood flow velocity in the middle cerebral artery is elevated by habitual exercise across different ages (Ainslie, Cotter et al., 2008). In the present study, it is also found that old rats showed significantly decreased rCBF when compared to rCBF of young rats. Interestingly, our results significantly showed that training exercise program used in the present study could increase cerebral blood flow. This finding is agree with the previous idea which suggested that the increased rCBF associated with training are mediated, at least in part, by local changes within the vasculature (Ainslie, Cotter et al., 2008). It has been reported that physical exercise ameliorates age-induced impairment of angiogenesis and VEGF level in several tissues (Ding, Luan et al., 2004, Iemitsu, Maeda et al., 2006).

Exercise has also demonstrated to increase angiogenesis and cerebral perfusion (Ide and Secher 2000, Swain, Harris et al., 2003). Recently, Labandeira-Garcia's Lab showed that physical exercise reversed age-dependent decreases in the density of nigral microvessels and VEGF expression (Villar-Cheda, Sousa-Ribeiro et al., 2009). The current data provided that swimming training restored lower brain capillary vascularity, which data obtained from *in situ* study, as well as improved VEGF protein expression in cerebral microvessels of aging rats. The present study also demonstrated that there is a tendency for elevation of Flk-1 expression in trained-aged group, however, it was not statistically significant difference when compared to those in sedentary-aged group. Since the obtained brain microvessel fractions isolated from each animal in the present study is low (approximately one milligram wet weight), the protein yield is quite low. The low protein yield could consider the small

amount of Flk-1. It was a limitation for immunoassay study and statistics consideration. Thus, it could be suggested to pool the microvessel samples for determination of Flk-1 expression.

NO, produced by endothelial cells, is a regulating factor involved in CBF regulation (Atochin and Huang 2011). It is found that CBF attenuation was observed in parallel with downregulation of eNOS expression in spontaneously hypertensive rats (Jesmin, Togashi et al., 2004). Moreover, fluid shift and arterial blood pressure elevation was associated with diminished middle cerebral artery eNOS protein levels, lower CBF and higher cerebral vascular resistance (Wilkerson, Lesniewski et al., 2005). Physical training has been shown to improve endothelium-dependent vasodilation, in part via upregulation and increased phosphorylation of eNOS (Kojda, Cheng et al., 2001, Hambrecht, Adams et al., 2003). In old animals, exercise training ameliorated reduction in endothelium-dependent vasodilation in muscle arterioles with decreased eNOS mRNA and protein (Spier, Delp et al., 2004). Exercise training restored an age-related decrease in flow-induced vasodilation in muscle arterioles. By using L-NAME, the inhibition of vasodilator responses was increased after exercise training, suggesting that the exercise training-induced enhancement of flow-induced dilation occurs primarily through an NO mechanism (Spier, Delp et al., 2007). ***The present study is the first study showed that exercise training was capable to enhance eNOS expression markedly in brain microvessels in aging rats.***

Although the mechanism by which regular physical activity induces beneficial effects in the cerebral vasculature have not been elucidated, It is possible that repeated exposure to increase in blood flow and shear stress in specific regions of the brain during exercise plays a role (Padilla, Simmons et al., 2011). Study of Porter group showed that cerebral vessels in exercised animals had a smooth surface, which may facilitate laminar blood flow and make them less prone to thrombogenic events than sedentary vessels (Latimer, Searcy et al., 2011). Indeed, there is consistent evidence that blood flow is increased in some areas of the brain during physical activity (Ide and Secher 2000, Secher, Seifert et al., 2008). The increase in cerebral blood flow seems to be linked to the local vasodilator action of metabolites and/or perhaps to other local effects produced by increased neural activity during exercise

(Querido and Sheel 2007, Ogoh and Ainslie 2009). A number of literatures suggested that shear stress may also be a signal for endothelial adaptations particular in vasculatures of noncontracting tissues. The concept that shear can signal the endothelium to alter its phenotype/function is well supported by *in vitro* (Chatzizisis, Coskun et al., 2007, Siasos, Tousoulis et al., 2007, Davies 2009) and *in vivo* (Tinken, Thijssen et al., 2009, Green, Carter et al., 2010, Tinken, Thijssen et al., 2010) data. Many vascular bed flow-induced dilation is known to be mediated predominantly by the production of NO from eNOS (Davies 1995). For cerebral vasculature, it is reported that flow-induced vasodilation *in vivo* involves the activation of NOS and generation of NO (Paravicini, Miller et al., 2006). Recently, D'Amore group demonstrated that cultured human umbilical vein endothelial cells (HUVECs) exposed to shear stress showed an increase in VEGF and Flk-1 expressions compared with the static control. Fluid shear stress also exhibited regulation of endothelial sprouting in a NO-dependent manner *in vitro* (Song and Munn 2011). Moreover, increased capillary shear stress induces angiogensis was found *in vivo* study (Gee, Milkiewicz et al., 2010).

In the present study, the result of correlation analysis (**Figure 13**) confirmed that brain capillary vascularity was apparently found to be associated with both VEGF and eNOS expressions. Under the linear regression results, it presented that capillary vascularity decreases when VEGF and eNOS decrease. In addition, the correlation between VEGF and eNOS (**Figure 14**) also demonstrated that the relationship of VEGF and eNOS may be linked to microvascular alterations affected by aging and exercise training. Therefore, it is possible to conclude that the protective effect of exercise training on ameliorated brain microvascular rarefaction and hypoperfusion, in part, involved in closely the up-regulation of VEGF and eNOS expression.

It is well established that mitochondrial DNA accumulates mutations with aging (Muller, Eckert et al., 2010). A decrease in the mitochondrial antioxidant superoxide dismutase (MnSOD) has also been found in cerebrovasculature with increasing age (Tripathy, Yin et al., 2010). Exposure of brain endothelial cells to oxidized lipids increases NO and ROS production (Hamdheydari, Christov et al., 2003). Approximately 90% of cellular ROS production is attributable to mitochondria

(Muller, Eckert et al., 2010). Although the present study did not determine lipid peroxide level in either the brain tissue or isolated microvessels, however, the plasma MDA level is commonly used as an indicator of oxidative damage. Current data showed the relationship between brain capillary vascularity and plasma MDA level in all groups of animal. This negative correlation could be plausible that exercise training improved microvascular deterioration in the brain partly involves antioxidant mechanism.

Figure 15 is a diagram that proposed the overall possible mechanisms of exercise training on protecting brain microvasculature against aging process. From our finding it appeared that the effective mechanisms of exercise training medicated through the up-regulation of VEGF and eNOS expression in associated with oxidant-antioxidant balance.

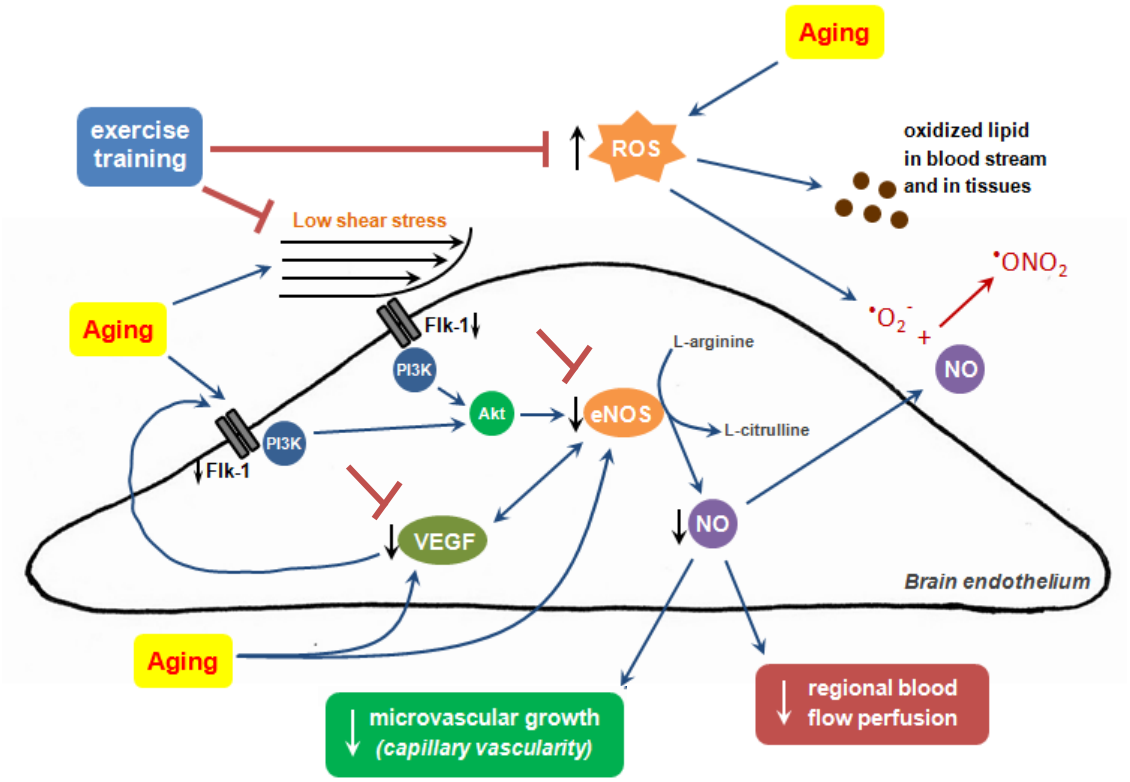


Figure 15 Proposed mechanism from the present study is that aging could increase ROS with the increase of lipid peroxidation product, MDA. Moreover, the cerebral microvascular changes with decreased VEGF and capillary vascularity (CV) were also observed in aged group. However, the exercise training could ameliorate age-induced cerebral microvascular deterioration by enhancing capillary vascularity, elevating regional blood flow perfusion, decreasing oxidative stress as well as increasing VEGF and eNOS expression.

CHAPTER VI
CONCLUSION

The present study aimed to investigate the effect of exercise training on *in situ* microvascular changes in aging rat brain. To understand the mechanism underlying in the exercise-mediated alteration of cerebral microvessels, correlations of brain capillary vascuarity and VEGF as well as eNOS expression in isolated brain microvessels were examined.

The main findings could be concluded as follows:

1. Swimming exercise training program employed in this study is effective and sufficient to cause endurance performance in aging rats, indicated by lower body weight and resting MAP as well as attenuated dyslipidemia and oxidative damage.
2. Aging reduced brain capillary vascularity and regional cerebral blood perfusion, and these alterations was ameliorated by exercise training.
3. Downregulation of VEGF and eNOS expressions were found in aging brain microvessels, and the key-regulated protein downregulations were improved by exercise training.
4. Linear relationship between brain capillary vascularity and plasma MDA level was observed in non-exercised and exercised old rats as well as young rats.
5. Either correlation of brain capillary vascularity and VEGF as well as eNOS expressions or correlation of VEGF and eNOS expressions in brain microvessels demonstrated a linear regression line in non-exercised and exercised aged rats as well as young rats.

In conclusion, results from the present study implied that antioxidant effects of exercise training could protect brain microvascular and blood perfusion against aging, particularly associated with its actions on VEGF and eNOS expressions.

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APPENDICES

APPENDIX A

List of presentations

Oral Presentation

1. Viboolvorakul S, Niimi H, Wongeak-in N, Eksakulkla S and Patumraj S. Effects of exercise training on muscle VEGF level and bone microvascular density in aging rats. The 11th Thai Society of Microcirculation Annual Meeting, Bangkok, Thailand, December 26, 2008.
2. Viboolvorakul S and Patumraj S. Exercise training ameliorates age-induced capillary rarefaction in the brain associated with VEGF and eNOS expressions. The 8th Asian Congress for Microcirculation, Bangkok, Thailand, October 26-28, 2011.

Poster presentation

1. Viboolvorakul S, Niimi H, Wongeak-in N, Eksakulkla S and Patumraj S. Effects of exercise training on muscle VEGF level and bone microvascular density in aging rats. The 7th Asian Congress for Microcirculation, Taishan, China, October 17-19, 2008.
2. Viboolvorakul S, Niimi H, Wongeak-in N, Eksakulkla S and Patumraj S. Exercise training attenuates age-induced bone and muscle microvascular suppression. The 2nd National Aging Conference, Bangkok, Thailand, February 9- 12, 2010.
3. Viboolvorakul S, Eksakulkla S, Wongeak-in N, Niimi H and Patumraj S. Exercise training could reduce age-induced microvascular impairment related to its anti-oxidant potential. The 9th World Congress for Microcirculation, Paris, France, September 26-28, 2010.
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APPENDIX B

Protocol for hematoxalin staining of smeared brain microvessels

Smear preparation

1. A drop of isolated brain microvessels fraction was placed on the center of slide (Fisherbrand® SuperFrost/Plus positively-charged microscope slides, *Cat. No. 12-550-15*).
2. The one slide (up-above) with one end flush was held on the down-below slide (has microvessel's drop) and gently wiped the edge of the up-above slide along the middle of the down-below slide's surface. The microvessels were smeared along the slide, making a layer thin enough to view clearly. After that, the smear was waited for air dry.
3. Once the smear was dried, it was fixed with ***95% alcohol*** overnight at room temperature (by immersing the slides in staining jar containing 95% alcohol).

Hematoxalin staining

1. Rinse slides in running tab water for 5 minutes.
   (Blot excess water from slide holder before going into hematoxalin)
2. Stain slides in ***hematoxalin*** for 3 minutes.
3. Rinse slides in running tab water for 3 minutes *(remove excess stain)*.
   (Blot excess water from slide holder before going into the next step)
4. Immerse slides in ***1% lithium carbonate*** (blueing solution) for ***10 dips*** (fast).
5. Rinse slides in running tab water for 3 minutes *(remove excess stain)*.
6. Dehydration
   - ***95% alcohol*** ***10 dips*** (*2 times*)
   - ***100% alcohol*** ***10 dips*** (*2 times*)

   (Blot excess ethanol before going into xylene)

7. Clear slides by immersing slides in ***xylene*** for ***10 dips*** (*2 times*).
   (Blot excess xylene before going into slide mounting)

Slide mounting *(performed every steps in hood)*

1. Immerse the slide in xylene jar.
   (Remove excess xylene using gauze before going to permount dropping)
2. Place a drop of ***Permount*** (xylene based) *(Fisher Scientific #SP15-100, histological mounting medium)* on the lower edge of the slide, taking care to leave no bubbles.
3. Drop coverslip over the stained smeared microvessels from a low angle with the edge of the slide (let fall gently onto the slide to avoid bubbles). Allow the Permount to spread beneath the coverslip, covering the stained smear microvessels.
4. Dry the slide overnight in the hood.

BIOGRAPHY

NAME	Sheepsumon Viboolvorakul
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AWARDS

1. Travel Award, the 7th Asian Congress for Microcirculation, Taishan, China, October 17-19, 2008.
2. Young Investigator Award, The 8th Asian Congress for Microcirculation, Bangkok, Thailand, October 26-28, 2011.

PUBLICATIONS

1. Viboolvorakul S, Niimi H, Wongeak-in N, Eksakulkla S and Patumraj S. Increased capillary vascularity in the femur of aged rats by exercise training. Microvascular Research (2009) 459-463. (IF = 3.0)
2. Viboolvorakul S, Eksakulkla S, Wongeak-in N, Niimi H and Patumraj S. Exercise training could reduce age-induced microvascular impairment related to its anti-oxidant potential. British Journal of Medicine & Medical Research (2011) 385-396. (IF = 0.89)