รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาผลของการฝึกออกกำลังกายต่อการลดลงของหลอดเลือดในสมองของหนูแก่: บทบาทของวีอีจีเอฟในการส่งสัญญาณการเกิดหลอดเลือดใหม่

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา สำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยรังสิต

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

การลดลงของการไหลเวียนเลือดและจำนวนหลอดเลือดขนาดเล็กในสมองพบได้ในวัยชรา ซึ่งสามารถนำไปสู่ภาวะที่สมองได้รับเลือดไม่เพียงพอได้ โดยพบว่าการลดลงของหลอดเลือดฝอยมีความสัมพันธ์กับการลดลงในการส่งสัญญาณของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ (วีอีจีเอฟ) การฝึกออกกำลังกายอย่างสม่ำเสมอส่งเสริมการไหลเวียนเลือดในสมองรวมถึงสามารถกระตุ้นขบวนการสร้างหลอดเลือดใหม่ในวัยชราได้ อย่างไรก็ตามกลไกที่อธิบายผลของการฝึกการออกกำลังกายต่อการเปลี่ยนแปลงของหลอดเลือดในสมองยังไม่ทราบแน่ชัด การศึกษาวิจัยนี้มีจุดประสงค์เพื่อศึกษาผลของการฝึกออกกำลังกายต่อการเปลี่ยนแปลงของหลอดเลือดในสมอง โดยมุ่งเน้นศึกษาบทบาทการส่งสัญญาณของวีอีจีเอฟในการส่งสัญญาณการเกิดหลอดเลือดใหม่ ในการทดลองใช้หนูขาวเพศผู้สายพันธุ์วิสตาร์ โดยแบ่งออกเป็น 3 กลุ่ม คือ 1) กลุ่มหนูวัยเจริญพันธุ์ 2) กลุ่มหนูแก่ไม่ได้ออกกำลังกาย 3) กลุ่มหนูแก่ได้รับการฝึกออกกำลังกายด้วยการว่ายน้ำ 5 วันต่อสัปดาห์ เป็นเวลา 8 สัปดาห์ โดยได้ทำการศึกษาการเปลี่ยนแปลงของหลอดเลือดที่สมอง ซึ่งแบ่งเป็น 1) วัดการไหลเวียนของเลือดที่สมอง 2) ฉีดสารเรืองแสงและส่องตรวจดูด้วยกล้องจุลทรรศน์แบบส่องกราดด้วยแสงเลเซอร์และบันทึกภาพหลอดเลือดสมองที่เรืองแสงเพื่อนำไปวิเคราะห์หาปริมาณหลอดเลือดขนาดเล็ก สำหรับการศึกษากลไกของการออกกำลังกายต่อการเปลี่ยนแปลงของหลอดเลือดสมองของหนูแก่ ได้ทำการแยกหลอดเลือดขนาดเล็กจากเนื้อสมองเพื่อนำมาตรวจหาปริมาณของวีอีจีเอฟ, เอฟเอลเค-1, พีไอ-3-เค และเอเคที ซึ่งเป็นสารที่เกี่ยวข้องกับการส่งสัญญาณการเกิดหลอดเลือดใหม่ โดยวิธีการวัดปฏิกิริยาแอนติบอดี-แอนติเจน ผลการวิจัยพบว่าการออกกำลังกายทำให้มีการเปลี่ยนแปลงทารสรีรวิทยาของหนูแก่ที่ดีขึ้นอย่างมีนัยสำคัญทางสถิติ คือน้ำหนักตัวและความดันเลือดลดลงเมื่อเทียบกับหนูแก่ไม่ได้ออกกำลังกาย นอกจากนี้ยังพบว่าการออกกำลังกายกระตุ้นการไหลเวียนเลือดและสร้างหลอดเลือดขนาดเล็กที่สมองของหนูแก่ที่ลดลงได้อย่างมีนัยสำคัญทางสถิติ อีกทั้งยังพบว่าการออกกำลังกายกระตุ้นการสร้างวีอีจีเอฟ, เอฟเอลเค-1, พีไอ-3-เค และเอเคทีซีในหลอดเลือดขนาดเล็กที่สมองของหนูแก่อย่างมีนัยสำคัญทางสถิติ ผลการวิจัยสรุปได้ว่าการออกกำลังกายสามารถกระตุ้นการไหลเวียนเลือดและสร้างหลอดเลือดขนาดเล็กที่สมองของหนูแก่ได้ โดยกลไกของการเปลี่ยนแปลงของหลอดเลือดที่สมองภายใต้อิทธิพลของออกกำลังกายส่วนหนึ่งผ่านการกระตุ้นการส่งสัญญาณของวีอีจีเอฟในการส่งสัญญาณการเกิดหลอดเลือดใหม่

Keywords: Exercise, Aging, Angiogenesis, Cerebral Microcrovascular

Abstract

During advancing age, reduction of basal blood flow and microvascular loss in the brain contributes tissue perfusion insufficiency. Capillary loss in aged tissues appears to be related to downregulation of vascular endothelial growth factor (VEGF) signaling. Regular exercise has been reported to have beneficial effects to the brain health, including promoted blood flow, enhanced angiogenesis in aging individuals. However, the underlying mechanisms are largely unknown. The present study aimed to investigate effect of exercise training on age-induced cerebrovascular alterations with modulation of VEGF signaling. Male Wistar rats were divided into three groups: sedentary-young, sedentary-age, and exercise-age. Exercise program included swimming training 5 days/week for 8 weeks. Physiological characteristics including body weight and mean arterial blood pressure 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, Flk-1, PI3K and Akt 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 exercise-aged rats. In addition, CV and rCBF significantly decreased in non-exercise old rats when compared to those in young rats, however, in exercise-aged rats, CV amd rCBF significantly increased when compared to those in old rats without training. Further, age induced significant downregulation of VEGF, Flk-1, PI3K and Akt expression in aged rats when compared to those in young rats. In addition, exercise significantly upregulated VEGF, Flk-1, PI3K and Akt level in exercise-aged rats when compared to those in non-exercise old rats. Therefore, it implied that effects of exercise training could protect brain microvascular and blood perfusion against aging, particularly associated with upregulation of VEGF angiogenic signaling cascade.

Keywords: Exercise, Aging, Angiogenesis, Cerebral Microcrovascular

Introduction

The number of elderly in the world is growing rapidly. Thailand also experienced a rapid and extensive growth. The rapid growth of global aging population has profound implications for many aspects of human health. The Ministry of Public Health reported that the cerebrovascular disease is the most important leading cause of death in elderly persons.

Structural and functional alterations in cerebral vasculature are of pivotal importance in the pathogenesis of the brain. Cerebrovascular dysfunction has been demonstrated a key contributor to age-related brain pathogenesis. Moreover, aging is associated with a reduction in resting cerebral blood flow and a dysfunction of mechanisms regulating cerebral circulation. These alterations reduce cerebral perfusion and increase susceptibility of the brain to vascular insufficiency and ischemic injury.

Microvascular density is an important determinant of the capacity for blood flow through a region of brain tissue. In aging, brain regions exhibit marked capillary loss and increase in microvascular tortuosity, leading to increase resistance to flow and reduce tissue perfusion. The mechanism responsible for suppression of capillary in aging appears to be related to a reduced expression of vascular endothelial growth factor (VEGF), known as a potent angiogenic growth factor.

VEGF signaling pathway is considered a major stimulating factor in the angiogenesis process. VEGF receptor 2 (VEGFR2/Flk-1) has an important role in VEGF-mediated angiogenesis. VEGF activates angiogenic signaling cascade which promotes angiogenesis in association with phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway. VEGF-dependent phosphorylation of Akt via Flk-1 receptor results in the activation of endothelial nitric oxide synthase (eNOS), which finally leads to enhanced nitric oxide (NO) production.

Exercise training has been shown to induce angiogenesis as well as VEGF expression in the brain, suggesting that regular exercise decreased incidence of cerebrovascular events. Exercise training has demonstrated significant improvement of angiogenesis related to expression of VEGF in aged rat brain. However, the molecular mechanisms underlying the exercise-induced improvement of angiogenesis in the aged brain are largely unknown. The study of alteration of molecular expression of VEGF and its receptors and the angiogenic signaling pathway in the brain by exercise training in aging remains a significant challenge. Therefore, the present study aims to investigate effect of exercise training on age-induced changes in cerebral microvascular networks, and also to determine whether VEGF angiogenic signaling cascade participates in this underlying molecular mechanism of exercise training or not.

Objectives

1. To examine the effect of exercise training on aging-induced microvascular deterioration in rat brain.
2. To investigate the possible mechanisms underlying the beneficial effects of exercise traininig on modulation of VEGF angiogenic signaling cascade in rat brain microvessels.

Methods

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.

Rats were randomly divided into 3 groups as follows: Group 1) sedentary-young (aged 4 months), rats were subjected to the same swim environment as the exercised-aged animals, except they remained in their cages; Group 2) sedentary-aged (aged 22 months), 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 3: exercise-aged group (aged 22 months), rats were individually swum in cylindrical tanks 1 hour/day, 5 days/week for 8 weeks.

The present swimming exercise protocol is a nonimpact endurance exercise with moderate intensity. 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, 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. Thereafter, the trained-aged group continued swam for 7 week. Therefore, the exercised-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-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. After 8-week swimming training, sedentary-aged and exercised-aged animals were rested for at least 24 hour before they conducted to the experiment.

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). Rat's body temperature was maintained at 37°C. The rat was tracheotomized and was mechanically ventilated with room air with supplemental oxygen by an animal ventilator. A catheter was placed into the right femoral artery to obtain a blood sample for measurement of blood gas. The arterial blood gases were monitored throughout the experiment, and the blood gas values were recorded and maintained stable within normal ranges. 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 stereotaxic apparatus. When the animal head was fixed in the stereotaxic frame, the skull was exposed. Using a low-speed drill, 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. A custom-made stainless metal ring (8 mm diameter) was then sealed over the cranial window using dental cement. The cerebral cortex was suffused with artificial cerebral spinal fluid.

After surgical preparation of the cerebral cortex exposure, measurement of regional cerebral blood flow (rCBF) was performed using a laser Doppler perfusion monitoring unit with laser Doppler probe interfaced to a labtop equipped with data-acquisition software. Laser Doppler flowmetry is a noninvasive method of determining tissue perfusion and is now finding widespread use. 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.

To visualize microvessels, 0.2 ml of fluorescein isothiocyanate-dextran was injected intravenously. The microvascular network was imaged using a laser scanning confocal microscopy system, including an upright fluorescence microscope equipped with epi-illuminator for fluorescence provided by a high pressure mercury lamp 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. The cerebrovascular network was recorded by a computer-based frame grabber 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.

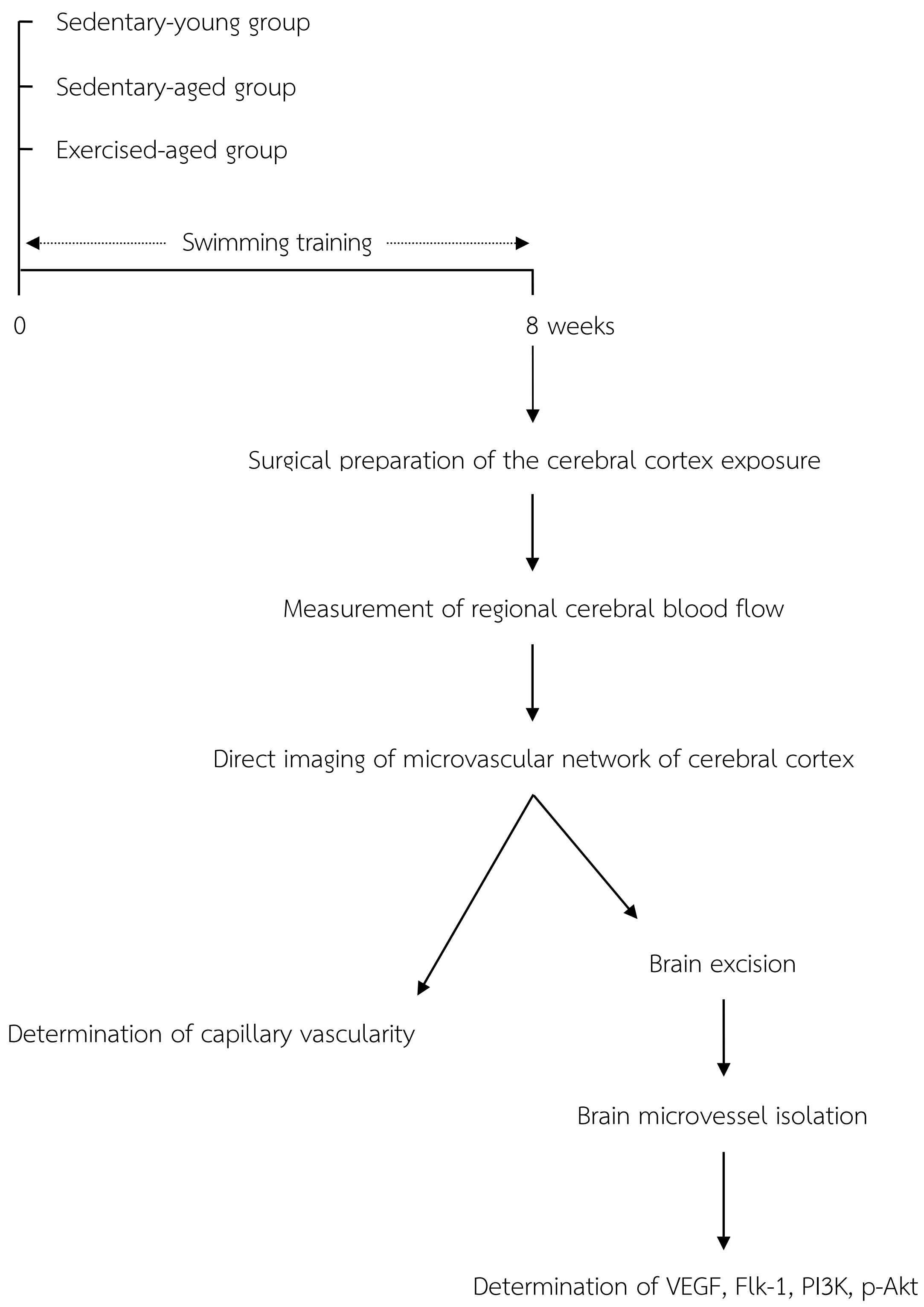

Figure A Schematic diagram showing the protocol for study the effects of exercise training on microvascular changes in aging rat brain.

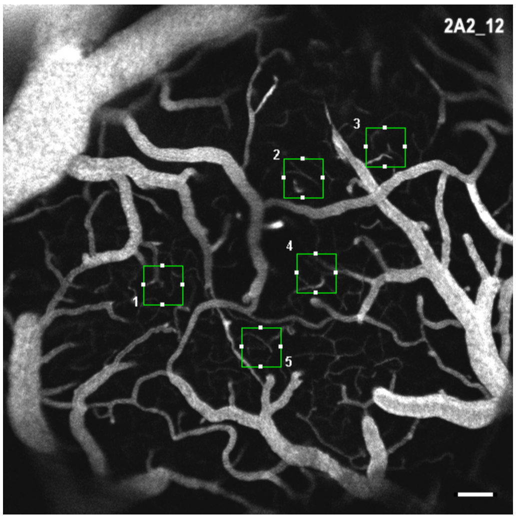

Figure B Grey-scale fluorescent image of microvasculature in the cortical surface. Five rectangular boxes were located to cover area of microvessel (diameter < 10 μm).

The recorded fluorescent images (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 image software calculated the percentage of capillary vascularity using the following equation:

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

After the imaging, a cannula was inserted into the apex of the left ventricle to allow perfusion of the brain with ice-cold phosphate buffer saline containing heparin, and then the brain was then removed. 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 at 3,000 rpm, 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. The isolated brain microvessels were used for determining of VEGF level by immunoassay technique.

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. The microvessel preparations were free of contamination by other cell fragments.

Isolated brain microvessels were homogenized in 500 μl of ice-cold RIPA buffer containing protease inhibitor cocktail. The homogenate was centrifuged at 1,000 xg for 5 minutes at 4°C. The supernatant as a postnuclear fraction. Protein concentration of postnuclear supernatant was determined using bicinchoninic acid assay. The supernatant aliquots were used for quantification of VEGF, Flk-1, PI3K and phosphor-Akt levels by commercial immunoassay kits.

For statistical analysis, results were 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.

Results

Table 1 shows body weight significantly increased in sedentary-aged (701.50±10.69 g) and exercised-aged rats (589.33±13.19 g) compared to sedentary-young rats (491.33±1.76). However, the body weight of exercised-aged group was significantly lower when compared to sedentary-aged group. The mean arterial blood pressure (MAP) was significantly higher in the sedentary-aged (137.70±2.64 mmHg) and exercised-aged (114.60±3.72 mmHg) group compared to the sedentary-young (94.10±4.34 mmHg) group (Table 1). However, the exercised-aged rats were significantly declined in MAP compared to the sedentary-aged rats (Table 1).

Table 1 Body weight and mean arterial blood pressure in sedentary-young (SE-Young), sedentary-aged (SE-Aged) and exercised-aged (EX-Age) groups.

	SE-Young	SE-Age	EX-Age
Body weight (g)	491.33±1.76	701.50±10.69*	589.33±13.19*,#
MAP (mmHg)	94.10±4.34	137.70±2.64*	114.60±3.72*,#

Values are expressed as the mean+SEM; n = 5 rats. *P<0.05, significantly different from the SE-Young group. #P<0.05, significantly different from the SE-Age group.

Figure 1 shows representative the examples of microvascular network visualized by FITC-dextran under a laser-scanning fluorescence confocal microscope from sedentary-young, sedentary-aged and exercised-aged groups. In sedentary-young group, microvascular network exhibited rich of capillary (Figure 1A). In contrast, the capillary network in aged rats without exercise shows markedly reduced density of capillaries (Figure 1B) when compared to the sedentary-young rats. However, in aged rats with exercise training, capillaries appeared to be greater in density (Figure 1C), developed by exercise training.

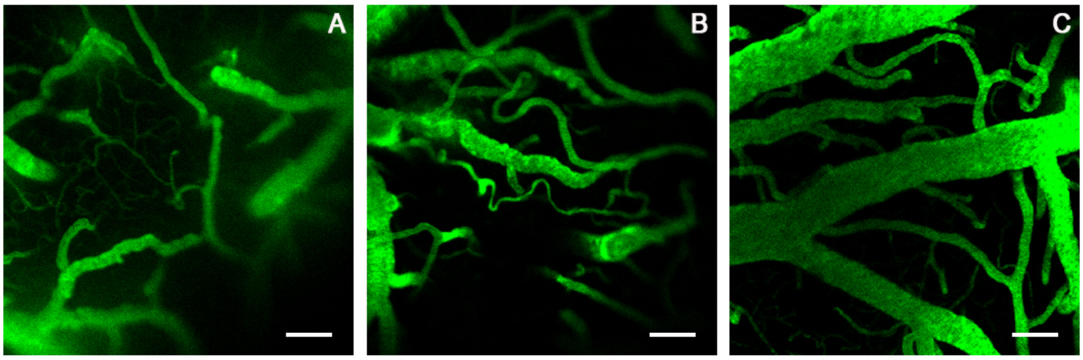

Figure 1 Representative fluorescent image of microvascular networks from sedentary-young (A), sedentary-aged (B) and exercised-aged (C) groups. Scale bar represents 100 μm.

Capillary vascularity in the brain was significantly lower in the sedentary-aged rats (15.86±1.25%) compared with the sedentary-young rats (39.25±2.18%) and was significantly higher in the exercised-aged group (29.81±1.64%) than in the sedentary-aged rats (Figure 2). As similar to alteration of the capillary vascularity, rCBF was significantly lower in the sedentary-aged rats (135.00±14.74 PU) compared with the sedentary-young rats (334.30±35.00 PU) and was significantly higher in the exercised-aged group (255.80±13.17 PU) (Figure 3).

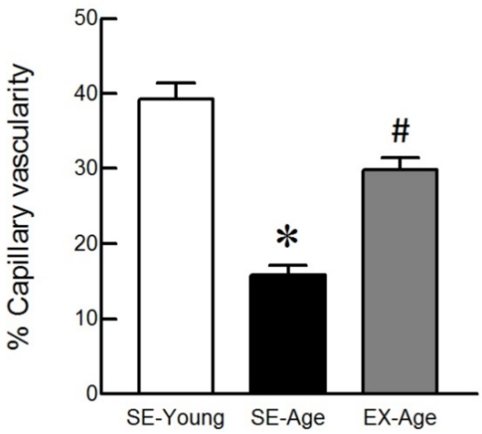

Figure 2 Capillary vascularity in the cortical brain from sedentary-young (SE-Young), sedentary-aged (SE-Age) and exercised-aged (EX-Age) groups. *P<0.05, significantly different from the SE-Young group. #P<0.05, significantly different from the SE-Age group.

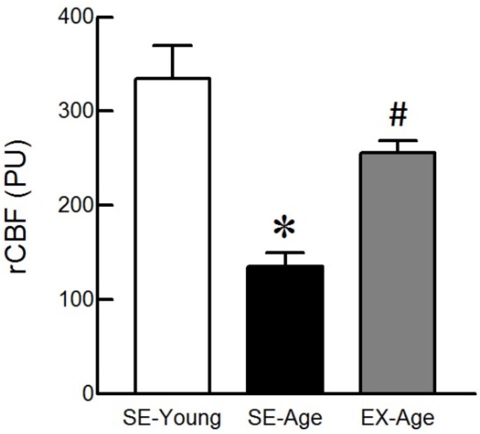

Figure 3 Regional cerebral blood flow (rCBF) from sedentary-young (SE-Young), sedentary-aged (SE-Age) and exercised-aged (EX-Age) groups. *P<0.05, significantly different from the SE-Young group. #P<0.05, significantly different from the SE-Age group.

The level of VEGF in the isolated brain microvessels was significantly lower in the sedentary-aged rats (19$\pm$0.24 pg/mg protein) compared with the sedentary-young rats (36$\pm$2.5 pg/mg protein) and was significantly higher in the exercised-aged group (30$\pm$0.5 pg/mg protein) than in the sedentary-aged rats (Figure 4). Corresponding to the change in the VEGF level, the Flk-1 expression was significantly lower in the sedentary-aged rats (0.11$\pm$0.02 pg/mg protein) compared with the sedentary-young rats (0.35$\pm$0.04 pg/mg protein) and was significantly higher in the exercised-aged group (0.17$\pm$0.01 pg/mg protein) (Figure 5).

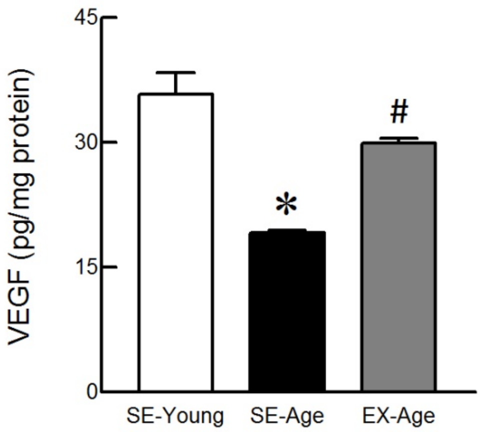

Figure 4 Level of vascular endothelial growth factor (VEGF) in the isolated brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Age) and exercised-aged (EX-Age) groups. *P<0.05, significantly different from the SE-Young group. #P<0.05, significantly different from the SE-Age group.

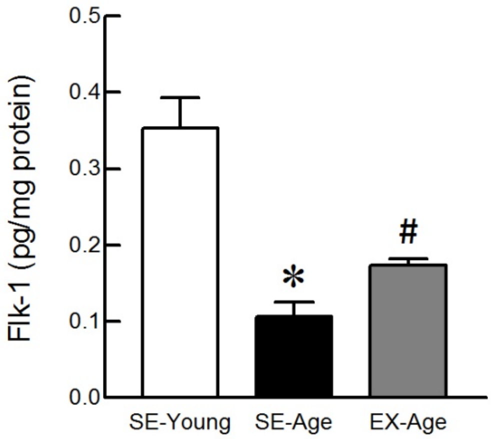

Figure 5 Level of VEGF receptor 2 (Flk-1) in the isolated brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Age) and exercised-aged (EX-Age) groups. *P<0.05, significantly different from the SE-Young group. #P<0.05, significantly different from the SE-Age group.

The level of PI3K in the isolated brain microvessels was significantly lower in the sedentary-aged rats (3,000$\pm$510 pg/mg protein) compared with the sedentary-young rats (5,300$\pm$240 pg/mg protein) (Figure 6). The level of PI3K in the exercised-aged group (3,700$\pm$330 pg/mg protein) was higher than that in the sedentary-aged group, however, there was no significant difference in the level of PI3K between both groups (Figure 6).

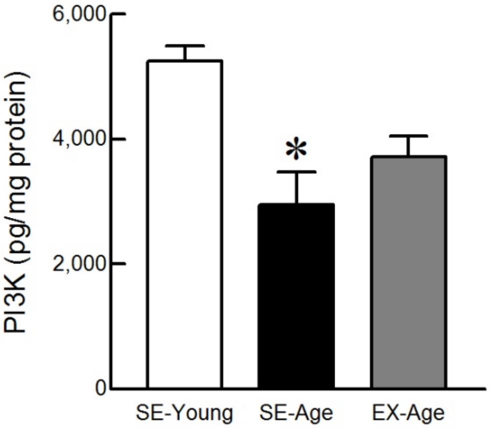

Figure 6 Level of PI3K in the isolated brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Age) and exercised-aged (EX-Age) groups. *P<0.05, significantly different from the SE-Young group.

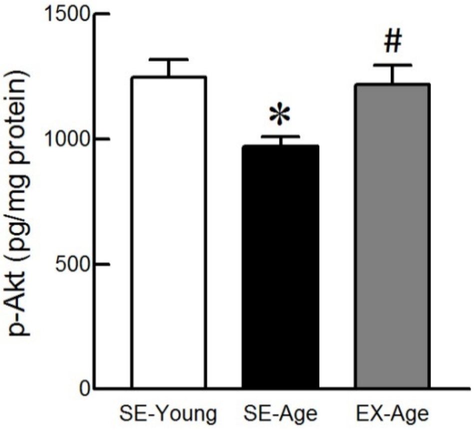

Figure 7 Level of phospho-Akt (p-Akt) in the isolated brain microvessels of sedentary-young (SE-Young), sedentary-aged (SE-Age) and exercised-aged (EX-Age) groups. *P<0.05, significantly different from the SE-Young group. #P<0.05, significantly different from the SE-Age group.

The level of phosphor-Akt was significantly lower in the sedentary-aged rats (970±40 pg/mg protein) compared with the sedentary-young rats (1,200±71 pg/mg protein) and was significantly higher in the exercised-aged group (1,200±76 pg/mg protein) (Figure 7).

Discussion

In the present study, performing in vivo experiment, revealed the protective effects of swimming exercise training on brain microvascular changes in advancing age. Deterioration of capillary vascularity as well as regional blood perfusion was found in aging brain. Upregulation of VEGF, Flk-1, PI3K and Akt, key angiogenic proteins, may play, in part, a role in the protective effect of exercise training in amelioration of brain microvascular perfusion during aging. Therefore, from these findings of the present study, it could imply that the protective effect of exercise training on upregulations of VEGF and key angiogenic proteins expression represent as considerable factors contributing to the possible underlying mechanism against age-induced alteration of brain microvessels.

The present study demonstrated age induced alteration in physiological characteristics, including body weight and MAP (Table 1). 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. It was found that the sedentary-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 exercised-aged group significantly 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. Elastin content is 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 occurred in sedentary-aged rats (Table 1). In present study, MAP was significantly lower in exercised-aged rats when compare to sedentary-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).

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

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 VEGF angiogenic signaling proteins, in aging rat brain. Lower brain capillary vascularity and regional blood perfusion as well as downregulation of VEGF and Flk-1 exhibited with age, however, physical activity attenuated these unfavourable results associated with its antioxidant and angiogenic properties (Figure 1-7).

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 group (Figure 3). The study of de la Torre and colleagues revealed that cerebral neurovascular dysfunction in relation to bioavailability of NO (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 extracerebral 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 group had a significantly lower brain capillary vascularity, as an index of capillary density, compared with the young group (Figure 1, 2).

The present study demonstrated that preparation of isolation brain microvessel yielded tubular vascular structure with diameters ranging from capillaries to small arterioles. 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, PI3K and phosphor-Akt 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, PI3K and Akt expressions (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 signaling protein 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 angiogenic protein expressions was found in the aging rats.

The angiogenic action of VEGF is mainly mediated by VEGF receptor 2 (Flk-1) which is primarily expressed in the endothelial cells. Activation of the tyrosine kinase receptor Flk-1, PI3K and phosphor-Akt 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 5) 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 involve 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 exerciseed-aged group.

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. 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).

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Output ที่ได้จากโครงการ

1. อยู่ระหว่างดำเนินการเตรียมส่งผลงานเพื่อตีพิมพ์ในวารสารนานาชาติเรื่อง Exercise training alters VEGF angiogenic signaling in aging cerebral microvasculature
2. นำเสนอผลงานในที่ประชุมวิชาการ 3 ครั้ง

    2.1 S. Viboolvorakul, S. Patumraj.
    "Exercise training attenuates age-related microvascular deterioration in rats" ในการประชุมวิชาการสามัญสมาคมหลอดเลือดจุลภาคไทยประจำปี 2556 วันที่ 20 ธันวาคม 2556 ณ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

    2.2 S. Viboolvorakul, M. Sakhakorn, S. Patumraj.
    "Exercise training ameliorates brain, bone and skin microvascular deterioration in aged rats" ในการประชุมวิชาการแห่งชาติด้านผู้สูงวัยและผู้สูงอายุ ครั้งที่ 3 : อาเซียนสูงวัยอย่างทรงพลัง ปี 2558 วันที่ 24 พฤษภาคม 2558 ณ โรงพยาบาลจุฬาลงกรณ์ สภากาชาดไทย

    2.3 S. Viboolvorakul, M. Sakhakorn, S. Patumraj.
    "Exercise training ameliorates microvascular deterioration and VEGF signaling downregulation in aging rat brain" ในการประชุม the 10th World Congress for Microcirculation (Young Investigator Award Symposium) ระหว่างวันที่ 25-27 กันยายน 2558 ณ Kyoto International Conference Center ประเทศญี่ปุ่น

3. เผยแพร่ผลงานวิชาการในจดหมายข่าวของสมาคมหลอดเลือดจุลภาคไทย

    Viboolvorakul S. Sakhakorn M. Patumraj S. Exercise training ameliorates microvascular deterioration and VEGF signaling downregulation in aging rat brain. Newsletter of the Thai Society for Microcircualtion (ISSN1513-3303), Number 1, Volume 17, 2015, p.2-4.

The 10th World Congress for Microcirculation

Young Investigators Award

Presented to

Sheepsumon Viboolvorakul

This award is given for outstanding achievement by a young investigator in the field of Microcirculation. This individual has demonstrated excellence and originality in the submitted paper entitled:

"Exercise training ameliorates microvascular deterioration and VEGF signaling downregulation in aging rat brain"

Presented at Kyoto, Japan

September 26, 2015

Chairperson, Awards Committee

President, 10th World Congress for Microcirculation

Review

EXERCISE TRAINING AMELIORATES MICROVASCULAR DETERIORATION AND VEGF SIGNALING DOWNREGULATION IN AGING RAT BRAIN

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The number of elderly in the world is growing rapidly. Thailand also experienced a rapid and extensive growth. The rapid growth of global aging population has profound implications for many aspects of human health. A cerebrovascular abnormality is the one of leading cause of death in Thailand [1]. Moreover, the cerebrovascular disease is the most important leading cause of death in elderly persons.

Structural and functional alterations in cerebral vasculature are of pivotal importance in the pathogenesis of a variety of diseases affecting the brain. Cerebrovascular dysfunction is a key contributor to age-related brain pathogenesis [2]. Reduction of basal cerebral blood perfusion during aging 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. In aging, brain regions exhibit marked capillary loss and increase in microvascular tortuosity, leading to increase resistance to flow and reduce tissue perfusion [3]. 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 [4]. VEGF signaling pathway is a major stimulating factor in both physiological and pathological angiogenesis process [5]. VEGF receptor 2 (VEGFR2) plays an important role in VEGFmediated angiogenesis. Age-impaired angiogenesis associated with VEGF decline have been reported in several tissues [6].

Regular exercise has been shown to reduce risk of cerebrovascular and cardiovascular events. Accumulating evidence suggests that exercise training induced brain vascularization, augmented angiogenesis and increased basal cerebral blood flow (CBF). Increased brain vascularization, and hence blood flow, might prove to be an effective strategy to minimize or delay cerebrovascular events with age [7]. Studies have demonstrated that exercise training ameliorated impaired angiogenesis and reduced VEGF level in the brain with advancing age [8, 9]. However, the mechanisms underlying the exercise-induced improvement of vascular deterioration in the aged brain are largely unknown. It is interesting to investigate effects of exercise training on age-induced cerebro-microvascular alterations with modulation of VEGF signaling.

Male Wistar rats were divided into 3 groups; sedentary-young (4 months), sedentary-aged (22 months) and exercised-aged (22 months). Exercise program included swimming training 5 days/week for 8 weeks. In situ study of brain microvascular networks was performed to determine regional blood flow (CBF) (by Doppler flowmetry) and microvascular vascularity (MV) (using a laser scanning confocal fluorescent microscopy) (Figure 1). Level of VEGF, VEGFR2, Akt and PI3K in isolated brain microvessels were determined by immunoassay.

MV and CBF were significantly lower in the sedentary-aged rats compared with the sedentary-young rats, whereas the exercised-aged rat was significantly higher than the sedentary-aged rats. The protein level of VEGF and VEGFR2 were significantly lower in the sedentary-aged rats compared with the sedentary-young rats, whereas those in the exercised-aged rats were significantly higher than those in the sedentary-aged rats. The expression of PI3K and eNOS corresponded to the alterations in the VEGF and VEGFR2 levels. These findings suggest that exercise training ameliorates cerebromicrovascular deterioration and VEGF signaling downregulation during aging. Figure 2 is the concluded idea taken from our findings at this point.

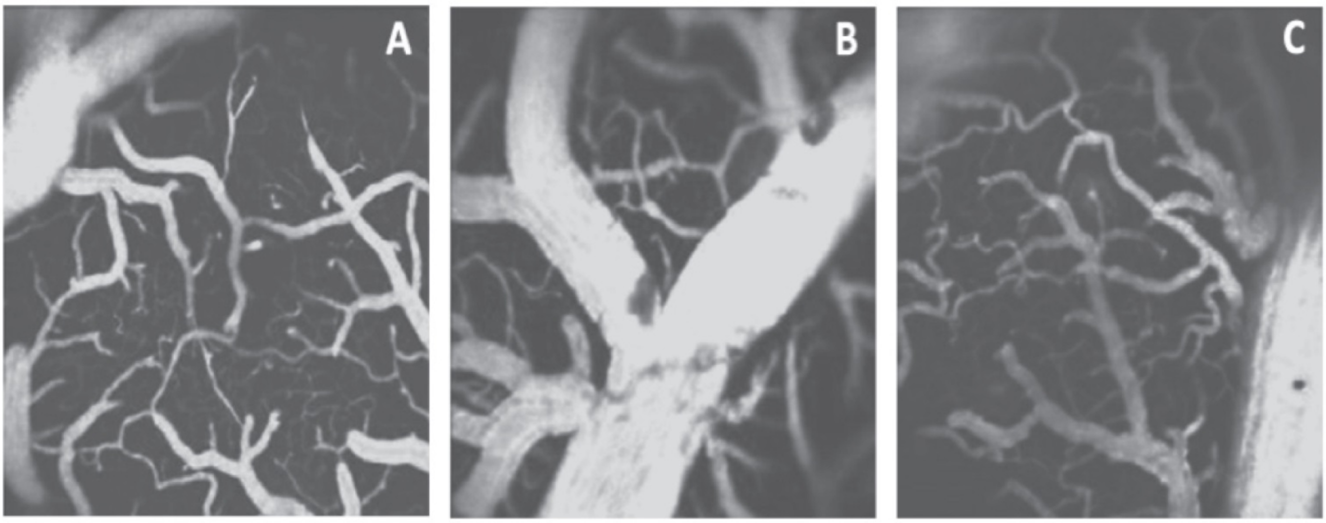

Figure 1. Microvascular vascularity (MV) observed in sedentary-young (4 months), sedentary-aged (22 months) and exercised-aged (22 months) by using a laser scanning confocal fluorescent microscopy.

Summary

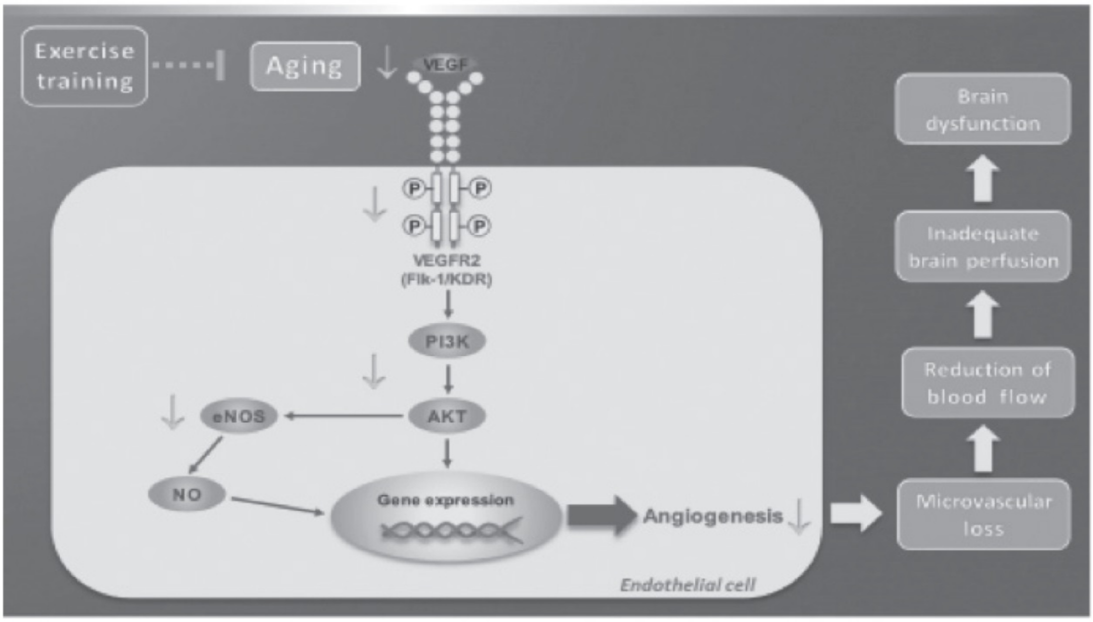

Figure 2. The concluded idea taken from our findings suggested that exercise training could ameliorate cerebromicrovascular deterioration and VEGF signaling downregulation during aging. And the expression of PI3K and eNOS are corresponded to the alterations in the VEGF and VEGFR2 levels. .

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