Electroacupuncture effects on cortical neurons, as well as Janus kinase 2-signal transducer and activator of transcription 3 signal transduction pathway, in a rat model of cerebral ischemia

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Abstract
The present study established a model of focal cerebral ischemia through heat-coagulation induced occlusion of the middle cerebral artery. Following electroacupuncture at Baihui (GV20) and Dazhui (GV14), or intracerebroventricular infusion of AG490, a Janus kinase 2 phosphorylation inhibitor, the amount of necrotic or degenerated neurons in the ischemic cerebral cortex decreased, neuronal swelling was ameliorated, and expression of phosphorylated Janus kinase 2 and signal transducer and activator of transcription 3 decreased. Results confirmed that electroacupuncture promoted neuronal repair in the cerebral cortex by reducing expression of phosphorylated Janus kinase 2 and signal transducer and activator of transcription 3, as well as weakening the phosphorylated activated Janus kinase 2-STAT3 signal transduction pathway.

Key Words: cerebral ischemia; JAK2-STAT3; neurons; electroacupuncture; AG490

INTRODUCTION
The Janus kinase-signal transducer and activator of transcription (JAK-STAT) system consists of Janus kinase (JAK) and signal transducer and activator of transcription (STAT). Following cerebral ischemia, a large amount of cytokines and growth factors are released, which could activate the JAK-STAT signal transduction system[1-3]. The JAK-STAT signal transduction system, in particular the JAK2-STAT3 signal transduction pathway, plays an important role in regulating neuronal apoptosis following cerebral ischemia[4]. Intracerebroventricular infusion of AG490, a Janus kinase 2 phosphorylation inhibitor, in rats prevents post-ischemic JAK2 and STAT3 phosphorylation and significantly reduces infarct volume, the number of apoptotic cells, and neurological deficits[5]. Intracerebral injection of siRNA specific for STAT3 leads to reduced STAT3 mRNA expression and phosphorylation, as well as decreased infarct volume, fewer apoptotic cells, and improved neurological function following transient middle cerebral artery occlusion[6]. In addition, phosphorylated-JAK2 (p-JAK2) and p-STAT3 protein expression remains unchanged following ischemic reperfusion. AG490 is an inhibitor of JAK2 receptor, and application of AG490 has been shown to inhibit JAK2 activation, resulting in decreased STAT3 phosphorylated levels[5-6]. AG490 has also been shown to reduce the amount of neuronal apoptosis[7]. Electroacupuncture plays an important role in neuroprotection in the cerebral striatum following cerebral ischemia, and these protective mechanisms could contribute to anti-oxidative stress and gene expression changes due to electroacupuncture[8]. Electroacupuncture has been shown to increase Bcl-2 protein expression, which inhibits neuronal apoptosis, in the ischemic penumbra of rats with cerebral ischemia. This suggests that acupuncture provides neuroprotection following cerebral ischemia[9]. However, recent studies have not elucidated the functional mechanisms of the JAK2-STAT3 signal transduction pathway affected by acupuncture following cerebral ischemia. In the present study, the effects of electroacupuncture and AG490 on neuronal ultrastructure in the ischemic cortex of rats with focal cerebral ischemia were observed by transmission electron microscope. In addition, p-JAK2 and p-STAT3 expression was observed in the ischemic cortex by fluorescent
immunohistochemistry and laser confocal scanning microscopy to evaluate the significance of JAK2 and STAT3 activation in focal ischemia-induced neuronal damage. The mechanisms of action of electroacupuncture will provide further insight into treatment paradigms for ischemic cerebral diseases.

RESULTS

Quantitative analysis of experimental animals
A total of 200 Sprague Dawley rats were randomly assigned to sham-surgery, model, electroacupuncture, AG490, and electroacupuncture + AG490 groups, with 40 animals in each group. Except for the sham-surgery group, focal cerebral ischemia was established in the remaining groups. AG490 group rats were subjected to an intracerebroventricular infusion of AG490 20 minutes prior to middle cerebral artery occlusion. The electroacupuncture group was treated with electroacupuncture at Baihui (GV20) and Dazhui (GV14) 1 hour post-middle cerebral artery occlusion. The electroacupuncture + AG490 group was intracerebroventricularly injected with AG490 20 minutes prior to middle cerebral artery occlusion and electroacupuncture at Baihui and Dazhui 1 hour post-middle cerebral artery occlusion. All rats were included in the final analysis.

Electroacupuncture and AG490 improved neuronal ultrastructure in the ischemic cortex
In the sham-surgery group, neurons were normal without swelling. Euchromatin was plentiful and evenly distributed, and few heterochromatin fibers were observed in the nuclei. The nuclear membranes were clear, nucleoli were regular, and there were many cytoplasmic organelles. Mitochondria were normal with complete cristae (Figure 1A). At 2 hours post-ischemia, neuronal nuclei were swollen and deformed, chromatins were unevenly distributed, and mitochondria and cytoplasm were swollen. At 1 and 3 days post-cerebral ischemia, the model group exhibited cytoplasmic swelling, loss of organelles, significantly distended mitochondria, loss of part of the mitochondrial cristae, and expanded rough endoplasmic reticulum with serious degranulation. Some neurons exhibited chromatin condensation, as well as aggregation at the periphery of the nuclei and nuclear condensation. The electroacupuncture, AG490, and electroacupuncture + AG490 groups exhibited similar changes in neuronal ultrastructure in the ischemic cortex at the same time points. Compared with the model group, there were less degenerative and necrotic neurons in the remaining three groups. In addition, there were less swollen neurons than in the model groups, as well as loss of a small part of mitochondrial cristae and some slightly irregular nuclei (Figures 1B–F). At 7 days post-cerebral ischemia, there were less degenerative and necrotic neurons in the electroacupuncture, AG490, and electroacupuncture + AG490 groups compared with the model groups. Some nuclei were slightly irregular, and parts of the heterochromatin aggregated at the nuclear periphery. At 21 days, except for the sham-surgery group, changes of neuronal ultrastructure were not obvious.

Figure 1 Neurological evaluation of rats using the Neurological Deficit Scale
(A) Neurological deficit scale in the sham-surgery group is normal.
(B) Neurological deficit scale in the model group is normal.
(C) Mitochondria (arrows) in the sham-surgery group are barely visible in the neurons.
Mitochondria (arrows) are swollen, and part of the cristae is barely visible in the electroacupuncture (D), AG490 (E), and electroacupuncture + AG490 (F) groups.

In addition, the neurons were slightly distended, the cell outline was clear, several mitochondrial cristae integrated after fracturing, and there was sufficient organelles. These
results suggested that post-cerebral ischemia, electroacupuncture and AG490 intervention ameliorated neuronal swelling, increased the number of mitochondriae, and promoted neuronal repair in the cerebral cortex.

**Electroacupuncture and AG490 reduced p-JAK2 and p-STAT3 expression in the ischemic cortex**

Immunofluorescence results showed that p-JAK2 expression was primarily in the neuronal cytoplasm of the ischemic cortex, with no obvious expression in the nuclei. p-STAT3 was primarily expressed in neuronal nuclei, with little expression in the cytoplasm. At different times in the sham-surgery group, there was little p-JAK2 or p-STAT3 expression. However, in the model group, some p-JAK2 and p-STAT3 expression was detected in the cerebral cortex at 2 hours post-ischemia. p-JAK2 and p-STAT3 expression peaked at 1 day post-ischemia, but decreased by 3 days. Compared with the sham-surgery group at the same time point of ischemia, p-JAK2 and p-STAT3 expression in the model group was significantly greater ($P < 0.01$). Similar changes existed between the electroacupuncture and AG490 groups, with exception to the 2-hour and 21-day groups. Compared with the model group at the same time point of ischemia, p-JAK2 expression decreased in the electroacupuncture and AG490 groups ($P < 0.05$). Compared with the model groups, p-JAK2 expression significantly decreased in the electroacupuncture + AG490 group at the same ischemic time ($P < 0.05$). In the electroacupuncture group, p-STAT3 significantly decreased compared with model group at 1 and 3 days post-ischemia ($P < 0.01$, respectively). In the AG490 group, p-STAT3 expression significantly decreased compared with the model group at 2 hours, as well as 1 and 3 days, post-ischemia ($P < 0.01$, respectively). Except for the 21-day subgroup, p-STAT3 expression was less in the electroacupuncture + AG490 group at same time point than in with model group ($P < 0.05$; Figure 2, Tables 1, 2). p-JAK2 and p-STAT3 overexpression following cerebral ischemia, as well as abnormal activation of the JAK2-STAT3 signal transduction pathway, served as a mechanism of aggravated cerebral injury. Electroacupuncture treatment was shown to down-regulate p-JAK2 and p-STAT3 expression following cerebral ischemia, as well as decrease phosphorylated activation, thereby blocking abnormal activation of the JAK2-STAT3 signal transduction pathway.

![Figure 2](image)

**Figure 2**  Expression of phosphorylated JAK2 (p-JAK2) or STAT3 (p-STAT3) in ischemic brain tissues at 1 day post-cerebral ischemia, as determined by laser confocal scanning microscopy (immunofluorescent staining, × 400).

Hoechst 33258 was used to label the nuclei. p-JAK2 is primarily expressed in the neuronal cytoplasm of the ischemic cortex (green, arrows). p-STAT3 is primarily expressed in neuronal nuclei (red, arrows). JAK: Janus kinase; STAT: signal transducer and activator of transcription.

| Group                     | Time post-ischemia |
|---------------------------|--------------------|
|                           | 2 hours            | 1 day              | 3 days            | 7 days            | 21 days           |
| Sham-surgery              | 53.36±14.18        | 75.33±16.84        | 65.91±20.94       | 62.77±19.45      | 59.64±14.18       |
| Model                     | 241.68±27.72       | 621.47±67.38       | 546.14±70.46      | 404.90±54.26      | 213.43±38.90      |
| Electroacupuncture        | 225.99±39.50       | 533.58±59.15       | 442.56±42.53      | 335.84±38.44      | 194.80±38.90      |
| AG490                     | 232.27±40.68       | 461.39±61.60       | 439.42±63.77      | 317.01±40.24      | 207.16±47.64      |
| Electroacupuncture + AG490| 182.05±45.61       | 451.98±55.87       | 373.51±40.24      | 276.21±38.90      | 166.35±43.63      |

Data are expressed as mean ± SD from six rats in each group (least significant difference t-test). *P < 0.01, vs. sham-surgery group; **P < 0.05; ***P < 0.01, vs. model group. JAK: Janus kinase.
**DISCUSSION**

Cellular apoptosis due to cerebral ischemia is related to several cytokines, such as interleukin, caspase, c-fos, and tumor necrosis factor-α. The JAK-STAT signal transduction system plays an important role in signal transduction induced by these cytokines\(^{[10]}\). This signal transduction system and the regulated mechanisms following cerebral ischemia have been shown to inhibit the inflammatory course and improve neuronal injury in the ischemic region, as well as provide a novel method for treating cerebral ischemia\(^{[10]-[11]}\).

The intracerebroventricular infusion of rats with AG490 significantly reduces infarct volume following cerebral ischemia, as well as significantly reduces cellular apoptosis and ameliorates neurological deficits\(^{[10]}\). Interventions of acupuncture on the JAK-STAT signal transduction system following cerebral ischemia/reperfusion results in up-regulated STAT3 protein expression in the hippocampus, as well as increased activation and nuclei translocation of STAT3, thereby providing neuroprotection\(^{[12]}\). STAT1 mRNA expression increases at 3 days post-cerebral ischemia, and electroacupuncture has been shown to inhibit this expression\(^{[13]}\). Increased STAT1 could be a critical signal for inducing apoptosis, and inhibition by electroacupuncture could be serve as an important pathway for reducing apoptosis and improving cerebral function following cerebral ischemia\(^{[13]}\). The marginal zone of the ischemic neuronal ultrastructure, which included mitochondrial swelling, cristae destruction, and degeneration of ribosomes, was less in the electroacupuncture group than in the control groups. These results suggested that electroacupuncture reduced cerebral infarct size and marginal zone neuronal apoptosis, thereby protecting ischemic neurons\(^{[14]}\).

In the present study, transmission electron microscope was used to observe the effects of electroacupuncture and AG490 on neuronal ultrastructure in the ischemic cortex of rats with focal cerebral ischemia. Results showed significantly swollen neurons, loss of organelles, and unevenly distributed nuclear chromatin. The amount of neuronal degeneration and necrosis in the cerebral ischemic cortex in the electroacupuncture, AG490, and electroacupuncture + AG490 groups was less than in the model groups, and the degree of neuronal swelling was also less than in the model group. These results suggested that following cerebral ischemia, electroacupuncture and AG490 intervention alleviated neuronal swelling, increased the number of mitochondriae, and promoted neuronal repair in the cerebral cortex.

Immunofluorescence results revealed no p-JAK2 and p-STAT3 expression in the sham-surgery group, which suggested that p-JAK2 and p-STAT3 remained inactive or weak. However, following cerebral ischemia, p-JAK2 and p-STAT3 expression increased, and these changes occurred almost simultaneously in post-focal cerebral ischemia. These results demonstrated that expression and activation of p-JAK2 and p-STAT3 following cerebral ischemia played an important role in neuronal death induced by cerebral ischemia. In addition, electroacupuncture functional mechanisms on JAK2-STAT3 at different times after cerebral ischemia were analyzed and compared with intracerebroventricular infusion of AG490. Results showed that electroacupuncture effectively inhibited over-expression of p-JAK2 and p-STAT3, as well as abnormal activation of the JAK2-STAT3 signal transduction pathway. This could be one of the critical mechanisms by which electroacupuncture protects against cerebral ischemia and inhibits neuronal apoptosis.

### MATERIALS AND METHODS

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
The experiment was performed at the Laboratory Animal Center and Laboratory of Electron Microscopy, Guangzhou University of Chinese Medicine, China from March 2010 to May 2011.

**Materials**
A total of 200 adult, male, specific pathogen-free, Sprague Dawley rats, aged 42–49 days and weighing 180–240 g, were provided by the Laboratory Animal Center of Guangzhou University of Chinese Medicine (license No. SCXK (Yue) 2008-0020). The rats were...
Experimental animals were sacrificed after 2 hours, housed at 22 ± 2°C, with 65% relative humidity and free access to food, and were exposed daily to 12 hours of light. All experimental procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[16].

**Methods**

**Establishment of a focal cerebral ischemia model**

Heat coagulation-induced middle cerebral artery occlusion was used to establish a model of focal cerebral ischemia. Rats were initially intraperitoneally anesthetized with a 10% chloral hydrate injection (330 mg/kg) and were fixed in a right upper lateral position on the surgical table. An incision was made at the median position between the ear and eye to isolate the temporal muscle. The middle cerebral artery was exposed and occluded using a heated stainless steel wire. In the sham-surgery group, the middle cerebral arteries were exposed without occlusion.

**Intracerebroventricular infusion of AG490**

AG490 (Merck, Darmstadt, Germany), 250 μm in 3% dimethyl sulphoxide, was continuously infused into the lateral ventricles of rat cohorts. The drugs were filled into 25-μL osmotic minipumps that contained 10 μL solution. Each pump was connected to an Alzet brain infusion stainless steel cannula via peristaltic tubing and was primed overnight at 37°C to ensure immediate delivery post-implantation. The cannula was stereotaxically implanted into the lateral ventricle (bregma, 0.8 mm posterior, −4.8 mm dorsoventral, −1.5 mm lateral) and secured to the skull with a needle. The pump was placed in the skin fold on the neck. The cannula and pump implantation was conducted under chloral hydrate injection anesthesia. The intracerebroventricular infusion lasted for 10 minutes, and the needle was maintained for 2 minutes. Penicillin was used for disinfection following infusion. The middle cerebral artery of the AG490 group and electroacupuncture + AG490 group was occluded for 20 minutes following intracerebroventricular infusion[4].

**Electroacupuncture treatment**

Rats were electroacupuncture at 1 hour post-focal cerebral ischemia. The acupoints of Baihui and Dazhui in the Du meridian were selected, as previously described[17]. Baihui is located in the median of the parietal bone, and Dazhui is located in the median of the back, below the spinous process of the seventh cervical and first thoracic vertebrae. A 0.3 mm × 25 mm needle (Suzhou Medical Equipment Factory, Suzhou, China) was inserted approximately 15 mm ventral to Baihui and inserted vertically into Dazhui at a depth of 7.5 mm. Acupoints were connected to a G-6805 electric acupuncture apparatus (Qingdao Xinsheng Medical Instrument Factory, Qingdao, China; 4/20 Hz, 1–2 mA for 30 minutes, once daily).

**Sample collection and transmission electron microscope analysis**

Experimental animals were sacrificed after 2 hours, and 1, 3, 7, and 21 days post-surgery. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. The chest was opened to expose the heart for left ventricle aortic cannulation. A total of 200 mL normal saline and 300 mL 4% paraformaldehyde/phosphate-buffered saline (pH 7.4) was perfused. Brain tissues from two rats in each group were collected, and 1 mm × 1 mm × 1 mm cerebral cortex tissue samples were harvested from the marginal zone of the focal cerebral ischemia. The tissues were immediately fixed in 2.5% glutaraldehyde overnight at 4°C, rinsed four times in 0.01 M phosphate buffered saline for 15 minutes each, post-fixed in 1% osmium tetroxide solution for 1 hour, and rinsed four times in phosphate buffered saline for 15 minutes each. The tissues were then dehydrated in a gradient series of acetone (50%, 70%, and 90%) for 15 minutes each, followed by three incubations in 100% acetone (15 minutes each). The tissues were then soaked in glutaraldehyde: araldite (1: 1) for 1 hour, glutaraldehyde:araldite (1: 2) for 2 hours, and araldite overnight. All samples were embedded in araldite, polymerized at 72°C for 8 hours, and sectioned into 90-nm thick slices. Sections were screened using a 200-μm mesh copper screen and stained with uranyl acetate and lead citrate prior to H-7500 transmission electronic microscopy (JEOL, Tokyo, Japan). A sheet of copper screen was used to examine one sample from each rat, and negatives were amplified 12 000 times for printing.

**Immunofluorescence of p-JAK2 and p-STAT3 expression in the ischemic cortex**

Brain tissues from six rats in each group were collected, immediately fixed in 4% paraformaldehyde for 24 hours, and soaked in 20% sucrose solution until the tissues sank. Frozen sections were cut using a cryostat freezing microtome (HM550, Microm, Walldorf, Germany). Coronal sections were made horizontally, 20 μm in diameter, followed by p-JAK2 and p-STAT3 immunofluorescent staining. The sections were fixed in acetone for 15 minutes, rinsed three times in 0.01 M phosphate buffered saline (pH 7.4, 10 minutes each), permeabilized in 0.3% Triton X-100 for 5 minutes, rinsed three times in phosphate buffered saline for 5 minutes each, then blocked in 5% bovine serum albumin for 30 minutes. The sections were then incubated with rabbit anti-p-JAK2 polyclonal antibody (1: 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-p-STAT3 polyclonal antibody (1: 200; Abnova, California, USA) overnight at 4°C. The negative control was incubated only with phosphate buffered saline. On the following day, sections were rinsed three times with phosphate buffered saline (5 minutes each), followed by incubations with goat anti-rabbit IgG/fluorescein isothiocyanate (1: 50; Bioss, Wuhan, China) or goat anti-rabbit IgG/RBITC (1: 50; Bioss) for 1 hour at 37°C. Sections were rinsed three times with phosphate buffered saline, 5 minutes each, and nuclear staining was performed with Hoechst 33258 (1: 8; BLKW, Beijing.
China) for 5 minutes, followed by dehydration and blocking. LSM 510 META laser confocal scanning microscope, laser confocal scanning microscopy (Zeiss, Oberkochen, Germany) was used to detect p-JAK2 and p-STAT3 expression in the cerebral cortex, and images were analyzed for average fluorescence intensity. Excitation wavelength of fluorescein isothiocyanate and fluorophore rhodamine B isothiocyanate were 490 and 550 nm respectively, and images were scanned at a resolution of 1 024 × 1 024 pixels. LSM510 Image Examiner software (Zeiss) was used to analyze p-JAK2 and p-STAT3 expression. Relatively dense regions of cells were selected for continuous scanning of five visual fields, and the average fluorescent intensity values were regarded as relative expression levels of p-JAK2 and p-STAT3.

Statistical analysis
Data were expressed as mean ± SD. SPSS 13.0 software (SPSS, Chicago, IL, USA) was used to perform one-way analysis of variance. Least significant difference t-test was used for comparison between groups, and P < 0.05 was regarded as statistically significant.

Author contributions: Nenggui Xu was responsible for funding and authorized the present study. Rong Liu wrote the manuscript. Rong Liu, Wei Yi, and Kangbai Huang designed and performed the present study, and also participated in data analysis and statistics.

Conflicts of interest: None declared.

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