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The Effect of Electroacupuncture on the Expression of p53 mRNA in Newborn Rats following Hypoxic-ischemic Encephalopathy

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[Abstract] Aim: To explore the neuroprotective effects of electroacupuncture on the expression of p53 in newborn rats following hypoxic-ischemic encephalopathy (HIE).**Method:** The hypoxic-ischemic models were made in new-born SD rats aged 7 days by colligating bilateral common carotid artery(CCA). All rats were randomly divided into sham-surgery group, model group and electroacupuncture group (EA), and the successfully established rat models were subdivided in to 1d, 3d, 7d, 21d,three time-phases. The changes of morphology in the injured cortex were observed by pathology; the real-time PCR was used to determine the expression level of P53mRNA in the injured cortex. **Results:** HE and pathological staining indicated that the nerve cells are arranged in order, the structure is normal, and the cell outline and the nucleus are clear at 21d in EA group. The expression of p53mRNA in 1d, 3d, 7d, 21d, four points in EA group were significantly increased than those in model group ($P<0.05$). **Conclusion:** EA plays a neuroprotective effect through promoting the expression of p53mRNA in hypoxic-ischemic brain tissuein rats.

[keywords] Electroacupuncture; Hypoxic-ischemic Encephalopathy; Neuroprotective effect; Acupoints; p53; Rats

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INTRODUCTION

Neonatal hypoxic-ischemic encephalopathy(HIE) is the main reason of the death of perinatal asphyxia, which has high disability and mortality, and is the main cause of mental retardation, epilepsy, cerebral palsy, blindness, and so on[1].When the cells were stimulated by DNA damage, hypoxia, or high temperature and other environmental stimuli, the gene of mouse double microsomal-2 gene(MDM2) was inhibited by the ubiquitin-proteasome dependent degradation pathway, which resulted in the degradation of p53 and from the reduction in nuclear transfer, nuclear p53 accumulation, involved in DNA damage repair[2,3]. It is well known that the acupuncture is a traditional Chinese treatments, which have a long history in the world.It has been used as a treatment or as an adjuvant modality for patients with stroke. It has becoming more and more popular in the western countries[4,5].In recent years, studies have shown that acupuncture can participate in cerebral ischemia area in nerve repair, protect and repair damaged neurons, play a protective effect on brain tissue[6].Therefore, this study hopes to explore the neuroprotective mechanism of acupunctureon hypoxic ischemic brain damage in young rats with HIE model, so as to provide experimental the oratical basis for the acupuncture to intervent neonatal HIE.

MATERIALS AND METHODS

MATERIALS

Experimental animals

Experiments were performed at the Experimental Center, Qingdao University Medical College, from September 2014 to May 2015.

A total of mother tape sixteen broods(including mothers, 8 to 9 newborn rats per brood) Sprague-Dawley(SD) rats aged 7d of either sex, weighing 10.5 to 15.0g(average weight, 12.36±1.3g), were provided by Experimental Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20140001). All rats were housed at the ambient temperature of 20-25°C, and fed with their mothers. All experimental procedures were performed in accordance with the the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by theMinistry of Science and Technology of the People's Republic of China.

Animal grouping

A total of 157 experimental animals feeding in the conditions with house temperature control in 20 - 25°C, light-dark cycle 12h, breast feeding. The rats were randomly divided into sham-surgery group (25 cases) and HIE group (127 cases)followed by HIE model operations, there are 51 rats died during the modeling, and finally, 76 rats were successfully established as the HIE models, Then the successfully established HIE model rats were randomly divided into model group38 rats and the acupuncture group 38 rats.After the operation 1d, 3d, 7d, 21d of four time points, 6 rats were randomly select from each group respectively to collect the brain tissue for detecting the indicators.

Reagents

cDNA: PrimeScriptTM RT reagent Kit with gDNA Eraser kit, purchased from Takara company;SYBR® Premix Ex TaqTM II (Takara), purchased from Takara company;Other biochemical reagents were purchased from Sigma company.Other biochemical reagents were purchased from Sigma company.

Major equipment

Low temperature refrigerator (Haier,-20°C),Ultra-low temperature freezer (Thermo, -80°C),autoclave (JWFU, AMA440), Rat stereotactic instrument, G6805-IIelectric acupuncture apparatus,Conventional PCR instrument (Biometra, T-gradient),Lowspeed centrifuge (Eppendorf, KA-1000),Electronic balance (Shanghai Instrument Co., Ltd., FA),Drying box (Shanghai test instrumentfactory,101A-2type),Ultra pure water meter (pro PS-9000703 LABCONCO-Water),Gel image Analyzer(Bk-Rad,Gel Doc TMEQ170-8060),ABI 7500 automated quantitative PCR instrument (ABI, USA).

METHODS

Establishment of HIE animal model

The HIE model was established by bilateral common carotid artery (CCA) ligation method incombination with hypoxic treatment described by Rice[7]. Has numbered 127 in neonatal SD rats at first, and then animals were anesthetized by inhaling ether. In the supine position,a midline ventral incision was made to expose the CCA, which were carefully separated and ligated of bilateral CCA, severed blood vessels, suture the skin(ligated using a 5/0 silk suture), placed in a transparent airtight containers of homemade,After a two-hour restoration, and then we put them in a sealed transparent vessel for a warm bathe at 37°C. Then the vessel was passed into gas with low content of oxygen (including 8% of oxygen and 92% of nitrogen) at the velocity of 1 L/min for 1.5 h.The survivors were kept warm for another one hour and then received behavioral tests. At last,the survivors were put back to the cage and kept warm. The rats still unconscious or dead were removed.For the sham-surgery group, after anesthesia, the bilateral CCAwere separated without ligation and the incision was sewed up without any hypoxic treatment. Behavioral tests were made four hours later, and rats in this group showed no behavioral abnormality.

Intervention of different processing methods

According to the randomized block,total of 76 successful model rats were included in the survey and were divided into 38 rats of the acupuncture group and model group respectively.

(1)Sham-surgery group:The bilateral CCA were separated,without ligation, and the rats were without hypoxia after incisions sutured.

(2) Model group:The bilateral CCA were separated and ligated, and incisions were sutured. Then the vessel was passed into gas with low content of oxygen, but without any other treatment.

(3) EA group: Animal model making just the same as model groups. However,in the EA model group,four acupoints including Baihui (GV20), Dazhui (GV14), Quchi (LI11) and Yongquan (KI1) were chosen for treatment. Localization of the acupoints was based on the International Standard Scheme for acupoint names of acupuncture drafted by the experimental acupuncture branch of the China Acupuncture Academy[8]. Four acupoints were chosen, where the position according to the ordinary acupoints for acupuncture in rats[9].From the second day after ischemia-hypoxia, rats were treated with a 0.5 inch needle, along the skin acupuncture "Baihui" 5mm(Baihuiacupoint is located in the center of the parietal bone).Pierce" Dazhui"5mm(Dazhuiacupoint is located between the seventh cervical vertebra and the first thoracic vertebra, just in the center of the back). Straight on both sides "Quchi" 8-10mm(Quchiacupoint in the midpoint of the line between the outer end of the elbow stripes and the epicondyle of the humerus),and rapidly inserted into "Yongquan" without leaving the needle. The two acupoints "Baihui" and "Quchi" were connected with G-6805 electric acupuncture apparatus (Shanghai Huayi Medical Instrument Factory, China), and receive electroacupuncture for 10 minutes with the continuous wave at the frequency of 5 to 10Hz with the local tissue shivering slightly, and with the voltage between 3 to 5V. The acupuncture therapy was given once a day for consecutive 1d, 3d, 7d and 21d respectively.

Hematoxylin-eosin (HE) staining for determination of neurologic damage

Rats were anesthetized by inhaling ether at the indicated time. The left ventricle was cannulated and perfused with phosphate-buffered saline (PBS) (preheated at 37°C), and then perfused and fixed with 4% (w/v) paraformaldehyde (in 0.1 M PBS (pH 7.4), precooled at 4°C) firstly at full speed till convulsion of the limbs ceased, then perfusion was kept at the velocity of 1ml/min. Then, the brain was quickly separated with the cerebellum and the brainstem removed and placed in 4% paraformaldehyde for 24 hours at 4°C. The specimens were dehydrated with 20% sucrose and frozen. The frozen sections were then serially cut into 20 μm thick coronal slices. HE staining was performed according to the standard protocol. The sections were observed under a light microscope (Nikon, Tokyo, Japan) using a magnification of ×400, and photographed.

Detection of P53mRNA expression in the injured cortex by realtime RT-PCR

Six rats in each group were randomly selected. Total RNA was extracted from the injured cortex (about 1mm × 1mm × 1mm) of rats using Trizol (In vitro gen, USA) according to the manufacturer's instructions. After that the total RNA was examined by agarose gel electrophoresis, the concentration was determined by UV spectrophotometer, and finally stored at -20°C. The primers were designed by Shanghai Sangon Company. PCR were performed by reference to the instructions of SYBR®Premix Ex Taq^{TM} II (Takara) kit. Forward primer forP53: 5'-GCCATCTACAAGAAGTCACAACAC-3', Reverse primer for p53: 5'-CTGTCGTCCAGATACTCAGCATAC-3', product size 146bp; Forward primer for GADPH: 5'-GAAGGTGAAGGTCGGAGT-3'; Reverse primer for GADPH: 5'-GAAGATGGTGATGGGATTTC-3', product size 168bp. Prime Script^{TM} RT reagent kit with gDNA Eraser (Takara) kit was used to synthesize cDNA. Reaction procedure is:95°C,30s;95°C,5s;60°C,34s; 40 cycles. Then reverse transcription was cDNA, and the PCR reaction was carried out by agarose gel electrophoresis, gel imaging system photographed and electrophoretic band analysis. The results were processed using $2^{-\Delta\Delta Ct}$ method, significant analysis *t*-test using SPSS software.

Statistical Analysis

SPSS 18.0 software was applied for the statistical analysis. The data was expressed as mean ±SD. Factorial analysis was applied in the multi-group comparison after the homogeneity test of variances; and *t*-test were applied for the two-group comparison. Values were considered to be significant when *P* was less than 0.05. □

RESULTS

Electroacupuncture treatment significantly ameliorates neuronal damage caused by hypoxic-ischemic injury

In the sham-surgery group, HE staining showed clear cell morphology, structure of neuron cells arranged in order, no bubble formation, clear nuclear membrane, cytoplasm is pale red, nuclei blue, nucleolus large, round, nucleoli Center(Fig.1 A1-4).

In the control model group, model 1d mirror blurry, visible signs of nerve edema and necrosis, sieve mesh, derangement of cell structure and neuronal cytoplasmic condensation dark cell clearance, vacuolar degeneration of the cytoplasm, and nucleolus disappear(Fig.1B1). At 3rd day, the nerve cell necrosis have increased markedly, and structural derangement, triangles and polygons, nucleus membrane fuzzy, kernels are not clear, with cellular structure disappears, many nuclear pyknosis and cracking(Fig.1B2).7d to 21d mirror is clear, fuzzy nucleus membrane, vacuolization, hyperchromatic nuclei condensation, swelling and degeneration of nerve cells, endoplasmic reticulum dilated(Fig.1B3-4).

In the EA group, The pathological changes of nerve cells in the EA group were similar to that in the model group, swelling of the nerve cells can be observed, endoplasmic reticulum, mitochondria swell(Fig.1C1). At 3d, EA group's Vision is not clear, massive swelling of the nerve cells, degeneration, necrosis, necrosis of cells are polygonal, but less severe than the control model group (Fig.1C2). At 7th day, it can be seen in the state of nerve cells is clear, the hierarchical structure of nerve cells arranged in an orderly manner, and a small amount of proliferation of glial cells, The extent of the lesion was reduced (Fig.1C3). At 21st day of EA group, can clearly be seen in the view of neurons arranged in order, the nerve cell degeneration and necrosis was not obvious, light swelling of nerve cells, neurogliacells increased, outline and nucleoli than clear (Fig.1C4).

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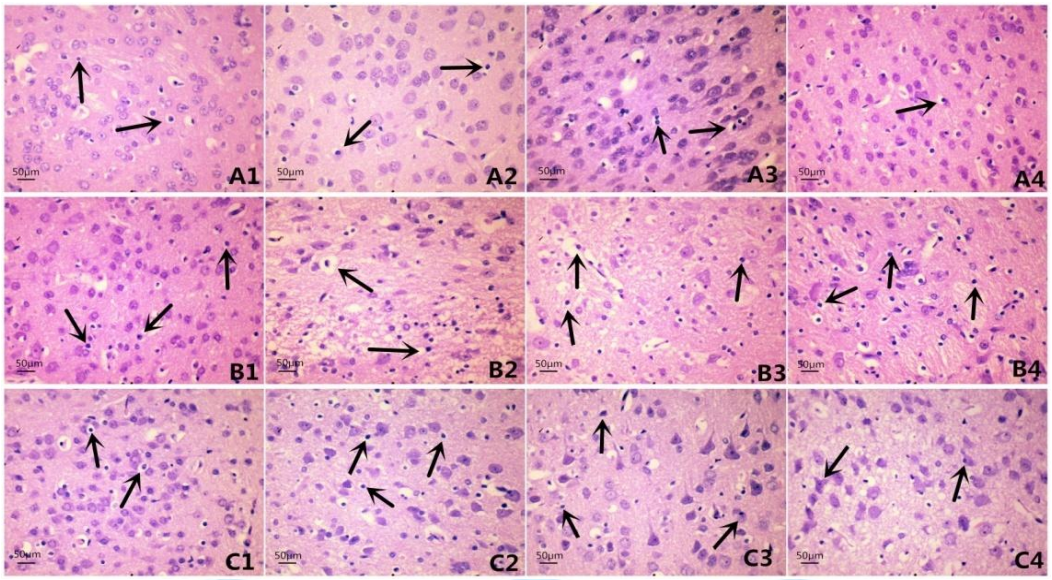

Figure 1 Cell structure in the cortex of rats (optical microscope ,HE staining x 400)

Expression changes of p53mRNA in the Cerebral Cortex of Rats from three experimental groups

P53mRNA expression levels were detected with real-time RT-PCR. At different periods of brain tissue of young rats in each group are shown in Table 1, Figure 2.Compared with that of the sham-surgery group, expression of p53mRNA in 1d began to increase in the model group, 3d to 7d continued to increase significantly (P<0.05), the level of p53mRNA continued increasing at 3d and 7d significantly (P<0.05)compared with sham operation group.In EA group,the expression of p53mRNAalso began to increase at 1d, and continued to increase significantly at 3d, 7d, 21d compared with the control group (P<0.05).The expression of p53mRNA can be further promoted after EA treatment.

Table 1 Expression changes of p53mRNA in the Cerebral Cortex of Rats（$\bar{x}$±s）

组别	N	1d	3d	7d	21d
假手术组	6	1.0770±0.1148	1.1282±0.1247	1.0091±0.1708	1.0291±0.2097
模型组	6	2.3809±0.2227*	5.147±0.0305*	6.1579±0.0142*	7.3103±0.4869*
电针组	6	2.7501±0.1315*Δ	6.5323±0.0732*Δ	8.4324±0.1491*Δ	10.0341±0.1347*Δ
P		0.032	0.026	0.017	0.013

Note: The data was expressed as mean ±standard error (n=6); *Compared with sham-surgery group, P<0.05; Compared with model group, P<0.05.

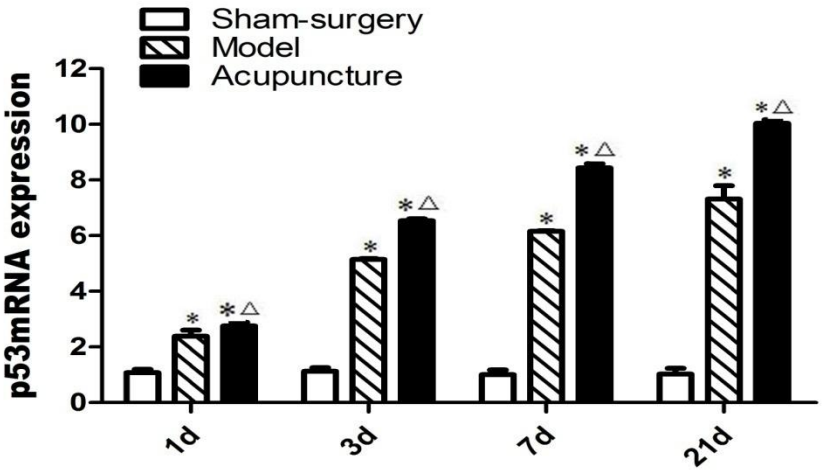

Figure 2 p53mRNA expression in cerebral cortex of rats at different time

DISCUSSION

The human p53 gene is composed of 11 exons and 10 inner exons, located in the 17 region of chromosome 31, and it's called 17p13.1, the total length of about 20kb, and encoding a molecular weight of 53kd nuclei of phosphorylated protein[10], Its transcription translation p53 protein encoded by 393 amino acid residues, and contain multiple functional domains, could bind to specific DNA sequences and activates transcription.A large number of studies have shown[11] that p53 regulates cell cycle and repair DNA damage, and maintenance of genome stability, inhibit the growth of tumorsthrough a series of signal transduction pathways. In cellular stress (such as hypoxia, ischemia, radiation, etc),preventing gene mutation and tumor suppression, it plays a critical role in the process. Normally, MDM2 as a negative regulator of p53 factor, which can effect on protein concentration and activity, mdm2 and p53 constitute a degradation-trans activation circulation path[12], p53 and its regulation factors product interactions and remained at a low level. When cell DNA damage, hypoxia or temperature and other environmental stimuli,MDM2 catalytic degradation of the ubiquitin-proteasome dependent degradation pathway is inhibited,and leading to the degradation of p53 and the reduction of nuclear p53 aggregation. The research have shown[13]that activation of signal transduction p53 could regulates cell to repair itself, prevent DNA damage and the p53 effect.

In this experiment, the expression of p53 in neurons of model rats began increase-regulated from 1d to 21d after cerebral ischemia-hypoxia, but the increasing trend slowed down gradually, which suggesting that the body launch the mechanism of self-protection and self-healing after cerebral ischemia-hypoxia.In the absence of interventions with hypoxia-ischemia time, rats gradually reduces this ability to self-maintenance, but compared with sham-surgery group, there are still significant differences, prompts HIE self-healing brain protection of rats. The change of p53mRNA in EA group showed an upward trend continuing significant changes, compared with the group, 3d, 7d,and 21d were significant. Speculation of acupuncture on hypoxic-ischemic brain damage of possible mechanisms for ubiquitin-proteasome-dependent degradation pathway is inhibited, bringing together nuclear p53, thereby involved in DNA damage repair and promote axon growth, so that its protective effects on damaged brain cells.

According to the results of this experiment, electro-acupuncture plays a protection role in hypoxic-ischemic brain damage by increasing-regulating the expression of p53. Description electro-acupuncture by promoting the expression of p53, on hypoxic-ischemic brain damaged rats play a protective role. And the result has great significance to the further research of the mechanism of electro-acupuncture on hypoxic-ischemic encephalopathy.

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