Electroacupuncture exerts anti-inflammatory effects in cerebral ischemia-reperfusion injured rats via suppression of the TLR4/NF-κB pathway

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Abstract. Inflammatory response has been shown to play a critical role in brain damage after cerebral ischemia-reperfusion (I/R) injury, which is tightly regulated by the Toll-like receptor (TLR)4/nuclear factor (NF)-κB pathway; therefore, suppression of TLR4/NF-κB signaling has become a promising target for the anti-inflammatory treatment in ischemic stroke. Acupuncture has been used as a complementary and alternative therapy practice that supplements conventional medicine. Numerous studies have demonstrated the clinical efficacy of acupuncture in stroke rehabilitation. However, the precise mechanism of its neuroprotective effect remains poorly understood. Using a focal cerebral I/R injured rat model, in the present study we evaluated the neuroprotective and anti-inflammatory activities of electroacupuncture at Quchi and Zusanli, and investigated the underlying molecular mechanisms. We found that electroacupuncture at Quchi (LI11) and Zusanli (ST36) acupoints significantly improved the ischemia-associated scores of neurological deficits, reduced cerebral infarction and alleviated inflammatory responses. Moreover, the crucial signaling molecules in the TLR4/NF-κB signaling pathway were regulated by acupuncture, which coincided with suppressed secretion levels of inflammatory cytokines such as TNF-α, IL-1β and IL-6. Our data suggest that electroacupuncture exerts a neuroprotective function in ischemic stroke through inhibition of TLR4/NF-κB-mediated inflammation.

Introduction

Ischemic stroke is one of the most common causes of mortality and complex disability in adults worldwide (1-3). Despite improvements in the interventional techniques and pharmacological agents, there are currently no exact effective drugs and techniques to manage post-stroke rehabilitation or recovery. Acupuncture originated in ancient China and is among the oldest healing methodologies in the world. As part of traditional Chinese medicine (TCM), acupuncture has been used for thousands of years as a complementary and alternative therapy practice that supplements conventional medicine in many oriental countries as well as recently in Western society (4). A large number of studies have demonstrated the clinical efficacy of acupuncture in stroke rehabilitation (5-9). Recently, we reported that electroacupuncture alleviates neurological deficits possibly by promoting the proliferation and differentiation of nerve stem cells (10). Additionally, based on a number of documents and materials, the Zusanli (ST36) and Quchi (LI11) acupoints were commonly used in China to clinically treat stroke (11,12). However, the precise mechanism of its neuroprotective effect remains largely unknown. Inflammatory response has been shown to play a critical role in brain damage after cerebral ischemia-reperfusion (I/R) injury (13,14), which is tightly regulated by Toll-like receptors (TLRs). The transmembrane TLRs are a family of pattern-recognition receptors (PRRs) which enable the innate and adaptive immune systems to recognize pathogen-associated molecular patterns (PAMPs). TLRs contain an intracellular Toll-interleukin 1 receptor (TIR) domain and exert their functions by interacting with TIR domain-containing adaptor proteins such as MyD88, Mal, TRIF and TRAM (15,16). To date, over 13 members of the TLR family have been identified in mammals (17), of which TLR4 is the best studied. TLR4 recognizes LPS from Gram-negative bacteria and various host-derived molecules, such as heat-shock proteins,

fibronectin, hyaluronic acid and heparan sulfate (18-20). Upon ligand binding, TLR4 undergoes a conformational change and dimerizes, which then recruits TIR domain-containing adaptor proteins, transducing the immune-related signals to the nucleus via transcription factors such as nuclear factor-κB (NF-κB). As one of the most important nuclear transcription factors, NF-κB is involved in the control of many critical physiological processes, such as cell proliferation, apoptosis, and especially in pathways of the immune and inflammatory responses. In unstimulated cells, NF-κB is sequestered in the cytosol via interaction with inhibitory IκB proteins. However, when cells receive pathological stimuli, IκB proteins could be phosphorylated by IκB kinase (IKK) that is activated by various mechanisms including TLR4-mediated immune signaling. Phosphorylation of IκB proteins results in their ubiquitination and degradation, which in turn releases sequestered NF-κB, leading to its translocation to the nucleus where it induces the expression of various pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6. Therefore, suppression of the TLR4/NF-κB pathway has become a promising target for the anti-inflammatory treatment in ischemic stroke.

Using a focal cerebral I/R injured rat model, in the present study we evaluated the neuroprotective and anti-inflammatory activities of electroacupuncture at Quchi and Zusanli, and investigated the underlying molecular mechanisms.

Materials and methods

Materials and reagents. TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). TLR4, NF-κB p65, p-IκB and β-actin antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Rat TNF-α, IL-1 and IL-6 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. Adult male Sprague-Dawley rats (with an initial body weight of 200-250 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle and free access to food and water. All animal procedures were strictly in accordance with 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 I/R injured rat model and animal grouping. The I/R injured model was generated by occlusion of the middle cerebral artery (MCA). Prior to surgery, rats were allowed access to water but fasted for 24 h and the surgical procedures were performed as previously described by Longa *et al* (21) with slight modifications. Briefly, after a rat was anesthetized with 10% chloral hydrate by intraperitoneal injection (300 mg/kg), the left common carotid artery (CCA), the left external carotid artery (ECA) and the internal carotid artery (ICA) were exposed and isolated through a midline

cervical incision. An embolus was advanced through the ICA to the MCA until mild resistance was encountered (20±2 mm), thereby occluding the origin of the MCA. The cervical incision was closed with a silk suture. Reperfusion was achieved by withdrawing the intraluminal occlusive embolus to restore blood supply to the MCA area after 2 h of ischemia. For the rats in the sham group, the left CCA, ECA and ICA were exposed, but no ligations and occlusions were performed. The rectal temperature of rats was maintained at 37°C throughout the surgical procedures.

Animals were randomly divided into three groups (n=8): the sham operation control group (SC), the ischemia control group (IC) and the electroacupuncture group (EA). All rats were studied for behavioral parameters at 2 and 24 h after I/R, and then sacrificed for subsequent experiments.

EA stimulation. EA stimulation was applied to the acupuncture points of Quchi (LI11) and Zusanli (ST36) on the right paralyzed limb by using an EA stimulator instrument (Model G6805; SMIF, Shanghai, China) after recovery from operation (2 h after I/R treatment). Two stainless steel acupuncture needles 0.3 mm in diameter, which were connected with the output terminals, were inserted at a depth of 2-3 mm into the above-mentioned acupuncture points. The stimulation with 1- or 20-Hz frequencies was generated at an intensity of the muscle twitch threshold (the muscle twitch threshold was approximately 0.01 mA).

Neurological assessment. Neurological function was evaluated at 2 and 24 h after I/R in a blinded fashion according to a standard scoring system on a four-point scale (21): score 0, no apparent deficits; score 1, failure to extend the right forepaw fully; score 2, circling to the right; score 3, falling to the right; score 4, loss of walking. In brief, rats scoring 1-3 points indicated successful model establishment.

Measurement of the infarct volumes. Following cerebral I/R injury for 24 h, rats were euthanized under deep anesthesia using 10% chloral hydrate and perfused transcardiacally with 0.9% NaCl. The brains of all rats were quickly removed for dissecting into five coronal blocks at a thickness of 2 mm/section. The fresh slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) solution in phosphate-buffered saline (PBS) (Hyclone, Beijing, China) at 37°C for 20 min. The normal area of brain was stained dark red based on intact mitochondrial function, whereas the infarct area remained unstained. Stained slices were photographed by a high-resolution digital camera (Cannon sx20). Using a computerized image analysis system (Motic Med 6.0 System), the total volume of infarction was determined by integration of the areas from the sections.

Hematoxylin and eosin (H&E) staining of the brain. Rats were anesthetized and perfused transcardiacally with 0.9% NaCl and 4% paraformaldehyde through the left ventricle. After the brain was removed, it was fixed in cold 4% paraformaldehyde at 4°C for 24 h. After dehydration, the specimens were embedded in paraffin, cut into 5-μm sections and stained with H&E. The histological morphology of each brain specimen was examined under optical microscopy.

Direct immunofluorescence analysis of NF-κB p65 nuclear translocation. The paraffin sections of brain tissues were treated with microwave heat-induced epitope retrieval. After the specimens were washed three times in PBS (pH 7.4), they were incubated for 1 h at 37°C with a 150 dilution of rabbit anti-rat NF-κB p65 antibody (green). The wash step was repeated. Nuclei of all cells were counterstained with DAPI. After three washes with PBS, the tissues were mounted in Prolong Gold Antifade reagent. Images were collected by a confocal fluorescence microscope (Leiss LSM710) with a magnification of x200.

Western blot analysis. Total proteins were extracted from the infarct cortex and protein concentrations were determined by BCA assay. Samples, containing 50 µg proteins, were separated by electrophoresis on 12% SDS-polyacrylamide gels. Subsequently, proteins were transferred onto PVDF membranes in a Tris-glycine transfer buffer. The membranes were blocked for 2 h with 5% nonfat dry milk at room temperature and detected with rabbit anti-TLR4, anti-NF-κB p65, anti-p-IκB and anti-β-actin antibodies (at a dilution of 1:1,000) at 4°C overnight, followed by incubation with the appropriate HRP-conjugated secondary antibody for 50 min. The bands were visualized with enhanced chemiluminescence, and images were captured using a Bio-Image Analysis System (Bio-Rad, Hercules, CA, USA).

RNA extraction and RT-PCR. Total-RNA from cerebral tissues were isolated with the TRIzol reagent according to the manufacturer's instructions. Oligo(dT)-primed RNA (1 µg) was reverse transcribed into cDNA, which was then used to determine the amount of TLR4, NF-κB p65 mRNA by PCR. The primer sequences and the sizes of the amplification were, for β-actin: forward, 5'-ACT GGC ATT GTG ATG GAC TC-3' and reverse, 5'-CAG CAC TGT GTT GGC ATA GA-3'; TLR4: forward, 5'-GGA CTC TGC CCT GCC ACC ATT TA-3' and reverse, 5'-CTT GTG C CCT GTG AGG TCG TTG A-3'; NF-κB p65: forward, 5'-GTG CAG AAA GAA GAC ATTG AGG TG-3' and reverse, 5'-AGG CTA GGG TCA GCG TAT GG-3'. Samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation system (Model Gel Doc 2000; Bio-Rad).

Determination of the serum level of TNF-α, IL-1β and IL-6 by ELISA. Animal blood was obtained aseptically from abdominal aorta. Blood-containing tubes were allowed to stand at room temperature for 2 h, and sera were obtained by centrifugation at 3,000 x g for 20 min in 4°C. The serum was assayed for levels of TNF-α, IL-1β and IL-6 by ELISA kits (Shanghai XiTang Biological Technology Co., Ltd.) according to the manufacture's instructions. Briefly, the wells were coated with 100 µl capture antibody 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 TNF-α, IL-1β or IL-6 standard and test samples were added and incubated for 1 h at 37°C. After repeated washes, the substrate was added and incubated for 20 min at room temperature. Absorbance at 450 nm wavelength was measured, and protein concentration was determined by interpolation onto absorbance curves generated by recombinant TNF-α, IL-1β or IL-6 protein standards using an ELISA reader (Model ELX800; BioTek, USA).

Table I. Neurological assessment of rats.

Group (n=8)	2 h after IR	24 h after IR
SC	0	0
IC	2.38±0.74	2.13±0.83
EA	2.50±0.53	1.38±0.52[a]

SC, sham operation control; IC, ischemic control; EA, electroacupuncture. Data shown as averages ± SE from 8 individual rats in each group. [a]P<0.05 vs. the IC group.

Statistical analysis. Statistical data are expressed as the means ± SD. Statistical analysis was performed with the Student's t-test and ANOVA using the SPSS package for Windows (Version 16.0). Differences with P<0.05 were considered statistically significant.

Results

Electroacupuncture at the acupoints of Zusanli (ST36) and Quchi (LI11) displays neuroprotective activity in I/R injured rats. The neuroprotective effect of electroacupuncture was examined by neurological assessment and evaluation of cerebral infarct volume. Rats in the IC and EA groups displayed obvious manifestation of neurological deficits and cerebral infarction, whereas rats in the SC group did not show any signs of cerebral injury (P<0.05 vs. the SC group), indicating the success of the model construction (Table I and Fig. 1). In addition, prior to electric stimulation, the rats in the EA group did not show any remarkable difference in clinical evaluation compared with the IC group rats. However, 24 h after electroacupuncture at the acupoints of Zusanli (ST36) and Quchi (LI11) the neurological function was improved and the cerebral infarct volumes were reduced significantly (P<0.05 vs. the IC group), demonstrating the therapeutic efficacy of electroacupuncture against cerebral I/R injury.

Electroacupuncture alleviates cerebral inflammation in cerebral I/R injured rats. Brain damage caused by cerebral I/R is partly attributed to secondary injury from inflammation for a short time after the onset of cerebral ischemia, therefore developing anti-inflammation therapies at the early stage of ischemia has become an attractive strategy to combat cerebral lesion. To determine the anti-inflammatory efficacy of acupuncture in ischemic cerebral tissues, the I/R-induced cerebral histopathological changes and the degree of inflammatory cell infiltration in infarct areas were observed under optical microscope after H&E staining. As expected, no histopathological abnormalities and inflammatory cells were observed in the SC group rats. By contrast, in the infarct core zone of the IC group rats the glial and neuron cells appeared shrunken and showed condensed nuclei, which, however, was ameliorated by electroacupuncture (Fig. 2). Moreover, compared to the IC group, much fewer inflammatory cells were infiltrated into the cerebral infarct areas in the EA group, suggesting that electroacupuncture alleviates I/R-mediated cerebral inflammation.
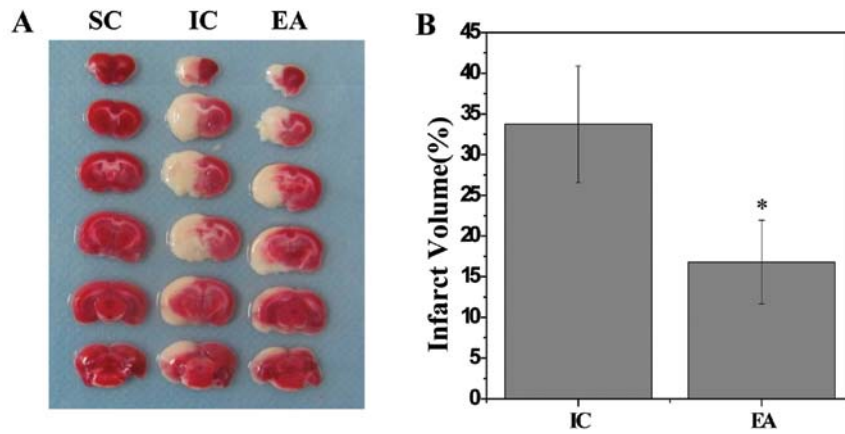
Figure 1. Effect of electroacupuncture at the Quchi (LI11) and Zusanli (ST36) acupoints on cerebral infarction in cerebral ischemia/reperfusion (I/R) injured rats. (A) At the end of the experiment, cerebral tissues from each group were coronally sectioned into 2-mm slices and then processed for TTC staining. The photographs are representative images captured by a high-resolution digital camera. SC, sham operation control; IC, ischemic control; EA, electroacupuncture. (B) Infarct volume was quantified with the 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 three individual rats in each group. *P<0.05 vs. the IC group.
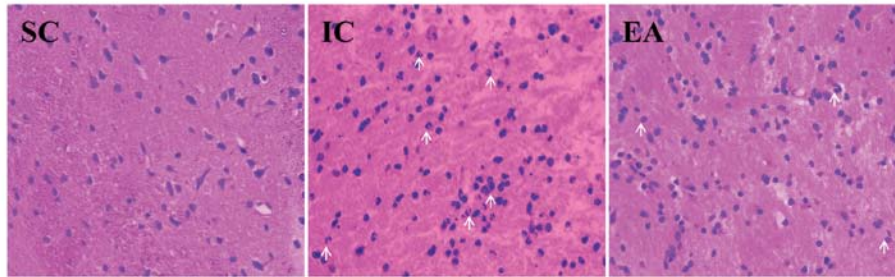

Figure 2. Effect of electroacupuncture on cerebral inflammation in cerebral I/R injured rats. At the end of the experiment, cerebral tissues from each group (n=5) were processed for hematoxylin and eosin (H&E) staining. I/R-induced cerebral histopathological changes and the degree of inflammatory cell infiltration in infarct areas were observed under an optical microscope at a magnification of x200. SC, sham operation control; IC, ischemic control; EA, electroacupuncture.
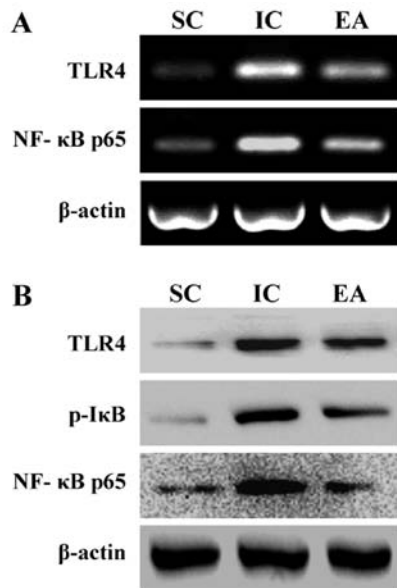

Figure 3. Effect of electroacupuncture on the TLR4/NF-κB pathway in cerebral I/R injured rats. (A) The mRNA levels of TLR4 and NF-κB in ischemic cerebral tissues were determined by RT-PCR. (B) The protein expression levels of TLR4, NF-κB and the phosphorylation of IκB were analyzed by western blotting. GAPDH and β-actin were used as the internal control for RT-PCR and western blotting, respectively. Data are representative of five individual rats in each group. SC, sham operation control; IC, ischemic control; EA, electroacupuncture.

Electroacupuncture suppresses the activation of the TLR4/NF-κB pathway in cerebral I/R injured rats. TLR4 can activate a common signaling pathway by inducing IκB phosphorylation and degradation and culminating the translocation of NF-κB from cytoplasm into nucleus, which arouse downstream-associated pro-inflammatory cytokines. To explore the anti-inflammatory mechanism of electroacupuncture, we examined its effect on the TLR4/NF-κB signaling pathway in ischemic cerebral tissues. The expression of TLR4 and NF-κB p65 as well as the phosphorylation level of IκB were significantly increased in the IC group compared with those in the SC group, which, however, was significantly neutralized by electroacupuncture (Fig. 3). To further verify these results, we evaluated the effect of electroacupuncture on nuclear translocation of NF-κB which is a critical step for NF-κB activation. The NF-κB p65 subunit was visualized by immunofluorescence staining and the cells were counterstained with DAPI; NF-κB nuclear translocation was recognized by the co-localization of p65 subunit with DAPI. Cerebral I/R injury resulted in the nuclear translocation of NF-κB p65 subunit, which was not observed in the sham operation group (Fig. 4). However, electroacupuncture profoundly inhibited I/R-induced NF-κB nuclear translocation. This indicates that the anti-inflammatory effect of electroacupuncture at Zusanli and Quchi is mediated by suppression of TLR4/NF-κB signaling in cerebral I/R injured rats.
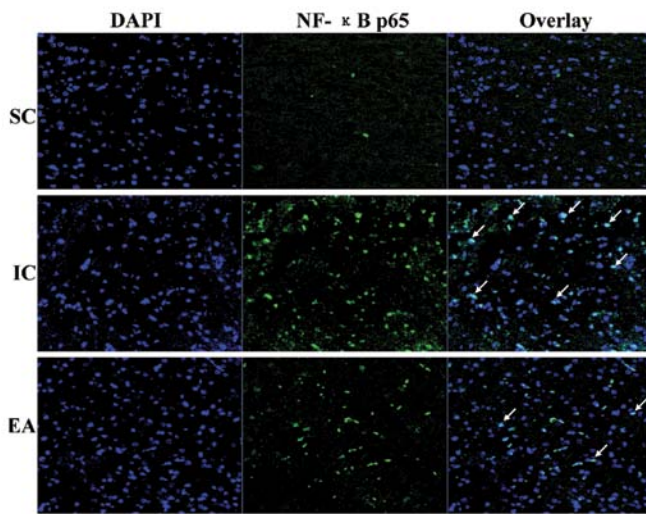
Figure 4. Effect of electroacupuncture on the nuclear translocation of NF-κB p65 subunit in cerebral I/R injured rats. At the end of the experiment, cerebral tissues were processed for immunofluorescence assay. The NF-κB p65 subunit was visualized by immunofluorescence staining (green) and the cells were counterstained with DAPI (blue). NF-κB nuclear translocation was represented by the co-localization of p65 subunit with DAPI (indicated by white arrows). Images were captured under a confocal fluorescence microscope (Leiss LSM710) with a magnification of x200. Images are representative of five individual rats in each group. SC, sham operation control; IC, ischemic control; EA, electroacupuncture.
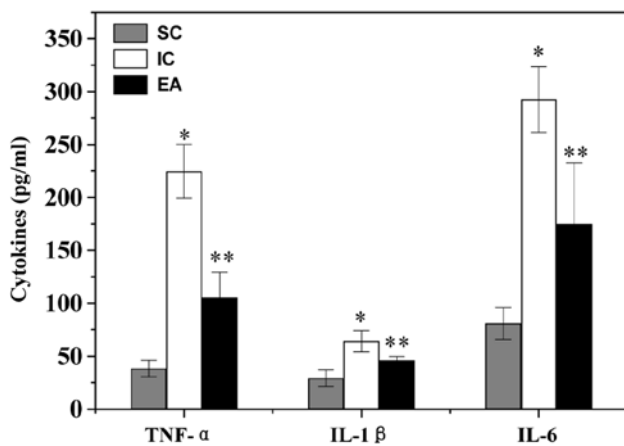

Figure 5. Effect of electroacupuncture on the secretion level of pro-inflammatory cytokines in cerebral I/R injured rats. The serum levels of TNF-α, IL-1β and IL-6 were examined by ELISA. Data are averages with SE (error bars) from five individual rats in each group. *$P<0.05$ vs. the SC group; **$P<0.05$ vs. the IC group. SC, sham operation control; IC, ischemic control; EA, electroacupuncture.

Electroacupuncture regulates the secretion level of inflammatory cytokines in cerebral I/R injured rats. To identify the downstream effectors in the TLR4/NF-κB pathway, the secretion level of cytokines was examined by ELISA. The serum level of pro-inflammatory TNF-α, IL-1β and IL-6 in the IC group was significantly increased, compared to that in the SC group ($P<0.05$) (Fig. 5). However, electroacupuncture at Zusanli and Quchi profoundly inhibited I/R-induced secretion of TNF-α, IL-1β and IL-6 ($P<0.05$ vs. the IC group).

Discussion

Acupuncture has served as a major complementary and alternative therapy that supplements conventional medicine and has been used in patients with acute ischemic stroke (5-9,22). On the basis of a multitude of documents and materials, the Zusanli (ST36) and Quchi (LI11) acupoints were commonly used in China to clinically treat cerebral ischemia-reperfusion (I/R) injury (11,12). In the present study, by using the model of MCAO followed by reperfusion which is a classical model of cerebral I/R, we demonstrated that electroacupuncture at Zusanli and Quchi displayed neuroprotective effects as evidenced by improving neurological deficits and reducing cerebral infarct volume.

Brain damage caused by cerebral I/R is partly attributed to secondary injury from inflammation for a short time after the onset of cerebral ischemia (23). Therefore, developing anti-inflammation therapies at the early stage of ischemia has been an attractive strategy to combat cerebral lesion. Acupuncture has been reported to exert anti-inflammatory effects in several non-infectious disease models of the nervous system, such as spinal cord injury and amyotrophic lateral sclerosis (24,25). To determine the anti-inflammatory efficacy of acupuncture in cerebral I/R, we visualized the degree of inflammation in cerebral I/R cord by H&E-staining. The results confirmed that electroacupuncture at Zusanli and Quchi for only 24 h already attenuated the histopathological changes infiltration of neutrophils in the cerebral infarct areas.

Depending on the cerebral I/R injury, TLR4 can activate a common signaling pathway by triggering IκB phosphorylation and degradation and culminating the translocation of NF-κB from cytoplasm into nucleus, which arouse downstream-associated pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 that in turn induce inflammation response (26-28). In the present study, we found that the TLR4/NF-κB pathway was activated at 24 h after cerebral I/R injury, which was consistent with previous studies (29-31). However, the activation of TLR4/NF-κB signaling induced by cerebral ischemia was neutralized by systemic electroacupuncture intervention through downregulating important target genes of the TLR4/NF-κB pathway. As expected, we also found that electroacupuncture decreased the serum levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6).

In conclusion, we report for the first time that electroacupuncture at the Quchi (LI11) and Zusanli (ST36) acupoints on the paralyzed limb reduces ischemic brain damage, improves neurological deficits and exerts anti-inflammation function against ischemic stroke via inhibition of the TLR4/NF-κB pathway. These results suggest that electroacupuncture may be a potential therapeutic modality for cerebral ischemia.

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