Neuroprotective effects of electro acupuncture on hypoxic-ischemic encephalopathy in newborn rats Ass

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Abstract: Hypoxic-ischemic encephalopathy (HIE) is a common and potentially devastating condition in the neonate, associated with high mortality and morbidity. Effective treatment options are limited and therefore alternative therapies such as acupuncture are increasingly used. Previous studies have shown that electro acupuncture promoted proliferation of neural progenitor cell and increased expression of neurotrophic factor in HIE. However, effects of electro acupuncture on downstream signaling pathways have been rarely researched. So, in the present study, we aimed to evaluate the neuroprotective effects of electro acupuncture on HIE and to further investigate the role of GDNF family receptor member RET and its key downstream PI3-K/Akt pathway in the process. A rat HIE model was constructed by the left common carotid artery (LCCA) ligation method in combination with hypoxic treatment. Considering that Baihui (GV20), Dazhui (GV14), Quchi (LI11) and Yongquan (KI1) are commonly used in clinics for stroke treatment and are easy to locate, we chose the above four acupoints as the combination for electro acupuncture treatment which was performed once a day for different time periods. Hematoxylin-eosin (HE) staining and transmission electron microscopy results showed that electro acupuncture could ameliorate neurologic damage and alleviate the degenerative changes of ultra structure 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. These findings suggest that electro acupuncture shows neuroprotective effects in HIE, which at least in part is attributed to activation of PI3-K/Akt signaling pathway.

Keywords: hypoxic-ischemic encephalopathy; electro acupuncture; neurotrophic factor; acupoint; neurologic damage; degenerative changes; GDNF; RET; Akt; neuroprotective effect.

INTRODUCTION

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 (Shanka 2012; Parker and Kenner 2012). Despite recent advances in obstetric and neonatal care, HIE remains a major problem worldwide, associated with high mortality and morbidity (Jacobs *et al*., 2013). There has been amounting research progress in HIE over the last two decades and many target molecules have been found (Pimentel-Coelho PM *et al*., 2012). However, therapeutic interventions are still limited, and there are no specific treatments proven to decrease brain damage from HIE (Lai and Yang, 2011; Fatemi *et al*., 2009; Jacobs *et al*., 2011).

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 (MacPherson *et al*., 2012; Fang *et al*., 2012; Ji and Zhang, 2009; Zhou *et al*., 2009). Accumulating evidence indicates neuroprotective effects of acupunture on hypoxic-ischemic injury. The established research on the neuro-physiological correlates of acupuncture 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 (Manni *et al*., 2010; Yu *et al*., 2011; Joh *et al*., 2010). It was shown that in cerebral ischemic injury, electro acupuncture played an important role in functional reorganization and cerebral compensation (Yi *et al*., 2006; Li *et al*., 2012; Gao *et al*., 2011), 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) (Wang *et al*., 2005; Yun *et al*., 2002; Liang *et al*., 2003; Hoke *et al*., 2000; Liang *et al*., 2002).

In response to cerebral ischemia, activated ascrocytes synthesize and release many neurotrophic factors, as a

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compensatory self-protection response. Among which GDNF was elaborately researched (Ikeda *et al*., 2002; Ikeda *et al*., 2000). GDNF treatment was 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 (Katsuragi *et al*., 2005; Katsuragi *et al*., 2005; Katsuragi *et al*., 2011; Wang *et al*., 2012). It was reported that GDNF could promote survival of many kinds of neurons, such as dopamine-producing (DA) neurons, motor neurons, primary sensory neurons and sympathetic neurons, etc. and promote the formation and survival of synapses (Airaksinen and Shaarma, 2002; Sariola and Shaarma, 2003; Lucini *et al*., 2011). Furthermore, it was the most potent trophic factor for spinal motoneurons. GDNF exerts its neuroprotective role via interaction with its receptor GFRα, leading to dimerization of GFRα. Then the complex, containing GDNF and GFRα dimers, brings two molecules of RET together, triggering transphosphorylation of specific tyrosine residues in their tyrosine kinase domains and activation of several intracellular signaling pathways, such as PI3-K/Akt pathway (Guillou *et al*., 2011; Korsak *et al*., 2012; Farhi *et al*., 2010; Perrinjaquet *et al*., 2010). PI3-K/Akt is an important pathway related to cell fate determination. Akt is the main target kinase of PI3-K, involved in many biological processes, such as cell survival, proliferation and metabolism. Especially its activation was shown to inhibit cell apoptosis and promote cell survival (Wang *et al*., 2012; Wang *et al*., 2007).

Recently 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 interventing mechanisms involved have been rarely reported. So in the present study, using a rat HIE model we evaluated *in vivo* therapeutic efficacy of electroacupuncture and 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.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

Experiments were performed at the Experimental Animal Center, Guangzhou University of Traditional Chinese Medicine, from September 2009 to May 2010.

Materials

A total of 114 seven-day-old SD rats (belonging to 11 broods with the mothers included, 10 to 11 newborn rats per brood), male or female, specific pathogen-free (SPF) grade, weighing 11.0 to 15.8g (average weight, 13.2±1.8g), were provided by the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine (No. SYXK (Yue) 2009-0001). 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 the People's Republic of China.

METHODS

Establishment of HIE animal model

The HIE model was constructed according to the LCCA ligation method in combination with hypoxic treatment described by Rice (Rice *et al*., 1981). 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 LCCA was separated, ligated using a 5/0 silk suture, and then the incision was sewed up. After a two-hour restoration, the rat was put in a sealed transparent vessel and set in the warm bathe with the temperature of 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 1L/min for 2.5 hours. The survivors were kept warm for another one hour 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 four hours later, and rats in this group showed no behavioral abnormality.

Electro acupuncture treatment

In the electro acupuncture model group, four acupoints including Baihui (GV20), Dazhui (GV14), Quchi (LI11) and Yongquan (KI1) 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 (Zheng *et al*., 2009). Baihui (GV20) acupoint is located in the center of the parietal bone. Dazhui (GV14) is located between the seventh cervical vertebra and the first thoracic vertebra, just in the center of the back. Quchi (LI11) is in the midpoint of the line between the outer end of the elbow stripes and the epicondyle of the humerus, and Yongquan (KI1) is located in the plantar anterior third (toes excluded). A needle 0.5 inch in length was inserted horizontally backwards into Baihui (GV20) at a depth of 5mm, perpendicularly inserted into Dazhui (GV14) for 5mm in depth, perpendicularly inserted into Quchi (LI11) for 10mm in depth and rapidly inserted into Yongquan (KI1) without leaving the needle in, respectively. The two acupoints Baihui (GV20) and Quchi (LI11) were

connected with G-6805 electric acupuncture apparatus (Shanghai Huayi Medical Instrument Factory, China), and receive electroacupuncture for 10 minutes with local tissue shivering slightly (frequency 5-10Hz, voltage 3-5V). The acupuncture therapy was given once a day for consecutive 1d, 3d, 7d and 21d respectively. The other two groups (the sham-surgery group and the control model group) were raised under the same condition, but didn't receive any treatment.

Hematoxylin-eosin (HE) 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 °C), and then perfused and fixed with 4% (w/v) paraformaldehyde (in 0.1 M PBS (pH 7.4), precooled at 4°C) firstly at full speed till convulsion of the limbs ceased, then perfusion was kept at the velocity of 1ml/min. Then, the brain was quickly separated with cerebellum and brainstem removed and placed in 4% paraformaldehyde for 24 hours at 4 °C. The specimens were dehydrated with 20% sucrose and frozen. The frozen sections were then serially cut into 20 μm thick coronal slices. HE staining was performed according to the standard protocol (Longo UG *et al*., 2009). Briefly, 1. Fix for 10~30s, then rinse in running tap water for 1~2s. 2. Stain in hemotoxylin for 30~60s at 60 °C, then rinse in running tap water for 5~10s. 3. Decolorized in 1% acid alcohol for 1~3s, then rinse in running tap water for 1~2s. 4. Blue in 1% NH3 solution for 5~10s, then rinse in running tap water for 15~30s. 5. Stain in eosin for 30~60s, then rinse in running tap water for 1~2s. 6. Dehydrate in 80% and 95% EtOH for 1~2s, respectively, and in 100% EtOH for 2~3s, twice. 7. Immerse in dimethylbenzene for 2~3s, twice. 8. Seal in neutral gum. The slides were observed under a light microscope (Nikon, Tokyo, Japan) using a magnification of ×200, and photographed.

Electron microscopy for cortical ultra structures

Rats anesthetized with ether were transcardially perfused with 4% (w/v) para formaldehyde in 0.1 M 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°C. Then, specimens were post-fixed in 1% (w/v) OsO4 for one hour. 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 realtime RT-PCR

Total RNA was extracted from the injured cortex (about 1mm × 1mm × 1mm) of rats using Trizol (*In vitro* gen, 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. Realtime RT-PCR was performed using the ABI 7500 realtime 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 GAPDH: 5'-TGG TCT ACA TGT TCC AGT ATG ACT-3' ; Reverse primer for GAPDH: 5'-CCA TTT GAT GTT AGC GGG ATC TC-3'. GAPDH was used as an internal control. The standard procedure is as follows, 95°C 5min, then 95°C 10sec, 60°C 40sec for 40 cycles.

Detection of Akt protein expression in the injured cortex by western blot analysis

Total protein extracts were prepared from rat brains as previously described (Liu H *et al*., 2013). In brief, rats were anesthetized with ether and rapidly decapitated. Brain was quickly separated with cerebellum and brainstem removed and placed on ice in 10volumes of cold homogenization buffer (50mmol/L Tris, 120mmol/L NaCl, pH 7.4) with protease inhibitors (Sigma, USA). The tissue was then homogenized and stored at -80°C. Protein concentrations were determined using the Bradford method (Bio-Rad, USA). Equal amounts of protein (50 μg/lane) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). After being blocked in blocking buffer for 1~2h at room temperature, the filters were incubated with the following primary antibodies overnight at 4□: anti-Akt rabbit polyclonal antibody (Cell Signaling, USA). Anti-GAPDH antibody (Sigma, USA). GAPDH was used as an internal loading control. After being washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 2h at room temperature, the immune complexes were visualized with a chemilumine scence reagent. Western blots were quantified densitometrically with Quantity One software (Bio-Rad, USA), and the intensity values were normalized to GAPDH.

STATISTICAL ANALYSIS

All data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Measurement data were expressed as mean ± SD. Comparisons among several groups were made using one-way analysis of variance under equal variances and Kruskal-wallis H analysis under unequal variances. Comparisons between groups were made using SNK-q test under equal variances and Dunnett's T3 test under unequal variances. $P<0.05$ (two-tail) was considered statistically significant.

RESULTS

Quantitative analysis of experimental animals

A total of 114 seven-day-old SD rats (belonging to 11 broods with the mothers included, breast feeding, 10 to 11 newborn rats per brood) were maintained in a temperature-controlled (20 to 25°C) facility with a 12h light/12h dark cycle. Among which four weighing less than 11g were excluded. Each brood was randomly assigned into two groups, sham-surgery group and HIE model group. Totally there were 30 rats in the former group and 80 rats in the latter group. After the HIE modeling operation, 7 rats died, with the death rate of 8.75%, and 3 rats didn't show obvious hemiplasia, which were excluded. Finally 70 rats were successfully established as the HIE model with the successful rate of 87.5%. Then the successfully established rats were randomly divided into the control model group and the electro acupuncture model group, with 35 rats in each group. And both groups were subdivided into four subgroups, with 9, 9, 9 and 8 rats in each subgroup in terms of four time periods of 1d, 3d, 7d and 21d for electro acupuncture treatment post-surgery.

Electro acupuncture treatment significantly ameliorates neurologic damage caused by hypoxic-ischemic injury

In the sham-surgery group, HE staining showed normal structures of cortex with clear organizational structure, normal cell outline, clear nucleolus and cell nucleus located in the center (fig. 1A). In the control model group, hypoxic-ischemic injury for 1d to 3d induced neurologic damage with obvious signs of necrosis and degeneration of neurons, such as mesh-like structure, irregular arrangements of neurons, concentrated cytoplasm, vacuolation of cytoplasm, karyopyknosis, and nucleolus loss (fig. 1B, 1D). 7d to 21d post-surgery, severe neuron loss along with proliferation of surrounding astrocytes was observed (fig. 1F, 1H).

Electro acupuncture for 3d showed swelling and degeneration of neurons but less severe than the control model group (fig. 1E). And electro acupuncture treatment for 7d to 21d showed less signs of necrosis and degeneration of neurons that is relatively clear organizational structure, regular arrangements of neurons, normal cell outline and relatively clear nucleolus (fig. 1G, 1I).

(A) Sham-surgery group; (B)-(D)-(F)-(H) 1d, 3d, 7d and 21d after the modeling operation in the control model group respectively; (C)-(E)-(G)-(I) 1d, 3d, 7d and 21d electro acupuncture treatment in the electro acupuncture model group respectively.

Electro acupuncture treatment significantly protected cortical neurons against hypoxic-ischemic injury.

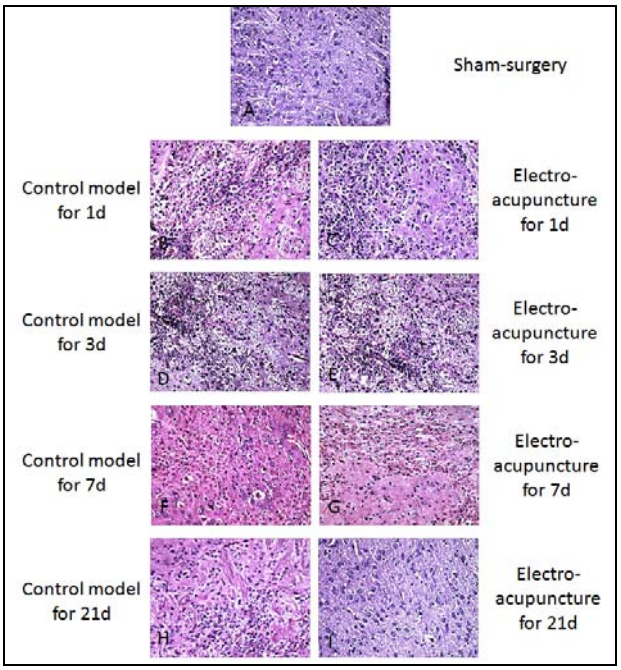

Fig. 1: Effect of electro acupuncture treatment on neurologic damage in the injured cortex of rat with HIE (Hematoxylin-eosin staining, optical microscope, ×200).

Electro acupuncture treatment protects ultra structure of cortical neurons against the degenerative alterations

As was shown in fig. 2A and 2B, ultra structure of cortical neurons was kept normal in the sham-surgery group with clear nuclear membrane, big and round nucleolus, evenly distributed cytoplasm, normal mitochondria and rough endoplasmic reticulum. In the control model group, cortical neurons went through gradual changes from degenerative alterations to self-repair. Hypoxic-ischemic injury for 1d to 3d induced degenerative alterations as nuclear membrane disruption, uneven distribution of nucleoplasm, indistinct nucleolus, formation of micro bubbles surrounding the nucleus, vacuolation of cytoplasm, mitochondria swelling and rough endoplasmic reticulum dilating (figs. 2C and 2E). 7d to 21d post-surgery, cortical neurons showed signs of self-repair with the above changes decreased in severity (figs. 2G and 2I). Electro acupuncture treatment promoted self-repair of cortical neurons. 3d treatment dramatically decreased the severity of degenerative alterations compared with the control model group (fig. 2F), and electro acupuncture treatment for longer period improved the ultra structure of cortical neurons for better (figs. 2H and 2J).

(A)-(B) Sham-surgery group; (C)-(E)-(G)-(I) 1d, 3d, 7d and 21d after the modeling operation in the control model group respectively; (D)-(F)-(H)-(J) 1d, 3d, 7d and 21d electro acupuncture treatment in the electro acupuncture model group respectively.

Electro acupuncture treatment protected the ultra structure of cortical neurons against degenerative alterations.

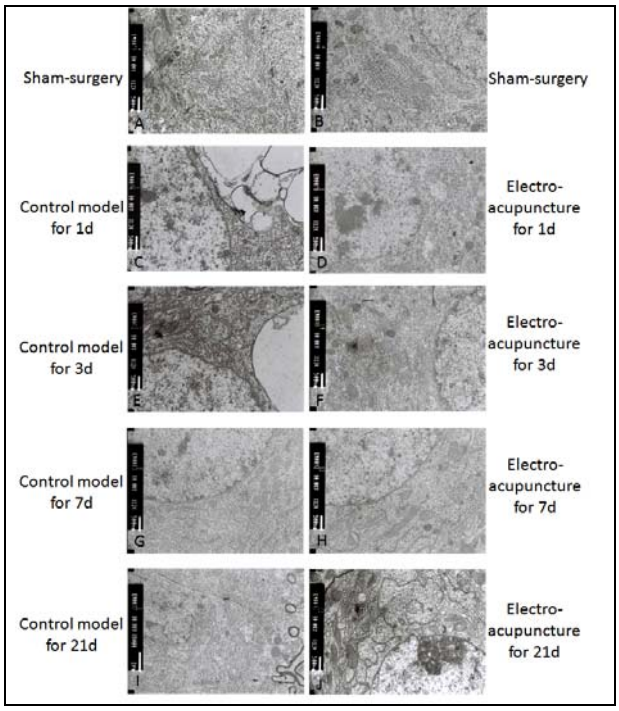

Fig. 2: Effect of electro acupuncture treatment on ultra structure of cortical neurons in rat with HIE (transmission electron microscopy, scale bar: 500 nm).

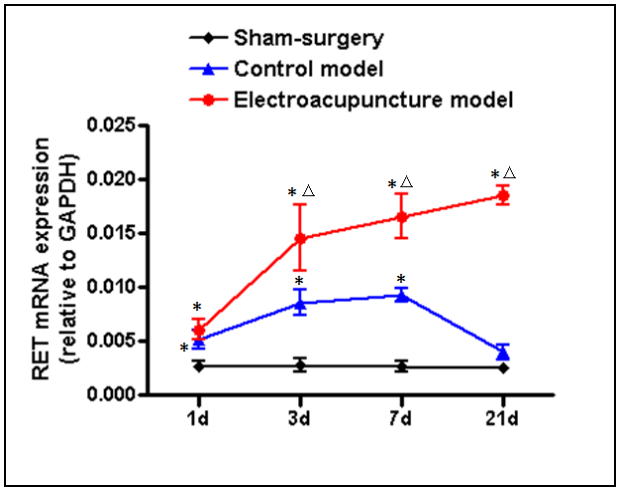

Fig. 3: Effect of electro acupuncture treatment on RET mRNA expression in the injured cortex of rat with HIE (realtime RT-PCR).

Expression changes of RET mRNA in the cerebral cortex of rats from three experimental groups

RET mRNA expression levels were detected with realtime RT-PCR. As was shown in fig. 3, compared with that of the sham-surgery group, expression levels of RET in the cerebral cortex with hypoxic-ischemic injury from the control model group increased at the first day post-surgery ($P<0.05$), continued increasing three days later and reached the peak at the 7th day ($P<0.05$). By contrast, its expression decreased at the 21st day, with no significant difference ($P>0.05$ vs the sham-surgery group). In the electro acupuncture model 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 group, electro acupuncture treatment increased RET mRNA dramatically at the 3rd, 7th and 21st day ($P<0.05$), though there was no significant difference at the 1st day.

RET mRNA expression increased at the 1st, 3rd and 7th day, and decreased to the normal state at the 21st day in the control model group, compared with the sham-surgery group. Electro acupuncture treatment further promoted RET mRNA expression, especially after 21d treatment.

RNA was normalized to the internal control GAPDH. Data are expressed as mean ± SD (n=6 rats per group, *$P<0.05$ vs the corresponding sham-surgery group; △ $P<0.05$ vs the corresponding control model group).

Expression changes of Akt protein in the cerebral cortex of rats from three experimental groups

Akt expression was analyzed by western blotting. In comparison with the sham-surgery group, Akt expression at the protein level was significantly increased both in the control model group ($P<0.05$) and in the electro acupuncture model group ($P<0.05$) at the 1st day post-surgery. But there was no statistical significance between the control model group and the electro acupuncture model group ($P>0.05$) (fig. 4A).

At the 3rd day post-surgery, Akt expression was significantly increased both in the control model group ($P<0.05$) and in the electro acupuncture model group ($P<0.05$), compared with that of the sham-surgery group. Furthermore, elecro acupuncture treatment for three days increased Akt expression more dramatically than in the control model group ($P<0.05$) (fig. 4B).

Just like the above result at the 3rd day, Akt expression at the 7th day post-surgery was significantly increased both in the control model group ($P<0.05$) and in the electro acupuncture model group ($P<0.05$), compared with that of the sham-surgery group. Furthermore, electro acupuncture treatment for seven days increased Akt expression more dramatically than in the control model group ($P<0.05$) (fig. 4C).

At the 21st day post-surgery, no more increase of Akt expression was detected in the control model group ($P>0.05$). But in the electro acupuncture model group, Akt expression was significantly increased compared with that of the sham-surgery group ($P<0.05$) or with that of the control model group ($P<0.05$) (fig. 4D).

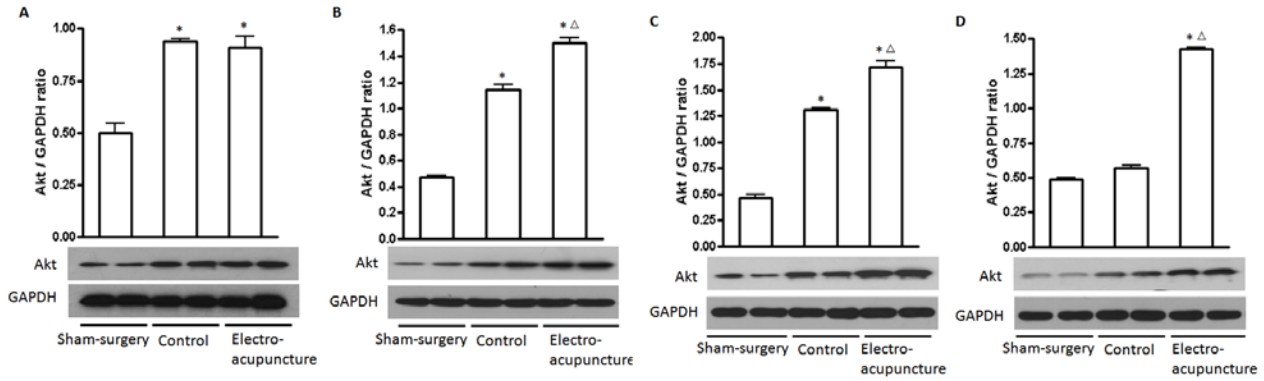

Fig. 4: Effect of electro acupuncture treatment on Akt protein expression in the injured cortex of rat with HIE (Western blot).

(A)-(D) Akt protein expression at the 1st, 3rd, 7th and 21st day post-surgery respectively. Akt protein expression increased at the 1st, 3rd and 7th day and decreased to the normal state at the 21st day in the control model group, compared with the sham-surgery group. Electro acupuncture treatment further promoted Akt expression, especially after 21d treatment.

Bar graphs show densitometric analysis of western blot of Akt protein. The densitometric quantification was normalized to the internal control GAPDH. Data are expressed as mean ± SD (n=6 rats per group, * $P<0.05$ vs the sham-surgery group; △ $P<0.05$ vs the control model group). The immunoblots are representative of three independent experiments.

DISCUSSION

HIE was 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 the present study, we constructed a HIE animal model to evaluate the *in vivo* therapeutic efficacy of electro acupuncture and to further investigate the role of RET and its key downstream PI3-K/Akt pathway in the process. For the first time, our results showed that electro acupuncture at Baihui (GV20), Dazhui (GV14), Quchi (LI11) and Yongquan (KI1) dramatically ameliorated neurologic damage and alleviated the degenerative changes of ultra structure of cortical neurons in rats subjected to HIE, which is partly attributed to increased expression of RET and Akt.

HIE model was constructed using seven-day-old rat, since cerebral development of seven-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 (Rice *et al*., 2012). Since then, the model has been widely used for its simple operation, high successful rate, low mortality, high reliability and good reproducibility.

Acupuncture, as one of the major components of traditional Chinese medicine, has been used for more than 1000 years as a treatment or as an adjuvant modality for patients with stroke. Acupoint combination is a complicated issue. Among different acupoints, Baihui (GV20) and Dazhui (GV14) 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 (Fang *et al*., 2012). 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 (Kim *et al*., 2011; Chuang *et al*., 2007; Liu *et al*., 2013; Liu *et al*., 2012). Quchi (LI11) is the He-Sea point of the large intestine channel of Hand-Yangming. Electro acupuncture at Quchi (LI11) and Zusanli (ST36) 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 (Lan *et al*., 2012; Hong *et al*., 2013; Chen *et al*., 2012; Zhao *et al*., 2010). Yongquan (KI1) is the significant Jing-Well point of the kidney channel of Foot-Shaoyin. Using in combination with Baihui (GV20), it was shown to improve energy and intelligence, clear the brain collaterals and coordinate the channels and collaterals

(Xue *et al*., 2009; Xue *et al*., 2011). Acupuncture at Quchi (LI11) and Yongquan (KI1) was reported to promote functional recovery of the extremities after neuron injury (Ji *et al*., 2009; Zhou *et al*., 2009). Considering that Baihui (GV20), Dazhui (GV14), Quchi (LI11) and Yongquan (KI1) 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.

Many animal studies have indicated neuroprotective effect of acupunture on hypoxic-ischemic injury. Electro acupuncture applied at bilateral acupoints (Quchi, Waiguan, Huantiao, and Zusanli) was shown to enhance cell proliferation and neuronal differentiation in young rat hippocampus, providing a theoretical basis for the clinical application of acupuncture to cerebral injury rehabilitation in children (Gao *et al*., 2011). Yi *et al* found that electro acupuncture could protect synaptic ultra structure, promote the expression of P38, GAP-43, NGF and BDNF in the ischemic cerebral cortex, and thus improve synaptic plasticity in cerebral ischemic rats (Yi *et al*., 2006). Li *et al* reported that electro acupuncture combined with transcranial magnetic stimulation improved learning and memory function of rats with cerebral infarction by inhibiting neuron cell apoptosis (Li *et al*., 2012). In our research, HE staining and electron microscopy results showed that electro acupuncture treatment significantly ameliorated neurologic damage caused by hypoxic-ischemic injury and protected ultra structure of cortical neurons against the degenerative alterations, suggesting that electro acupuncture at Baihui (GV20), Dazhui (GV14), Quchi (LI11) and Yongquan (KI1) exerts neuroprotective function in HIE, which is in accordance with previous studies.

GDNF, belonging to distant members of the transforming growth factor-β (TGFβ) superfamily, is one of the most potent neurotrophic factor. GDNF mRNA, widely distributed in the nervous system, have been detected in striatum, cortex, hippocampus and spinal cord in adults by RT-PCR. GDNF is a small-secreted protein which is crucial for the development and maintenance of distinct sets of central and peripheral neurons. It has been reported to potently promote the survival of many types of neurons. GDNF is specifically trophic to DA neurons, which can promote survival and prevent apoptosis of DA neurons, promote morphological differentiation of DA neurons, and increase the ingestion of DA (Aoi *et al*., 2001). It is also the most potent trophic factor for motor neurons. During development, GDNF is produced primarily by Schwann cells. 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 (Garces *et al*., 2000). Conversely, motor neuron survival is promoted by muscle-specific over expression of GDNF or by GDNF treatment in utero, indicating that GDNF is indeed a physiological survival factor for a subpopulation of motor neurons (Oppenheim *et al*., 2000). GDNF expression was induced in ischemic brain injury, which was a self-protection 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 electro acupuncture 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 electro acupuncture on HIE.

GDNF exerts its neuroprotective effects through the classic growth factor signaling pathway (Baloh RH *et al*., 2000). All GDNF family ligands (GFLs) signal through the RET receptor tyrosine kinase, which was first discovered as a proto-oncogene. It is activated only if the GFL is first bound to a novel class of proteins, known as GDNF-family receptor-α (GFR α) receptors, which are linked to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor. RET is a single-pass transmembrane protein that contains four cadherin-like repeats in the extracellular domain and a typical intracellular tyrosine kinase domain. GFL-GFR α binding to the extracellular domain of RET leads to activation of the intracellular tyrosine kinase domain, which then further activate downstream signaling pathways to promote cell survival and cell differentiation (Trupp *et al*., 1996; Jing *et al*., 1996; Takahashi and Cooper, 1987). PI3-K/Akt activation is one of the most important downstream pathway of RET. It 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 (Wang *et al*., 2012; Wang *et al*., 2007). 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* found that Akt was activated in both non-preconditioned and preconditioned groups after ischemia for 1 hr, 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 (Nakajima *et al*., 2004). And it was reported that transgenic mouse over expressing the active Akt reduced the volume of infarct area by 35% after middle cerebral artery occlusion compared to the wild-type littermate (Ohba N *et al*., 2004). Chen A *et al* reported that using a focal cerebral ischemia/reperfusion injured rat model, electro acupuncture 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

electro acupuncture at Quchi and Zusanli acupoints exerts neuroprotective function in ischemic stroke via activation of the PI3K/Akt pathway (Chen A *et al*., 2012). In the present study, for the first time, 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 interventing effect of electro acupuncture. Results showed that electro acupuncture 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 (Chen A *et al*., 2012). 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 electro acupuncture exerts its neuroprotective function at least in part through activation of RET-Akt pathway. Nevertheless, we cannot rule out the possibility that other mechanisms are involved in the process.

CONCLUSION

In conclusion, our data provide evidence that electro acupuncture 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, electro acupuncture may become a potential therapeutic strategy for HIE in the neonate.

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