Basic Investigations

Effects of Electroacupuncture on Hippocampal and Cortical Apoptosis in A Mouse Model of Cerebral Ischemia-reperfusion Injury

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Objective: To observe the effects of electroacupuncture on hippocampal and cortical apoptosis in a mouse model of cerebral ischemia-reperfusion injury.

Methods: Mouse models established by repeated cerebral ischemia-reperfusion, followed by electroacupuncture at Shenshu, Geshu, and Baihui points. The control group mice were intragastrically administered Hydergine. On day 1 and 7 post-treatment, hippocampal and cortical apoptosis was detected by terminal-deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL), and apoptosis images in the hippocampal CA1 zone and cortical area were analyzed.

Results: In the model group, apoptotic cells were detected one day after treatment and some cellular fibers were disarrayed. By day 7 post-treatment, there was an increase in the number of apoptotic cells in the hippocampal CA1 region. In addition, there were apoptotic cells in the cortical area, the cortical layers were thinner with localized neuronal loss and sieve-like lymphocyte infiltration, as well as glial cell proliferation and visible infarct lesions. However, in the Hydergine and electroacupuncture groups, there was a small number of apoptotic cells. At 7 days post-treatment in the model group, field number, numerical density on area, and surface density were increased. However, in the Hydergine and electroacupuncture groups these parameters were decreased (P<0.01), with a significant difference between the two treatment groups (P<0.01).

Conclusion: Electroacupuncture treatment inhibited apoptosis and provided neuroprotection.

Keywords: electroacupuncture; cerebral ischemia-reperfusion; hippocampus; cortex; neurons; apoptosis; image analysis

Cerebral ischemia-reperfusion injuries induce apoptosis in neural cells1-4 and result in hippocampal5-18 and cortical injury.7,19-24 Acupuncture has been shown to inhibit cerebral ischemia-reperfusion injuries,25-32 and electroacupuncture is thought to further inhibit injuries32 and prevent spinal neuronal apoptosis in rat models.33 Through the use of mouse models of cerebral ischemia-reperfusion injury, the present study aims to analyze in situ apoptosis of hippocampal and cortical neurons to determine whether electroacupuncture protects neurons by inhibiting apoptosis.

MATERIALS AND METHODS

Drugs and Reagents

Hydergine (co-manufactured by Huajin Pharmaceutical Factory of Tianjin, China and Beijing Novartis Pharma, Beijing, China); terminal-deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) kit (Roche Inc., Basel, Swiss Confederation); poly-L-lysine and 3,3-diaminobenzidine (DAB) (Hebei Bohai Bioengineering, Shijiazhuang, China).

Experimental Animals

A total of 40 healthy, male, Kunming mice, weighing 25–28 g, were provided by the Experimental Animal Center of Hebei Medical University (Certificate No.: Experimental Animal Center of Hebei Province 601050).

Experimental Design

After housing the mice at 18–22°C for one week (12-h day/12-h night light cycle in a clean animal laboratory), the mice were randomly assigned to four groups: sham surgery, model, Hydergine, and electroacupuncture, with 10 mice in each group. As previously described,34-35 a model of cerebral ischemia-reperfusion was replicated, and the mice received the appropriate treatments after recovery on the same day as surgery.

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Histopathological Study
At 1 and 7 days post-treatment, three mice from each group, respectively, were sacrificed by decapitation. The entire brain was quickly removed and placed on ice. Brain tissue from the optic chiasma to the corpus albicans was placed into 4% paraformaldehyde/0.1 mol phosphate-buffered saline (PBS), pH 7.0-7.6, containing 0.1% diethylpyrocarbonate (DEPC). The tissue was then paraffin-embedded, and 5-μm thick coronal sections were made. The first section of every three consecutive sections was kept for further use.

TUNEL Staining of Hippocampal and Cortical Tissue
Paraffin sections underwent conventional dehydrating, followed by incubation at room temperature for 15 min with proteinase K (20 μg/mL in Tris/HCl, pH 7.4-8.0), two wash steps in PBS, and incubation with 50 μL TUNEL reaction mixture onto section for 60 min in 37 °C in a humidity chamber (to prevent evaporation and guarantee uniform distribution of TUNEL reaction mixture, sealed with a coverslip during the incubation process). The sections were then washed three times with PBS, wiped dry, incubated with 50 μL converter-POD for 30 min at 37 °C in a humidity chamber, washed three times with PBS, incubated with 50 μL DAB substrate solution at room temperature for 10 min, and washed three times with PBS. Hematoxylin counterstain was used for 5 minutes, and the sections were subsequently dehydrated, cleared, and mounted. Apoptosis-positive nuclei appeared as darkly stained, pyknotic nuclei.

Image Analysis of Apoptotic Cells in the Hippocampal CA1 Area
A HPIAS-1000-type high-resolution color graphic pathological analysis system (HPIAS-1000-type, Thousands Screen Image Engineering Company at Tongji Medical University, Tongji, China) was used to analyze TUNEL images. As previously described,36 three mice from each group were used for analysis, and 3 sections from each mouse were analyzed. Apoptotic cells from two fields of view from the hippocampal CA1 area (left and right hemisphere) were quantified. In total, 36 recordings were analyzed for each group. Measurements were performed in a rectangular window: [(0, 0), (575, 767)]; forty-fold the pixel length 0.2073 μm. Quantitative analysis of field number (Fn) of apoptotic cells in area CA1 of hippocampus, numerical density of area (Na), and surface density (Sv) were measured.

Fn = number of apoptotic cell in an image
Na = number of apoptotic cells in each field in measurement window divided by reference area
Sv = 4π × (Bx/Ac), Bx is reference area, Ac is total perimeter of apoptotic cell.

Statistical Analysis
Experimental data are expressed as mean ± standard deviation (X ± s), and analysis was performed using SPSS for Windows software (version 11.5; SPSS Inc, Chicago, IL, USA). Comparisons between groups were performed using single-factor analysis of variance, and Student-Newman-Keuls test was used for pairwise comparisons for homogeneity of variance among the groups. Tamhane’s T2 test was used for heterogeneity of variance. P<0.05 and 0.01 were considered significant.

RESULTS

In situ Cell Apoptosis Detection of Hippocampal and Cortical Tissue
TUNEL staining in hippocampal CA1 area In the sham group, pyramidal cells were arranged in multiple layers and appeared as round, blue-stained cells with clear nucleoli. The fibers were densely arranged in neat rows, with no apoptotic cells (Figure 1). One day after surgery, apoptotic cells were detected in the model group, and the cells presented with nuclear condensation and disarranged fibers (Figure 2). At 7 days after surgery, the number of apoptotic cells significantly increased; the cells exhibited nuclear condensation, brownish-yellow, and disordered nerve fibers (Figure 3). In contrast, only a small number of apoptotic cells were visible in the Hydergine and electroacupuncture groups (Figure 4 and 5).

TUNEL staining in the cerebral cortex In the sham group, apoptotic cells were not detected (Figure 6). One day after surgery, apoptotic cells were detected in the model group (Figure 7). At 7 days after surgery, a large number of apoptotic cells appeared; the cells exhibited nuclear condensation, brownish-yellow, the cortical layer was thinner with localized neuronal loss and sievelike lymphocyte infiltration, as well as glial cell proliferation and visible infarct lesions (Figure 8). In contrast, only a small number of apoptotic cells were visible in the Hydergine and electroacupuncture groups (Figure 9 and 10).

Image Analysis of Apoptotic Cells in the Hippocampal CA1 Area
Field parameters of apoptotic cells in hippocampal CA1 area 7 days after surgery (Table 1).

DISCUSSION
Cellular apoptosis plays a crucial role in cerebral ischemia, involving both acute focal cerebral ischemia and delayed neuronal death after transient ischemia. The process of gene-regulated active cell death is complex and correlates with multiple gene expressions and interactions under different stimulating signals.37 Following transient ischemia, necrosis occurs immediately in neural cells of the ischemic core region. However, surrounding the ischemic core region, delayed neuronal degeneration typically occurs after 1–2 days, and previous studies have shown that this delayed cellular degeneration is apoptosis.38 Dynamic
observation of changes in cortical and striatal neural cell death revealed apoptotic cells in the striatal edema area at one hour after cerebral ischemia-reperfusion. With prolonged reperfusion time, the number of apoptotic cells increased and began to appear in the striatum and cerebral cortex. After reperfusion for 24-48 hours, a peak number of apoptotic cells was detected. The number of apoptotic cells has been shown to significantly increase at 24 hours after acute cerebral ischemia-reperfusion, and further increases after 48 and 72 hours; while the number of survived neurons gradually decreases with prolonged reperfusion time. These results suggest that neural cell apoptosis plays an important role in cerebral ischemia-reperfusion injury.

Figure 1. Sham group, hippocampus CA1 zone TUNEL staining (×400)

Figure 5. Hydergine group at 7 day after surgery, hippocampus CA1 zone TUNEL staining (×400)

Figure 2. Model group at 1 day after surgery, hippocampus CA1 zone TUNEL staining (×400)

Figure 6. Sham group, cortical zone TUNEL staining (×400)

Figure 3. Model group at 7 days after surgery, hippocampus CA1 zone TUNEL staining (×400)

Figure 7. Model group at 1 day after surgery, cortical zone TUNEL staining (×400)

Figure 4. Electroacupuncture group at 7 days after surgery hippocampus CA1 zone TUNEL (×400)

Figure 8. Model group at 7 days after surgery cortical zone TUNEL staining (×400)
Cerebral cortical injury has been previously shown in rat models during cerebral ischemia-reperfusion, and the number of apoptotic cells also increases in the hippocampus. In addition, at 7 days after cerebral ischemia-reperfusion in mice, the cerebral cortex becomes thin, with nuclear condensation in some neural cells. In addition, localized neuronal loss is apparent, and a sieve-like structure and glial cell proliferation are observed. Under microscopic observation, findings at day 15 and 30 were similar to day 7 after cerebral ischemia-reperfusion. Cellular loss in the hippocampal CA1 area was more significant with prolonged time. At 30 days after ischemia-reperfusion, almost all cells in the hippocampal CA area were gone, with increased proliferation of glial cells. There was also significant cell loss in the CA2 and CA3 areas, as manifested by delayed necrosis of hippocampal pyramidal cells. In addition, previous studies of rat models of cerebral ischemia-reperfusion injury, protein expression of c-fos, caspase-3, and Bax was increased in the hippocampal area, as well as an increase in apoptotic cells. These results suggested that cerebral ischemia-reperfusion most likely induced neural cell apoptosis.

Activation of apoptotic signaling transduction is dependent upon intensity, action mode, and duration of different in vivo stresses or stimuli. Overlapping pathways can be identified between various apoptotic signals. During cerebral ischemia, in addition to the classical death receptor (Fas/FasL-mediated pro-apoptotic signaling pathway) and mitochondrial pathway, the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathway plays an important role in signaling transduction from membrane receptors into cells, thereby regulating apoptosis and cell proliferation, as well as expressions of some important genes. In the hippocampus of cerebral ischemia-reperfusion mice, NO increases, levels of thiobarbituric acid reactive substance (TBARS) significantly increases, and production of reactive oxygen species (ROS) significantly increases, indicating neural injury.

Cerebral ischemia-reperfusion has been shown to elevate cyclooxygenase-2 (COX-2) expression and activate p38 and p42/44 MAPK pathways, as well as nuclear factor-kappa B (NF-kappa B), which results in oxidative stress and inflammatory injury. The LR4-mediated MyD88-dependent signaling pathway is activated by cerebral ischemia-reperfusion, which could involve ischemia-reperfusion injury via up-regulation of NF-kappa B and TNF-α. In right-side cortical ischemia-reperfusion injury, increased osteopontin (iOPN) in cells indicates that the response is associated with iOPN. Global cerebral ischemia-reperfusion has been shown to increase activation of the glutamate receptor 6(GluR6)-mediated c-Jun N-terminal kinase (JNK) signaling pathway, and S-nitrosylation of GluR6 facilitates assembly of the GluR6-PSD95-MLK3 signaling pathway, which subsequently activates MLK3 and downstream signaling pathways to induce cerebral ischemic injury.

Results from previous studies demonstrated that electroacupuncture exhibits beneficial effects on learning and memory functions in cerebral ischemia-reperfusion animal models, such as inhibition of learning and memory impairment in mice, as demonstrated in platform and water maze experiments. In addition, neuronal loss is reduced after acupuncture treatment.

The present study replicated a mouse model of repeated
cerebral ischemia-reperfusion to induce damage to the hippocampus and cortex. In the model group, apoptosis was detected in the CA1 area and cortex at one day after surgery, and the number of apoptotic cells further increased by 7 days after surgery. Based on image analysis in the model group, Fn, Na, and Sv of apoptotic cells increased in the CA1 area, suggesting that the number of apoptotic cells increased with nuclear condensation. After 7 days of treatment in the electroacupuncture group, Fn, Na and Sv all decreased, suggesting a significantly reduced number of apoptotic cells, which was consistent with previous results. These results suggested that electroacupuncture inhibited apoptosis in hippocampal and cortical regions.

Results from the present study demonstrated that electroacupuncture exhibited multi-pathway and multi-dimensional effects by inhibiting cerebral ischemia-reperfusion-induced apoptosis in hippocampal and cortical regions. Electroacupuncture has been shown to provide neuronal protection by influencing neuronal ultrastructure of ischemia-reperfusion lesions and increasing the energy supply and metabolic functions of ischemic regions. Electroacupuncture has also been shown to promote angiogenesis by mobilizing endothelial progenitor cells in peripheral blood, reducing activity of inducible nitric oxide synthase, and decreasing expression of vascular endothelial growth factor to improve cerebral ischemia. In addition, electroacupuncture inhibits neural cell apoptosis in cat models of dorsal root ganglion block by down-regulating Bax protein expression and up-regulating Bcl-2 protein expression. Scalp electroacupuncture has been shown to reduce leukocyte infiltration following middle cerebral artery occlusion reperfusion, as well as down-regulate TNF-α, IL-1β, COX-2, and NF-κB expression in plasma and ischemic brain tissues. In addition, IL-10 and transforming growth factor-β1 expression decreases, thereby reducing the immune inflammatory response, which results in ameliorated cerebral ischemia-reperfusion injury and recovery of cerebral nervous functions in rat models. Moreover, electroacupuncture reduces apoptosis of hippocampal cells by up-regulating HSP70, c-fos and bcl-2 expressions and down-regulating Bax expression. Furthermore, a previous study indicated that the mitochondrial membrane potential level of cortical cells in cerebral ischemia-reperfusion rats was significantly lowered with a much significantly higher apoptotic rate; after treatment with electroacupuncture, the mitochondrial membrane potential was increased and apoptosis was inhibited.

In conclusion, results from the present study suggested that electroacupuncture treatment protected neurons in a mouse model of cerebral ischemia-reperfusion injury by reducing neuronal apoptosis.

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