Research on the Effect of Electroacupuncture on MDM2 Expression in a Hypoxic-Ischemic Encephalopathy Rat Model*

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ABSTRACT: Aim: To explore the effect of electroacupuncture (EA) on the expression of mdm2 following hypoxic-ischemic encephalopathy (HIE). **Method:** The hypoxic-ischemic models were established by colligating bilateral common carotid artery in new-bore SD rats aged 7 days. All rats were randomly divided into sham-surgery, model, sham-EA, EA, antagonist and EA plus antagonist group which subdivided 1d, 3d, 7d, and 21d time-phases. Ultra-structure of neurons was observed using TEM, real-time PCR was used to determine the level of mdm2 mRNA, and the expression of mdm2 was tested with Western Blot. **Results:** TEM shown that At 21d, the damnification of neurons had evidently recovery with electroacupuncture treatment. The down-regulation of mdm2 was observed in electroacupuncture group, which was significantly lower than that in model groups ($P<0.05$). **Conclusion:** Electroacupuncture could obviously lighten the damage of ultrastructure of neurons to protect the neurons from HIE by inhibiting the expression of mdm2.

Keywords: Electroacupuncture; Hypoxic-Ischemic Encephalopathy; MDM2; Rats

INTRODUCTION

Hypoxic-ischemic encephalopathy is the brain injury in the neonate caused by perinatal asphyxia, can result in varying degrees of residual symptoms, even acute neonatal death and chronic nervous system damage[1]. HIE can lead to DNA damage and mutation of neurons, and the gene expression of mdm2 in neurons has been reported[2]. As an effective treatment on the nervous system dysfunction left by HIE, acupuncture can promote the proliferation, differentiation of neural stem cells, axonal sprouting, growth and extension, so as to achieve the effect of building neural networks [3]. In this study, the authors aimed at exploring the effect and mechanism of electroacupuncture on hypoxic-ischemic encephalopathy by observing the effect of electroacupuncture on mdm2 expression in neurons of HIE rats, and provide theoratical and experimental basis for clinical treatment.

MATERIALS AND METHODS
Experimental animals
Total of 250 Sprague-Dawley rats aged 7d of either sex, weighing 14.0 to 18.0g, were provided by

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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.

Animal grouping

Each brood was randomly divided into two groups, sham-surgery group (25 rats) and HIE model group (225 rats). Among the 190 HIE rat models, 80 were firstly injected with 5μl wortmannin (dissolved in dimethyl sulfoxide, 20 μg/kg) through left lateral ventricles, and 0.5h later received HIE modeling operation. The other 145 rats were received the modeling operation directly. After the surgery, 100 rats died and finally 125 rats were successfully established as the HIE models. Then the successfully models rats were randomly divided into five groups: model group, EA group, sham-EA group, antagonist group and antagonist plus EA group consisting of 25 rats in each group. And each of the five groups was subdivided into four subgroups in terms of four time periods of 1st, 3th, 7th and 21th day for EA treatment post-surgery, with 6-7 rats in each subgroup for the test of index.

Reagents

Mdm2 monoclonal antibody was purchased from Abcam company (ab3110); Goat anti-mouse secondary antibody was purchased from Maixin Biotech company;Other biochemical reagents were purchased from Sigma Co. Lid.; Wortmannin, DMSO (dimethyl sulfoxide) were purchased from J & K technology Co. Ltd. Beijing; SYBR® Premix Ex TaqTM II (Takara) was purchased from Takara Company; Chloroform, isopropanol, ethanol were provided by Beyotime Institute of Biotechnology.

Major equipment

Conventional PCR instrument (Biometra, T-gradient), low-speed centrifuge (Eppendorf, KA-1000), electrophoresis apparatus (Bio-Rad), gel image analyzer (Bk-Rad, Gel Doc TMEQ 170-8060), water bath oscillator (Zhejiang Jintan Kaiyuan experimental Instrument Factory, THZ-82A), ABI 7500 automated quantitative PCR instrument (ABI, USA), ultra-low temperature freezer (Thermo, -80℃), autoclave (JWFU ， AMA440), rat stereotaxic apparatus, G-6805 electro-acupuncture device.

Establishment of models

Rats was anesthetized with ether and fixed later. The model was established by bilateral common carotid artery (CCA) ligation according to the improved method of Rice[4]. In addition to the sham group, the other five groups were dealt with hypoxia: The rats were put in a sealed transparent vessel for a warm bathe at 37℃. 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 2.0 h. The survivors were put back to the cage and kept warm. The rats still unconscious or dead were removed.

Intervention of electroacupuncture

① Sham-surgery group: Bilateral CCA were separated without ligation, and after incision surued without hypoxia. ② Model group: Bilateral CCA were separated and ligatured, but without any treatment. ③ EA group: Four acupoints including Baihui, Dazhui, Quchi and Yongquan were chosen, positioned according to the ordinary acupoints for acupuncture in rats[5]. From the second day after ischemia hypoxia, rats were treated with electroacupuncture. Baihui acupoint is located in the center of the parietal bone. Dazhui is located between the seventh cervical vertebra and the first thoracic vertebra, just in the center of the back. Quchi is located in the midpoint of the line between the outer end of the elbow stripes and the epicondyle of the humerus, and Yongquan is located in the plantar anterior third (toes excluded). A needle of 0.5 inches in length was inserted horizontally backwards into Baihui at a depth of 5mm, perpendicularly inserted into Dazhui for 5mm in depth, perpendicularly inserted into Quchi for 10mm in depth and rapidly inserted into Yongquan without leaving the needle in for two to three times till minor bleeding, respectively. Both acupoints Baihui and Quchi were

connected with G-6805 electric acupuncture apparatus, and received EA for 10 min with local tissue shivering slightly (asymmetric bidirectional continuous pulse waves, frequency 5–10 Hz, voltage 3–5V). The acupuncture therapy was given once a day. ④ Sham-EA group: Needles were inserted into the point about 1 cm away from the corresponding acupoints and the other treatment was just the same as the EA group. ⑤ Antagonist group: 5μl wortmannin (dissolved in dimethyl sulfoxide, 20 μg/kg) was injected into left lateral ventricle with the guide of Stereotaxic instrument. And 30 minutes later, rats were began to built the models of HIE. ⑥ Antagonist plus EA group: 5μl wortmannin (dissolved in dimethyl sulfoxide, 20 μg/kg) was injected into left lateral ventricle with the guide of Stereotaxic instrument. And 30 minutes later, rats were began to built the models of HIE. After the establishment of models, the rats received acupuncture treatment just as the EA group.

Detection Indexes

Transmission electron microscopy (TEM): Six rats in each group at 1st, 3th, 7th and 21th day after hypoxic-ischemic injury were randomly selected respectively and anesthetized with 10% chloral hydrate. Then 100ml normal saline was perfused into the heart and the whole brain was taken out. Small amount of fresh tissue was cut from cortex around fontanelle into some small pieces of about 1mm×1mm×1mm, and fixed in 2.5% glutaraldehyde for 24 hours. Then specimens were post-fixed in 1% OsO4 for 1h. And then dehydrated with gradient of acetone, embedded in Epon812 epoxy resin. The embedded blocks were cut by the ultramicrotome into ultrathin sections of 50nm thickness. The sections were stained with uranyl acetate and lead citrate. Finally the ultrastructure of neurons was observed under the transmission electron microscope.

Reverse-Transcription Polymerase Chain Reaction(RT-PCR): Six rats in each group were randomly selected. Total RNA was extracted from the injured cortex of each rat using Trizol kit 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 TaqTM II (Takara) kit. M2m2 primers: sense 5'-CTA TCG GGT CAC AGT CTA TCA GG-3'; antisense 5'-AGT TCT CAC GAA GGG TCC AAC-3', product size 161bp; GADPH primers: sense 5'-GAA GGT GAA GGT CGG AGT-3'; antisense 5'-GAA GAT GGT GAT GGG ATT TC-3', product size 168bp. PrimeScriptTM RT reagent kit with gDNA Eraser (Takara) kit was used to synthesize cDNA. The PCR products were separated by 2% agarose gel electrophoresis and visualized by gel-imaging system. The gray value of all mRNA bands were detected by gel-imaging analysis system. The relative value of mRNA (RVM）(the gray value of mdm2 mRNA / the gray value of GAPDH) presented the abundance of mdm2 mRNA, which were determined 3 times to count the average.

Western-Blot: Six rats in each group were randomly selected. And the total protein was extracted from the brain tissue. The objective proteins mdm2 and GAPDH were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto the polyvinylidene fluoride (PVDF) membranes. Then the PVDF membrane was blocked in 5% skimmed milk solution at 4°C overnight at room temperature. After that the PVDF membrane was incubated with mdm2 primary antibody for 3h at room temperature, then incubated with goat anti-mouse second antibody for 1h at room temperature. During each step, the PVDF membrane must be rinsed sufficiently to avoid nonspecific coloration. The gel film images was developed in A-B mixed developing agent and scanned with Gel Imaging System. The gray value of all protein bands were measured and analysed with the Quantity one software. The relative value of protein (RVP) = the gray value of mdm2 / the gray value of GAPDH,

which were determined 3 times to count the average.

Statistical Analysis

SPSS 17.0 software was applied for the statistical analysis. The data was expressed as mean ± standard error ($\bar{x} \pm s$). 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

Ultrastructure of neurons (Fig.1)

Sham-surgery group: The structure of neurons in sham-surgery group was regular with distinct outline and cytoplasm, and the chromatin was uniform (Fig. 1 A).

Model group: At 1st day after hypoxic-ischemic injury, the neurons have integrated membrane, unevenly distributed chromatin and mild swelling mitochondrion (Fig.1 B1). At 3rd day, the damage of neurons increased with blurred membrane, dissolved nuclear membrane, agglutinated chromatin, and a large number of vacuoles. Apoptotic neurons can be observed (Fig.1 B2). For the self-healing of neurons, at 7th day after hypoxic-ischemic injury, the membrane of neurons was intact with a little loose nucleoplasm and uniform chromatin (Fig.1 B3). The morphology of neurons at 21st day after hypoxic-ischemic injury was similar to that of 7th day (Fig.1 B4).

Sham-EA group: The pathological change of neurons at each period of sham-EA group was similar to that of model group (Fig.1 C1-4).

EA group: At 1st day after hypoxic-ischemic injury, the ultrastructure of neurons was similar to that of model group, and the cell body was slightly swelling with blurred nuclear membrane, indistinct nucleolus and micro-bubbles around nucleus can be observed (Fig.1 D1). Slightly swelling cell body, vague nuclear membrane and nucleolus and perinuclear micro-vacuoles could also be observed in neurons at the subgroup of 3rd day, but the degree of neurons damage was lighter than that of model group (Fig.1 D2). At 7th day, the swelling of cell body was lightened, and the nuclear membrane and nucleolus became clear (Fig.1 D3). At 21st day of EA group, the ultrastructure of neurons was similar to that of sham-surgery group. The membrane of neurons was intact with a little loose nucleoplasm and generally uniform chromatin. And a few of slightly swelling mitochondria and dilated rough endoplasmic reticulum can be observed (Fig.1 D4).

Antagonist plus EA group: Compared with neurons at each period of EA group, signs of necrosis and degeneration were more serious than those in antagonist plus EA group (Fig.1 E1-4).

Antagonist group: The damage of neurons at each period was more serious than that of model group. The cytomembrane and nuclear membrane were indistinct, karyopyknosis, chromatin condensation, a large number of vacuoles in cytoplasm, dilated rough endoplasmic reticulum and degranulation were more obvious (Fig.1 F1-4).

The results of TEM showed that: at 3rd day after hypoxic-ischemic injury, the damage of neurons was obvious, and the damage became alleviated with the extension of time. Compared with model group, the damage of neurons in EA group was slighter, suggesting that electroacupuncture can alleviate the damage of neurons caused by Hypoxic-ischemic encephalopathy.
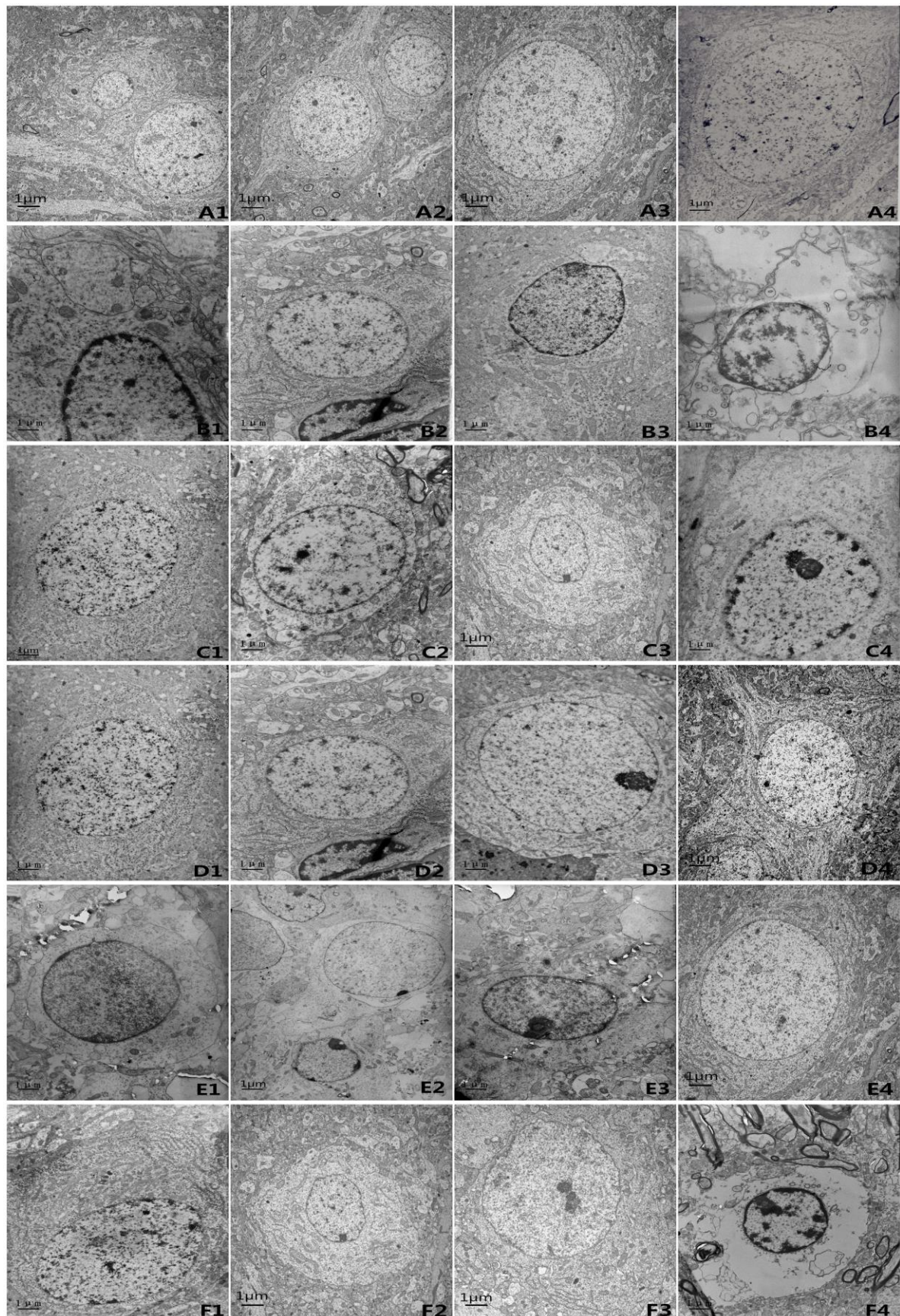
Fig. 1 Ultrastructure of cortical neurons, TEMX5K

RT-PCR

Compared with sham-surgery group, the expression of mdm2 mRNA at the 1st day decreased significantly ($P<0.05$); the level of mdm2 mRNA continued decreasing at the 3rd day, and reached the bottom on the 7th day, and the expression of mdm2 mRNA among the 1st, 3rd, 7th day in model group has significant difference compared with model group ($P<0.05$); and on the 21st day the down-regulated trend slowed, but there was still significant difference between model group and sham-surgery ($P<0.05$). The expression trend of of mdm2 mRNA in sham-EA group was similar to that of model group, and the level of mdm2 mRNA was less than that of sham-surgery group ($P<0.05$). In EA group, the expression of mdm2 was down-regulated from the 1st day, and continued decreasing till 21 day. The level of mdm2 mRNA at each period of EA group was obviously lower than that of model group ($P<0.05$). The expressions of mdm2 mRNA at 1st, 3rd, 7th day in antagonist plus EA group was less than those in sham-surgery group ($P<0.05$), and at the 21st day the level of mdm2 was less than that in model group ($P<0.05$). (Tab.1, Fig.2)

Tab.1 The expression of mdm2 mRNA of hypoxia-ischemia brain tissue（$\overline{x}$±s）

Group	1d	3d	7d	21d
sham-surgery	1.0061±0.0136	1.0061±0.0136	1.0143±0.0338	1.0129±0.0210
model	0.9411±0.0432*	0.731±0.0244*	0.6458±0.0162*	0.5549±0.0149*
sham-EA	0.9591±0.0134*	0.7336±0.0160*	0.6576±0.0138*	0.5587±0.0115*
EA	0.8500±0.0132*△▲	0.6432±0.0273*△▲	0.5932±0.0149*△▲	0.4634±0.0135*△▲
antagonist	0.9703±0.0121	0.9224±0.0123	0.823±0.0125	0.7791±0.0136
antagonist＋EA	0.8757±0.0312*	0.6824±0.0369*	0.6235±0.0510*	0.5002±0.0155*△▲
P	0.04	0.03	0.02	0.02

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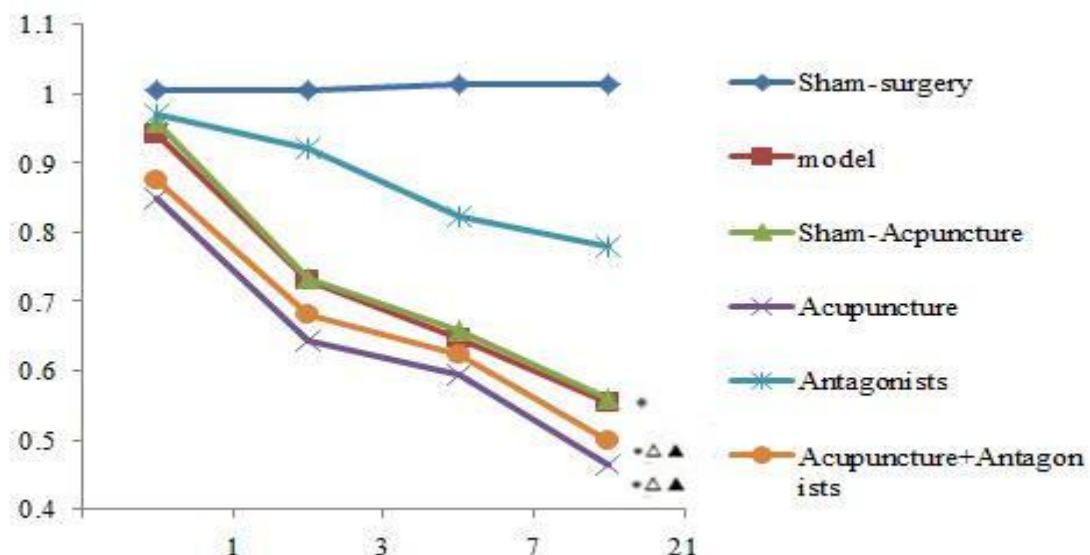

Fig.2 The expression of mdm2 mRNA at different periods of each group

Note: *Compared with sham-surgery group, $P<0.05$; △Compared with model group, $P<0.05$; ▲Compared with antagonist group, $P<0.05$.

Western Blot

The expression of protein mdm2 in model group, sham-EA group, antagonist plus EA group and EA group began to decrease at the 1st day, and continued to decrease till 21 day, while the level of protein mdm2 was obviously than that of sham-surgery ($P<$0.05). At the 1st, 3rd, 7th, 21st day, the expression of protein mdm2 showed significant difference between EA group and model group ($P<$0.05). (Fig. 2)
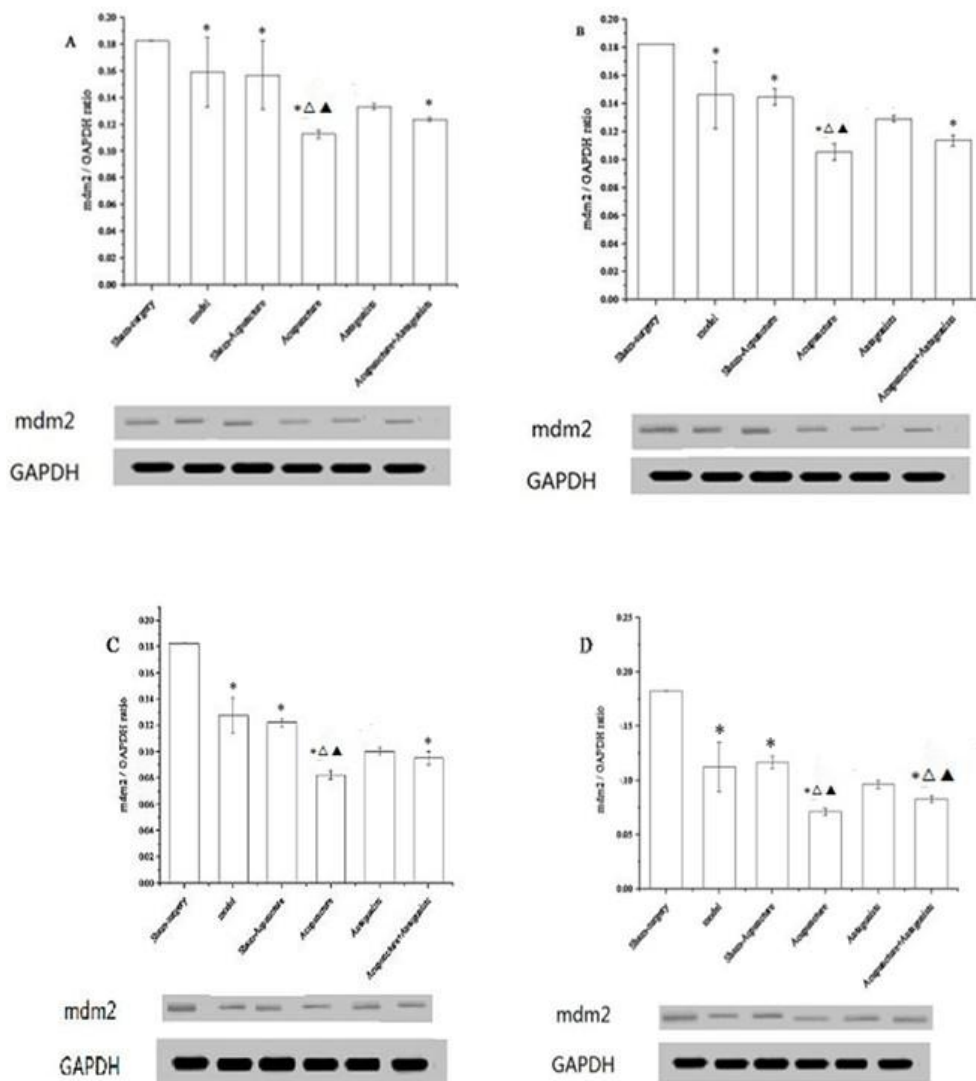

Fig.3 the expression of protein mdm2 at different periods of each group

A: 1st day; B: 3rd day; C: 7th day; D: 21st day

Note: *Compared with sham-surgery group, $P<$0.05; △Compared with model group, $P<$0.05; ▲Compared with antagonist group, $P<$0.05.

Discussion

As a conservative proto-oncogene[6], murine double minute 2 (mdm2) gene can inhibit the transcriptional activity of p53[7]and inhibit the DNA repair of damaged cells[8]by promoting the degradation of p53. Mdm2 is widely researched in the field of cancer with few studies reported in hypoxic-ischemic areas. The overexpression of mdm2 for some incentives could led to abnormal hyperplasia and canceration of cells[9,10]. As a negative regulator of p53 factor, mdm2 and p53 constitute a degradation-trans activation circulation path[11]. When cerebral

ischemia and hypoxia occurs, the level of mdm2 down-regulated which could promote the accumulated of p53 in the nucleus. And the increased p53 could promote the repair of DNA and prevent the mutation of cells, thus playing an important role in the repair of neurons. With distribution in both neuron axons and growth cones, the inhibition of p53 can lead to the disintegration of axonal growth cones and the elimination of the amino acid sequence of p53 in the nucleus, furtherly suggest that p53 can promote the growth of axons[12].

In this experiment, the expression of mdm2 in the neurons of model rats began to be down-regulated from 1st day to 21th day after cerebral ischemia-hypoxia, but the downward trend slowed down gradually, suggesting that the body launch the mechanism of self-protection and self-healing after cerebral ischemia-hypoxia, but the protection mechanism decreases with the extension of time. In EA group, the level of mdm2 present a significant downward trend, and in 3th, 7th, 21th day subgroups the level of mdm2 showed significant difference compared with corresponding model subgroups. So it was speculated that the possible mechanism of electroacupuncture on hypoxic-ischemic brain damage is about the trans circulation pathway between mdm2 and p53. Electroacupuncture can promote the increasing of p53 by down-regulating the expression of mdm2, thus promoting the DNA repair and achieving lasting repair function to damaged neurons.

According to the results of this experiment, electroacupuncture plays a protection role in hypoxic-ischemic brain damage by down-regulating the expression of mdm2. And the result has great significance to the further research of the mechanism of electroacupuncture on hypoxic-ischemic encephalopathy.

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