

Electroacupuncture ameliorates learning and memory in rats with cerebral ischemia-reperfusion injury by inhibiting oxidative stress and promoting p-CREB expression in the hippocampus

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Abstract. The present study aimed to investigate the mechanisms by which electroacupuncture (EA) ameliorates learning and memory in rats with cerebral ischemic-reperfusion (I/R) injury. Focal cerebral ischemia was induced in adult male Sprague-Dawley (SD) rats by transient middle cerebral artery occlusion (MCAO). Following MCAO surgery, the rats received EA at the Shenting (DU24) and Baihui (DU20) acupoints. The results of the present study demonstrated that treatment with EA significantly ameliorated neurological deficits and reduced cerebral infarct volume ($P < 0.05$). In addition, EA improved the learning and memory ability of the rats, and markedly activated the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) signaling pathway, resulting in the inhibition of cerebral cell apoptosis in the ischemic penumbra. Furthermore, EA increased the activity of superoxide dismutase and glutathione peroxidase, the protein expression levels of phosphorylated-CREB and B-cell lymphoma 2 (Bcl-2), and the mRNA expression levels of Bcl-2. Conversely, EA decreased the levels of malondialdehyde and inhibited the expression levels of Bcl2-associated X protein. The results of the present study suggest that treatment with EA may result in the amelioration of learning and memory ability in rats with cerebral I/R injury.

Introduction

Cognitive function is regarded as the ability to objectively understand ideas. Cognitive function is composed of numerous cognitive domains, including memory, calculation, orientation

in time and space, structural ability, ability to perform tasks, and language comprehension and application (1-3). Previous studies have suggested that cognitive impairment has a detrimental effect on the recovery of motor function and the ability of impaired patients to perform daily activities, which is an important factor restricting the comprehensive rehabilitation of stroke patients (4-7). Stroke is a leading cause of mortality and permanent disability, and two-thirds of all strokes are considered ischemic (8). In addition, the incidence of cognitive impairment following a stroke may reach $\leq 65\%$ (9). Furthermore, in ~ 10 to 40% of patients with mild cognitive impairment, the condition may develop into dementia within a year (10-14). There is therefore an urgent requirement for the early detection of cognitive impairment, so that the mental and physical functions of patients can be fully recovered and dementia can be prevented.

Although the pathogenesis of stroke and post-stroke disabilities are complex (15), apoptosis has been suggested as one of the major pathways that may lead to cell death in brain injury following an ischemic stroke (16). In addition, oxidative stress caused by reactive oxygen species (ROS) has long been implicated in neurotoxicity following cerebral ischemic-reperfusion (I/R), and may ultimately result in the initiation of pathways that lead to apoptotic cell death (17). Since free radicals significantly affect the pathogenesis of cerebral ischemic injuries, high levels of free radicals may cause injury to the brain and detrimentally influence nervous function, learning ability, and memory. Therefore, the identification of strategies that suppress, and increase the clearance rate, of free radicals is important in the treatment of ischemic stroke.

The activation of the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) transcription factor has been reported to protect neuronal cells in cerebral ischemia (18,19) Furthermore, CREB and the CRE-mediated system are associated with learning and memory, and B-cell lymphoma 2 (Bcl-2) has a pivotal role in the control of cell death. Bcl-2 has been shown to be upregulated by ischemic tolerance, and its expression is regulated by CREB (20). Therefore, activation of CREB phosphorylation can increase

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Bcl-2 expression, which results in protection of the neuronal cells, and ameliorates learning and memory following cerebral ischemia.

Acupuncture is a simple, convenient and cost-effective treatment strategy originating from ancient China, which has been widely used for thousands of years to treat various diseases (21-23). Previous studies have demonstrated the clinical efficacy of acupuncture in stroke and rehabilitation of post-stroke cognitive impairment (24-27). Two acupoints located on the Du meridian; Baihui (DU20) and Shenting (DU24), are considered the most effective locations and have been commonly used in the treatment of cognitive impairment (28,29). In addition, electroacupuncture (EA), which uses fixed frequency and intensity instead of the traditional twisting and extracting techniques, has advantages including stability, strong persistence, and reduced variability and error between practitioners (30). However, the precise mechanism underlying the neuroprotective effects of EA on cognitive impairment remains unclear. Therefore, the present study aimed to evaluate the therapeutic efficacy of EA against post-stroke cognitive impairment. The underlying molecular mechanisms were investigated using a focal cerebral I/R-injured rat model.

Materials and methods

Animals. Healthy adult male Sprague-Dawley (SD) rats weighing 250-280 g were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and housed in pathogen-free conditions with a 12 h light/dark cycle. All experiments were performed strictly in accordance with the International Ethical Guidelines. The rats had *ad libitum* access to food and water during the experiment. The present study was approved by the Institutional Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine (Fuzhou, China).

Establishment of the cerebral I/R-injured rat model. Middle cerebral artery occlusion (MCAO) was used to establish a cerebral I/R-injured rat model, as previously described (31). Briefly, the rats were anesthetized using 10% chloral hydrate (300 mg/kg; Shanghai Chemical Reagent Co., Ltd., Shanghai, China) injected intraperitoneally. The left common carotid artery, the left external carotid artery, and the internal carotid artery (ICA) were then carefully exposed following a midline neck incision. Approximately 18 to 22 mm of nylon surgical thread (Beijing Sunbio Biotech Co., Ltd., Beijing, China) was inserted into the ICA until the blunted distal end met resistance, in order to block the left middle cerebral artery (MCA). The thread was removed following 2 h of occlusion to restore the blood supply to the MCA area, and reperfusion was achieved. Following awakening, the neurological deficit scores of the rats were assessed, prior to their random division into two groups (n=24/group): An ischemia (MCAO) control group, and an MCAO + EA group. The rats in the sham group (n=24) were subjected to the procedure as described above, without the occlusion of the MCA. Following the surgery, the rats were allowed to recover in prewarmed cages.

EA treatment. Following I/R injury, the rats in the EA group received EA treatment. Acupuncture needles, 0.3 mm

in diameter, were inserted at a depth of 2 to 3 mm into the heads of the rats at the Baihui (DU20) and Shenting (DU24) acupoints. Stimulation was then generated using EA apparatus (model G6805; Suzhou Medical Appliance Factory, Suzhou, China), and the stimulation parameters were set as follows: 5 and 20 Hz at 1-3 mA, dispersed for 30 min once daily. The treatment was performed 2 h following I/R treatment and was continued until the animals were sacrificed by 10% chloral hydrate intraperitoneal injection and decapitation, 7 days after the operation.

Assessment of neurological deficit scores. The neurological deficit score was assessed in a single-blind manner, as previously described by Chen *et al* (31). The neurological deficit scores were assessed on the first, third, fifth and seventh day following I/R injury. The scores were determined as follows: Score 0 indicated no neurological deficit; score 1, (failure to fully extend the right forepaw) indicated mild deficits; score 2, (circling of the right forepaw); score 3, (falling on the right forepaw) indicated moderate deficits; and score 4, (failure to walk) indicated severe deficits. Rats with scores 0 or 4 were excluded from the experiment.

Measurement of cerebral infarct volume. Following completion of the experiment, the rats were sacrificed and their brains rapidly collected. The brain tissue was coronally sectioned into slices 2 mm thick, prior to being stained with a 2% solution of tetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 20 min. The sections were subsequently fixed with 4% paraformaldehyde, as previously described (32). Normal tissue was stained deep red, whereas the infarct area was stained a pale gray color. The stained sections were scanned using a Canon SX20 high-resolution digital camera (Canon, Inc., Tokyo, Japan), and the infarct volume was quantified using the Motic Med 6.0 System (Motic Incorporation, Ltd., Causeway Bay, Hong Kong). The infarct volume was expressed as a percentage of the contralateral hemisphere volume.

Assessment of cognitive function. From the third day following surgery, the spatial learning and memory abilities (33-35) of the rats were investigated by subjecting them to a Morris water maze (Chinese Academy of Sciences, Beijing, China), a circular tank with a diameter of 120 cm and a height of 50 cm. The tank was filled with 22±1°C water to a depth of 30 cm. A circular escape platform, measuring 6 cm in diameter and 28 cm in height, was submerged 2 cm below the surface of the water. The tank was divided into four quadrants: Northeast, southeast, southwest, and northwest. These points served as the starting positions at which each rat was lowered gently into the water, its head facing the wall of the water maze. Morris water maze tasks include orientation, navigation and space exploration trials. In the first set of trials, each rat was placed in the water at four equidistant locations to the platform. If the rat arrived at the platform within the 90 sec time restriction and remained on it for 3 sec, it was considered to have found the platform and was scored on the time taken to complete the task, as well as the length of the chosen route. However, if the rat was unable to find the platform within 90 sec, it was placed on the platform for 10 sec and given a time score of

90 sec. The computer recorded the time taken and the length of the route by which each rat found the safe platform, and each day the average result of the time taken and the length of the route taken for the four quadrants were assessed for each rat. The duration of the first set of trials was 5 days, with the experiment performed once daily.

The second part of the experiment was performed on the seventh day following surgery. Briefly, the ability of each rat to remember the position of the platform was evaluated by measuring the time in which each rat found the platform within the 90 sec time restriction. Following the trials, the rats were thoroughly dried with a hair dryer and returned to their cages.

Determination of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities, and malondialdehyde (MDA) levels. The ischemic brain hippocampus of each rat was collected on the seventh day following MCAO. The brains were rinsed, weighed, and homogenized in 9 volumes of 9 g/l ice-cold saline for 10 min using a Dounce Tissue Grinder (Kimble Chase Life Science and Research Products LLC, Vineland, NJ, USA). The supernatant homogenate was collected following centrifugation at 12,000 x g for 10 min at 4°C. The total protein concentrations were then determined using a Bradford protein assay (Novagen, Inc., Madison, WI, USA). The SOD and GSHPx activities, and the MDA levels were measured using assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The brains of the rats were removed immediately following decapitation, and the ischemic brain tissues were dissected and maintained at -80°C until use. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and the Oligo(dT)-primed RNA (1 µg) was reverse transcribed into cDNA, according to the manufacturer's instructions (Fermentas, Thermo Fisher Scientific, Pittsburgh, PA, USA). The cDNA was subsequently used to determine the expression levels of Bcl-2 and Bax mRNA by PCR using Taq DNA polymerase (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA), and β-actin was used as an internal control. The primer sequence were as follows: Bcl-2, forward, 5'-GGTGGTGGAGGAAGTCTTCA-3'; and reverse, 5'-GAGCAGCGTCTTCAGAGACA-3'; Bax forward, 5'-GAGCAGCGTCTTCAGAGACA-3'; and reverse, 5'-TCACGGAGGAAGTCCAGTGT-3'; and β-actin forward, 5'-ACTGGCATTGTGATGGACTC-3'; and reverse, 5'-CAGCACTGTGTTGGCATAGA-3' (Shanghai Institute of Bioengineering, Shanghai, China). The PCR products were analyzed on a 1.5% agarose gel and examined using a gel documentation system (Model Gel Doc 2000; Bio-Rad Laboratories Inc., Hercules, CA, USA).

Western blot analysis. The left cerebral hippocampal tissues were collected and triturated in a radioimmunoprecipitation assay buffer (Fansbio, Guangzhou, China), and the proteins were quantified using a bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). The protein lysates were separated by electrophoresis by 12% SDS-PAGE, prior

to being transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), which were blocked for 2 h with 5% non fat dry milk at room temperature. The blots were then incubated with primary antibodies targeting Bcl-2 (1:1,000; cat. no. 15071; Cell Signaling Technology, Inc., Danvers, MA, USA), Bax (1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), phosphorylated (p)-CREB (1:1,000; cat. no. 9198; Cell Signaling Technology, Inc.), and β-actin (1:4,000; cat. no. AA-128; Beyotime Institute of Biotechnology, Haimen, China) overnight at 4°C, prior to incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000; goat anti-rabbit IgG; cat. no. 611-1322-0500; Rockland Immunochemicals Inc., Pottstown, PA, USA) for 1 h at room temperature. The bands were visualized with enhanced chemiluminescence (Amersham, GE Healthcare, Piscataway, NJ, USA), and the images were captured using a Bio-Image Analysis system (Bio-Rad Laboratories Inc.).

Statistical analysis. The data are expressed as the mean ± standard deviation (SD) and statistically analyzed by one-way analysis of variance using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA), prior to being subjected to a post hoc least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

EA reduces neurological deficits and infarct volume in rats following MCAO. To evaluate whether EA at the Baihui (DU20) and Shenting (DU24) acupoints attenuated ischemic brain injury, the neurological scores were determined at various time points following a stroke. As hypothesized, the rats in the sham group did not exhibit any manifestations of neurological deficits, whereas all of the rats in the MCAO and MCAO + EA groups exhibited clear symptoms of cerebral injury (Fig. 1). However, the neurological function scores were significantly improved in the MCAO + EA group, as compared with the MCAO group (P<0.05). To further verify these results, the effects of EA on cerebral infarction were evaluated. As shown in Fig. 2, the infarct volume was measured using TTC staining. Normal tissue was stained deep red, whereas the infarct area was a pale cream color. There was a statistically significant decrease in infarct volume in the MCAO + EA group, as compared with the MCAO group (P<0.05).

EA ameliorates cognitive impairment in cerebral I/R-injured rats. A Morris water maze test was performed on the third to seventh day following MCAO surgery. As shown in Fig. 3, the rats in the MCAO group had longer latency periods and took longer routes to reach the hidden platform. In addition, the number of times that the MCAO rats crossed the location of the platform was significantly lower, as compared with the rats in the sham group (P<0.05). However, the rats in the EA group had a shorter latency and route length, and the number of times they crossed the platform was higher, as compared with the MCAO group (Fig. 3).

Effects of EA on MDA content and the activity of antioxidant enzymes. To evaluate whether EA affects oxidative stress damage, the MDA content and the activities of antioxidant

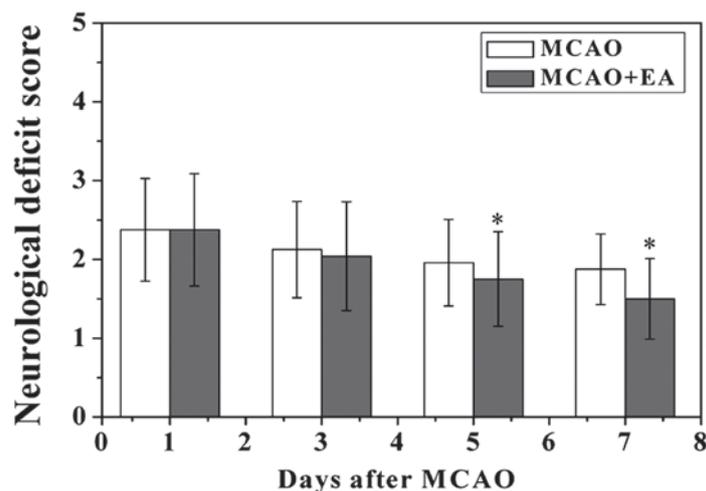


Figure 1. Effects of electroacupuncture (EA) at the Baihui (DU20) and Shenting (DU24) acupoints on the neurological deficits in cerebral ischemia-reperfusion-injured rats. The neurological deficit score was determined, and the data are presented as the mean \pm standard deviation from 18 individual rats. * $P < 0.05$, vs. MCAO group. MCAO, middle cerebral artery occlusion.

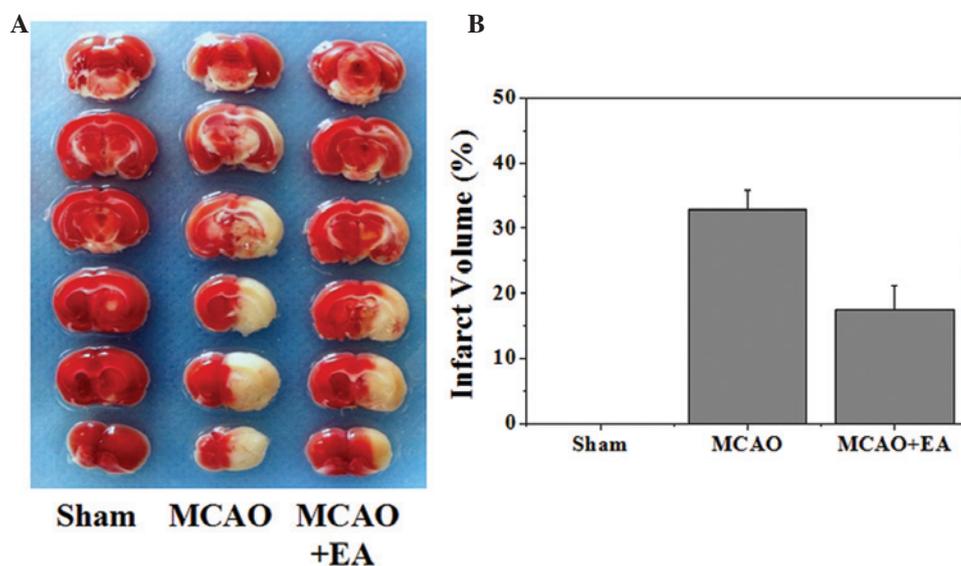


Figure 2. Effects of electroacupuncture (EA) at the Baihui (DU20) and Shenting (DU24) acupoints on cerebral infarction following cerebral ischemia-reperfusion. (A) Tetrazolium chloride staining was used to indicate cerebral infarct volume of Sham, middle cerebral artery occlusion (MCAO) and MCAO + EA groups. The representative captured images were taken using a high-resolution digital camera. (B) Infarct volume was quantified using Motic Med 6.0 system and is represented as a percentage of the total brain volume. Data are presented as the mean \pm standard deviation from six individual rats in each group. * $P < 0.05$, vs. MCAO group.

enzymes in the hippocampus were investigated. MDA content, an index of lipid peroxidation was significantly increased following cerebral I/R injury ($P < 0.05$), whereas MDA content was significantly decreased after EA treatment, as compared with the MCAO group ($P < 0.05$). Furthermore, the activities of the antioxidant enzymes SOD and GSHPx were decreased in the MCAO group, as compared with the sham group ($P < 0.05$). However, EA treatment induced a significant elevation of SOD and GSHPx activities as compared with the MCAO group ($P < 0.05$) (Fig. 4).

Effects of EA on p-CREB and apoptosis-associated factors. To investigate the mechanism underlying the anti-apoptotic effects of EA, western blot analysis was used to examine the effects of EA on the immunoreactivity of p-CREB in

the hippocampus. As shown in Fig. 5, a significant decrease in the immunoreactivity of p-CREB was observed in the hippocampus following MCAO ($P < 0.05$). Conversely, EA significantly attenuated the decrease in the immunoreactivity of p-CREB ($P < 0.05$). Western blotting and RT-qPCR were used to evaluate both the protein and mRNA expression levels of the vital target genes Bcl-2 and Bax. As shown in Fig. 6, EA treatment significantly ($P < 0.05$) increased the mRNA expression levels of Bcl-2 and decreased the mRNA expression levels of Bax caused by the cerebral I/R injury.

Discussion

EA is a core component of traditional Chinese medicine, which is recognized as an effective treatment for numerous

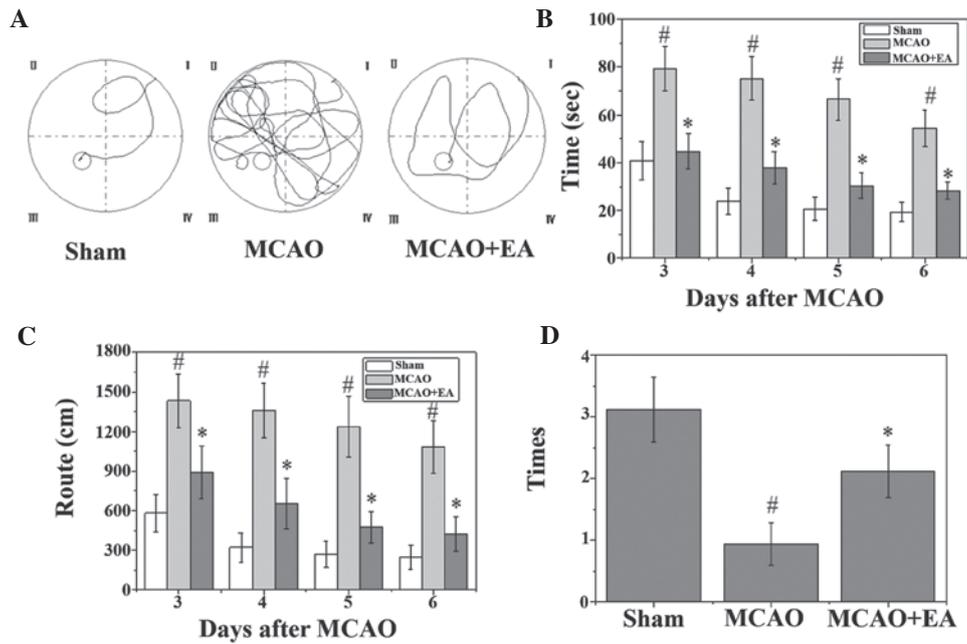


Figure 3. Effects of electroacupuncture (EA) on cognitive impairment in cerebral ischemia-reperfusion (I/R) injured rats. The learning and memory ability of the rats was determined using a Morris water maze test on days 3-6 following I/R injury. (A) Tracing images from the Morris water maze test. (B and C) Time taken for the rats to find the platform (within 90 sec) and the length of the routes. (D) The number of times the rats passed through the area in which the platform was located on the 7th day after I/R injury. Data are presented as the mean \pm standard deviation in each group. [#]P<0.05, vs. Sham group; ^{*}P<0.05, vs. MCAO group.

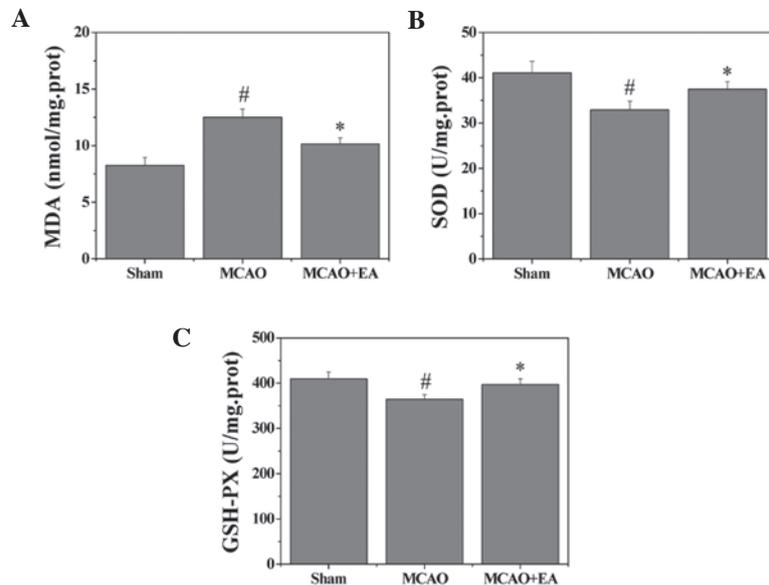


Figure 4. Electroacupuncture (EA) ameliorates oxidative stress following cerebral ischemia-reperfusion (I/R) injury. EA significantly decreased the levels of (A) malondialdehyde (MDA), and increased (B) superoxide dismutase (SOD), and (C) enhanced glutathione peroxidase (GSH-Px) activities in I/R rats. Data are presented as the mean \pm standard deviation from six individual rats in each group. [#]P<0.05, vs. sham group; ^{*}P<0.05, vs. middle cerebral artery occlusion (MCAO) group.

chronic diseases, with no side effects. Numerous studies have demonstrated the clinical efficacy of acupuncture in stroke and cognitive impairment (28). The Baihui (DU20) and Shenting (DU24) acupoints are situated on the Du meridian, which is considered to be beneficial to human health, good spirits, and memory function. The results of the present study demonstrated that EA on the Baihui and Shenting acupoints could significantly ameliorate neurological deficits

and reduce cerebral infarct volume. Consistent with previous reports (27,36,37), a Morris water maze test revealed that EA improved the learning and memory ability of rats with cerebral I/R injury, demonstrating the therapeutic efficacy of EA against post-stroke cognitive impairment.

The most effective treatment for acute ischemic stroke is reperfusion of the ischemic penumbra. However, I/R injury often leads to secondary damage. Therefore, anti-reperfusion

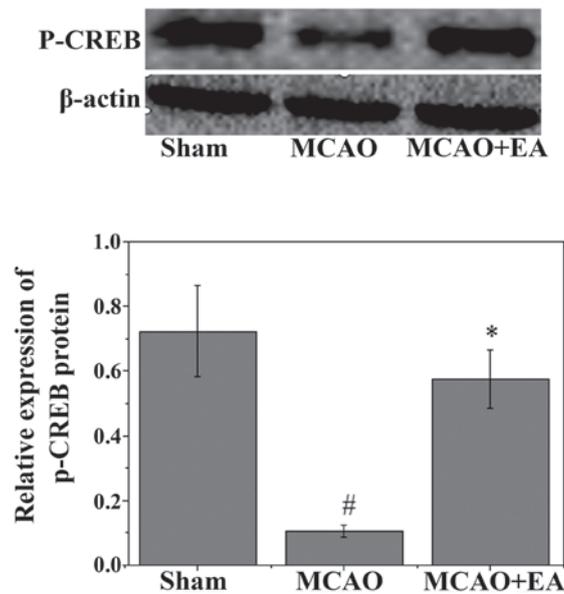


Figure 5. Effects of electroacupuncture (EA) on the expression levels of phosphorylated-cyclic adenosine monophosphate (cAMP) response element-binding protein (p-CREB) in cerebral ischemia-reperfusion-injured rats. The protein expression levels of p-CREB were analyzed by western blotting. β -actin was used as an internal control for western blotting. The data are representative of six individual rats in each group. Data are presented as the mean \pm standard deviation. [#] $P < 0.05$, vs. sham group; ^{*} $P < 0.05$, vs. middle cerebral artery occlusion (MCAO) group.

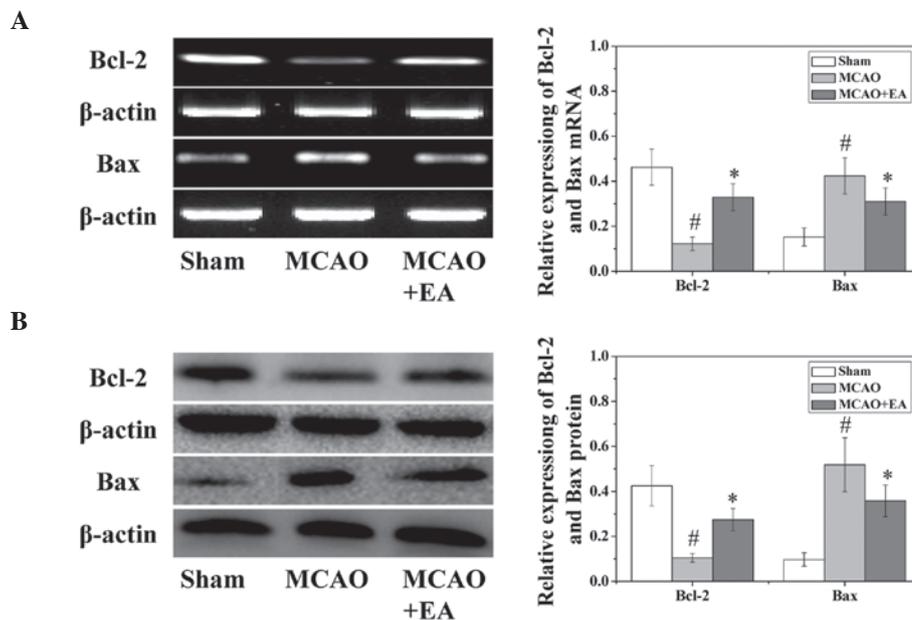


Figure 6. Effects of EA on the expression levels of apoptosis-associated proteins in cerebral ischemia-reperfusion-injured rats. The protein and mRNA expression levels of B cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein were determined by (A) reverse transcription quantitative polymerase chain reaction and (B) western blotting 7 days following cerebral ischemia. β -actin was used as an internal control. The data are representative of six individual rats in each group. Data are presented as the mean \pm standard deviation. [#] $P < 0.05$, vs. sham group; ^{*} $P < 0.05$, vs. middle cerebral artery occlusion (MCAO) group.

injury and neuroprotection are critical for stroke management. Furthermore, oxidative stress has been well-established as the main mechanism underlying I/R injury, and reactive oxygen species (ROS) produced in the mitochondria have an important role in regulating the neurocyte apoptotic pathway during I/R (38).

Under physiological conditions, ROS are generated at low levels, controlled by endogenous antioxidants, such as SOD and GSHPx (39). However, the sudden overproduction of ROS

during cerebral I/R leads to oxidative stress, which results in cell damage in nervous tissue. This may lead to the induction of chain reactions, such as membrane lipid peroxidation (17). ROS produce MDA, a toxic end-product of lipid peroxidation, and MDA levels directly reflect the rate and extent of lipid peroxidation (40). SOD and GSHPx enzymes are thought to act as free radical scavengers that may prevent the deleterious stroke-induced ROS generation (41); therefore, their expression levels may directly reflect the capacity of the brain tissue

to eliminate free radicals. The results of the present study demonstrated that EA could protect the brain tissue from damage by stimulating SOD and GSHPx activity and by decreasing the levels of MDA.

Apoptosis is one of the predominant types of neurocyte death in the ischemic penumbra during the progression of an ischemic stroke. A previous study (42) demonstrated that memory impairment in MCAO rats was associated with neuronal apoptosis in the hippocampus. In addition, apoptosis was reportedly suppressed by the enhanced expression of Bcl-2 (43,44). The expression of Bcl-2 is mediated by CREB (45), and p-CREB decreases Bax expression (46). In brain tissue, CREB is associated with learning, memory, and dendritic transmission. When hippocampal CREB activity decreases due to cerebral ischemia, impairment of learning and memory ability occurs. Furthermore, CREB knockout mice were reported to exhibit memory impairment (47), with the relative level of CREB activity at the time of learning being a key factor in determining whether a neuron was recruited into the memory trace. The present study demonstrated that compared with the MCAO group, treatment with EA could increase the immunoreactivity of p-CREB and Bcl-2, and decrease the immunoreactivity of Bax. These results suggested that EA is associated with pro-apoptotic activity and the amelioration of learning and memory ability, which may be mediated via activation of CREB phosphorylation.

In conclusion, the present study demonstrated that EA on the Baihui (DU20) and Shenting (DU24) acupoints was able to improve cognitive impairment following cerebral ischemia. The protective effects of EA were associated with an anti-apoptotic mechanism, via activation of CREB, and inhibition of oxidative stress. These results indicate that EA may have therapeutic potential for the treatment of post-stroke cognitive impairment.

Acknowledgements

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