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Electroacupuncture Inhibits Apoptosis of Peri-Ischemic Regions via Modulating p38, Extracellular Signal-Regulated Kinase (ERK1/2), and c-Jun N Terminal Kinases (JNK) in Cerebral Ischemia-Reperfusion-Injured Rats

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Background: Previous studies suggested that inhibition of apoptosis prevents the dysfunction of ischemia-reperfusion injury. In the pathogenesis of ischemia-reperfusion injury, JNK/ERK1/2 and p38 play an essential role in regulation of cell apoptosis. Electroacupuncture (EA), a form of acupuncture, has demonstrated superiority in preventing ischemia-reperfusion injury, but the underlying mechanism is unclear. In the present study, we explored the effects of electroacupuncture at Shenting (GV24) and Baihui (GV20) acupoints on focal cerebral ischemia-reperfusion (MCAO) rats, and explored whether JNK/ERK1/2- and p38-mediated cell apoptosis are involved.





Material/Methods: The rats were divided into a sham operation control group, an ischemia group, and an electroacupuncture group with acupuncture applied for 10 days (30 min per day). TTC staining was used to calculate the ischemic brain volume. TUNEL staining and transmission electron microscopy were used to detect cell apoptosis. Western blot analysis and Bio-Plex were used to detect JNK, p38, ERK1/2, Bcl-2, and Bax protein expression.

Results: We found that electroacupuncture at day 10 significantly reduced cerebral infarction. In addition, electroacupuncture suppressed activation of JNK and p38, while enhancing the activation of ERK1/2 in the peri-ischemic regions. Consequently, the effect of electroacupuncture on these pathways resulted in the inhibition of apoptosis, which was demonstrated by TUNEL and transmission electron microscopy. We found that electroacupuncture upregulated the anti-apoptotic Bcl-2/Bax ratio in peri-ischemic regions.

Conclusions: Our findings suggest that inhibition of cell apoptosis via regulating multiple signaling pathways might be a mechanism whereby electroacupuncture has a positive therapeutic effect on post-stroke impairment.

MeSH Keywords: **Acupuncture • Apoptosis Regulatory Proteins • Stroke**

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Background

Apoptosis, a genetically programmed cell death process, can be regulated by pro- and anti-apoptotic proteins such as the Bcl-2 family and Fas system [1,2] and plays an important role in the progression of cerebral ischemia-reperfusion injury. Several studies have confirmed that the inhibition cell apoptosis can reduce ischemia-reperfusion injury [3,4]. In the mitochondrial signaling pathway, Bcl-2 family members play a key role in regulating apoptosis, and changes in the balance of Bcl-2 and Bax may result in either inhibition or induction of cell apoptosis [5,6]. There are many activating signaling pathways that regulate apoptosis during post-stroke, such as the ERK1/2, JNK, and p38 signaling pathways [7–9].

Ischemia-reperfusion injury causes overproduction of free radicals, which can include triggers of apoptosis by activating lipid peroxidation and mediating Ca²⁺ channel signals, altering membrane phospholipids [10]. In the mitochondrial signaling pathway, overproduction of free radicals is followed by release of apoptotic proteins such as cytochrome c (CytC) into the cytosol. CytC interacts with the apoptotic protease to form an apoptosome, which serves as a scaffold to bring the initiator caspase molecules together. Caspase-9 and caspase-8 are activated in the pathway, leading to cleavage and activation of procaspase-3 [11–13], which is one of the important apoptosis-related regulatory factors. In the process, the Bcl-2 protein family plays an important role in regulating apoptosis [5]. Many apoptosis-regulating signaling pathways are activated during cerebral ischemia-reperfusion, such as ERK1/2, JNK, and p38 signaling pathways. These pathways play a key role in regulating apoptosis [14–16]. ERK is a signaling molecule that activates gene expression [17]. Many studies have reported that ERK1/2 activation promotes cerebral cell proliferation and regulates ischemia-induced apoptosis by adjusting Bcl-2 family expression [18,19]. C-Jun N-terminal kinases (JNK) also play an important role in apoptosis by interacting with the mitochondrial apoptotic machinery [20,21]. p38 is responsive to stress stimuli and is involved in cell apoptosis [22] through activation of poly-ADP-ribose polymerase (PARP) and caspase-9-mediated mechanisms [23]. These signaling pathways regulate apoptosis, which mainly involves the Bcl-2 family, such as Bcl-2/Bax proteins, which plays an important role in anti-apoptosis.

In preclinical studies, activation of anti-apoptotic mechanisms has become a promising strategy for treatment of post-stroke impairment [24]. In Traditional Chinese Medicine (TCM), acupuncture has relatively significant benefits and play an important role in the treatment of central nervous system diseases [25]. Electroacupuncture (EA), with electro-stimulation [26] in which 2 needles are inserted as electrodes to pass an electric current, is one style of acupuncture [27]. EA has the advantage

of setting stimulation frequency and intensity objectively and quantifiably. Previous study have suggested that, compared to manual acupuncture, EA was more effective in some situations, particularly when strong, continued stimulation is required [28]. It has been reported that EA can produce more widespread functional magnetic resonance imaging signal increase in anterior middle cingulate cortex [29]. Wu et al. have found that, compared to sham EA, real EA elicited significantly higher activation over the hypothalamus and primary somatosensory-motor cortex and deactivation over the rostral segment of anterior cingulate cortex [30]. Shenting (GV24) and Baihui (GV20) are Du Meridian acupoints and play a crucial in the nervous system modulation in TCM. It is generally believed that the physiological effects of electroacupuncture are caused by the mechanical or electrical stimulation of the autonomic nervous system via induction of the somatosensory pathway or release of neuroactive mediators from immune cells [31].

Many studies have shown that acupuncture at Baihui and Shenting acupoints can significantly improve impaired functions such as motor control and cognition of post-stroke patients [32]. Our previous study indicated that electroacupuncture at Baihui and Shenting can improve neurological deficit scores and ameliorate cognitive impairment in ischemic rats via inhibiting NF- κ B-mediated neuronal cell apoptosis, which ameliorated cognitive impairment in ischemic rats [33]. However, since TCMs are considered to be multi-component and multi-targeted agents [34], it is unclear whether other signaling pathways are activated. In this study, we used an MCAO model to investigate the mechanisms underlying the effects of electroacupuncture at Baihui and Shenting acupoints. In additional, Huang et al. conducted a systematic review and found mild stimulation, which means the reinforcing method in TCM theory is adopted widely including improving functional impairment of post-stroke [35]. Shu et al. found EA at GV26 acupoints and right sulcus auriculae posterior with the frequency of 2 and 20 Hz for 30 min, alternatively (disperse-dense waves), and intensity strong enough to only elicit slight twitches of the orofacial areas can significantly decreased neurological deficit scores and the volume of cerebral infarction, and relax the autophagy and apoptosis in cerebral ischemia reperfusion injury rats [36]. Our previous study suggested electroacupuncture at Shenting and Baihui with the parameter of 30 min/d and disperse-dense waves of 1/20 Hz of frequency can significantly downregulated the expression of Nogo-A and NgR in the hippocampus of the cerebral ischemia side [37], improve neurological deficit score, and reduce cerebral infarct volumes in cerebral ischemia-reperfusion injured rats [38,39]. In our previous study, we also found the number of times that rats crossed the location of the platform in the Morris water maze test was significantly increased compared to the MCAO group, which suggests that electroacupuncture ameliorated cognitive impairment in

cerebral ischemia-reperfusion-injured rats by EA at Shenting (GV24) and Baihui (GV20) with the above parameter for 10 days, and also significantly suppressed the ischemia-reperfusion induced activation of NF- κ B signaling in ischemic cerebral tissues [39]. Therefore, EA at Shenting and Baihui with the parameter of 30 min/d and disperse-dense waves of 1/20 Hz of frequency for 10 days may be an effective parameter to improve post-stroke dysfunction. In the present study, we used electroacupuncture using the above parameter to explore the underlying mechanisms. We hypothesized that electroacupuncture at Shenting (GV24) and Baihui (GV20) acupoints inhibits cell apoptosis in cerebral ischemia-reperfusion injury rats via modulating p38, ERK $\frac{1}{2}$, and JNK.

Material and Methods

Material and reagents

TUNEL assay kits were obtained from Promega (Madison, WI, USA). All antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Cell Signaling Technology, Inc (Beverly, MA, USA). Bio-Plex phosphoprotein assay kits were purchased from Bio-Rad Laboratories (Hercules, CA, USA). 2,3,5-Triphenyltetrazolium chloride (TTC) and all the additional chemicals used, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Animals and ethics

Sixty adult male Sprague–Dawley rats (250–280 g) were supplied by SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were housed in specific pathogen-free rooms in the Central Laboratory of Fujian University of Traditional Chinese Medicine (Fuzhou, China) at 22°C with a 12–12 h light/dark cycle. Food and water were provided freely. The rats were randomly divided into a sham operation control group (sham group, n=20) and an ischemia group (n=40) by use of a computer-generated randomization schedule. All animals were treated strictly according to the National Institutes of Health guide concerning the Care and Use of Laboratory Animals and international ethical guidelines. The study was approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (Ethics Committee reference number: Fujian university of Traditional Chinese Medicine [2015] Ethics Committee reference number [008]; Fuzhou, China).

The middle cerebral artery occlusion model (MCAO), which was developed by Longa et al. as a reliable method for studying reversible regional ischemia in rats without craniectomy model [40], has been widely used in ischemia-reperfusion injury studies [41,42]. In the present study, we established the cerebral ischemia-reperfusion model by MCAO. Briefly, all rats

were anesthetized with 10% chloral hydrate (300 mg/Kg; i.p.). The left common carotid artery (CCA), the left external carotid artery (ECA), and the internal carotid artery (ICA) were exposed via a neck incision. The ECA was blocked proximal to the branch of the ECA and ICA. The middle cerebral artery (MCA) was occluded with an embolus made from fishing line with 0.28–0.30-mm diameter through a small incision in the ICA. Reperfusion was achieved by removing the line after 120 min. The body temperatures of the animals were maintained at 37°C during surgery. Only the CCA, ICA, and ECA were isolated, without endovascular occlusion in the sham group.

Twenty-four hours after reperfusion, the rats were evaluated in blinded fashion using the neurological deficit score, which is used to examine the neurologic function of the rats after MCAO [40]: 0=no deficit, 1=failure to extend the right forepaw fully, 2=circling to the right, 3=falling to the right, and 4=no walking and depressed level of consciousness. The rats with scores of 1–3 were included into the experiment and were randomly divided into the ischemia control group (MCAO group, n=19) or the electroacupuncture group (EA+MCAO group, n=19) (1 rat was excluded due to the neurological deficit score=0 and 1 rat died during the operation) by use of a computer-generated randomization schedule. In the EA group, electroacupuncture at Shenting (GV24) and Baihui (GV20) acupoints with dense disperse wave (1 or 20 Hz, 1 mA, adjusted to the muscle twitch threshold) was performed for 10 days (30 min per day) after the MCAO operation with the electroacupuncture apparatus (SDZ-II, Huatuo, Suzhou, China). In the MCAO and the sham groups, no intervention was given to the rats. There were 56 rats at the end of the experiment (n=20 in sham group, n=17 in MCAO group, n=19 in EA+MCAO group), and 2 rats died during the experiment in MCAO group. Four rats each group were used for TTC staining, 4 rats in each group were used for TUNEL staining, 4 rats each group were used for transmission electron microscopy, and 4 rats each group were used in both Western blotting analysis and Bio-Plex phosphoprotein assay.

Biochemical staining

TTC staining

Triphenyltetrazole oxide (TTC) staining was used to evaluate the cerebral infarction area from the morphological level. Ten days after the operation, the rats were anesthetized (i.p.) with 10% chloral hydrate, then the anesthetized animals were sacrificed by cervical dislocation. The brains were quickly removed and sectioned into five 2-mm coronal slices, and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 20 min and fixed with 10% buffered formalin solution at 4°C, followed by scanning with a high-definition camera (Canon SX20, Tokyo, Japan). The infarct volume was determined with the Motic Med 6.0 System (Motic, China). The

percentage of ischemic brain was calculated by taking the volume of infarcted ischemia, dividing by the total brain volume, and multiplying by 100.

TUNEL staining

In the present study, we used the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), a method for detecting DNA fragmentation by labeling the 3'-hydroxyl termini in the double-strand DNA breaks generated during apoptosis, which is widely used to identify and quantify apoptotic cells [43,44] to examine effects of EA on the apoptosis level of the MCAO rats. The peri-ischemic region brain tissues were fixed with 4% paraformaldehyde and cut into 5- μ m-thick coronal sections after being paraffin-embedded. The nuclei of all cells were stained by DAPI and the green fluorescence of apoptotic cells were detected using a confocal fluorescence Carl Zeiss microscope (LSM 710, Thornwood, NY, USA), according to the TUNEL assay kit manufacturer's protocol. Apoptotic cells were counted in 4 randomly selected areas at a magnification of 400 \times , and the percent apoptotic cells were calculated as the ratio of green-stained cells to the blue fluorescence cells.

Transmission electron microscopy

Transmission electron microscopy was used to observe cellular and ultrastructural changes of neurons after EA intervention. The fixative-perfused brain specimens were fixed with a mixture of 5% glutaraldehyde and 10% paraformaldehyde for 72 h at 4°C, followed by 1% osmium tetroxide in cacodylate buffer for 2 h at room temperature. After washing with PBS, the samples were dehydrated with the standard samples and embedded in an Epon-Araldite 618 mixture. The brain was sectioned into 80-nm modular ultrathin slices and observed with a H70 electron microscope operating at 80 kV. The photos were obtained on an HP digital CCD camera (SIS4million Volex).

Western blotting analysis

To qualitatively assess the effective of EA on the BCL-2 and Bax protein expression, which plays a key role in the progress of the modulation of post-stroke, we used Western blotting analysis. After total protein of the peri-ischemic region brains tissues was collected and quantified by bicinchoninic acid (BCA) assay, the lysates were separated by SDS-PAGE gels and transferred onto PVDF. The membrane was then incubated with primary antibodies against Bcl-2, Bax, and β -actin (1: 1000; Cell Signaling Technology) overnight at 4°C. A computer scan was performed and processed by use of the Bio-Image Analysis System (Bio-Rad, Hercules, CA, USA) after the corresponding secondary antibody (1: 5000) was added (n=4/group).

Bio-Plex phosphoprotein assay

In the present study, we used the Bio-Plex phosphoprotein assay to test the phosphoprotein level of ERK1/2, JNK, and p38. Compared to other protein detection methods, the Bio-Plex assays was shown to be robust and reliable and can reduce sample volume and time of experiment [45]. After the total protein of peri-ischemic hippocampal tissue of the ischemic side was collected and quantified by bicinchoninic acid (BCA) assay, the presence of p-ERK1/2, p-JNK, and p-p38 was detected using a bead-based multiplex assay for phosphoproteins (Bio-Plex Phosphoprotein assay, Bio-Rad Laboratories). Data were collected and analyzed using the Bio-Plex 200 suspension array system (Bio-Rad).

Statistical analysis

Data are presented as means with standard errors of the mean (mean \pm SEM). All data were analyzed using IBM SPSS 24.0 (IBM Inc., Armonk, NY, USA) statistics software. Statistical analysis was performed with the *t* test and one-way analysis of variance (ANOVA). We performed post hoc analysis by Fisher's least significant difference (LSD) or Games-Howell test. Statistical significance was determined by $p < 0.05$.

Results

Effects of electroacupuncture at Baihui and Shenting acupoints on cerebral infarct volumes

As shown in Figure 1, the cerebral infarct volumes in the MCAO and EA+MCAO groups were significantly increased compared to the sham group ($p < 0.01$), indicating successful development of an I/R injury model. At day 10, there were significant decreases in cerebral infarction volumes of the EA+MCAO group ($p < 0.05$) ($p < 0.05$, vs. the MCAO group), showing that EA provided significant therapeutic efficacy in preventing cerebral I/R injury.

Effects of electroacupuncture at Baihui and Shenting acupoints on inhibiting apoptosis

As shown in Figure 2A, the percentage of TUNEL-positive cells in the peri-ischemic region cortex in the MCAO group ($70 \pm 0.8\%$) was significantly increased compared to the sham group ($11 \pm 0.5\%$), indicating that ischemia-reperfusion can induce apoptosis in cells by day 10. The percentages were significantly decreased ($21 \pm 0.9\%$) in the EA+MCAO group, suggesting the anti-apoptotic activity of EA *in vivo*.

As shown by the SEM in Figure 2B in the sham group, there were abundant mitochondria and intact mitochondrial crests were found. The large axon can be seen and the chromatin

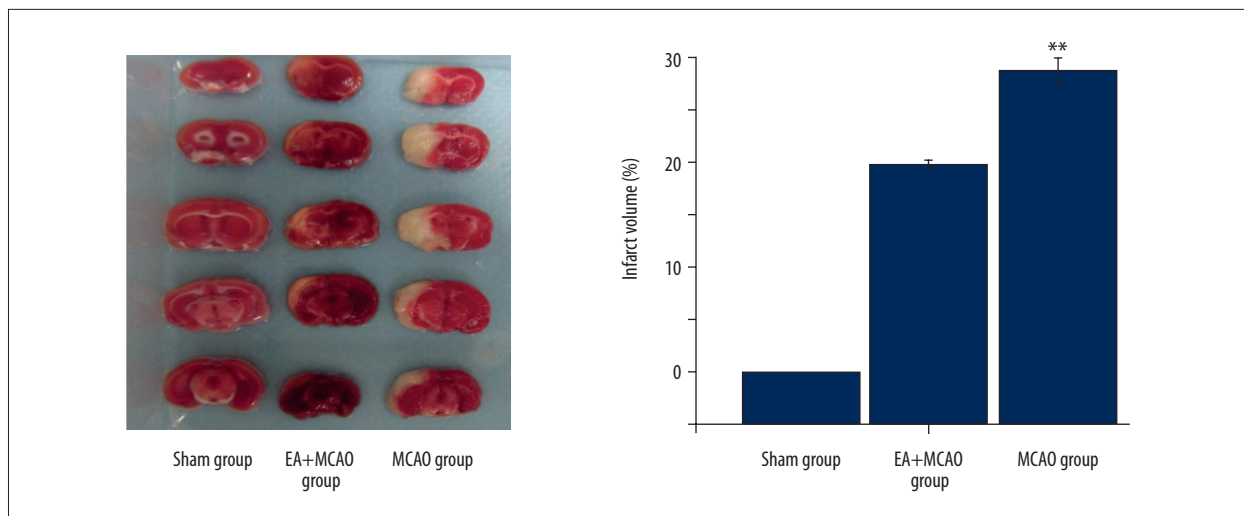


Figure 1. Effects of electroacupuncture at Baihui and Shenting on the cerebral infarction in injured rats. The cerebral infarct volumes in the MCAO group and EA+MCAO group were significantly lower, while EA treatment for 10 days significantly reduced the cerebral infarction volumes ($p < 0.01$, vs. the MCAO group) (*, EA+MCAO group vs. MCAO group; ** $p < 0.01$) ($n = 4$ in each group).

was uniformly distributed. In the MCAO group, the euchromatin was found at the edge of the neuronal nuclei, the cells appeared smaller than in the sham group, and the electronic density was deep, which are the early apoptotic signals. In the EA+MCAO group, the cell membrane was partly ruptured and the mitochondria were slightly damaged. No changes in the rough endoplasmic reticulum and axon were observed in the EA+MCAO group. The cell nucleoli were complete and chromatin was uniformly distributed.

Effects of electroacupuncture at Baihui and Shenting acupoints at the translational level of Bcl-2 and Bax

To further explore the mechanism of the anti-apoptotic effect of EA, we evaluated the protein levels of Bcl-2 and Bax, which are essential anti-apoptotic and pro-apoptotic mediators. As shown in Figure 3A and 3B, EA treatment profoundly upregulated the Bcl-2/Bax ratio at translational levels after cerebral injury (vs. MCAO group).

Effects of electroacupuncture at Baihui and Shenting acupoints on the levels of ERK1/2, JNK, and p38 in MCAO rats

To further investigate the mechanism of the anti-apoptotic activity of electroacupuncture, we investigated the phosphorylation level of ERK1/2, JNK, and p38 in the peri-ischemic region cortex. As shown in Figure 4, the phosphorylation level of ERK1/2 (the ratio of phosphorylation ERK1/2 and total ERK 1/2, p-ERK1/2/t-ERK1/2) was significantly increased, whereas that of JNK (p-JNK/t-JNK) and p38 (p-p38/t-p38) was significantly reduced (Figure 4, $p < 0.05$, vs. MCAO group). These data

suggest that EA modulated the activation of multiple apoptotic signaling pathways.

Discussion

In the present study we investigated the therapeutic efficacy of EA at Shenting and Baihui acupoints after ischemia-reperfusion injury and explored the possible mechanism by which it affects apoptosis. We found that EA significantly decreased cerebral suppressed activation of the JNK and p38 signaling pathways while enhancing the activation of ERK1/2. Consequently, the effect of electroacupuncture at Baihui and Shenting acupoints on these pathways resulted in the inhibition of apoptosis, which was demonstrated by transmission electron microscopy and Western blot analysis.

Electroacupuncture, a mainstay treatment in TCM for a variety of diseases, is a special form of acupuncture therapy, and is based on traditional acupuncture [46–48]. Compared with traditional acupuncture, electroacupuncture has advantages of being readily controlled, standardized, and objectively measured. Electroacupuncture preconditioning attenuates ischemic brain injury by activation of the adenosine monophosphate-activated protein kinase signaling pathway. Several studies showed that the physiological effects of electroacupuncture are caused by the mechanical and electrical stimulation of the autonomic nervous system via induction of robust axon reflex or release neuroactive mediators from immune cells [49,50]. Baihui and Shenting, 2 important acupoints of the Du meridian, have been demonstrated in the improvement in post-stroke functional impairment [51], which was also confirmed by our previous study [33].

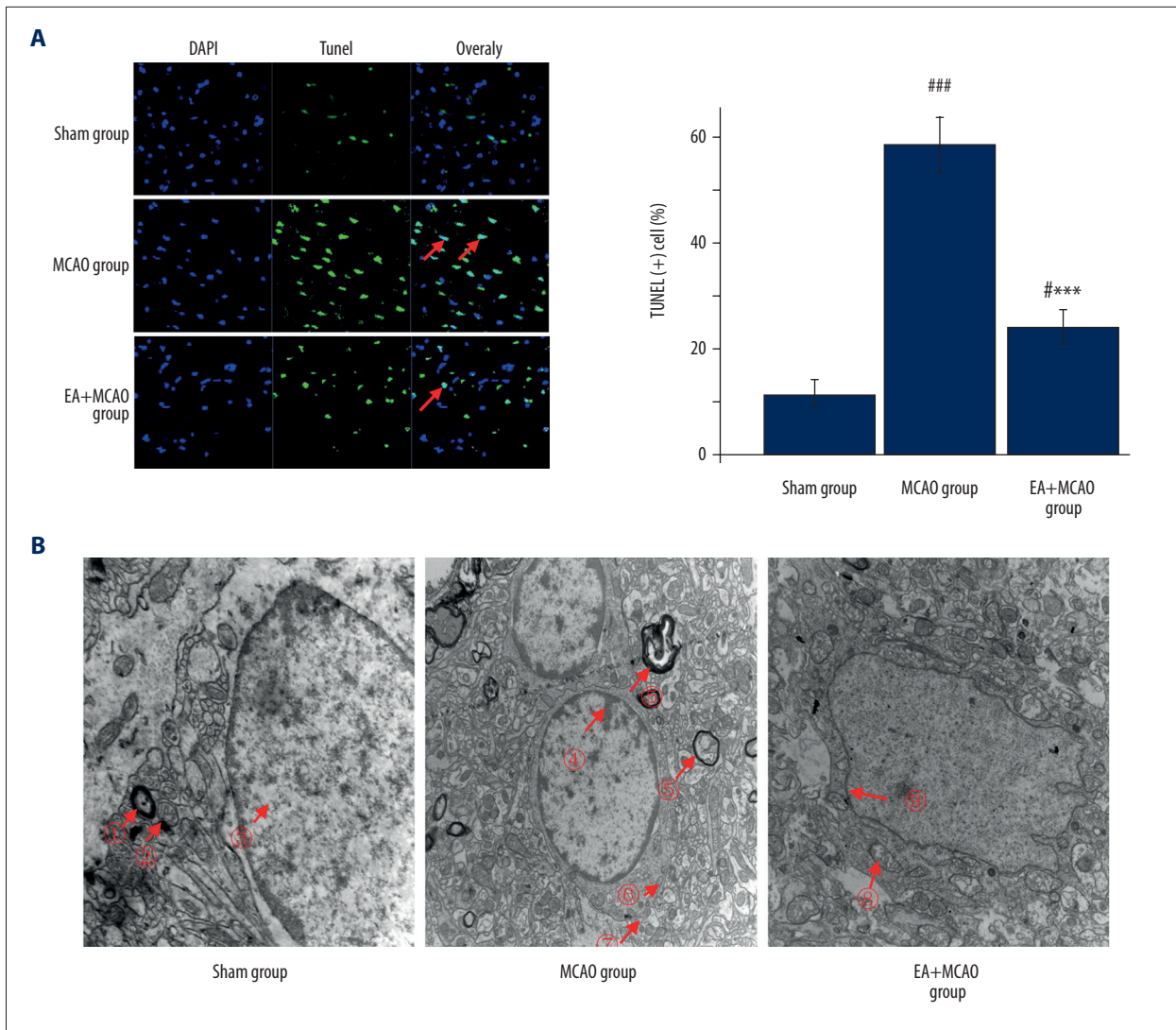


Figure 2. Effects of electroacupuncture on inhibiting cell apoptosis. **(A)** The percentage of TUNEL-positive cells in the peri-ischemic regions in the MCAO group was significantly increased compared with the sham group ($p < 0.001$). However, the percentage was significantly decreased in the EA +MCAO group ($p < 0.001$). ($n = 4$ in each group; *, vs. MCAO group; *** $p < 0.001$; #, vs. sham group; # $p < 0.05$ /### $p < 0.001$). **(B)** In the sham group, ① large axon; ② mitochondria; ③ chromatin. In the MCAO group, ④ chromatin; ⑤ cell myelin; ⑥ mitochondria; ⑦ cells mitochondria. In the EA+MCAO group, ⑧ mitochondria; ⑨ chromatin ($n = 4$ in each group).

Based on Traditional Chinese Medical theory, Du meridians are associated with the regulation of a number of brain functions and are used to treat brain disease [53,54]. The Baihui acupoint has 3 Yang and 5 convergences, and Shenting is the convergence of qi and blood, and both of them can activate spirit and resuscitate the brain in Traditional Chinese Medicine [55].

Oxidative stress after stroke leads to an increase in reactive species production, resulting in DNA fragmentation, and is thought to be one of the major factors that induce apoptosis in the peri-ischemic region [56]. Apoptosis in the ischemic penumbra or peri-infarct region play a key role in the progress of

cerebral ischemia-reperfusion injury [57]. After acute ischemic stroke, oxidative stress immediately occurred and then oxidative stress induced lipid peroxidation and mediating Ca^{2+} channel signals. Apoptotic proteins such as cytochrome c (CytC) are released into the cytosol, which can interact with the apoptotic protease to form an apoptosome, and finally results in neuronal apoptosis in post-ischemic stroke [58,59]. In the present study, we showed that the number of TUNEL-positive apoptotic cells and the phenomenon of small cells and the electronic density deep in the peri-inchemic regions were increased after MCAO and were inhibited by electroacupuncture. This indicates that electroacupuncture might inhibit apoptosis.

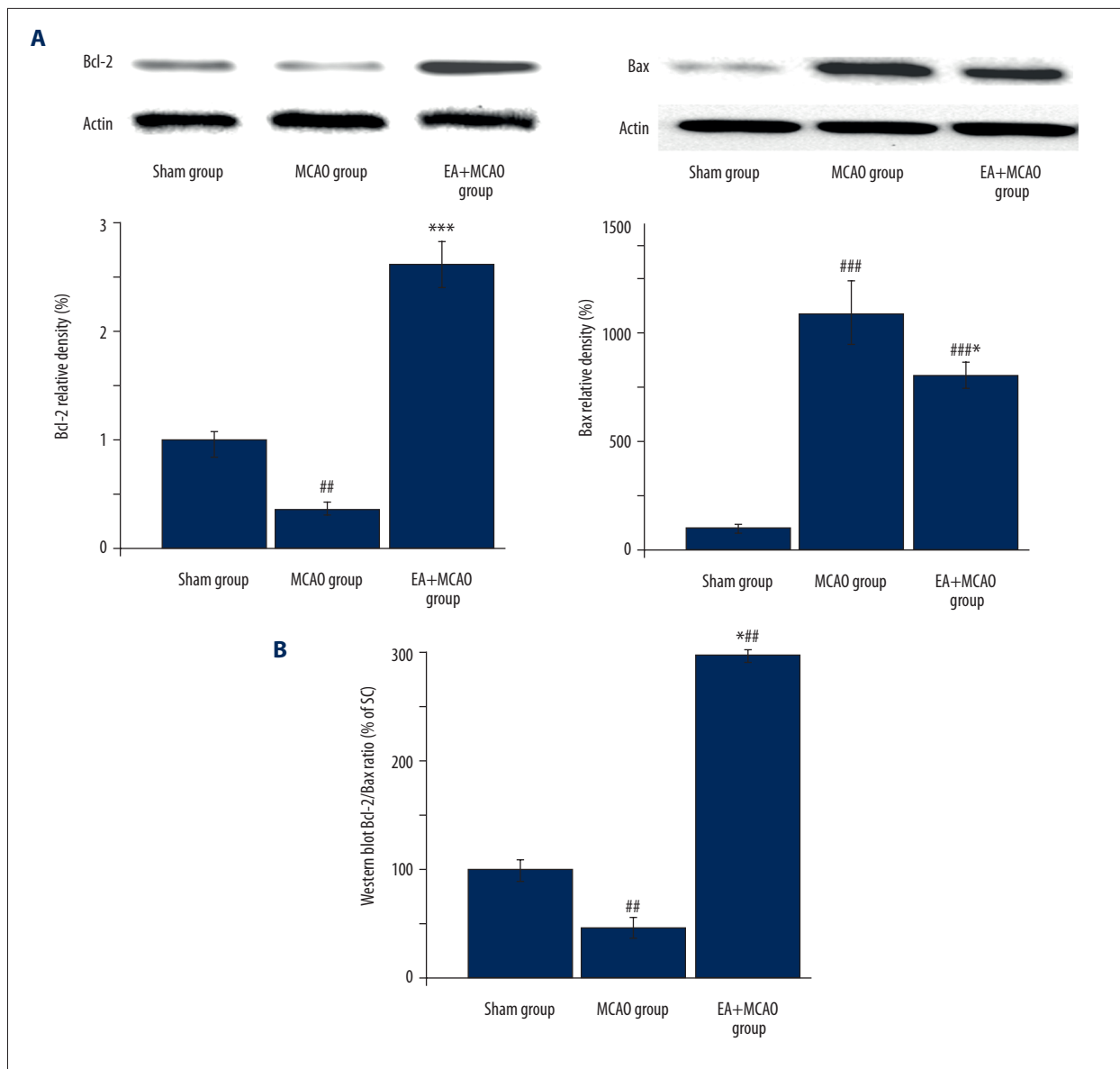


Figure 3. Electroacupuncture modulated the apoptosis factors of Bcl-2 and Bax in injured rats. At translational levels, EA treatment profoundly inhibited the model construction-mediated down-regulation of Bcl-2 and Bax and the Bcl-2/Bax ratio (data are expressed as the percentage of the value of sham group) (*, vs. MCAO group; * p<0.05/** p<0.01/***/ p<0.001; #, vs. sham group; ## p<0.01/### p<0.001) (n=4 in each group).

To further explore the underlying anti-apoptotic mechanism of electroacupuncture, we examined the effects of EA on several signaling pathways that modulate apoptosis after ischemia-reperfusion injury. Ischemia and reperfusion generate large quantities of ROS products, which activates a number of signaling pathways such as ERK, JNK, and p38 [60]. These pathways play a key role in regulating apoptosis. ERK1/2/C-Jun N-terminal kinases (JNKs) and the p38 mitogen-activated protein kinases activation play a major role in ischemia-induced apoptosis through regulation of Bax/Bcl-2/Bcl-xL expression [61,62]. In the present study, ERK1/2, JNK, and p38

pathways were activated after cerebral I/R injury, which was demonstrated by Bio-Plex analysis. The results are consistent with previous results [62], while EA significantly further upregulated ERK1/2 in I/R-injured brain tissues and inhibited JNK and p38 activation. Activation of these signaling pathways upregulated the ratio of Bcl-2/Bax, which plays an important anti-apoptotic role. The results demonstrated that inhibiting multiple cellular pathways regulating cell apoptosis is one of the possible mechanisms by which electroacupuncture reduces injury in post-stroke rats.

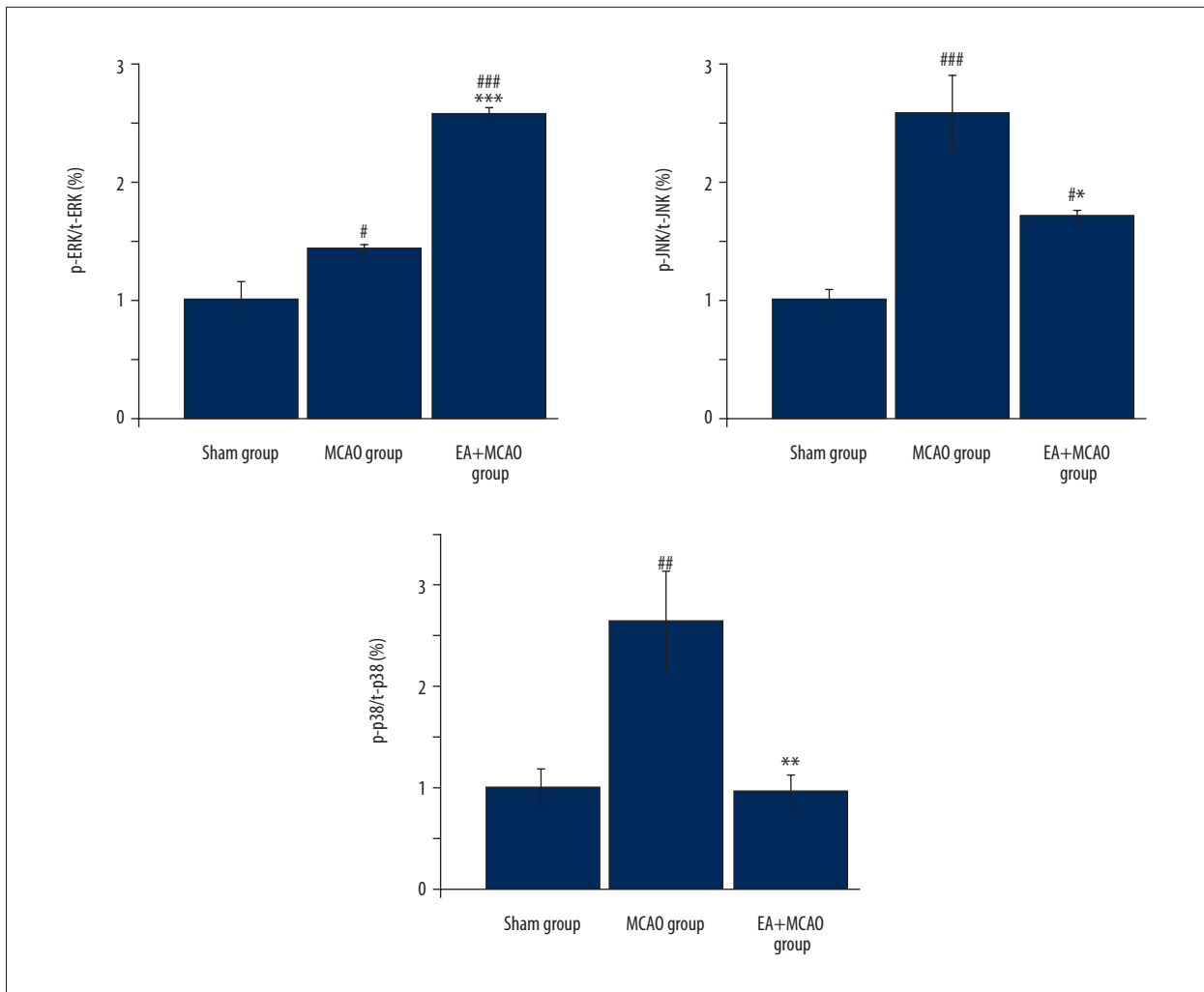


Figure 4. Electroacupuncture at Baihui and Shenting modulated ERK, JNK, and p38 pathways in focal cerebral ischemia-reperfusion (MCAO) rats. After EA treatment, the phosphorylation level of ERK1/2 (p-ERK1/2/t-ERK1/2) in peri-ischemic regions was significantly increased, whereas that of JNK (p-JNK/t-JNK) and p38 (p-p38/t-p38) was significantly reduced (n=4 in each group) (*, vs. MCAO group; * p<0.05/** p<0.01/*** p<0.001; #, vs. sham group; # p<0.05/## p<0.01/### p<0.001).

Conclusions

This study showed possible mechanisms by which electroacupuncture at GV20 and GV24 promotes functional recovery in post-stroke rats. Electroacupuncture mediates neuronal cell apoptosis via multiple cellular pathways such as JNK, ERK, and P38. Further studies are needed to determine other protective mechanisms of electroacupuncture.

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Conflict of interest

None.

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