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Original article

Exercise training improves age-related changes in cerebral capillary vascularity through the upregulation of PI3K / Akt signaling

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Background: Currently, the number of the elderly has been rising sharply worldwide. Seemingly, age-induced cerebral endothelial dysfunction may lead to vascular abnormality that can later progress to cerebrovascular and neurodegenerative diseases. Moreover, several evidences have shown that age-related oxidative stress and decline in cellular function appeared in the vascular system both in humans and laboratory animals. The role of exercise training in the regulation of age-related oxidative stress and endothelial functions have been reported.
Objectives: To investigate whether exercise training can prevent age-induced cerebral endothelial dysfunction associated to PI3K/Akt signaling.
Methods: Male Wistar rats were randomly divided into 3 groups: sedentary-young group (SE-Young, 4 months), sedentary-aged group (SE-Aged, 22 - 24 months), and swimming trained-aged group (ET-Aged, 22 - 24 months), which individually swam 1 hour/day, 5 days/week for 8 weeks. After 8 weeks of the exercise period, the rats took rest for 24 hours. After a phosphate buffer saline (PBS) perfusion, brain was used for determining CD31 by immunohistochemistry. Vascular endothelial growth factor (VEGF), phospho-Akt level (p-Akt), and malondialdehyde (MDA) levels in the brain were measured by enzyme-linked immunosorbent assay.
Results: The aged rats' physiological characteristics had significant alteration when compared to the young group ($P < 0.05$). However, ET-Aged rats showed significantly reduced resting mean arterial blood pressure when compared to the young group ($P < 0.05$). This study also showed that exercise could upregulate VEGF, p-Akt level, and increase CD31 in the ET-aged group. Furthermore, tissue MDA in ET-Aged rats was significantly reduced when compared to SE-Aged rats ($P < 0.05$).
Conclusion: Our findings imply that exercise training protected age-induced cerebral endothelial dysfunction associated with its effects of oxidative stress and PI3K/Akt signaling.

Keywords: Aging, exercise training, CD31, Akt.

Nearly 47.5 million people worldwide suffer from dementia with 7.7 million more new cases every year. The general population aged 60 years and above with dementia is estimated between 5 to 8 per 100 people. The total number of people with dementia has been predicted to increase to 75.6 million in 2030 and almost 135.5 million by 2050. (Dementia-Who Health Organization; http://www.who.int > Newsroom > Fact sheets). As people live longer, reduction in certain cognitive abilities is expected due to increasing age. Interestingly, vasculature changes have been reported regarding the close correlation with cerebrovascular disease and neurodegeneration. Dementia and Alzheimer's disease were associated with marked alterations in both cerebrovascular structure and function.

Endothelial functions play an important role in the controls of vascular tone, permeability, inflammation, remodeling, and angiogenesis. It was believed that endothelial dysfunction was a key that deterioration of aging brain. Many studies showed that the

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endothelial dysfunction associated with aging could cause the impairment of endothelium-dependent vasodilator, impaired modulation of vascular growth and dysregulation of vascular remodeling and then decreased capillary density in the brains. This reduced brain capillary density could cause a lowered cerebral blood flow and hypoperfusion which led to brains' lack of oxygen, glucose and nutrient supply. Therefore, it could ultimately affect cognitive function and behavior and lead to brain degeneration and age-related cognitive disorders. [1 – 3]

Interestingly, exercise training was shown to decrease oxidative stress and improve angiogenesis through the up-regulation of vascular endothelial growth factor (VEGF) mRNA and protein in brains.[4] The researchers indicated that when exercise training increased blood vessel density, it helped to restore blood flow and to modify vascular shear stress.[4] Shear stress was known as a crucial factor for maintaining vascular tone. It played the role as the most important physiologic activator of nitric oxide (NO) production as seen from the activation of phosphoinositide 3-kinase (PI3K/Akt) pathway which was the underlying mechanism showing that shear stress stimulated NO production. [5, 6]

Therefore, the reason why exercise training could prevent cerebrovascular deterioration in aging might be able to explain, through the effect of exercise training on mediated VEGF signaling cascade regulation, which improved the mechanism of the angiogenic adaptation in the brain. [7] However, few studies have addressed the direct effect of exercise training on aged brain capillary density associated with the VEGF and the PI3k/Akt pathway. Therefore, the aim of this study was to investigate whether exercise training can prevent age-induced cerebral endothelial dysfunction associated to PI3K/Akt signaling or not.

Materials and methods

Animal preparation

Male Wistar rats (8-week-old) from the National Laboratory Animal Center of Salaya campus, Mahidol University (Nakornpathom, Thailand) were divided into three groups: sedentary-young (SE-Young, aged 4 - 6 months, n = 5), sedentary-aged (SE-Aged, 23 - 24 months, n = 8), and exercise trained-aged (ET-Aged, n = 8) rats. This study has been approved by the Ethics Committee on the Care and Use of Laboratory Animals, Faculty of Medicine, Chulalongkorn University (NO.14/2559). The present study was conducted in accordance with the guidelines for laboratory animals established by the National Research Council of Thailand (1999).

The effects of exercise training in the aging group were determined and compared between sedentary-aged (SE-Aged, placed in the swimming tanks with 5-cm water depth for 30 minutes/day and 5 days/week for 8 weeks, n = 5) and the exercise trained-aged (ET-Aged, 60 minutes/day and 5 days/week for 8 weeks, n = 8) rats. To minimize the possible stress effects associated with cold or hot water exposure, the SE-Aged group was trained according to the modified methods of an exercise training program by Iemitsu M, *et al*. and Viboolvorakul S, *et al*. [8, 9]

The swimming exercise protocol involved non-impact endurance exercise with moderate intensity and was modified from the methods of Iemitsu M, *et al*., and also from Viboolvorakul S, *et al*. [8, 9] Each day, the animals were transported to an exercise training room and swam individually in cylindrical tanks with a diameter and height of 50 and 65 cm, respectively, with water at a depth of 50 - 55 cm. The rats were exercised once per day between 2:00 and 4:00 p.m. for 5 days/week. The animals swam for 15 minutes/day for the first 2 days, and the swimming time was then gradually increased each week from 15 to 60 minutes/day. Thus, the trained-aged group received 8 weeks of swim training. To minimize the stress associated with exposure to hot or cold water, the water temperature was kept at 33 - 36°C. At the end of each training session, the rats were dried with a towel and hair dryer. The sedentary-young and sedentary-aged animals were transported to the same training room but remained in their cages during the training hour and were handled daily.

Malondialdehyde measurement

Malondialdehyde (MDA) level, a biomarker for lipid peroxidation, was estimated at the endpoint of experiment. As for the test, rats were used to measure oxidative stress in the aging brain tissue. After the rat was perfused and sacrificed under anesthesia, the data were obtained from the whole brain tissue. The supernatants of both tissue samples were used to analyze MDA levels by a TBARS assay kit (Cayman Chemical Co, USA) according to the manufacturer's instruction. This protocol was adapted from the study of Wang X, et al. [10] The results of MDA were expressed in nM/mg protein unit.

VEGF and p-Akt immunoassay

Based on the manufacturer's instructions, the supernatant aliquots were used for analyzing the tissue VEGF level, p-Akt protein contents by enzyme-linked immunosorbent assay (ELISA) (R&D system, USA; Cusabio Biotech, China).

For the VEGF immunoassay, all reagents, standards and samples were prepared beforehand. Each well was filled with 50 μL of Assay Diluent RD1N. Then, 50 μL of standard and sample were added per well. The plate was covered with a plate sealer and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker, set at 500 ± 50 rpm. Then, the plate was washed by filling Wash Buffer (400 mL) to each well using a multichannel pipette. The process was repeated four times for a total of 5 washes. After washing the plate, 100 μL of mouse VEGF conjugate was added to each well. A plate sealer was applied and the plate incubated for 2 hours at room temperature on the shaker. Then, the same plate washing steps were done again. 100 μL of substrate solution was added to each well afterwards. The plate was incubated for 30 minutes at room temperature in the box to protect from light. After incubation, 100 μL of stop solution was added to each well. The optical density was evaluated using an automated microplate reader at 450 nm with wavelength set to 540 nm or 570 nm.

For the p-Akt immunoassay, the capture antibody was immediately diluted to a working concentration of 6.0 mg/mL in phosphate buffer saline (PBS) without carrier protein before coating a 96 well microplate with 100 μL per well of the diluted capture antibody. Then, the plate was sealed and incubated overnight at room temperature.

100 μL of standards were added to the plate and samples per well and incubated for 2 hours at room temperature. To wash the plate, wash buffer (400 μL) was added to each well, repeating the process twice for a total of 3 washes. After washing the plate, 100 μL of the diluted detection antibody was added and incubated for 2 hours at room temperature. Then, the same plate washing steps were repeated. 100 μL of diluted Streptavidin-HRP was added to each well afterwards. The plate was incubated for 20 minutes at room temperature. The plates were washed again with the same steps before adding 100 μL of substrate solution to each well and incubating for 20 minutes at room temperature in a box to protect from light. After incubation, 50 μL of stop solution was added to each well. The optical density was determined with an automated microplate reader at 450 nm with wavelength set to 540 nm or 570 nm.

CD31 Immunohistochemistry

In this study, an immunohistochemistry method [11, 12] was used to detect the expression of CD31 of the aging brain. After ice-cold phosphate buffer saline (PBS) containing heparin was used for perfusing all rats from each group through the left cardiac ventricle, the brain was removed and the forebrain cut by brain matrix, 7 mm from the start point and cut 2 mm thick and fixed in 4% paraformaldehyde (pH 7.4) for 24 hrs. The brain sections were embedded in a paraffin box and cut at 2 μm before placing on slides and put into a 60°C oven overnight. After de-paraffinization and rehydration, the slides went through an antigen retrieval process (DaKo, US) by warming the slides at 100% power for 5 min and 30% power for 10 min. After blocking sections in 3% H_2O_2, nonspecific protein-blocking was performed on the sections using diluent antibody (Dako, US). The sections were incubated in primary antibody which were rabbit polyclonal antibody CD31 (Cat: RB 10333P, 1:500 dilution, Thermo scientific) overnight at 4°C. Then, the process of incubation with secondary antibody (anti-rabbit, DAKO) for 30 min at room temperature was conducted. A light microscope (Nikon eclipse TS100, Japan) was used to photograph the number of CD31 immunoreactive cells at 40 × magnification in eight areas of the brain, 4 pictures per area. The number of positive cells were analyzed by the Image-Pro plus 6.0.

Statistical analysis

The data were expressed as the mean ± standard error of mean (SEM). Any significant differences between groups were determined using one-way analysis of variance (one-way ANOVA), and differences between pairs of means were evaluated by the least significant difference test. To evaluate the difference between the SE-Aged and ET-Aged groups, Student's *t* - test for unpaired values was used. Differences were statistically significant if the *P*-value was less than 0.05. The data were analyzed using SPSS 16.0 for Windows (SPSS Inc., USA).

Results

All physical adaptations including body weight, mean arterial blood pressure, systolic blood pressure, and diastolic blood pressure of the sedentary-young group (SE-Young), sedentary-aged group (SE-Aged) and the trained-aged group (ET-Aged) are summarized in Table 1.

Table 1. Body weight (g), mean arterial blood pressure (mmHg), systolic blood pressure (mmHg), diastolic blood pressure (mmHg) and brain tissue malondialdehyde (nM/mg protein) in the SE-Young, SE-Aged, and ET-Aged groups.

	SE-Young	SE-Aged	ET-Aged
Body weight (g)	500.67 ± 9.25 (5)	711 ± 22.98* (8)	729.33 ± 15.45 (8)
Mean arterial blood pressure (mmHg)	102.12 ± 4.51 (5)	125.54 ± 5.28* (8)	107.32 ± 1.96 (8)
Systolic blood pressure (mmHg)	114.17 ± 5.85 (5)	141.33 ± 4.29* (8)	120.67 ± 2.27# (8)
Diastolic blood pressure (mmHg)	96.11 ± 3.91 (5)	117.67 ± 5.98* (8)	100.67 ± 1.87# (8)
Plasma malondialdehyde (nM/mg protein)	2.46 ± 0.11 (5)	3.41 ± 0.31* (8)	2.65 ± 0.26# (8)

Values are expressed as the mean ± SEM, and the number of rats is shown in parentheses.
*$P < 0.05$; significantly different from the SE-Young group
#$P < 0.05$; significantly different from the SE-Aged group

Mean arterial blood pressure

Resting blood pressure was measured with a pressure transducer (Statham, USA) which connected to a polygraph system (Nihon Koden, Japan). In Table 1, the systolic blood pressure of the sedentary-aged group was significantly higher than that of the sedentary-young group, while the systolic blood pressure of the trained-aged group was significantly lower than that of the sedentary-aged group. Because of an 8-week exercise training program, the diastolic blood pressure of the trained-aged group was significantly decreased when compared to the sedentary-aged group ($P < 0.05$). It also showed significantly higher mean arterial blood pressure (MAP) in the sedentary-aged group than in the sedentary-young group. The MAP of the trained-aged group significantly decreased when compared to the sedentary-aged group ($P < 0.05$).

Malondialdehyde (MDA)

MDA levels, which are the indicator of oxidative stress in the brain tissue, of the sedentary-young rats, the sedentary-aged rats, and the trained-aged rats are summarized in Table 1. In this study, the MDA levels were significantly elevated in the sedentary-aged group when compared to sedentary-young group. In contrasted, MDA levels were significantly reduced in the trained-aged group compared to the sedentary-aged group ($P < 0.05$).

Vascular endothelial growth factor (VEGF)

VEGF protein levels in brain tissue homogenate of rats in the sedentary-young group, the sedentary-aged group, and the trained-aged group are summarized in Figure 1. It can be seen that VEGF levels in the sedentary-aged group were significantly lower than those in the sedentary-young group while the effect of the exercise training significantly increased VEGF level in the trained-aged rats compared to the sedentary-aged group ($P < 0.05$).

p-Akt level

As shown in Figure 2, p-Akt protein levels in the sedentary-aged group were significantly lower than the sedentary-young group. The exercise training caused the p-Akt level in the trained-aged group to significantly elevate when compared to the sedentary-aged group ($P < 0.05$).

CD31

The expression of CD31 was detected by immunohistochemistry. According to Figure 3A, the white arrows point at the positive CD31 which were stained a brown color in 8 brain areas of each group. As in Figure 3B, the results were represented by the percentage of positive cell/ total area. The positive stained cells in the whole cortex in the sedentary-aged group was significantly decreased when compared to the sedentary-young group ($P < 0.05$) while the effect of the exercise training in the trained-aged rats significantly improved the number of CD31 positive staining cells when compared to the sedentary-aged group ($P < 0.05$). Image-Pro plus 6.0 software was used to confirm the results of CD31 expression.

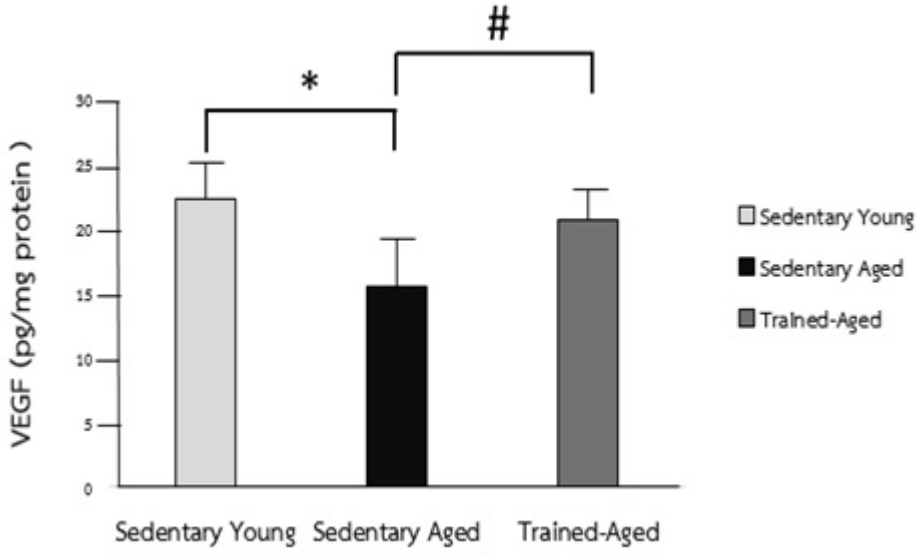

Figure 1. The effect of exercise training on vascular endothelial growth factor protein (VEGF) levels in the sedentary-young group (SE-Young), the sedentary-aged group (SE-Aged), and the trained-aged group (ET-Aged).
*$P < 0.05$; significant differences from sedentary-young group
#$P < 0.05$; significant differences from sedentary-aged group

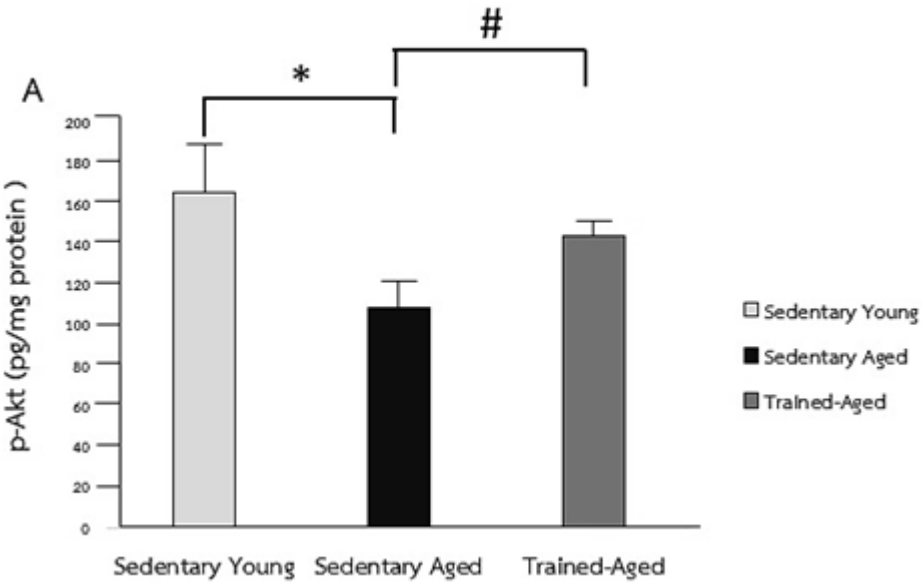

Figure 2. The effect of exercise training on p-Akt in the sedentary-young group (SE-Young), the sedentary-aged group (SE-Aged), and the trained-aged group (ET-Aged).
*$P < 0.05$; significant differences from sedentary-young group
#$P < 0.05$; significant differences from sedentary-aged group

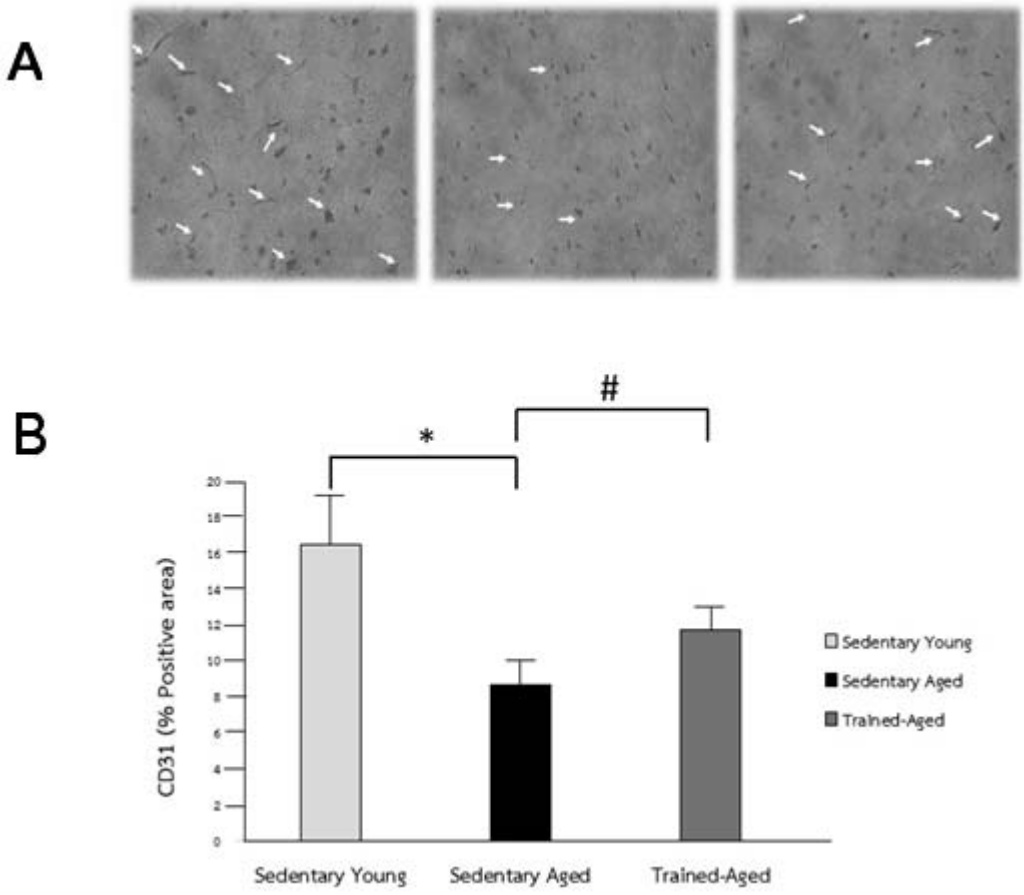

Figure 3. Effect of exercise training on CD31 expression (A) using an immunohistochemistry assay and observed under a light microscope with 40 × magnification as indicated by white arrows. (B) The result represented by percentage of positive cell/ total area of sedentary-young group (SE-Young, n = 5), the sedentary-aged group (SE-Aged, n = 8), and the trained-aged group (ET-Aged, n = 8).
*$P < 0.05$; significant differences from sedentary-young group
#$P < 0.05$; significant differences from sedentary-aged group

Discussion

In the present study, the protective effects of moderate exercise training on brain microvessels against age-induced microvascular changes were revealed. It was shown that the preventing mechanism might be associated with stabilized levels of VEGF, and p-Akt expression. Moreover, tissue MDA was reduced, indicating a decrease in oxidative damage.

The results show that the changes in physiological characteristics (Table 1) including body weight, systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure were age-related. These negative alterations could be improved by the 8-week swimming exercise training protocol used in this study.

The sedentary-aged rats had a markedly higher body weights than the sedentary-young rats. Aging was generally associated with increases in total adiposity from middle age until old age. Higher body fat was the most relevant change leading to increased body weight and a reduction of the basal metabolic rate,[14, 15] a sedentary life-style was also a major risk for weight gain. [16] Thus, it could be concluded that the increased weight in aging was due to less physical activity. This may perpetuate the decline in muscle strength leading to muscle atrophy that attenuates resting metabolic rates to promote weight gain. [17, 18] However, in the present study, exercise training did not change the body weight between the trained-aged rats and the sedentary-aged rats (Table 1). The sedentary-aged group still had a markedly higher body weight than the trained-aged group at 24 months of age. The result above was similar to the research of Caponi PW, *et al.* [20] According to Caponi, aerobic

exercise training did not change the body weight in hypertensive rats, but it increased the insulin sensitivity. Viboolvorakul S, *et al*. and Kusunoki M, *et al*. [9, 19] also found that exercise training could reduce body fat, plasma total cholesterol, and triglycerides, and improve high density lipoprotein (HDL) cholesterol in aging rats. These effects may be related to the fact that exercise increases muscle strength, leading to higher basal and exercise metabolic rates.

Aging and hypertension were associated with an increased incidence of coronary and cerebrovascular disease. [21, 22] Cardiovascular complications were mostly related to changes in both vascular structure and function including endothelial dysfunction, increased oxidative stress, vascular remodeling, and decreased compliance. [22 - 24] Alternations of the endothelium and smooth muscle cells occurred by structural and functional abnormalities in the vasculature. When the interaction between these two important cell layers of the vascular wall changed, it influenced modulate arterial stiffness. [24] Many findings show that dysregulated vascular tone happened because age-associated endothelial dysfunction decreased NO bioavailability but raised endothelin-1 production. This increased arterial stiffness which further contributed to higher SBP. [25, 26]

Similarly, the result of this study shows that the systolic blood pressure of the sedentary-aged group was higher than the sedentary-young group. This may be because large artery stiffening is associated with systolic blood pressure in aging. High systolic blood pressure could damage small vessels in the heart and brain and caused abnormal remodeling and rarefaction in those small vessels. These changes raised the resistance to mean arterial blood pressure. [27, 29] In addition, diastolic blood pressure increased in the sedentary-aged group compared to the sedentary-young group suggesting that this alteration of arterials was linked to the idea of endothelial dysfunction as mention above.

The present study also showed that after the 8-week exercise training, MAP was significantly decreased in the trained-aged group compared to the sedentary-aged group. According to animal studies, it was shown that attenuation of vascular oxidative stress associated with aerobic exercise induced antioxidant defenses by downregulation superoxide dismutase (SOD). [30 - 32] Moreover, exercise training was found to increase shear stress that restored NO bioactivity and reduce blood pressure by increasing arterial distensibility, arterial compliance and decrease sympathetic tone. [33]

PI3K/Akt signaling was confirmed to be decreased in brains of 24-month-old rats when compared to 6-month-old rats. The decrease of PI3K/AKT signaling was also observed in various organs of older mice and aged mice such as skeletal muscle. [34 - 35] Age-associated impairment of Akt phosphorylation in primary rat hepatocytes and cardiac muscles were remediated by alpha-lipoic acid through PI3 kinase, PTEN, and PP2. [36 - 37] For age-related death-survival balance in myocardium; an immunohistochemical and a biochemical study were reported in pancreatic tissues of mice and humans and in the kidneys, lungs and livers of mice. [38 - 39]

Tomobe K, *et al*. suggested that in 10-month-old mice, the total nuclear factor erythroid 2-like factor 2 (commonly known as Nrf2) in livers was decreased in response to a decreased AKT phosphorylation when compared to normal aged mice. [40] Similarly, the present study showed decreased phospho-Akt protein levels in the sedentary-aged group in comparison to the sedentary-young group. This may further imply that the protective effect of exercise training may be associated with the interactions between the PI3K/Akt pathway and the Nrf2-dependent antioxidant system.

In the present study, endothelial dysfunction in aging was studied by staining with the immunohistochemistry of CD31. CD31 is known as a reliable marker to identify endothelial cells. Thus, its expression was widely used as a way to understand endothelial cells distribution. [41, 42] The result of this study showed that positive staining in the whole cortex in the sedentary-aged group was significantly lower in comparison with the sedentary-young group. There were many evidences suggesting that in aging, endothelial dysfunction is related to endothelium-dependent NO-mediated vasodilatation impairment. Increased reactive oxygen species levels could inactivate NO in aged vascular by promoting endothelial dysfunction in both aged adults [43, 44] and older laboratory animals. [45 - 46]

The results from this study reveal that VEGF levels in the sedentary-aged group decreased when compared to the sedentary-young group. Similarly, there was evidence of the reduction of VEGFR2 expression with aging in the cerebral vessels. [47] The distribution of VEGFR2 in neurons instead of blood

vessels has also been reported. If the VEGFR2 expression in brain microvessels was decreased, it could be assumed that VEGF receptors may have a role in angiogenesis regulation.[48-49] Exercise training did not only enhance VEGF and downstream signaling through Akt to increase NO ability for angiogenesis process, but also vascular vasomotor from these processes kept endothelial function. This was because the percentage of positive CD31 in the trained-aged rats was significantly improved compared to the sedentary-aged group. The adhesion molecule CD31/PECAM-1 was pointed as a detector for shear stress.[50] There was a study showing that a mechanosensory complex at endothelial cell-cell junctions which were composed of PECAM-1 and VEGFR2 was capable of fluid shear stress detection. In this complex, the role of PECAM-1 was the mechanotransducer, implicating shear stress-dependent VEGFR2 activation. This may also suggest that exercise training induced shear stress through this process. Exercise training was also reported to activate Nrf2 translocation for nucleus in order to regulate the antioxidant defense system via the mechanotransducer process of shear stress through PI3K/Akt pathway signaling in endothelial cells.[51]

Increased shear stress from exercise training also stimulated an AKT1 level. Many studies have shown that the physiological hypertrophy induced by exercise training and the regulation of normal cardiac growth[52] required AKT1 was for development.[53] Moreover, upstream PI3K could activate AKT1 induced by swimming exercise through phosphorylation in the left ventricle.[54] Hence, exercise training was another way to stimulate PI3K/Akt pathway for maintaining Nrf2/Antioxidant response element (ARE) signaling. This led to the transcription process of antioxidant enzymes that protect the cerebral endothelial in the brain from ROS. In this study, the malondialdehyde level in the trained-aged group was significantly lower than the sedentary-aged group.

Conclusion

Our findings indicate that the effective mechanisms of exercise training on age-induced brain microvascular changes involve the up-regulation of VEGF and p-Akt expression in association with changes in the oxidant-antioxidant balance.

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Conflict of interest

The authors, hereby, declare no conflict of interest.

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