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Acupuncture combined with exercise training at different time points on nerve repair of cerebral ischemia-reperfusion injury in rats and its effects on the expressions of Nestin, bFGF and EGF

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Abstract. – OBJECTIVE: The aim of the study was to observe the neuroreparative effect of electroacupuncture in rats with cerebral ischemia-reperfusion injury, and to explore the difference in the therapeutic effect of acupuncture on different acupoint groups after cerebral ischemia-reperfusion.

MATERIALS AND METHODS: Experimental rats were randomly divided into: sham operation group, model group, electroacupuncture group, rehabilitation group, and Diankang group (electroacupuncture + rehabilitation training). There were 24 rats in each group, and the focal cerebral ischemia-reperfusion model was established by Zea-Longa suture method. After modeling, it took 4 hours to electroacupuncture at Baihui and Dazhui points, which was used to observe the changes of nerve function in rats with signs of keel nerve function defect. Protein expression was detected by immunohistochemistry.

RESULTS: Compared with the model group, the EA 3d, 7d, 10d groups and the rehabilitation group had no significant difference in promoting the expression of Nestin ($p>0.05$). There was a significant difference ($p<0.01$). After cerebral ischemia-reperfusion injury, the expression of bFGF and EGF on the ischemic side was stronger. The peak of bFGF expression appeared earlier, and the peak of EGF expression appeared later. The expression of bFGF and EGF in cerebral ischemic cortex at different time points of ischemia in electroacupuncture group, rehabilitation group and Diankang group was increased, and the response was enhanced. The effect of Diankang group on the upregulation of bFGF and EGF was more significant ($p<0.01$, $p<0.05$).

CONCLUSIONS: Under the influence of different effects, Diankang is superior to simple treatment in improving ischemic neurological dysfunction. This may be related to the fact that Diankang can promote the proliferation of neural stem cells and the expression of neurotrophic factors on the ischemic side of the rat brain.

Key Words:
miRNA-106, Pediatric osteosarcoma, PI3K/AKT signaling pathway.

Introduction

Cerebrovascular disease is a common disease with high morbidity, morbidity, mortality and recurrence rate, which seriously threatens human health. Neurons are particularly sensitive to ischemic injury, so ischemic stroke is the most common cerebrovascular disease, and the incidence of ischemic stroke has been on the rise in recent years[1,2]. Traditional acupuncture and moxibustion has a significant effect on the treatment of cerebral ischemic diseases[3]. After ischemic stroke, neurogenesis is regulated by neuroplasticity signals. In the past, most scholars had focused on the research of neuroplasticity and restoring nerve cell function[4], ignoring the effect of neural stem cells in the marginal zone of cerebral infarction.

NSCs, existing in the nervous system, can be proliferated and differentiated into neurons, astrocytes and oligodendrocytes specific for preneural somatic cells; its basic biological characteristics include self-renewal ability and multi-directional differentiation potential[5-7]. Nestin is the sixth category of intermediate filament protein that only exists in neuroectodermal epithelial stem cells[8]. Nestin is the sixth category of intermediate fila-

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ment protein that only exists in neuroectodermal epithelial stem cells[7]. It belongs to a family of fibroskeletal proteins that indicate the degree of cell differentiation. Its expression begins with neural plate formation and disappears after neurons begin to migrate and fully differentiate. It is a cellular antigen expressed by immature nerve cells and is a currently recognized NSC marker. The presence of NSC can be identified by detecting the expression of Nestin[9]. BFGF, a mitogenic cationic polypeptide consisting of 155 amino acids, plays an important role in the development of the nervous system and the maintenance of normal nerve function; it can promote the repair and regeneration of neuron damage[10,11]. EGF is a polypeptide consisting of 53 amino acids, which is expressed in both the developing brains and adult brains, can stimulate the proliferation of various cells (including astrocytes) through its receptors[12-15]. Studies[16-18] at home and abroad have shown that the synergistic effect between bFGF and EGF can improve the efficiency of neural stem cell proliferation and differentiation.

The combined treatment mode of electroacupuncture and modern rehabilitation medicine participates in the repair of nerve function by mobilizing the proliferation of endogenous neural stem cells (NSCs). It also improves the microenvironment after ischemia in the brain region, thereby promoting the proliferation of nerve cells, and ultimately promotes the repair of damaged nerve function. In this experiment, it was observed the effects that the electroacupuncture combined with rehabilitation training had on the proliferation of neural stem cells and the expression level of neurotrophic factors in the brain of rats with middle cerebral artery embolism. This study aims at expanding the use of comprehensive treatment methods to promote the recovery of brain function from the perspective of nerve cell proliferation and differentiation and providing a new theoretical basis for the combination and application of traditional therapy and modern rehabilitation methods.

Materials and Methods

Experimental Materials

Animals and Groups

A total of 120 healthy male SD rats, weighing 280-350 g, were purchased from the Laboratory Animal Center of Nanjing Medical University, License number: SCXK(Su) 2008_0004. The animals were free to drink and feed during the experiment. They were fasted for 8 h and allowed to drink water before surgery. 120 rats were randomly divided into 5 groups: sham operation group, model group, electroacupuncture group, rehabilitation group and Diankang group (electroacupuncture + rehabilitation training). There were 24 animals in each group, and 8 animals at each of 3 different time points 3 d, 7 d and 10 d. In the sham operation group, only the common carotid artery was separated, and no suture embolization was performed. This experiment had been approved by the Experimental Animal Ethics Committee of Anhui University of Traditional Chinese Medicine (Ethics Approval Number: AHUCM-rats-2020006).

Main Reagents and Instruments

Mouse anti-Nestin monoclonal antibody, rabbit anti-basic fibroblast growth factor (bFGF) polyclonal antibody, rabbit anti-epidermal growth factor (EGF) polyclonal antibody were purchased from Boorson (Beijing, China). Immunohistochemical staining streptavidin-biotin complex kit, DAB reagent was purchased from Zhongshan Golden Bridge (Beijing, China). DZF-6030 Electric heating constant temperature blast drying oven was purchased from Shanghai Xinmiao Medical Equipment Manufacturing Co., Ltd (Shanghai, China). TS-12A Biological Tissue Automatic Dehydrator, CS-VI type spreader was purchased from Hubei Xiaogan Hongye Medical Instrument Co., Ltd (Hubei Xiaogan, China). Paraffin Tissue Microtome was purchased from Leica (München, Germany). Model BX51 Olympus Microscope was purchased from Olympus (Tokyo, Japan).

Rehabilitation Training Equipment

Roller Mesh Trainer

A circular mesh apparatus with 1.0 m long and 60 cm in diameter, had 4 compartments in the middle for training 4 rats simultaneously. This device had a fixed base and a hand crank at one end for training by hand cranking at 5 r/min, which was used to train the rats in grasping, rotating, walking and other movements.

Balance Beam Training

A square wooden stick with a length of 170 cm and a width of 2 cm was made, placed flat at 7 cm from the ground, and used as a balance beam

to let the rats walk on it, mainly to evaluate and train the balance function of the rats.

Rotor Training

The midpoint of a wooden stick with 150 cm long and 4.5 cm in diameter was fixed on a 3 r/min rotator, which was used to assess and train the dynamic balance of the rats by alternating left and right rotation.

Mesh Screen Training

The mesh belt was 50 cm×40 cm, and the mesh was 1 cm×1 cm. The left and right sides and the top of the screen were framed with 25 cm high wooden boards. The height of the screen was 80 cm from the ground, and the bottom was covered with a 12 cm thick sponge. The screen was placed horizontally with the rat on top. Then, one end of it slowly raised and the screen was turned into a vertical position within 2 s. After holding for 5 s and observe whether the rat fell off the screen or grasped the screen with its front grasp. The screen training is mainly used to objectively evaluate the grasping ability and muscle strength of the rat forepaw.

Operation Steps

Make a Model

The animal mode of middle cerebral artery ischemia-reperfusion was established by referring to Longa et al[19] to make external carotid artery suture method. The brief surgical steps are as follows: A 10% chloral hydrate (300 mL/kg) was injected into the rat's abdominal cavity to anesthetize it. The rat was fixed with supine position and disinfected after its hair was shaved. All branches of the external carotid artery were electrocoagulated, and the main trunk of the external carotid artery was cut off and left free. The common and internal carotid arteries were temporarily clamped with a miniature non-destructive arterial clamp. A "V" shaped incision was made at the stump of the external carotid artery and a pre-prepared PA with varnish at the tip was inserted rapidly into the internal carotid artery approximately (18.0±0.5) mm (until a little resistance is felt). After the operative area was cleaned, the full layers were sutured, and the incision was closed. The blocking time was 2 hours. The PA line was withdrawn for about 10 mm during reperfusion, and the withdrawal was stopped when there was a sensation of falling out. The withdrawn PA line was cut about 10 mm to prevent the animal from grasping the line off when it came to life, causing death by hemorrhage.

0-Point Scoring Method for Signs of Neurological Deficits

Referring to the zero-point scoring method of Longa et al[19] for neurological deficit signs, neurological deficit signs were scored after reperfusion of 4 h, 3 d, 7 d, and 10 d within 2 hours of ischemia. Whether the model of cerebral ischemia in rats was successful was judged by the neurological function score. Zero point was indicated as no signs of neurological injury; 1 point as inability to fully extend the contralateral front paw; 2 points as turning the body in a circle towards the hemiplegic side during voluntary movement; 3 points as tilting the body towards the contralateral side during voluntary movement; 4 points as inability to walk spontaneously and loss of consciousness. The rats with 1-3 points were selected. Selection criteria: Although the rats showed signs of cerebral ischemia after the model was prepared, however, those who have one of the following factors were excluded (Table I).

If the number of animals in each experimental group was less than the predetermined number due to the above factors, it was supplemented by random sampling.

Electroacupuncture and Rehabilitation Training

The rats in the electroacupuncture group were acupunctured at Baihui and Dazhui, the positioning of each point is based on the experimental animal points developed by Koo ST and Yin CS et al[20]After reperfusion, the rats were acupunctured with the No. 30 milli-needles of 0.5 inch. The millineedles were connected to electro-acupuncture stimulators. The frequency of the electro-acupuncture stimulators was 5~50 Hz. The intensity was decided by the fact that the

Table I. Filter criteria.

Exclusion criteria
1. Neurological symptom scores of 0 and 4
2. Subarachnoid hemorrhage was found when the rat brain was taken
3. HE staining of frozen sections of brain tissue without ischemic pathological changes
4. Rats that died before the observation time point

rat's limbs were lightly shaken without hissing or struggling, and the electro-acupuncture was applied once a day for 20 mins each time. After the evaluation of various indicators after the operation, the rats in the rehabilitation group were placed in the roller mesh trainer every day for rotation training, balance beam training, walking training on the rotarod, and screen training, lasting for a total of 30 mins, which was carried out 5 times a week. The rats of Electro-acupuncture of the Diankang group were acupunctured like those of the electro-acupuncture group. They carried out rehabilitation training like the rehabilitation group. After electroacupuncture, the sham operation group and the model group were not treated. After modeling, neurological function evaluation was given to the 5 groups before sacrifice at 3 d, 7 d, and 10 d.

Tissue Processing

At the respective time points, eight rats in each group were anesthetized (10% chloral hydrate intraperitoneal injection, 300 mg/kg). The rat's thorax was opened and a catheter was inserted from the left ventricle apical to the aortic arch, and a small incision was made through the right auricle. Approximately 300 mL of 0.9% saline at 37°C was injected rapidly into the aorta until the fluid from the right atrium became clear. Approximately 300 mL of 4% paraformaldehyde was injected and the rat was fixed by cardiac perfusion. The first 1/3 was rapidly perfused, and the last 2/3 was slowly perfused until the rat's limbs and tail were rigid. The brain was then taken out immediately, and the preoptic chiasm and cerebellum tissues were removed, which were fixed overnight with 4% paraformaldehyde. Brain tissues were taken out after having been fixed in 4% paraformaldehyde solution for 1 week. Dehydrated, dipped in wax, embedded, sliced, spread, baked and deparaffinized for immunohistochemical staining.

Primary Measurement Index

Immunohistochemical Staining and Observation of Nestin, bFGF and EGF

Staining for Nestin, bFGF and EGF immunohistochemistry was performed according to the kit instructions, respectively, using the immunohistochemical SABC method. All rat brain specimens were serially coronally sectioned, and three sections were selected at contiguous levels (frontoparietal cortex). The three different angles with the same magnification (×400) were selected in each of the three sections in the frontoparietal cortex. The number of Nestin-immunopositive cells, the total area of bFGF and EGF-positive cells and the mean optical density were calculated by image processing system of each angle (Total area or mean optical density was indicated in total number of pixels). Evaluation criteria: cells-stained brown was regarded as positive, and the average percentage of positive cells in each section was calculated.

Statistical Analysis

All data were indicated as mean ± standard deviation ($x \pm s$). Data statistics were processed by SPSS 16.0 for windows software package (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used, and Student Newman-Keuls test was used to compare the means between two groups. $p<0.05$ and $p<0.01$ indicated that the difference was statistically significant.

Results

Comparison of the Number of Positive Cells Expressing Nestin Protein in Fronto-Parietal Cortex of Rats in Each Group

The results are shown in Table II. Compared with the model group, the number of Nestin-posi-

Table II. Comparison of the number of Nestin-positive cells in the ischemic cortex of each group ($\bar{x} \pm s$).

Group	3d	7d	10d
Simulation operation group	2.26 ± 1.16	2.87 ± 1.68	2.52 ± 2.04
Model group	5.35 ± 1.24**	13.42 ± 3.50**	11.32 ± 2.27**
Electroacupuncture group	12.74 ± 2.45**#	22.25 ± 3.53**#	20.52 ± 2.32**##
Rehabilitation group	10.12 ± 1.46**#	19.44 ± 2.82**#	18.26 ± 2.52**#
Diankang group	13.22 ± 2.28**##	26.33 ± 2.54**##Δ	22.06 ± 3.18**##

Compared with the sham operation group, **$p<0.01$; compared with the model group, #$p<0.05$, ##$p < 0.01$; compared with the electroacupuncture group and the rehabilitation group, Δ$p < 0.05$.

tive cells in the fronto-parietal cortex of the three treatment groups at three time points was significantly different ($p<0.01$, $p<0.05$). Compared with the electroacupuncture group and the rehabilitation group, the number of Nestin-positive cells in the Diankang group was higher at 7 days ($p<0.05$). There was no significant difference in the number of positive cells at 3 d and 10 d ($p>0.05$). There was no significant difference in the number of positive cells at three time points between the electroacupuncture group and the rehabilitation group ($p>0.05$).

Comparison of the Total Area of FGF Protein-Positive Cells in the Fronto-Parietal Cortex of Rats in Each Group and Comparison of the Mean Optical Density of bFGF Protein Expression in Fronto-Parietal Cortex of Rats in Each Group

The results are shown in Table III. It can be seen from Table III that, compared with model group, the total area and average optical density of bFGF-positive cells in the three treatment groups were significantly increased at 3 d, 7 d, and 10 d ($p<0.01$). When the three treatment groups were compared, there was no statistically significant difference in the total area and mean optical density of bFGF-positive cells at 3 d, 7 d and 10 d in the electroacupuncture and rehabilitation groups ($p>0.05$). The total area of bFGF positive cells at 3 d, 7 d and 10 d and the mean optical density of bFGF positive cells at 3 d and 7 d were higher in the Diankang group compared with the electroacupuncture group and rehabilitation group ($p<0.05$, $p<0.01$).

Comparison of the Total Area of Positive Cells Expressing EGF Protein in the Fronto-Parietal Cortex of Rats in Each Group and Comparison of the Mean Optical Density of EGF Protein Expression in Fronto-Parietal Cortex of Rats in Each Group

It can be seen from Table IV that, compared with model group, there were significant differences in the total area and average optical density of EGF-positive cells at 7d and 10d in the three treatment groups and the model group ($p<0.01$, $p<0.05$). There was no significant difference in the total area and average optical density of EGF-positive cells at three time points in the electroacupuncture group and the rehabilitation group ($p>0.05$). At 7d and 10d, compared with the electroacupuncture group and the rehabilitation group, the total area and average optical density of EGF positive cells in the Diankang group were statistically significant ($p<0.01$, $p<0.05$).

Discussion

This experiment observed the expression of Nestin immunopositive cells existed in and around ischemic cortex after cerebral ischemia. With the increase of time, the number of Nestin immunopositive cells also increased to a certain extent. This suggests that cerebral ischemia may act as a stimulating factor to enhance the proliferation of resting NSCs and increase the number of Nestin-positive cells in the ischemic side of the brain. Using of traditional acupuncture therapy and modern rehabilitation therapy can promote the proliferation of NSC and increase the expression of bFGF and EGF after cerebral ischemia injury. This suggests that electroacupuncture and rehabilitation training may promote the proliferation and differentiation of NSCs by affecting the expression of neurotrophic factors, thereby promoting the survival and repair of damaged neurons in the brain. This experiment also found that the integrated treatment of acupuncture combined with rehabilitation training was more effective than acupuncture alone or rehabilitation training alone, which suggests that the use of multiple therapies within a given treatment period is an important way to treat cerebral ischemic disease and to improve neurological dysfunction caused by cerebral ischemia.

Any study has certain limitations. There are still some shortcomings in this study. First of all, there are few basic research results of electroacupuncture combined with rehabilitation training to prevent cerebral infarction are few. Secondly, in terms of this experiment, the sample size is relatively small, and the self-made rat rehabilitation equipment is relatively simple, which makes certain shortcomings in the experimental process. Although NSC proliferation was observed in this experiment and the expression of bFGF and EGF was increased after ischemic injury, the proliferation of cells in the brain induced by focal cerebral ischemia may not be a direct result of the local stress response after cerebral ischemic injury. The proliferation of cells in the brain induced by focal cerebral ischemia may be caused by changes in certain factors in the brain

Table III. Comparison of the total area of bFGF-positive cells in each group (10^3, $\bar{x} \pm s$) and comparison of the average optical density of bFGF-positive cells in each group ($\bar{x} \pm s$).

Group	Comparison of the total area of bFGF-positive cells in each group			Comparison of the average ptical density of bFGF-positive cells in each group		
	3d	7d	10d	3d	7d	10d
Simulation operation group	3.87 ± 0.66	3.36 ± 0.47	3.07 ± 0.69	0.06 ± 0.02	0.04 ± 0.02	0.04 ± 0.02
Model group	7.54 ± 0.32**	7.16 ± 0.98**	6.98 ± 1.41**	0.16 ± 0.03**	0.14 ± 0.02**	0.13 ± 0.01**
Electroacupuncture group	14.41 ± 0.44**##	12.27 ± 0.76**##	11.85 ± 0.84**##	0.24 ± 0.01**##	0.21 ± 0.03**##	0.20 ± 0.02**##
Rehabilitation group	15.37 ± 0.62**##	13.65 ± 0.51**##	12.96 ± 0.46**##	0.23 ± 0.02**##	0.20 ± 0.02**##	0.20 ± 0.01**##
Diankang group	20.36 ± 0.68**##ΔΔ	18.40 ± 0.91**##ΔΔ	16.72 ± 0.82**##Δ	0.28 ± 0.02**##Δ	0.25 ± 0.03**##Δ	0.22 ± 0.02**##

Comparison of the total area of bFGF-positive cells in each group: Compared with the sham operation group, $^{**}p < 0.01$; compared with the model group, $^{\#\#}p < 0.01$; compared with the electroacupuncture group and the rehabilitation group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$. Comparison of the average optical density of bFGF-positive cells in each group: Compared with sham operation group, $^{**}p < 0.01$; compared with model group, $^{\#\#}p < 0.01$; compared with the electroacupuncture group and rehabilitation group, $^{\Delta}p < 0.05$.

Table IV. Comparison of the total area of EGF-positive cells in each group ($\times 10^3$, $\bar{x} \pm s$) and comparison of the average optical density of EGF-positive cells in each group ($\bar{x} \pm s$).

Group	Comparison of the total area of EGF-positive cells in each group			Comparison of the average optical density of EGF-positive cells in each group		
	3d	7d	10d	3d	7d	10d
Simulation operation group	3.84 ± 0.93	4.01 ± 1.27	3.98 ± 0.76	0.0721 ± 0.01	0.0813 ± 0.02	0.08 ± 0.01
Model group	8.47 ± 0.83**	8.71 ± 1.13**	8.66 ± 1.05**	0.1172 ± 0.02**	0.1333 ± 0.02**	0.1242 ± 0.02**
Electroacupuncture group	10.72 ± 1 .53**	14.25 ± 1.48**#	13.17 ± 1.04**#	0.1492 ± 0.02**	0.1637 ± 0.01**#	0.1489 ± 0.03**#
Rehabilitation group	9.76 ± 0.82**	14.03 ± 1.35**#	12.2 ± 1.15**#	0.1583 ± 0.03**	0.1664 ± 0.01**#	0.1527 ± 0.02**#
Diankang group	10.03 ± 2.18**	17.48 ± 2.77**## Δ	16.46 ± 2.02**##Δ	0.1562 ± 0.02**	0.2088 ± 0.02**##ΔΔ	0.1812 ± 0.01**##Δ

Comparison of the total area of EGF-positive cells in each group: Compared with the sham operation group, $^{**}p < 0.01$; compared with the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with the electroacupuncture group and the rehabilitation group, $^{\Delta}p < 0.05$; Comparison of the average optical density of EGF-positive cells in each group: Compared with the sham operation group, $^{**}p < 0.01$; compared with the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with the electroacupuncture group and the rehabilitation group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$.

or the release of signaling substances, which act on the cells in the brain through different pathways causing changes in the proliferation of cells in the brain. The signal transduction mechanisms stimulating autologous NSC proliferation *in situ* after cerebral ischemia are unclear and will be an important part of our next research.

Conclusions

The results of this study show that the use of multiple means to treat cerebral ischemic diseases can promote the structural and functional recovery of neurons in ischemic areas. The protective mechanism of EA combined with rehabilitation training on ischemic brain injury may be related to the proliferation of endogenous neural stem cells and the expression level of related proteins. At the same time, the combination of traditional therapy and modern rehabilitation methods has a certain difference in the treatment of cerebral ischemia, and the combination of the two methods may be more effective than the single treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

WeiLan prepared the manuscript, provided the study concept and design, and analyzed and interpreted the data. ZhenLi and HeyanLi conducted experimental modeling and functional training of rats, and ZongshengSong provided acupuncture treatment. All authors read and approved the final version of the manuscript.

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