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Effects of progesterone on glutamate transporter 2 and gamma-aminobutyric acid transporter 1 expression in the developing rat brain after recurrent seizures*☆

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Abstract
Seizures were induced by flurothyl inhalation. Rats were intramuscularly treated with progesterone after each seizure. Results demonstrated that glutamate transporter 2 and γ-aminobutyric acid transporter 1 expression levels were significantly increased in the cerebral cortex and hippocampus of the developing rat brain following recurrent seizures. After progesterone treatment, glutamate transporter 2 protein expression was upregulated, but γ-aminobutyric acid transporter 1 levels decreased. These results suggest that glutamate transporter 2 and γ-aminobutyric acid transporter 1 are involved in the pathological processes of epilepsy. Progesterone can help maintain a balance between excitatory and inhibitory systems by modulating the amino acid transporter system, and protect the developing brain after recurrent seizures.

Key Words
progesterone; seizure, transporter, glutamate, γ-aminobutyric acid

Research Highlights
Immunohistochemistry and western blot revealed that progesterone promoted glutamate transporter 2 expression and inhibited γ-aminobutyric acid transporter 1 expression in the cerebral cortex and hippocampus following recurrent seizures.

Abbreviations
GABA, γ-aminobutyric acid; GLT-1, glutamate transporter 2; GAT-1, GABA transporter 1

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INTRODUCTION

Seizures are strongly associated with an imbalance of excitatory and inhibitory systems in the central nervous system[1]. Following a seizure, excitatory amino acid levels increase in the synaptic cleft. Cytotoxicity can induce neuronal necrosis and apoptosis. Amino acid transporters can remove glutamate and γ-aminobutyric acid (GABA) in the central nervous system, especially glutamate transporter 2 (GLT-1) and GABA transporter 1 (GAT-1)[2-4]. GLT-1, mainly expresses in astrocytes, maintains a steady state of excitatory amino acids, and participates in neuronal differentiation, survival, synaptic plasticity formation and glial cell proliferation[5]. Expression levels of GLT-1 have been shown to remain unchanged after a seizure[6]. However, this may be due to the seizure model used, type of seizure, and the specific age being studied. GAT-1 expression is present

throughout neurons and astrocytes, where it rapidly uptakes GABA in the synaptic cleft and terminates inhibitory synaptic transmission, and participates in neuronal repair and synaptic plasticity[7].

Progesterone, a neuroactive steroid synthesized and secreted by nerve tissue, can protect the brain by suppressing neural cell apoptosis, promoting nerve growth, and relieving inflammatory swelling[8-10]. Progesterone has been clinically used in the treatment of epilepsy, cerebral trauma, stroke and other nervous system diseases. Progesterone is involved in neuronal maturation, neural plasticity, nervous excitation, mitochondrial function, and neurotransmitter synthesis and transport by progesterone receptors in the hypothalamus, hippocampus and limbic system[11-12]. Progesterone maintains normal brain function by regulating transmitter release and ion channel function in the neurotransmitter system[13]. To date, research on glutamate and GABA receptors has mainly focused on the regulatory effects of progesterone on the central neurotransmitter system[14-15]. However, few studies have focused on the effects of progesterone on amino acid transporter levels in the developing brain following seizures. This study sought to investigate the effects of progesterone on GLT-1 and GAT-1 levels in the rat cerebral cortex and hippocampus following recurrent seizures in the developing brain. In addition, we aimed to explain the pathogenesis of seizure-induced excitotoxic brain injury and the mechanism of action of progesterone.

RESULTS

Quantitative analysis of experimental animals

A total of 72 Sprague-Dawley neonatal rats were equally and randomly divided into control, seizure and progesterone groups. Seizures were induced by flurothyl inhalation in the seizure and progesterone groups. The rats from the progesterone group were intramuscularly treated with progesterone after each seizure. Eight rats from each group were analyzed at 1, 3 and 7 days following the last seizure. All rats were included in the final analysis.

Behaviors of flurothyl-induced seizure rats

Rats from the seizure group experienced dysphoria, shaking heads, yelping, walloping every 1–2 minutes, and tonic-clonic seizures lasting 5–8 minutes, with an interval of 3–5 minutes. They were not conscious during each intermission. The rats were lethargic and out of breath for 30 minutes following seizures, after which rats returned to normal activity. Dyskinesia and abnormal behavior were not observed at 7 days following the last seizure. Spontaneous convulsion was not detectable in each group.

GLT-1 expression in the rat cerebral cortex and hippocampus

Immunohistochemistry demonstrated that GLT-1 was widely expressed in cerebral cortical and hippocampal neurons. Microscopy revealed elliptical neuronal cells, blue cytoplasm, and brown cell membranes (GLT-1 expression) in the control group. GLT-1 expression was significantly greater following recurrent seizures compared with the control group in the rat cerebral cortex and hippocampus ($P < 0.01$). GLT-1 expression was increased in the rat cerebral cortex and hippocampus following intramuscular injection of progesterone, and significant differences were detectable between the progesterone and seizure groups ($P < 0.01$). GLT-1 expression was similar between the rat cerebral cortex and hippocampus ($P > 0.05$; Figures 1, 2).

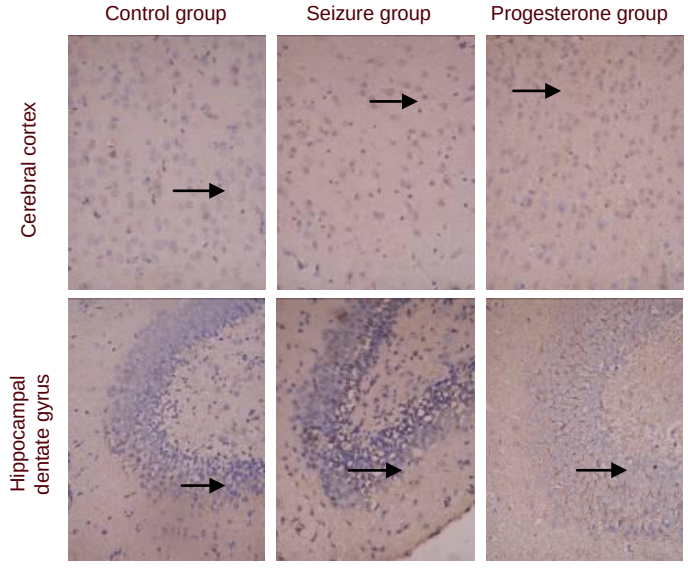


Figure 1 Glutamate transporter 2 (GLT-1) expression in the rat cerebral cortex and hippocampus at 3 days following recurrent seizures (immunohistochemistry, × 200).

Progesterone was given following each seizure in the progesterone group.

A brown color indicates GLT-1 expression, which was mainly expressed on cell membranes. Arrows show GLT-1-positive cells.

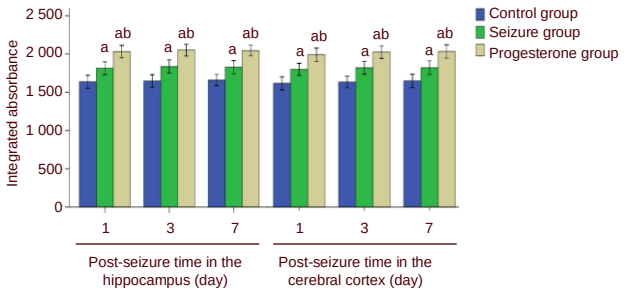


Figure 2 Glutamate transporter 2 (GLT-1) expression in the rat cerebral cortex and hippocampus (immunohistochemistry, × 200).

Data are expressed as mean ± SD. Mean value among groups was compared using one-way analysis of variance and least significant difference *t*-test. [a]*P* < 0.01, *vs*. control group; [b]*P* < 0.01, *vs*. seizure group.

Western blot revealed that GLT-1 protein expression was present in neuronal membranes of cerebral cortical and hippocampal cells in the developing brain of neonatal rats. GLT-1 protein expression did not change over time in the rat cerebral cortex and hippocampus (*P* > 0.05). GLT-1 expression was significantly higher following recurrent seizures compared with the control group in the cerebral cortex and hippocampus (*P* < 0.01). GLT-1 expression was upregulated in the cerebral cortex and hippocampus following progesterone treatment. Significant differences were observed between the progesterone and seizure groups (*P* < 0.01). GLT-1 expression was similar between the rat cerebral cortex and hippocampus (*P* > 0.05; Figure 3).

GAT-1 expression in the rat cerebral cortex and hippocampus

Immunohistochemistry demonstrated that GAT-1 was widely expressed in the brain. GAT-1 protein expression was significantly greater following recurrent seizures compared with the control group in the rat cerebral cortex and hippocampus (*P* < 0.01). GAT-1 expression was significantly lower in the rat cerebral cortex and hippocampus following intramuscular injection of progesterone compared with the seizure group (*P* < 0.01). GAT-1 expression was similar between the rat cerebral cortex and hippocampus (*P* > 0.05; Figures 4, 5).

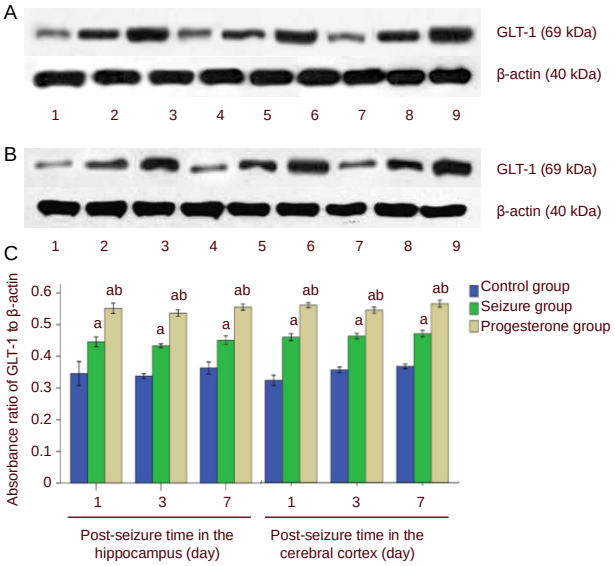


Figure 3 Glutamate transporter 2 (GLT-1) expression in the rat hippocampus (A) and cerebral cortex (B) (western blot).

Lanes 1, 4, 7: control group at 1, 3, 7 days, respectively; Lanes 2, 5, 8: seizure group at 1, 3, 7 days, respectively; Lanes 3, 6, 9: progesterone group at 1, 3, 7 days, respectively.

(C) Data are expressed as mean ± SD. Intergroup and intragroup mean values were compared using one-way analysis of variance and least significant difference *t*-test. [a]*P* < 0.01, *vs*. control group; [b]*P* < 0.01, *vs*. seizure group.

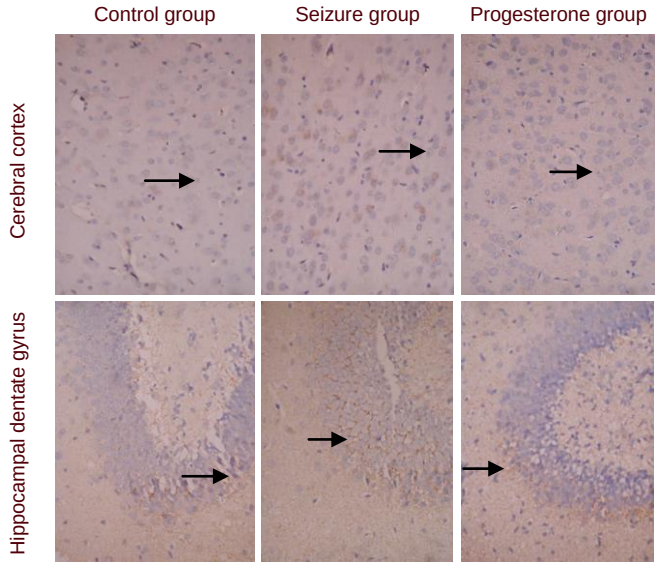


Figure 4 γ-aminobutyric acid transporter 1 (GAT-1) expression in the rat cerebral cortex and hippocampus at 3 days following recurrent seizures (immunohistochemistry, × 200).

A brown color indicates GAT-1 expression, which is mainly expressed on cell membranes. Arrows show GAT-1-positive cells.

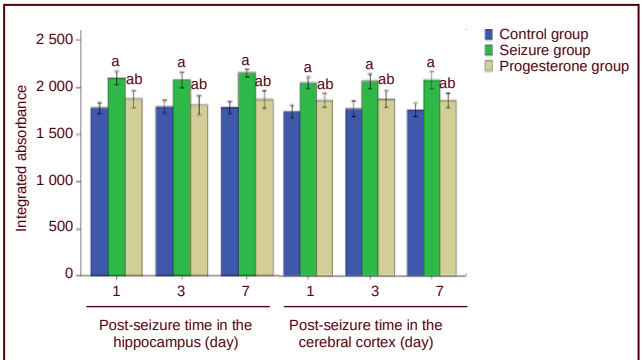


Figure 5 γ-aminobutyric acid transporter 1 (GAT-1) expression in the rat cerebral cortex and hippocampus (immunohistochemistry).

Data are expressed as mean ± SD. Mean value among groups was compared using one-way analysis of variance and least significant difference *t*-test. [a]*P* < 0.01, *vs*. control group; [b]*P* < 0.01, *vs*. seizure group.

Western blot revealed that GAT-1 protein expression was detected in the cerebral cortex and hippocampus of the developing brain of neonatal rats. GAT-1 protein expression did not change over time in the rat cerebral cortex and hippocampus (*P* > 0.05). GAT-1 expression was significantly higher following recurrent seizures compared with the control group in the cerebral cortex and hippocampus (*P* < 0.01). GAT-1 expression was downregulated in the cerebral cortex and hippocampus following progesterone treatment. Significant differences were observed between the progesterone and seizure groups (*P* < 0.01). GAT-1 expression was similar between the rat cerebral cortex and hippocampus (*P* > 0.05; Figure 6).

DISCUSSION

Changes in GAT-1 expression are not consistent in the brains of various seizure models[16-17]. The effect of GAT-1 expression on seizures remains controversial. Mice that lack GAT-1 experience behavior and character disorder, decreased learning and memory abilities, and have a high sensitivity to pentylenetetrazol-induced epilepsy[18-20]. Results from this study have demonstrated that GLT-1 and GAT-1 protein levels gradually increase in the developing brain, which was consistent with a previous study[6]. Our results are most likely associated with astrocyte and neuronal hyperplasia in the developing brain. There was no significant difference in GLT-1 and GAT-1 protein expression between the cerebral cortex and hippocampus, suggesting no obvious specificity of GLT-1 and GAT-1 expression in the cerebral cortex and hippocampus. GLT-1 and GAT-1 expression was significantly increased following recurrent seizures, which was identical to previous results[21-24]. GLT-1 activation accelerated the uptake and clearance of glutamate in the synaptic cleft, and reduced neuronal injury following seizures[25]. These changes are probably associated with compensatory mechanisms of self-protection in the central nervous system.

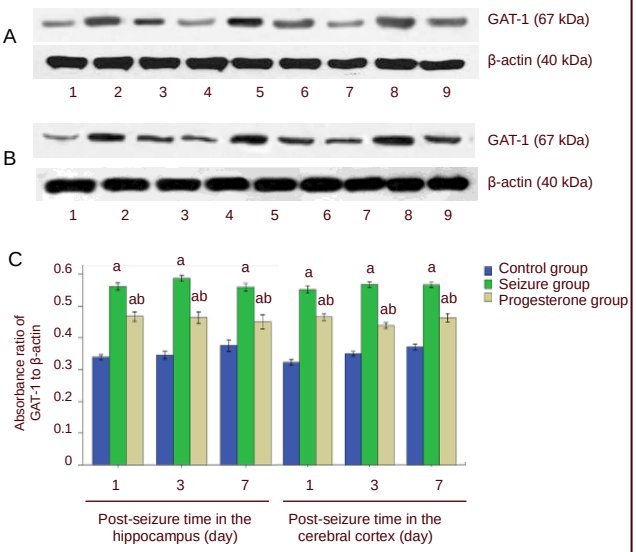


Figure 6 γ-aminobutyric acid transporter 1 (GAT-1) expression in the rat hippocampus (A) and cerebral cortex (B) (western blot).

Lanes 1, 4, 7: control group at 1, 3, 7 days, respectively; Lanes 2, 5, 8: seizure group at 1, 3, 7 days, respectively; Lanes 3, 6, 9: progesterone group at 1, 3, 7 days, respectively.

(C) Data are expressed as mean ± SD. Intergroup mean values were compared using one-way analysis of variance and least significant difference-*t* test. [a]*P* < 0.01, *vs*. control group; [b]*P* < 0.01, *vs*. seizure group.

Upregulated GAT-1 expression accelerated extracellular GABA uptake, maintained glutamate balance, and delayed the occurrence of epilepsy. Moreover, GAT-1 diminished the concentration of inhibitory amino acids in the intercellular space, decreased the inhibitory function of neural networks, accelerated the occurrence and extension of epileptic seizures and aggravated brain injury[26]. Nevertheless, some pathological factors induced the conversion of GLT-1 and GAT-1 function. GLT-1 released glutamate from cells[27] to intensify excitotoxicity, while GAT-1 released GABA into the synaptic cleft to suppress the excitatory function of neural networks[28]. Results from this study have confirmed that the upregulation of GLT-1 and GAT-1 levels is probably associated with immature brain function. Nervous system function was disordered under stress conditions. We presumed that the balance of excitatory and inhibitory systems in the brain was damaged, and the body tried to achieve a balance in other levels to reduce seizure-induced brain injury. If this

mechanism existed for a long time, it would decrease the seizure threshold, promote the onset of epilepsy, and finally aggravate brain injury.

Previous studies have revealed that progesterone delayed seizures following amygdala kindling, shortened the duration taken for rats to search and climb the platform in the Morris water maze in kainic acid-kindled rats, improved spatial memory[29-30], and regulated immunocompetence of GABAergic interneurons in the brain of rats with pentetrazole-induced epilepsy. In the present study, progesterone upregulated GLT-1 levels and downregulated GAT-1 expression following seizures, reduced glutamate accumulation in the synaptic cleft, increased the concentration of extracellular inhibitory amino acids to recover the balance of the excitatory and inhibitory systems in the central nervous system, and finally inhibited seizures[31]. Taken together, no significant difference in GLT-1 and GAT-1 levels was detected in the cerebral cortex and hippocampus following progesterone treatment, suggesting that progesterone regulated GLT-1 and GAT-1 expression and that the regulatory effects were identical.

In summary, GLT-1 and GAT-1 exert important effects on the pathological process of brain injury following seizures, and our results have provided new potential targets for epilepsy therapy[32]. Progesterone regulated neurotransmitter release and uptake, maintained the balance of the excitatory and inhibitory systems in the central nervous system, and relieved recurrent seizure-induced brain injury in the developing brain by upregulating GLT-1 expression and downregulating GAT-1 expression. The above results provide evidence for the antiepileptic action of progesterone and provide theoretical insights for the development of new antiepileptic drugs.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

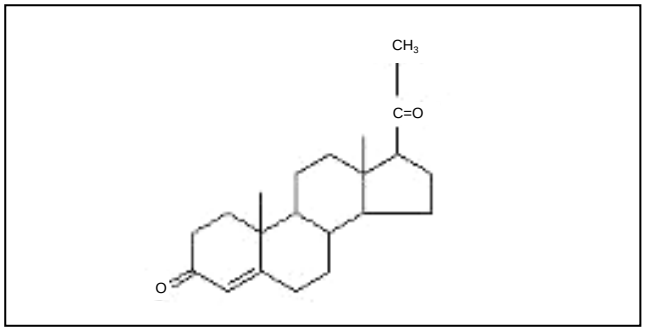
Time and setting

Animal experiments were performed at the Animal Laboratory of Hunan People's Hospital, China, from November 2010 to January 2011. Immunohistochemistry and western blotting experiments were conducted at the Department of Pediatrics, Second Xiangya Hospital, Central South University, China from February to June 2011.

Materials

A total of 72 clean, healthy, Sprague-Dawley rats aged 7 days and weighing 12.50 ± 4.05 g, both genders, were supplied by the Experimental Animal Nursery, College of Animal Technology, Hunan Agricultural University, China (License No. SYXK (Xiang) 2003-0003). The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[33].

Progesterone was purchased from the Xianju Pharmaceutical, Zhejiang Province, China (production lot No. 101121). The structural formula is as follows:



Methods

Establishment of models of recurrent seizures

Rats aged 7 days were placed in a rectangular box (40 cm × 20 cm × 20 cm). Flurothyl (0.1 mL; Sigma-Aldrich Inc, St. Louis, MO, USA) was dropped on the filter paper in the box through the injection orifice on the top of the box. The orifice was sealed. The recovery conditions were observed at 30 minutes following seizures. Seizures were induced once a day, for 6 consecutive days[34]. The rats from the control group did not inhale flurothyl.

Intramuscular injection of progesterone

The rats from the progesterone group were intramuscularly treated with 16 mg/kg progesterone after each seizure for 6 consecutive days. Saline (0.2 mL) was given to rats from the control and seizure groups.

Immunohistochemical staining of GLT-1 and GAT-1 expression in the rat brain

Eight rats from each group were intraperitoneally injected with 10% (v/v) chloral hydrate (0.5 mL/kg) at 1, 3, and 7 days following the last seizure. Rats were decapitated and the left cerebral hemisphere containing the cortex and hippocampus was obtained to prepare paraffin sections[35]. Hydrogen peroxide (3% (v/v)) was used to inactivate endogenous peroxidase. The sections were blocked using 10% (v/v) normal goat serum at 37°C for 30 minutes, incubated in rabbit anti-GLT-1 and GAT-1 polyclonal antibody (1:400; Abcam, Cambridge, MA,

USA) at 4°C overnight, washed in PBS, followed by incubation in horseradish peroxidase-labeled goat secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) at 37°C for 30 minutes. After a wash in PBS, the sections were developed with diaminobenzidine, counterstained with hematoxylin, dehydrated, permeabilized, mounted and tested under a microscope. Two sections were randomly selected from each rat. Two fields were randomly obtained from each section under 400 × magnification. Accumulative absorbance values were calculated using Image-pro plus 6.0 image analysis software (Media Cybernetics Inc, Silver Spring, MD, USA).

Preparation of neural cell membrane

The right cerebral cortex and hippocampus were homogenized in 10 times volume of buffer, supplemented with 0.154 M KCl, 50 mM Tris•HCl buffer, pH 7.4, and centrifuged at 1 000 r/min for 20 minutes. The supernatant was centrifuged at 12 000 r/min for 20 minutes. After removal of the supernatant, the remaining precipitates were resuspended in 1 mL homogenate buffer. The above mentioned procedure was conducted at 4°C. Protein concentrations were measured using the Coomassie brilliant blue method. The specimens were stored at –20°C until ready for western blot [36].

Western blot for GLT-1 and GAT-1 in neural cell membranes from the rat cerebral cortex and hippocampus

A 10% (w/v) sodium dodecyl sulfate polyacrylamide gel was prepared. Samples (25 μL) were mixed with loading buffer, boiled at 100°C for 3 minutes, and electrophoresed. Following electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane at 300 mA for 70 minutes. The membranes were blocked in 5% (w/v) nonfat milk for 2 hours, cut into 3 mm-wide strips, placed in the loading slot, and then incubated with rabbit anti-rat GLT-1, GAT-1 (GLT-1 1:500, GAT-1 1:1 000, Abcam, USA), or mouse anti-β-actin (internal control) monoclonal antibodies (1:4 000; Proteintech Inc, USA) at 4°C overnight. The membranes were washed with Tris buffered saline. Blocking buffer was added to dilute horseradish peroxidase-labeled goat anti-rabbit IgG (1:3 000; Zhongshan Golden Bridge Biotechnology) at 37°C for 60 minutes, followed by a wash in Tris-buffered saline-Tween. The membranes were treated with enhanced chemiluminescence reagent for 3 minutes, and then exposed to X-ray film in a dark box for several seconds to several minutes, followed by developing. The absorbance values of target protein were analyzed using Gel-Pro 4.0 gel image analysis software (Media Cybernetics Inc). The absorbance ratio of target protein to internal reference was represented as the relative amount of target protein[37].

Statistical analysis

Data were statistically evaluated using SPSS 17.0 software (SPSS, Chicago, IL, USA), and expressed as mean ± SD. Intergroup and intragroup comparisons were performed using one-way analysis of variance (α = 0.05). A value of $P < 0.05$ was considered statistically significant. Least significant difference *t*-test was used to identify the differences between intergroup means.

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Author contributions: Lingjuan Liu participated in study design, statistical analysis and manuscript writing. Lingjuan Liu and Yu Huang performed the experiments. Liqun Liu and Tao Bo provided technical guidance. Dingan Mao evaluated the study and was in charge of manuscript authorization. Liqun Liu obtained the funding.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Hunan Provincial People's Hospital, China.

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