Mechanism of cerebral acupuncture for neuronal cell apoptosis via BrdU and Nestin in hypoxic-ischemic brain-damaged immature rats

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Original Research

Abstract: In this study, the effects of scalp acupuncture on the number of apoptotic cells and apoptotic indexes were determined in rats with intrauterine hypoxic-ischemic brain damage (HIBD), and the inhibitory mechanism of mitochondrial pathway, mediated apoptosis in the repair of brain ischemia and hypoxia was examined. A clip was applied to the uterine artery for 5 min before a cesarean section was performed in a rat model. The rats were divided into normal group (n = 24), model group (n = 24), and acupuncture group (n = 24). Rats in the acupuncture group were treated with continuous acupuncture on the head beginning on the 28th day after birth. Rats were sacrificed at days 35, 42 and 49, and samples were analyzed by TUNEL staining of cells obtained from the cerebral cortex. The number of apoptotic cells in each group (n = 8) was determined and compared. An immunohistochemical assay of cerebral cortex cells was performed to examine BrdU and Nestin expression. Compared with the normal group, the number of apoptotic cells in the model group was significantly higher and increased over time, (P < 0.05). BrdU expression was higher in the acupuncture group than in the model group, and the number of apoptotic cells was significantly lower in the acupuncture group than the model group, and decrease over time. BrdU expression decreased, while Nestin expression significantly increased. Acupuncture can reduce the number of apoptotic cells in the cerebral cortex. The expression of BrdU and Nestin inhibited the apoptosis of neural stem cells, promoting the repair of injured neurons via neural stem cells.

Key words: HIBD; Apoptotic cell; BrdU; Nestin; Neural stem cells; Acupuncture; Jin’s three-needle technique.

*Qing Yuan and Xing-er Li contributed equally to this study.

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Introduction

Hypoxic-ischemic brain damage (HIBD) is the leading cause of neonatal cerebral palsy, mental retardation, autism, and epileptic encephalopathy. Epidemiological studies have shown that perinatal intrauterine asphyxia is the main cause of neonatal HIBD (1). A number of studies have shown that acupuncture can effectively treat various diseases, such as neck pain (2-3), knee pain (4-5), hypomenorrhea (6), primary dysmenorrhea (7), impotence (8), pressure ulcers (9), stroke (10-11) and autism (12). After decades of practice and accumulation of knowledge, needle acupuncture treatment using three points (collectively referred to as «Jin») has been shown to be successful for a wide range of diseases and syndromes. A large number of preclinical studies have found that needling scalp stimu-
model of whole brain hypoxic ischemic brain damage was examined by estimating BrdU and Nestin expression with respect to acupuncture treatment. Changes in the two indexes and the expression of apoptotic cells in the cerebral cortex were observed, and the mechanisms by which acupuncture induces cerebral ischemia and hypoxia injury repair are discussed.

Material and Methods

Experimental animals and groups
Forty-two healthy pregnant specific pathogen-free Sprague-Dawley rats [SCXK (Guangdong) 2006-0015], weighing 210–290 g, were provided by the Animal Centre of the Southern Medical University. All rats were kept in ambient conditions with a 12-h light/12-h dark cycle, a temperature of 22–24°C, and a humidity of 58–70%. Sterile surgeries were conducted in the Experiment Center of Guangzhou University of Chinese Medicine Animal Laboratory [SYXK (Guangdong) 2008-0001]. All rats were provided humane care according to the 3R principles of experimental animals. (The 3Rs, defined as Replacement, Reduction, and Refinement, are fundamental principles driving ethical research, testing, and education using animals).

Neonatal rats were randomly assigned to 3 groups. In the normal group, neonatal rats underwent natural deliveries (n = 24). In the model group, neonatal rats underwent a 5-min-delayed cesarean section (n = 24). In the acupuncture group, neonatal rats underwent a 5-min-delayed cesarean section and received acupuncture treatment (n = 24). All baby rats in every group were sub-divided into an additional 3 groups (n = 8) according to the sacrifice date (35 d, 42 d, or 49 d after birth).

Establishment of the neonatal hypoxic-ischemic brain-damaged (HIBD) rat model
Pregnant rat models were received a 5-min-delayed cesarean section using the Bjelke method (18). On d21 of gestation, 21 pregnant rats were subjected to neck dislocation without anesthesia. They were placed in a supine position on a surgical board with the abdominal cavity exposed along the ventral midline to the uterus. The uterine cornual arteries on both sides were clamped for 5 min using 4 pairs of forceps. At the same time, sterile gauze soaked in a 37°C saline solution was placed on the exposed uterus for 5 min. The uterus was cut open and infant rats were removed within 2 min; the umbilical cord was retained. The amniotic fluid in the nose and mouth of the rat was cleaned with dry cotton. The infant rats were kept in a 37°C water bath tray. The baby rats were wiped with distilled water-soaked cotton swabs. Only the infant rats that curled up and turned from pale to pink within 30 min were selected for our study. These neonatal rats were breastfed by the other 21 pregnant rats who delivered fetuses naturally.

Neuroethological behavioral testing of newborn rats, including righting reflex tests, trapeze tests, and inclined plane tests

Righting reflex test: The 2-day-old rats were examined by righting reflex tests. The newborn 2-day-old rats were set in a supine position after lactation by female mice. The reaction time of the righting reflex was recorded in seconds. The rats that did not exhibit a righting reflex within 1 min were considered failures, and values of 60 s were recorded. Experiments were repeated 10 times in a row and the number of successes was recorded.

Trapeze test: Newborn rats hung from a level glass rod (0.5 cm in diameter) using their front legs, hanging at 45 cm height. The time until rats dropped was recorded in seconds. Tests were repeated 3 times in a row and the longest time was used as the score.

Inclined plane test: Newborn rats were set on a 45° incline with their heads down. The time until the rats used torque to reposition their bodies such that their heads upright and the turning angle was >135° in the vertical direction was recorded in seconds. The rats that could not accomplish this task successfully were considered failures, and values were recorded as 180 s.

Reagents and instruments
The following reagents and equipment were used in the study: In Situ Cell Death AP Kit (Batch number: 12463500; Roche, Branchburg, NJ, USA), rabbit anti-Nestin antibody (Batch number: N5413; Sigma, St. Louis, MO, USA), two rabbit resistance ABC Kits (Batch number: 7201212; OriGene Technologies Company, Beijing, China), TP1020 biological tissue automatic dehydrator, EG1140 biological tissue paraffin embedding machine, RM2255 rotary paraffin full-automatic slicer (Leica, Wetzlar, Germany), and a BX50 biological tissue microscope (Olympus, Tokyo, Japan).

Acupuncture treatment
The newborn rats in the acupuncture group received acupuncture intervention. The newborn rats in the normal and model groups did not receive any intervention and were fixed in the same way. According to Jin’s 3-needle technique theory, the 3-Occipital Points (Naohu ‘DU17’, both Naohong ‘GB19’ points), the 3-Points in the Temporal Area (1st, 2st, and 3st acupoints in the temporal area), the 3-Points for Intelligence (Shenting ‘DU24’, both Ben- shen ‘GB13’ points) were selected. The locations are described in «Experimental acupuncture» (19). Beauty acupuncture needles were used (0.25 mm × 10 m, Huato; Medical Instrument Co., Ltd., Suzhou, China) and needled points were applied subcutaneously. Use the twisting technique, continuous, uniform twisting was applied for 10 s with a torsional amplitude of ±180° and a frequency of 120 times/min, retaining the needle. All acupuncture treatments were performed by the same person. Acupuncture was applied at various time points (i.e., at 35, 42, and 49 days after birth).

Animal sacrifice and tissue manipulations
After behavioral testing, the rats were injected with 50 mg/kg 5-bromo-2’-deoxyuridine (BrdU) (Sigma) intraperitoneally. Two hours later, neonatal rats were anesthetized with chloral hydrate (450 mg/kg, intraperitoneally) and sacrificed. Their heads were cut off and the brains were removed from the skulls. Brain tissue was flushed with 50 ml of 0.9% saline, followed by tissue fixation with 10% formaldehyde solution for 24 h. Tissue obtained, from the optic chiasm coronal plane to the hippocampal formation was dehydrated, embedded in paraffin wax, and cut into 4-μm sections using the Leica TP1020 tissue processor, Leica EG1140 modular tissue embedding center, and Lei-
ca RM2255 rotary microtome.

**TUNEL staining**

Apoptotic cells were detected using the In Situ Cell Death AP Kit(Roche) following the manufacturer’s instructions. Sections were dewaxed in pure xylene, rehydrated using graded ethanol, washed with water and phosphate-buffered saline (PBS), immersed in 0.1% Triton X-100 solution for 8 min, and washed with water and PBS again. The labeling mixture was added for 1h at 37°C, then cells were washed with PBS, the conversion liquid was added for 30 min at 37°C, and cells were washed with PBS again. The BCIP/NBT solution was added for color development, and the chromogenic control was observed under a microscope for about 10 min and, washed with water and PBS. When sections were dry, GVA aqueous mounting solution was added.

Cell counting: Brain tissue was observed under a microscope with a 40× objective lens (Olympus BX50). The pyramidal cell layer of the cerebral cortex was located in the lateral parietal lobe of the brain. Three consecutive fields were photographed and analyzed using Guangzhou Mingmei image processing software. The total area of the statistical field was 1768185.44 μm². The number of apoptotic cells was manually counted. The optical densities of the BrdU+ and Nestin+ cells were examined using image processing software developed by the Beijing University of Aeronautics and Astronautics. Average data obtained from 6 pictures were used for statistical analyses.

**Immunohistochemistry**

NSCs and neurogenic cell proliferation were detected using monoclonal anti-BrdU (1:50; Sigma) and anti-Nestin antibodies (1:150; Sigma) following the manufacturer’s instructions. Briefly, sections were dewaxed and antigens were restored by microwaving; they were then blocked in goat serum and incubated with the primary antibodies at 4°C overnight. Sections were subsequently incubated for 20 min at 37°C with VECTASTAIN ABC Kit Rabbit IgG secondary antibodies (1:200; Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China). Then 3, 3’-diaminobenzidine was added for color development, and a light orange color was interpreted as a positive signal. The sections were washed and hematoxylin was added to re-colorize. After the sections were dried, neutral balsam was added and coverslips were placed on slides.

**Statistical analysis**

All data are expressed as means ± SD. To compare differences in means among groups, one-way ANOVA was implemented by SPSS 20.0. Statistical significance was reached when P<0.05.

**Results**

**Behavioral tests of neonatal HIBD rats at 14 d after birth**

*Evaluation of newborn rats by the righting reflex test on the second day after birth*

Newborn rats in the model group exhibited longer righting reflex test times than rats in the normal group (P < 0.01). These results indicated that rats in the model group exhibited nervous system retardation (Table 1, Figure 1).

*Newborn rats were examined by trapeze tests and inclined plane tests at 14 d after birth*

Newborn rats in the model group exhibited earlier release times than rats in the normal group in trapeze tests at 14 d after birth (P < 0.01). Newborn rats in the model group exhibited longer response times than rats in the normal group in inclined plane tests at 14 d after birth (P < 0.01). These differences indicated reduced muscle strength and coordination in rats in the model group compared with the normal group (Table 2, Figure 2).

**Effect of acupuncture on nerve cell apoptosis in HIBD neonatal rats**

The number of apoptotic cells in newborn rat brain tissue slices at each point-time after birth was compared among the three groups. The model rats are received acupuncture treatment.

**Table 1.** The shortest time until rats turned right and number of successes for 2-day-old newborn rats (X ± S).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Shortest time until turning right</th>
<th>Number of successes</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>8.64 ± 14.21</td>
<td>6.97 ± 3.46</td>
</tr>
<tr>
<td>model</td>
<td>16.24 ± 21.97**</td>
<td>3.82 ± 3.49**</td>
</tr>
<tr>
<td>F</td>
<td>11.900</td>
<td>47.114</td>
</tr>
<tr>
<td>P</td>
<td>0.000*</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Notes: ▲Rank and inspection, analysis of variance, compared with the normal group, **P < 0.01.

**Table 2.** Trapeze tests and inclined plane tests for 14-day-old newborn rats(X ± S).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Trapeze test duration (s)</th>
<th>Inclined plane test duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>18.67 ± 10.4</td>
<td>25.81 ± 46.44</td>
</tr>
<tr>
<td>model</td>
<td>12.17 ± 8.84**</td>
<td>75.32 ± 78.73**</td>
</tr>
<tr>
<td>F</td>
<td>10.411</td>
<td>5.075</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Notes: Analysis of variance, compared with the normal group, **P < 0.01.
puncture from day 28 to 35, the number of apoptotic cells was higher in the model group than the normal group (P < 0.01). Compared with the model group, the number of apoptotic cells was significantly lower in the acupuncture group (P < 0.01). On day 42 and 49, the number of apoptotic cells in the acupuncture group was significantly lower than that of the model group (P < 0.05). These results show that acupuncture can inhibit apoptosis for cortex vertebral body cells of HIBD newborn rats and can promote the repair of apoptotic cells. Specifically, acupuncture reduced the number of apoptotic cells in HIBD rats after one week of treatment (Table 3, Figure 3).

**Effects of acupuncture on newborn neural cells in neonatal HIBD rats: BrdU-positive and Nestin-positive cells in each group at various time points**

BrdU-positive cells were estimated based on optical density in brain tissue for each group on various days after birth. On the 49th day, the average optical density of BrdU-positive cells was lower in the acupuncture group than the model group (P < 0.01). These results indicate that acupuncture inhibited BrdU-positive cells in the cerebral cortex of neonatal rats with intrauterine HIBD. At 35–49 d, BrdU-positive cells exhibited a decreasing trend in the acupuncture group and normal group, but an increasing trend in the model group. This indicated that acupuncture can regulate and repair normal functions in brains after HIBD (Table 4, Figure 4).

Nestin-positive cells in brain tissues obtained from newborn rats based on optical density were compared on different days after birth. At 35–49 d, Nestin-positive cells exhibited an increasing trend in the acupuncture group. At d 42, there were more Nestin-positive cells in the acupuncture group (after 14 d of treatment) than the model group (P > 0.05). Compared with the model group, the optical density of BrdU-positive cells increased in the acupuncture group (P < 0.01). These results indicated that acupuncture can promote the proliferation of NSCs in the cerebral cortex. Although the onset of effect was slow, it
Average optical density of Nestin-positive cells in different groups at various time points (X ± S).

<table>
<thead>
<tr>
<th>Group (n = 24)</th>
<th>Average optical density of Nestin-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35 d (n = 8)</td>
</tr>
<tr>
<td>normal</td>
<td>0.51 ± 0.13</td>
</tr>
<tr>
<td>model</td>
<td>0.37 ± 0.09</td>
</tr>
<tr>
<td>acupuncture</td>
<td>0.29 ± 0.21**</td>
</tr>
<tr>
<td>F</td>
<td>3.616</td>
</tr>
<tr>
<td>P</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>42 d (n = 8)</td>
</tr>
<tr>
<td>normal</td>
<td>0.48 ± 0.19</td>
</tr>
<tr>
<td>model</td>
<td>0.32 ± 0.22**</td>
</tr>
<tr>
<td>acupuncture</td>
<td>0.37 ± 0.20</td>
</tr>
<tr>
<td>F</td>
<td>3.243</td>
</tr>
<tr>
<td>P</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>49 d (n = 8)</td>
</tr>
<tr>
<td>normal</td>
<td>0.16 ± 0.15</td>
</tr>
<tr>
<td>model</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td>acupuncture</td>
<td>0.36 ± 0.16**</td>
</tr>
<tr>
<td>F</td>
<td>9.415</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Notes: PS: Analysis of variance. Compared with the normal group, **P < 0.01. Compared with the model group,**#P < 0.01.

Behavioral testing (i.e., righting reflex, trapeze, and inclined plane tests) was performed to evaluate the HIBD models. Newborn rats in the model group had longer righting reflex times than rats in the normal group. Newborn rats in the model group had fewer successful righting reflex tests (out of 10 trials) than rats in the normal group. Rats in the model group had faster release times than rats in the normal group in trapeze tests, and the inclined plane test completion time was long in the model group than the normal group. These results show that the nervous system of rats in the model group developed more slowly than that of rats in the normal group, and muscle strength and coordination of model group rats are worse than those of normal rats. Combined, these results indicate that the model of 5-min delayed cesarean neonatal rats with intrauterine distress HIBD was successful.

Apoptosis is a programmed process of cell death that is genetically controlled. It is active in response to internal and external stimuli (26). In our study, compared with the model group, the number of apoptotic cells was significantly reduced (P < 0.01). This shows that acupuncture can inhibit cortex vertebral body cell apoptosis and promote the repair of apoptotic cells in HIBD newborn rats, which is related to the mechanism by which acupuncture inhibits intrauterine HIBD rat neural cell apoptosis and promotes repair apoptotic cells. The number of apoptotic cells was lower in the acupuncture group than the model group at 7 days after acupuncture treatment (P < 0.01) and higher than the normal group. However, at 14 days after acupuncture treatment, the number of apoptotic cells was lower in the acupuncture group than the normal group and the groups had similar trends over time. We inferred that acupuncture may inhibit cortex vertebral body cell apoptosis in HIBD newborn rats, and this effect may be strengthened over time.

BrdU, is related to thymus diseases; it is a thymidine analogue (27). After the injection of animals with BrdU, the cell cycle is stopped at the S phase (28). Suqing Q found that after hypoxic ischemic brain damage, the NSCs of newborn rats were activated, resulting in high proliferation rates (29). In this study, in the normal group, BrdU-positive cells decreased over time. Accordingly, the vertebral body cell proliferation capacity decreased gradually during this period. This presumably reflects the development of normal rat NSCs, which eventually no longer proliferate or differentiate. On the 49th day, the average optical density of BrdU-positive cells in the acupuncture group was lower than that in the model group (P < 0.01). At days 35-49, the acupuncture group and normal group exhibited

Discussion

We have devoted more than ten years of clinical research to the treatment of children with encephalopathy using Jin’s three-needle technique and observed high efficiency (20-22). However, few published clinical studies have examined the use of acupuncture for neonatal hypoxic-ischemic encephalopathy intervention and the underlying mechanisms. In this study, hypoxic-ischemic encephalopathy caused by intrapartum asphyxia changed the neuronal environment. NSC apoptosis in the brain cortex of HIBD newborn rats impeded the repair of damaged neurons (17,23-25). We inferred that the inhibition or reduction of the number of apoptotic nerve cells and NSCs and improvements in neural stem cell survival rate were key factors for the treatment of ischemic-hypoxic brain damage. Accordingly, it is important to study the mechanism by which acupuncture inhibits mitochondrial pathway-mediated apoptosis and promote ischemia-anoxia brain nerve cell repair. In this study, perinatal intrauterine HIBD of newborn rats was adopted as a model, in which a 5-min delay in cesarean delivery led to brain nerve cells apoptosis. The acupuncture points used in this experiment were the «3-Occipital Points,» «3-Points in the Temporal Area,» and «3-Points for Intelligence» of Jin’s 3-needle technique, and these points are effective for the treatment of encephalopathy in children. The experimental results showed that acupuncture can inhibit the apoptosis of cerebral cortex cells, promote NSCs to repair damaged neurons, and reverse the damage caused by ischemia and anoxia in the brain. These results support our inferences based on preliminary clinical observations.

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a decreasing trend, while the model group exhibited an increase. This indicated that acupuncture can regulate and repair functions after HIBD. We thought that acupuncture might inhibit intrauterine HIBD by reducing newborn rat neural cell apoptosis and promoting the restoration of damaged neurons via the inhibition of the expression of BrdU-positive cells, the inhibition of glial cells proliferation, and a reduction in the proliferation of new positive neurons in the damaged brain area.

Nestin is a NSC-specific VI intermediate filament protein. It is a marker of NSCs. Experimental studies have shown that acupuncture can promote the expression of Nestin in the ependyma and hippocampal dentate gyrus in animal models of cerebral ischemia (30). Rats in the normal group expressed similar levels of Nestin at 35 and 42 d, but exhibited a sharp decline at 49 d. We surmised that this was due to the formation of new neurons from NSCs of neonatal rats, which exhibited strong growth and differentiation until 42 d day after birth, after which nervous system development in neonatal rats reached a mature stage, NSCs disappeared, and spinal cell proliferation decreased. For 21 days of acupuncture (i.e., the 49 d group), in the cerebral cortex of neonatal rats with intrauterine HIBD, expression of Nestin-positive cells increased significantly compared with the control group (P < 0.01). These results suggest that acupuncture promotes the expression of NSCs from the cerebral cortex and hypoxic-ischemic brain damage can affect the expression and differentiation of NSCs, thereby impeding the normal development of neuronal maturation. By promoting the expression of NSCs from the cerebral cortex and differentiation to new neurons, acupuncture can protect and repair damaged neurons to treat hypoxic-ischemic encephalopathy.

Jin’s three-needle acupuncture was used to treat neonatal rats with intrauterine distress HIBD caused by 5-min delayed cesarean. Acupuncture was able to reduce the number of apoptotic cells in the cerebral cortex of neonatal rats with hypoxia-ischemia. This inhibitory effect on apoptosis may be related to the number of BrdU-positive cells and Nestin-positive cells in the spinal cell layers of the cerebral cortex; NSCs may function to protect and repair damaged neurons, promote brain development, and reverse the damage. We believe that acupuncture can inhibit the expression of BrdU-positive cells in the cerebral cortex, inhibit the proliferation of glial cells in the brain, and activate neural stem cells to promote the differentiation and maturation cells in brain damage conditions. Acupuncture can also promote the expression of Nestin in the cerebral cortex, and increase the level of newborn NSCs in damaged areas, repairing damaged neurons and activating endogenous NSCs to inhibit apoptosis. These mechanisms might explain how acupuncture inhibits apoptosis in neonatal rats with intrauterine HIBD. However, the inhibition of apoptosis involves multiple factors; accordingly, we examined changes in various apoptosis-related genes and proteins. Recent research has shown that cell autophagy is another important mechanism of acupuncture treatment in response to hypoxia deficiency brain injuries (31-32).

Our research on the use of the acupuncture to treat nerve cell apoptosis in HIBD newborn rats, has been declared a new National Science Fund subject. We are currently extending these analyses to examine the mechanism by which acupuncture treats HIBD newborn rats at the level of cell autophagy and the relationship between apoptosis and autophagy in a distressed HIBD newborn rat model. These results will clarify the mechanisms of hypoxic-ischemic brain injury.

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