

Electroacupuncture at the Quchi and Zusanli acupoints exerts neuroprotective role in cerebral ischemia-reperfusion injured rats via activation of the PI3K/Akt pathway

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Abstract. The PI3K/Akt pathway, a critical mediator of cell survival, is suppressed in cerebral ischemia/reperfusion (I/R) injury; therefore, it is a major focus in treatment of ischemic stroke. Acupuncture has long been used in China to clinically treat stroke. However, the precise mechanism of its neuroprotective activities remains largely unknown. Using a focal cerebral I/R injured rat model, in the present study we evaluated the *in vivo* therapeutic efficacy of electroacupuncture and investigated the underlying molecular mechanisms. We found that electroacupuncture at Quchi (LI11) and Zusanli (ST36) acupoints on the contralateral paralyzed limb significantly improved neurological deficits and cerebral infarction. In addition, electroacupuncture profoundly activated PI3K/Akt signaling in ischemic cerebral tissues. Consequently, the upregulatory effect of electroacupuncture on PI3K/Akt activation resulted in the inhibition of cerebral cell apoptosis. Moreover, electroacupuncture increased the serum secretion levels of the PI3K activators BDNF and GDNF, as well as

upregulated the anti-apoptotic Bcl-2/Bax ratio in ischemic cerebrum. Our data suggest that electroacupuncture at Quchi and Zusanli acupoints exerts neuroprotective function in ischemic stroke via activation of the PI3K/Akt pathway.

Introduction

Stroke, a medical emergency characterized by the rapid loss of brain function due to disturbance in brain blood supply, is currently the leading cause of serious and long-term disability in adults, as well as the second most common cause of death worldwide, ranking after heart disease and before cancer (1,2). Ischemia, which is caused by thrombosis blocking the blood flow to brain, is one of the major causative factors for the pathogenesis of stroke. Therapeutic approaches of ischemic stroke include removing the blood clot by thrombolysis or thrombectomy, minimizing clot enlargement or preventing new clot formation with medications, recovering any lost function with rehabilitation modalities such as physical therapy and occupational therapy.

Acupuncture has long been used in China to treat various diseases including stroke and currently it is also increasingly practiced in the western society (3). A large number of studies have demonstrated the clinical efficacy of acupuncture in stroke rehabilitation (4-7). Recently, we reported that electroacupuncture may alleviate neurological deficits via promoting the proliferation and differentiation of nerve stem cells (8). Moreover, on the basis of a large amount of documents and materials, the Zusanli (ST36) and Quchi (LI11) acupoints were commonly used in China to clinically treat stroke. However, the precise mechanism of neuroprotective effect of electroacupuncture at Quchi and Zusanli remains largely unclear.

PI3K/Akt pathway is essential for cell growth, proliferation, differentiation, motility, survival and intracellular trafficking (9,10). Phosphatidylinositol 3-kinase (PI3K) can be activated by cytokines including neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). Activated PI3K is able to phosphorylate PI(4)P and PI(4,5)P₂ at the 3 position hydroxyl group of the inositol ring to generate PI(3,4)P₂ and PI(3,4,5)

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Abbreviations: PI3K, phosphoinositide 3-kinase; IR, ischemia/reperfusion; MCAO, middle cerebral artery occlusion; EA, electroacupuncture; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; TTC, 2,3,5-triphenyl tetra-zolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Key words: apoptosis, electroacupuncture, Zusanli (ST36), Quchi (LI11) acupoints, cerebral ischemia/reperfusion, PI3K/Akt pathway

P3, respectively. These lipids serve as plasma membrane docking sites for proteins that contain pleckstrin-homology (PH) domains, such as Akt and its upstream activator PDK1. Upon activation of PI3K, both Akt and PDK1 translocate to the plasma membrane, where they bind directly to PI(3,4)P2 and PI(3,4,5)P3 via PH domain. The colocalization of PDK1 and Akt results in the phosphorylation of Akt, which in turn promotes cell survival by increasing the expression of anti-apoptotic Bcl-2 and downregulating the pro-apoptotic Bax expression. It has been shown that the activation of PI3K/Akt pathway could exert a neuroprotective role through inhibiting cell apoptosis in cerebral ischemia (11-13).

To elucidate the neuroprotective mechanism of electroacupuncture at Quchi and Zusanli, in the present study we investigate its effect on PI3K/Akt pathway in cerebral ischemia by using a focal cerebral ischemia/reperfusion injured rat model.

Materials and methods

Materials and reagents. Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). SuperScript II reverse transcriptase and TUNEL assay kit were provided by Promega (Madison, WI, USA). PI3K, Akt, phospho-Akt (Thr³⁰⁸), Bcl-2, Bax and β -actin antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling (Beverly, MA, USA). Rat BDNF and GDNF ELISA kits were purchased from Shanghai Xitang Biological Technology Co., Ltd. (Shanghai, China). All the other chemicals used, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Animals. Male Sprague-Dawley rats (with an initial body weight of ~250 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with a 12 h light/dark cycle. Food and water were given *ad libitum* throughout the experiment. All animal treatments were strictly in accordance with the international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

Establishment of the cerebral ischemia-reperfusion (I/R) injured rat model and animal grouping. I/R injured model was established by middle cerebral artery occlusion (MCAO) as previously described (14). Briefly, after a rat was anesthetized with 10% chloral hydrate by intraperitoneal injection, the left common carotid artery (CCA), the left external carotid artery (ECA) and internal carotid artery (ICA) were carefully exposed by a midline neck incision. The left middle cerebral artery was occluded by introducing an embolus through the ICA. Focal cerebral ischemia started until the tip of the catheter reached the origin of the MCA (~18-22 mm). Reperfusion was achieved by pulling out the thread after 120 min of occlusion to restore blood supply to the MCA area, and the left CCA and ECA were ligated. The rectal temperature of rats was maintained at 37°C throughout the surgical procedures. After operation the rats were allowed to recover in pre-warmed cages.

The rats were randomly divided into 3 groups (n=8) as follows: i) sham operation control group (SC): rats underwent a neck dissection and coagulation of the external carotid artery, but no occlusion of the middle cerebral artery; ii) ischemia control group (IC): the blood flow of left middle cerebral artery was blocked for 120 min, followed by reperfusion; iii) electroacupuncture group (EA): the treatment of ischemia/reperfusion (I/R) was same as that in IC group. After recovery from operation (2 h after I/R treatment), rats received electroacupuncture for 30 min daily. The acupuncture needles (0.3 mm diameter) were inserted 2-3 mm deep into the Quchi (LI11) and Zusanli (ST36) acupoints on the right paralyzed limb. Then stimulation was generated by the EA apparatus (Model G6805, SMIF, Shanghai, China) and the stimulation parameter were set as disperse wave of 1 and 20 Hz.

Evaluation of neurological deficit scores. At 2 or 24 h after ischemia/reperfusion, the neurological deficit score was examined in a blinded fashion as described before (14): score 0 represented no neurological deficit; score 1 (failure to extend the right forepaw fully) represented mild deficits; both score 2 (circling to the right) and score 3 (falling to the right) represented moderate deficits; score 4 (loss of walking) represented severe deficits. In brief, the rats scored 0 or 4 were eliminated out of the experiment.

Measurement of cerebral infarct volume. After cerebral I/R injury for 24 h, rats were anaesthetized with 10% chloral hydrate by intraperitoneal injection. The rat was perfused transcardially with 0.9% NaCl and the brain was removed. The brain of each rat was coronally sectioned into 2-mm-thick slices. The slices were stained with 2% TTC solution (Sigma, St. Louis, MO, USA) at 37°C for 20 min and then fixed with 10% buffered formalin solution. Stained slices were photographed by a high-resolution digital camera (Cannon sx20), and the infarct volume was quantified with Motic Med 6.0 System, which was represented as a percentage of the total brain volume.

In situ apoptosis detection by TUNEL staining. Rats were anesthetized and perfused transcardially with 0.9% NaCl and 4% paraformaldehyde through the left ventricle and the brain was removed. Samples were fixed in cold 4% paraformaldehyde and then processed into 5- μ m-thick sections. *In situ* apoptosis was analyzed by TUNEL assay kit (Promega) according to the manufacturer's instructions. Nuclei of all cells were visualized by DAPI staining and the green fluorescence of apoptotic cells was detected by a confocal fluorescence microscope (Leiss LSM 710). Apoptotic cells were counted at four arbitrarily selected microscopic fields at a magnification of 200x. Apoptotic rate was expressed as the ratio of green-stained cells to the blue DAPI-stained total cells.

Western blot analysis. Ischemic cerebral tissues were homogenized in non-denaturing lysis buffer and centrifuged at 15,000 x g for 15 min followed by determination of protein concentration in supernatants. Protein lysates were separated by 12% SDS-PAGE gels and then electrophoretically transferred onto PVDF membranes. The membranes were blocked for 2 h with 5% non-fat dry milk and then probed with primary antibodies against PI3K, Akt, pAkt, Bcl-2, Bax and β -actin

Table I. Neurological deficit score.

Group (n=8)	2 h after IR	24 h after IR
SC	0	0
IC	2.63±0.74	2.25±0.70
EA	2.38±0.52	1.50±0.54 ^a

SC, sham operation control; IC, ischemic control; EA, electroacupuncture. Data shown as averages ± SE from 8 individual rats in each group. ^aP<0.05 vs. with IC group.

(at a dilution of 1:1,000) overnight at 4°C, followed by incubation with appropriate HRP-conjugated secondary antibody for 50 min. Blots were developed using enhanced chemiluminescence, and images were taken using a Bio-Image Analysis System (Bio-Rad Laboratories, Hercules, CA, USA).

RNA extraction and RT-PCR analysis. Total RNA was isolated from cerebral tissues with TRIzol Reagent. Oligo(dT)-primed RNA (1 µg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of Bcl-2 and Bax by PCR with Taq DNA polymerase (Fermentas). β-actin was used as an internal control. The sequences of the primers used for amplification of Bcl-2, Bax and β-actin transcripts are as follows: Bcl-2 forward 5'-CGG GAG AAC AGG GTA TGA-3' and reverse 5'-CAG GCT GGA AGG AGA AGA T-3'; Bax forward 5'-GTT GCC CTC TTC TAC TTT GC-3' and reverse 5'-ATG GTC ACT GTC TGC CAT G-3'; β-actin forward 5'-ACT GGC ATT GTG ATG GAC TC-3' and reverse 5'-CAG CAC TGT GTT GGC ATA GA-3'. Samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation System (Bio-Rad Laboratories, Model Gel Doc 2000, USA).

Detection the level of BDNF and GDNF in serum by ELISA. Animal blood was obtained aseptically from abdominal aorta. Blood-containing tubes were allowed to stand at room temperature for 2 h, and serums were obtained by centrifugation at 3000 x g for 20 min in 4°C. The serum level of BDNF and GDNF was measured using ELISA kits (Xitang, Shanghai, China) according to the manufacturer's instructions. The wells were coated with 100 µl capture antibody diluted in coating buffer. The plate was sealed and incubated overnight at 4°C. After three washes, the wells were blocked with 200 µl assay diluents at room temperature for 1 h, followed by another three washes. Then, 100 µl diluted BDNF or GDNF standard and test samples were added and incubated for 2 h at room temperature. After repeated washes, the substrate was added and incubated for 20 min at room temperature, and the absorbance was measured at 450 nm using an ELISA reader (BioTek, Model ELx 800, USA).

Statistical analysis. All data were the means of at least three determinations and data were analyzed using the SPSS package for Windows (Version 16.0). Statistical analysis of the data was

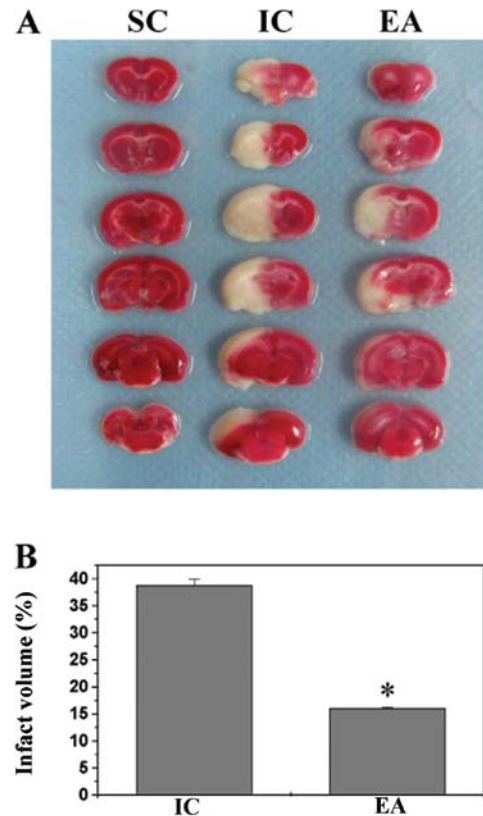


Figure 1. Effect of electroacupuncture at Quchi (LI11) and Zusanli (ST36) acupoints on cerebral infarction in cerebral ischemia/reperfusion (I/R) injured rats. (A) At the end of the experiment, cerebral tissues from each group were coronally sectioned into 2-mm-thick slices and then processed TTC staining. The photographs were taken by a high-resolution digital camera. Images are representative of three independent experiments. SC, sham operation control; IC, ischemic control; EA, electroacupuncture. (B) Infarct volume was quantified with Motic Med 6.0 System, which was represented as a percentage of the total brain volume. Data shown are averages with SE (error bars) from 3 individual rats in each group. *P<0.05, vs. IC group.

performed with the Student's t-test and ANOVA. Differences with P<0.05 were considered statistically significant.

Results

Electroacupuncture at acupoints of Zusanli (ST36) and Quchi (LI11) improve neurological deficits and reduce infarct volumes in cerebral ischemia-reperfusion (I/R) injured rats. After establishing the I/R injured rat model by MCAO on the left side, rats received electroacupuncture at Zusanli and Quchi acupoints on the right paralyzed limbs. The neuroprotective effect of electroacupuncture was examined by evaluating neurological deficit scores and cerebral infarct volume. As shown in Table I and Fig. 1, compared with sham operation group (SC) rats that did not show any signs of cerebral injury, all rats in both ischemia control (IC) and electroacupuncture (EA) groups displayed obvious manifestation of neurological deficits and cerebral infarction (P<0.05, vs. SC group), indicating the success of model construction. There was no significant difference in clinical evaluation between IC and EA groups before electric stimulation. However, electroacupuncture at Zusanli and Quchi for 24 h significantly ameliorated neurological deficit scores and reduced cerebral infarct volumes (P<0.05,

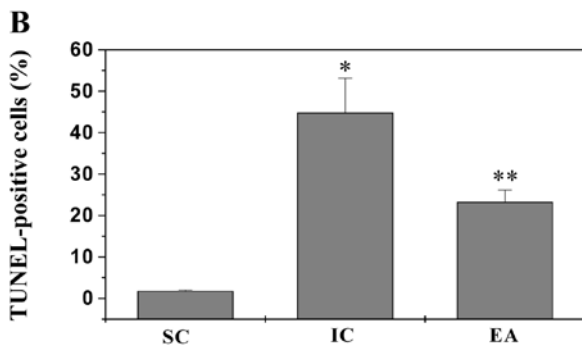
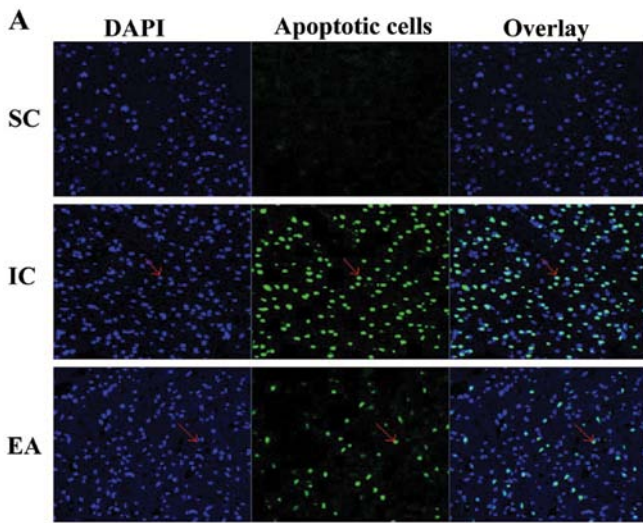


Figure 2. Effect of electroacupuncture on cerebral cell apoptosis in cerebral I/R injured rats by TUNEL staining. At the end of the experiment, cerebral tissues from each group (n=5) were processed for TUNEL assay. Nuclei of all cells were visualized by DAPI staining and the green fluorescence of apoptotic cells was detected by a confocal fluorescence microscope. Apoptotic cells were counted at four arbitrarily selected microscopic fields at a magnification of x200. Apoptotic rate was expressed as the ratio of green-stained cells to the blue DAPI-stained total cells. Data are averages with SE (error bars). *P<0.05, vs. SC group; **P<0.05, vs. IC group.

vs. IC group), demonstrating the therapeutic efficacy of electroacupuncture against cerebral I/R injury.

Electroacupuncture inhibits cerebral cell apoptosis in cerebral I/R injured rats. To determine the mechanism of neuroprotective action of electroacupuncture, we examined its anti-apoptotic activity in cerebral I/R injured rats via TUNEL. Data in Fig. 2 show 1.78±0.19%, 44.82±8.31% and 23.3±2.86% TUNEL-positive cells in SC, IC and EA rat groups, respectively, suggesting that electroacupuncture at Zusanli and Quchi inhibits ischemia-mediated cerebral cell apoptosis *in vivo*.

Electroacupuncture activates PI3K/Akt pathway in cerebral I/R injured rats. PI3K/Akt signaling pathway plays an important role in cell survival. The phosphorylation of Akt (pAkt) upon activation of PI3K regulates the expression of various target genes, leading to the inhibition of cell apoptosis. We therefore examined the effect of electroacupuncture on PI3K expression and Akt phosphorylation in ischemic cerebral tissues using western blotting. As shown in Fig. 3, PI3K protein

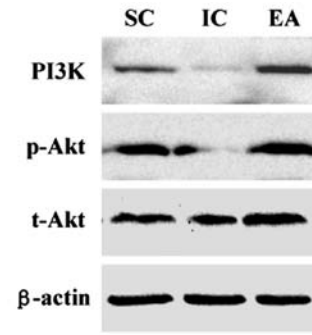


Figure 3. Effect of electroacupuncture on PI3K/Akt pathway in cerebral I/R injured rats. The levels of PI3K protein expression and Akt phosphorylation in ischemic cerebral tissues were determined by western blot analysis. β -actin was used as the internal control. Data are representative of 5 individual rats in each group.

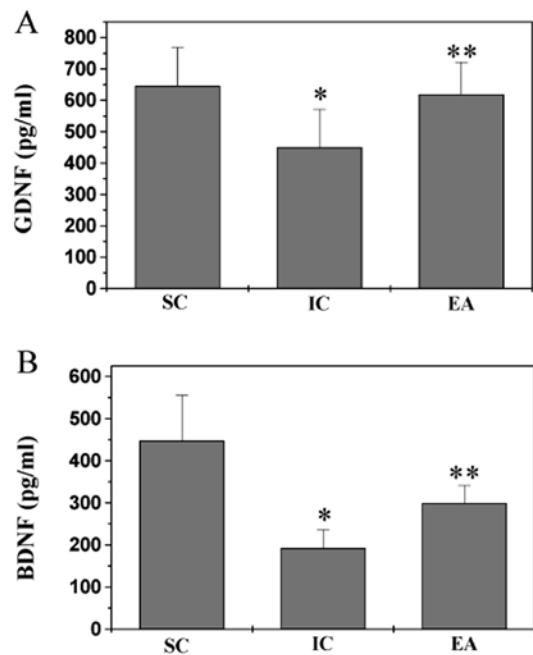


Figure 4. Effect of electroacupuncture on the secretion level of BDNF and GDNF in serum of cerebral I/R injured rats. The serum levels of BDNF and GDNF were examined by ELISA. Data are averages with SE (error bars) five individual rats in each group. *P<0.05, vs. SC group; **P<0.05, vs. IC group.

expression and the phosphorylation level of Akt in IC group were significantly reduced compared with those in SC group (P<0.05), consistent with previous studies that in models of cerebral ischemia Akt phosphorylation profoundly decreases 24 h after reperfusion (11,13). However, electroacupuncture at Zusanli and Quchi significantly neutralized the effect of model construction, increasing both PI3K protein expression and Akt phosphorylation levels in ischemic cerebral tissues (P<0.05, vs. IC group).

Electroacupuncture increases BDNF and GDNF secretion levels and the anti-apoptotic Bcl-2/Bax ratio in cerebral I/R injured rats. BDNF and GDNF are critical activators of PI3K in cerebrum. By using ELISA we found that the serum levels of BDNF and GDNF in IC group were significantly decreased,

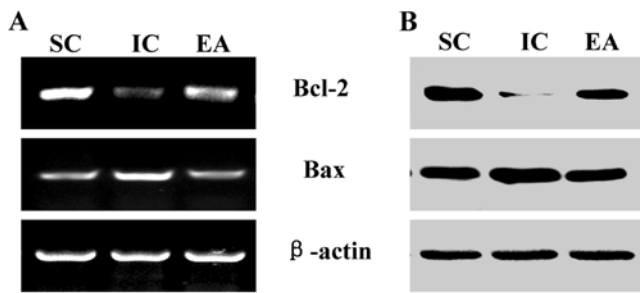


Figure 5. Effect of electroacupuncture on the expression of Bcl-2 and Bax in cerebral I/R injured rats. (A) The mRNA levels of Bcl-2 and Bax in ischemic cerebral tissues were determined by RT-PCR. (B) The protein expression levels of Bcl-2 and Bax were analyzed by western blotting. β -actin was used as the internal controls for both RT-PCR or western blot analyses. Data are representative of five individual rats in each group.

compared to that in the SC group ($P < 0.05$), which, however, was neutralized by electroacupuncture at Zusanli and Quchi (Fig. 4; $P < 0.05$, vs. IC group). Bcl-2 family proteins are key regulators of apoptosis, functioning as either suppressors such as Bcl-2, or promoters such as Bax. Both anti-apoptotic Bcl-2 and pro-apoptotic Bax are important target genes of PI3K/Akt signaling pathway. To further explore the mechanism of electroacupuncture's anti-apoptotic activity, we performed RT-PCR and western blot analyses to respectively examine the mRNA and protein expression of Bcl-2 and Bax in ischemic cerebral tissues. As shown in Fig. 5A and B, electroacupuncture at Zusanli and Quchi profoundly inhibited the model construction-mediated downregulation of anti-apoptotic Bcl-2/Bax ratio, at both transcriptional and translational levels.

Discussion

PI3K/Akt pathway is a critical mediator of cell survival, which exerts anti-apoptotic function via inhibiting the pro-apoptotic Bax/Bcl-2 ratio (15-17). A large number of reports indicate that the suppression of PI3K/Akt pathway is strongly associated with the progression of cerebral ischemia/reperfusion (I/R) injury, increasing the infarct size and promoting cerebral cell death (18,19). Therefore, inhibiting cerebral cell apoptosis via activation of PI3K/Akt signaling has been a promising strategy for the treatment of ischemic stroke. Acupuncture is an alternative medicine methodology that has long been used in China to treat various diseases. Previous studies have demonstrated the clinical efficacy of acupuncture in stroke rehabilitation. On the basis of a large amount of documents and materials, the Zusanli (ST36) and Quchi (LI11) acupoints were commonly used in China to clinically treat stroke. However, the mode of action of its neuroprotective activities remains poorly understood.

By using a focal cerebral ischemia/reperfusion rat model, in the present study we demonstrated that electroacupuncture at Zusanli and Quchi for only 24 h already displayed neuroprotective effect as evidenced by improving neurological deficits and reducing cerebral infarct volume. In addition, we found that PI3K/Akt pathway was suppressed 24 h after cerebral I/R injury, which was consistent with previous studies (11,13). However, electroacupuncture significantly neutralized the effect of cerebral ischemia, activating PI3K/

Akt signaling in ischemic cerebral tissues. Consequently, the upregulatory effect of electroacupuncture on PI3K/Akt activation resulted in the inhibition of cerebral cell apoptosis. Neurotrophic factors, such as BDNF and GDNF, are critical activators for PI3K (20,21), and anti-apoptotic Bcl-2 and pro-apoptotic Bax are important target genes of PI3K/Akt pathway (22). As expected we found that electroacupuncture increased the serum secretion levels of both BDNF and GDNF, as well as upregulated the anti-apoptotic Bcl-2/Bax ratio in cerebral ischemic.

In conclusion, here we reported for the first time that electroacupuncture at Quchi (LI11) and Zusanli (ST36) acupoints on the contralateral paralyzed limb exerts neuroprotective function in ischemic stroke via activation of PI3K/Akt pathway. These results suggest that electroacupuncture may be a potential therapeutic modality for cerebral ischemia.

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