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Effects of genistein on neuronal apoptosis, and expression of Bcl-2 and Bax proteins in the hippocampus of ovariectomized rats★

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Abstract
Genistein is one of several isoflavones that has a structure similar to 17β-estradiol, has a strong antioxidant effect, and a high affinity to estrogen receptors. At 15 weeks after ovariectomy, the expression of Bcl-2 in the hippocampus of rats decreased and Bax expression increased, with an obvious upregulation of apoptosis. However, intraperitoneal injection of genistein or 17β-estradiol for 15 consecutive weeks from the second day after operation upregulated Bcl-2 protein expression, downregulated Bax protein expression, and attenuated hippocampal neuron apoptosis. Our experimental findings indicate that long-term intervention with genistein can lead to a decrease in apoptosis in hippocampal neurons following ovariectomy, upregulate the expression of Bcl-2, and downregulate the expression of Bax. In addition, genistein and 17β-estradiol play equal anti-apoptotic and neuroprotective roles.

Key Words
ovariectomized model; rats; hippocampus; apoptosis; Bcl-2; Bax; genistein; 17β-estradiol; brain injury; neural regeneration

Research Highlights
(1) Genistein can inhibit neuronal apoptosis in the hippocampus of ovariectomized rats.
(2) Genistein can increase the expression of Bcl-2 and decrease the expression of Bax in the hippocampus of ovariectomized rats.
(3) Genistein and 17β-estradiol show an equal protective effect in ovariectomized rats.

Abbreviations
AD, Alzheimer's disease; OVX, ovariectomy

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INTRODUCTION

Epidemiological research has found that the prevalence of Alzheimer's disease (AD) in menopausal women is 1.5–3.0 times higher than that in men[1], which indicates that low estrogen levels after menopause may have a close relationship with AD onset[2]. Meanwhile, the benefit of improving cognition, and reducing the risk and severity of dementia shown by estrogen replacement therapy on menopausal women[3] further confirms the potential protective effect of estrogen in the occurrence and progress of AD. The hippocampus is predominantly affected in AD. The extensive loss of neurons in the hippocampus is a typical

characteristics of AD, in which apoptotic cell death plays an important role[4-6]. In addition, recent studies have shown that the hippocampus expresses high levels of estrogen receptors, and that it is an important target organ for estrogen[7]. These high levels of estrogen receptors suggest that estrogen may play a protective role in AD *via* decreasing apoptotic cell death of hippocampal neurons.

Previous studies have shown that estrogen (17β-estradiol) is capable of ameliorating neuronal apoptosis[8], but the detailed mechanism of its protective effect remains unclear. Phytoestrogen has been proposed as an alternative to estrogen with similar actions in neuronal protection with lower side effects[9]. Genistein, one of several known isoflavones, with a structure similar to 17β-estradiol, is commonly used as the primary candidate for phytoestrogen due to its strong antioxidant properties and high affinity to estrogen receptors[10-11]. However, there is a paucity of information on the effects of genistein on neuronal apoptosis in the hippocampus of ovariectomized (OVX) rats in the literature. Bcl-2 and Bax protein, two important apoptosis-related proteins of the Bcl-2 protein family, which have been reported to change expression levels in the hippocampus of AD patients, are thought to be involved in the neuronal apoptosis observed in AD brains[12]. Thus, we examined the effects of genistein and 17β-estradiol on apoptosis, and the expression of Bcl-2 and Bax proteins in the hippocampus of OVX rats.

RESULTS

Quantitative analysis of experimental animals

A total of 130 healthy female Sprague-Dawley rats were randomly assigned to five groups: control group (n = 10), sham group (n = 30), OVX group (n = 30), OVX + genistein group (Gen group; n = 30), and OVX + 17β-estradiol group (Est group; n = 30). The 10 rats from the control group were immediately sacrificed at 7 months of age. The other four groups were further divided into 3-week subgroups (n = 10) and 15-week subgroups (n = 20) according to the time of sacrifice after operation. Rats in the OVX, Gen, and Est groups underwent OVX surgery, while rats in the sham group received a fake operation. From the second day after operation, rats in the Gen group and Est group were given a corresponding dose of genestein and 17β-estradiol through intraperitoneal injection till the day before death. At the end of the experiment, the number of animals in the control, sham (3-week subgroup, 15-week subgroup), OVX (3-week subgroup, 15-week subgroup), Gen (3-week subgroup, 15-week subgroup), and Est (3-week subgroup, 15-week subgroup) groups were 10, 10 and 17, 10 and 16, 10 and 19, and 9 and 13, respectively. Sixteen rats died at different times after operation, which may be partly associated with inappropriate surgical operation or infection. However, we could not determine the precise cause of death. A total of 114 rats were included in the final analysis.

Effects of genistein and 17β-estradiol on neuronal morphology in the hippocampus of OVX rats

Hematoxylin-eosin staining was used to observe the morphology of neurons in the hippocampus. All subregions of the hippocampus (CA1, CA2, CA3, CA4 and dentate gyrus) were observed in the study for TUNEL and immunohistochemistry. Images of the CA3 region were selected as representative images to show changes in the hippocampus. The morphology of cells in the control group and all the 3-week subgroups from the other four groups remained normal. At 15 weeks after operation, apoptosis was evident in the hippocampus of the OVX group, such as cells with a semilunar nucleus, nuclear fragmentation, and chromatin condensation with the loss of neurons. Meanwhile, after corresponding treatments with genistein or 17β-estradiol, only a few apoptotic changes were visible, such as cell body shrinking, trachychromatic nuclei and karyopyknosis. There were no obvious differences between the treatments (Figure 1, supplementary Figure 1 online).

Effects of genistein and 17β-estradiol on cell apoptosis in the hippocampus of OVX rats

TUNEL was used to estimate the extent of apoptosis. Apoptotic death was confirmed by the presence of TUNEL-positive cells which stained brown. As shown in Figure 2, few TUNEL-positive cells were seen in the control group. At 3 weeks after operation, a few TUNEL-positive cells were observed in the sham, Est, Gen and OVX groups. While at 15 weeks after operation, there were a large numbers of TUNEL-positive cells in the OVX group, yet only a few in the Sham, Est, and Gen groups (supplementary Figure 2 online).

Results revealed that at 3 weeks after operation, there were no significant differences in apoptotic index in the control, sham, Est, Gen and OVX groups (P > 0.05; Table 1). At 15 weeks after operation, the apoptotic index in the OVX group was significantly higher than that in the control, sham, Est, and Gen groups (P < 0.05). However, following treatment with genistein or 17β-estradiol, the apoptotic index in the rat hippocampus (Gen and Est

groups) decreased to almost normal levels. There was no difference between the treatments (Table 1).

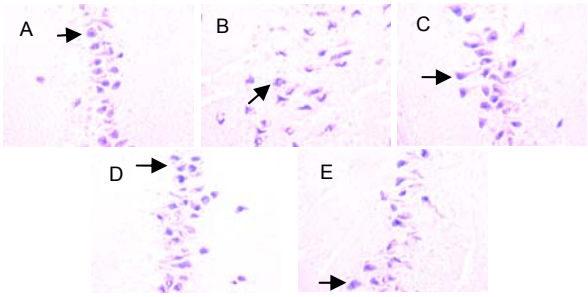

Figure 1 Morphology of neurons (arrows) in the rat hippocampal CA3 region (hematoxylin-eosin staining, × 400).

(A) Representative images of the hippocampus in the control group show that neurons had a normal, giant pyramidal shape, with light red plasma and blue round nucleus.

(B) Few nuclei of normal morphology can be found in the CA3 region of the hippocampus in the ovariectomy group at 15 weeks. Karyopyknosis, chromatin margination and karyolobism were prevalent in cells.

(C) Neurons in the sham group at 15 weeks remained normal.

(D) Most neurons in ovariectomy + genistein group at 15 weeks tended to appear normal. Dyskaryosis and nuclei with vacuoles can be seen in a few cells.

(E) Neurons in the ovariectomy + 17β-estradiol group at 15 weeks tended to appear normal. Dyskaryosis can be seen in only a few cells, and almost no nuclei had vacuoles.

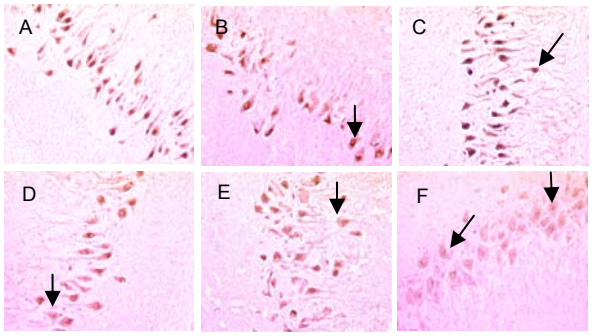

Figure 2 Apoptosis of neurons (arrows) in the rat hippocampal CA3 region (TUNEL method, × 400).

(A) Representative images of the hippocampus in the control group show few TUNEL-positive cells.

(B) Few TUNEL-positive cells can be seen in the sham group at 3 weeks.

(C) Few TUNEL-positive cells can be seen in the sham group at 15 weeks.

(D) Few TUNEL-positive cells can be seen in the ovariectomy + genistein group at 15 weeks.

(E) Few TUNEL-positive cells can be seen in the ovariectomy + 17β-estradiol group at 15 weeks.

(F) A large number of TUNEL-positive cells can be seen in ovariectomy group at 15 weeks.

Effects of genistein and 17β-estradiol on Bcl-2 and Bax expression in the hippocampus of OVX rats

Immunohistochemistry revealed that Bcl-2 and Bax immunoreactive products presented as brown-yellow fine granules, located in the cytoplasm. Massive expression of Bcl-2 protein and minimal expression of Bax was observed in the control group, and in the other groups at 3 weeks after operation (Figure 3, supplementary Figures 3, 4 online), with no significant difference in the percentage of Bcl-2-positive cells or Bax-positive cells between these groups ($P > 0.05$; Tables 2, 3). At 15 weeks after operation, Bcl-2 protein expression decreased and Bax expression increased in the OVX group ($P < 0.05$), whereas treatment with genistein or 17β-estradiol reversed this change, retaining the expression of Bcl-2 and Bax in the hippocampus close to the normal levels ($P > 0.05$; Tables 2, 3).

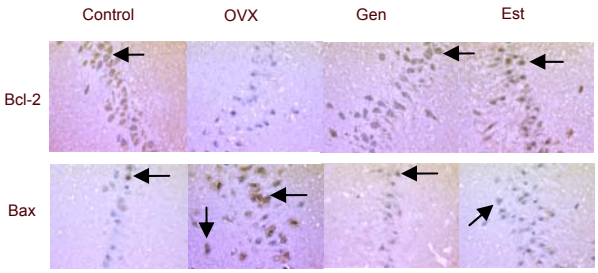

Figure 3 Bcl-2 and Bax expression (arrows) in the rat hippocampal CA3 region (immunohistochemistry, × 400).

Massive expression of Bcl-2 and few Bax-positive cells can be seen in the control group. Scarce Bcl-2 expression can be seen in the ovariectomy group (OVX) at 15 weeks. Expression of Bax was upregulated in the ovariectomy group at 15 weeks.

The expression levels of Bcl-2 and Bax in the ovariectomy + genistein (Gen) and ovariectomy + 17β-estradiol groups (Est) at 15 weeks were similar to the control.

DISCUSSION

Apoptosis or programmed cell death is regulated by various factors[13-18]. Studies have shown that the anti-apoptotic effect of estrogen is biologically relevant to many harmful processes, with one of the most important being its affect on the expression of apoptosis-related genes[19]. The Bcl-2 family is one of the most well known protein families, with Bcl-2 being a key regulator in endogenous apoptosis. Bcl-2 and Bax are characteristic anti-apoptotic and pro-apoptotic genes, respectively. Bcl-2 can inhibit apoptosis induced by multiple factors, and is widely expressed in the central nervous system[20].

Table 1 Effects of genistein and 17β-estradiol on the apoptotic index (%) in sub-regions of the hippocampus in ovariectomized rats

Group	*n*	3 weeks after operation				
		CA1	CA2	CA3	CA4	Dentate gyrus
Control	10	3.81±1.29	4.90±1.28	4.81±1.33	4.80±1.32	3.79±1.29
Sham	10	4.11±1.34	5.21±1.32	5.24±1.31	4.95±1.30	4.39±1.31
OVX	10	4.52±1.31	5.69±1.27	6.01±1.29	5.52±1.31	5.04±1.30
Gen	10	4.37±1.33	5.44±1.30	5.71±1.28	5.29±1.29	4.82±1.32
Est	9	4.28±1.31	5.35±1.29	5.49±1.30	5.17±1.28	4.51±1.29
Group	*n*	15 weeks after operation				
		CA1	CA2	CA3	CA4	Dentate gyrus
Control	10	3.81±1.29	4.90±1.28	4.81±1.33	4.80±1.32	3.79±1.29
Sham	17	4.39±1.33	5.13±1.31	5.19±1.30	5.20±1.31	4.51±1.30
OVX	16	54.43±8.66[a]	76.55±7.39[a]	78.97±6.77[a]	71.05±8.04[a]	58.98±6.95[a]
Gen	19	5.03±1.30	6.05±1.34	6.04±1.31	6.02±1.33	5.07±1.32
Est	13	4.85±1.34	5.81±1.33	5.88±1.33	5.77±1.31	4.85±1.34

Apoptotic index = number of TUNEL-positive cells/(number of TUNEL-positive cells + number of TUNEL-negative cells) × 100%. Data are expressed as mean ± SD. [a]*P* < 0.05, *vs*. Control, Sham, Gen and Est groups (one-way analyses of variance). OVX: Ovariectomy group; Gen: ovariectomy + genistein group; Est: ovariectomy + 17β-estradiol group.

Table 2 Effects of genistein and 17β-estradiol on percentage of Bcl-2 positive cells (%) in the hippocampai sub-regions in ovariectomized rats

Group	*n*	3 weeks after operation				
		CA1	CA2	CA3	CA4	Dentate gyrus
Control	10	92.84±5.28	95.28±3.02	97.79±2.17	91.23±7.00	93.35±5.68
Sham	10	93.48±7.25	94.34±4.42	96.37±3.51	93.12±6.43	92.55±4.89
OVX	10	90.48±7.25	89.27±4.43	91.24±5.57	88.91±5.36	88.09±4.33
Gen	10	91.32±6.25	92.31±4.43	93.37±4.22	90.19±5.33	90.67±5.18
Est	9	92.42±6.25	93.37±5.64	95.13±3.68	92.47±4.86	91.98±6.74
Group	*n*	15 weeks after operation				
		CA1	CA2	CA3	CA4	Dentate gyrus
Control	10	92.84±5.28	95.28±3.02	97.79±2.17	91.23±7.00	93.35±5.68
Sham	17	93.07±6.21	95.37±4.13	96.89±3.51	92.21±6.61	92.38±5.38
OVX	16	36.63±6.90[a]	27.34±5.39[a]	24.09±4.35[a]	24.48±6.21[a]	43.99±5.07[a]
Gen	19	90.32±5.35	91.51±6.47	92.75±3.82	89.88±6.37	89.34±4.65
Est	13	91.87±7.27	93.07±8.06	93.81±4.39	90.38±7.09	90.66±7.45

Percentage of Bcl-2-positive cells = number of Bcl-2-positive cells/(number of Bcl-2-positive cells + number of Bcl-2-negative cells) × 100%. Data are expressed as mean ± SD. [a]*P* < 0.05, *vs*. Control, Sham, Gen and Est groups (one-way analyses of variance). OVX: Ovariectomy group; Gen: ovariectomy + genistein group; Est: ovariectomy + 17β-estradiol group.

In contrast, Bax induces apoptosis. As Bcl-2 expression is upregulated, the ratio of Bcl-2/Bax increase, which induces the increase of the homodimer of Bcl-2, resulting in cell survival. In contrast, as Bax expression increases, the ratio of Bcl-2/Bax decreases, resulting in cell death[21]. The distribution of estrogen receptors is closely related to the pathological region of AD[22]. Recent studies have found that estrogen may affect the expression of Bcl-2 through the regulation of estrogen receptors, particularly estrogen receptor β[23], resulting in inhibition of apoptosis[24]. In the present study, we monitored the expression of Bcl-2 and Bax in the hippocampus. We found that there was minimal apoptotic cell death in all groups at 3 weeks, with no notable change in the expression of Bcl-2 and Bax. At 15 weeks after operation, apoptosis was observed in OVX rats, with an obvious decline in Bcl-2 and an increase in Bax, compared with those at 3 weeks. However, a low dose of genistein and 17β-estradiol blocked this change, with a significant upregulation in the expression of Bcl-2 and a downregulation in the expression of Bax in rats. There was no difference between the Gen and Est groups. These results suggest that long-term estrogen deficiency can lead to an increase in Bax and a decrease in Bcl-2, leading to a decrease in the ratio of Bcl-2/Bax and the induction of apoptosis in hippocampal neurons in OVX rats.

Table 3 Effects of genistein and 17β-estradiol on percentage of Bax-positive cells (%) in the hippocampal sub-regions in ovariectomized rats

Group	*n*	3 weeks after operation				
		CA1	CA2	CA3	CA4	Dentate gyrus
Control	10	5.37±1.85	5.65±1.90	5.96±1.79	5.68±1.90	5.56±1.84
Sham	10	5.52±1.83	5.73±1.77	6.03±1.91	6.21±1.89	5.71±1.78
OVX	10	6.00±1.79	6.28±2.03	7.07±1.86	7.06±1.83	6.87±1.85
Gen	10	5.96±1.78	6.01±1.94	6.48±1.83	6.80±1.85	6.26±1.88
Est	9	5.82±1.81	5.87±1.86	6.25±1.87	6.59±1.94	6.03±1.92
Group	*n*	15 weeks after operation				
		CA1	CA2	CA3	CA4	Dentate gyrus
Control	10	5.37±1.85	5.65±1.90	5.96±1.79	5.68±1.90	5.56±1.84
Sham	17	5.61±1.91	5.98±1.92	6.23±1.94	6.53±1.94	5.86±1.89
OVX	16	91.33±2.73[a]	92.85±1.15[a]	98.41±1.59[a]	98.15±1.85[a]	97.30±3.22[a]
Gen	19	6.59±2.01	6.83±1.88	7.06±1.88	7.15±1.87	6.78±2.08
Est	13	6.35±1.94	6.40±1.91	6.69±1.85	6.81±1.96	6.27±1.94

Percentage of Bax-positive cells = number of Bax-positive cells/(number of Bax-positive cells + number of Bax-negative cells) × 100%. Data are expressed as mean ± SD. [a]$P < 0.05$, *vs*. Control, Sham, Gen and Est groups (one-way analyses of variance). OVX: Ovariectomy group; Gen: ovariectomy + genistein group; Est: ovariectomy + 17β-estradiol group.

Therefore, the reduction in estrogen may involve this mechanism to induce apoptosis. Genistein and 17β-estradiol may act by increasing the expression of Bcl-2 and decreasing the expression of Bax, resulting in a neuroprotective effect. This protective effect may also come from the stimulation of estrogen receptor β, which activates the estrogen response element of the Bcl-2 gene, and then increases transcription and translation to upregulate the expression of Bcl-2. Recent studies have shown that estrogen may also act on estrogen receptors on the cell membrane to affect binding and release of Bcl-2 and related proteins, which ultimately effects the levels of these proteins and has a biological effect[25]. However, the way in which estrogen conducts its signal and affects the expression of Bcl-2 and Bax still needs further study. With further clinical use of estrogen replacement therapy on menopause-related diseases, side effects such as increasing the risk of tumor disease limit its clinical administration. The type of estrogen used in estrogen replacement therapy may account for these side effects, as estrogen derived from animals have a higher affinity with both estrogen receptor α and estrogen receptor β. Estrogen receptor α is predominantly distributed in the uterus, breast, ovaries and other endocrine organs. Therefore, exposure to estrogen may increase the risk of tumors through the stimulation of estrogen receptor α[26]. Genistein, one of the most common phytoestrogens, which is widely contained in vegetables, fruit, cereals, and beans, is natural, has few side effects, and has a higher affinity with estrogen receptor β over estrogen receptor α[27]. Accordingly, it may reduce the risk of tumors by avoiding the above endocrine organs or antagonize some of the effects of estrogen[28-29]. However, there is still a lack of clinical data regarding the use of genistein to prevent and treat AD.

In summary, the present study demonstrated that genistein and 17β-estradiol can reduce apoptosis in hippocampal neurons in OVX rats, and that both treatments have an equal neuroprotective effect.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

This study was performed from January 2006 to December 2010 at the Institute of Endocrinology and Metabolism Laboratory and the Animal Experiment Center of the Second Xiangya Hospital, Central South University, China.

Materials

Animals

A total of 130 clean healthy female Sprague-Dawley rats (clean grade), aged 7 months and weighing 293.1 ± 10.2 g, with no history of reproduction or breast-feeding, with no liver disease or skeletal deformities, were supplied by the Research Animal Center of the Second Xiangya Hospital, Central South University, China (license No. SYXK (Xiang) 2004-0013). The experimental rats were housed individually in plastic cages under standard environment conditions (12-hour light/dark cycle lighting on 8:00 a.m.,

21–25°C, 50–70% humidity), fed with standard rat feed (22 g/rat per day), and were allowed free access to water. The experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[30].

Drugs

Genistein (5,7-Dihydroxy-3-(4-hydroxyphenyl) chromen-4-one, $C_{15}H_{10}O_5$, 98.5% purity, laboratory reagent) was purchased from Huabei Pharmaceutical Company (Shijiazhuang, Hebei Province, China). First, genestin was dissolved in dimethyl sulfoxide, and then diluted in polyethylene glycol 200 (dimethyl sulfoxide: polyethylene glycol 200 = 1:4) to obtain a working solubility (5 mg/mL) of genestein. 17β-estradiol (3,17β-Dihydroxy-1,3,5(10)-estratriene 17-acetate, $C_{20}H_{26}O_3$, ≥ 99% purity, analytical reagent) was from Sigma (St. Louis, MO, USA). 17β-estradiol was dissolved in dimethyl sulfoxide, and was then diluted in polyethylene glycol 200 (dimethyl sulfoxide:polyethylene glycol 200 = 1:4) to get a working solubility (10 μg/mL) of 17β-estradiol.

Methods

Establishment of OVX rat models

Rats in the OVX, Gen, and Est groups were anesthetized by intraperitoneal injection with 3% pentobarbital (0.1 mL/100 mg), and were the subjected to OVX operation[31]. Rats in the sham group received fake operation, removal of a small piece of fat (the volume of fat was equal to the removal of tissue in OVX rats), with the bilateral ovary reserved.

Intraperitoneal injection of genistein and 17β-estradiol

From the second day after OVX surgery, rats in the Gen group and Est group were injected with genestein (5 mg/kg)[32-34] and 17β-estradiol (10 μg/kg), respectively, once per day[35] into the abdominal cavity. Rats in the sham and OVX groups were injected in the abdominal cavity with the same volume (1 mL/kg, about 0.3 mL) of dimethyl sulfoxide/polyethylene glycol 200 (1:4). Rats in the control group were not given any treatment.

Preparation of specimens

All rats were anesthetized by intraperitoneal injection with 3% (v/v) pentobarbital (0.1 mL/100 mg). After anesthesia, rats were decapitated, and the entire brain was immediately collected. The brains were routinely perfused and fixed with PBS containing 40 g/L citromint for 24 hours, routinely dehydrated, cleared, dipped in wax, and embedded in paraffin. Brain tissues were sliced into consecutive coronal sections at a thickness of 8 μm from the superior natis to the optic chiasm according to the atlas by Paxinos and Watson[36], mounted onto polylysine-coated glass slides. Slices of the hippocampus were prepared for further observation.

Morphology of hippocampal cell detected by hematoxylin-eosin staining

A total of four paraffin sections from each rat were selected and subjected to de-waxing and hydration, followed by Harris hematoxylin staining for 10 minutes: 75% hydrochloric acid, alcohol differentiation for 5 seconds, acidified eosin alcohol counterstaining for 1 minute, dehydration, clearing, gum mounting, and then were observed by a bright field microscope (Leica London, United Kingdom). All the sub-regions of the hippocampus (CA1, CA2, CA3, CA4 and dentate gyrus) were observed in the study, including for TUNEL and immunohistochemistry.

Hippocampal cell apoptosis detected by TUNEL

Slices were treated using TUNEL to observe apoptosis[37]. Paraffin sections were de-waxed and hydrated, followed by 3% H_2O_2 at room temperature for 10 minutes. The sections were then digested with fresh diluted Proteinase K (1:200) at 37°C for 15 minutes. Then, 1 μL TDT and DIG-d-UTP, together with 18 μL marker buffer, were added to each section at 37°C for 2 hours. The sections were then blocked at room temperature for 30 minutes, followed by incubation with biotinylated anti-digoxin antibody (1:100) at 37°C for 30 minutes, SABC (1:100) at 37°C for 30 minutes, diaminobenzidine (Beijing Zhongshan Biotechnology Co., Ltd.) coloration, and observation by bright field microscopy (Leica). Cells with brown-yellow fine granules present in the nucleus were recognized as TUNEL-positive cells. Four slices were selected from each specimen and three visual fields were selected from each slice, and then were observed under high-power magnification (400 ×) to quantify the number of TUNEL-positive cells. The apoptotic index was calculated as follows: apoptotic index = number of TUNEL-positive cells/(number of TUNEL-positive cells + number of TUNEL-negative cells) × 100%.

Bcl-2 and Bax expression in the hippocampus detected by immunohistochemical staining

The expression of Bcl-2 and Bax protein expression in the hippocampus was determined using the streptavidin/peroxidase immunohistochemical method[38]. Sections were incubated with rabbit anti-rat Bcl-2 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Bax

(1:100; Santa Cruz Biotechnology) antibodies at 4°C overnight. PBS instead of primary antibody served as negative control. Tissue was incubated with goat anti-rabbit IgG (1:200; Beijing Zhongshan Goldenbridge Biotechnology, Beijing, China) at 37°C for 15 minutes. A positive reaction was indicated by the appearance of brown-yellow coarse or fine particles, which were diffusely distributed. Cells were colored with DAB. Nuclei were counterstained with hematoxylin, followed by gradient ethanol dehydration, xylene transparency, and neutral gum mounting, and observed under a bright field microscope (Leica). Positive immunohistochemical results were quantitatively analyzed using a Leica image analysis system (Leica DMLA/software Version3.0). Bcl-2 and Bax-positive slices were randomly selected from the three visual fields in all the sub-hippocampal areas (400 × magnification). The percentage of positive cells was calculated using the following formula: The percentage of positive cells = number of positive cells/(number of positive cells + number of negative cells) × 100%. Four slices were selected from each specimen and three visual fields were selected from each slice to obtain the average values. The analysis of the average gray value of the expression of Bcl-2 and Bax are shown in supplementary Tables 1, 2 online.

Statistical analysis

All data are expressed as mean ± SD and are analyzed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA) by multiple comparison using one-way analyses of variance. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Yun Peng was in charge of the study design, acquisition and analysis of data, drafting of the paper and statistical treatment. Bo Jiang was responsible for the funding, had full access to the study concept and design, oversaw the experiments, and checked the paper. Huiling Wu participated in experimental animal breeding, statistical analysis and processing. Ruchun Dai provided information support. Liming Tan provided validation and guidance of the study, and helpful discussion regarding the manuscript. All authors approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Hunan Ethics Committees of Animal Research in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

Supplementary information: Supplementary data associated with this article can be found in the online version, by visiting www.nrronline.org.

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