Neuroprotection of Glycyrrhizin against Ischemic Vascular Dementia *in Vivo* and Glutamate-induced Damage *in Vitro*

HOU Yuan-yuan[1], YANG Yang[1,2], YAO Yang[2], BAI Gang[1,2*]

1. College of Pharmacy, Nankai University, Tianjin 300071, China
2. College of Life Sciences, Nankai University, Tianjin 300071, China

Abstract: **Objective** To evaluate the neuroprotective effects of glycyrrhizin (GL) on vascular dementia (VaD) *in vivo* and Glutamate-induced damage *in vitro*. **Methods** Male Wistar rats were subjected to permanent occlusion of the bilateral common carotid arteries. On d 15 postsurgery, rat cognition was assessed using the Morris water maze. The activity of superoxide dismutase and the content of malondialdehyde of brains were also measured. Brain damage was evaluated histologically using HE staining. *In vitro* cell viability was examined in PC12 cells exposed to Glutamate, and mRNA levels of Bcl-2 and Bax were assessed. **Results** GL (20 mg/kg for 12 d) improved the performance of learning and memory of VaD rats, decreased the level of lipid peroxidation, and attenuated the pathological alterations in the hippocampal CA1 and CA3 areas. Moreover, GL (0.6 mmol/L) could protect PC12 cell lines from injury induced by Glutamate (10 mmol/L) and inhibit apoptosis of neuronal cells. **Conclusion** The present findings suggest that GL may have therapeutic potential in treating VaD.

Key words: glycyrrhizin; neuroprotection; vascular dementia

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Introduction

With a growing elderly population, aging-related diseases, such as hypertension, arteriosclerosis, and different forms of dementia are also increasing. Vascular dementia (VaD) remains the second-most common form of dementia in the elderly after Alzheimer's disease (Jorm and Jolley, 1998; Rockwood *et al*, 2000; Stewart, 2007). As the number of people aged 65 and older is expected to rise to 800 million by 2025, VaD may be a growing health crisis among the elderly population. VaD results from ischemic injury or sustained oligemia to brain regions associated with impairment of cognitive function, memory, and behavior (Román, 2003). It is not a singular disease, but rather a group of conditions with different pathological and pathophysiological mechanisms with abnormal levels of glucose, cholinergic substances, reactive oxygen species, and other metabolic substrates that can initiate and sustain the cascade of neuropathological events (Desmond, 2004; Chan, 2001; Ni *et al*, 1995). Permanent occlusion of bilateral common carotid arteries (2VO) in rat has emerged as a suitable experimental model for chronic cerebral hypoperfusion; it allows simultaneous investigation of cerebral blood flow, learning ability, and histopathological changes (Sarti, Pantoni, and Inzitari, 2002; Zhou, Zhang, and Tang, 2001; Ni *et al*, 1994). In this study, we used a 2VO rat model to assess the potential therapeutic value of glycyrrhizin (GL) in VaD.

GL, one of the major bioactive compounds in *Glycyrrhizae Radix* et *Rhizoma*, is composed of one molecule of glycyrrhetinic acid, which has a steroid-like structure and two molecules of glucuronic acid. GL has diverse biological activities, including anti-inflammatory, anti-viral, anti-liver cancer, anti-oxidative, immunomodulatory, cardioprotective, and hepatoprotective activities (Matsui *et al*, 2004; Duan and Ji, 2007; Kinjo *et al*, 2003; Makino *et al*, 2006; Gumpricht *et al*, 2005; Kim *et al*, 2004). However, little research has been conducted to determine whether GL has

* Corresponding author: Bai G Address: College of Pharmacy, Nankai University, 94 Weijin Road, Nankai District, Tianjin 300071, China
Tel / Fax: +86-22-2350 6792 E-mail: gangbai@nankai.edu.cn
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effects on cerebral ischemia-induced memory impairment. Therefore, we evaluated the effects of GL on the cognition-enhancing and brain damage prevention in permanent occlusion of 2VO rats.

Materials and methods

Animals

Eighteen male Wistar rats (weighing 250 ± 10 g) were obtained from the Academy of Military Medical Science (Beijing, China). The animals were housed in groups of six per cage under a 12/12 h light-dark cycle, with food and water continuously available. All procedures and experiments were performed according to the protocols approved by the Animal Care Committee of the Animal Center in Nankai University.

Materials

GL was purchased from Shanxi Huike Pharmaceutical Co., Ltd (Batch no: Gu20050915; purity ≥ 95%). Glutamate was obtained from Afar Aesar (St Louis, MO). Cell culture medium and reagents were purchased from GibcoBRL Life Technologies (Gaithersburg, MD). Reagent kits were provided by Nanjing Jianchen Institute of Biological Engineering (Nanjing, China).

Animal model and treatments

Eighteen rats were randomly divided into three groups with six rats in each group: vehicle, Sham, and GL-treated groups. The rats were subjected to 2VO surgery to induce chronic cerebral ischemia and mimic the VaD models as described previously (Farkas, Luiten, and Bari, 2007). Under chloral hydrate (350 mg/kg ip) anesthesia, 2VO rats were exposed and carefully separated from the carotid sheath and the cervical sympathetic and vagus nerves through a ventral cervical incision. The 2VO of each global ischemic group were ligated with silk thread. Control animals in the Sham group had the 2VO exposed but received no further manipulation. The rats were placed on a heating pad during recovery from anesthesia to maintain the body temperature at (37.5 ± 0.5) ℃. Fourteen days after surgery, the GL-treated group received GL (20 mg/kg, ip) dissolved in dimethyl sulfoxide (DMSO) daily for 12 d. The Sham group and the vehicle group were ip injected with the same volume of DMSO. During the behavior tests, drugs or DMSO were administered 30 min before the trials.

Morris water maze

The spatial learning and memory performance of rats were evaluated using the Morris water maze from d 8 after administration (Yu *et al*, 2005). The maze apparatus consists of a circular pool and a circular hidden platform (10 cm in diameter). The pool was 1.2 m in diameter and 40 cm in depth and was filled with water (23 ± 1) ℃ to a depth of 25 cm, while the platform was supported by a base resting on the bottom of the pool 1.5 cm below the water surface and hidden from view. At the beginning of each day, the water was made white opaque by adding 3 kg of non-fat milk to prevent the animals from seeing the submerged platform. Every spatial sign around the maze was held constant throughout the test. For descriptive data collection, the pool was divided in four zones by the software (Ethovision 2.0, Noldus, Wagenigen, Netherlands). The swimming activity of each rat was monitored by a video camera linked to a computer through an image analyzer. Each rat received two trials everyday and the test lasted for 5 d. The escape latency (time to reach the platform) was used to assess learning and memory performance. The retention of spatial memory was assessed on the d 6 of swimming training by the spatial probe test. The swimming trace and distance in the target zone where the platform had been set were used to evaluate cognitive performance.

Antioxidant activity

After the last behavioral test, rats in all groups were decapitated immediately. The brains were removed quickly and stored at –80 ℃ until assayed for antioxidant activity measurement. Estimation of lipid peroxidation was done by measuring the malondialdehyde (MDA)-lipid peroxidation product. MDA content was determined by the modified thiobarbituric acid (TBA) method (Ohkawa, Ohishi, and Yagi, 1979), and superoxide dismutase (SOD) activity was detected as described previously (Sun, Oberley, and Li, 1998). Based on these methods, the SOD activity and MDA level were measured using the reagent kits according to recommendation from the manufacture.

Histopathological examination

After the brains of rats were removed, coronal sections were cut into approximately 4 μm sections and stained with hematoxylin and eosin as described previously (Pappas *et al*, 1996). In the hippocampal CA1 and CA3 area, viable neurons were defined as

neurons in which a clear nucleus could be seen. Ischemic damaged neurons exhibit features including pyknosis and shrunken cell bodies.

Cell viability assay

The differentiated rat pheochromocytoma (PC12) cell line was maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 ℃ in 5% CO_2. Cells were seeded in 96-well plates and grown to a confluence of 60%–70%. Cells were incubated with Glutamate (10 mmol/L), with or without GL (0.03 mmol/L, 0.3 mmol/L, and 0.6 mmol/L) for 24 or 48 h. At the end of drug treatments, the culture medium was replaced by a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (5 mg/mL) in fresh medium. After incubation at 37 ℃ for 4 h, the supernatants were discarded and 150 μL of DMSO was added and mixed thoroughly to dissolve the resultant dark blue crystal. The absorbance in each well was determined with an automatic plate reader at 490 nm.

Real-time quantitative PCR

Total RNA in cultured PC12 cell was extracted using the RNAex reagent and systems (Watson Biotech, Shanghai, China). The potential contaminating genomic DNA was treated with DNase Ⅰ using a DNA-free kit (Ambion, Austin, TX). The cDNA was synthesized using oligo (dT)18 primer and ReverTra Ace Moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's recommendations. The quantitative real time PCR (qPCR) primers were 5'-ATCTTCTCCTTCCAGCCTGA-3' (forward) and 5'-TGCAGCTGACTGGACATCTC-3' (reverse) for bcl-2; 5'-CTGCAGAGGATGATTGCT GA-3' (forward) and 5'-GAGGAAGTCCAGTGTCC AGC-3' (reverse) for bax. The specificity of the PCR product was verified by RT-PCR. In PC12 cells, the expected sizes of the generated fragments are 386 base pairs (bp) for bcl-2, 206 bp for bax, and 292 bp for GAPDH. The qPCR was performed using gene-specific primers on a Chromo4 Four-color Real Time PCR system (MJ Research, San Francisco, CA) using SYBR Green Realtime Master Mix (Toyobo). The relative gene expression was evaluated using the comparative cycle threshold method (Livak and Schmittgen, 2001).

Statistics analysis

Data are presented as $\bar{x} \pm s$. Statistical analysis of the data for multiple comparisons was performed one-way by ANOVA. For single comparison, differences were determined by Student's t test. $P < 0.05$ was considered statistically significant.

Results

Cognitive function

Rats in all groups became more efficient at finding the platform on successive trials during the 5-day water maze trial (Fig. 1A), but showed differences in their progression with time ($P < 0.05$). The intra-group latencies significantly declined on d 2, 4, and 5 in the Sham and GL-treated groups. The GL-treated and Sham groups were similar on d 1 and 3.

GL affected performance in the probe test. The vehicle group spent 27.2% of their time in the platform quadrant, a decrease of 28.0% ($P < 0.05$ *vs* 37.8% in Sham) (Fig. 1B). GL rats spent 33.7% of their time in the platform quadrant, an increase of 23.9% ($P < 0.05$ *vs* vehicle) with no difference from Sham group ($P > 0.05$).

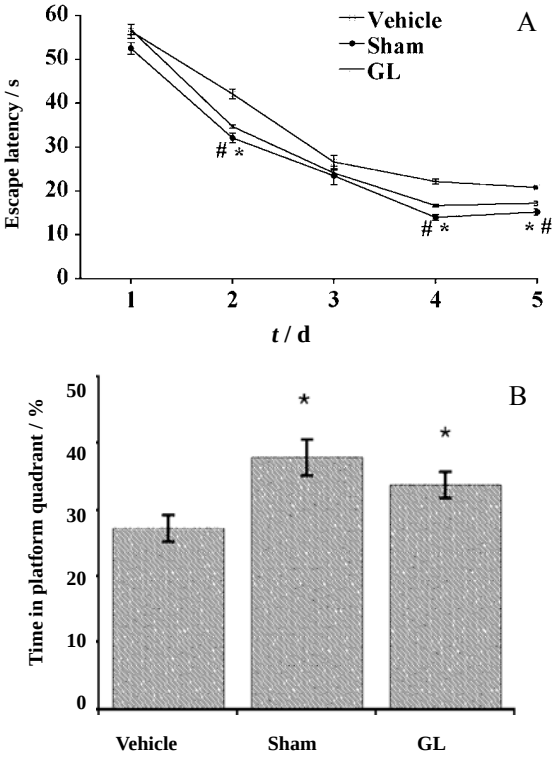

Fig. 1 Effect of GL on the Morris water maze performance of rats (n = 6)

A: Daily escape latency means to find the hidden platform

$^{*}P < 0.05$ Sham group *vs* vehicle group

$^{\#}P < 0.05$ GL-treated group *vs* vehicle group

B: The percentage of time spent in the platform quadrant, results are showed as $\bar{x} \pm s$

$^{*}P < 0.05$ *vs* vehicle group

Antioxidant activity

We then measured the antioxidant effect of GL by measuring MDA content and SOD activity in ischemic brain tissue. MDA content significantly increased in the vehicle group, (32.2 ± 7.7) nmol/mg protein in cerebral cortex and (54.6 ± 9.1) nmol/mg protein in hippocampus, compared with the Sham group (20.3 ± 4.3) nmol/mg protein in cerebral cortex and (30.3 ± 8.7) nmol/mg protein in hippocampus, whereas GL treatment attenuated this increase (24.6 ± 3.8) nmol/mg protein in cerebral cortex and (33.7 ± 10.2) nmol/mg protein in hippocampus; $P < 0.05$ *vs* vehicle (Fig. 2A). SOD activity in the Sham group was higher, (467.3 ± 47.8) nU/mg protein in the cerebral cortex and (226.5 ± 26.7) nU/mg protein in the hippocampus than vehicle-treated animals (337.5 ± 35.9) nU/mg protein and (161.2 ± 13.5) nU/mg protein, respectively ($P < 0.05$). GL treatment restored SOD activity (438.2 ± 31.5) nU/mg protein in cortex and (213.5±20.2) nU/mg protein in hippocampus ($P < 0.05$, Fig. 2B).

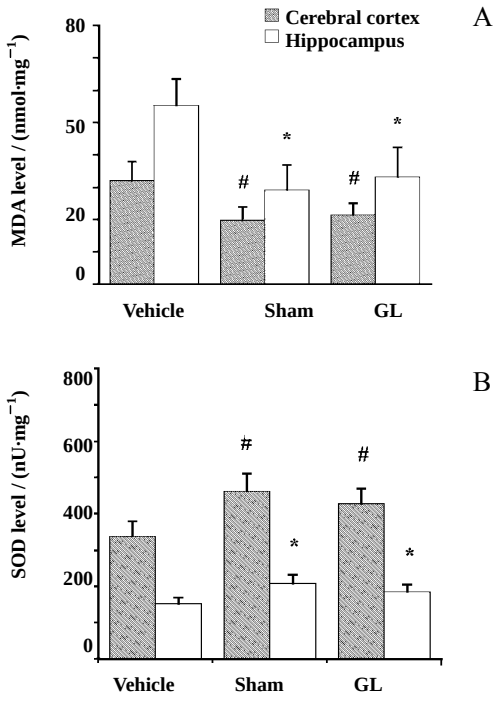

Fig. 2 The activity of SOD and the content of MDA in cerebral cortex and hippocampus

A: The content of MDA in cerebral cortex and hippocampus

B: The activity of SOD in cerebral cortex and hippocampus

The decreased activity of SOD and the increased level of MDA were significantly corrected by GL ($P < 0.05$, $n = 6$). Results are showed as $\overline{X} \pm s$ ($n = 6$)

[*] $P < 0.05$ *vs* the vehicle group in cerebral cortex

[#] $P < 0.05$ *vs* the vehicle group in hippocampus

Histopathological examination

GL prevented the histological injury induced by chronic cerebral ischemia (Fig. 3). In the vehicle group, 2VO caused neuron injury and reduced cell counts in the hippocampal CA1 and CA3 areas. Pyramidal cellular nuclei shrank and showed cellular edema. Furthermore, the pyramidal layer was disordered and irregular. GL reduced this abnormal morphology.

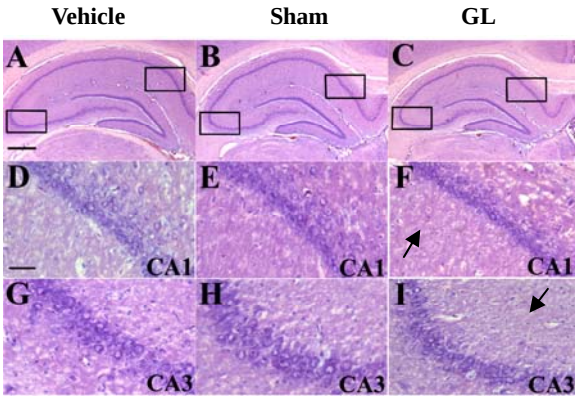

Fig. 3 HE staining in hippocampus after 26 d global cerebral ischemia

Neurons were observed in CA1 and CA3 areas (D–I).The abnormal morphology in hippocampal CA1 and CA3 areas was significantly relieved in the GL-treated group (F and I) compared with the vehicle group ischemia controls (D and G). Histological injury was marked by arrow in the pictures. A–C, D–I were shown at the same magnification, respectively

Cell viability

Excess Glutamate, a cell permeable oxidant, is cytotoxic (Sattler and Tymianski, 2001). We measured Glutamate-induced toxicity in PC12 cells using 10 mmol/L Glutamate for 24 and 48 h, which decreased viability by 59% ± 7.6% and 42.8% ± 3.1%, respectively. GL (0.03–0.6 mmol/L) dose- and time-dependently improved cell viability after Glutamate treatment (Fig. 4).

mRNA expression of Bcl-2 and Bax

Glutamate (10 mmol/L) treatment also increased Bax mRNA levels, peaking at 12 h (Fig. 5A), and the time point used for subsequent treatments. GL (0.6 mmol/L) blocked this increase in Bax mRNA. In contrast, Glutamate reduced Bcl-2 mRNA levels after 12 h (Fig. 5B), and GL could reverse this decrease.

Discussion

The present study demonstrated that cerebral ischemia produced severe deficits in performance on the Morris water maze as well as neuronal cell damage

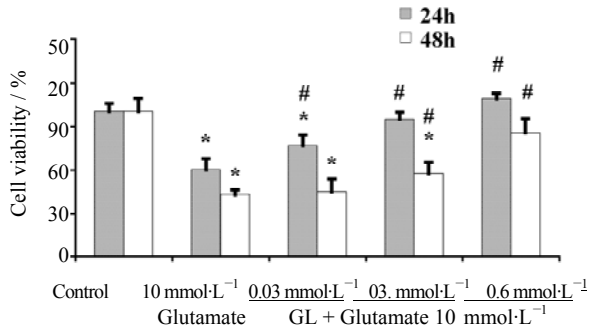

Fig. 4 Effects of GL on Glutamate-induced neurotoxicity in cultured PC12 cells

Cells were treated with 10 mmol·L^{-1} Glutamate for 24 and 48 h with or without GL. Data are expressed as percentage of values in untreated control cultures, and are $\bar{X} \pm s$ ($n = 6$)

$^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* cells exposed to Glutamate alone

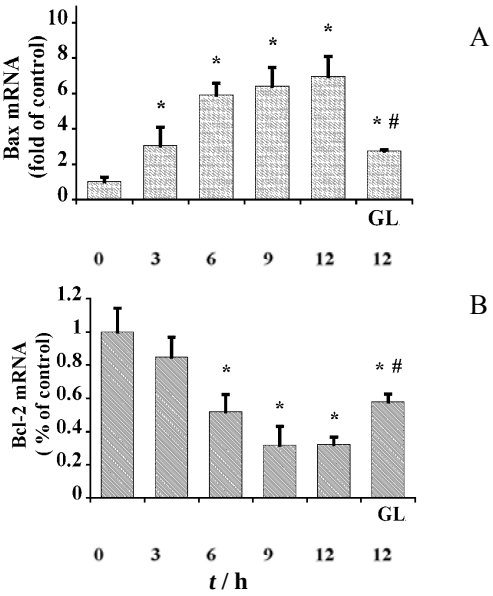

Fig. 5 qPCR resluts of Bax mRNA (A) and Bcl-2 mRNA (B) levels

Data are expressed as $\bar{X} \pm s$ ($n = 3$). $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* cells exposed to Glutamate alone for 12 h

in the hippocampus and cerebral cortex. Treatment with GL attenuated ischemia-induced learning and memory deficits and protected against cell damage in the brain. Neuronal apoptosis might cause this memory impairment.

VaD can show severe, progressive cognitive impairment, and neuropathological changes clinically (Desmond, 2004). Therefore, choosing a suitable animal model with long-lasting and/or progressive cognitive deficits and neuronal damage is very important in preclinical studies for the efficacy of potential neuroprotective drugs. The 2VO model is the most common model for understanding the pathophysiology of VaD and evaluating the therapeutic potential of drugs (Sarti, Pantoni, and Inzitari, 2002; Ni *et al*, 1994). The Morris water maze was used to test cognitive function, where the hidden platform trial measured acquisition and the probe trail measured retention (Yu *et al*, 2005). Postischemic 20 mg/kg per day GL ip administration significantly decreased the latency of 2VO rats to reach the platform compared with vehicle control and increased the time of 2VO rats to spend swimming in the platform quadrant, which suggested that GL treatment could improve cognitive dysfunction caused by chronic cerebral hypoperfusion.

One of the most vulnerable regions for cell death after ischemic insults is the hippocampus, which plays a major role in learning and memory (Butler *et al*, 2002). Histologically, hippocampal CA1 pyramidal cells, which are Glutamatergic or aspartatergic, show marked necrosis after transient ischemia (Matsumoto *et al*, 1991). Reducing neuronal damage in the hippocampus can improve memory performance in the Morris water maze (Block, Pergande, and Schwarz, 1997). Here, we observed neuropathological alterations in the hippocampus of rats after permanent occlusion of 2VO, but GL treatment reduced this damage. GL treatment also blocked the decrease in pyramidal cell number induced by 2VO.

The pathogenesis of VaD is very complex and still not clear. The symptoms of VaD may correlate with cortical or hippocampal injury induced by the increase of free radicals (Klejman *et al*, 2005). Lipid peroxidation from free-radical generation induced by chronic cerebral hypoperfusion damages membranes (Bassett and Montine, 2003). Oxidative stress caused increased intracellular reactive oxygen species (ROS) contributes to degenerative disorders, including premature aging, Alzheimer's disease, and VaD (Beckman *et al*, 1994). MDA is produced by free radical-catalyzed peroxidation of unsaturated fatty acids in cell membrane and can disrupt protein synthesis, leading to obvious cognitive deficits (Huang *et al*, 2008). SOD can scavenge excessive free radicals and protect cells from free radicals (Beckman *et al*, 1994). Free radical production could increase MDA content but decrease SOD activity, as we found here. These changes paralleled cognitive impairment and histopathological

changes, and GL ameliorated these changes, indicating antioxidant activity.

The PC12 cell is a well-characterized neuronal model, originally isolated from a catecholamine-secreting tumor (pheochromocytoma) in rats. Glutamate is a major excitatory neurotransmitter that modulates synaptic plasticity, neuronal development, and excitation *via* the activation of Glutamate receptors (Conn, 2003). *N*-methy-*D*-aspartate (NMDA) receptors, a subset of Glutamate receptors, could conduct Ca^{2+}, leading to a direct increase in intracellular Ca^{2+} through Ca^{2+} from the extracellular environment. Exposure to high concentration of Glutamate is excitotoxic to PC12 cells *via* calcium entry (Choi and Rothman, 1990). Under physiological conditions such as periods of patterned Glutamatergic synaptic activity, the increase in intracellular calcium could promote the synaptic stabilization and strengthening (Huettner, 2003). Under pathological conditions, such as prolonged exposure to high concentration of Glutamate, excessive amounts of intracellular calcium are toxic, causing excitotoxic reactions (Naarala *et al*, 1995). Thus, excessive Glutamate accumulation in the extracellular space contributes primarily to neuronal injury, with high concentration of Glutamate often used to mimic neuronal excitotoxicity *in vitro*. Our data provide strong evidence that treatment with GL (0.03–0.6 mmol/L) for 24 h could protect PC12 cells from Glutamate toxicity, indicating the neuroprotective effects of GL.

Cerebral ischemia might be related to cell apoptosis (Greco *et al*, 2007). Early cell changes that occur during apoptosis are associated with mitochondrial changes mediated by the Bcl-2 family of proteins, including the anti-apoptotic Bcl-2 and pro-apoptotic Bax protein (Stewart and Pietenpol, 2001). Bcl-2 promotes cell survival and Bax accelerates apoptotic cell death. Here, GL could block changes in mRNA levels of Bcl-2 and Bax following Glutamate-induced apoptosis, providing a potential mechanism for reducing apoptotic cell death.

In conclusion, GL could improve chronic cerebral ischemic-induced cognitive impairment, increase endogenous antioxidant defenses, decrease pathological alterations in the hippocampal CA1 and CA3 areas, attenuate neuron injuries, and protect PC12 cells from excitotoxicity induced by Glutamate. These findings provide experimental evidence for the application of GL in the treatment of VaD.

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