A ginkgo biloba extract promotes proliferation of endogenous neural stem cells in vascular dementia rats★

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Abstract

The ginkgo biloba extract EGb761 improves memory loss and cognitive impairments in patients with senile dementia. It also promotes proliferation of neural stem cells in the subventricular zone in Parkinson's disease model mice and in the hippocampal zone of young epileptic rats. However, it remains unclear whether EGb761 enhances proliferation of endogenous neural stem cells in the brain of rats with vascular dementia. In this study, a vascular dementia model was established by repeatedly clipping and reperfusing the bilateral common carotid arteries of rats in combination with an intraperitoneal injection of a sodium nitroprusside solution. Seven days after establishing the model, rats were intragastrically given EGb761 at 50 mg/kg per day. Learning and memory abilities were assessed using the Morris water maze and proliferation of endogenous neural stem cells in the subventricular zone and dentate gyrus were labeled by 5-bromo-2-deoxyuridine immunofluorescence in all rats at 15 days, and 1, 2, and 4 months after model establishment. The escape latencies in Morris water maze tests of rats with vascular dementia after EGb761 treatment were significantly shorter than the model group. Immunofluorescence staining showed that the number and proliferation of 5-bromo-2-deoxyuridine-positive cells in the subventricular zone and dentate gyrus of the EGb761-treated group were significantly higher than in the model group. These experimental findings suggest that EGb761 enhances proliferation of neural stem cells in the subventricular zone and dentate gyrus, and significantly improves learning and memory in rats with vascular dementia.

Key Words

neural regeneration; traditional Chinese medicine; ginkgo biloba extract; EGb761; vascular dementia; neural stem cells; subventricular zone; dentate gyrus; learning and memory; grants-supported paper; neuroregeneration

Research Highlights

(1) Dynamic changes in endogenous neural stem cell proliferation in the subventricular zone and dentate gyrus of rats with vascular dementia were observed over 4 months. Neural stem cells began to proliferate following vascular dementia induced by cerebral ischemia and reperfusion injury. The proliferation reached a peak at 15 days and then gradually returned to normal levels.
(2) The ginkgo biloba extract EGb761 promoted the proliferation of neural stem cells in the subventricular zone and dentate gyrus of rats with vascular dementia, with a peak observed at 1 month. The cells continued to proliferate at 4 months.
(3) EGb761 had similar promoting effects on the proliferation of neural stem cells in the subventricular zone and dentate gyrus of rats with vascular dementia, especially in the subventricular zone.

INTRODUCTION

Neural stem cells proliferate in the subventricular zone and hippocampal dentate gyrus of adult mammals[1-2]. However, the number of endogenous neural stem cells is insufficient to prevent cerebral ischemia/reperfusion injuries such as vascular dementia, so it is important to stimulate endogenous neural stem cell proliferation and differentiation.

The ginkgo biloba extract EGb761 effectively and safely treats memory loss and cognitive impairments in patients with senile dementia[3-6]; however, its nootropic mechanisms have not been elucidated. EGb761 can reduce persistent neuronal loss in the hippocampal CA1 area of rats with vascular dementia, increase the rate of induction of long-term potentiation[7], upregulate hippocampal synaptophysin expression[8], and improve the neurogranin and synapsin I levels and phosphorylation[9-10]. Furthermore, EGb761 promotes pathological synaptic structure and transmission plasticity and improves learning and memory deficits in vascular dementia. Ginkgo biloba extract treatment promotes neural stem cell proliferation and migration in the subventricular zone of Parkinson's disease mice[11], induces neural stem cell proliferation and differentiation in the hippocampus, and improves learning and memory in young epileptic rats[12]. However, no long-term observations have been presented. It remains controversial whether EGb761 promotes proliferation of endogenous neural stem cells in the brains of rats with vascular dementia, thus facilitating nerve repair and regeneration. In this study, we observed the effects of EGb761 on proliferation of neural stem cells in the subventricular zone and dentate gyrus of rats with vascular dementia, in a broader attempt to provide insights into vascular dementia treatment.

RESULTS

Quantitative analysis of experimental animals

Sixty adult rats were randomly and equally divided into sham operation, model, and EGb761 treatment groups. In the model and EGb761 treatment groups, a vascular dementia model was established by repeatedly clipping and reperfusing the bilateral common carotid arteries in combination with an intraperitoneal injection of a sodium nitroprusside solution. The sham operation group underwent the same operation with no clipping of the common carotid arteries and injection of the sodium

nitroprusside solution. The EGb761-treated rats were intragastrically given ginkgo leaf tablet suspensions after establishing the model. The sham operation and model groups were given normal saline. All 60 rats were included in the analyses.

EGb761 improved learning and memory in rats with vascular dementia

The Morris water maze tests showed that the escape latencies of model group rats were longer than those of the sham-operated rats at 15 days, and 1, 2, and 4 months after establishing the model ($P < 0.01$). The escape latencies of EGb761-treated rats were significantly shorter than those of the model group rats ($P < 0.01$), but longer than those of the sham operation group rats at each time point ($P < 0.01$). This indicates that EGb761 significantly improved learning and memory in rats with vascular dementia, without complete recovery compared with the sham-operated rats. In the model group, escape latencies at 2 and 4 months were longer than that at 15 days ($P < 0.01$). In the EGb761 treatment group, escape latencies at 2 and 4 months were shorter than that at 15 days ($P < 0.05$, $P < 0.01$). Our findings suggest that learning and memory deficits occurred and were aggravated over time in the model group rats, and gradually improved following EGb761 administration (Figure 1).
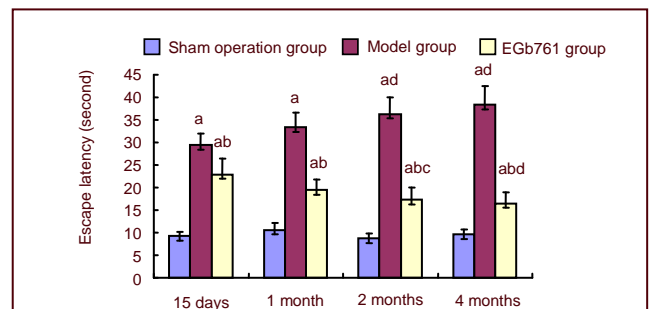

Figure 1   Effects of EGb761 on learning and memory abilities in rats with vascular dementia (Morris water maze).

Data are expressed as mean ± SD. There were 20 rats in each group at each time point. [a]$P < 0.01$, *vs.* sham operation group; [b]$P < 0.01$, *vs.* model group; [c]$P < 0.05$, [d]$P < 0.01$, *vs.* 15 days.

The Morris water maze navigation test was performed at 15 days and 1, 2, and 4 months after establishing the model. In this test, a shorter escape latency indicates stronger spatial learning and memory. Differences among the groups were compared using one-way analysis of variance, and significant differences between the two groups were compared using the Student-Newman-Keuls test.

EGb761 promoted proliferation of neural stem cells in the rat dentate gyrus

Only a small amount of 5-bromo-2-deoxyuridine

(BrdU)-positive cells were visible in the hippocampal dentate gyrus of sham-operated rats, and their cell nuclei were stained pale green. These cells were sparsely distributed mainly in the dentate gyrus subgranular zone and portal area. A large number of BrdU-positive cells in the model group rats were observed at 15 days after establishing the model, showing a linear discontinuous arrangement along the subgranular zone, and accumulation of BrdU-positive cells was occasionally seen. There were two kinds of morphology of BrdU-positive cells. One was clustered and exhibited an irregular nucleus shape of varying sizes. Another was found as one or two BrdU-positive cells, showing a regular round or oval-shaped nucleus of uniform size, with rod-shaped or paired nuclei occasionally visible. At 1 month after establishing the model, the number of BrdU-positive cells in the dentate gyrus was significantly reduced and they were scattered, but still higher than in the sham operation group, which showed few positive cells in the granule cell layer. At 2 and 4 months, occasional BrdU-positive cells were observed in the dentate gyrus. In the EGb761 treatment group, BrdU-positive cells in the dentate gyrus increased at 15 days, showing a paired, clustered, and dispersed distribution, with cells mainly located in the subgranular zone and portal zone. At 1 month, the cell number increased significantly, with a clustered distribution and deep staining. The majority of positive cells were located in the subgranular zone, portal area, and granule cell layer. At 2 and 4 months, the cell number decreased gradually, clustering was rarely seen, and few BrdU-positive cells were scattered at 4 months (Figure 2).

Cell counts showed that there was no significant difference in the sham operation group at each time point ($P > 0.05$). The number of BrdU-positive cells in the model group at 15 days was significantly higher than that at 1, 2, and 4 months ($P < 0.01$). As the EGb761 treatment time proceeded, the number of BrdU-positive cells showed a unimodal change, reaching a peak at 1 month and then decreasing at 2 and 4 months ($P < 0.01$). After EGb761 treatment, BrdU-positive cells proliferated vigorously and persistently, and the peak proliferation was higher than in the model group. The number of cells was also higher than in the model group at each time point except for 4 months ($P < 0.05$, $P < 0.01$; Table 1). These findings suggest that BrdU-positive cells proliferated transiently in the model group. In contrast, EGb761 promoted sustained neural stem cell proliferation in the dentate gyrus of rats with vascular dementia.
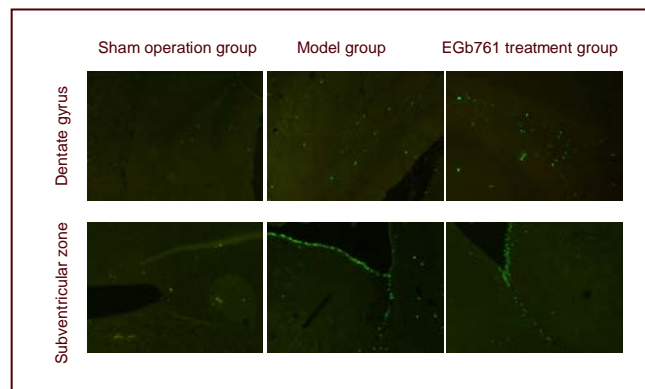

Figure 2 Effects of EGb761 on the expression of BrdU-positive cells in the hippocampal dentate gyrus and subventircular zone of vascular dementia rats (immunofluorescence staining, × 100).

Five rats in each group were euthanized at 1 month after modeling. Whole-brain coronal sections were labeled with BrdU for immunofluorescence staining, positive cells in the dentate gyrus were observed under a fluorescence microscope, and the presence of green particles in the nucleus was the marker of BrdU-positive cells. BrdU: 5-Bromo-2-deoxyuridine.

Table 1 Effect of EGb761 on the number of BrdU-positive cells in the dentate gyrus of rats with vascular dementia

Group	Time after modeling			
	15 days	1 month	2 months	4 months
Sham	5.8±1.8	5.8±1.3	8.0±3.3	8.0±3.1
Model	31.0±5.6[a]	11.4±5.5[ad]	8.2±3.3[d]	8.2±3.8[d]
EGb761	40.0±4.5[ab]	59.0±4.4[acd]	17.8±5.3[acde]	11.4±5.0[de]

Data are expressed as mean ± SD; there were five rats in each group at each time point. [a]$P < 0.01$, *vs.* sham operation (sham) group; [b]$P < 0.05$, [c]$P < 0.01$, *vs.* model group; [d]$P < 0.01$, *vs.* 15 days; [e]$P < 0.01$, *vs.* 1 month. The number of positive cells was detected with MiPrd image analysis system, and the average value in each rat was measured on three sections. Difference among groups was compared using one-way analysis of variance, and significant differences between the two groups were compared using the Student-Newman-Keuls test. BrdU: 5-Bromo-2-deoxyuridine.

EGb761 promoted proliferation of neural stem cells in the rat subventricular zone

In the sham operation group, a small amount of BrdU-positive cells were visible in the subventricular zone and BrdU was mainly expressed in the nuclei. The BrdU-positive cells were stained pale green, and showed small, oval, irregular nuclei. These cells were sparsely distributed, mainly in the dorsolateral horn and lateral wall, and few BrdU-positive cells were seen in the inner wall. In the model group, there were more BrdU-positive cells than in the sham operation group at 15 days. These cells were clustered along the lateral ventricle wall and the cell number was higher in the dorsolateral horn. Their cell nuclei were dyed dark green, and showed varying

sizes and circular, oval or irregular shapes. At 1 month, the cell number was lower, with a stratified distribution. At 2 and 4 months, the cell number was significantly lower, and stratification disappeared. In the EGb761 treatment group, the number of BrdU-positive cells was significantly higher than in the model group at 15 days, showing marked cluster aggregation and a multi-layer distribution. The paired rod-shaped or oval positive cells increased, with deeply stained nuclei and large sizes. At 1 month, the cell number significantly increased, clustering was more apparent, and some positive cells were located in the anterior lateral horn. At 2 and 4 months, the cell number was slightly decreased, but still relatively high (Figure 2).

Table 2 Effects of EGb761 on the number of BrdU-positive cells in the subventricular zone of rats with vascular dementia

| Group | Time after modeling | | | |
	15 days	1 month	2 months	4 months
Sham	52.8±8.0	54.2±8.3	53.8±6.5	54.6±6.4
Model	106.0±13.3[a]	94.4±8.1[a]	56.4±9.9[ef]	57.4±7.9[ef]
EGb761	146.6±14.9[ac]	197.6±20.5[ace]	79.2±9.1[acef]	71.8±9.7[bdef]

Data are expressed as mean ± SD, and there were five rats in each group at each time point. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* sham operation (sham) group; [c]$P < 0.01$, [d]$P < 0.05$, *vs.* model group; [e]$P < 0.01$, *vs.* 15 days; [f]$P < 0.01$, *vs.* 1 month. The number of positive cells was detected with the MiPrd image analysis system, and the average value in each rat was measured on three sections. Differences among groups were compared using one-way analysis of variance, and significant differences between the two groups were compared using the Student-Newman-Keuls test. BrdU: 5-Bromo-2-deoxyuridine.

Cell counting showed that BrdU-positive cells in the subventricular zone of model group rats gradually decreased as cerebral ischemia/reperfusion proceeded. In the EGb761 treatment group, the cell number reached a peak at 1 month and then gradually decreased, and there was no significant difference between 2 and 4 months ($P > 0.05$). The EGb761 treatment group showed more BrdU-positive cells than the model group at each time point ($P < 0.05$, $P < 0.01$; Table 2). Our findings suggest that EGb761 promotes proliferation of BrdU-positive cells in the subventricular zone of rats with vascular dementia, and this effect lasts at least 4 months.

DISCUSSION

Under physiological conditions, neural stem cells are static in the adult mammalian brain. Brain tissue injuries or microenvironment changes may activate neural stem cells and trigger cell proliferation[1]. Ischemia induces neurogenesis in the neocortex of adult rats[13]. In a rat model of transient forebrain ischemia and a gerbil model of transient global ischemia, neurogenesis[14] was observed in the dentate gyrus. The number of proliferating cells in the ipsilateral cortex and subependymal regions of rats with focal cerebral ischemia significantly increased at 2–14 days after ischemia, and reached a peak at 7 days, whereas the number of BrdU-positive cells in the dentate gyrus did not significantly increase[15]. Li and colleagues[16] found that BrdU-positive cells were visible in the ependyma, subventricular zone, and choroid plexus at 2–14 days after focal cerebral ischemia/reperfusion. Mild hypoxia (2.5–5% oxygen concentration) in the human body can significantly promote the proliferation and differentiation of human neural stem cells[17]. Thus, neural stem cells proliferate following cerebral ischemia and hypoxia; however, neurogenesis results are inconsistent and lack long-term dynamic observations.

BrdU is a thymine derivative that mimics thymidine such that it is incorporated into the newly synthesized DNA strands during the S phase of the cell division cycle, and is permanently labeled within cells[18]. A classic method in neurogenesis research is labeling of proliferating cells according to BrdU characteristics[18]. In this study, the proliferation of neural stem cells in the subventricular zone and dentate gyrus of rats with vascular dementia was detected with the BrdU labeling method and immunofluorescence at 15 days, 1, 2, and 4 months. The results indicate that BrdU-positive cells in the subventricular zone and dentate gyrus were activated and proliferating in the model group rats after cerebral ischemia/reperfusion. This proliferation peaked and then decreased gradually, indicating spatial and temporal regulation. This transient proliferation is the result of emergent compensation after cerebral ischemia, and may functionally compensate for neuronal loss caused by cerebral ischemia/reperfusion. Furthermore, BrdU-positive cells showed two kinds of morphology, which is related to the differentiation of neural stem cells. Cells with large nuclei, deep dye uptake, and round shapes differentiated into neurons. Those with small nuclei and oval or irregular shapes may differentiate into glial cells. Paired, rod-shaped nuclei are markers of cell proliferation and differentiation. Because of the limited supply of endogenous neural stem cells, these cells are insufficient to protect brain tissue structure and functions from cerebral ischemia/reperfusion. Therefore, drugs or measures to regulate the migration, differentiation, and proliferation of endogenous neural

stem cells are compelling in broad attempts to enhance brain plasticity and provide new avenues for repair of vascular dementia and neural regeneration.

EGb761 is composed of 24% flavonoids and 6% terpene lactone compounds, which easily cross the blood-brain barrier, exert neuroprotective effects[19], and play an important role in improving vascular dementia. Growing evidence shows that EGb761 obviously improves the cognitive impairments seen in vascular dementia patients[20]. Long-term EGb761 administration ameliorates cognitive disorders and loss of hippocampal neurons in gerbil models of vascular dementia[6]. Furthermore, EGb761 leads to the repair of synaptic structures and functions and protects against neuronal loss in vascular dementia[7-10]. Our Morris water maze findings show that escape latencies in the EGb761 treated rats at each time point were significantly shorter than that in the model group, but still longer than those in the sham operation group. In the EGb761 treatment group, escape latencies at 2 and 4 months were significantly shorter than those at 15 days, indicating that EGb761 improves spatial learning and memory dysfunction in rats with vascular dementia, and this therapeutic effect was more apparent as the treatment time proceeded. This is consistent with previous results and clinical observations[7-10, 20]. The mechanisms underlying EGb761-mediated reversal of cognitive disorders in vascular dementia have been investigated. EGb761 increased cholinergic fiber density in the hippocampal CA1 region of rats with vascular dementia[21], upregulated postsynaptic density-95 and NMDAR1 protein expression in the hippocampus, inhibited hippocampal neuron apoptosis, attenuated damage to neurons in the hippocampal CA1 region after cerebral ischemia/reperfusion[22-23], increased somatostatin and cholecystokinin expression in the hippocampus of vascular dementia mice[24], and improved learning and memory to a certain extent. In vascular dementia patients, EGb761 reduces serum β-Ap content and increases vascular endothelial growth factor content[25]. These experimental findings indicate that EGb761 may improve learning and memory disruptions in vascular dementia patients or animal models through multi-channel, multi-targeted effects.

The existing researches focus on the large-scale clinical efficacy and safety of EGb761 in vascular dementia[3-5, 20]. EGb761 is a promoter of endogenous neural stem cell proliferation in young epileptic rats with Parkinson's disease and of cultured neural stem cells from these animals *in vitro* [11-12]. High-dose EGb761 can stimulate the proliferation of mesenchymal stem cells[26]. However, little research has focused on the effects of EGb761 on endogenous neural stem cells in vascular dementia.

In this study, we found that the effects of EGb761 on promotion of proliferation were more apparent in the subventricular zone than in the dentate gyrus. The reason for this regional difference may be because (1) the majority of neural stem cells in the subventricular zone are static, after vascular dementia occurs, and the cells begin to proliferate and remain viable for a long time. Alternatively, (2) the subventricular zone and dentate gyrus are susceptible to cerebral ischemia, especially compared with the hippocampus, and the hippocampal dentate gyrus is more prone to damage following vascular dementia (including damage to neural stem cells). Therefore, these differences may have allowed EGb761 to produce significantly more proliferation of neural stem cells in the subventricular zone compared with the dentate gyrus.

There are a number of possible mechanisms by which EGb761 promotes neural stem cell proliferation in a rat model of vascular dementia. (1) Inhibition of neural stem cell apoptosis: EGb761 may upregulate Bcl-2 protein expression in hippocampus of rats with vascular dementia, down-regulate Bax protein expression[27], inhibit apoptosis of hippocampal cells, protect cell chromosomes from fracture, and support survival of neural stem cells. (2) Anti-lipid peroxidation and excitotoxicity: EGb761 may improve cerebral blood flow, block lipid peroxidation, prevent toxicity of excitatory amino acids and calcium overload after vascular dementia occurs, thus protecting neural stem cells from damage[6]. (3) Change in the redox state in neural stem cells: EGb761 may strengthen mitochondrial oxidative phosphorylation capacity, and promote energy metabolism of neural stem cells. (4) Regulate growth of neural stem cells and promote gene regulation: Neural stem cells in neonatal Sprague-Dawley rats proliferate vigorously and show increased cyclin D1 expression after hypoxia[28]. Activation of the Akt and extracellular signal-regulated kinase signal pathway[29] and death receptor CD95[30] inhibition can increase the survival of neural stem cells under the ischemic state. Our findings also suggest that EGb761 increases postsynaptic receptor protein and microtubule-associated protein in the hippocampus and cortex of normal young mice[27]. Furthermore, EGb761 upregulates long-term expression levels of synapse- associated proteins (such as synaptophysin, synapsin I, and neurogranin) in vascular

dementia[7-10]. (5) Improve the microenvironment of neural stem cell proliferation. However, whether neural stem cells differentiate into neurons deserves further study.

In this study, we observed the proliferation of neural stem cells in rats with vascular dementia over 4 months. After ischemia and reperfusion, neural stem cells vigorously proliferated, peaked, and then gradually returned to normal levels. This evidence suggests that there is an optimal phase (time-window) for promoting the proliferation of neural stem cells. EGb761 administration can regulate neural stem cell proliferation, migration and differentiation, and promotes cell proliferation and differentiation in rats with vascular dementia. The newborn cells establish synaptic connections with surrounding cells, and integrate into the original neural network to enhance synaptic plasticity, promote the recovery of brain tissue, and improve learning and memory in rats with vascular dementia.

MATERIALS AND METHODS

Design
One-way, randomized, controlled animal experiments.

Time and setting
Experiments were performed in the Medical Research Center of Weifang Medical University in China from September 2010 to May 2011.

Materials
Animals
Healthy male Sprague-Dawley rats, aged 3–4 months, weighing 250 ± 30 g, were provided by Experimental Animal Center of the 89 Hospital of PLA, with license No. SCXK (Lu) 20050017. Rats were housed at 18–26°C, in 40–60% humidity, and under a 12-hour light/dark cycle, and they were allowed free access to food and water. All experimental procedures were in accordance with the *Guideline for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology. Great efforts were undertaken to reduce the number of experimental animals, and to reduce pain and suffering.

Drugs
Ginkgo leaf tablets (trade name *Yinkeluo*; Haiwang Pharmaceutical Co., Ltd., Shenzhen, Guangdong Province, China), mainly composed of extract EGb761 containing 24% total flavonol glycosides and 6% terpene lactones were used. Each 40 mg tablet contained 9.6 mg

total flavonoids and 2.4 mg terpene lactones. Each rat was treated with 50 mg/kg of ginkgo leaf tablets in 5 mL normal saline.

Methods
Model establishment and drug administration
Vascular dementia models were established with bilateral common carotid arteries repeated occlusion and injection of sodium nitroprusside as previously described[3]. In brief, rats were anesthetized with 10% chloral hydrate, a cervical anterior median incision was made, the bilateral common carotid arteries were separated, and the rats were given an intraperitoneal sodium nitroprusside solution (injected at 2.5 mg/kg in saline). Then, the bilateral common carotid arteries were occluded with a non-invasive clamp for 10 minutes and reperfused for 10 minutes, repeated three times. The wound was sutured and rats were returned to the cage. In the sham operation group, the bilateral common carotid arteries were only isolated, with no occlusion or injection, and the remaining treatment was the same as that in the model group. In the EGb761 treatment group, rats were injected with ginkgo leaf tablets suspension (5 mL; 50 mg/kg) at day 7, while the sham operation group and model group were given an equal volume of normal saline, once per day.

Morris water maze tests
Rats in each group were euthanized at 15 days and 1, 2, and 4 months after establishing the model. Over 6 days before euthanasia, escape latencies of the rats were tested in the Morris water maze, once per day, and the average value of the six tests was calculated. The escape latency reflects spatial learning and memory in rats, with shorter escape latencies indicating better learning and memory.

Cerebral perfusion fixation and specimen collection
Five rats in each group were killed at 15 days and 1, 2, and 4 months after establishing the model. At day 4 prior to death, S phase cells were labeled with BrdU, and rats were intraperitoneally injected with BrdU (Sigma, St. Louis, MO, USA; 10 mg/L) at a dose of 50 mg/kg, twice per day for 3 days. Twenty-four hours after the last injection, rats were euthanized under 10% chloral hydrate anesthesia. Brain tissue was harvested and fixed in 4% paraformaldehyde for 36 hours, rinsed with tap water for 2 hours, dehydrated in a gradient of ethanol and xylene transparent, and embedded in paraffin. The brain was sliced into 5-μm-thick coronal slices using a type BT-320 microtome (Botai Electronic Technology Co., Ltd., Xiaogan, Hubei Province, China), and toasted at

60°C in an oven (GZX-DH202-1-BS-II; Wanrui Laboratory Equipment Co., Ltd., Shanghai, China) for 2–3 hours. Finally, immunofluorescence detection was performed.

Immunofluorescence staining

Brain tissue sections were dewaxed and placed in citrate buffer for antigen retrieval by microwave heating, and infused with 2 mol/L HCl at 37°C for 30 minutes (DNA degenerates, BrdU exposes and binds with the antibody), then blocked with normal goat serum. After the sections were incubated at room temperature for 20 minutes, excessive serum was discarded and sections were incubated with mouse anti-BrdU antibody (1:700; Millipore, Billerica, MA, USA) at 37°C for 2 hours, and with FITC-labeled goat anti-mouse antibody (1:200; Zhongshan Biotech Corporation, Beijing, China) in the dark at 37°C for 30 minutes. Sections were rinsed with PBS three times between each step, and the sections were mounted and stored at 4°C in the dark. Finally, brain sections were photographed under DMIRE2 Leica fluorescence microscopy (Leica, Heidelberg, Germany), and cells with green nuclei were BrdU-positive cells. The number of BrdU-positive cells in the subventricular zone and dentate gyrus was calculated in five fields at 400 × magnification using an MiPrd image analysis system (Image-Pro Plus 6; Media Cybernetics, Silver Spring, MD, USA). Each rat was examined over three brain sections, and the average value was measured.

Statistical analysis

All data are expressed as mean ± SD and were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Differences among the groups were compared with one-way analysis of variance, while those between two groups were compared with the Student-Newman-Keuls test. $P < 0.05$ was considered to be statistically significant.

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