

# Electro-acupuncture at Conception and Governor vessels and transplantation of umbilical cord blood-derived mesenchymal stem cells for treating cerebral ischemia/reperfusion injury

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*doi:10.4103/1673-5374.125334*

*http://www.nrronline.org/*

*Accepted: 2013-11-25*

## Abstract

Mesenchymal stem cell transplantation is a novel means of treating cerebral ischemia/reperfusion, and can promote angiogenesis and neurological functional recovery. Acupuncture at Conception and Governor vessels also has positive effects as a treatment for cerebral ischemia/reperfusion. Therefore, we hypothesized that electro-acupuncture at Conception and Governor vessels plus mesenchymal stem cell transplantation may have better therapeutic effects on the promotion of angiogenesis and recovery of neurological function than either treatment alone. In the present study, human umbilical cord blood-derived mesenchymal stem cells were isolated, cultured, identified and intracranially transplanted into the striatum and subcortex of rats at 24 hours following cerebral ischemia/reperfusion. Subsequently, rats were electro-acupunctured at Conception and Governor vessels at 24 hours after transplantation. Modified neurological severity scores and immunohistochemistry findings revealed that the combined interventions of electro-acupuncture and mesenchymal stem cell transplantation clearly improved neurological impairment and up-regulated vascular endothelial growth factor expression around the ischemic focus. The combined intervention provided a better outcome than mesenchymal stem cell transplantation alone. These findings demonstrate that electro-acupuncture at Conception and Governor vessels and mesenchymal stem cell transplantation have synergistic effects on promoting neurological function recovery and angiogenesis in rats after cerebral ischemia/reperfusion.

*Key Words: nerve regeneration; acupuncture; human umbilical cord blood-derived mesenchymal stem cells; electro-acupuncture; cerebral ischemia/reperfusion; vascular endothelial growth factor; angiogenesis; Conception vessel; Governor vessel; modified neurological severity score; NSFC grant; neural regeneration*

*Funding: This study was supported by the National Natural Science Foundation of China, No. 81072877; Key Laboratory Project of Condition and Platform Construction Plan of Shenzhen Scientific Research Fund, No. CXB201111250113A; Shenzhen Scientific and Technology Development Program, No. 201203149*

*Yu HB, Chen PD, Yang ZX, Luo WS, Pi M, Wu YG, Wang L. Electro-acupuncture at Conception and Governor vessels and transplantation of umbilical cord blood-derived mesenchymal stem cells for treating cerebral ischemia/reperfusion injury. Neural Regen Res. 2014;9(1):84-91.*

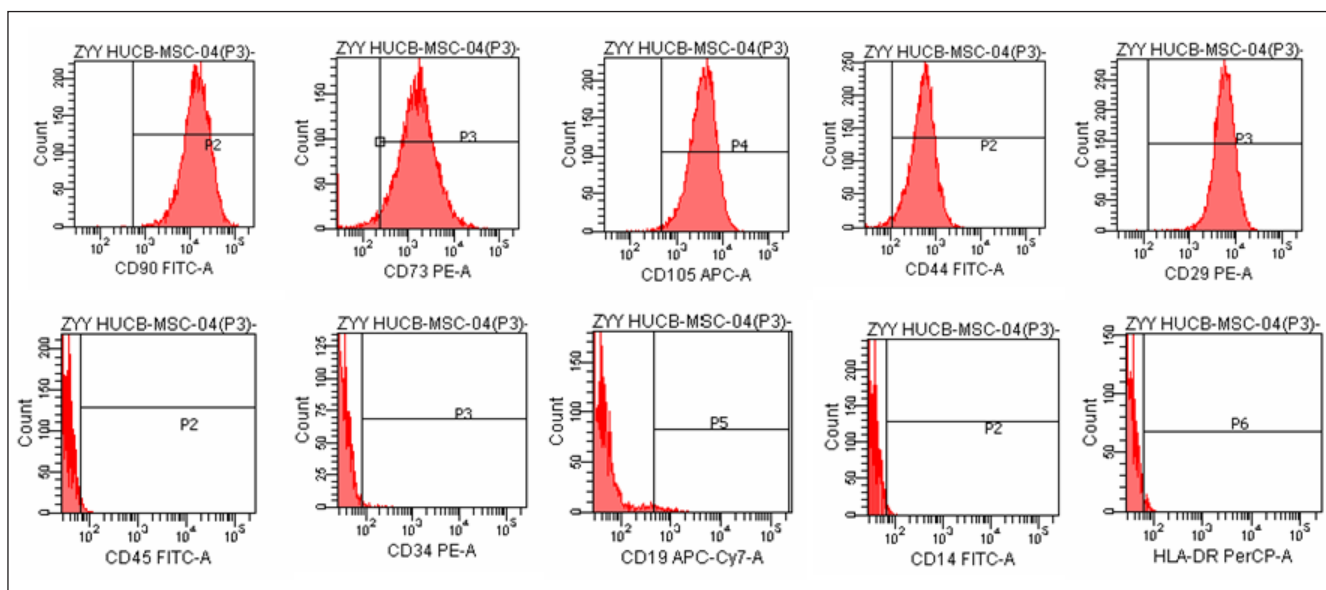
## Introduction

Despite advances in medical treatment, cerebral ischemia/reperfusion is still a major cause of adult morbidity, mortality and disability, and the only approved therapy is thrombolysis within 3.0–4.5 hours after symptom onset<sup>[1-2]</sup>. Besides thrombolysis and neuroprotection, vascular protection is also of significance for the treatment of cerebral ischemia injury<sup>[3]</sup>. Vascular endothelial growth factor, a potent angiogenic and neurotrophic factor, is known to be involved in the regulation of the vascular permeability of the blood-brain barrier<sup>[4-5]</sup>.

In recent years, progress in stem cell biology has opened up avenues to therapeutic strategies for the treatment of

neurological diseases. Mesenchymal stem cells are an attractive candidate for cell therapy since they are readily obtained and can be expanded *in vitro*. Some transplanted mesenchymal stem cells differentiate into neuron-like cells and endothelial cells in recipient brains<sup>[6]</sup>. In addition, these cells can be induced to differentiate into vascular endothelial cells showing up-regulated vascular endothelial growth factor expression, with autocrine and paracrine modes of action, in the brains of cerebral ischemia/reperfusion rats; consequently, they promote angiogenesis and ameliorate neurological functional deficits<sup>[7-10]</sup>.

Acupuncture has been widely applied as a treatment or as an adjuvant modality for apoplexy patients in China for



**Figure 1** Flow cytometric analysis of human umbilical cord blood-derived mesenchymal stem cells at passage 3.

The cells highly expressed CD90, CD73, CD105, CD44 and CD29, but expressed low levels of CD45, CD34, CD19, CD14 and human leukocyte antigen-DR.

centuries. A previous study by our laboratory<sup>[11]</sup> showed that electro-acupuncture at Conception and Governor vessels continuously promotes the proliferation and differentiation of endogenous neural stem cells into neurons in cerebral ischemia/reperfusion rats, which is beneficial for neural regeneration<sup>[12]</sup>. Recent studies have also found that electro-acupuncture induces up-regulation of vascular endothelial growth factor expression and reduces neurological deficit scores in cerebral ischemia/reperfusion rats<sup>[13-14]</sup>. Therefore, we hypothesized that electro-acupuncture at Conception and Governor vessels may have additive or synergetic effects with mesenchymal stem cell transplantation, on the up-regulation of vascular endothelial growth factor expression and improved neurological functional recovery, which could provide a novel treatment for cerebral ischemia/reperfusion.

To investigate the combinatorial effects of electro-acupuncture and mesenchymal stem cells in the treatment of cerebral ischemia/reperfusion, human umbilical cord blood-derived mesenchymal stem cells (HUCB-MSCs) were isolated, cultured and transplanted into the brains of cerebral ischemia/reperfusion rats. Electro-acupuncture was also applied. We examined their combinatorial effects on neurological severity scores and vascular endothelial growth factor expression in the rats.

## Results

### Quantitative analysis of experimental animals

A total of 96 rats were equally and randomly divided into the following four groups: a sham group (no ischemia/reperfusion), model group (ischemia/reperfusion plus PBS transplantation), HUCB-MSC group (ischemia/reperfusion plus mesenchymal stem cells transplantation), and electro-acupuncture group (ischemia/reperfusion, mesenchymal stem

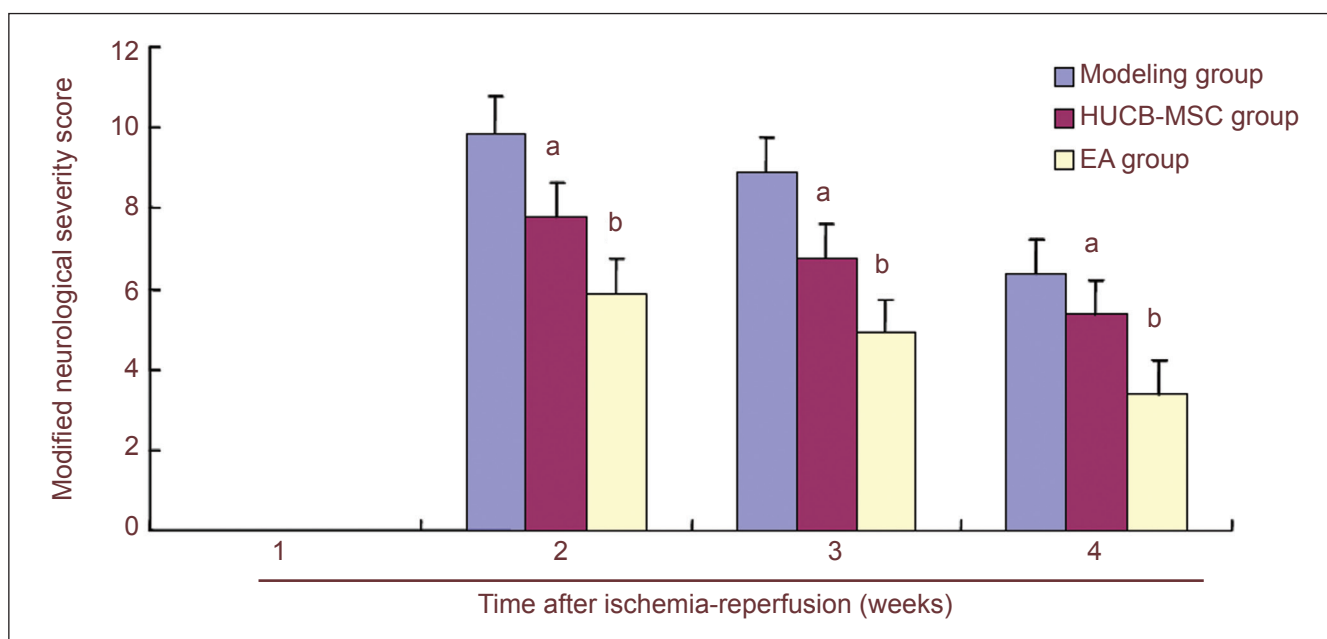
cells transplantation and electro-acupuncture). In terms of the time period after cerebral ischemia/reperfusion, each group was further divided into three subgroups of 7, 14 and 28 days. Thus, each subgroup contained eight rats. All 96 rats were involved in the final analysis.

### Identification of HUCB-MSCs

According to the identification criteria of mesenchymal stem cells proposed by the International Society for Cellular Therapy<sup>[15]</sup>, we selected specific cell surface markers to identify these cells. Flow cytometric analysis revealed that HUCB-MSCs at passage three highly expressed mesenchymal stem cell antigens, including CD90 (100%), CD73 (96.4%) and CD105 (99.5%), and adhesion molecules CD44 (100%) and CD29 (98.2%), but expressed low levels of CD45 (0.9%), CD34 (0.3%), CD19 (0.9%), CD14 (0.6%) and human leukocyte antigen-DR (0.8%). These findings indicate that the major population of the adherent cells cultured was HUCB-MSCs (Figure 1).

### Electro-acupuncture and HUCB-MSC transplantation improved neurological deficits of rats following cerebral ischemia/reperfusion

The modified neurological severity score in the sham group was 0 because no neurological deficits were found. All rat models of cerebral ischemia/reperfusion injury in the other three groups showed severe neurological deficits, which gradually improved as time proceeded. The HUCB-MSC group had a lower score than the model group on days 7, 14 and 28 ( $P < 0.01$ ), but a higher score than the electro-acupuncture group ( $P < 0.01$ ). These findings suggest that electro-acupuncture combined with HUCB-MSC treatment is superior to HUCB-MSC transplantation alone in improving neurological functions (Figure 2).



**Figure 2** Effect of electro-acupuncture (EA) plus human umbilical cord blood-derived mesenchymal stem cell (HUCB-MSC) treatments on neurological function in cerebral ischemia rats.

The results are represented as the modified neurological severe scores, and higher scores represent more serious nerve injuries. Data are expressed as mean  $\pm$  SD of eight rats for each group. <sup>a</sup> $P < 0.01$ , vs. model group; <sup>b</sup> $P < 0.01$ , vs. HUCB-MSC group (one-way analysis of variance and the least significant difference test).

### Electro-acupuncture and HUCB-MSC transplantation up-regulated vascular endothelial growth factor expression around the ischemic focus in rats following cerebral ischemia/reperfusion

Under a light microscope, vascular endothelial growth factor-positive cells were observed with yellow or brown cytoplasm. There were no significant differences in the numbers of positive cells in the sham group among the various time points (Figure 3). However, the numbers of vascular endothelial growth factor-positive cells in the other three groups gradually decreased from a peak at 7 days after injury, being significantly decreased at 14 days and further decreased at 28 days after cerebral ischemia/reperfusion ( $P < 0.01$ ; Figure 3).

The numbers of vascular endothelial growth factor-positive cells in the HUCB-MSC and electro-acupuncture groups were significantly higher than that in the model group at 7, 14 and 28 days ( $P < 0.01$ ). There was no significant difference in cell numbers between the HUCB-MSC group and the electro-acupuncture group at 7 days ( $P > 0.05$ ). However, with prolonging of the reperfusion period, there were significantly more vascular endothelial growth factor-positive cells in the electro-acupuncture group compared with HUCB-MSCs group at 14 and 28 days ( $P < 0.05$ ; Figure 3).

### Discussion

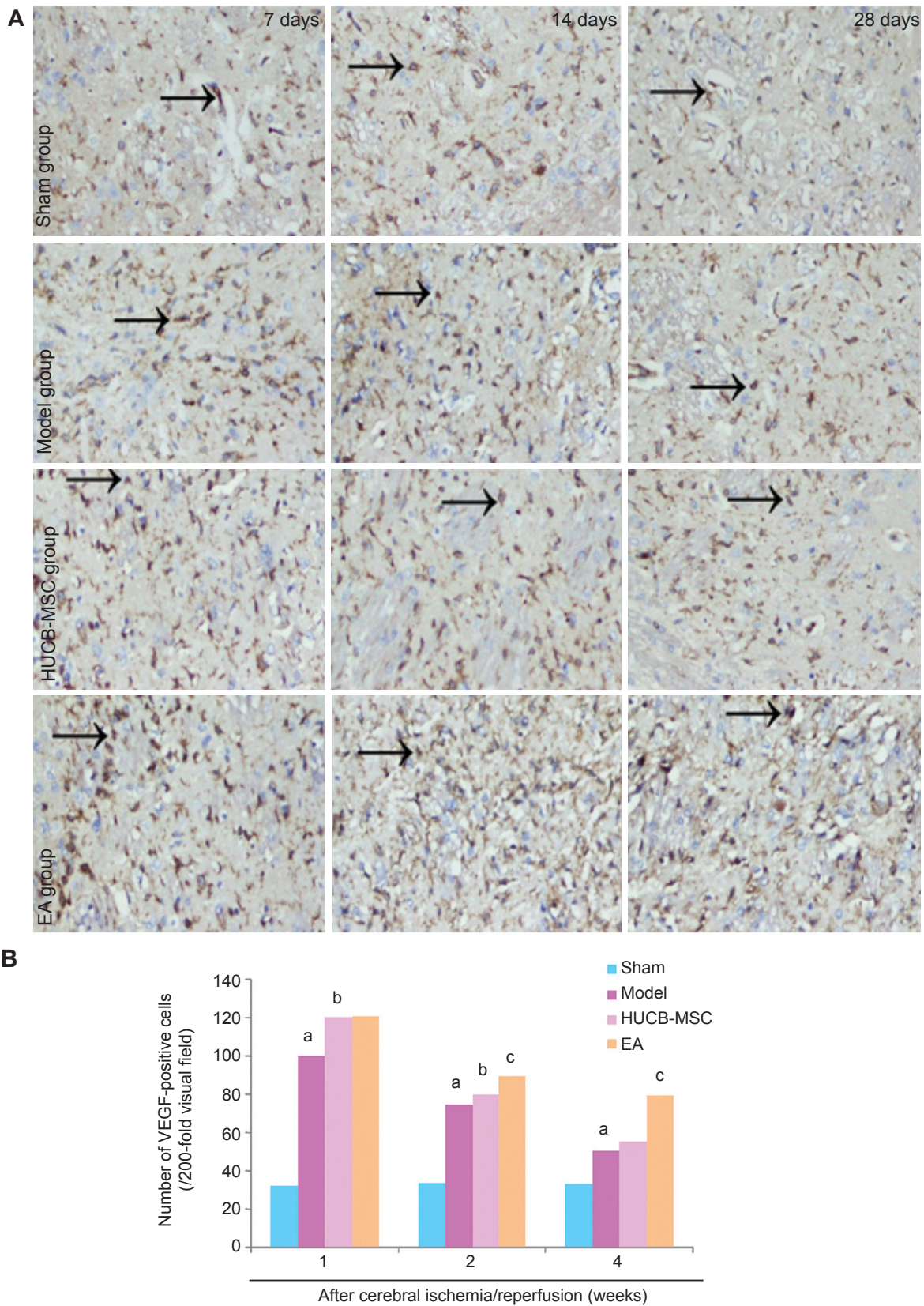
Mesenchymal stem cells offer promise for cell therapy procedures owing to their multipotent abilities, which means they could be induced to secrete diverse cytokines and growth factors and promote neurological functional recovery following cerebral ischemia/reperfusion injuries<sup>[6]</sup>. Compared

with human bone marrow-derived MSCs, HUCB-MSCs have many advantages such as an abundant source that can be accessed without pain, easy harvesting, low immunogenicity and a low possibility of being polluted by viruses and bacteria<sup>[7]</sup>. Accordingly, in the present study, we selected the HUCB-MSCs. Our data indicated that the cultured adherent HUCB-MSCs exhibited a spindle shape and expressed mesenchymal stem cell-specific markers.

In our previous studies, electro-acupuncture at Conception and Governor vessels continuously promoted the proliferation and differentiation of neural stem cells into neurons in cerebral ischemia/reperfusion rats<sup>[11]</sup>. Furthermore, electro-acupuncture at the Conception vessel up-regulated the expression levels of neurotrophic factors such as basic fibroblast growth factor and epidermal growth factor<sup>[16-17]</sup>. Accumulating evidence suggests that electro-acupuncture at the Governor vessel enhances vascular endothelial growth factor expression and inhibits cellular apoptosis following acute cerebral ischemia/reperfusion injuries<sup>[14, 18-19]</sup>. Based on classic acupuncture texts and other generally acknowledged traditional Chinese medicine sources as well as the results of clinical practice<sup>[20-22]</sup>, we selected Qihai (CV6), Guanyuan (CV4) and Chengjiang (CV24) acupoints from the Conception vessel and Baihui (GV20), Dazhui (GV14) and Shuigou (GV26) acupoints from the Governor vessel in the present study.

Vascular endothelial growth factor is a chemokine and specific mitogen of endothelial cells, exerting its biological functions *via* three related receptor tyrosine kinases (Flt-1, Flk-2 and Flt-4), promoting endotheliosis *in vitro* and inducing angiogenesis *in vivo*<sup>[23]</sup>. In addition, it has been shown





**Figure 3** Effect of electro-acupuncture (EA) plus human umbilical cord blood-derived mesenchymal stem cells (HUCB-MSCs) on the number of vascular endothelial growth factor (VEGF)-positive cells around the ischemic focus in rats.

(A) Representative photographs of VEGF expression around the ischemic focus (immunohistochemistry,  $\times 200$ ). Compared with the sham group at 7, 14 and 28 days, the numbers of VEGF-positive cells (arrows) were significantly higher in the model group, HUCB-MSC group and EA group at 7, 14 and 28 days.

(B) Histogram showing the numbers of VEGF-positive cells. Data are expressed as mean  $\pm$  SD of eight rats in each group. <sup>a</sup> $P < 0.01$ , vs. sham group; <sup>b</sup> $P < 0.01$ , vs. model group; <sup>c</sup> $P < 0.01$ , vs. HUCB-MSC group (one-way analysis of variance and the least significant difference test).

to stimulate axonal outgrowth and enhance cell viability<sup>[24]</sup>, promote the proliferation and differentiation of endothelial cells, and inhibit cellular apoptosis *via* inducing Bcl-2 and Survivin expression and down-regulating caspase-3 expression<sup>[25-26]</sup>. Moreover vascular endothelial growth factor acts directly on neural stem cells and repairs the brain through inducing other growth factors such as brain-derived neurotrophic factor<sup>[27-28]</sup>. Hence, vascular endothelial growth factor plays a role in angiogenesis and has a neurotrophic effect in the treatment of cerebral ischemia/reperfusion. The results from the present study reveal that vascular endothelial growth factor expression is up-regulated following cerebral ischemia/reperfusion, and that electro-acupuncture plus HUCB-MSCs transplantation can maintain this increase in vascular endothelial growth factor expression. This evidence is consistent with the results of previous studies showing beneficial effects of human bone marrow-derived mesenchymal stem cells in experimental cerebral ischemic models<sup>[29]</sup>. With prolonging of the reperfusion period, the effects were superior to that of HUCB-MSC transplantation alone.

A number of studies have shown functional improvement in focal cerebral ischemia/reperfusion rats following intracerebral delivery of mesenchymal stem cells<sup>[30]</sup>. However, the mechanisms by which mesenchymal stem cell transplantation improves stroke deficits remain uncertain. Diverse mechanisms may be responsible for properties such as increasing neovascularization, integrating into the host circuitry, reducing apoptosis of host cells, inducing host brain plasticity, and attenuating inflammation<sup>[31-32]</sup>. Accumulating evidence indicates that mesenchymal stem cells protect against cerebral injuries and promote angiogenesis *via* differentiating into vascular endothelial cells, extracellular matrix or laminin<sup>[33]</sup>. Interestingly, the modified neurological severity score results in this study showed that electro-acupuncture plus HUCB-MSC transplantation better improved neurological functions than did HUCB-MSC transplantation alone. This may be a consequence of the up-regulation of vascular endothelial growth factor expression. Considering the angiogenic effects of electro-acupuncture and mesenchymal stem cells, we tentatively infer that electro-acupuncture has additional or synergetic effects on mesenchymal stem cell transplantation, to further improve neurological functions following cerebral ischemia/reperfusion. According to traditional Chinese medicine theories, the Conception vessel governs embryonic pregnancy and controls the growth and development of the fetus, and may enhance the expression levels of endogenous neurotrophic factors such as vascular endothelial growth factor and promote functional recovery. The Governor vessel manages and activates *yang qi*, and inhibits cellular apoptosis following cerebral ischemia/reperfusion injuries<sup>[34]</sup>.

In conclusion, electro-acupuncture enhances the therapeutic potency of mesenchymal stem cells including neurological improvement, possibly through induction of angiogenesis. A combination of electro-acupuncture plus HUCB-MSC transplantation represents a new potential therapeutic strategy for the treatment of cerebral ischemia/reperfusion.

However, the present study lacked a group receiving electro-acupuncture only, so we could not rule out the possi-

bility that electro-acupuncture alone would have a similar action to the combined treatments; this possibility needs further investigation. In addition, the mechanisms of action of the combined treatments for cerebral ischemia/reperfusion are not entirely understood. Further studies addressing the combinatorial effects on the transformation of mesenchymal stem cells into neurons, neurotrophic factor expression and cellular apoptosis are warranted.

## Materials and Methods

### Design

A randomized, controlled, animal experiment.

### Time and setting

The experiments were performed in the Research Center for Neural Engineering, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, China from June 2011 to October 2012.

### Materials

#### Animals

Ninety-six adult male Sprague-Dawley rats, weighing 220–250 g were purchased from Guangdong Provincial Experimental Animal Center, Guangdong Province, China (license No. SCXK (Yue) 2008-0002). The rats were housed (four or five per cage) at  $23 \pm 1^\circ\text{C}$ , in an alternating 12-hour light/dark cycle, and allowed free access to food and water. All experimental protocols and procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China<sup>[35]</sup>.

#### Cells

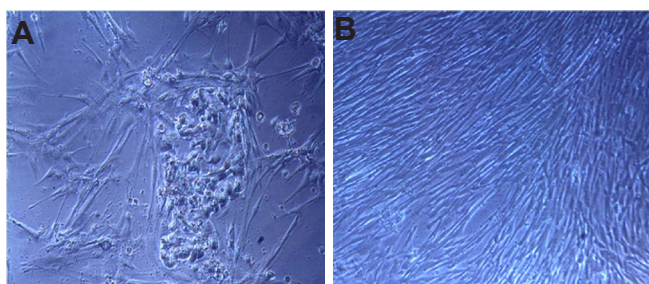
Human umbilical cord blood samples were collected from the umbilical veins of deliveries, from the Maternal Department, Affiliated Shenzhen Traditional Chinese Medicine Hospital, Guangzhou University of Chinese Medicine, China, with informed written maternal consent. Parturient infected with hepatitis virus, HIV or *Treponema pallidum*, and samples from fetuses with deformities, were excluded from the study.

### Methods

#### Isolation and culture of HUCB-MSCs

With 75% alcohol disinfection, the needle of an umbilical cord blood collection bag containing 0.74 g of sodium citrate (Sichuan Nigale Biomedical Co., Ltd., Sichuan Province, China) was inserted into the umbilical vein near placentas and human umbilical cord blood was collected by gravity. When the blood was collected, we put the bag lower than the umbilical cord. According to rules of gravity, the blood flows from the umbilical cord (higher position) to the bag (lower position). Isolation and expansion of HUCB-MSCs were conducted as previously reported<sup>[36]</sup>. In brief, mononuclear cells were isolated in lymphocyte separation by Ficoll-Hypaque gradient centrifugation (specific gravity  $1.077 \text{ g/cm}^3$ ; Pharmacia, Piscataway, NJ, USA). The separated mononuclear cells were washed and suspended in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Gibco, Car-





**Figure 4** Morphology of human umbilical cord blood-derived mesenchymal stem cells at primary passage at days 11 (A) and 28 (B). The monolayer of bipolar spindle-like cells with a whirl pool-like array could be seen after 4–5 days in culture.

lsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin-streptomycin (Hyclone, Logan, UT, USA), 1% glutamine (Gibco), 10 ng/mL human epidermal growth factor (ProSpec, Ness-Ziona, Israel) and 10 ng/mL recombinant human fibroblast growth factor-basic (PeproTech, USA). The culture medium served as the maintenance and expansion system and cells were seeded at  $1 \times 10^6$  cells/cm<sup>2</sup> onto 75-cm<sup>2</sup> plastic culture flasks (Corning Incorporated, NY, USA). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After 1 week, non-adherent cells were removed. The medium was replaced with the same fresh medium twice a week, and the onset of fibroblast-like adherent cells was observed. At 3–4 weeks, when the monolayer of mesenchymal stem cell colonies reached 80% confluence, cells were trypsinized with 0.25% trypsin-EDTA (Gibco), washed, resuspended in culture medium and subcultured at 3,000 cells/cm<sup>2</sup> (Figure 4). Mesenchymal stem cells of each umbilical cord blood harvested were expanded *ex vivo* by successive subcultivation under the same condition. HUCB-MSCs at passage three, at  $1 \times 10^5$ /μL, were used for transplantation.

Before transplantation, flow cytometric analyses of HUCB-MSCs at passage 3 were performed to identify the cells. In brief, cell suspensions were washed twice with PBS. For direct assays, aliquots of cells at  $2 \times 10^5$ /mL were immunolabeled at 4°C for 30 minutes with the following mouse anti-human monoclonal antibodies (7 μL each, 0.2 mg/mL): phycoerythrin-conjugated CD73, CD29, CD34, fluorescein isothiocyanate-conjugated CD90, CD14, CD44, CD45, allophycocyanin-conjugated CD19, CD105 and Peridinin-Chlorophyll-Protein Complex-conjugated human leukocyte antigen-DR. All antibodies were purchased from Becton Dickinson (BD) Biosciences (San Diego, CA, USA) and the labeled cells were analyzed using a FACSCalibur flow cytometer (Aria, BD Biosciences).

#### Establishing cerebral ischemia/reperfusion models

Transient cerebral ischemia/reperfusion was induced as described previously<sup>[37]</sup>. Briefly, after anesthesia with intraperitoneal injection of 300 mg/kg chloral hydrate, the rats were placed in a supine position. Using a surgical microscope (STEMI DV4, USA), the skin was incised along the midline of the neck and the right common carotid artery, external carotid artery and internal carotid artery were exposed through a ventral midline incision. The occipital artery and

thyroid artery were electrocauterized; and the right external carotid artery distal to the heart was ligated. The common carotid artery and internal carotid artery distal to the heart were then clamped. The right external carotid artery was cut off at the ligation site, and the internal carotid artery and external carotid artery were intersected and aligned. A gap was made at the bifurcation of the external carotid artery adjacent to the common carotid artery, and a 4-0 monofilament suture with its tip rounded by heating near a flame was inserted from the incision and slowly advanced to the middle cerebral artery (depth  $18 \pm 2$  mm). Rectal temperature was maintained at 37°C throughout the surgical procedure until the anesthesia ended. After 2 hours, the animals were re-anesthetized, the filament was withdrawn out of internal carotid artery for reperfusion, and the wound was sutured. Model establishment was considered successful with the appearance of contralateral hemiplegia (limb disturbance such as flexion and adduction) and contralateral circling while walking. For the sham surgery group, the operating procedure was the same as described above without filament insertion into the internal carotid artery.

#### HUCB-MSC transplantation

HUCB-MSCs at passage 3 were harvested, suspended in PBS and placed in an ice bath before cell transplantation. Transplantation of cells was conducted as previously reported with some modification<sup>[38]</sup>. Namely, after 24 hours of reperfusion, rats were anesthetized and tethered to a stereotaxic apparatus (World Precision Instruments Inc., Sarasota, FL, USA). A piece of paper was used to cover the two eyes, and the cranium was exposed through a midline skin incision. A burr hole was made using a small high-speed dental drill, and a suspension of 10 μL of cells containing a total of 10<sup>6</sup> HUCB-MSCs was injected stereotactically into the right striatum (anteroposterior = 0 mm, mediolateral = 2.0 mm, dorsoventral = 4.5 mm) and right subcortex (anteroposterior = 0 mm, mediolateral = 2.0 mm, dorsoventral = 2.0 mm) using a 25-μL microsyringe (Hamilton, Switzerland), with 7 μL being injected into the striatum and 3 μL into the subcortex. Cells were injected at a speed of 1 μL/minute with an automatic microinjection pump and the needle was left *in situ* for 5 minutes post-injection before being slowly removed. An equal volume of PBS was administered in the model group using the same procedure.

#### Electro-acupuncture treatment

At 24 hours after transplantation, rats in the electro-acupuncture group were electro-acupunctured at *Shuigou* (GV26; 1 mm below nasal tip, depth of insertion: 1 mm, upward), *Baihui* (GV20; center of parietal bone, depth of insertion: 4 mm, backward), *Dazhui* (GV14; below the spinous process of the 7<sup>th</sup> cervical and 1<sup>st</sup> thoracic vertebrae, depth of insertion: 5 mm, vertically), *Chengjiang* (CV24, 1 mm below pubic margin of low lip, depth of insertion: 1 mm, downward), *Guanyuan* (CV4, 25 mm below the umbilicus, depth of insertion: 5 mm, vertically) and *Qihai* (CV6, middle point between the umbilicus and *Guanyuan*, depth of insertion: 5 mm, vertically) according to the document<sup>[39]</sup>. Huanqiu Brand sterile acu-

puncture needles (0.3 mm in diameter and 25 mm in length, Suzhou Acupuncture Supplies, Suzhou, Jiangsu Province, China) were used by Doctor Chen, who has 5 years of acupuncture experience. In addition, electrical stimulation was given from a medical electro-acupuncture apparatus (type SDZ-II, Suzhou Medical Appliance Factory, Suzhou, Jiangsu Province, China), with a frequency of 30 Hz disperse waves and 100 Hz dense waves and 5 V intensity for 20 minutes below the level that induced visible muscle contraction.

#### Neurological function assessment

Neurological function was assessed on days 7, 14 and 28 using a modified neurological severity score by two individuals blinded to the experimental groups, as described previously<sup>[40-41]</sup>. In brief, this score is derived by assessing animals for hemiparesis (response to raising the rat by the tail or placing the rat on a flat surface), sensory deficits (*i.e.*, visual, tactile, placing, proprioception), beam balance tests (response to placement and posture on a narrow beam and time before dropping), absent reflexes (pinna, corneal, startle), and abnormal movement (seizure, myoclonus, myodystony). One point is awarded for the inability to perform a task or for the lack of a tested reflex. Neurological functions were graded on a scale of 0 to 18 (normal score = 0; maximal deficit score = 18), and higher scores represent a more serious nerve injury.

#### Immunohistochemistry for vascular endothelial growth factor

After anesthesia, rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde (pH 7.4; Sigma-Aldrich, St. Louis, MO, USA). Brains were removed carefully and fixed in paraformaldehyde solution. After paraffin embedding, a series of adjacent 3- $\mu$ m-thick coronal cryostat sections were cut from the brain tissue (anteroposterior = -1.0 to 1.0 from the needle hole). Following a series of procedures, including xylene dewaxing, gradient ethanol hydration and tap water washing, the slices were boiled in antigen retrieval solution (EDTA buffer, pH 6.0). The glass slides were cooled to room temperature and rinsed three times with PBS. The sections were incubated in mouse anti-vascular endothelial growth factor monoclonal antibody (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The following day, the slices were washed three times with PBS for 5 minutes each time. Each slice was incubated with 50  $\mu$ L MaxVision™ reagent (containing goat anti-mouse IgG complex; Fuzhou Maxim Biotech, Fuzhou, Fujian Province, China) at room temperature for 20 minutes, followed by three PBS rinses. Each slice was supplemented with 100  $\mu$ L of enhanced fresh 3,3'-diaminobenzidine solution (Fuzhou Maxim Biotech, China). The sections were rinsed with tap water, counterstained with hematoxylin, rinsed with tap water, and then processed by gradient ethanol hydration, xylene transparency, and mild gum mounting. Then, the region around the cerebral ischemia focus was observed under a light microscope (Leica DC, Germany) with 200  $\times$  magnification. The mean number of positive cells per six non-overlapping fields from each rat was counted.

#### Statistical analysis

Measurement data were analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA), and are expressed as mean  $\pm$  SD. Significance was analyzed using one-way analysis of variance and the least significant difference test, and a level of  $P < 0.05$  was considered statistically significant.

**Acknowledgments:** We would like to show our deepest gratitude to Professor Wang LP (Research Center for Neural Engineering, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences), Professor Xiong YQ, Professor Liu XL, Ms. Mu GP (Department of Central Laboratory, Shenzhen Traditional Chinese Medicine Hospital), Professor Shao MM (Department of Pathology, Shenzhen Traditional Chinese Medicine Hospital) and Professor Feng J (Department of Maternity, Shenzhen Traditional Chinese Medicine Hospital) for excellent advanced technical assistance.

**Author contributions:** Yu HB conceived and designed the study, conducted experiments, collected and interpreted the data, and wrote the manuscript. Chen PD conceived and designed the study and wrote the manuscript. Yang ZX conducted the experiments, and collected and interpreted the data. Luo WS conceived the study, interpreted the data, and wrote the manuscript. Pi M assisted with study implementation. Wu YG and Wang L conducted the experiments, and collected and interpreted the data. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Peer review:** The present study indicated that electro-acupuncture at Conception and Governor vessels combined with HUCB-MS-C transplantation could up-regulate VEGF expression and improve neurological deficit function for cerebral ischemia/reperfusion rats, which provide novel ideas and experimental evidence for transformed medicine research in the combined treatments for cerebral ischemia.

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*Copyedited by McGowan D, Zhang DS, Wang XM, Wang J, Yang Y, Li CH, Song LP, Liu WJ, Zhao M*