

# Large-conductance $\text{Ca}^{2+}$ -activated $\text{K}^+$ channel involvement in suppression of cerebral ischemia/reperfusion injury after electroacupuncture at *Shuigou* (GV26) acupoint in rats

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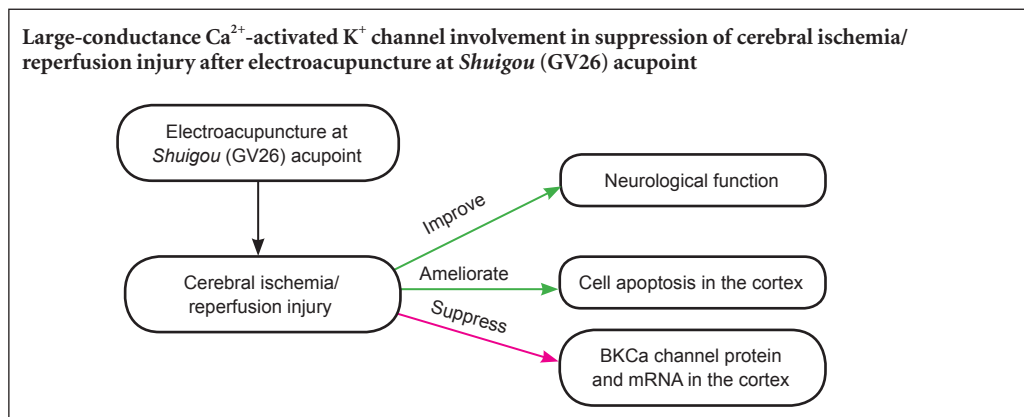
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## Graphical Abstract



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## Abstract

Excess activation and expression of large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (BKCa channels) may be an important mechanism for delayed neuronal death after cerebral ischemia/reperfusion injury. Electroacupuncture can regulate BKCa channels after cerebral ischemia/reperfusion injury, but the precise mechanism remains unclear. In this study, we established a rat model of cerebral ischemia/reperfusion injury. Model rats received electroacupuncture of 1 mA and 2 Hz at *Shuigou* (GV26) for 10 minutes, once every 12 hours for a total of six times in 72 hours. We found that in cerebral ischemia/reperfusion injury rats, ischemic changes in the cerebral cortex were mitigated after electroacupuncture. Moreover, BKCa channel protein and mRNA expression were reduced in the cerebral cortex and neurological function noticeably improved. These changes did not occur after electroacupuncture at a non-acupoint (5 mm lateral to the left side of *Shuigou*). Thus, our findings indicate that electroacupuncture at *Shuigou* improves neurological function in rats following cerebral ischemia/reperfusion injury, and may be associated with down-regulation of BKCa channel protein and mRNA expression. Additionally, our results suggest that the *Shuigou* acupoint has functional specificity.

**Key Words:** nerve regeneration; electroacupuncture; *Shuigou* (GV26) acupoint; stroke; cerebral ischemia/reperfusion injury; large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels; channel protein; neural regeneration

## Introduction

Large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels (BKCa channels) are broadly expressed in mammalian neurons (Adams et al., 1982; Maue and Dionne, 1987). Due to this specific distribution, BKCa channels play an important role in regulating neuronal excitability (Marrion and Tavalin, 1998; Isaacson and Murphy, 2001; Grunnet and Kaufmann, 2004). The main mechanism underlying cerebral ischemia/reperfusion injury is excitatory amino acid toxicity and  $\text{Ca}^{2+}$  overload, resulting in a series of cascade reactions that lead to delayed neuronal necrosis (Lo et al., 2003). At this stage, neuronal membrane excitability is reduced in the ischemic penumbra. *In vitro* intracellular recordings show that spontaneous discharge frequency and cell membrane excitability are persistently reduced after ischemia/reperfusion, and consequently trigger delayed neuronal death. BKCa channels are activated by  $\text{Ca}^{2+}$  and membrane depolarization in the rising phase of action potentials. Further, they can mediate action potential repolarization and fast afterhyperpolarization potentials, thereby altering neuronal excitability (Gao et al., 1999). Chen et al. (2013) suggested that excess activation and expression of BKCa channels are important neuronal apoptosis factors of cerebral ischemia and reperfusion injury. Therefore, enhanced activity of  $\text{K}^{+}$  channels, especially BKCa channels, may be an important neuronal injury mechanism following cerebral ischemia.

Acupuncture has been used to treat cerebrovascular disease for over 2000 years in China, and its efficacy has been demonstrated in modern clinical studies (Li et al., 2014a; Küçük et al., 2015; Yang and Liu, 2015; Zhang et al., 2015a, b). Electroacupuncture (EA) is used in rehabilitation after stroke (Chen et al., 2014; Li et al., 2014b; Qi et al., 2014; Tang et al., 2014; Zhang et al., 2014). The *Shuigou* (GV26) acupoint is an acupuncture point in the meridian named Governor Vessel, and is commonly used for stroke (Li et al., 2006; He and Shen, 2007; Wu and Liu, 2008). Our previous orthogonal design study showed that the optimal EA parameters at *Shuigou* are 1 mA and 2 Hz (Han et al., 2011). However, it is not known if EA improves neurological deficits by inhibiting BKCa channel overexpression at the site of cerebral ischemia/reperfusion injury. Thus, to provide basic evidence for clinical treatment, we examined the relationship between recovery of neurological function and BKCa channel protein and mRNA expression after EA at *Shuigou* in rats with cerebral ischemia/reperfusion injury.

## Materials and Methods

### Ethics statement

This study was approved by the Animal Ethics Committee, Tianjin University of Traditional Chinese Medicine in China (No. TCM-LAEC2014008), and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Institutional Animal Care and Use Committee approval was obtained prior to initiation of the animal studies. Precautions were taken to minimize the suffering and number of animals used in each experiment.

### Animals

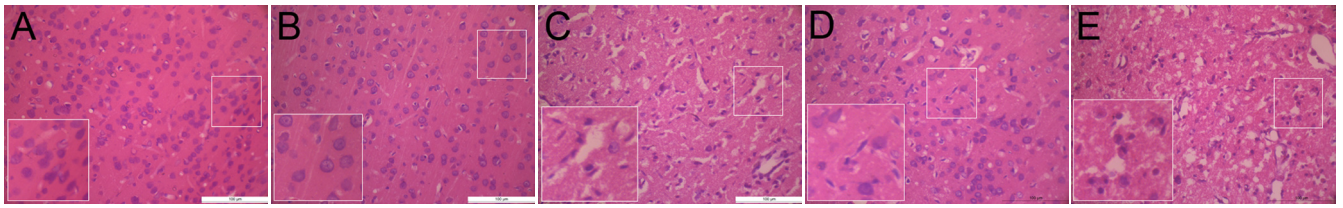
Ninety healthy adult specific-pathogen-free male Wistar rats weighing 200–250 g were provided by the Experimental Animal Center of Chinese Academy of Military Medical Sciences (license No. SCXK 2012-0004). Rats were housed at  $22 \pm 1^{\circ}\text{C}$  in humidity of  $55 \pm 5\%$ , with light from 7:00 to 19:00. They were allowed free access to food and water. The rats were equally and randomly assigned to control, sham surgery, middle cerebral artery occlusion/reperfusion (MCAo/R), *Shuigou* (MCAo/R + EA at *Shuigou*), and non-acupoint (MCAo/R + EA at non-acupoint) groups. Rats with unqualified neurological scores or that died within 72 hours were replaced by new rats.

### Establishment of a rat ischemia/reperfusion model

The ischemia/reperfusion model was established in accordance with the modified method of Longa et al. (1989). After fasting for 12 hours and water deprivation for 6 hours, rats were intraperitoneally anesthetized with 10% chloral hydrate (3.5 mL/kg). A single fiber probe from a DRT4 laser Doppler flowmeter (Moor Instruments, Devon, UK) was fixed on the rat parietal bone, specifically, 2 mm posterior to the anterior fontanelle and 5 mm lateral to the left side of the median cleft. The rat was fixed in the supine position, and cerebral blood flow was monitored for 5 minutes to provide a baseline value. A median incision was made on the neck to dissociate the left common carotid artery and internal and external carotid arteries. The common carotid artery and internal carotid artery were occluded, and the distal end of the external carotid artery was transected. A nylon thread (0.26 mm diameter) was inserted into the middle cerebral artery from the external to the internal carotid artery until resistance was felt. The thread was inserted for 18–20 mm. Successful occlusion was determined by a  $> 70\%$  reduction in cerebral blood flow. The external carotid artery was occluded and the common carotid artery opened. Real-time cerebral blood flow was monitored. Two hours later, the thread was removed. An increase in cerebral blood flow indicated successful perfusion. In the sham surgery group, the procedure was the same as for the MCAo/R group except for thread insertion. The control group did not undergo any treatment.

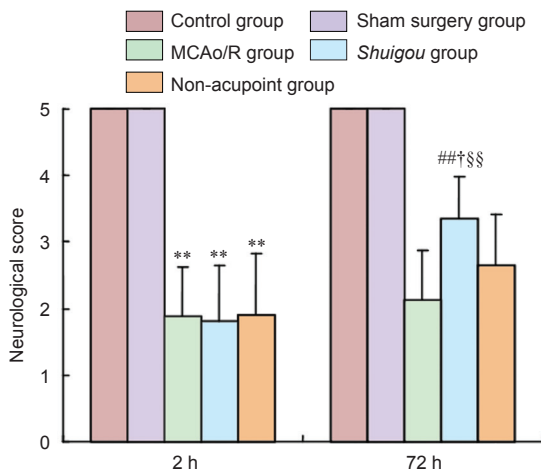
### Neurological evaluation

In accordance with a previous method (Zausinger et al., 2000, 2003), neurological function was scored at 2 hours after ischemia/reperfusion. Rats scoring 0, 4, or 5 points and dead rats were excluded. At 72 hours, neurological function was scored again in all surviving rats. Score 0: no spontaneous walking; score 1: rotates to the contralateral side of the lesion during free walking; score 2: rotates to the contralateral side of the lesion when the tail is held; score 3: decreased resistance to lateral pressure on the contralateral side of the lesion; score 4: cannot extend front paws on the contralateral side of the lesion, even body flexion to the contralateral side; score 5: no neurological deficit.



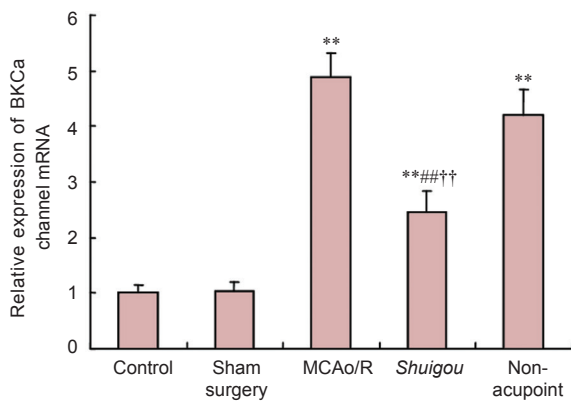
**Figure 2** Effect of electroacupuncture at *Shuigou* (GV26) on histomorphology in the cerebral cortex of rats following ischemia/reperfusion (hematoxylin-eosin staining).

(A, B) Control and sham surgery groups: no pathological injury is visible in the cerebral cortex of rats. (C–E) MCAo/R, *Shuigou*, and non-acupoint groups, respectively: cell number is strikingly reduced. Sparse cytoplasm, light staining, and pyknosis are observed. Nucleoli are small or absent. The degree of injury is milder in the *Shuigou* group than that in the MCAo/R and non-acupoint groups. The box in the lower left corner shows enlarged images. Scale bars: 100  $\mu$ m. MCAo/R: Middle cerebral artery occlusion/reperfusion.



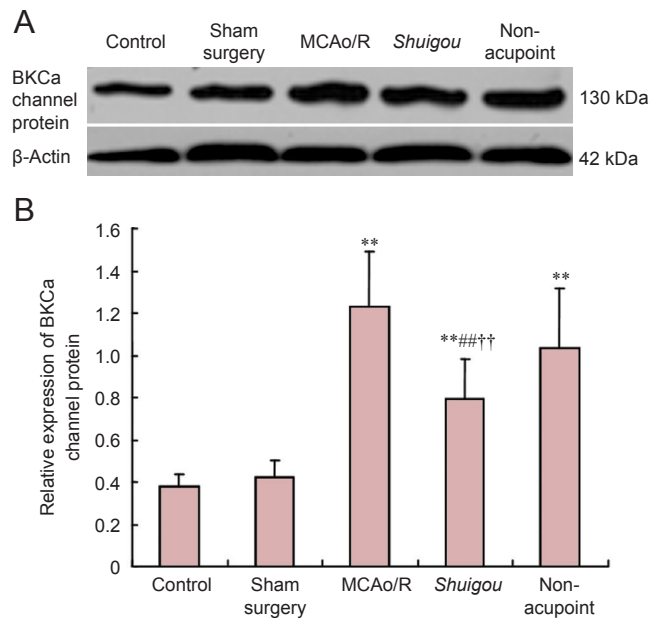
**Figure 1** Effect of electroacupoint at *Shuigou* (GV26) on neurological function in rats following ischemia/reperfusion injury.

Lower neurological scores indicate poorer neurological function. All data are expressed as the mean  $\pm$  SD ( $n = 6$ ). One-way analysis of variance was used to compare intergroup differences. The least significant difference test was used for paired comparisons among groups. \*\* $P < 0.01$ , vs. control group; ### $P < 0.01$ , vs. MCAo/R group; † $P < 0.05$ , vs. non-acupoint group; §§ $P < 0.01$ , vs. 2 hours. MCAo/R: Middle cerebral artery occlusion/reperfusion; h: hours.



**Figure 4** Electroacupuncture at the *Shuigou* (GV26) acupoint affects BKCa channel mRNA expression in the rat cerebral cortex at 72 hours after ischemia/reperfusion (quantitative real-time PCR).

Data are expressed as the mean  $\pm$  SD ( $n = 6$ ; one-way analysis of variance and least significant difference test). \*\* $P < 0.01$ , vs. control group; ### $P < 0.01$ , vs. MCAo/R group; †† $P < 0.01$ , vs. non-acupoint group. MCAo/R: Middle cerebral artery occlusion/reperfusion; BKCa: large-conductance  $Ca^{2+}$ -activated  $K^+$  channels.



**Figure 3** Electroacupuncture at the *Shuigou* (GV26) acupoint affects BKCa channel protein expression in the rat cerebral cortex at 72 hours after ischemia/reperfusion (western blot assay).

(A) Western blot assay. (B) Quantification of BKCa channel protein expression: BKCa channel protein expression is shown as the optical density ratio of BKCa channel protein to  $\beta$ -actin. Data are expressed as the mean  $\pm$  SD ( $n = 6$ ; one-way analysis of variance and the least significant difference test). \*\* $P < 0.01$ , vs. control group; ### $P < 0.01$ , vs. MCAo/R group; †† $P < 0.01$ , vs. non-acupoint group. MCAo/R: Middle cerebral artery occlusion/reperfusion; BKCa: large-conductance  $Ca^{2+}$ -activated  $K^+$  channels.

**EA treatment**

In accordance with *Experimental Acupuncture Science* (Guo and Fang, 2012), *Shuigou* is located below the rat nasal septum, at the junction of the lower two thirds and upper one third of the cleft lip midline. The non-acupoint was 5 mm lateral to the left side of *Shuigou*. Two hours after ischemia/reperfusion, rats in the *Shuigou* and non-acupoint groups underwent EA with an acupuncture needle of 0.30 mm diameter (Hwato, Suzhou, Jingsu Province, China). The needle was vertically punctured towards the nasal septum at a depth of 2 mm for 10 minutes, once every 12 hours, for a total of six times within 72 hours. Needles were connected to the Han’s acupoint nerve stimulator (HANS-200E; GENSUN, Nanjing, Jiangsu Province, China), at a frequency of 2 Hz,

current of 1 mA, and with a continuous wave.

### Tissue preparation

Seventy-two hours after ischemia/reperfusion, six rats from each group were anesthetized with 10% chloral hydrate. The heart was perfused with 200 mL ice-cold physiological saline, and pre-fixed with 200 mL 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA). The rats were then decapitated, and the brains removed and fixed in 4% paraformaldehyde overnight. Coronal sections of the optic chiasma on the injured side and 4 mm posterior to the optic chiasma were dehydrated through a graded alcohol series, permeabilized with xylene, and embedded in paraffin. Two 4  $\mu$ m-thick sections of each tissue were cut using a microtome (Leica, Wetzlar, Germany) for hematoxylin-eosin staining. After anesthesia, the remaining 12 rats in each group were decapitated and the brains placed on ice. After washing with 0.01 M ice-cold phosphate buffered saline, the cortex at the site of the left middle cerebral artery was placed in liquid nitrogen into a freezing tube, and stored in a  $-80^{\circ}\text{C}$  low-temperature freezer until further western blot assay and quantitative real-time polymerase chain reaction (PCR).

### Hematoxylin-eosin staining

Paraffin sections were dewaxed, hydrated through a graded alcohol series, stained with hematoxylin for 15 minutes, washed with running water for 5 minutes, stained with eosin for 1 minute, dehydrated through a graded alcohol series, and permeabilized with xylene. After air-drying, sections were mounted with neutral resin, observed and photographed using a light microscope (Leica).

### Western blot assay

The cerebral cortex from six rats in each group was lysed in 1 mL of ice-cold radioimmunoprecipitation assay buffer containing protease inhibitors (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Total protein was quantified using a bicinchoninic acid protein assay kit (Santa Cruz Biotechnology Inc.). An equal volume of protein underwent sodium dodecyl sulphate-polyacrylamide gel electrophoresis and was then transferred onto polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk, incubated with rabbit BKCa- $\alpha$  (1:500; Santa Cruz Biotechnology Inc.) and  $\beta$ -actin antibody (1:1,000; Santa Cruz Biotechnology Inc.) at  $4^{\circ}\text{C}$  overnight, and then horseradish peroxidase-labeled goat anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology Inc.) at room temperature for 2 hours, followed by enhanced chemiluminescence (Thermo Fisher Scientific Inc., Waltham, MA, USA). Membranes were visualized and photographed using a gel imaging system (Bio-Rad, Hercules, CA, USA). Optical density values were determined using Quantity One image analyzer software (Bio-Rad).  $\beta$ -Actin served as the internal reference.

### Quantitative real-time PCR

The cerebral cortex was obtained from six rats in each group.

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Purity and integrity were assessed using an ultraviolet spectrophotometer (Thermo Fisher Scientific Inc.) and agarose gel electrophoresis. Upstream and downstream primers for the BKCa- $\alpha$  subunit were 5'-CGG ACT TAG GGG ATG GTG GT-3' and 3'-GGG GGA TTG GTG ATG ACG TA-5', respectively. Upstream and downstream primers for  $\beta$ -actin were 5'-CCG TAA AGA CCT CTA TGC CAA CA-3' and 5'-TAG GAG CCA GGG CAG TAA TC-3' (TaKaRa Bio Inc., Kusatsu, Japan), respectively. cDNA was synthesized using a reverse transcription kit (TaKaRa Bio Inc.). PCR was performed using a PCR system (Eppendorf, Hamburg, Germany) with a SYBR green fluorophore (Bio-Rad). BKCa- $\alpha$  and  $\beta$ -actin reactions for each sample were detected in three wells. A reaction volume of 25  $\mu$ L included 12.5  $\mu$ L iQ SYBR Green SuperMix, 10  $\mu$ M upstream/downstream primers (1  $\mu$ L), and 1  $\mu$ L cDNA template (1  $\mu$ g/mL).  $\beta$ -Actin served as the reference. BKCa- $\alpha$  mRNA expression was analyzed using the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen, 2001).

### Statistical analysis

All data are expressed as the mean  $\pm$  SD and were analyzed using SPSS 19.0 software (IBM, Armonk, NY, USA). One-way analysis of variance was used to compare intergroup differences. The least significant difference test was used for paired comparisons among groups. A value of  $P < 0.05$  was considered statistically significant.

## Results

### EA improved neurological function in rats after ischemia/reperfusion for 72 hours

Rats in the control and sham surgery groups scored 5 for neurological function. Neurological scores were similar between the MCAo/R, *Shuigou*, and non-acupoint groups at 2 hours after injury ( $P > 0.05$ ). Moreover, they were considerably lower than in the control group ( $P < 0.01$ ). In the MCAo/R and non-acupoint groups, neurological score slightly improved at 72 hours after ischemia/reperfusion, although mean neurological score was similar to that obtained at 2 hours ( $P > 0.05$ ). In the *Shuigou* group, neurological score was significantly higher at 72 hours compared with 2 hours ( $P < 0.01$ ). Additionally, neurological score was higher in the *Shuigou* group than the MCAo/R and non-acupoint groups ( $P < 0.01$  or  $P < 0.05$ ; **Figure 1**).

### EA ameliorated rat cortex histomorphology after ischemia/reperfusion

Hematoxylin-eosin staining demonstrated that 72 hours after injury, rat cerebral cortex had an intact histomorphology, with distinct cell boundaries, abundant cytoplasm, and big, round nuclei. Moreover, the nucleus was centrally located in the control and sham surgery groups. In the MCAo/R group, cell number was remarkably reduced. Additionally, cells exhibited sparse cytoplasm, light staining, pyknosis, and many vacuoles. Nucleoli became small or disappeared. In the *Shuigou* group, sparse cytoplasm and pyknosis were observed. The degree of



injury was milder in the *Shuigou* group compared with the MCAo/R and non-acupoint groups (Figure 2).

#### EA suppressed BKCa channel protein in rat cortex after ischemia/reperfusion

Western blot assays revealed that 72 hours after injury, BKCa channel protein expression was significantly higher in the MCAo/R, *Shuigou*, and non-acupoint groups compared with the control and sham surgery groups ( $P < 0.01$ ). BKCa channel protein expression was significantly lower in the *Shuigou* group compared with the MCAo/R and non-acupoint groups ( $P < 0.01$ ). BKCa channel protein expression was similar between the non-acupoint and MCAo/R groups ( $P > 0.05$ ; Figure 3).

#### EA suppressed BKCa channel mRNA in rat cortex after ischemia/reperfusion

BKCa channel mRNA expression followed the same trend as BKCa channel protein expression in each group. BKCa channel mRNA expression was significantly higher in the MCAo/R, *Shuigou*, and non-acupoint groups compared with the control and sham surgery groups ( $P < 0.01$ ). BKCa channel mRNA expression was significantly lower in the *Shuigou* group compared with the MCAo/R and non-acupoint groups ( $P < 0.01$ ). BKCa channel mRNA expression was similar between the non-acupoint and MCAo/R groups ( $P > 0.05$ ; Figure 4).

### Discussion

*Shuigou* is the main acupoint of *Xingnao Kaiqiao* acupuncture therapy. Several studies have confirmed that *Shuigou* plays a positive role in recovery of neurological function after stroke (Lin et al., 2010; Chang et al., 2012, 2014; Shen et al., 2012; Li et al., 2013). Animal studies have shown that acupuncture at the *Shuigou* acupoint regulates vascular elasticity, promotes blood circulation within the brain, increases perfusion volume, improves ischemia-induced abnormal blood flow, and protects nerve cells (Wang et al., 2009; Zhou et al., 2011). In our previous orthogonal design study, EA using the optimal stimulation parameters of 1 mA and 2 Hz markedly improved neurological impairment and motor evoked potentials in rats subjected to middle cerebral artery occlusion (Han et al., 2011). Therefore, we used 1 mA and 2 Hz in this study. After 72 hours of intervention, neurological function had noticeably improved in the *Shuigou* group, but not in the MCAo/R and non-acupoint groups. Histomorphological examination revealed greater recovery of injured tissue in the *Shuigou* group compared with the MCAo/R and non-acupoint groups.

BKCa channel protein and mRNA expression in rat cerebral cortex were significantly higher in the MCAo/R group compared with the control and sham surgery groups, consistent with a previous study (Chen et al. 2013). However, BKCa channel protein and mRNA expression were obviously diminished after EA at *Shuigou*. Thus, our neurological score and histopathological findings show that neurological impairment and pathological injury are likely associated with

high BKCa channel expression, but EA at the *Shuigou* acupoint can reverse this phenomenon. These results indicate that EA at *Shuigou* is specific in recovery of neurological function and reducing BKCa channel protein and mRNA expression after ischemia/reperfusion.

Gribkoff et al. (2001) suggested that BKCa channel activation can protect ischemic neurons. Peri-infarct depolarization occurs over 2–24 hours after permanent middle cerebral artery occlusion, resulting in calcium overload and neuronal death (Hartings et al., 2003). Activation of BKCa channels can lead to membrane repolarization and subsequent neuronal protection. Neuronal depolarization only occurs during ischemia, and membrane repolarization occurs immediately during reperfusion (Gao et al., 1998; Xu et al., 1999). It is inferred that neuronal depolarization recovers after ischemia/reperfusion, but BKCa channels are still in a state of excess activation, which may cause a large amount of potassium efflux and membrane hyperpolarization, reduce neuronal excitability, and lead to neuronal apoptosis or death.

In summary, EA at the *Shuigou* acupoint (1 mA and 2 Hz) improved neurological function and pathological injury in rats with ischemia/reperfusion injury, possibly by decreasing BKCa protein and mRNA expression at the injury site.

**Author contributions:** SW and YS were responsible for study design and conception. SW obtained the funding. YW, HPL, ZL and YYC participated in animal experiments. YW and YS were in charge of data analysis and statistical processing. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Plagiarism check:** This paper was screened twice using Cross-Check to verify originality before publication.

**Peer review:** This paper was double-blinded and stringently reviewed by international expert reviewers.

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