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Acupuncture at *Baihui* and *Dazhui* reduces brain cell apoptosis in heroin readdicts

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Abstract

Acupuncture at *Baihui* (GV20) and *Dazhui* (GV14) reduces neuronal loss and attenuates ultrastructural damage in cerebral ischemic rats. However, whether acupuncture can treat addiction and prevent readdiction through changes to brain cell ultrastructure remains unknown. In this study, cell apoptosis was observed in the hippocampus and frontal lobe of heroin readdicted rats by electron microscopy. Immunohistochemical staining displayed a reduction in Bcl-2 expression and an increase in Bax expression in the hippocampus and frontal lobe. After rats were given acupuncture at *Baihui* and *Dazhui*, the pathological damage in the hippocampus and frontal lobe was significantly reduced, Bcl-2 expression was upregulated and Bax expression was downregulated. Acupuncture exerted a similar effect with methadone, a commonly used drug for clinical treatment of drug addiction. Experimental findings suggest that acupuncture at *Dazhui* and *Baihui* can prevent brain cell apoptosis in heroin readdicted rats.

Key Words: nerve regeneration; traditional Chinese medicine; acupuncture; heroin readdiction; brain injury; ultrastructure; Bcl-2; Bax; apoptosis; neuroprotection; NSFC grant; neural regeneration

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Introduction

Heroin addiction and readdiction can cause extensive damage to the brain, and result in irreversible damage, with longer drug abuse triggering severe brain damage[1-6]. Brain injury caused by heroin addiction and readdiction is highly involved in nerve cell apoptosis and nerve demyelination[7-10]. Apoptosis in the brain is a common symptom of heroin addicted rats and ultrastructural pathological changes are apparent in neurons of the midbrain ventral tegmental area and the nucleus accumbens, which plays an important role in the reward circuit. Therefore, apoptosis is a dominant mode of neuronal death caused by heroin addiction[7-10]. Accumulating evidence has shown ultrastructural changes in the cerebral cortex, hypothalamus, hippocampus, and pituitary gland of heroin addicted rats, rhesus monkeys and humans[11-17]. Ultrastructural changes such as degeneration, necrosis, and apoptosis of neurons, axons and dendrites are known to be found in the brain of heroin addicted rats, including the prefrontal cortex, nucleus accumbens, hippocampus, midbrain ventral tegmental area and hypothalamus[18-22].

Acupuncture has therapeutic effects on cerebral ischemia, dementia, epilepsy and other brain diseases, and also functions to repair the nervous system. *Dazhui* (GV14) and *Baihui* (GV20) are the preferred acupoints for treatment[23-29]. Acupuncture at *Baihui* and *Dazhui* improves oxygen atmosphere in the brain of cerebral ischemic rats, inhibits abnormal activation of the JAK2-STAT3 signal transduction pathways, and attenuates oxidative stress-induced brain injury[30-32]. In addition, acupuncture therapy can regulate the expression of apoptosis-related genes in the hippocampus, increase the number of nestin-positive cells, attenuate ultrastructural injury in ischemic neurons, endothelial cells, myelin and glial cells, improve cerebral cortex ultrastructural damage, and exert neuroprotective effects[33-36]. Acupuncture at *Baihui* and *Dazhui* contributes to the regulation of expression of various proteins, receptors and enzymes in the blood and hippocampus, modulates synaptic number and structure, promotes nerve regeneration and hippocampal long-term potentiation, accelerates synaptic transmission in hippocampal neurons, improves learning and memory ability of diabetic rats and dementia rats, and slows brain aging[37-44]. Finally, acupuncture at *Baihui* and *Dazhui* is responsible for inhibiting the reduced activity of mitochondrial complexes, protecting brain mitochondrial function, decreasing neuro-

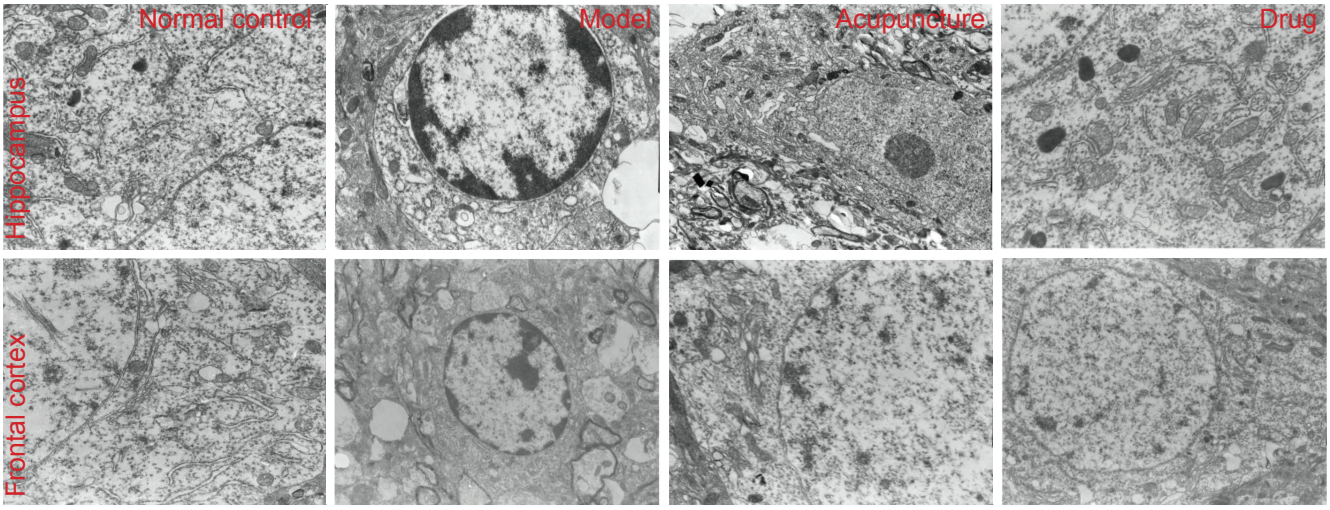

Figure 1 Effect of acupuncture on the morphology of nerve cells in the frontal cortex and hippocampus of heroin readdicted rats (transmission electron microscopy, × 10,000).

In the normal control group, the nuclear membrane in hippocampal nerve cells was clearly visible, there were abundant rough endoplasmic reticulum, ribosomes and mitochondria, mitochondrial cristae were clear; fewer ribosomes were observed in the frontal cortex, some mitochondria degenerated, and rough endoplasmic reticulum mildly expanded. In the model group, hippocampal neuronal cells were apoptotic, chromatin aggregated and rough endoplasmic reticulum expanded, partial mitochondrial expansion and vacuolar degeneration were observed, intermembrane space widened; the frontal lobe also exhibited nerve cell apoptosis, nuclear chromatin aggregation, and enlarged perinuclear gaps. In the acupuncture group, hippocampal nerve cells had clear nuclear membranes, expanded rough endoplasmic reticulum, intact mitochondria; frontal lobe nerve cells had clear nuclear membranes, partial mitochondrial degeneration, and abundant rough endoplasmic reticulum. In the drug group, hippocampal nerve cells had clear nuclear membranes, there were a large number of mitochondria with clear mitochondrial cristae, rough endoplasmic reticulum mildly dilated; frontal lobe nerve cells also presented clear nuclear membranes, the ribosomes and rough endoplasmic reticulum were abundant, and a few mitochondria appeared degenerated.

nal loss, and attenuating ultrastructural damage in Parkinson's disease mice[45-46].

Acupuncture therapy exerts a similar effect with other withdrawal therapies for the treatment of heroin toxic encephalopathy[47-55]. However, whether acupuncture improves central nervous system damage caused by heroin toxicity remains unknown. A review of relevant literatures revealed the influence of acupuncture on brain ultrastructure in heroin addicts[56-58]. However, to date, no studies have investigated if the role of acupuncture is mediated by changes to brain ultrastructure.

In this study, heroin readdiction was produced through repeated exposure and detoxification in rats, which more closely mimics the long-term or repeated drug addiction state in humans than other withdrawal models (one exposure and detoxification). We acupunctured heroin readdicted rats at *Baihui* and *Dazhui*, and then compared rats to methadone treated rats, a commonly used drug for the clinical treatment of drug addiction, in an effort to explore the neuroprotective effect of acupuncture on the brain of heroin readdicted rats at a cellular level.

Results

Quantitative analysis of experimental animals

Forty rats were randomly divided into four groups: normal control, model, acupuncture, and drug. Except for the normal control group, rats in the other three groups were intramuscularly injected with increasing doses of heroin into the hindlimb for 8 successive days, followed by a 5-day withdrawal. The injection and withdrawal were given three times to establish a heroin readdiction model. Rats in the acupuncture group were treated by acupuncture during the detoxification period, while those in the drug group were treated with methadone. No rats died during the experiment.

Acupuncture improved the morphology of nerve cells in the frontal cortex and hippocampus of heroin readdicted rats

Under transmission electron microscopy, we found clear nuclear membranes, normal structure of rough endoplasmic reticulum, ribosomes and cell nuclei, abundant rough endoplasmic reticulum and mitochondria, as well as mild expansion of rough endoplasmic reticulum in the hippocampus and frontal lobe of normal control rats.

In model rats, the frontal cortex and hippocampal neurons exhibited nuclear pyknosis, chromatin margination, rough endoplasmic reticulum expansion, mitochondria degeneration, widened perinuclear gaps, and apparent apoptosis. In the acupuncture group, frontal cortex and hippocampal neurons had clear nuclear membranes and abundant mitochondria and rough endoplasmic reticulum, rough endoplasmic reticulum structure was normal or mildly dilated, and some mitochondrial vacuolar degeneration was observed. Neuronal morphology in the frontal cortex and hippocampus was similar between the acupuncture and drug group (Figure 1).

Acupuncture at *Baihui* and *Dazhui* enhanced Bcl-2 expression in frontal cortex and hippocampal nerve cells of heroin readdicted rats

Immunohistochemical staining showed that the number of

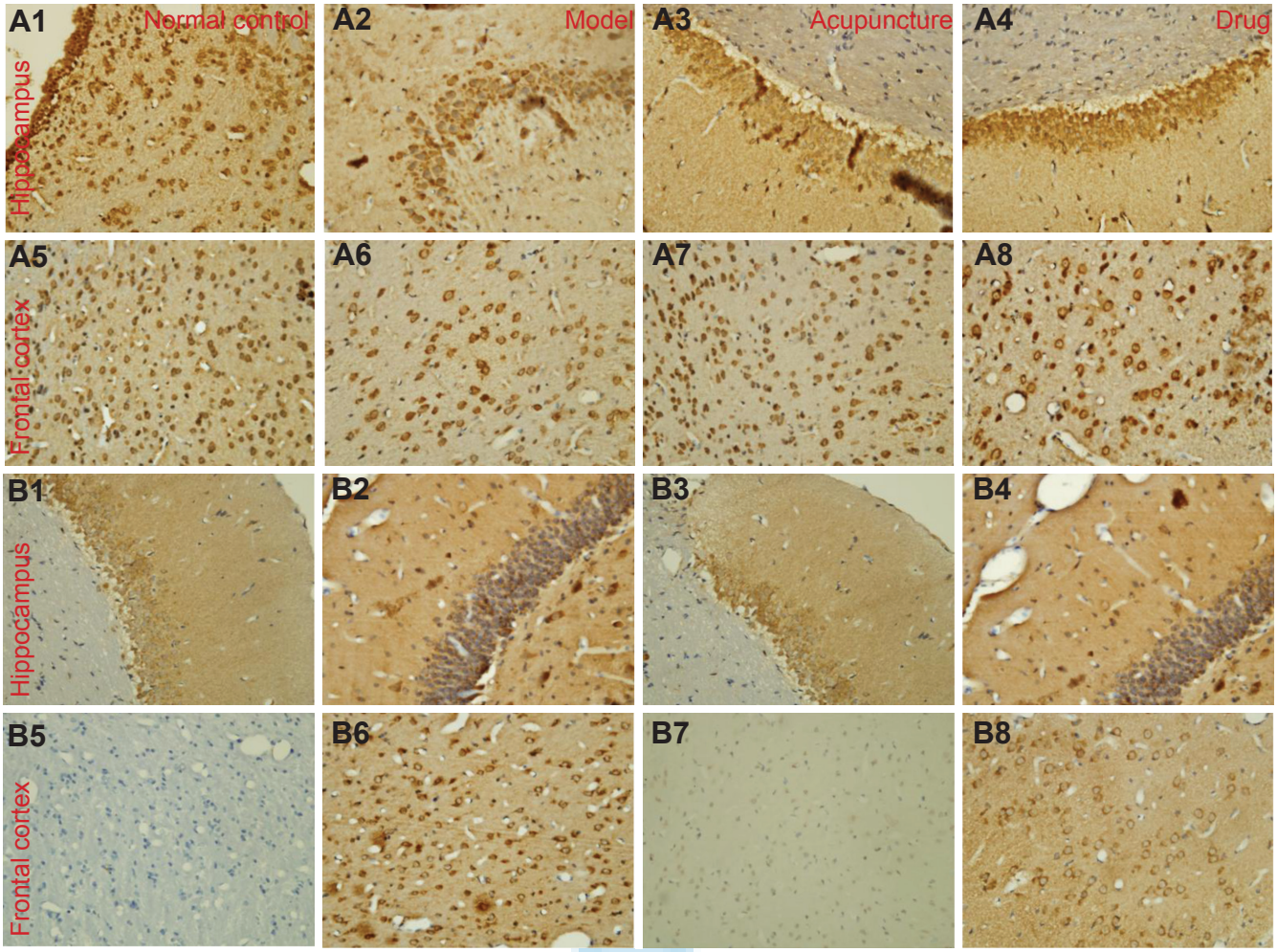

Figure 2 Effect of acupuncture at *Baihui* and *Dazhui* on Bcl-2 (A1–A8) and Bax (B1–B8) expression in the frontal lobe and hippocampus of heroin readdicted rats (immunohistochemical staining, × 400).
Bcl-2 expression in the hippocampus and frontal lobe of model rats was decreased, while Bax epxression was increased compared with the normal control group. Both acupuncture and drug therapy increased Bcl-2 expression but decreased Bax expression in the frontal cortex and hippocampus of heroin readdicted rats. Bcl-2- and Bax-positive expression appeared brown.

Bcl-2-positive cells and Bcl-2 expression in the frontal cortex and hippocampus of heroin readdicted rats significantly decreased compared with the normal control group ($P < 0.01$). In the acupuncture and drug groups, the number of Bcl-2 positive cells and Bcl-2 expression significantly increased compared with the model group ($P < 0.05$). In addition, the acupuncture group showed more Bcl-2-positive cells and higher Bcl-2 expression than the drug group ($P < 0.01$ or $P < 0.05$; Figure 2A, Table 1).

Acupuncture at *Baihui* and *Dazhui* reduced Bax expression in frontal lobe and hippocampal nerve cells of heroin readdicted rats

Immunohistochemical staining showed that the number of Bax-positive cells and Bax expression in the frontal cortex and hippocampus of heroin readdicted rats significantly increased compared with the normal control group ($P < 0.01$). In the acupuncture and drug groups, the number of Bax-positive cells and Bax expression significantly decreased compared with the model group ($P < 0.05$). In addition, the acupuncture group showed more Bax-positive cells and higher Bax expression than the drug group ($P < 0.01$ or $P < 0.05$; Figure 2B, Table 1).

Discussion

In this study, we observed ultrastructural damage in the hippocampus and prefrontal cortex of heroin readdicted rats. Once the stability of the cerebral environment is destroyed, it inevitably affects the basic function and hinders information transmission between the brain and the periphery[59-60]. After the nerve nuclear group in the limbic system is partially destroyed, neuroendocrine immune function may change[59-60], resulting in withdrawal symptoms.

Our findings showed that ultrastructure pathological damage to brain nerve cells of heroin readdicted rats was markedly attenuated after acupuncture. This evidence indicates that acupuncture can reverse heroin readdiction-caused brain injury to some extent. Furthermore acupuncture has the potential to maintain mitochondria and rough endoplasmic reticulum function, promote the transport of proteins synthesized by membrane-bound ribosomes, and help to synthesize ATP, which are conducive to providing energy for

Table 1 Effect of acupuncture at *Baihui* and *Dazhui* on Bcl-2- and Bax-positive cells in the frontal lobe and hippocampus of heroin readdicted rats

Group	Frontal lobe		Hippocampus	
	Number of positive cells/400 × magnification	Absorbance	Number of positive cells/400 × magnification	Absorbance
Bcl-2				
Normal control	424.0±66.0	0.418±0.006	630.3±44.1	0.425±0.008
Model	235.0±36.3[a]	0.320±0.005[a]	140.0±43.6[a]	0.285±0.057[a]
Acupuncture	407.7±38.9[bde]	0.410±0.026[df]	260.7±36.5[ad]	0.324±0.027[ad]
Drug	301.0±38.3[a]	0.351±0.046[ad]	263.3±41.4[ad]	0.326±0.017[ad]
Bax				
Normal control	191.7±25.5	0.125±0.006	172.3±7.0	0.271±0.007
Model	458.7±22.1[a]	0.356±0.041[a]	513.7±28.7[a]	0.493±0.043[a]
Acupuncture	331.3±20.2[ac]	0.307±0.021[ae]	200.7±32.8[cf]	0.346±0.015[bce]
Drug	297.0±17.4[ac]	0.229±0.021[ac]	310.0±27.0[ac]	0.444±0.041[ad]

Data are expressed as mean ± SD. There were two rats in each group. Differences between groups were compared using one-way analysis of variance. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* normal control group; [c]$P < 0.01$, [d]$P < 0.05$, *vs.* model group; [e]$P < 0.01$, [f]$P < 0.05$, *vs.* drug group.

a variety of physiological activities of cells and restoring the function of nerve nuclear groups in the hippocampus and prefrontal cortex[37-46]. Based on the aforementioned evidence, we speculate that acupuncture may potentially restore learning and memory disorders, cognitive disorders, and mood disorders caused by heroin, and prevent readdiction.

In this study, heroin readdiction caused the downregulation of Bcl-2 expression in the frontal lobe and hippocampus, while increased proapoptotic Bax expression levels. Acupuncture at *Baihui* and *Dazhui* increased Bcl-2 expression in the frontal lobe and hippocampus of heroin readdicted rats, while decreased Bax expression levels. Excess Bax produces Bax-Bax homodimers and induces apoptosis; the decline of Bax expression levels leads to the generation of heterodimers with Bcl-2, thus inhibiting apoptosis[61-64]. Electron microscopy results showed that acupuncture can prevent brain cell apoptosis in heroin readdicted rats, which is most likely mediated by altering cell ultrastructure, regulating the expression of the apoptosis-related genes Bcl-2 and Bax, and changing Bcl-2/Bax ratio. However apoptosis is a complex physiological process involving many factors and mechanisms.

We also compared the therapeutic effect of two therapies (acupuncture *vs.* methadone) in the treatment of heroin readdiction. The results showed consistent variations in brain cell ultrastructure and apoptosis-related genes (Bcl-2 and Bax) in the two groups. Acupuncture was more effective than methadone on local lesions.

Overall, our study has provided a partial mechanism for the neuroprotective effect of acupuncture on brain cell death in heroin readdicted rats, and provided insights on the possibility of using acupuncture for the treatment of heroin readdiction. However, further studies are needed to understand how acupuncture prevents apoptosis of brain cells in heroin readdicted rats.

Materials and Methods

Design

A randomized controlled animal experiment.

Time and setting

Experiments were performed from May to June in 2010 at Research Institute of Acupuncture and Meridian, Anhui University of Traditional Chinese Medicine, Anhui Key Laboratory of Foundation and Technology of Acupuncture and Moxibustion (Cultivating Base), China.

Materials

Animals

Forty Wistar rats of clean grade, aged 4 months, half male and half female, weighing 200–220 g, were provided by the Experimental Center of Nanjing Medical University, China, under the permission number of SCXK (Su) 2008-0004. Prior to experimentation, rats were given a normal diet for 7 days to adapt to vivarium conditions, under a natural lighting cycle, at 25°C and 58–60% humidity. All experimental procedures were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[65].

Drugs

Heroin powder (purity 90%; chemical name: diacetylmorphine; Figure 3A), was provided by the Narcotics Control Commission of Anhui Province, China. Methadone hydrochloride is chemically described as 6-(dimethylamino)-4,4-diphenyl-3-heptanone hydrochloride (Figure 3B), and was provided by Tianjin Central Pharmaceutical Co., Ltd., Tianjin, China. The approval No. was (97) X-313 (2), lot No. was 090919, and the specification was 10 mg/10 mL.

Methods

Establishment of heroin readdiction model

Rats were given an intramuscular injection of heroin into the hindlimb for 8 successive days. The injection dose was initially 0.8 mg/kg on day 1 and gradually increased to 3.6 mg/kg on day 8, with a 0.4 mg/kg increment per day. Heroin was dissolved in saline and 0.5 mL was given to each rat per day[21]. At days 4–8, each rat received two injections of heroin, as the exposure (addiction) process. After 8 days

of injection, the intramuscular injection of heroin was withdrawn for 5 days (detoxification). The exposure and detoxification procedures were repeated three times to establish heroin readdiction models[66-67]. In the normal control group, rats were given intramuscular saline 0.5 mL per day.

Acupuncture intervention

Each rat was treated with acupuncture during the detoxification period. According to the Rat Brain in Stereotaxic Coordinates[68], we selected *Baihui* (parietal bone) and *Dazhui* (between the seventh cervical vertebrae and the first thoracic vertebrae, in the middle of the back). Rats were fixed in a special holder and remained awake. A 25-mm-long stainless steel needle (Suzhou Medical Supplies Factory, Suzhou, Jiangsu Province, China) was flatly inserted into the *Baihui* and *Dazhui* points, to a depth of 12 mm, then the needle was retained for 30 minutes. The acupuncture therapy was given at 8:00 daily for 5 successive days.

Methadone intervention

Each rat was treated with methadone at decreasing doses for 5 days during the detoxification period. The daily dose of methadone was 0.4, 0.3, 0.2, 0.1, 0 mg per day.

Immunohistochemical detection of Bcl-2 and Bax expression in the frontal cortex and hippocampus

Two rats in each group were anesthetized with 10% (v/v) chloral hydrate *via* peritoneal injection. The chest was opened, and a small incision was made on the right atrial appendage. The catheter was then engaged *via* the left ventricle into the aorta and the rat was rapidly perfused with 250 mL saline until the effluent from the right atrial appendage became clear, followed by a slow infusion of 4% (w/v) paraformaldehyde for 30 minutes[69]. The frontal cortex and hippocampus were harvested and fixed in 4% (w/v) paraformaldehyde for 1 week. Subsequently, paraffin sections were dewaxed and placed in 3% (v/v) H_2O_2 methanol solution at room temperature for 10–20 minutes. After three washes with distilled water, endogenous peroxidase was blocked. The sections were immersed in 0.01 mol/L citrate buffer (pH 6.0) and heated to boiling on an electric heater twice with an interval of 5 minutes. After cooling to room temperature, sections were rinsed using 0.1 mol/L PBS (pH 7.3) twice and incubated with normal goat blocking solution (1:20) at room temperature for 20 minutes to block non-specific binding sites.

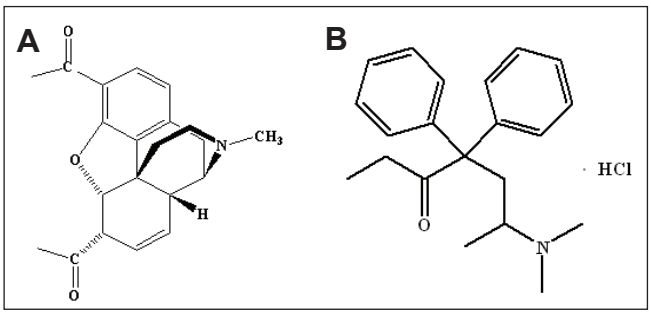

Figure 3 Chemical structure of heroin (A) and methadone hydrochloride (B).

After excess liquid was removed, sections were incubated with rabbit anti-rat Bcl-2 monoclonal antibody (1:100; Boster Biological Engineering Co., Ltd., Wuhan, Hubei Province, China) and rabbit anti-rat Bax monoclonal antibody (1:100; Boster Biological Engineering Co., Ltd.) in a wet box at 4°C overnight. The negative control was treated with PBS instead of primary antibody. Sections were then incubated with biotinylated goat anti-rabbit IgG and SABC at 37°C for 30 minutes. Between each step, sections were rinsed using 0.1 mol/L PBS (pH 7.3) twice for 5 minutes each. Subsequently, sections were developed with 3,3′-diaminobenzidine for 5–20 minutes, washed with distilled water and counterstained with hematoxylin for 10 seconds, followed by dehydration, and mounting. Using the DP801 morphological microscopic image analysis system (Jetta Technology Development Co., Ltd., Nanjing, Jiangsu Province, China), five random sections of each rat were examined at five different visual fields (400 × magnification). Positively stained cells showing brown particles were counted in each field and averaged to obtain the mean absorbance values.

Electron microscopy observation of prefrontal cortex and hippocampal ultrastructure

After the experiment was complete, rats were sacrificed by cervical dislocation, and prefrontal cortex and hippocampal tissue were cut according to the Rat Brain Stereotaxic Coordinates[69] on ice. The specimens were then cut into 1-mm^3 blocks and fixed in 1% (v/v) osmium tetroxide fixative, then serially sliced into thin slices at 70 nm thickness for transmission electron microscopy (JEOL, Tokyo, Japan) observation.

Statistical analysis

Data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL, USA) and results were expressed as mean ± SD. Differences among groups were compared using one-way analysis of variance. A $P < 0.05$ value was considered statistically significant.

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Author contributions: *Hou XR was responsible for the study concept and design, writing the manuscript and was in charge of funds. Song XG supervised the manuscript, guided the study, and was in charge of funds. Lv H, Xie GC and Cai XH were responsible for data acquisition and integration. Zhang RJ performed statistical analysis. All authors approved the final version of the manuscript.*

Conflicts of interest: *None declared.*

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