

Electroacupuncture pretreatment attenuates blood-brain barrier disruption following cerebral ischemia/reperfusion

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Abstract. Disruption of the blood-brain barrier (BBB) and subsequent brain edema are major contributors to the pathogenesis of ischemic stroke, however, current clinical therapeutic methods remains unsatisfactory. Electroacupuncture (EA) pretreatment has a protective effect against cerebral ischemia/reperfusion (I/R). However, the underlying mechanisms remain to be fully elucidated. In the present study, the effect of EA pretreatment on BBB disruption was investigated in a focal I/R rat model. Male Sprague-Dawley rats (280-320 g) were pretreated with EA at the acupoint 'Baihui' (GV20) 30 min/day, for five days consecutively prior to focal cerebral I/R, which was induced by middle cerebral artery occlusion (MCAO) for 2 h. The results demonstrated that the infarction volume, brain water content and neurological deficits increased in the MCAO model rats at 3 h and 24 h post-reperfusion, and were attenuated significantly by EA pretreatment. Furthermore, electron microscopy examination confirmed a reduction in brain edema reduction in the EA pretreated rats. Western blot analysis revealed that the tight junction proteins between endothelial cells, including claudin-5, occludin, were significantly degraded, while the protein expression of phosphorylated (p-) caveolin-1 and p-Akt increased following reperfusion, all of which were alleviated by EA pretreatment. However, no significant differences were observed in the expression of caveolin-1 or Akt. Overall, the results demonstrated that EA pretreatment significantly reduced BBB permeability and brain edema, which were correlated with alleviation of the degradation of tight junction proteins and inhibition of the expression of p-caveolin-1 in the endothelial cells.

Introduction

Stroke is the third leading cause of mortality in industrialized countries (1). Acute ischemic stroke, resulting from sudden blood vessel occlusion by a thrombus or embolism, is the most common form of stroke (1). Different mechanisms are involved in the pathogenesis of ischemic stroke, and ischemic injury and post-ischemia/reperfusion (I/R), induced by thrombolytic therapy, always leads to disruption in the blood-brain barrier (BBB), resulting in the development of brain edema and subsequent damage (2). Thus, methods to protect the BBB may assist in developing therapeutic strategies for the treatment of ischemic stroke.

The BBB is formed by endothelial cells, tight junctions (TJs), pericytes, astrocytes and other extracellular matrix components (3). BBB is essential for maintaining appropriate neural function, and for protecting the central nervous system from injury and disease, which tightly regulates the movement of molecules, ions and cells between the blood and the central nervous system (4,5).

TJs are important BBB structural components, which seal the gaps between adjacent endothelial cells and thus maintain paracellular permeability (6). Claudin and occludin are key transmembrane proteins forming this seal (7). Alterations in the distribution or loss of TJ proteins is frequently observed in ischemic cerebral microvessels, resulting in compromised BBB integrity (8,9).

Caveolae are small vesicular invaginations of the plasmamembrane, which have been implicated in endocytosis, vesicular trafficking and signal transduction (10). The principal structural proteins of caveolae are the caveolins, which consist of three distinct proteins, caveolin-1, -2 and -3. Caveolin-1 is particularly abundant in endothelial cells and has been implicated in the pathogenesis of cerebral I/R injury (11). However, the phosphorylation of caveolin-1 at tyrosine14 is required for the regulation of caveolae formation and function (12-14). Furthermore, it has been suggested that phosphorylation of caveolin-1 may be one of the factors associated with early BBB breakdown and brain edema in brain injury (15).

In Asia, and particularly China, electroacupuncture (EA), a novel therapy based on traditional acupuncture combined with modern electrotherapy, is a general method used for the treatment of cerebrovascular diseases (16). Several experimental and clinical studies have discussed the effect of EA

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on regulating different brain and heart diseases (17). There is evidence that EA significantly promotes the recovery of neurological function and, thus, improves patient quality of life (18,19). However, the exact mechanisms underlying the effects of EA pretreatment on BBB require elucidation.

Therefore, the present study was designed to investigate the role of EA pretreatment in BBB disruption following I/R, with a focus on caveolae and TJs. This was investigated using a rat stroke model of middle cerebral artery occlusion (MCAO), and the effect of EA pretreatment on BBB permeability and on the expression levels of caveolin-1, phosphorylated (p)-caveolin-1, claudin-5 and occludin were determined.

Materials and methods

Animals. The experimental procedure used in the present study was approved by the Ethics Committee for Animal Experimentation of Nanjing University of Chinese Medicine (Nanjing, China) and all procedures were performed in accordance with the National Institutes of Health Guidelines for Animal Research (20). Male Sprague-Dawley rats weighing between 280 and 300 g were provided by the Experimental Animal Center of Nanjing Medical University. The rats were housed in cages under controlled conditions, with a 12 h light/dark cycle, temperature at $22\pm 2^{\circ}\text{C}$ and humidity at 60-70% for at least one week prior to surgery, and received food and water *ad libitum*. The rats were fasted 12 h prior to surgery, but were allowed free access to water. A total of 140 rats were randomly divided into five groups: Sham group (S); 3 h post-I/R group (I/R3 h), 24 h post-I/R group (I/R24 h); EA pretreated 3 h post-I/R group (EA+I/R3 h); and EA pretreated 24 h post-I/R group (EA+I/R24 h). Each group contained 28 rats. All the rats were assessed between the point of ischemia and 24 h post-reperfusion.

MCAO model. Transient focal cerebral ischemia was induced by MCAO. Anesthetization was induced using pentobarbital sodium (40 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally. The right common carotid artery and the right external carotid artery were exposed through a ventral midline neck incision, and were ligated proximally and temporarily. A 2.0 monofilament nylon suture (Doccol Corporation, Redlands, CA, USA), with its tip rounded through heating in a flame, was inserted into the common carotid artery through an arteriotomy, just beneath the carotid bifurcation, and was advanced into the internal carotid artery ~18-20 mm distal to the carotid bifurcation, until mild resistance indicated occlusion of the origin of the anterior cerebral artery and the MCA. Following 2 h of ischemia, reperfusion was accomplished by withdrawing the suture. The incision was closed and the animals were allowed to recover from the surgery. In the sham group, the same surgery was performed, but without occlusion of the MCA. The rectal temperature was maintained at $38\pm 0.5^{\circ}\text{C}$ throughout the procedure using a thermostat-controlled heating pad (Doccol Corporation).

Cerebral blood flow (CBF) of the MCA was measured using laser Doppler flowmetry. A flexible fiber-optic probe was affixed to the skull over the cortex supplied by the proximal part of the MCA (2 mm caudal to the bregma and 6 mm lateral to the middle). Rats exhibiting <80% reduction in CBF in the

core of the MCA area were excluded from further investigation. Besides the sham group (S), the other four groups all had rats excluded (I/R3 h, n=3; I/R24 h, n=2; EA+I/R3 h, n=2; EA+I/R24 h, n=2).

EA pretreatment. According to the Experimental Animals Meridians Mapping, Baihui (GV20), which is located at the vertex of the parietal bone, that is, the midpoint of the connecting line between the auricular apices, was selected (18,19). The rats in EA pretreatment groups were anesthetized and stimulated using a 1 mA current, with a density-sparse wave of 2/15 Hz, for 30 min/day for five consecutive days. This was performed using a Hwato Electronic Acupuncture Treatment instrument (SDZ-V; Suzhou Medical Appliances Co., Ltd., Suzhou, China).

Neurobehavioral evaluation. A modified neurologic deficit score, described by Longa *et al* (21) was used for neurological assessment and was scored as follows: 0, no deficit; 1, failure to extend left forepaw fully; 2, circling to the left; 3, falling to the left; 4, no spontaneous walking with a depressed level of consciousness. Rats scoring between 2 and 3 were included in the subsequent experiments.

Brain water content. Following neurobehavioral evaluation, the rats (n=5) were sacrificed and decapitated after an intraperitoneal injection of 40 mg/kg pentobarbital sodium, followed by removal of the brains. The brains were rapidly separated into left and right cerebral hemispheres, and the right cerebral hemispheres were weighed (wet weight) using an electronic balance (Ohaus Corporation, Parsippany, NY, USA). Subsequently, the brains were dried for 24 h at 100°C in order to obtain the dry weight. The brain water content was calculated as follows: Brain water content = [(wet weight - dry weight) / wet weight] x 100. This was used as an index for brain edema (22).

Infarct volume assessment. Following decapitation, the brains (n=8) were removed and frozen at -20°C for 15 min. The brains were then sliced using a plastic matrix (2 mm thickness; Sigma-Aldrich) and stained using 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) in 0.1 mol/l phosphate buffer (Sigma-Aldrich) for 30 min at 37°C to evaluate the infarct volume (23). The infarcted tissue remained unstained (white), whereas normal tissues were stained red. The infarct volume was calculated as follows: Infarct volume = contralateral hemisphere volume - non-infarcted volume of the ipsilateral hemisphere / contralateral hemisphere volume (24).

Ultrastructure examination. The rats (n=5) were anesthetized with 40 mg/kg intraperitoneal pentobarbital sodium, then were perfused with pre-cooled phosphate-buffered saline (PBS; pH 7.4), followed by PBS containing 4% paraformaldehyde (Sigma-Aldrich) and 0.25% glutaraldehyde (Sigma-Aldrich). The brain was sectioned into three slices, starting 3 mm from the anterior tip of the frontal lobe in the coronal plane. The slices were 3, 4, and 3 mm thick from front to back, respectively. The middle slice was cut longitudinally in the ischemic hemisphere 2 mm from the midline then a transverse diagonal cut was made at the 2 o'clock position to

separate the core from the penumbra. A 1 mm thick coronal slice from the cortex penumbra area was removed. The slice was placed in fresh prepared 2.5% glutaraldehyde overnight at 4°C. Following rinsing with 0.1 mol/l PBS three times, the slice was post-fixed in 1% osmium tetroxide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h, dehydrated in graded ethanol (Sigma-Aldrich), and embedded in epoxy resin (Sigma-Aldrich). Polymerization was performed at 80°C for 24 h. Blocks were cut from the slice using a Reichert/Leica Ultracut S Ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) into ultrathin sections (60-70 nm), which were then post-stained with uranyl acetate (Sigma-Aldrich) and lead citrate (Sigma-Aldrich), and examined using a Hitachi 7100 electron microscope (Nikon, Corporation, Tokyo, Japan).

Western blot analysis. To analyze the expression of proteins, the rats (n=5) were decapitated and brains were removed. The ischemic cortices, and corresponding cortices of the sham rats, were rapidly dissected and frozen on dry ice (Sigma-Aldrich). Total protein samples were extracted using radioimmunoprecipitation assay (RIPA; Sigma-Aldrich) buffer for determination of the expression of claudin-5, occludin, caveolin-1 and Akt. To determine the expression of p-caveolin-1 and p-Akt, protease inhibitor cocktail tablets (Sigma-Aldrich) were added, at a ratio of 1 tablet/10 ml RIPA buffer. The protein concentrations were determined using a spectrophotometer (UV-2540; Shimadzu Corporation, Kyoto, Japan). Equivalent quantities of proteins (70 µg) from each sample were separated using 10% SDS-PAGE (Sigma-Aldrich) and subsequently transferred onto a nitrocellulose membrane (Sigma-Aldrich). The membranes were then incubated overnight at 4°C with the following rabbit primary antibodies: Claudin-5 (1:1,000; cat.no. ab53765; Abcam), occludin (1:1,000; cat.no. ab31721, Abcam), caveolin-1 (1:1,000; cat. no. 3238; Cell signaling Technology, Inc., Danvers, MA, USA), Akt (1:1,000; cat.no. 9272; Cell signaling Technology, Inc.), p-caveolin-1 (1:1,000; cat. no. 3251; Cell signaling Technology, Inc.) and p-Akt (1:1,000; cat. no. 9275; Cell signaling Technology, Inc.), followed by incubation with the respective horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:5,000; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 60 min at room temperature. Immunoreactivity was detected using an enhanced chemiluminescent autoradiography system (sc-2048 Western Blotting Luminol Reagent; Santa Cruz Biotechnology, Inc.). Each blot was re-probed with β-actin (1:5,000; cat. no. sc-130657; Santa Cruz Biotechnology, Inc.), following stripping by heat and detergent to remove the antibodies from the membrane, to provide a control for the load variations between the samples. Autoradiographic films (Santa Cruz Biotechnology, Inc.) were used for the final determination of protein expression using SigmaScan 5.0 (Sigma-Aldrich) and normalized to the relative optical density obtained for β-actin.

Statistical analysis. SPSS 19.0 for Windows (IBM SPSS, Armonk, NY, USA) was used to performed statistical analysis. All values are expressed as the mean ± standard deviation. Data were analyzed using one-way analysis of variance, and inter-group differences were detected using Student-Newman-Keuls

post-hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

EA pretreatment reduces infarction volume and neurological deficits. A modified neurologic deficit score, described by Longa *et al* (21), was used for neurological assessment of the different groups. The rats in the I/R3 h group exhibited severe neurological deficits compared with the sham group. Following 24 h reperfusion without any pretreatment, the neurological deficits remained unchanged, while EA pretreatment significantly improved the neurological deficits induced by I/R.

TTC staining was used to reveal cerebral infarcts, with normal brain tissues stained red, and infarct lesions remaining unstained (white). Compared with the sham group, the brain infarct volume increased significantly in the I/R3 h and I/R24 h groups. In the EA+I/R groups, these I/R-induced cerebral infarcts were reduced significantly and dose-dependently (Fig. 1).

EA pretreatment improves brain water content. The brain water content was significantly increased in the I/R groups, compared with the sham group. In addition, EA pretreatment significantly reduced the brain water content, compared with the I/R3 h and I/R24 h groups. (P<0.05; Fig. 2).

Taken together, these findings demonstrated that EA pretreatment attenuated the I/R-induced increase in BBB permeability.

EA pretreatment ameliorates cerebral microvasculature. In order to determine the mechanisms underlying the role of EA pretreatment in maintaining BBB permeability, transmission electron microscopy was used to identify the morphological changes of cerebral microvasculature in the cortex in all the groups. In the sham group, the cerebral microvasculature was relatively normal, with normal caveolae, and the TJs localized between endothelial cells as continuous lines. By contrast, in the I/R groups, the caveolae in the endothelial cells were increased, and the continuous lines of the TJs became ill-defined, appearing as dotted lines, indicating degradation of the TJ proteins. However, these observations were improved by EA pretreatment. These results were also confirmed using western blotting (Fig. 3).

EA pretreatment alleviates the degradation of TJ proteins. Western blotting was used to evaluate the expression levels of claudin-5 and occludin. Compared with the sham group, I/R induced a significant decrease in claudin-5 and occludin. Following EA pretreatment, the expression of these two TJ proteins increased significantly. No significant differences were observed between the EA+I/R3 h group and the EA+I/R24 h group (Fig. 4).

EA pretreatment alleviates the increase of p-caveolin-1. Western blotting was used to evaluate the expression of caveolin-1 and p-caveolin-1. In terms of caveolin-1, no significant differences were identified among the treatment groups. However, the expression of p-caveolin-1 was significantly increased following I/R compared with that of the sham

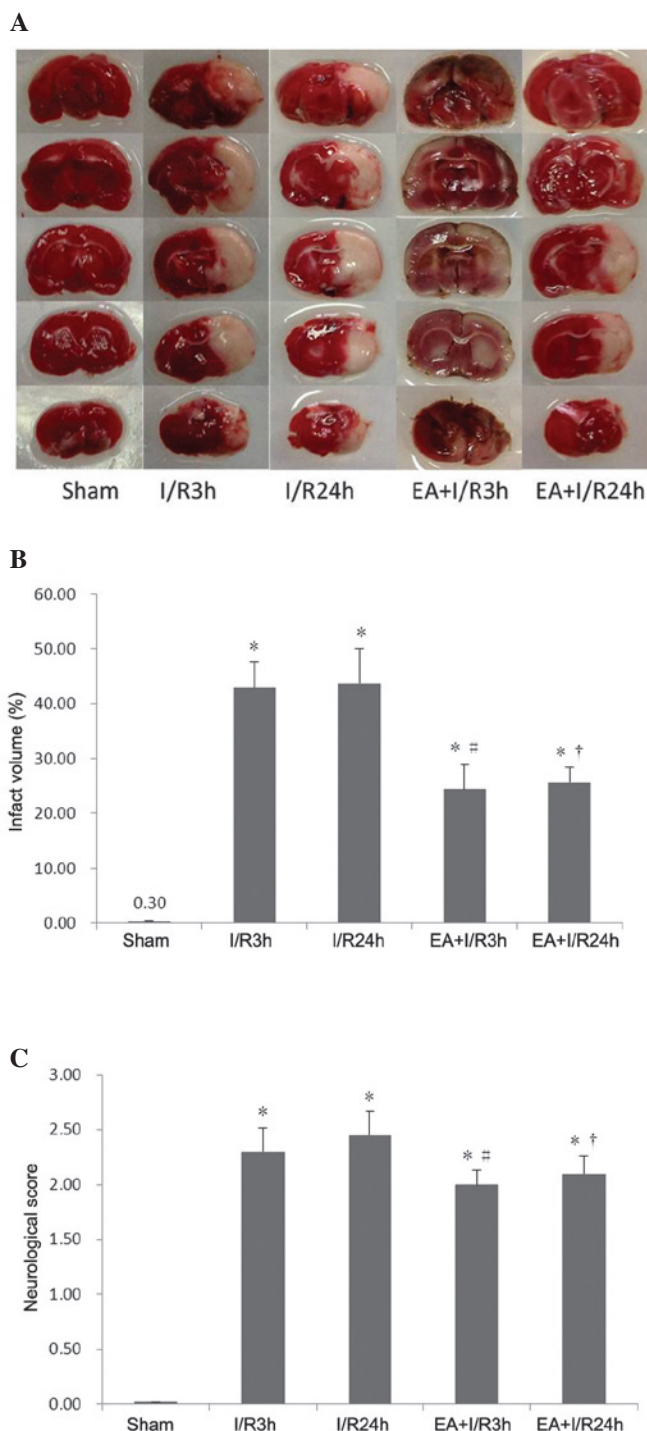


Figure 1. EA pretreatment reduces brain injury and improves neurological outcome. (A) Representative images of rat brain 2,3,5-triphenyltetrazolium chloride staining in different groups, red stain, normal brain tissues, white stain, infarct lesions (n=8). (B) Quantitative analysis of infarct size in different groups (n=8). (C) Neurological scores of animals in different groups (n=5). Values are expressed as the mean \pm standard deviation. * P <0.05, vs. sham group; # P <0.05, vs. I/R3 h group; † P <0.05, vs. I/R24 h group. EA, electroacupuncture; I/R, ischemia/reperfusion.

group, while EA pretreatment significantly relieved this effect (Fig. 5).

EA pretreatment alleviates the increase of p-Akt. Western blotting was used to evaluate the expression of Akt and p-Akt.

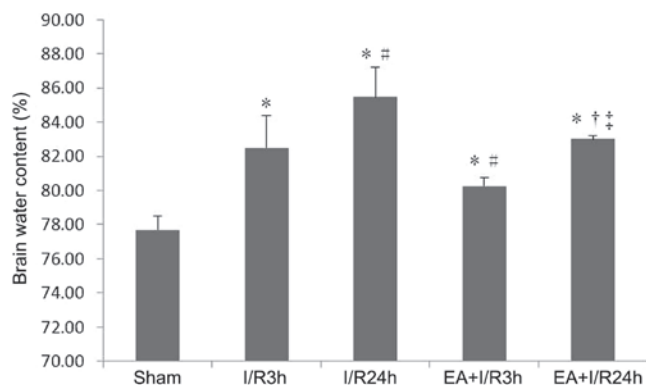


Figure 2. EA pretreatment reduces brain water content in rats (n=5). Values are expressed as the mean \pm standard deviation. * P <0.05, compared with the sham group; # P <0.05, compared with the I/R3 h group; † P <0.05, compared with the I/R24 h group; ‡ P <0.05, compared with the EA+I/R3 h group. EA, electroacupuncture; I/R, ischemia/reperfusion.

Similar to caveolin-1, no significant differences were observed among the groups for Akt. However, the expression of p-Akt was significantly increased following I/R compared with that of the sham group, while EA pretreatment significantly relieved this effect (Fig. 6).

Discussion

In the present study, the effects and possible mechanisms of EA pretreatment on BBB permeability following focal cerebral I/R were investigated. Initially, EA pretreatment was observed to effectively reduce cerebral infarct volume and improved the neurobehavioral scores, alleviating the ischemic damage. EA pretreatment also ameliorated brain water content and cerebral microvasculature, and reduced the degradation of TJ proteins, including claudin-5 and occludin. Furthermore, EA pretreatment reduced the increased expression of p-caveolin-1 and p-Akt in the endothelial cells.

In countries, including China, EA at Baihui (GV 20) (25-27), an acupoint of the DU series, has been commonly used for treating cerebrovascular diseases. A number of studies have demonstrated the protective effects of EA pretreatment in cerebral ischemia (18). The present study and previous studies have reported that EA pretreatment reduced the cerebral infarct volume and improved neurobehavioral scores following transient MCAO. However, the effects of EA pretreatment on BBB disruption associated with cerebral I/R remain to be fully elucidated.

Ischemic stroke and subsequent reperfusion cause severe clinical complications, including brain edema (28). In the present study, following 3 h of reperfusion and 2 h subsequent ischemia, marked brain edema was observed on examination of brain water content, which was maintained until 24 h of reperfusion. In addition, EA pretreatment significantly alleviated brain edema, indicating EA pretreatment as a therapeutic strategy for ischemia.

It is well-known that the BBB is a highly specialized structure between the blood circulation and the CNS, maintaining the appropriate environment for appropriate neural function and protecting the CNS from injury and disease (29). Following a period of ischemia, the BBB is broken down, resulting in

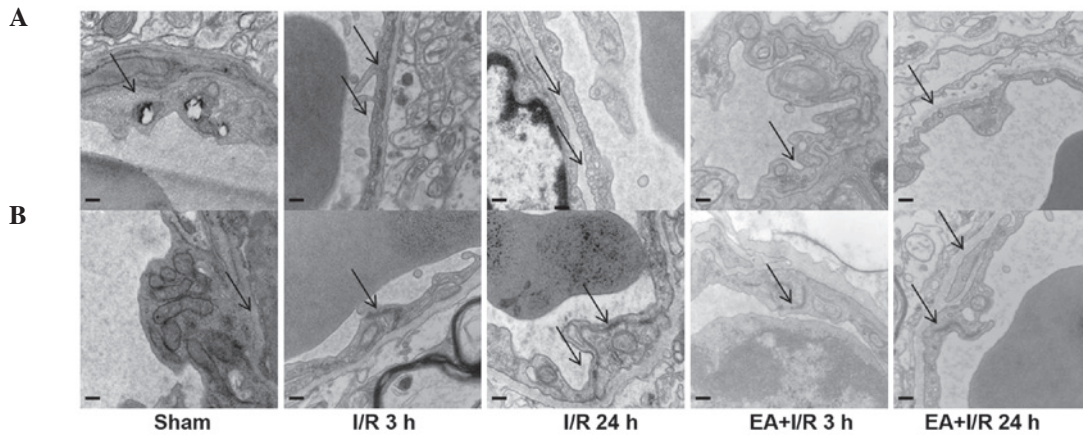


Figure 3. Ultrastructure of microvessels in the cortex penumbra area of rats (n=5). The representative transmission electron micrographs of capillaries in the cortex penumbra area are presented. Scale bar=0.2 μ m. (A) Arrows indicate caveolae in the endothelial cells. (B) Arrows indicate tight junctions between the endothelial cells. EA, electroacupuncture; I/R, ischemia/reperfusion.

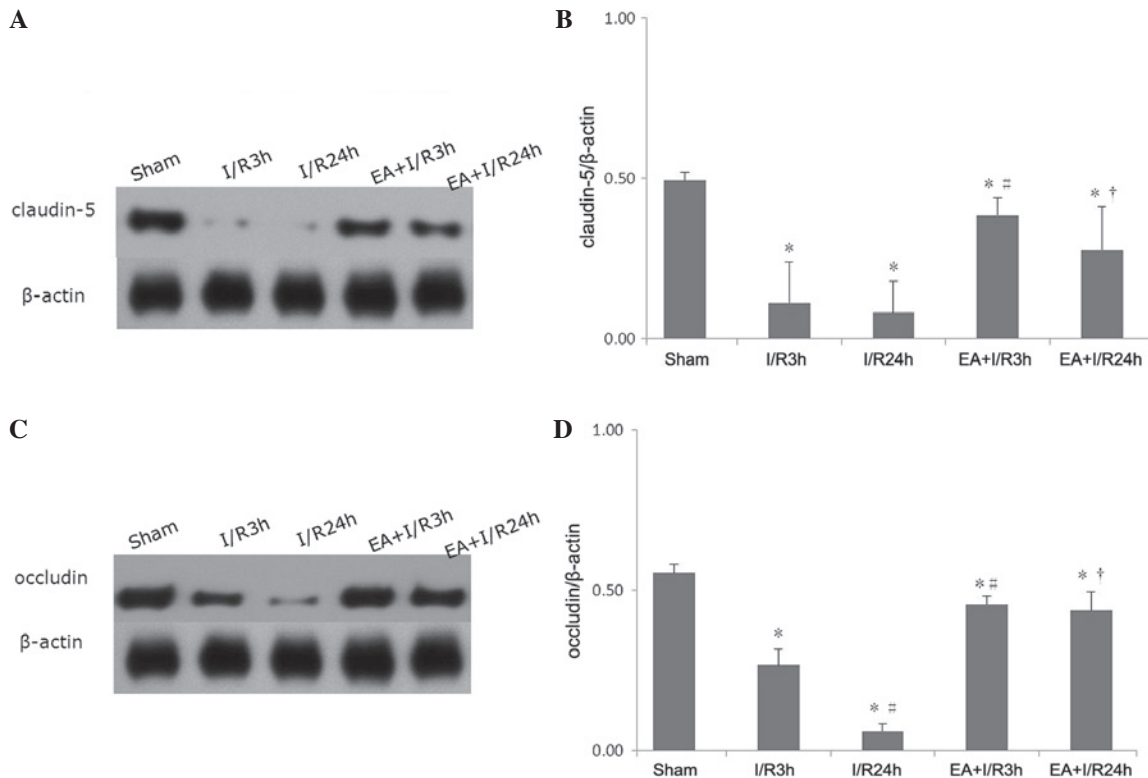


Figure 4. EA pretreatment alleviates degradation of claudin-5 and occludin tight junction proteins in rat vascular endothelial cells. (A) Representative western blots (n=5) and (B) quantitative analysis (n=5) of claudin-5. (C) Representative western blots (n=5) and (D) quantitative analysis (n=5) of occludin. Values are expressed as the mean \pm standard deviation. *P<0.05 compared with the sham group; #P<0.05 compared with the I/R 3 h group; †P<0.05 compared with the I/R 24 h group n=5. EA, electroacupuncture; I/R, ischemia/reperfusion.

vasogenic brain edema. It is suggested that two pathways are involved in endothelial cells, which affect BBB permeability: Tight junctions, which mediate paraendothelial transport, and caveolae, which mediate transcellular traffic (30,31). The present study demonstrated, using western blotting and electron microscopy, that these two pathways may be involved in I/R induced BBB interruption, and was alleviated by EA pretreatment.

During BBB breakdown associated with cerebral I/R, TJ protein degradation is a critical step. Occludin, the first

integral transmembrane protein, may act as a primary shock-absorber, mediating TJ responses to acute vascular dynamics changes (32). Claudin-5, a TJ protein with four transmembrane domains, is particularly important in regulating paracellular permeability for small solutes across the BBB (33). In the present study, western blotting demonstrated that the protein expression levels of occludin and claudin-5 decreased following cerebral I/R, and were partly relieved by EA pretreatment. In addition, electron microscopy revealed that the appearance of TJs were ill-defined following cerebral

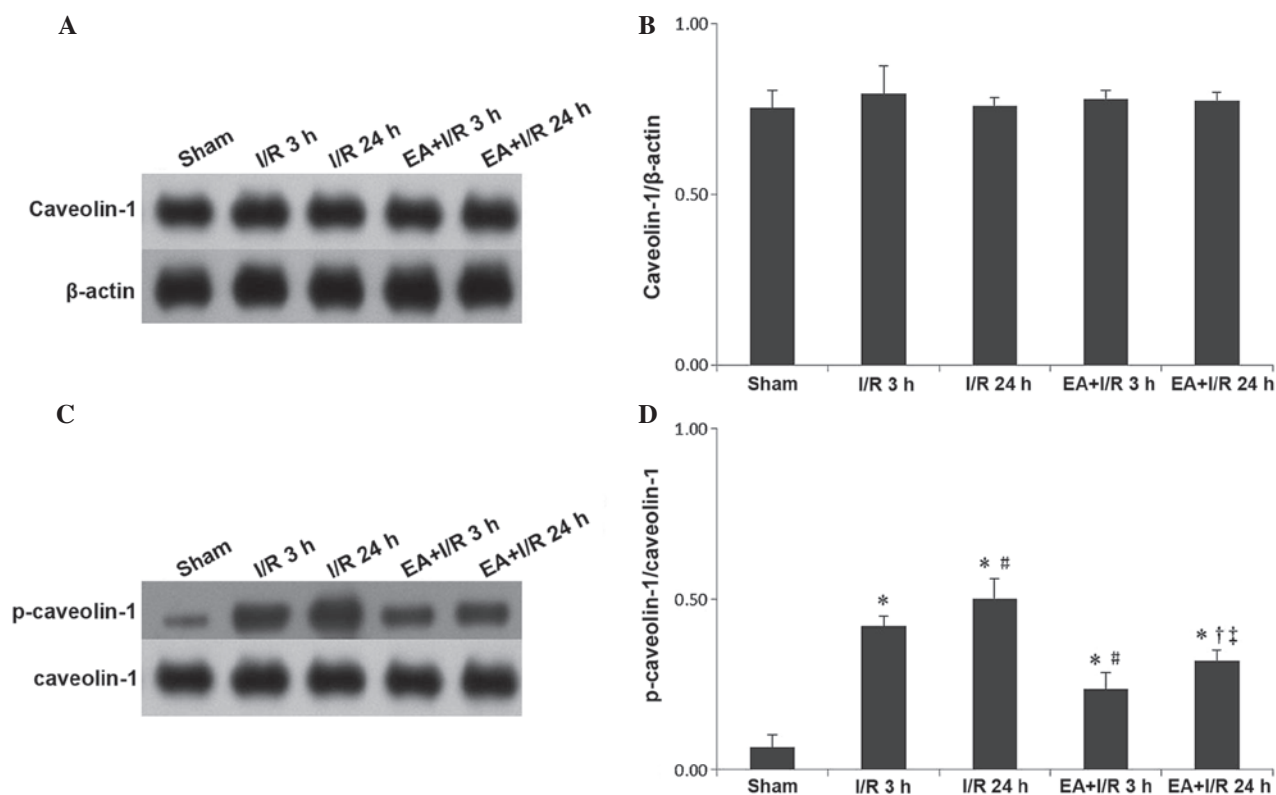


Figure 5. EA pretreatment inhibits the protein expression of p-caveolin-1 in the endothelium subsequent to I/R. (A) Representative western blots (n=5) and (B) quantitative analysis (n=5) of the expression of caveolin-1. (C) Representative western blots (n=5) and (D) quantitative analysis (n=5) of the expression of p-caveolin-1. The values are expressed as the mean \pm standard deviation. * P <0.05, compared with the sham group; # P <0.05, compared with the I/R3 h group; † P <0.05, compared with the I/R24 h group; ‡ P <0.05, compared with the EA+I/R3 h group. EA, electroacupuncture; I/R, ischemia/reperfusion; p-, phosphorylated.

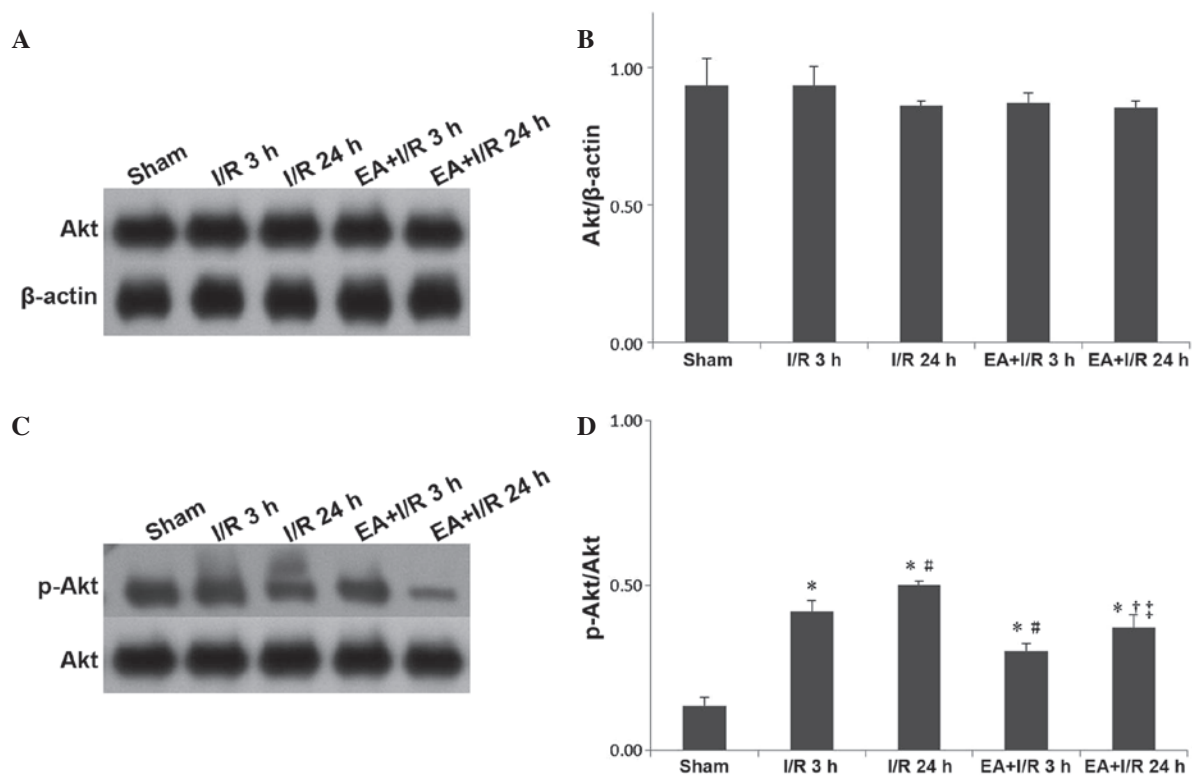


Figure 6. EA pretreatment inhibits the protein expression of p-Akt in the endothelium subsequent to I/R. (A) Representative western blots of Akt (n=5) and (B) quantitative analysis (n=5) of the expression of Akt. (C) Representative western blots of p-Akt (n=5) and (D) quantitative analysis (n=5) of the expression of p-Akt. The values are expressed as the mean \pm standard deviation. * P <0.05, compared with the sham group; # P <0.05, compared with the I/R3 h group; † P <0.05, compared with the I/R24 h group; ‡ P <0.05, compared with the EA+I/R3 h group. EA, electroacupuncture; I/R, ischemia/reperfusion; p-, phosphorylated.

I/R, and this was relieved by EA pretreatment. Therefore, the protective action of EA pretreatment on BBB permeability is possibly associated with paraendothelial transport.

Caveolin-1 is known to be important in vesicular trafficking by transcytosis, endocytosis and potocytosis (10). Previous studies have demonstrated that early BBB breakdown may be associated with increased or decreased expression of caveolin-1, using several experimental models (34-37). In the present study, the association between EA pretreatment and caveolin-1 was examined. Therefore, the effects of EA pretreatment on the expression of caveolin-1 following cerebral I/R were investigated. However, no significant differences in caveolin-1 were observed among the groups.

The phosphorylation of caveolin-1 at tyrosine14 is required to regulate caveolae formation and function (12,15), and the present study demonstrated that the expression of p-caveolin-1 was significantly increased following I/R compared with that in the sham group, while EA pretreatment significantly relieved this effect. These results suggested that p-caveolin-1 signaling increased the density of caveolae and caused transcytosis of proteins, leading to BBB breakdown and brain edema following cerebral I/R. These effects were reversed by EA pretreatment.

In the endothelium, caveolin-1 regulates nitric oxide signaling by binding to and inhibiting endothelial nitric oxide synthase (eNOS). Activation of the Akt kinase leads to eNOS activation and its dissociation from caveolin-1 (10). Therefore, the expression of Akt and p-Akt were also investigated in the present study. Similar to caveolin-1, no significant differences were observed among the groups for Akt. However, the expression of p-Akt was significantly increased following I/R compared with that of the sham rats, while EA pretreatment significantly relieved this effect. It was hypothesized that the increased expression of p-Akt may be due to the phosphorylation of caveolin-1, leading to the downstream activation of phosphatidylinositol 3-kinase and Akt. There may be a correlation between p-caveolin-1 and p-Akt.

In conclusion, the present study demonstrated that EA pretreatment was capable of protecting against I/R-induced BBB disruption in rats, partly by interference in the degradation of TJ protein and caveolin-1-mediated signal transmission in vascular endothelial cells. These findings suggested that EA pretreatment may provide novel strategies for the clinical treatment of I/R injury, as an alternative approach to alleviate severe brain edema. This requires further investigation.

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