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Spatial Memory Disturbance Following Transient Brain Ischemia is Associated with Vascular Remodeling in Hippocampus

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A number of studies have investigated the effects of ischemic injury on functional and cellular characteristics of hippocampus. There is only a limited study on vascular remodeling of it. The present study aimed at examining vascular remodeling in hippocampus and spatial memory disturbances after transient brain ischemia. Male Wistar rats were randomly divided into four groups, i.e. sham operated (SHAM), transient brain ischemia with 1 day reperfusion (IR1), 3 day reperfusion (IR3), and 10 days reperfusion (IR10) groups. Transient brain ischemia was induced by bilateral common carotid artery occlusion (BCCAO). The spatial memory test was performed using the Morris water maze (MWM) in SHAM and IR10 groups. The rats were euthanized at day 1, 3 or 10 after BCCAO depending on the groups. The mRNA expressions of SOD2, Bcl-2, NeuN, eNOS, endothelin-1 (ET-1), CD31, VE-cadherin and vascular remodeling of the hippocampus were examined. There were deteriorations of spatial learning ability in IR10 group. The percentages of SOD2 and Bcl-2, the expression of NeuN, decreased and the vascular remodeling was observed in the ischemic groups. The eNOS and CD31 expressions were less in IR10, the VE-cadherin expression was less in all ischemic groups than in SHAM group, while ET-1 expression in IR1 group was higher than any other groups. The spatial memory deterioration after BCCAO is associated with vascular remodeling in hippocampus, characterized by lumen narrowing and smooth muscle thickening of microvessels.

Coronary heart disease, heart failure, and cardiovascular shock are very common disorders found in many regions of the world. In Europe, around 350,000 people suffer from heart attack each year [1], whereas in the USA, the incidence of heart attack is about 200,000 people each year [2]. In 2005, the World Health Organization (WHO) claimed that cardiovascular diseases were responsible for 5.7 million deaths out of 58 million total deaths [3]. Sudden reduction of blood supplies in these conditions induces ischemia reperfusion injuries and functional disturbances [1, 2, 4]. The brain, including the hippocampus, is the most vulnerable organ in the body to ischemic reperfusion injury [5, 6]. The pyramidal cells of the hippocampus, particularly those in the CA1 region, will easily die -termed delayed neuronal death- when they are deprived from the blood supply [1, 2, 7]. In turn this causes functional disturbances, which include memory deficits [1, 8-11], since hippocampus is well-known to play a pivotal role in the spatial memory regulation [12].

Transient brain ischemia of the brain implicates alterations in apoptosis, oxidative stress, and neuronal histology, all of which result in delayed neuronal deaths [8, 9, 13-15]. The increase of the level of oxidative stress is also considered to cause vascular remodeling [16], which is defined as an adaptive structural alteration process of vessels in response to hemodynamic conditions [17]. Several studies using various model of ischemia in rodents have reported the effects of ischemia on the lumen, medial, and intimal areas of the internal carotid artery [18], the lumen/wall thickness ratio of the medial cerebral artery [16], the medial cerebral artery thickening [19], and the

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vascular density of the hippocampus [20]. All of these vascular changes affect the brain arterial diameter, which is critical in the pathology of transient brain ischemia due to its association with the risk of death in cardiovascular cases [21]. The integrity of arterial diameter depends on endothelial nitric oxide synthase (eNOS), which is a signal transduction molecule that protects brain circulation by maintaining vascular permeability [22, 23], promoting vasodilation[24] and also depends on vasoconstrictor like endothelin-1 (ET-1) [25]. ET-1 is a potent vasoconstrictor which causes ischemia injury and functional deficits in learning and memory [26].

eNOS expression is influenced by a variety of mediators, one of which is platelet endothelial cell adhesion molecule-1 (PECAM-1) or CD31. This mediator is expressed enormously on the surface of endothelial cells [17] and commonly used as a tissue endothelial marker for evaluating the vascularization changes in an experimental stroke model [27], but not in transient brain ischemia paradigm using bilateral common carotid artery occlusion (BCCAO) model. Endothelial cells also express VE-cadherin [28, 29] which is an adhesion molecule in the endothelial cells junction [30].

Despite the importance of all of these vascular aspects in the pathogenesis of transient brain ischemia, there is a lack of studies on these vascular parameters, including the vascular lumen and wall thickness of the hippocampus. The aim of the present study was to investigate vascular remodeling in hippocampus and possible spatial memory disturbances of rats, subsequent to transient brain ischemia.

MATERIAL AND METHODS

Animals and treatments

The present experiment was approved by the Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada (approval number KE/FK/554/EC). Male Wistar rats, weighing 200-400g, were obtained from the Animal House (LPPT) and the Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada. The animals were randomly divided into four groups: (1) sham operated group (SHAM), (2) BCCAO with 1 day reperfusion (IR1), (3) BCCAO with 3 days reperfusion (IR3), and (4) BCCAO with 10 days reperfusion (IR10). The rats were maintained in 12/12-hour light/dark cycle. Food pellets and water were given ad libitum.

Bilateral common carotid arteries occlusion (BCCAO) was performed to induce transient brain ischemia. The protocol was based on that of previous studies [6, 31-35]. Briefly, the rats were anasthesized using 100mg/kg bw of Ketamine anaesthetic (PT Guardian Pharmatama, Jakarta, Indonesia). Once the rats were under anaesthesia, the anterior midline of the necks of the rats were opened and the common carotid arteries were exposed and clamped using non-traumatic vascular clamps (Dieffenbach, World Precision Instruments, USA). The clamps were left obstructing the blood flow of both common carotid arteries for 20 minutes before they were removed. Sham operations were carried out on the rats of SHAM group. After the surgery, the SHAM and IR10 groups were kept in the recovery phase for two days before being tested for the spatial memory. The rats were sacrificed on day 1 (IR1), day 3 (IR3), and day 10 (IR10 and SHAM).

Morris water maze test

The spatial memory test using the Morris water maze (MWM) was conducted 2 days following BCCAO on the SHAM and IR10 groups, each of which consisted of eight animals. The protocol used for the test was that described in previous study [36]. The equipments of the test consisted of a circular tank (1.5 m in diameter and 0.4 m in height) and a white-painted circular platform (diameter 13 cm, height 16.5 cm). The platform was placed in the middle of a randomly selected quadrant and remained in the same place throughout the experiment for any given rat. The tank was virtually divided into four quadrants designated as A, B, C and D. Eight starting points were marked at equal distances around the outer surface of the tank wall. Several different colored pictures were also attached on the curtain hanging around the tank. These pictures served as distal cues for the rats in finding the platform. The pool was filled with a mixture of water and milk to hide and keep the platform 1.5 cm below the water surface.

One day before the MWM test, the animals were placed in the test room to acclimatize to their surroundings. A number between one and eight was randomly selected to indicate the starting points of any given rat in every trial. The first phase of the test was an escape acquisition test. The trial was initiated by putting any given rat at the starting point with its head facing the inner-wall of the pool. The rat was forced to swim and was expected to accidentally find the escape platform and climb onto it. The rat was left on the platform for 20 seconds before being removed, dried, and returned into its holding cage. The time and trajectory traveled from the start until climbing onto the platform was recorded as the escape latency and path lengths, respectively. The path length of the rat's swimming trajectory was measured using a curvimeter (Comcurve 10; Koizumi Sokki Mfg, Nagaoka-shi, Nigata, Japan). The next trial started 1 minute after the rat was removed from the platform. The maximum duration for each trial was 1 minute. Each rat underwent 4 trials per day for four consecutive days (16

trials in total for the escape acquisition phase). A day after the last escape latency test, a memory retention test (the second phase) was performed to assess the long-term memory of the rats. The procedure for this memory retention test was exactly the same as that of the escape acquisition test except that the platform was removed from the tank. Any given rat underwent one trial only (one minute) in the test. The time and path lengths spent in the target quadrant were recorded and presented as percentages of the whole time and path lengths in any given trial. Visible platform tests to detect any possible sensorimotor deficits were performed in the next day. The tests consisted of three trials (60 seconds each) for any given rat [36]. All trials were recorded with a video camera connected to a laptop computer.

Tissue preparation

In the following day after the last MWM trial, which was the tenth day after the global brain ischemia induction, the rats were terminated. The rats were decapitated after deeply anaesthetized with chloroform (Merck, Germany, Cat.#1024451000). The cerebrum of any given rat was detached from the skull. The right hippocampus was isolated from the right hemisphere of cerebrum, kept in RNAlater® Stabilization Solution (Thermo Fisher Scientific, USA, Cat.#AM7021), and stored in -20ºC refrigerator. The left hemisphere was soaked in 10% formaldehyde in phosphate buffer solution (PBS) for 24 hours. On the subsequent day, the left hippocampus was isolated from the left hemisphere and immersed in 70% ethanol solution before being embedded in a paraffin block. The block was cut using a Leica RM 2235 microtome (Leica Biosystems, Wetzlar, Germany) at 3 μm nominal thickness. The hippocampal sections were then mounted onto slides.

Immunohistochemistry

Five paraffin blocks containing hippocampal tissues from each group were taken for further analyses. The examinations on Bcl-2 antiapoptotic protein, SOD2 antioxidant enzyme, and smooth muscle cells of hippocampal arteries were performed using immunohistochemistry. For this purpose, the hippocampal sections were deparaffinized, heated in citrate buffer solution for 20 minutes, and incubated in 3% H_2O_2 in methanol for 15 minutes. Next step was blocking using background sniper from the Starr Trek HRP Universal Detection Kit (Biocare Medical®, USA, Cat.#STUHRP700H). Afterwards, the sections were incubated in primary antibody anti-Bcl2 (Bioss, Cat.#bs-0032R, 1:100), anti-SOD2 (Bioss,Cat.#bs-1080R, 1:100), and antibody alpha-Smooth Muscle Actin (α-SMA, Sigma, Cat.#A2547, 1:300) overnight at 4ºC. On the following day, the sections were incubated in appropriate secondary antibodies for an hour and incubated with *avidin*-HRP from the Starr Trek HRP Universal Detection Kit (Biocare Medical®, USA, Cat.#STUHRP700H) for 30 minutes. The sections were stained with DAB solution and counterstained with hematoxylin.

The observation on hippocampal pyramidal cells was carried out in ten fields of view per hippocampus under a digital camera (Optilab, Miconos, Indonesia) connected to a light microscope (Olympus, USA) and a computer with 400x magnification. The pyramidal cells in the CA1 region which expressed anti-Bcl-2 and anti-SOD2 antibodies were counted and calculated as percentages to the number of all pyramidal cells in the respective CA1 region. Cells that positively express Bcl-2 and SOD2 are stained brown in the cytoplasm.

Vascular remodeling quantification

The vascular remodeling in the hippocampal arteries was assessed by quantifying the lumen area, wall area, wall thickness and lumen/wall areas ratio using αSMA immunostaining. The measurements were performed on 10 intra-hippocampal arteries, which expressed anti-αSMA antibody, with a range of diameters of between 10 and 50 μm. The calculations of lumen area, vessel area, lumen perimeter and vessel perimeter were done using ImageJ software program (NIH Image; National Institutes of Health, Bethesda, MD) as described in a previous study [37]. The wall area was measured by subtracting the lumen area from the vessel area. The data of the wall and lumen areas were used to calculate the ratio between the lumen and wall areas. The lumen and vessel perimeters were measured, and the center perimeter was calculated as the average of lumen and vessel perimeters. The wall thickness was obtained by dividing the wall area with the center perimeter.

RNA Extraction and PCR

Five hippocampal tissue samples from each group were taken for further analyses. The right hippocampus that was previously stored in RNAlater® Stabilization Solution (Thermo Fisher Scientific, USA, Cat.#AM7021) was cut in half. The RNA of the hippocampal tissue was extracted using RNAiso PLUS (Takara bio., Tokyo, Japan, Cat. #9108/9109). The cDNA was made from 1 μl RNA, which concentration was 1000 ng/μl, using a Rever Trace Kit (TOYOBO Co., Japan, Cat.#TRT-101) in a 20 μL reaction with random primer. The cycling conditions were 30ºC for 10 min, 42ºC for 60 min, and 99ºC for 5 min. The cDNA was diluted 4 times prior to PCR reaction.

The effect of ischemic reperfusion injury on hippocampal neurons was investigated on NeuN (a neuronal marker). In addition, the effect of the injury on hippocampal microvasculatures was examined on CD31, which is a marker of endothelial cells, VE-cadherin, and eNOS enzyme. The hippocampal cDNA was added with specific primers for NeuN, CD31, VE-cadherin, and eNOS, as follows: NeuN forward GCAGATGAAGCAGCACAGA C, NeuN reverse TGAACCGGAAGGGGA TGTTG, CD31 forward CCCAGTGACATTCACAGACA and CD31 reverse ACCTTGACCCTCAGGATCTC, VE-cadherin forward ATGAGAATGACAACGCCCCA and VE-cadherin reverse GCGGTATTGTCGTGGTTGTTG. While eNOS forward CCGGCGCTACGAAGAATG; and eNOS reverse AGTGCCACGGATGGAAATT. GAPDH, β-actin and hypoxanthine guanine phosphoribosyl transferase (HPRT1) served as housekeeping genes with the sequence of primers as follows: GAPDH forward TCTCGCTCCTGGAAGATGGTGA; GAPDH reverse GGCACAGTCAAGGCTGAGAATG, β-actin forward GCAGATGTGGATCAGCAAGC, β-actin reverse GGTGTAAAACGCAGCTCAGTAA, HPRT1 forward AGACGTTCTAGTCCTGTGGC, HPRT1 reverse ATCAAAAGGGACGCAGCAAC. The quantitative real-time PCR was performed using KAPA SYBR® Fast Mastermix (2x) Universal (Kapa Biosystems, KK4600, Massachusetts USA) in a real time PCR machine Biorad CFX96 (Bio-Rad, USA). The real-time cycling condition was as follows: an initial denaturation at 94° C for 2 min followed by 40 cycles of denaturation at 94ºC for 10 sec, an annealing at 60ºC for 60 sec for NeuN, 58ºC for 20 sec for CD31, 56ºC for 20 sec for VE-cadherin, and 54ºC for 20 sec for eNOS, an extension at 72ºC for 1 min, and a final elongation cycle at 72ºC for 10 min. The relative quantification of target genes mRNA expression was performed using ΔΔCT method.

Reverse transcriptase PCR was conducted to measure the mRNA levels of ET-1. GAPDH was used as a housekeeping gene. The sequences of the primers used were as follows: ET-1 forward GCTCCTGCTCCTCCTTGATG; and ET-1 reverse CTCGCTCTATGTAAGTCATGG. GAPDH forward TCTCGCTCCTGGAAGATGGTGA; GAPDH reverse GGCACAGTCAAGGCTGAGAATG. The PCR condition was as follows: an initial denaturation at 94ºC for 2 min followed by 40 cycles of denaturation at 94ºC for 10 sec, an annealing at 64ºC for 1 min, an extension at 72ºC for 1 min, and a final elongation cycle at 72ºC for 10 min. The PCR condition for GAPDH was as follows: an initial denaturation at 94ºC for 2 min followed by 30 cycles of denaturation at 94ºC for 10 sec, an annealing at 60ºC for 20 sec, an extension at 72ºC for 1 min, and a final elongation cycle at 72ºC for 10 min. The PCR products were analyzed with electrophoresis on 2% agarose gel (Agarose S; Nippon Gene, Tokyo, Japan). The levels of mRNA were estimated by calculating the intensity ratio of ET-1 mRNA/GAPDH mRNA using ImageJ software program (NIH Image; National Institutes of Health, Bethesda, MD).

Statistical analyses

The statistical analyses were carried out using SPSS software version 20 or Sigmastat (version 4.0) software for two-way repeated measures ANOVA. The normality and variance of the data were tested using Saphiro-Wilk and Levene test, respectively. The significance level was set at $p<0.05$. The data of the escape latency and path length in escape acquisition tests were analyzed using two-way repeated measures ANOVA, followed by post hoc Holm-Sidak test. The data of memory retention test and visible platform test were analyzed using independent t-test if the data were normal and homogenous, otherwise the data were analyzed using Mann-Whitney test. One-way ANOVA procedure was used to compare between the groups the means of the expression of SOD2, Bcl-2, lumen area, wall thickness, lumen area/wall area ratio, as well as mRNA levels of NeuN, CD31, VE-cadherin, eNOS and ET-1, after being log10 transformed whenever needed to normalize the data. The One-way ANOVA was followed by Least Significant Difference (LSD) post-hoc test whenever necessary. Kruskal-Wallis test was conducted when the data were not normally distributed, not homogenous, and failed to be transformed. The test was followed by Mann-Whitney post hoc test.

RESULTS

Transient brain ischemia induced spatial memory deterioration

The spatial memory of rats after transient brain ischemia was evaluated using MWM procedure which consisted of escape acquisition, memory retention, and visible platform tests. Figure 1 presents the data of the performance of the rats of the SHAM and IR10 groups in the MWM procedure. In the third trial (Figure 1A and 1B) and fifth trial (Figure 1B), the SHAM group followed a longer time to reach the platform than IR10 group. However, the IR10 group generally learned worse and traveled at a longer time and distance than the SHAM group in the subsequent trials. This can be clearly seen in trial 7, where the IR10 group traveled at a significantly longer time (Figure 1A) and in trial 7 and trial 9 in trajectory (Figure 1B) than the SHAM group. The two way repeated measure ANOVA showed significant main effects of trial on latency and path length ($p<0.001$), but no significant main effect of groups on latency ($p=0.972$) and path length ($p=0.809$). However, there were

significant main effects of trial x groups interaction on latency (p=0.040) and path length (p=0.034). Post-hoc tests using Holm-Sidak method showed that in trial 7, the escape latency and path length of IR10 group were longer than SHAM group. In trial 9, the path length of IR10 group was longer than SHAM group.

The memory retention test showed no significant difference between the SHAM and IR10 groups (Figure 1C) using independent t-test. There were also no significant differences in the escape latency and path length during the visible platform test, which means that all rats had the same level of sensory and motor ability. This indicates that the differences in the escape latency and path length in the escape acquisition phase were purely due to the difference in memory ability.

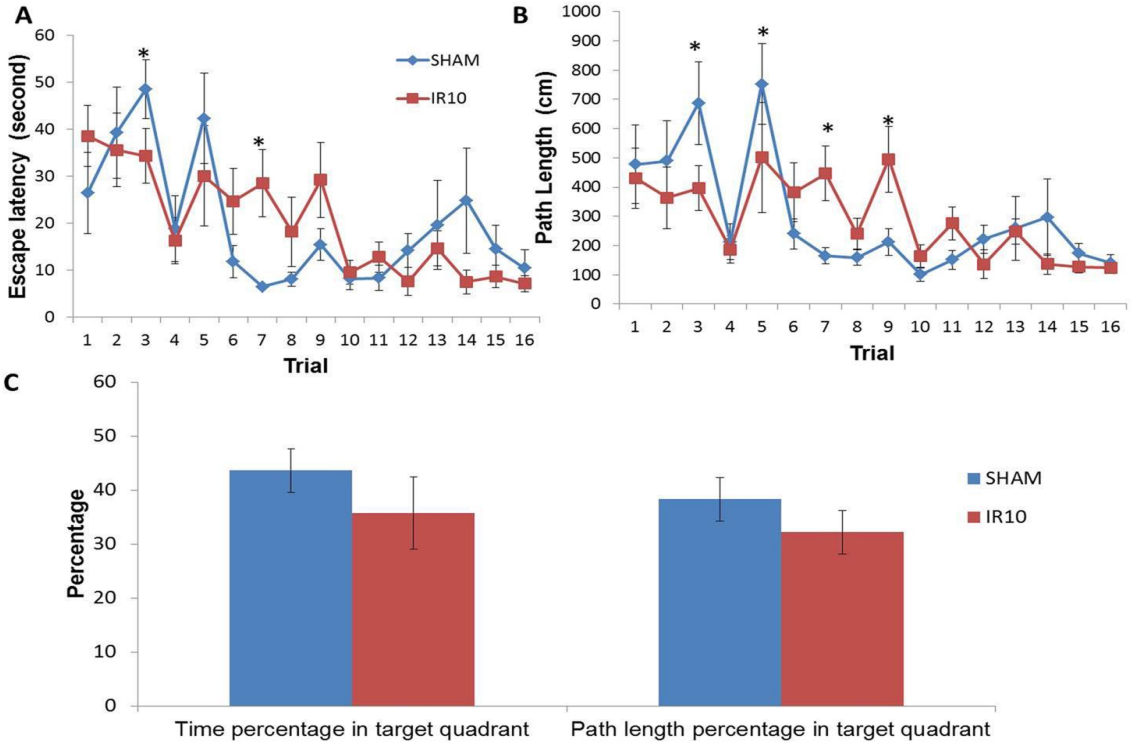

Figure 1. Spatial memory disturbance after BCCAO. Means ± SEM of the escape latency (A) and path length (B) of rats of SHAM and IR10 groups during the escape acquisition phase of the Morris water maze test. Two way repeated ANOVA showed $p<0.001$ for trial, $p<0.05$ for group x trial interaction.* $p<0.05$ in post-hoc test. Means ± SEM of the percentages of time and path length spent in the target quadrant in the memory retention phase of MWM (C).

Transient brain ischemia decreased the expressions of Bcl-2 protein and SOD2 enzyme

The examinations on Bcl-2 antiapoptotic protein and SOD2 antioxidant enzyme were conducted to assess the effects of BCCAO on the apoptosis and oxidant status of pyramidal cells in the hippocampus. Figures 2A and B show examples of the expressions of Bcl-2 antiapoptotic protein and SOD2 antioxidant enzyme, respectively, in the pyramidal cells of the CA1 region of hippocampus. One-way ANOVA and Kruskal-Wallis tests of the data showed the percentages of the expressions of Bcl-2 protein and SOD2 enzyme revealed a significant main effect of group. The post-hoc test showed that the expressions of Bcl-2 protein of all ischemic groups were significantly lower than those of the SHAM group, while the expression of SOD2 enzyme were significantly lower in IR1 and IR3 groups compared to SHAM group (Figures 2C and D).

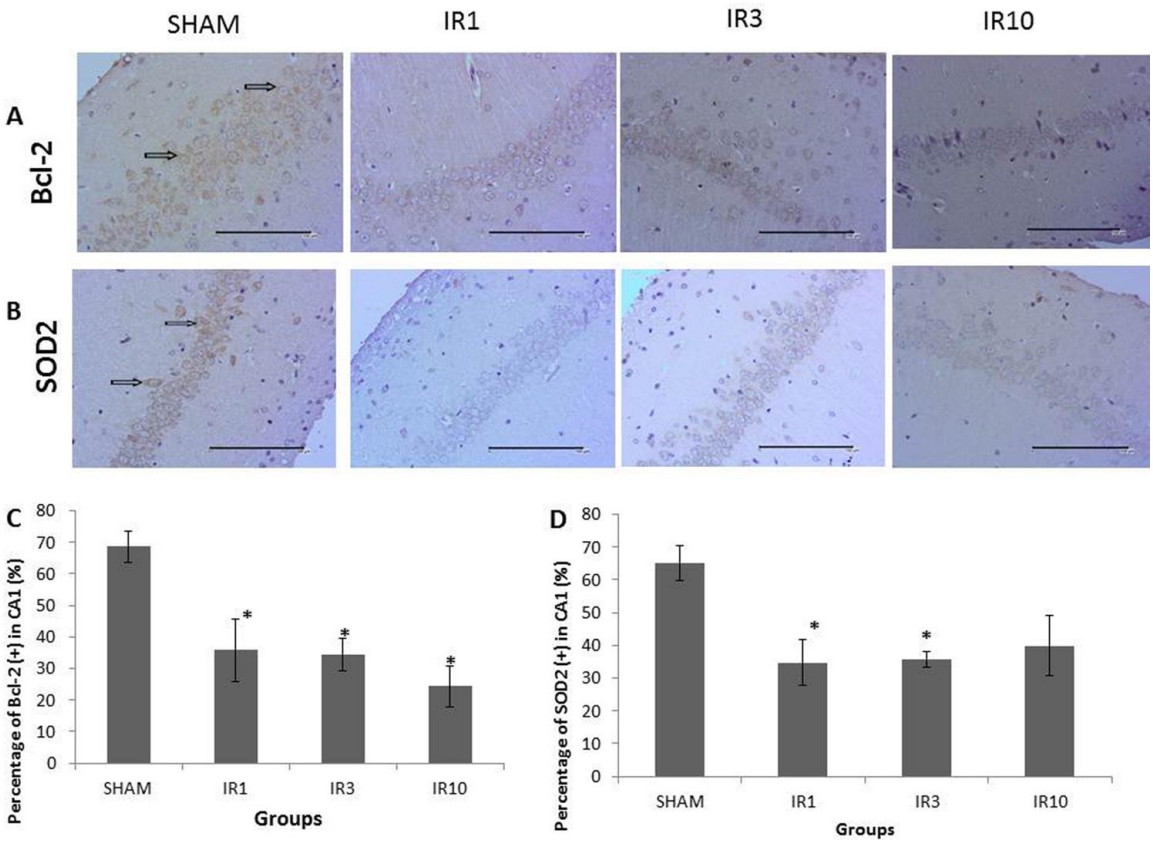

Figure 2. Immunohistochemistry of Bcl-2 and SOD2. Representative picture of immunohistochemistry of Bcl-2 protein (A) and SOD2 enzyme (B) on pyramidal cells of the CA1 region of hippocampus after BCCAO. Arrows point to examples of positive cells expressing Bcl-2 and SOD2 antibodies. BCCAO decreased the percentages of Bcl-2 (+) (C) and SOD2 (+)(D) expressions of pyramidal cells of the CA1 region of hippocampus. Values are expressed as means ± SEM. * $p<0.05$ compared to SHAM group.

Transient brain ischemia decreased the expression of NeuN

The degeneration of hippocampus after transient brain ischemia was evaluated using NeuN as a neuronal marker. It has been known that the decrease of NeuN (+) expression is associated with neuronal death[38]. Figure 3 presents the data of NeuN expression using GAPDH, β-actin, and HPRT1 to normalize the expressions. The mRNA expressions of NeuN/GAPDH, NeuN/β-actin and NeuN/HPRT1 were analyzed using Kruskal-Wallis test followed by post hoc Mann-Whitney test. The NeuN/β-actin expression of IR1 and IR3 groups were significantly lower than that of the SHAM group. In addition, the NeuN/HPRT1 expression of IR3 was significantly lower than the SHAM group.

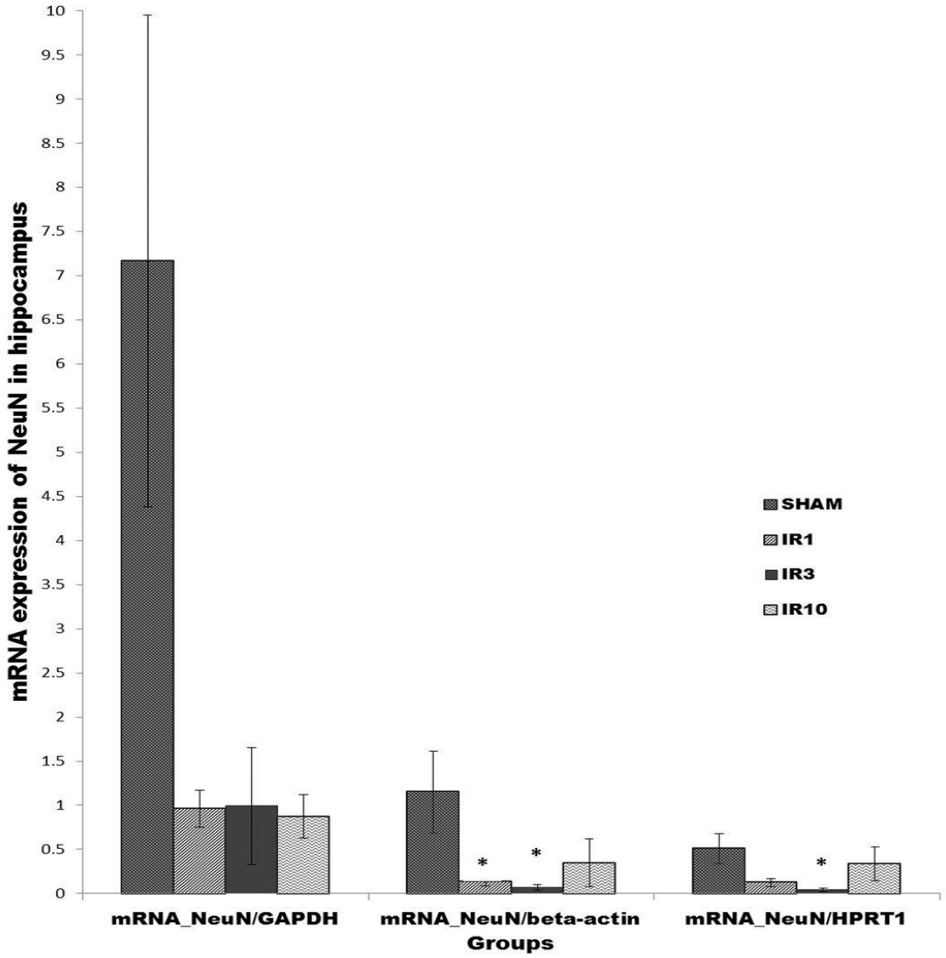

Figure 3. mRNA expression of NeuN in hippocampus. The means ± SEM of mRNA expression of NeuN/GAPDH, NeuN/β-actin and NeuN/HPRT1. BCCAO reduced the mRNA expression of NeuN. *p<0.05 compared to the SHAM group

Transient brain ischemia decreased the expressions of CD31 and VE-cadherin

The mRNA expressions of CD31 and VE-cadherin were used to assess the endothelial cell damage [17, 39] and vascular integrity [28, 29, 40], respectively. Figure 4 presents the data of the expression of CD31 and VE-cadherin as compared to GAPDH, β-actin, and HPRT1 as housekeeping genes. One-way ANOVA of these data showed that there was a significant main effect of group in the CD31 and VE-cadherin expressions. The post-hoc LSD test of these data revealed that the CD31/GAPDH expression of the IR10 group was significantly lower than that of the SHAM and IR3 groups. Furthermore, VE-cadherin/GAPDH and VE-cadherin/HPRT1 expressions in all ischemic groups were significantly lower than that of the SHAM group.

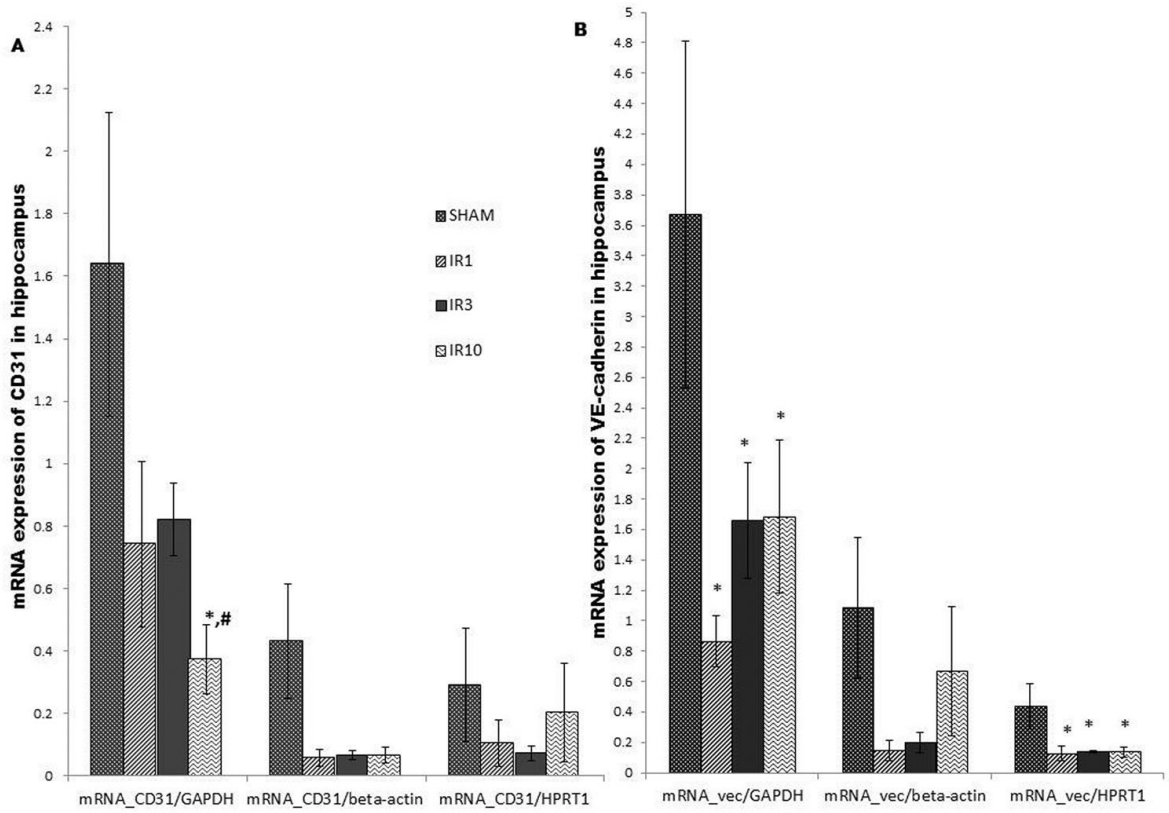

Figure 4. mRNA expression of CD31 and VE-cadherin in hippocampus. The means ± SEM of mRNA expression of CD31/GAPDH, CD31/β-actin and CD31/HPRT1 (A), and the means ± SEM of mRNA expression of VE-cadherin/GAPDH, VE-cadherin/β-actin and VE-cadherin/HPRT1 (B). BCCAO reduced mRNA expression of CD31 and VE-cadherin. *p<0.05 compared to SHAM group, # p<0.05 compared to IR3 group.

Transient brain ischemia induced vascular remodeling in hippocampus

The vascular remodeling after transient brain ischemia was assessed using immunohistochemistry staining of hippocampal arteries. Lumen/wall area ratio was measured as an indicator of vascular resistance [16]. Figure 5A shows the examples of the vascular remodeling occurring in the microvasculatures of rats of the IR1, IR3, and IR10 groups as compared to that of the SHAM group. Figure 5B shows the data of the microvessels' lumen area, wall thickness, and lumen/wall area ratios. Statistical analysis of these data revealed a significant main effect of group on the lumen area, wall thickness, and lumen/wall area ratios. The post-hoc tests of the data showed that the lumen areas of the IR1 and IR10 groups were significantly smaller than those of the SHAM group, while the wall thicknesses of the IR1 and IR3 groups were thicker than those of the SHAM group and IR10 group. Accordingly, the lumen/wall area ratios of all ischemic groups were considerably lower than those of the SHAM group (Figure 5B).

Vascular remodeling was also evaluated using the mRNA expression of eNOS as vasodilator and ET-1 as vasoconstrictor. Figure 5C demonstrates the data of the eNOS/GAPDH, eNOS/β-actin and eNOS/HPRT1 expressions ratio. One-way ANOVA of these data showed that there was a significant main effect of group in this ratio. The post-hoc LSD test of these data revealed that the eNOS/GAPDH expressions ratio of all ischemic groups were lower than that of the SHAM group after log transformation. Moreover, eNOS/GAPDH expression in IR10 group was also lower than IR3 group. Non parametric tests of the data of eNOS/ β-actin expression showed that the expressions in all ischemic groups were lower than that of the SHAM group.

Figure 5D shows the data of mRNA expression of ET-1/GAPDH as measured using reverse transcriptase PCR. One-way ANOVA of these data showed that there was a significant difference between groups. Post-hoc LSD test revealed that the expression of IR1 group was higher than SHAM, IR3 and IR10 groups.

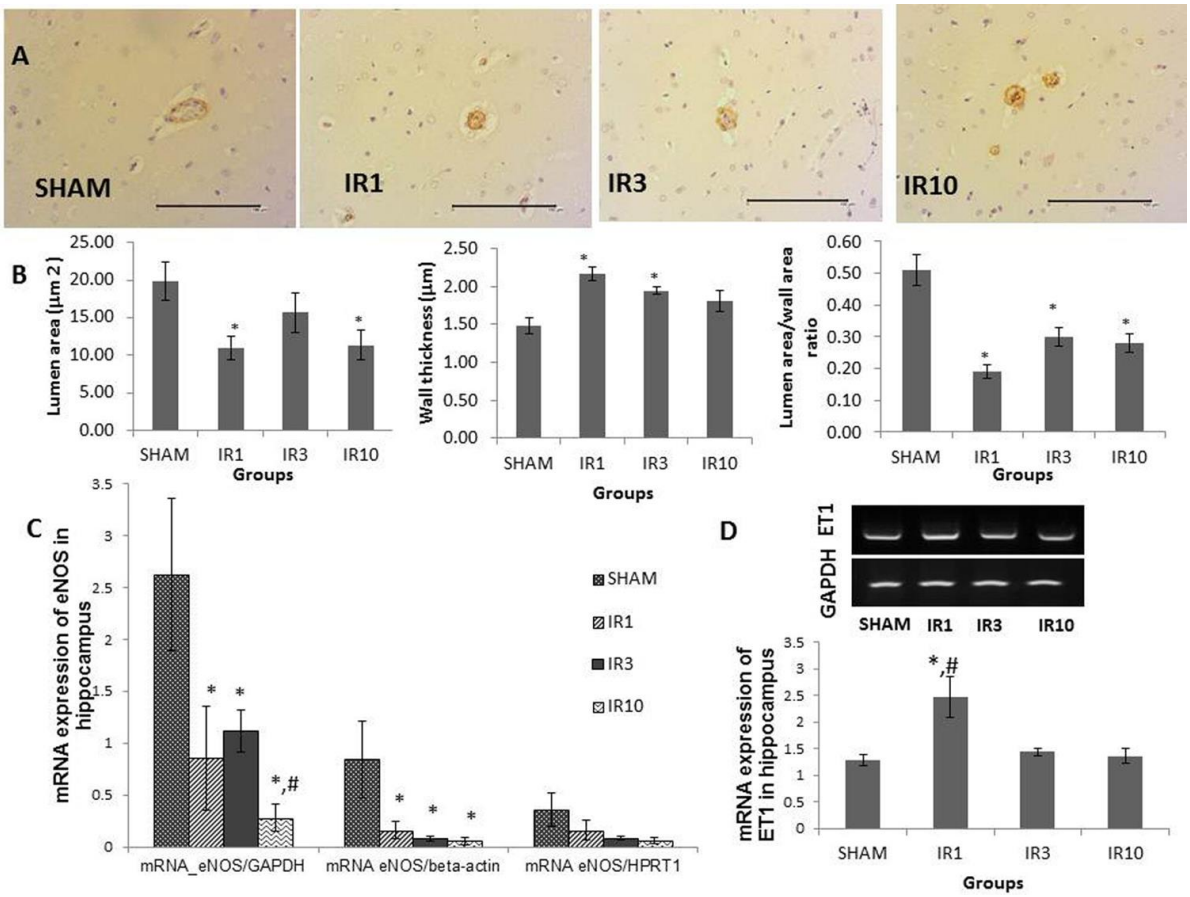

Figure 5. Vascular remodeling in hippocampus after BCCAO. The examples of αSMA-stained specimens which show the vascular remodeling in the hippocampus of the rats (A). The means ± SEM of lumen area, wall thickness, and lumen area/wall area ratios of the hippocampal microvessels stained with αSMA. BCCAO decreased lumen/wall area ratio (B). The means ± SEM of the eNOS/GAPDH, eNOS/β-actin and eNOS/HPRT1 mRNA expressions ratio of rats as examined using realtime PCR. BCCAO decreased the mRNA expression of eNOS (C). The means ± SEM of the ET-1/GAPDH mRNA expressions ratio of rats as examined using reverse transcriptase PCR. BCCAO increased the mRNA expression of ET-1 in IR1 group (D). * $p<0.05$ compared to the SHAM group, # $p<0.05$ compared to the IR3 group (C) or compared to the IR3 and IR10 groups (D).

DISCUSSION

The main finding of the present study was that transient brain ischemia using BCCAO model significantly decreased the spatial learning ability but not the long-term memory of rats. This corresponded with the significant increase of ET-1, the decrease of eNOS, CD31 and VE-cadherin expressions, as well as the lumen area, which accompanied the increase of the wall thickness of the hippocampal microvessels. In agreement with these findings was the reduction of the lumen/wall area ratio. In addition, the expression of NeuN mRNA, and the expression of Bcl-2 antiapoptotic protein and SOD2 antioxidant enzyme also decreased.

The BCCAO surgery procedure performed in this study seems to be successful in inducing transient brain ischemia which in turn causes a decrease in the spatial memory function. This finding is in agreement with several other studies that found the memory deficits in MWM tests and the damage in the hippocampal CA1 area as a result of the BCCAO procedure [1, 4, 8-11]. It has been observed in the present study that the learning performance of the rats of the ischemic group tended to be worse than that of the SHAM group from trial 6 onwards (see Figure 1). This is in contrast to the longer term memory performance, in which both groups performed at a similar level. These differences in the learning phase of MWM between both groups of rats might have stemmed from the possible damage of hippocampal CA1 region which was more severely affected by transient brain ischemia than the CA2-CA3 region. The CA1 region of the hippocampus, but not the CA2-CA3 region, plays an important role in the learning process [41], and consequently the damage on the CA1 region influences this process. Accordingly, several studies have reported that the CA1 region is more susceptible than the CA2-CA3 region in response to ischemia [8, 9, 42, 43].

Consistent with this finding was the decrease of the Bcl-2(+), SOD2(+) expressions of the hippocampal CA1 pyramidal cells and NeuN mRNA expression of the hippocampus of the ischemic rats which indicated a possible degeneration of the hippocampus. It has been suggested that ischemic reperfusion injury causes an increase in the level of free radicals that in turn induces apoptotic cell deaths [44]. In the present study, Bcl-2 expressions were lower in all of the ischemic groups compared to the SHAM group and thus there was an increase in apoptotic signals that lead to the cell deaths (Figure 2). We also found that the ischemic groups, especially IR1 and IR3 groups, had less expression of SOD2 in the pyramidal cells of the CA1 region of the hippocampus than the SHAM group (Figure 2). NeuN is a neuronal nuclear antigen which is commonly used as a specific neuronal marker. It has been known that the decrease of NeuN (+) expression is associated with neuronal death [38]. The present study showed the decrease of the expression of NeuN mRNA in the ischemic groups (Figure 3), which is in agreement with other studies [45, 46]. Those studies reported the decrease of NeuN expression after brain ischemia and found neuronal loss in some brain areas.

Endothelial cells are also susceptible to ischemia [47-49]. The present study examined the expression of endothelial cells, using CD31 as a marker [50]. Endothelial cells express CD31 on its surface [17] and this expression decreased in ischemic conditions [39]. This suggestion is in agreement with the results of our present study (Figure 4). In the acute phase of ischemia reperfusion injury, the levels of matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) increase. MMP is a proteolytic enzyme that is associated with microvascular damage in the early phase of stroke. These two MMP enzymes degrade the basal lamina and cause the disruption of the endothelial integrity [15, 51, 52]. In the sub-acute phase, an inflammation process also damages blood vessels. All of these processes are the causes of endothelial cell deaths in the later phase [15]. The finding in the present study that the expression of CD31 significantly decreased in the tenth day of reperfusion (Figure 4) is consistent with another study reporting that in transient brain ischemia, the expression of CD31 increased only until day 4 of reperfusion. After day 4, delayed neuronal death occurred and CD31 expression decreased. This is related to the process of neutrophils migration [43].

Microvessels have a capability to respond changes in their environment. In post stroke condition, non-demented vessels show a reactivity to hypoperfusion via remodeling [53]. During the vascular remodeling, endothelial cells respond to stimuli such as hypoxia and shear stress by dilating or constricting the vessels [17]. The present study showed that the ischemic reperfusion groups suffered from vascular remodeling from the first up to the tenth day of reperfusion. The vascular remodeling manifested as the luminal narrowing and microvessels wall thickening in the hippocampus of rats (Figure 5A and 5B). The lumen/wall area ratio was an indicator that reflects vascular resistance. It is an important factor for the determination of the progression of disease in cerebrovascular disturbances[16]. To our knowledge, there is a lack of studies assessing vascular remodeling in the hippocampus after BCCAO. The results of the current study confirmed that vascular remodeling, in the form of lumen narrowing and thickening, may have worsened the spatial memory of rats as indicated by the rats performance in the MWM test beginning from the sixth trial.

The narrowing of the blood vessels lumen might be due to the reduction in the eNOS expression that acts as a vasodilator (Figure 5C) and the increasing in ET-1 expression (Figure 5D). The imbalance of the levels of vasoconstrictors and vasodilators, which manifests in ET-1 up-regulation and eNOS down-regulation, might be the cause of the vascular remodeling following ischemia. This has been shown in a previous study that mice with ET-1 deletion from endothelial cells demonstrated an ameliorated kidney ischemic reperfusion through inflammation reduction and vascular remodeling [37]. ET-1 that is mostly secreted by endothelial cells is a potent vasoconstrictor and being up-regulated in the endothelial cells during ischemia [54, 55], especially in acute phase [15]. It is in accordance with the results of this study which states that the increased expression of ET-1 occurs in the IR1 group (Figure 5D). ET-1 considerably contributes to the pathogenesis of the ischemic brain damage [54, 55]. eNOS has an important role in regulating microvessel tone and reactivity [56]. A significant decrease in eNOS expression of the ischemic groups has been seen on the tenth day of reperfusion (Figure 4C). This finding lends support to several studies which reported that ischemic reperfusion injury [57, 58], hypoxic conditions [59], and oxygen glucose deprivation conditions during reperfusion injury [60], led to a decrease in the expression of eNOS. However, several other studies revealed that ischemic reperfusion injury increased eNOS expression [23, 61, 62], especially in the acute phase, and decreased within 24 hours [63] or seven days [23] of reperfusion. The exact reason of this discrepancy remains uncertain up to present.

The decrease of eNOS expression observed in the present study is supported by the data on the CD31 expression. Ischemia reperfusion injury, especially in day 10, brought about the decrease of CD31 expression (Figure 3). eNOS expression is influenced by a variety of mediators and acts as a response to various stimuli including hypoxia. An important mediator of eNOS expression is PECAM-1 or CD31 which is found in the vascular endothelium. CD31 affects eNOS phosphorylation. Therefore the down regulation of CD31 gives rise to the decrease of eNOS phosphorylation and eNOS expression [17].

The decrease of the expression of CD31 was parallel with the decrease of the expression of VE-cadherin (Figure 4). VE-cadherin is important in controlling and maintaining contacts between endothelial cells [30, 64], maintaining vascular integrity, and is required for the formation of vascular tissue [29, 40, 65]. It also plays a role in vascular remodeling [65] and maintains the stability of capillary tissues [29]. It has been reported that VE-cadherin knockout mice suffered from microvasculature disorganization and vascular integrity loss [66]. The decrease of the expressions of both CD31 and VE-cadherin observed in the present study may indicate the decrease of the blood vessels density. The destruction of junctional protein adherens such as VE-cadherin may cause endothelial cells apoptosis. This subsequently leads to microvascular rarefaction, which can be defined as reduced density of arterioles and capillary blood vessels [67]. The vascular rarefaction contributes to vascular resistance [68].

The present study revealed that BCCAO caused vascular remodeling which was characterized with microvessels lumen narrowing and vascular smooth muscle cells thickening. This vascular remodeling might be closely associated with the down regulation of eNOS, a potent vasodilator and up regulation of ET-1, a potent vasoconstrictor. The ischemic microvascular injuries were also confirmed by the decrease of CD31, an endothelial cells marker, and VE-cadherin, an adhesion molecule in the endothelial cells junction. Those changes were associated with the decrease of the neuronal marker in ischemic groups and the deterioration of the spatial memory of rats, particularly the spatial learning ability.

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