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Electroacupuncture stimulation of the brachial plexus trunk on the healthy side promotes brain-derived neurotrophic factor mRNA expression in the ischemic cerebral cortex of a rat model of cerebral ischemia/reperfusion injury☆

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Abstract
A rat model of cerebral ischemia/reperfusion was established by suture occlusion of the left middle cerebral artery. *In situ* hybridization results showed that the number of brain-derived neurotrophic factor mRNA-positive cells in the ischemic rat cerebral cortex increased after cerebral ischemia/reperfusion injury. Low frequency continuous wave electroacupuncture (frequency 2–6 Hz, current intensity 2 mA) stimulation of the brachial plexus trunk on the healthy (right) side increased the number of brain-derived neurotrophic factor mRNA-positive cells in the ischemic cerebral cortex 14 days after cerebral ischemia/reperfusion injury. At the same time, electroacupuncture stimulation of the healthy brachial plexus truck significantly decreased neurological function scores and alleviated neurological function deficits. These findings suggest that electroacupuncture stimulation of the brachial plexus trunk on the healthy (right) side can greatly increase brain-derived neurotrophic factor mRNA expression and improve neurological function.

Key Words
ischemia/reperfusion; brain-derived neurotrophic factor; electroacupuncture; brachial plexus trunk; cerebral cortex; *in situ* hybridization; neural regeneration

Research Highlights
Electroacupuncture stimulation of the brachial plexus trunk on the healthy side can significantly improve the neurological function of a rat model of cerebral ischemia/reperfusion injury and enhance brain-derived neurotrophic factor mRNA expression in the ischemic cerebral cortex 14 days after ischemia/reperfusion injury.

Abbreviations
MCAO, middle cerebral artery occlusion

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INTRODUCTION

There is evidence that brain-derived neurotrophic factor plays an important role in neural cell injury and repair after cerebral ischemia[1-2]. After cerebral ischemia, increased brain derived neurotrophic factor protein expression in the cerebral cortex, striatum, and hippocampus can greatly alleviate ischemic brain injury (such as cerebral edema) and inhibit neuronal apoptosis, resulting in a protective effect[3-5]. Studies have shown that electroacupuncture

can alleviate cerebral edema, decrease calcium ion inflow, and reduce inflammation and cell apoptosis in the brain tissue of rats after middle cerebral artery occlusion (MCAO) [6-10]. Moreover, electroacupuncture also exhibits good curative effects in the clinical treatment of acute cerebral ischemia[11]. Electroacupuncture stimulation of the nerve truck can also improve the neurological function of patients with stroke[12-13]. Little information is known about whether electroacupuncture stimulation of the nerve stem can influence brain derived neurotrophic factor mRNA expression levels in the brain tissue of rats subjected to MCAO. In this study, we monitored brain derived neurotrophic factor mRNA expression levels over time in the cerebral cortex of a rat model of cerebral ischemia/reperfusion injury established by MCAO. In addition, we monitored brain derived neurotrophic factor mRNA expression levels after electroacupuncture stimulation of the brachial plexus trunk on the healthy side.

RESULTS

Quantitative analysis of experimental animals

Sixty-eight healthy female adult Wistar rats were divided into a sham surgery group (n = 4), cerebral ischemia/reperfusion group (n = 32) and a brachial plexus trunk electroacupuncture group (electroacupuncture group, n = 32). Rats from the ischemia/reperfusion and electroacupuncture groups were subjected to suture occlusion of the left middle cerebral artery. Rats from the electroacupuncture group received electroacupuncture stimulation of the brachial plexus trunk after MCAO. Four rats from each group at each time point (2, 6, 12, 24 hours and 2, 3, 7 and 14 days after ischemia/reperfusion) were selected for subsequent examination and all rats were included in the final analysis.

Electroacupuncture stimulation of the brachial plexus trunk improved MCAO rat neurological function

After MCAO, rats exhibited varying degrees of neurological function deficits. At 12 hours after ischemia/reperfusion, there was a significant difference in neurological function deficit scores between the ischemia/reperfusion group and the electroacupuncture group (P < 0.05). In the following time periods, neurological function deficit scores in the electroacupuncture group were significantly reduced when compared with the ischemia/reperfusion group (P < 0.05; Table 1).

Electroacupuncture stimulation of the brachial plexus trunk on the healthy side increased brain derived neurotrophic factor mRNA expression in the ischemic cerebral cortex of MCAO rats

In situ hybridization showed that brain derived neurotrophic factor mRNA expression in the cerebral cortex was lower in the sham surgery group compared with the electroacupuncture and ischemia/reperfusion groups. The number of brain derived neurotrophic factor mRNA-positive cells in the ischemic rat cerebral cortex increased after ischemic injury for 1 hour and reperfusion for 2 hours, and peaked after reperfusion for 2 and 24 hours. Levels recovered to normal following reperfusion for 7 days (P < 0.05). After reperfusion for 6 hours, the number of brain derived neurotrophic factor mRNA-positive cells in the ischemic cerebral cortex in the electroacupuncture group were significantly greater than those in the ischemia/reperfusion group (P < 0.05), peaked after reperfusion for 24 hours, and decreased thereafter until reperfusion for 14 days (Table 2, Figure 1).

Table 1 Effects of electroacupuncture stimulation of the brachial plexus trunk on neurological function deficit scores in rats subjected to cerebral ischemia/reperfusion injury

Group	After reperfusion							
	2 hours	6 hours	12 hours	24 hours	2 days	3 days	7 days	14 days
Ischemia/reperfusion	2.96±0.98	2.76±0.56	2.80±0.48	2.75±0.42	2.62±0.56	2.40±0.46	2.05±0.41	1.47±0.38
Electroacupuncture	2.75±0.73	2.53±0.49	2.14±0.53[a]	2.18±0.38[a]	1.84±0.49[a]	1.58±0.34[a]	1.22±0.26[a]	1.02±0.21[a]

All data are expressed as mean ± SD of four rats from each group at each time point. Higher scores indicate more severe neurological function deficits. [a]P < 0.05, *vs*. ischemia/reperfusion group (two sample *t*-test).

Table 2 Effects of electroacupuncture stimulation of the brachial plexus trunk on brain-derived neurotrophic factor mRNA-positive cells (/400-fold visual field) in the ischemic rat cerebral cortex

Group	After reperfusion							
	2 hours	6 hours	12 hours	24 hours	2 days	3 days	7 days	14 days
Sham surgery	13.53±6.35							
Ischemia/reperfusion	64.50±5.45[a]	53.50±6.99[a]	54.13±3.94[a]	90.50±12.39[a]	72.00±5.17[a]	81.75±9.40[a]	17.00±4.29	10.75±3.19
Electroacupuncture	67.13±5.94[ab]	81.75±2.94[ab]	88.25±5.20[ab]	111.63±9.06[ac]	103.88±4.14[ab]	103.25±4.27[ab]	32.00±6.93[ab]	16.75±2.95[b]

All data are expressed as mean ± SD of four rats at each time point per group. [a]P < 0.01, *vs*. sham surgery group; [b]P < 0.05, *vs*. ischemia/reperfusion group (two sample *t*-test).

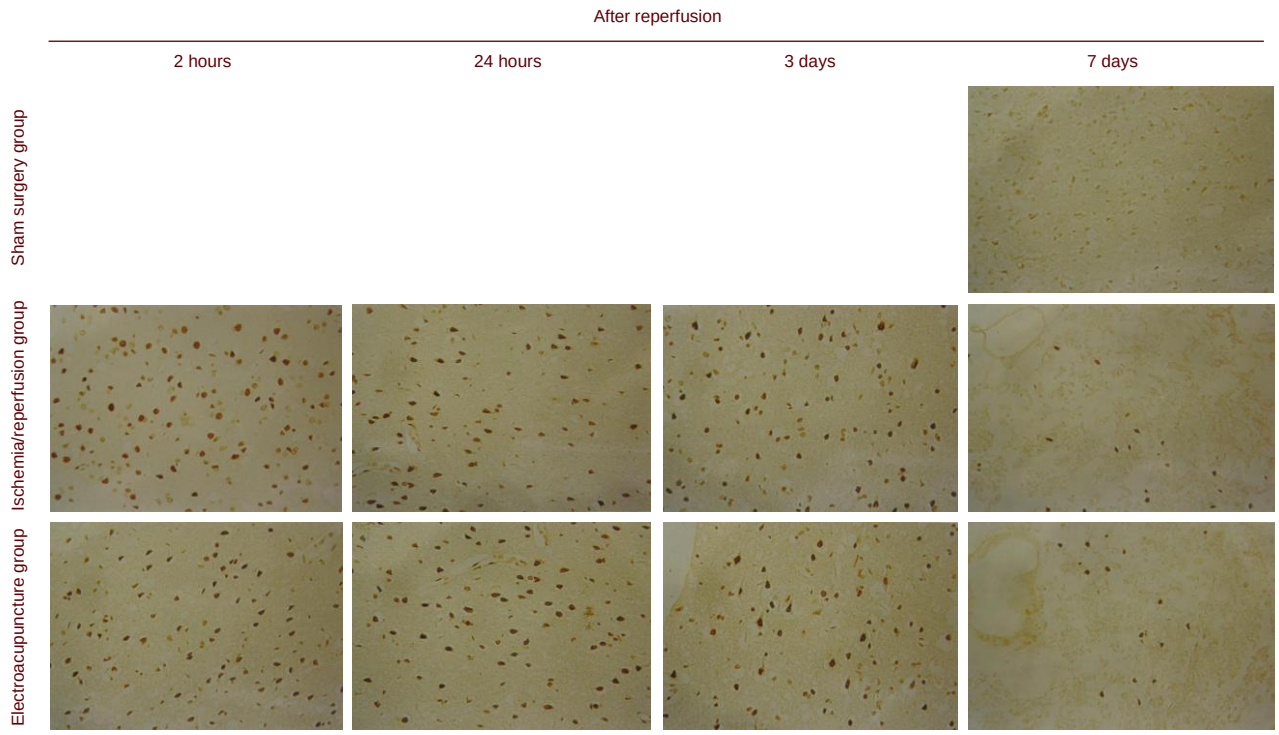

Figure 1 Brain-derived neurotrophic factor mRNA expression in the ischemic rat cerebral cortex (*in situ* hybridization, × 400).

Brain derived neurotrophic factor mRNA-positive expression appears brown/yellow. After electroacupuncture stimulation of the brachial plexus trunk, brain derived neurotrophic factor mRNA expression in the rat cerebral cortex increased.

DISCUSSION

Brain derived neurotrophic factor exhibits properties to enable the survival of multiple neurons, reduce the death of motor neurons, and directly promote axon growth, neuron repair and regeneration[14-16]. There is evidence that when cerebral ischemia/reperfusion injury occurs, the degree of injury of brain nerve cells is closely related to brain derived neurotrophic factor expression[1, 14, 17-18]. Results from this study showed that brain derived neurotrophic factor mRNA expression in the ischemic cerebral cortex was significantly increased at the early stage of ischemia/reperfusion, peaked after reperfusion for 24 hours, maintained high levels up to reperfusion for 3 days, and decreased to baseline levels after reperfusion for 7 days. Results also showed that brain derived neurotrophic factor mRNA expression in the cerebral cortex exhibited the first peak after reperfusion for 2 hours, and that the appearance of the first peak may be caused by the protective reaction of the cerebral cortex under the stressed state[15-16]; the second peak appears after reperfusion for 24 hours, and this peak is much higher and lasts for a longer period than the first peak, considering the self-repair process of neural cells is possibly related to the second peak[5, 15, 19].

Clinical evidence suggests that acupuncture exhibits better therapeutic effects in patients with acute cerebral ischemia[6], while electroacupuncture stimulation can interfere with the process of brain tissue injury after cerebral ischemia/reperfusion, alleviate cerebral edema after acute cerebral ischemia, and regulate intracellular calcium ion concentrations[20-21]. Electroacupuncture can increase the expression of nerve growth factor and brain derived neurotrophic factor in the peri-infarct cerebral cortex, exhibiting certain protective effects on cerebral ischemia[20-23].

In this study, we used electroacupuncture stimulation of the brachial plexus trunk. Results showed that after ischemia/reperfusion for 6 hours, electroacupuncture stimulation of the brachial plexus trunk can significantly increase brain derived neurotrophic factor mRNA expression until reperfusion for 14 days; at the same time, electroacupuncture stimulation of the brachial plexus trunk can significantly decrease the degree of neurological function deficits in rats with cerebral ischemia/reperfusion and thereby decrease neurological function scores. These findings suggest that electroacupuncture stimulation of the brachial plexus trunk exhibits similar therapeutic effects to acupoint electroacupuncture, and that direct stimulation of the brachial plexus trunk is easy to operate and be accepted by patients[7, 11, 13].

Increased brain derived neurotrophic factor levels *in vivo* exhibit brain protective effects by enhancing the activity

of the γ-aminobutyric acid system and counteracting the neurotoxicity of glutamate[24-25]. The phenomenon of propagation sensation along channels may be partly related to increased brain derived neurotrophic factor expression[26]. After repeated electroacupuncture of the forelimbs of animals, the topographical boundaries of the forelimb motor areas in the cerebral cortex were found to move towards whisker motor areas in the cerebral cortex, accompanied by alteration in movement threshold[27-28], suggesting that the properties of output motor cortex neurons changed rapidly with an alteration in afferent information. Based on this, electroacupuncture stimulation of the brachial plexus trunk can cause alteration of neuron output in the cerebral cortex, and thereby lead to alteration of brain derived neurotrophic factor mRNA expression. Nevertheless, the underlying mechanism requires further investigation.

Taken together, electroacupuncture stimulation of the brachial plexus trunk can greatly increase brain derived neurotrophic factor mRNA expression in the cerebral cortex in rats subjected to cerebral ischemia/reperfusion and promote the recovery of cerebral ischemia/reperfusion injury.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

This study was performed at the Central Laboratory, Affiliated Hospital of Qingdao University Medical College from March 2009 to December 2012.

Materials

Sixty-eight healthy female Wistar rats of clean grade, aged 5 months, weighing 253 ± 15 g, were provided by the Animal Cultivation Center, Qingdao Institute for Drug Control (license No. SCKK (Lu) 20100167). All experiments were performed at the Central Laboratory, Affiliated Hospital of Qingdao University Medical College, China. The environment temperature was kept at 22°C. Rats had free access to food (standard solid feedstuff produced by the Laboratory Animal Center, Qingdao University Medical College, China) and water. All experimental protocols were performed according to the *Guidance Suggestions for the Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of China[29].

Methods

Preparation of MCAO models

According to a modification of a previously described method[30-31], following anesthesia by 10% (v/v) chloral hydrate (300 mg/kg), a surgical nylon suture with a blunt end was inserted into the left external carotid artery, and entered into the inner segment of the internal carotid artery *via* the bifurcation of the common carotid artery and the outer segment of internal carotid artery, and advanced up to the bifurcation of the middle cerebral artery. After occlusion of the left middle cerebral artery for 60 minutes, the nylon suture was drawn out. In the sham surgery group, occlusion of the middle cerebral artery was omitted. After consciousness recovery for 30 minutes, neurological function deficits were scored according to a method described by Longa *et al* [30-31]. Rats with a score of 1–3 were considered to have undergone successful cerebral ischemia/reperfusion injury; otherwise, rats were refused for further experimentation. The lost rats were supplemented in time to ensure equal animal numbers in each group at each time point. Neurological function scoring was performed by an investigator who was blinded to group management.

Electroacupuncture stimulation of the brachial plexus trunk on the healthy side improved MCAO rat neurological function

After ischemia for 1 hour and reperfusion for 2, 6, 12, and 24 hours, and 2, 3, 7 and 14 days, rats from the electroacupuncture group were anesthetized and received electroacupuncture stimulation of the brachial plexus trunk. Precisely, a stainless steel acupuncture needle 0.4 mm in diameter, 25 mm in length (Suzhou Medical Supplies Factory Co., Ltd., Suzhou, Jiangsu Province, China) was punctured 6 mm towards the collarbone side from the right axillary fossa along the right midaxillary line until the rat brachial plexus trunk tissue was connected with a positive electrode. Another acupuncture needle was punctured 1 mm posterior to bregma and 1 mm left lateral to the midline[32], and was connected with a negative electrode. Each stimulation lasted for 30 minutes. Low-frequency continuous wave (frequency 2–6 Hz, current intensity 2 mA) electroacupuncture stimulation was performed using an electroacupuncture apparatus (G680522; Shanghai Tiancheng Science and Technology Co., Ltd, Shanghai, China).

Sample collection

Following anesthesia with 10% (v/v) chloral hydrate, a cannula was inserted from the left ventricle up to the ascending aorta for cardiac perfusion of approximately 300 mL of 0.9% (w/v) NaCl and approximately 400 mL 4% (w/v) paraformaldehyde (prepared with 0.01 M PBS). After perfusion, a craniotomy was performed for harvesting brain tissue. The brain tissue was resected in the region 1 mm anterior to bregma and 2 mm posterior to bregma to ensure collection of the infarcted cortex[32-33].

Thereafter, the brain tissue sample was fixed in pre-cooled 4 % (w/v) paraformaldehyde for 24 hours, routinely embedded in paraffin, and cut into 5 μm coronal sections.

Detection of brain derived neurotrophic factor mRNA expression in the rat cerebral cortex

After dehydration in a serious of gradient ethanol, paraffin sections were treated with 3% (v/v) H_2O_2 for 10 minutes, washed three times with distilled water, each for 2 minutes. The oligonucleotide probe (sequence 5'-GCA ACC AAA GTA TGA AAT AAC CAT AGT AAG-3') was provided by Boster Biotechnology, Wuhan, China. The sections were treated with diluted pepsin at 37°C and 20 minutes later, washed with 0.5 M PBS three times, each for 5 minutes. Subsequently, these sections were washed with double distilled water for 5 minutes, reacted with 20 μL prehybridization solution for 4 hours in the hybridization box, then with 20 μL hybridization solution for 10–12 hours at 40°C, and finally washed with 2 × sodium citrate for 10 minutes, 0.5 × sodium citrate for 15 minutes, and with 0.2 × sodium citrate for 15 minutes[34]. Diaminobenzidine coloration time was controlled through the use of an optical microscope (Shenzhen Winner Optical Co., Ltd., Shenzhen, Guangdong Province, China). PBS (0.01 M) rather than the oligonucleotide probe was used as a negative control. A high power lens (× 400) on the Olympus microscope camera (Wuxi Precision Machinery Co., Ltd., Wuxi, Jiangsu Province, China) was used for sample observation. Eight visual fields were randomly selected from the cerebral cortex on the ischemic side for counting brain derived neurotrophic factor mRNA-positive cells. The number of brain derived neurotrophic factor mRNA-positive cells per visual field was calculated[35-36].

Statistical analysis

All data were expressed as mean ± SD. The two sample *t*-test was performed using SPSS 10.0 software (SPSS, Chicago, IL, USA). A level of $P < 0.05$ was considered statistically significant.

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Conflicts of interest: None declared.

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