ACUPUNCTURE RESEARCH

Neuroprotective Effects of Electroacupuncture on Hypoxic-Ischemic Encephalopathy in Newborn Rats Are Associated with Increased Expression of GDNF-RET and Protein Kinase B*

XU Tao (徐 涛)[1], XU Neng-gui (许能贵)[2], YANG Zhong-hua (杨忠华)[2], WAN Yan-zhen (万言珍)[1], WU Qing-long (吴清龙)[2], and HUANG Kang-bai (黄康柏)[2]

ABSTRACT **Objective**: To explore the neuroprotective effects of electroacupuncture (EA) on hypoxic-ischemic encephalopathy (HIE) and to further investigate the role of glial cell line-derived neurotrophic factor (GDNF) family receptor member RET (rearranged during transfection) and its key downstream phosphatidylinositol 3 kinase (PI-3K)/protein kinase B (Akt) pathway in the process. **Methods**: A total of 220 seven-day-old SD rats (of either sex, from 22 broods) were randomly divided into two groups, one (30 rats) for sham-surgery group and the other (190 rats) for HIE model group. The HIE model was established using the left common carotid artery ligation method in combination with hypoxic treatment. The successfully established rats were randomly divided into five groups, including control model group, EA group, sham-EA group, antagonist group and antagonist plus electroacupuncture group, with 35 rats in each group. Baihui (GV 20), Dazhui (GV 14), Quchi (LI 11) and Yongquan (KI 1) acupoints were chosen for acupuncture. EA was performed at Baihui and Quchi for 10 min once a day for continuous 1, 3, 7 and 21 days, respectively. The rats were then killed after the operation and injured cerebral cortex was taken for the measurement of neurologic damage by hematoxylin-eosin (HE) staining and the degenerative changes of cortical ultrastructure by transmission electron microscopy. RET mRNA level and Akt protein level were detected by real-time reverse-transcription polymerase chain reaction (RT-PCR) and western blot analysis, respectively. **Results**: EA could ameliorate neurologic damage of the first somatic sensory area (S1Tr) and alleviate the degenerative changes of ultrastructure of cortical neurons in rats subjected to HIE. And the longer acupuncture treatment lasted, the better its therapeutic effect would be. This was accompanied by gradually increased expression of GDNF family receptor RET at the mRNA level and its downstream signaling Akt at the protein level in the ischemic cortex. **Conclusion**: EA has neuroprotective effects on HIE and could be a potential therapeutic strategy for HIE in the neonate. Activation of RET/Akt signaling pathway might be involved in this process.

KEYWORDS hypoxic-ischemic encephalopathy, electroacupuncture, glial cell line-derived neurotrophic factor, rearranged during transfection, protein kinase B

Hypoxic-ischemic encephalopathy (HIE) is one of the most important causes of brain injury in the neonate and can result in long-term devastating neurodevelopmental sequelae.[1,2] Despite recent advances in obstetric and neonatal care, HIE remains a major problem worldwide, associated with high mortality and morbidity.[3] There has been amounting research progress in HIE over the last two decades and many target molecules have been found. However, therapeutic interventions are still limited, and there are no specific treatments proven to decrease brain damage from HIE.

Acupuncture, as an alternative medicine methodology originating in ancient China, has been used for more than 1000 years as a treatment or as an adjuvant modality for patients with stroke. It has been frequently used in Asian countries and has become increasingly popular in the Western world. However, the precise mechanism of its neuroprotective effect remains poorly understood. The established research on the neuro-physiological correlates with acupuncture

*Supported by the 45th China Postdoctoral Science Foundation (No. 20090450856)
1. Traditional Chinese Medicine Department, Qingdao Women and Children Hospital, Qingdao, Shandong Province (266013), China; 2. College of Acupuncture and Moxibustion and Massage, Guangzhou University of Traditional Chinese Medicine, Guangzhou (510006), China
Correspondence to: Prof. XU Neng-gui, Tel: 86-20-39359833, E-mail: ngxu@tom.cn
DOI: 10.1007/s11655-015-1972-1

has pointed towards endogenous signaling molecules as the principal biological mediators of the therapeutic actions of this ancient technique. More recently, several classes of molecules, such as neurotransmitters, cytokines and growth factors, have also been identified as possible mediators for specific acupuncture effects.[4,5] It was shown that in cerebral ischemic injury, electroacupuncture (EA) played an important role in functional reorganization and cerebral compensation,[6,7] which might be in association with persistent increased expression of neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF).[8-10]

As a compensatory self-protecting response against ischemic injury, activated astrocytes synthesize and release many neurotrophic factors. Among them, well studied GDNF was crucial for the development and maintenance of distinct sets of central and peripheral neurons.[11,12] GDNF treatment has been shown to be effective not only in reducing brain damage but also in inhibiting learning and memory impairment, following hypoxic-ischemic insult in neonatal rats.[13,14] It has been established that GDNF exerts its neuroprotective effects through the classic growth factor signaling pathway,[15] mediated by the RET (rearranged during transfection) receptor tyrosine kinase, triggering activation of several intracellular signaling pathways, such as phosphatidylinositol 3 kinase (PI-3K)/protein kinase B pathway (Akt). Akt is the main target kinase of PI3-K, involved in many biological processes. Especially its activation was shown to inhibit cell apoptosis and promote cell survival.[16,17]

Recent research about therapeutic effect of acupuncture on cerebral ischemic injury has been focused on that acupuncture could promote proliferation of neural precursor cells and increase the expression of nerve growth factors. However, downstream effects, such as continuous influence of nerve growth factors and the intervening mechanisms involved, have been rarely reported. So in the present study, using a rat HIE model we evaluated *in vivo* therapeutic efficacy of EA. Four acupoints including Baihui (GV 20), Dazhui (GV14), Quchi (LI 11) and Yongquan (KI 1) were chosen, considering that the four acupoints are commonly used for clinical treatment and are easy to locate. And we further investigated the underlying molecular mechanisms, especially the role of GDNF family receptor member RET and its key downstream PI3-K/Akt pathway in the process.

METHODS

Animals

A total of 220 seven-day-old Sprague-Dawley rats of either sex from 22 broods (including mothers, 9 to 11 newborn rats per brood), at specific pathogen-free grade, weighing 11.0 to 15.8 g (average weight at 13.2 ± 1.8 g), were provided by the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine [No. SYXK (Yue) 2009-0001]. All experimental procedures were performed following the Guide for the Care and Use of Laboratory Animals, writen by the Ministry of Science and Technology of the People's Republic of China.

Establishment of HIE Animal Model

The HIE rat model was established using the left common carotid artery (LCCA) ligation method in combination with hypoxic treatment described by Rice, et al.[18] Briefly, animals were anesthetized by inhaling ether. In the supine position, a midline ventral incision was made to expose the LCCA, which was carefully separated from the vagus nerve. The separated LCCA was ligated using a 5/0 silk suture, and then the incision was sewed up. After a 2-h recovery, the rats were put in a sealed transparent vessel for a warm bathe at 37 ℃. Then the vessel was passed into gas with low content of oxygen (including 8% of oxygen and 92% of nitrogen) at the velocity of 1 L/min for 2.5 h. The survivors were kept warm for another 1 h and then received behavioral tests. For the sham-surgery group, after anesthesia, the LCCA was separated without ligation and the incision was sewed up without any hypoxic treatment. Behavioral tests were made 4 h later, and rats in this group showed no behavioral abnormality.

Animal Grouping

A total of 220 rats were maintained in a temperature-controlled (20 to 25 ℃) facility with a 12 h light/12 h dark cycle. Each brood was randomly divided into two groups, sham-surgery group and HIE model group. Totally there were 30 rats in the former group and 190 rats in the latter group. Among the 190 HIE rat models, 75 in the antagonist group were first given wortmannin (PI3K inhibitor in dimethyl sulfoxide, 20 μg/kg) treatment by lateral ventricle injection, and 0.5 h later received HIE modeling operation.

The other 115 rats received the modeling operation directly. After the surgery, 10 rats died and 5 rats were excluded as showing no obvious hemiplegia. Finally 175 rats (successful rate of 92.1%) were successfully established as the HIE models (70 in the antagonist group). Then the successfully established HIE model rats were divided into five groups, control model group, EA group, sham-EA group, antagonist group and antagonist plus EA group with 35 rats in each group. And each of the five groups was subdivided into four subgroups in terms of four time periods of 1, 3, 7 and 21 day for EA treatment post-surgery, with 9, 9, 9 and 8 rats in each subgroup.

EA Treatment

Four acupoints including Baihui, Dazhui, Quchi and Yongquan were chosen, positioned according to the ordinary acupoints for acupuncture in rats. Localization of the acupoints was based on the International Standard Scheme for Acupoint Names of Acupuncture drafted by the Experimental Acupuncture Branch of the China Acupuncture Academy.[19] Baihui acupoint is located in the center of the parietal bone. Dazhui is located between the seventh cervical vertebra and the first thoracic vertebra, just in the center of the back. Quchi is located in the midpoint of the line between the outer end of the elbow stripes and the epicondyle of the humerus, and Yongquan is located in the plantar anterior third (toes excluded). A needle of 0.5 inches in length was inserted horizontally backwards into Baihui at a depth of 5 mm, perpendicularly inserted into Dazhui for 5 mm in depth, perpendicularly inserted into Quchi for 10 mm in depth and rapidly inserted into Yongquan without leaving the needle in for three to four times till minor bleeding, respectively. Both acupoints Baihui and Quchi were connected with G-6805 electric acupuncture apparatus (Shanghai Huayi Medical Instrument Factory, China), and received EA for 10 min with local tissue shivering slightly (asymmetric bidirectional continuous pulse waves, frequency 5–10 Hz, voltage 3–5 V). The acupuncture therapy was given once a day for continuous 1, 3, 7 and 21 days respectively. The antagonist plus EA group received acupuncture treatment just as the EA group. And for the sham-EA group, needles were inserted into the point about 1 cm away from the corresponding acupoints and the other treatment was just the same as the EA group. The other two groups (control model group and antagonist group) and the sham-surgery group, were raised under the same condition, but didn't receive any treatment.

Hematoxylin-Eosin Staining for Determination of Neurologic Damage

Rats were anesthetized by inhaling ether at the indicated time. The left ventricle was cannulated and perfused with phosphate buffered saline (PBS) (preheated at 37 ℃), and then perfused and fixed with 4% (w/v) paraformaldehyde (in 0.1 mol/L PBS, pH 7.4, precooled at 4 ℃) firstly at full speed till convulsion of the limbs ceased, then perfusion was kept at the velocity of 1 mL/min. Then, the brain was quickly separated with cerebellum and brainstem removed and placed in 4% paraformaldehyde for 24 h at 4 ℃. The specimens were dehydrated with 20% sucrose and frozen. The frozen sections were then serially cut into 20 μm thick coronal slices. hamatoxylin-eosin (HE) staining was performed according to the standard protocol.[20] The sections were observed under a light microscope (Nikon, Tokyo, Japan) using a magnification of ×400, and photographed.

Electron Microscopy for Cortical Ultrastructures

Rats anesthetized with ether were transcardially perfused with 4% (w/v) paraformaldehyde in 0.1 mol/L PBS (pH 7.4). A small block of the cortical area was dissected and fixed in 2.5% (v/v) buffered glutaraldehyde overnight at 4 ℃. Then, specimens were post-fixed in 1% (w/v) OsO_4 for 1 h. After dehydration in acetone, specimens were embedded in epoxide resin and 60 nm thick sections were cut and stained with uranyl acetate (K&K laboratories, Inc., Jamaica, USA) and lead citrate. The sections were examined under a JEM-1200EX transmission electron microscope (JEOL, Ltd., Japan).

Detection of RET mRNA Expression in the Injured Cortex by Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from the injured cortex (about 1 mm × 1 mm × 1 mm) of rats using Trizol (Invitrogen, USA) according to the manufacturer's instructions. Two micrograms of total RNA were used to synthesize first-strand cDNA with M-MuLV reverse transcriptase (Fermentas, USA) using random primers. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed using the ABI 7500 real-time RT-PCR detection system (ABI, USA) with SYBR Green (Fermentas, USA). Primer sequences for specific genes are presented as follows. Forward primer for RET: 5'-GAAAACGCCTCCCAGAGTGA-3'; reverse primer for RET: 5'-CTGCAAGCCCCGTACAACTT-3';

forward primer for glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH): 5'-TGGTCTACATGTTCCAGTATGACT-3'; reverse primer for GAPDH: 5'-CCATTTGATGTTAGCGGGATCTC-3'. GAPDH was used as an internal control.

Detection of Akt Protein Expression in the Injured Cortex by Western Blot Analysis

Total protein extracts were prepared from rat brains as previously described. In brief, rats were anesthetized with ether and rapidly decapitated. Brain was quickly separated with cerebellum and brainstem removed and placed on ice in 10 volumes of cold homogenization buffer (50 mmol/L Tris, 120 mmol/L NaCl, pH 7.4) with protease inhibitors (Sigma, USA). The tissue was then homogenized and stored at –80 ℃. Protein concentrations were determined using the Bradford method (Bio-Rad, USA). Equal amounts of protein (50 μg/lane) were separated by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, USA). After being blocked, the filters were incubated with the following primary antibodies: anti-Akt rabbit polyclonal antibody (1:1000, Cell Signaling, USA). Anti-GAPDH antibody (1:3000, Sigma, USA). GAPDH was used as an internal loading control. After being washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000, Santa Cruz Biotechnology, USA) for 2 h at room temperature, the immune complexes were visualized with a chemiluminescence reagent. Western blots were quantified densitometrically with Quantity One software (Bio-Rad, USA), and the intensity values were normalized to GAPDH.

Statistical Analysis

All values were expressed as mean ± standard deviation. All data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Statistical analyses were performed by two-tailed unpaired Student *t*-test or by one-way analysis of variance as appropriate to determine statistical significance between the groups. $P<0.05$ was considered statistically significant.

RESULTS

EA Treatment Ameliorates Neurologic Damage Caused by Hypoxic-ischemic Injury

In the sham-surgery team, HE staining showed normal structures of cortex with clear organizational structure, normal cell outline, clear nucleolus and cell nucleus located in the center. In the control model group and the sham-EA group, hypoxic-ischemic injury induced neurologic damage with obvious necrosis and degeneration of neurons after 1–3 days, such as mesh-like structure, irregular arrangements of neurons, concentrated cytoplasm, vacuolation of cytoplasm, karyopyknosis, and nucleolus loss 7–21 days post-surgery, severe neuron loss along with proliferation of surrounding astrocytes was observed. After EA for 3 days, HE staining of the EA group showed swelling and degeneration of neurons but less severe than the control model group or the sham-EA group. And after EA treatment for 7–21 days, relatively clear organizational structure, regular arrangements of neurons, normal cell outline and relatively clear nucleolus were observed. In addition, in the antagonist plus EA group, signs of necrosis and degeneration of neurons were more severe than that of the EA group (Figure 1).

EA Treatment Protects Ultrastructure of Cortical Neurons against the Degenerative Alterations

In the sham-surgery group, clear nuclear membrane, big and round nucleolus, evenly distributed cytoplasm, normal mitochondria and rough endoplasmic reticulum were observed. In the control model group and the sham-EA group, cortical neurons went through gradual changes from degenerative alterations to self-repair. After 1–3 days hypoxic-ischemic injury, degenerative alterations, such as nuclear membrane disruption, uneven distribution of nucleoplasm, indistinct nucleolus, formation of microbubbles surrounding the nucleus, vacuolation of cytoplasm, mitochondria swelling and rough endoplasmic reticulum dilating, was induced. Seven to 21 days post-surgery, cortical neurons showed signs of self-repair with the above changes decreased in severity. EA treatment promoted self-repair of cortical neurons. Three days of treatment dramatically decreased the severity of degenerative alterations compared with the control model group or the sham-EA group. Moreover, EA treatment for longer periods improved the ultrastructure of cortical neurons for better. However, in the antagonist plus EA group, the protective effect of EA was impaired by wortmannin pretreatment, compared with the EA group (Figure 2).

Expression Changes of RET mRNA in the Cerebral Cortex of Rats from Different Groups

Compared with that of the sham-surgery group,

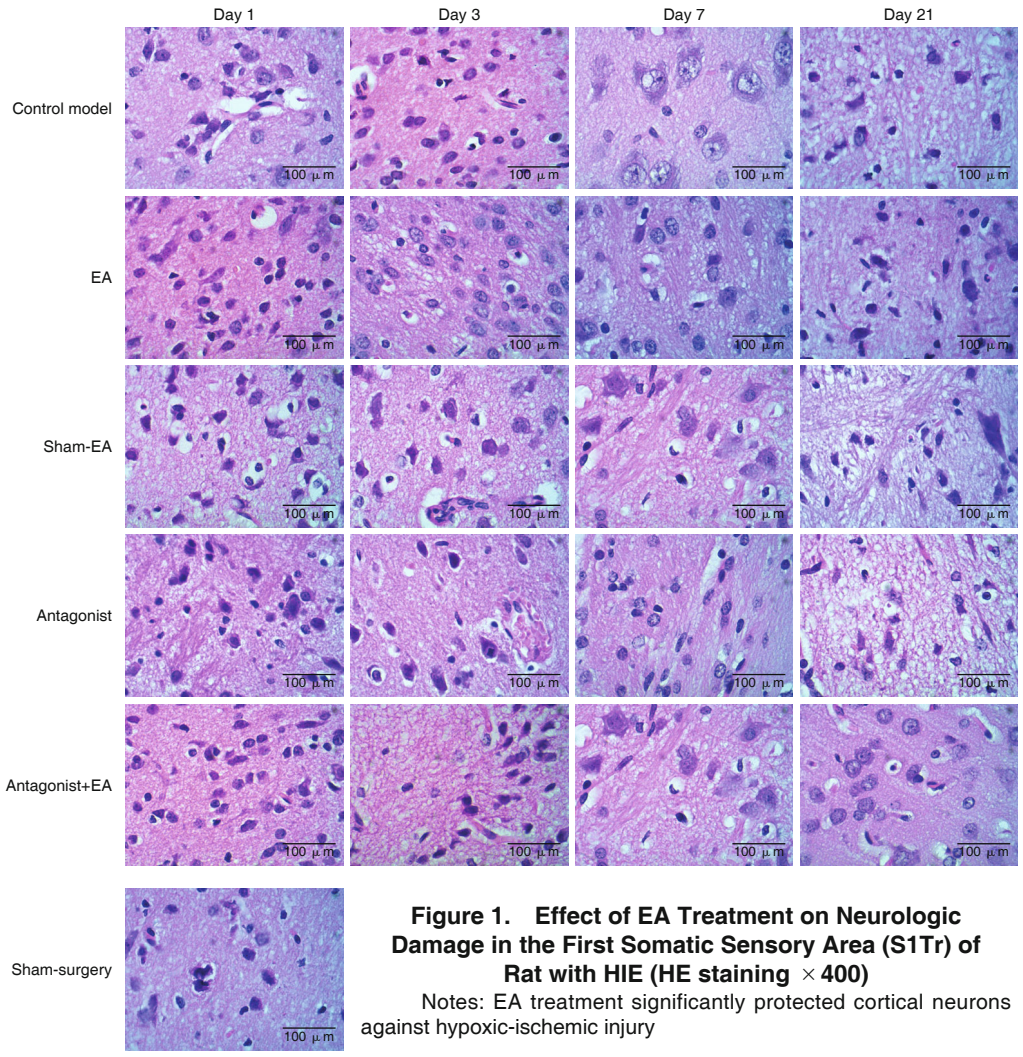

Figure 1. Effect of EA Treatment on Neurologic Damage in the First Somatic Sensory Area (S1Tr) of Rat with HIE (HE staining × 400)

Notes: EA treatment significantly protected cortical neurons against hypoxic-ischemic injury

expression levels of RET in the cerebral cortex with hypoxic-ischemic injury from the control model group and the sham-EA group increased on the 1 day post-surgery (P<0.05), and reached the peak at the 7 day (P<0.05). By contrast, its expression decreased on the 21st day, without significant difference (P>0.05). In the EA group and the antagonist plus EA group, RET expression also increased since the first day, and continued to rise till the 21st day (P<0.05 *vs*. the sham-surgery group). Furthermore, compared with that of the control model or the sham-EA group, EA group increased RET mRNA dramatically at the 3, 7 and 21 days (P<0.05). For the antagonist group, RET expression exhibited a similar but in a weaker degree of increase to that of the control model group (Figure 3).

Expression Changes of Akt Protein in the Cerebral Cortex of Rats from Different Groups

In comparison with the sham-surgery group, Akt expression at the protein level was significantly increased in all the other groups (P<0.05) on the 1st day post-surgery. But there was no statistical significance between the control model group and the EA group, between the EA group and the sham-EA group, and between the antagonist group and the antagonist plus EA group (P> 0.05, Figure 4A).

At the 3rd day post-surgery, Akt expression was significantly increased in all the other groups (P<0.05), compared with that of the sham-surgery group. Furthermore, Akt expression after three days of EA treatment increased more dramatically than that of either the control model group or the sham-EA group (P<0.05, Figure 4B).

Just like the above result at the 3rd day, Akt expression at the 7th day post-surgery was significantly increased in all the other groups (P<0.05), compared with that of the sham-surgery group. Furthermore, EA

Chin J Integr Med 2016 Jun;22(6):457-466

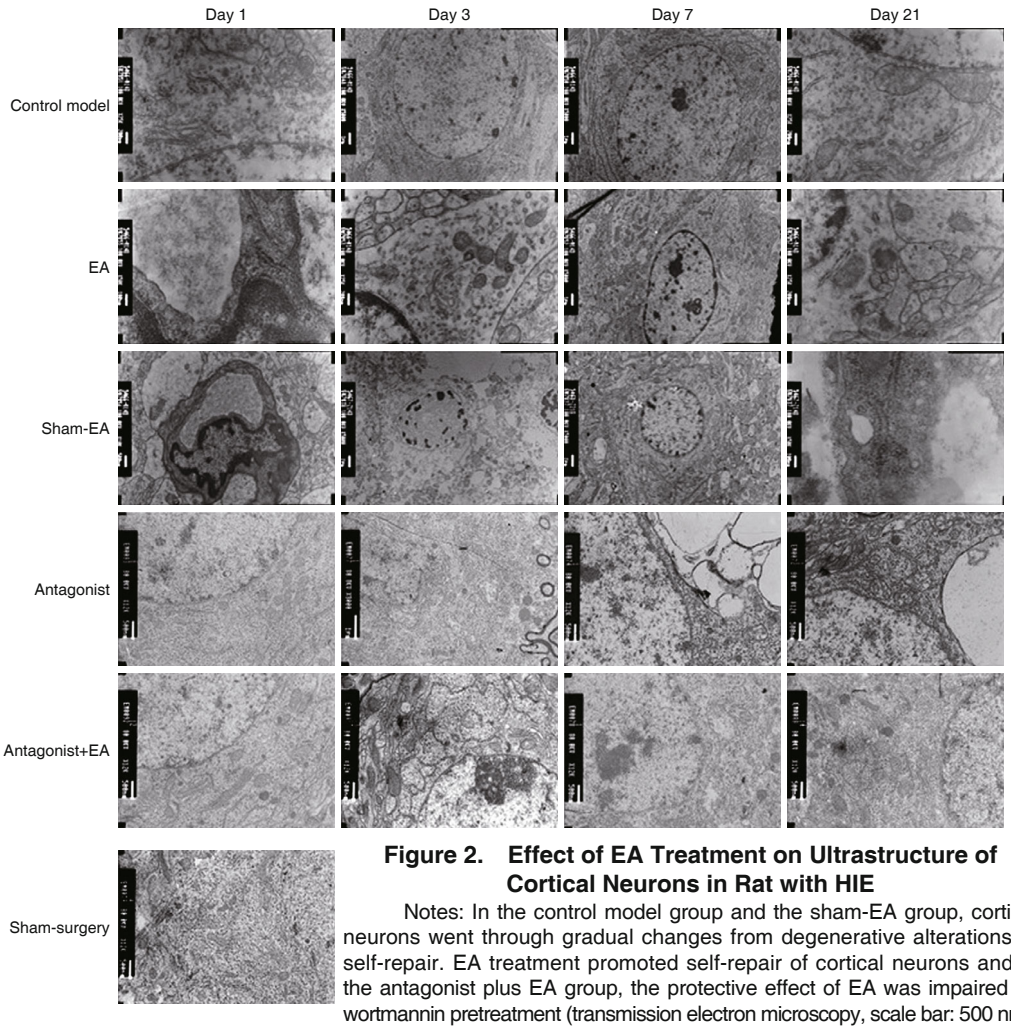

Figure 2. Effect of EA Treatment on Ultrastructure of Cortical Neurons in Rat with HIE

Notes: In the control model group and the sham-EA group, cortical neurons went through gradual changes from degenerative alterations to self-repair. EA treatment promoted self-repair of cortical neurons and in the antagonist plus EA group, the protective effect of EA was impaired by wortmannin pretreatment (transmission electron microscopy, scale bar: 500 nm).

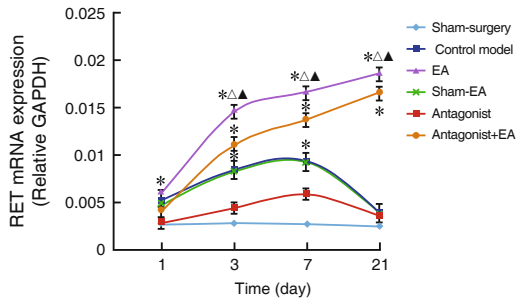

Figure 3. Effect of EA Treatment on RET mRNA Expression in the Injured Cortex of Rat with HIE by Realtime RT-PCR (n=6, $\bar{x} \pm s$)

Notes: $^{*}P$<0.05, compared with the sham-surgery group; $^{\triangle}P$<0.05, compared with the control model group; $^{\blacktriangle}P$<0.05, compared with the sham-EA group

treatment for seven days increased Akt expression more dramatically than in the control model group or in the sham-EA group (P<0.05, Figure 4C).

At the 21st day post-surgery, no increase of Akt expression was detected in the control model group or in the sham-EA group, compared with that of the sham-surgery group (P>0.05). But in the EA group, Akt expression was significantly increased compared with that of either the control model group or the sham-EA group (P<0.05). And acupuncture treatment in the antagonist plus EA group also increased Akt expression dramatically than in the antagonist group (P<0.05, Figure 4D).

DISCUSSION

HIE is reported to account for 25% of all neonatal deaths and contribute to significant financial and social burden. Effective treatment options for HIE are very limited and therefore alternative therapies such as acupuncture receives more and more attention. In this study, we established an HIE animal model to evaluate the *in vivo* therapeutic efficacy of EA and to further investigate the role of RET and its key downstream PI3-K/Akt pathway in the process. Our results showed that EA at Baihui, Dazhui, Quchi and

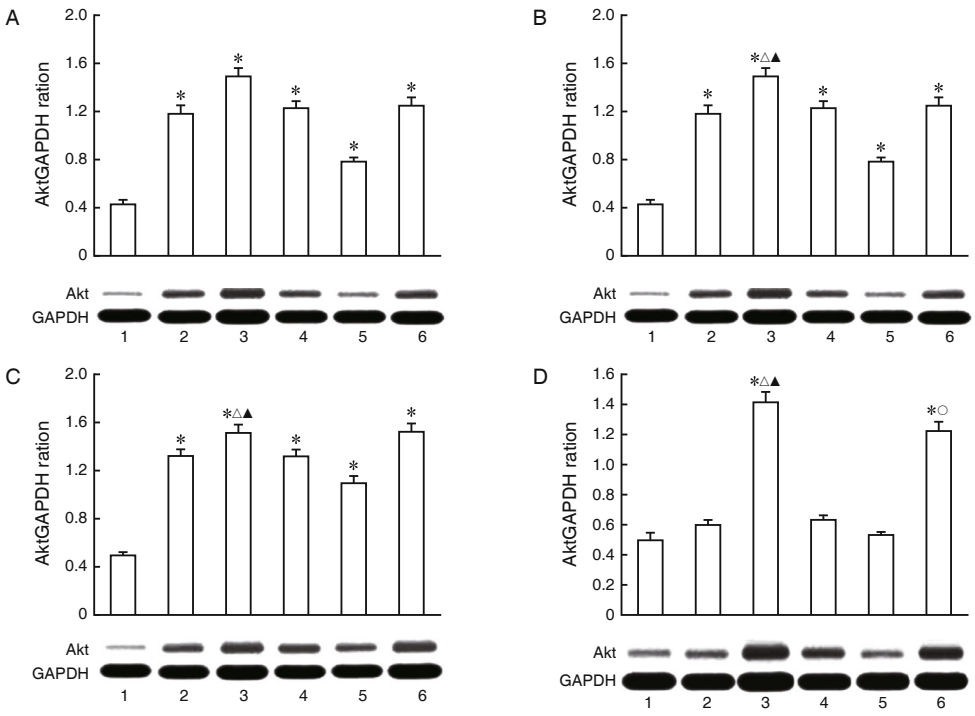

Figure 4. Effect of EA Treatment on Akt Protein Expression in the Injured Cortex of Rat with HIE by Western Blot Analysis ($\bar{x} \pm s$)

Notes: (A)-(D) Akt protein expression at the 1st, 3rd , 7th and 21st day post-surgery respectively. Lane 1: sham-surgery group; Lane 2: control model group; Lane 3: EA group; Lane 4: sham-EA group; Lane 5: antagonist group; Lane 6: antagonist plus EA group. Bar graphs show densitometric analysis of western blot of Akt protein. The densitometric quantification was normalized to the internal control GAPDH. The immunoblots are representative of three independent experiments. $^{*}P<0.05$, compared with the sham-surgery group; $^{\triangle}P<0.05$, compared with the control model group; $^{\blacktriangle}P<0.05$, compared with the sham-EA group; $^{\circ}P<0.05$, compared with the antagonist group

Yongquan in HIE rat models dramatically ameliorated neurologic damage of the first somatic sensory area (S1Tr) and alleviated the degenerative changes of ultrastructure of cortical neurons, which is partly attributed to increased expression of RET and Akt.

HIE model was constructed using 7-day-old rat, since cerebral development of 7-day-old rat is similar to that of the neonate. Internal carotid artery and vertebral artery of rats form Willis ring at the undersurface of cerebrum. Therefore ligation of one side of the common carotid arteries cannot induce ideal ischemic injury. In 1981, Rice firstly established a rat model of HIE by ligating the common carotid artery in combination with hypoxic treatment.[18] Since then, the model has been widely used for its simple operation, high successful rate, low mortality, high reliability and good reproducibility.

Acupuncture is a traditional therapy that has been widely applied for treatment of ischemic stroke. Acupoint combination is a complicated issue. Among different acupoints, Baihui and Dazhui are the main points of the Du Channel, which exhibits a combined supervising effect on the entire meridian system. Acupuncture at Baihui in combination with Dazhui promoted flow of qi and blood, resuscitated consciousness, recuperated depleted yang, and balanced yin and yang.[21] Amounting evidence of animal experiments have shown that acupuncture at the points located in the head and the neck could improve the blood circulation system of brain, dilate brain blood vessel, improve the microcirculation, ameliorate cerebral edema, and activate the repair function of cerebral neurons to promote functional recovery.[22-24] Quchi is the He-Sea point of the Large Intestine Channel of Hand-Yangming. EA at Quchi and Zusanli (ST 36) acupoints was reported to improve the ischemia-associated scores of neurological deficits, reduce cerebral infarction, alleviate inflammatory responses, promote neovascularization, inhibit neural cell apoptosis and promote neurological functional recovery in a focal cerebral ischemia-reperfusion injured rat model.[25,26] Yongquan is the significant Jing-Well point of the Kidney Channel of Foot-Shaoyin. Using in combination with Baihui, it was shown to improve energy and intelligence, clear the brain collaterals and coordinate the channels and

collaterals.[27,28] Acupuncture at Quchi and Yongquan was reported to promote functional recovery of the extremities after neuron injury.[29,30] Considering that Baihui, Dazhui, Quchi and Yongquan are commonly used in clinics for patient treatment and are easy to locate, we chose the combination of the above four acupoints in our experiments.

There are accumulating evidence indicating neuroprotective effects of acupuncture on hypoxic-ischemic injury.[31-33] In our research, HE staining and electron microscopy results showed that EA treatment significantly ameliorated neurologic damage of the first somatic sensory area (S1Tr) caused by hypoxic-ischemic injury and protected ultrastructure of cortical neurons against the degenerative alterations, suggesting that EA at Baihui, Dazhui, Quchi and Yongquan exerts neuroprotective function in HIE. Furthermore, PI3K inhibitor (wortmannin) pretreatment impaired the protective effect of EA, suggesting that PI3K-Akt pathway might contribute to the therapeutic effect of acupuncture.

GDNF, belonging to distant members of the transforming growth factor-β (TGFβ) superfamily, is one of the most potent neurotrophic factor. It is specifically trophic to DA neurons by promoting survival, preventing apoptosis, promoting morphological differentiation, and increasing the ingestion of DA.[34] It is also the most potent trophic factor for motor neurons. There is a substantial loss of spinal and cranial motor neurons, and a corresponding increase in dying cells, in GDNF- and GERα1-deficient mouse embryos compared with wild-type controls.[35] Conversely, motor neuron survival is promoted by muscle-specific overexpression of GDNF or by GDNF treatment in utero, indicating that GDNF is indeed a physiological survival factor for a subpopulation of motor neurons.[36] GDNF expression was induced in ischemic brain injury, which was a self-protecting response involved in self-repair of neurons. Our previous research also found that GDNF expression was induced by hypoxic-ischemic injury at the 1st, 3rd and 7th day, while decreased to the original level at the 21st day post-surgery, and EA treatment further promoted GDNF expression even at the 21st day post-surgery (data not shown), indicating that GDNF was involved in the neuroprotective effect of EA on HIE.

GDNF exerts its neuroprotective effects through the RET receptor tyrosine kinase, which was first discovered as a proto-oncogene. RET is a single-pass transmembrane protein that contains four cadherin-like repeats in the extracellular domain and a typical intracellular tyrosine kinase domain. PI3-K/Akt, as one of the most important downstream pathway of RET, is well known for its numerous and diverse physiological functions, involved in the regulation of cell metabolism, cell survival and proliferation, cell apoptosis and cell-cycle progression, etc.[16,17] As for the anti-apoptosis and pro-proliferation effects, activated Akt is reported to be one of the key signaling mediators. A brief period of ischemia induces ischemic tolerance reducing the cerebral infarction volume caused by subsequent lethal ischemia. Nakajima, et al[37] found that Akt was activated in both non-preconditioned and preconditioned groups after ischemia for 1 h, but the activation was long-lasting in the preconditioned rats, suggesting that the preconditioning-induced persistent activation of Akt in the penumbra region plays an important role in ischemic tolerance of the brain. And it was reported that transgenic mouse overexpressing the active Akt reduced the volume of infarct area by 35% after middle cerebral artery occlusion compared to the wild-type littermate.[38]

Chen, et al[26] reported that using a focal cerebral ischemia/reperfusion injured rat model, EA at Quchi and Zusanli acupoints profoundly activated PI3K/Akt signaling in ischemic cerebral tissues and increased the serum secretion levels of the PI3K activators BDNF and GDNF, suggesting that EA at Quchi and Zusanli acupoints exerts neuroprotective function in ischemic stroke via activation of the PI3K/Akt pathway. In this study, we investigated the expression changes of RET and Akt in the hypoxic-ischemic injured cerebral cortex at the 1st, 3rd, 7th and 21st day post-surgery and explored the intervening effect of EA. Results showed that EA treatment dramatically promoted persistent expression of RET at the mRNA level and expression of downstream Akt at the protein level in the cortex of rats subjected to HIE, which is in consistent with previous findings.[26] The expression changes of RET was in accordance with that of Akt. With the time extension of acupuncture treatment, expression levels of RET and Akt were kept in a continuous increase state, suggesting that the longer acupuncture treatment lasted, the better its therapeutic effect would be. Thus, these data suggest that EA exerts its neuroprotective function at least partially through

activation of RET-Akt pathway. Nevertheless, we cannot rule out the possibility that other mechanisms are involved in the process.

In conclusion, our data provide evidence that EA treatment protected cortical neurons against HIE-induced neurologic damage and degenerative changes in rats, which is in association with increased expression of RET and Akt. Therefore, EA may become a potential therapeutic strategy for HIE in the neonate.

REFERENCES

1. Shankaran S. Hypoxic-ischemic encephalopathy and novel strategies for neuroprotection. Clin Perinatol 2012;39:919-929.
2. Parker L, Kenner C. Neuroprotective strategies for hypoxic ischemic encephalopathy. Newborn Infant Nurs Rev 2012;12:8-11.
3. Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev 2013;1:CD003311.
4. Manni L, Albanesi M, Guaragna M, Barbaro Paparo S, Aloe L. Neurotrophins and acupuncture. Auton Neurosci 2010;157:9-17.
5. Joh TH, Park HJ, Kim SN, Lee H. Recent development of acupuncture on Parkinson's disease. Neurol Res 2010;32 Suppl 1:5-9.
6. Li M, Peng J, Song Y, Liang H, Mei Y, Fang Y. Electro-acupuncture combined with transcranial magnetic stimulation improves learning and memory function of rats with cerebral infarction by inhibiting neuron cell apoptosis. J Huazhong Univ Sci Technolog Med Sci (Chin) 2012;32:746-749.
7. Gao J, Wang S, Wang X, Zhu C. Electroacupuncture enhances cell proliferation and neuronal differentiation in young rat brains. Neurol Sci 2011;32:369-374.
8. Wang TT, Yuan Y, Kang Y, Yuan WL, Zhang HT, Wu LY, et al. Effects of acupuncture on the expression of glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (FGF-2/bFGF) in the left sixth lumbar dorsal root ganglion following removal of adjacent dorsal root ganglia. Neurosci Lett 2005;382:236-241.
9. Liang XB, Luo Y, Liu XY, Lu J, Li FQ, Wang Q, et al. Electroacupuncture improves behavior and upregulates GDNF mRNA in MFB transected rats. Neuroreport 2003;14:1177-1181.
10. Hoke A, Cheng C, Zochodne DW. Expression of glial cell line-derived neurotrophic factor family of growth factors in peripheral nerve injury in rats. Neuroreport 2000;11:1651-1654.
11. Ikeda T, Koo H, Xia YX, Ikenoue T, Choi BH. Bimodal upregulation of glial cell line-derived neurotrophic factor (GDNF) in the neonatal rat brain following ischemic/hypoxic injury. Int J Dev Neurosci 2002;20:555-562.
12. Ikeda T, Xia XY, Xia YX, Ikenoue T, Han B, Choi BH. Glial cell line-derived neurotrophic factor protects against ischemia/hypoxia-induced brain injury in neonatal rat. Acta Neuropathol 2000;100:161-167.
13. Katsuragi S, Ikeda T, Ikenoue T. A strategy to treat neonatal hypoxic encephalopathy using glial cell line-derived neurotrophic factor. No To Hattatsu 2011;43:265-272.
14. Wang X, Guo S, Lu S, Zhou J, Li J, Xia S. Ultrasound-induced release of GDNF from lipid coated microbubbles injected into striatum reduces hypoxic-ischemic injury in neonatal rats. Brain Res Bull 2012;88:495-500.
15. Baloh RH, Enomoto H, Johnson EM, Jr., Milbrandt J. The GDNF family ligands and receptors-implications for neural development. Curr Opin Neurobiol 2000;10:103-110.
16. Wang HQ, Xu YX, Zhu CQ. Upregulation of heme oxygenase-1 by acteoside through ERK and PI3 K/Akt pathway confer neuroprotection against beta-amyloid-induced neurotoxicity. Neurotox Res 2012;21:368-378.
17. Wang HJ, Cao JP, Yu JK, Gao DS. Role of PI3-K/Akt pathway and its effect on glial cell line-derived neurotrophic factor in midbrain dopamine cells. Acta Pharmacol Sin 2007;28:166-172.
18. Rice JE, 3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 1981;9:131-141.
19. Zheng GQ. Methodological standards for experimental research on stroke using scalp acupuncture. Acupunct Electrother Res 2009;34:1-13.
20. Longo UG, Franceschi F, Ruzzini L, Rabitti C, Morini S, Maffulli N, et al. Characteristics at haematoxylin and eosin staining of ruptures of the long head of the biceps tendon. Br J Sports Med 2009;43:603-607.
21. Fang Z, Ning J, Xiong C, Shulin Y. Effects of Electroacupuncture at head points on the function of cerebral motor areas in stroke patients: a PET study. Evid Based Complement Alternat Med 2012;2012:902413.
22. Kim M, Chung Y, Jung H, Park M, Han Y. Scalp electroacupuncture at the Baihui acupoint (DU 20) improves functional recovery in rats with cerebral ischemia. Neural Regen Res 2011;6:2822-2828.
23. Chuang C, Hsieh C, Li T, Lin J. Acupuncture stimulation at Baihui acupoint reduced cerebral infarct and increased dopamine levels in chronic cerebral hypoperfusion and ischemia-reperfusion injured Sprague-Dawley rats. Am J Chin Med 2007;35:779-791.
24. Liu H, Shen X, Tang H, Li J, Xiang T, Yu W. Using microPET imaging in quantitative verification of the

acupuncture effect in ischemia stroke treatment. Sci Rep 2013;3:1070.
25. Hong J, Wu G, Zou Y, Tao J, Chen L. Electroacupuncture promotes neurological functional recovery via the retinoic acid signaling pathway in rats following cerebral ischemia-reperfusion injury. Int J Mol Med 2013;31:225-231.
26. Chen A, Lin Z, Lan L, Xie G, Huang J, Lin J, et al. Electroacupuncture at the Quchi and Zusanli acupoints exerts neuroprotective role in cerebral ischemia-reperfusion injured rats via activation of the PI3K/Akt pathway. Int J Mol Med 2012;30:791-796.
27. Xue WG, Ge GL, Zhang Z, Xu H, Bai LM. Effect of electroacupuncture on the behavior and hippocampal ultrastructure in APP 695 V 717 I transgenic mice. Acupunct Res (Chin) 2009;34:309-314.
28. Xue WG, Zhang Z, Xu H, Wu HX, Bai LM. Effect of electroacupuncture on learning-memory ability, and Abeta and LRP1 immunoactivity in hippocampal sulcus microvessels in APP transgenic mice. Acupunct Res (Chin) 2011;36:95-100.
29. Ji XQ, Zhang ZL. Observation on therapeutic effect of nuchal acupuncture and abdominal acupuncture for treatment of stroke patients with spastic hemiplegia. Acupunct Res (Chin) 2009;29:961-965.
30. Zhou W, Liu H, Wang LP, Xu ZG, Feng YW, Lu H, et al. Effect of alternative administration of scalp-acupuncture, body-acupuncture and abdominal-acupuncture on the upper-limb dyscinesia in patients with stroke. Acupunct Res (Chin) 2009;34:128-131.
31. Wong V, Cheuk D, Chu V. Acupuncture for hypoxic ischemic encephalopathy in neonates. Cochrane Database Syst Rev 2013;1:CD007968.
32. Liu Y, Zou LP, Du JB, Wong V. Electroacupuncture protects against hypoxic-ischemic brain-damaged immature rat via hydrogen sulfide as a possible mediator. Neurosci Letters 2010;485:74-78.
33. Ding CH, Liu HR, Zhang SD, Ling YL. Treatment of hypoxic ischemic encephalopathy of newly born rats by acupuncture. Chin J Applied Physiol (Chin) 2005;21:200-201.
34. Aoi M, Date I, Tomita S, Ohmoto T. Single administration of GDNF into the striatum induced protection and repair of the nigrostriatal dopaminergic system in the intrastriatal 6-hydroxydopamine injection model of hemiparkinsonism. Restor Neurol Neurosci 2001;17:31-38.
35. Garces A, Haase G, Airaksinen MS, Livet J, Filippi P, deLapeyriere O. GFR alpha 1 is required for development of distinct subpopulations of motoneuron. J Neurosci 2000;20:4992-5000.
36. Oppenheim RW, Houenou LJ, Parsadanian AS, Prevette D, Snider WD, Shen L. Glial cell line-derived neurotrophic factor and developing mammalian motoneurons: regulation of programmed cell death among motoneuron subtypes. J Neurosci 2000;20:5001-5011.
37. Nakajima T, Iwabuchi S, Miyazaki H, Okuma Y, Kuwabara M, Nomura Y, et al. Preconditioning prevents ischemia-induced neuronal death through persistent Akt activation in the penumbra region of the rat brain. J Vet Med Sci 2004;66:521-527.
38. Ohba N, Kiryu-Seo S, Maeda M, Muraoka M, Ishii M, Kiyama H. Transgenic mouse overexpressing the Akt reduced the volume of infarct area after middle cerebral artery occlusion. Neurosci Lett 2004;359:159-162.

(Received December 12, 2013)
Edited by ZHANG Wen