Effects of Electroacupuncture Combined with Repetitive Transcranial Magnetic Stimulation on the Expression of Nestin in Neural Stem Cell after Focal Cerebral Ischemia in Adult Rats*

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Abstract **Objective:** To investigate the influence of electroacupuncture (EA) combined with repetitive transcranial magnetic stimulation(rTMS) on the temporal profile of nestin expression after induction of focal cerebral ischemia in adult rats and to explore the mechanism of EA combined with rTMS in treating ischemic brain injury. **Method:** The model of transient focal ischemia was produced by occlusion of middle cerebral artery. Seventy-five Wistar rats were randomly divided into normal group, model group, EA group, rTMS group and EA+rTMS group. The neurologic impairment rating and ability of learning and memory were observed at the 7th、14th and 28th d after infarction respectively. Meanwhile, Western blotting was used to observe the number of nestin expression positive cells. **Result:** Nestin-positive cells were found in cortex, subgranular zone (SGZ), subventricular zone (SVZ) of the ipsilateral side at different time points after cerebral ischemia. The number of nestin-positive cells peaked at the 7th d, began to decrease at the 14th d and was significantly higher in EA+rTMS group than that in model group ($P<0.05$), then almost reached normal at the 28th d. The improvement of neural motor function deficits as well as the indexes of learning and memory were more obvious in EA+rTMS group compared with model group ($P<0.01$, $P<0.05$). These effects were most obvious in EA+rTMS group compared with the EA and rTMS group ($P<0.05$). **Conclusion:** EA and rTMS possess the potency of building up and can increase the number of nestin-positive cells in some brain regions after focal cerebral ischemia, which might be one of the important mechanisms of EA combined with rTMS in treating ischemia brain injury.

Key words focal cerebral ischemia;neural stem cell; nestin; electroacupuncture; repetitive transcranial magnetic stimulation

Cerebral arterial thrombosis causes singularly adverse effects on the quality and duration of life. As traditional viewpoint, neural function deficits after cerebral ischemia are nonvolatil because cerebral neurons are terminally differentiated cells and they are impossible to restore once damaged. But the recent researches showed that neurogenesis occured in discrete regions of adult brain, including the rostral subventricular zone (SVZ) of lateral ventricles and the subgranular zone (SGZ) of dentate gyrus (DG). Neurons that arise in the SVZ travel via the rostral migratory stream to the olfactory bulb [1] and also enter association neocortex [2], and new neurons leaving the SGZ migrate into the adjacent DG granule cell layer. Moreover, stimulation of endogenous neural precursors in adult brain could provide therapeutic potential [3]. In the present study, we investigated the effects of electroacupuncture (EA) combined with repetitive transcranial magnetic stimulation (rTMS) on the temporal profile and cellular expression of nestin immunoreactivity from the 7th to 28th d after 2h of middle cerebral artery occlusion (MCAO) in adult rats.

1 MATERIALS AND METHODS

1.1 Modeling and grouping of animals

A total of 75 adult male Wistar rats weighing 180 to 220 g were employed and divided into normal group, model group, EA group, rTMS group and EA+rTMS group. Each group was divided into three subsets according to phase of the 7th, 14th and 28th d (n=5, per time point). Ischemia was induced by intraluminal MCA occlusion with a suture in accordance with Liao Wei-jing [4]. Briefly, the right external carotid artery was ligated with 5/0 silk suture and dissected distally, and the right internal carotid was isolated and separated from the vagus nerve. The extracranial branch of right internal carotid was ligated near its origin with suture.

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A length of 18.5–19.0mm 5/0 nylon suture with rounded tip was advanced from external carotid artery into the lumen of internal carotid artery until it blocked the origin of middle cerebral artery. The rectal temperature was controlled at 37℃ with a feedback regulated water heating system. Two h after MCAO, reperfusion was performed by withdrawal of suture until the tip cleared the internal carotid artery. Experimental rats were sacrificed at the 7th, 14th and 28th d of reperfusion (n=5, per time point) for Western blotting and immunohistochemistry detection.

Score and screening by Bederson standard[5] 24h after operation. 0: no behavioral deficiency; 1: the anterior limb stoop; 2: resisting ability of side-thrust descend and the anterior limb stoop; 3: the behavior was the same as score 2 but followed with spontaneous revolution. The rats of score 1–3 were for study.

1.2 Treatment

Treatment of EA was applied in EA group and Baihui (DU20) and Dazhui (DU14) were selected as the acupoints to perform EA with 0.30mm×40mm needles. Head And Neck Support (HANS) device was connected with needles by modulated frequency current. The general electric in tensity was ranging from 1 to 2mA and EA therapy was given for 30 min every day.Treatment of rTMS was applied in rTMS group, and the coil of magnetic stimulator(made in Denmark, Dantec Corp.) was sticked to the fixed head of rat and it's center located at the point 9mm in front of right auricula. The stimulus frequency was 0.5Hz and the intensity was 70% of the max output.Twenty continued stimulus every section, two sections per day. EA and rTMS were combined in EA+rTMS group, parameters and course of treatment were the same as EA and rTMS group. The therapy begun at 24h after operation and ended at 1d before sacrificing. The rats in normal and model groups were received no therapy.

1.3 Items and methods of observation

1.3.1 Neurologic impairment rating: Neurologic impairment rating was carried out before sacrificing at the 7th, 14th and 28th d according to Bederson standard[5].

1.3.2 Electro-jumping stand experiment: A passive avoidance reaction box sized of 50cm×10cm×20cm was used as jumping stand device. Alternating current of 40V was switched on after 3min accommodation 1d before sacrificing, then the time of jumping to security after shock was recorded as reaction period. The error was the number of shock within 5min. The reaction period and number of error were taken as learning results. Duplicate test was administered 24h later, switched on immediately as soon as the rat was placed on security stand. The time of jumping from stand to copper cage was taken as latency period and the error was the number of shock within 5min. The latency period and number of error were taken as memory results.

1.3.3 Western blotting: All rats were subjected to rapid transcardial perfusion with 200ml heparinized saline (25℃) at a pressure of 100 mmHg, and then were decapitated. Forebrain tissue was immediately obtained from interaural 12mm to interaural 2mm. Each specimen was dissected on a icy bed into hemisphere ipsilateral to the MCAO. The segments were quick-frozen in isopentane and dry ice and stored at −80℃ until homogenization. Then, these segments were thawed on ice, and wet weight was rapidly measured in grams. The pieces of tissue were homogenized by adding a 1:5 tissue weight to protein extraction buffer, containing 0.9% NaCl, 7mM β-mercaptoethanol, 50mM Tris-HCl (pH=7.5), 2mM EDTA, and 1% sodium dodecyl sulphate (SDS), in a glass homogenizer. Homogenate samples (5μg) were mixed with an equal volume of a sample buffer and heated at 95℃ for 5min and resolved by SDS, 7.5% polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Both rainbow colored protein molecular weight markers and enhanced chemiluminescence (ECL) protein molecular weight markers were used in each SDS-PAGE. Blots were blocked at room temperature for 1h in a blocking buffer, Tris buffered saline-Tween (TBS-T) containing 5% dried milk. Blots were incubated in the primary antibody, mouse anti-rat nestin (mAb, Rat-401), diluted in the blocking buffer (dilution: 1:500) for 1h at room temperature and were washed extensively with TBS-T. They were then incubated in a horseradish peroxidase-conjugated secondary antibody, diluted in the blocking buffer (dilution: 1:1000) for 1h and washed for an additional hour.

Detection was by enzyme linked electrochemiluminescence (ECL) according to the recommendations of the manufacturer.

1.4 Statistical analysis

All data were expressed as mean±standard deviation, and statistic analysis was performed with SPSS 10.0 software. Analysis of variance (ANOVA) was adopted in analyzing the data, and $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Comparison on the scores of neural motor function deficits

The scores in normal group were 0. The scores in EA, rTMS, EA+rTMS group were significantly lower as compared with model group, and the scores of EA+rTMS group were significantly lower as compared with EA, rTMS group at the 7th, 14th and 28th d (Table 1).

2.2 Experimental results of electro-jumping stand

There were significantly differences on every index of

learning and memory, $P<0.01$ result in EA, rTMS, EA+rTMS groups as compared with model group at the 7th, 14th and 28th d ($P<0.05$). The same results were seen in the comparison of EA+rTMS group with EA and rTMS groups($P<0.05$) (Table 2).

Table 1 Comparison on the scores of neural motor function deficits in every group ($\bar{x}\pm s$, n=5)

Group	the 7th d	the 14th d	the 28th d
Normal	0	0	0
Model	2.68±0.06	2.52±0.12	1.60±0.13
EA	1.96±0.09①	1.68±0.10①	0.59±0.15①
rTMS	1.97±0.09①	1.64±0.15①	0.52±0.14①
EA+rTMS	1.28±0.07①②	1.01±0.10①②	0.30±0.12①②

Note: ①compared with Model group, $P<0.01$; ②compared with EA, rTMS group

Table 2 Comparison of scores of learning and memory in electro-jumping stand in every group ($\bar{x}\pm s$, n=5)

Item/Group	the 7th d	the 14th d	the 28th d
learning result(reaction period, s)			
normal	13.88±9.26	14.63±8.46	13.76±7.96
model	78.28±8.76	49.52±7.12	37.93±8.14
EA	62.76±7.39①	34.68±7.15①	26.69±7.15①
rTMS	59.87±8.24①	32.74±8.14①	25.72±6.92①
EA+rTMS	49.24±9.07①②	25.01±8.10①②	15.50±7.34①②
learning result(number of error)			
normal	1.32±0.69	1.48±0.86	1.41±0.72
model	7.84±1.11	4.84±1.16	1.59±0.11
EA	6.86±1.62①	1.50±0.12①	0.97±0.15①
rTMS	6.87±1.34①	1.14±0.13①	0.98±0.13①
EA+rTMS	4.86±1.21①②	1.92±0.82①②	0.49±0.14①②
memory result(latency period, s)			
normal	271.36±17.22	267.88±32.86	269.93±33.37
model	40.07±7.35	67.57±20.19	127.36±22.76
EA	51.89±17.52①	158.31±34.64①	211.73±19.91①
rTMS	52.87±19.83①	187.57±32.37①	213.80±17.54①
EA+rTMS	82.94±8.86①②	246.26±42.32①②	261.70±18.45①②
memory result(number of error)			
normal	0.81±0.19	0.78±0.16	0.76±0.11
model	4.87±0.23	2.74±0.18	2.42±0.15
EA	3.63±0.09①	1.57±0.12①	1.48±0.72①
rTMS	3.56±0.08①	1.47±0.13①	1.42±0.72①
EA+rTMS	2.61±0.13①②	0.82±0.12①②	0.62±0.10①②

Note: ①compared with Model group, $P<0.05$; ②compared with EA and rTMS group, $P<0.05$

2.3 Western blotting

Incubation with the mAb nestin Rat-401 revealed weak staining lanes in normal brains. Nestin immunoreactivity peaked at the 7th d, began to decrease at the 14th d, and were significantly higher in EA, rTMS, EA +rTMS groups than that in model group ($P<0.05$), then almost reached normal at the 28th d in the ischemic hemispheres. There was no difference between EA group and rTMS group ($P>0.05$) (Figure 1—2).

3 DISCUSSION

Neural stem cells (NSCs) bring new prospect for treatment of cerebral arterial thrombosis because of their self-renewal ability and multipotential differentiation potency. However, research from domestic and abroad on ischemic region

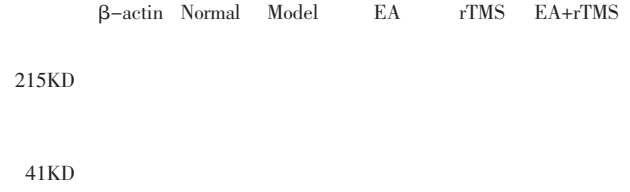

Figure 1 Representative nestin expression in 5μg of protein/lane of ipsilateral hemispheres

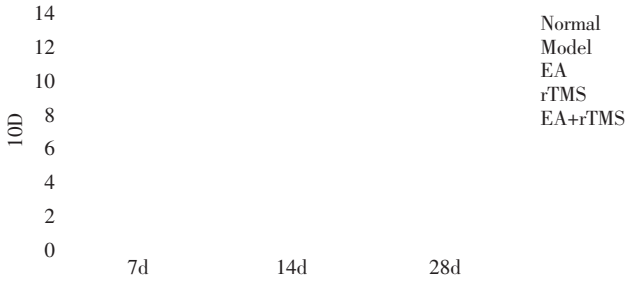

Figure 2 Time courses of optical densities of nestin immunoblots in the ipsilateral hemispheres from each group of rats

explantation of NSCs cultured in vitro showed low survival rate and noncontrollability of exogenous NSCs. So, there is great potentiality in the strategy of activating endogenous NSCs to treat cerebral arterial thrombosis. Many literatures reported that acupuncture could provide favourable regulated effects on related gene and neurotrophy factors after ischemia for recent years [6—8]. Our previous study showed that EA or EA combined with rTMS could promote the release of basic fibroblast growth factor (bFGF) and growth of NSCs[9—10].

Nestin is distinct from all other classes of intermediate filament proteins because it is abundantly and transiently present in multipotential stem cells of the developing central nervous system(CNS) in embryogenesis[11], e.g., neuroepithelial cells, radial glia, germinal matrix cells and vascular cells and then it declines in all but the vascular cells at later embryonic stages and the vascular cells and ependymal cells in the adult brain [12—13]. Nestin has been used to analyze proliferation of undifferentiated CNS cells in the developing rat nervous system and in immortalized CNS precursor cell lines[14—17].

Nestin expression is present in reactive astrocytes following induction of hippocampal lesions by kainic acid[18]. Nestin mRNA expression (by in situ hybridization) and nestin immunoreactivity are detected in reactive astrocytes at the 0—7th and 7th d, respectively, following 2h of focal cerebral ischemia and in reactive glial cells and some cortical neurons at the 7th—8th d after 10—20 min of global brain ischemia in adult rats[19].

The present study shows that neurologic impairment relieved in model group without any intervention, which indicates that cerebral ischemia can stimulate the potent of

self-proliferation of neural stem cells and illustrates brain tissue possess the ability of plasticity and self-recovery. EA and rTMS provide significant effects of improvement on the neurologic impairment and the ability of learning and memory at every time point. The study also showed that nestin immunoreactivity peaked at the 7th d, began to decrease at the 14th d, and were significantly higher in EA group, rTMS group, and EA combined with rTMS group than that in model group, then almost reached normal at the 28th d. So, it is concluded that treatment of EA combined with rTMS possess has the potency of building up in treating cerebral ischemia, can increase the number of nestin-positive cells in some brain regions after focal cerebral ischemia, which might be one of the important mechanisms of EA combined with rTMS in treating ischemia brain injury. However, the concrete mechanism is still not distinct and need to be further explored.

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