Effect of Electro-acupuncture on Proliferation and Differentiation of Endogenous Neural Stem Cells in Brain Tissues of Cerebral Ischemia/Reperfusion Injury Rats Based on Notch Signaling Pathway

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Abstract

Objective: The paper discussed the effect of electro-acupuncture (EA) on proliferation and differentiation of endogenous neural stem cells in brain tissues of cerebral ischemia-reperfusion injury (CIR/I) rats based on Notch signaling pathway. Methods: Among a total of 220 Wistar rats of SPF-grade, 32 were selected to constitute group A (The sham operation group) randomly. The others were established as the middle cerebral artery occlusion/reperfusion(MCAO/R) model according to the modified suture-occluded method of Zea Longa, then all model rats were divided into group B (The model group), group C (The DAPT group), group D (The EA group), group E (The EA + DAPT group) and group F (The EA + DMOS group), 32 rats in each group randomly. The rats in each group were further divided into four subgroups randomly according to the time of EA therapy, 8 rats in each. The manipulation of EA of group D, group E and group F was: 0.22mm diameter needles were inserted into the acupoints of Baihui(GV20) and Dazhui(GV14) to 5mm depth, then connected with the electric-acupuncture apparatus for 20mins, once a day. After the electro-acupuncture therapy, rats were sampled at 7d, 14d, 21d and 28d to respectively subgroup. The integrated optical density (IOD) of BrdU/DCX and BrdU/NeuN double-stained cells were determined by the method of Immunofluorescence. The expression of Notch1 protein was determined by method of Western Blot. Results: (1) The IOD of BrdU/DCX Double-stained Cells: Compared with group A, the IOD of BrdU/DCX double-stained cells of other groups increased significantly (P<0.01) at all time points. Group C was significantly lower than group B (P<0.05). Group D was significantly higher than group B, C and E (P<0.01). Group F was significantly higher than group E (P<0.01). (2) The IOD of BrdU/NeuN Double-stained Cells: Compared with group A, the IOD of BrdU/NeuN double-stained cells of other groups increased significantly (P<0.01) at all time points. Group C was significantly higher than group B (P<0.05). Group D was significantly higher than group B, C and E (P<0.01). Group E was significantly higher than group C (P<0.05). Group F was significantly higher than group E (P<0.01). (3) The Expression of Notch1 Protein: The level of Notch1 protein in group D was the highest, The next was group E and the lowest was group A. Conclusion: EA can promote the proliferation and differentiation of NSCs. Notch signaling pathway participates in the process of EA promoting the proliferation of NSCs at least to a certain extent; Notch signaling pathway does not participate in or is not mainly involved in the process of EA promoting the differentiation of NSCs. Our research also confirmed that EA therapy had a significant effect in the early and middle stages.
Keywords
Notch Signaling Pathway, Electro-acupuncture(EA), Cerebral Ischemia / reperfusion Injury(CIR/I).

1. Introduction
With the aging population, cerebrovascular disease(CVD) has become one of the main killers in China[1]. Of those survivors, 70%-80% have different degrees of disability[2]. In 2012, the Health Ministry of China survey showed that the stroke population was over 10 million, 80% of whom were ischemic stroke[3]. Nowadays, the prevention and treatment of CVD have become the focus in the medical area.

Neural stem cells (NSCs) are the pluripotent stem cells in the central nervous system, which have self-renewal ability and multidirectional differentiation potential, and can be induced into different mature brain cells, neurons, astrocytes, and oligodendrocytes. The proliferation and differentiation of the neural stem cells are regulated by various signaling pathways[4]. The Notch signaling pathway is a highly conserved signal transduction pathway in the evolutionary process in vertebrates as well as invertebrates, which can regulate the development and differentiation of various tissues and organs through intercellular interaction. The present studies show that the Notch signaling pathway participates in the process of the proliferation and differentiation of Endogenous NSCs [5,6,7].

Previous studies show that the cerebral ischemia/reperfusion injury can temporarily stimulate the proliferation and differentiation of NSCs, which can directly affect the remodeling of the central nervous system[8,9]. A large number of previous studies have confirmed that electro-acupuncture(EA) can improve the physical ability of ischemic stroke patients, improve their microcirculation, reduce the toxicity of NO, regulate the content of neuropeptide substances and neurotransmitters, inhibit the apoptosis of nerve cells, promote the expression of central GAP-43 and SYP, increase the remodeling of central nervous system, etc[10,11,12,13]. Nevertheless, the effect of EA on NSCs and their relationship with the Notch signaling pathway remains unclear. More researches are needed to clear the therapeutic mechanism of EA in treating ischemic stroke. This study was designed to discuss the effects of EA on the proliferation and differentiation of endogenous NSCs in cerebral ischemia/reperfusion injury(CIR/I) rats based on Notch signaling pathway.

2. Experimental Materials
2.1 Experimental Animals
220 healthy Wistar rats of SPF-grade, male, 3~4 months of age, weight 250 ~ 280g (provided by experimental animal center of Guangzhou University of Chinese Medicine, under animal certificate number: YXK (Guangdong) 2012-0117). All the experiments were carried out in the animal center of Medical department of Jinan University. The experiment operation were in accordance with the regulations of management. The feeding followed the principle of humanity. 32 rats were selected from 220 rats randomly to constitute the sham-operation group. The rest were established into the middle cerebral artery occlusion/reperfusion(MCAO/R) models according to the modified suture-occluded method of Zea Longa.

2.2 Main Reagents.
Monofilament nylon suture (2636,Cinontech, Beijing, China); Gamma-secretase Inhibitor (DAPT),Dimethyl sulfoxide(DMSO), Bromodeoxyuridine(BrdU)(Nuosi.tech, Guangzhou, China); Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0), 4',6-diamidino-2-phenylindole(DAPI), phosphate buffer saline(PBS), Anti-Doublecortin Rabbit pAb, Anti-BrdU Mouse mAb, Anti-NeuN Rabbit pAb, FITC conjugated Donkey Anti-goat IgG (H+L)(servicebio, Wuhan, China); HRP-labeled Goat Anti-Rat IgG (H+L), Electro-chemiluminescence(ECL), Bovine Serum Albumin(BSA), Bicinchoninic acid(BCA) Protein Assay Kit , Sodium Dodecyl Sulfate-Polyacrylamide
Gel Electrophoresis (SDS-PAGE), Anti-Notch1 Mouse mAb, Tris Buffered Saline (TBS) (Labgene, Guangzhou, China); Polyvinylidene fluoride (PVDF) (MilliporeSigma, US), etc.

2.3 Main Instruments
The microtome (RM2016, Leica, Germany), Microscope (XSP-C204, COIC, China), The inverted fluorescence microscope (Eclipse Ti-S, Nikon, Japan), Digital camera (DS-Ri1-U3, Nikon, Japan), The electric acupuncture apparatus (HANS-200E, Nanjing, China), Alpha Innotech (Alpha Ease FC, US), Image-Pro plus 6.0, etc.

3. Methods
3.1 Animal Model
The MCAO/R model was established according to the modified suture-occluded method of Zea Longa. Rats were anesthetized with 10% chloral hydrate (3.5ml/kg) by intraperitoneal injection. Neck disinfection and the right side incision were performed. The cervical vessels were separated gently. The right external carotid artery (ECA) and the common carotid artery (CCA) were ligated in turn. A "V" small incision was cut in the right CCA, and then a monofilament nylon suture was inserted into the skull. When there was resistance, it meant the suture had arrived at the middle cerebral artery (MCA). The length of insertion of the suture was about 1.8~2.0cm. After the thread was well fixed, the incision was stitched by layer. 2 hours later, the suture was pulled out of 1cm gently and the ends were cut off. The neurologic behavior of rats was scored in 2 hours after the rats woke up according to the Zea-Longa score. The rats with a score of 1~3 were included in this study. A total of 160 MCAO/R models were established successfully.

The animals of the sham-operation group were operated as follows: only the cervical vessels were separated and then the incision was stitched, without the operation of ligating vessels and inserting suture.

3.2 Groups
All 32 sham-operation models constituted Group A (The sham-operation group). All 160 MCAO/R models were randomly divided into Group B (The model group), Group C (The DAPT group), Group D (The EA group), Group E (The EA+DAPT group) and Group F (The EA + DMOS group), with 32 rats in each group. The rats in each group were randomly divided into subgroups of 7d, 14d, 21d and 28d, with 8 rats in each subgroup.

3.3 Interventions
Group A and B: 24 hours after the operation, all rats began to receive an intraperitoneal injection of BrdU (50mg/kg), twice a day with an interval of 8 hours, for 3 days.

Group C: The manipulation of the intraperitoneal injection of BrdU (50mg/kg) was the same as group A. At the same time, all rats began to receive an intraperitoneal injection of DAPT (100mg/kg), once a day for 7 days.

Group D: The manipulation of the intraperitoneal injection of BrdU (50mg/kg) was the same as group A. At the same time, all rats began to receive the EA therapy. The manipulation was: 0.22mm diameter needles were inserted into the acupoints of Baihui (GV20) and Dazhui (GV14) to 5mm depth, then connected with the electric-acupuncture apparatus with the frequency of 2/15HZ and the electrical current stimulation intensity of 1mA for 20min, once a day. The different subgroups had received the EA therapy for 7, 14, 21 and 28 days respectively.

Group E: The manipulation of the intraperitoneal injection of BrdU (50mg/kg) and DAPT (100mg/kg) was the same as group C. The manipulation of the EA therapy was the same as group D.

Group F: The manipulation of the intraperitoneal injection of BrdU (50mg/kg) was the same as group A. At the same time, all rats began to receive an intraperitoneal injection of DMOS (10ml/kg), once a day for 7 days. The manipulation of EA therapy was the same as group D.
3.4 Sampling and Processing
Rats in different subgroups were sampled on the 7th, 14th, 21th or 28th respectively. Rats were anesthetized with 10% chloral hydrate (3.5ml/kg) by intraperitoneal injection. The brain tissues was perfused with 4% paraformaldehyde solution continuously. The right hippocampus took out from cranial cavity was fixed it in 4% paraformaldehyde solution at room temperature for 4-6h. 5 samples were randomly selected from all 8 samples in each subgroup, and then these samples were rinsed with PBS, dehydrated with descending ethanol, embedded in paraffin and sectioned into 5μm-thick slices following conventional procedures. These paraffin section would be used to determinate the integrated optical density (IOD) of BrdU/DCX Double-stained cells.

The remaining 3 samples were not processed immediately and would be used to determinate the expression of Notch1 protein later on.

3.5 Immunofluorescence Assay
After deparaffinization and rehydration, the sections were placed in a container filled with EDTA solution (1mM, pH=9) for antigen retrieval. After the sections were slightly dried, histochemical pen was used to circle around the tissue (to prevent the antibody from flowing away), and 3% BSA was added to the circle to cover the tissue evenly, and the tissue was sealed at room temperature for 30 minutes. The section were incubated with the each of primary antibody mixtures (BrdU/DCX 1:50 or BrdU/NeuN 1:50) at 4℃ for 36h. Rinsed the sections with 0.01M PBS (pH=7.4) for 3 times again and incubated with the secondary antibody solution in a cassette at 37℃ for 50min. Rinsed the sections with 0.01M PBS (pH=7.4) 3 times once again, and stained by DAPI for 10min. Rinsed the sections 0.01M PBS (pH=7.4) 3 times again, then the sections were observed and photographed under the fluorescence microscope. The IOD of the Double-stained cells was analyzed by Image-Pro Plus 6 software.

3.6 Western Blot Experiment
The hippocampus tissues were rinsed with TBS buffer (pH=7.4) for 3 times, homogenized with cold saline and centrifugated to get supernatant for future determination. The supernatant was separated by 10% SDS–PAGE and transferred onto polyvinylidene fluoride membrane (PVDF). The blots were rinsed with Tris-buffered saline and 0.05% Tween-20, blocked with 5% milk in TBS buffer for 1 h and incubated with anti-Notch1 mouse mAb. Rinsed the blots with TBS buffer 3 times again, and then incubated them with the secondary antibody. Rocked gently for 30 min. Poured off the secondary antibody solution from membrane and washed twice for 10min with TTBS buffer. Poured off TTBS buffer from membrane and added developing reagent. Rocked PVDF gently, monitoring development. When the bands can be seen clearly, stop development by washing membrane with distilled water for 30min with 3 changes. The film was scanned and archived, and the image processing software was arranged and decolorized. The image analysis software processing system was used to analyze the optical density of the target band.

3.7 Statistical Analysis
Statistical analysis was performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The measurement data were represented as the mean values± standard deviation (X±s). The experimental results were evaluated using single factor analysis of variance (One-way ANOVA), A P value of less than 0.05 was considered to indicate statistical significance.

4. Results
4.1 Comparisons of The IOD of BrdU/DCX Double-stained Cells among Groups
In each group, the IOD of BrdU/DCX double-stained cells reached its peak on the 14th day and then decreased gradually. Compared with Group A, the IOD of BrdU/DCX double-stained cells of the other 5 groups was significantly increased at each time point (P<0.01). Compared Group B, the IOD of BrdU/DCX double-stained cells of Group C was significantly lower at each time point (P<0.05). Compared with Group D, the IOD of BrdU/DCX double-stained cells of Group B, C and E all
significantly decreased at each time point (P<0.01). Compared with Group F, the IOD of BrdU/DCX double-stained cells of Group E was significantly lower at each time point (P<0.05). Comparison between Group D and F revealed that there was no statistical difference at each time point (P>0.05). (Figure 1,2)

Figure 1: Comparisons of The IOD of BrdU/DCX Double-stained Cells among Groups.
*indicates P<0.01 (vs. Group A); #indicates P<0.05 (vs. Group B); △indicates P<0.05 (vs. Group B,C,E); □indicates P<0.05 (vs. Group F).

Figure 2: Photographs of BrdU/DCX Double-stained Cells of Different Group (×200)

4.2 Comparisons of The IOD of BrdU/NeuN Double-stained Cells among Groups
In each group, the IOD of BrdU/NeuN cells was the highest on the 21st day. Compared with Group A, the IOD of BrdU/NeuN cells of the other 5 groups was significantly increased at each time point (P<0.01). Compared with Group C, the IOD of BrdU/NeuN cells of Group B was significantly lower at each time point (P<0.05). Compared with Group D, the IOD of BrdU/NeuN cells of Group B, C, and E significantly decreased at each time point (P<0.01). Compared with Group F, the IOD of BrdU/NeuN cells of Group E was significantly lower at each time point (P<0.05). Comparison between
Group D and F revealed that there was no statistical difference at each time point (P>0.05). (Figure 3,4)

*indicates P<0.01 (vs. Group A); #indicates P<0.05 (vs. Group B); △indicates P<0.05 (vs. Group B,C,E); □indicates P<0.05 (vs. Group F).

4.3 Comparison of The Expression of Notch1 Protein among Groups

At each time point, the Notch1 protein expressed to a certain degree in hippocampus of rats in each group. In general, the level of Notch1 protein in group D was the highest, the next was group E and the lowest was group A. The results suggested that Notch1 protein was low-expressed when the cerebral ischemia and hypoxia were absent. Once the cerebral ischemia and hypoxia injury occurs, the Notch signaling pathway would be activated immediately and the expression of Notch1 protein began to increase. At the same time, the therapy of EA can further promote the expression of Notch1 protein, thereby stimulating the proliferation and differentiation of endogenous NSCs.
In the EA group, the expression of Notch1 protein was the highest on the 14th day, and then decreased gradually, which indicated that EA had a significant effect in the early and middle stages of cerebral ischemia and hypoxia. (Figure 5)

Figure 5: The Expression of Notch1 Protein of Different Groups

5. Discussion

Cerebral ischemia and hypoxia can cause a series of neuronal apoptosis and necrosis, leading to nervous system dysfunction. The key to clinical treatment of brain injury treatment is to restore the nerve function and remodel of the central nervous system through repair and regeneration of neurons[14,15]. NSCs have the potential of self-renewal and multi-directional differentiation, which can partly replace the damaged or dead nerve cells and promote the recovery of nerve function. In the central nervous system of adult mammals, NSCs are mainly concentrated in the subventricular zone (SVZ) of the lateral ventricular wall and the subgranular layer (SGZ) of the dentate gyrus of the hippocampus. NSCs are in a "dormant" state Normally, but when stimulated by pathological factors (such as cerebral ischemia, inflammation, etc.), these "dormant" NSCs are activated into the stage of proliferation and differentiation, migrate to specific areas and replace or repair damaged nerve cells, thereby improving the functional defect[16].

The proliferation and differentiation of NSCs is a complex process which is regulated by many factors[17]. The regulatory pathway and interaction mechanism of NSCs have not been fully elucidated by present, but numbers of study have shown that Notch signaling pathways are involved in the regulation of the proliferation and differentiation of NSCs after brain injury[18,19,20].

DAPT is a kind of gamma-secretase inhibitor, Which is the substrate of gamma-secretase that can inhibit the activity of Notch indirectly, thereby affecting cell signal proliferation and differentiation[21]. The IOD of BrdU/DCX and BrdU/NeuN double-stained cells in the hippocampus of the model group were significantly higher than those of the sham-operation group. The results suggest that cerebral ischemia/hypoxia injury can promote the proliferation and differentiation of NSCs in the injured area. The IOD of BrdU/DCX double-stained cells in the hippocampus of Group B was higher than that of Group C, but the IOD of BrdU/NeuN double-stained cells of Group B was lower than that of Group C. It was confirmed that the Notch signaling pathway was activated after cerebral ischemia/hypoxia injury and participated in the proliferation of NSCs.

The results also showed that EA could promote the expression of BrdU/DCX double-stained cells, while DAPT could inhibit the effect of EA therapy. It was proved that EA therapy could promote the proliferation of NSCs after cerebral ischemia/reperfusion injury through the Notch signaling pathway to some extent. At the same time, EA therapy could also promote the expression of BrdU/NeuN double-stained cells, confirming that EA could promote the differentiation of NSCs. However, the
addition of DAPT can reduce the effect of EA on promoting the differentiation of NSCs, which indicated that Notch signaling pathway did not participate in or was not mainly involved in the process of EA promoting differentiation of NSCs. DAPT may interfere with the process of differentiation of NSCs by EA through other signaling pathways. Therefore, we speculated that Notch signaling pathway participates in the process of EA promoting NSCs proliferation at least to a certain extent, while Notch signaling pathway does not participate in or is not mainly involved in the process of EA promoting NSCs differentiation. However, the specific mechanism of EA therapy and the relationship between EA therapy and DAPT need to be further studied.

The results also confirmed that the IOD of BrdU/DCX double-stained cells of Group D reached its peak on the 14th day and then decreased gradually, which indicated that EA had a significant effect in the early and middle stages.

In conclusion, the result suggested that Notch signaling pathway was involved in the therapy of EA in treating cerebral ischemia-hypoxia injury. EA can enhances the proliferation of NSCs through regulating the Notch signaling pathway, which could provide sufficient cell sources of NSCs differentiating into mature neurons. At the same time, EA may activate other signaling pathways to promote the differentiation of NSCs into mature neurons, so that damaged neurons can be repaired and replaced, which could promote the recovery of nerve system function objectively.

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References


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