# Effect of Gua Lou Gui Zhi decoction on focal cerebral ischemia-reperfusion injury through regulating the expression of excitatory amino acids and their receptors

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Received July 20, 2013; Accepted February 27, 2014

DOI: 10.3892/mmr.2014.2189

**Abstract.** Gua Lou Gui Zhi decotion (GLGZD) has been reported to be an effective treatment for post-apoplectic limb spasm in the clinic. The present study aimed to investigate whether GLGZD had an affect on cerebral injuries induced by middle cerebral artery occlusion (MCAO) in rats and its possible mechanism. High-performance liquid chromatography was performed to analyze GLGZD. Furthermore, a model was established to assess the efficacy of GLGZD. Neurological defect scores and screen tests were analyzed. Brain ischemic infarct volume was measured using 2,3,5-triphenyl tetrazolium chloride staining and glutamic acid (Glu), aspartic acid (Asp) and glycine (Gly) levels in the cerebrospinal fluid were measured using the Hitachi automatic amino acid analyzer. Immunohistochemistry was performed to determine the expression of the α-amino-3 -hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) glutamate receptors, and to analyze histopathological change. GLGZD was found to improve neurological performance and reduce infarct volumes in MCAO rats. In addition, GLGZD was observed to enhance motor performance, which was assessed using the screen test. Furthermore, GLGZD was found to reduce Glu, Asp and Gly levels in the cerebrospinal fluid and downregulate the protein expression of the AMPA and NMDA glutamate receptors. Thus, it was demonstrated that GLGZD may exert neuroprotective effects through the modulation of excitatory amino acids, and AMPA and NMDA receptor expression.

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*Abbreviations:* GLGZD, Gua Lou Gui Zhi decotion; MCAO, middle cerebral artery occlusion; CSF, cerebrospinal fluid; NMDA, N-methyl-D-aspartic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; TTC, 2,3,5-triphenyl tetrazolium chloride; EAAs, excitatory amino acids; CNS, central nervous system

*Key words:* cerebral ischemia/reperfusion, excitatory amino acids, N-methyl-D-aspartic acid receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor, Gua Lou Gui Zhi decotion

## Introduction

Stroke, a cerebrovascular disease, is defined as a sudden neurological deficiency in the brain, which is caused by either hemorrhage or a lack of blood supply in the brain, termed ischemia. A total of 80% of strokes are caused by arterial blockage and consequent ischemia and 20% are caused by hemorrhage. At present, stroke represents one of the three most dangerous diseases threatening human health (1). It has been reported that 70-80% of stroke survivors have a level of disability, particularly post-apoplectic limb spasm, which has become an increasingly serious social problem (2). Post-apoplectic limb spasm is one of the key difficulties, which is faced during rehabilitation therapy following stroke, due to its high rate of motor impairment, difficulty of treatment and importance for limb function (3). The identification of effective therapeutic approaches to treat post-apoplectic limb spasm is important for stroke rehabilitation.

It is well established that excitotoxicity is the major pathophysiological mechanism underlying ischemic stroke-induced brain injury (4). Furthermore, excitatory amino acid (EAA)-induced excitotoxicity has been proposed to be involved in post-apoplectic limb spasm. Ischemia-reperfusion (I/R)-induced injury has been reported to be associated with numerous biochemical events, a number of which may result from the release of EAAs and the activation of their receptors (5). When EAAs reach toxic levels, they contribute to increased excitability, which leads to injury (6). The associated ion channels open and cause excessive $Ca^{2+}$ influx, which stimulates a cascade of enzymatic processes, leading to irreversible injury (7). EAAs, including glutamic acid (Glu) and aspartic acid (Asp) are important neurotransmitters in the central nervous system (CNS) and have a major role in synaptic transmission. It is well established that EAAs are involved in the transmission of sensory information and motion instruction (8).

A large number of pharmacological agents have been used in attempt to improve motor impairment. These agents work through a variety of neurotransmitter- and receptor-associated

mechanisms (9). A number of these agents have been used in clinical trials with varying results; however, many of these agents exhibit considerable side effects. Traditional Chinese medicine has advantages compared with these other pharmacological agents, including lower side effects, thus more regular doses may be taken. Traditional Chinese medicines, including Buyang Huanwu Decotion (10) and Xiao-Xu-Ming decotion (11), have been used for stroke and muscular therapy to treat post-apoplectic limb spasm with the advantages of easy administration and few adverse reactions.

Gua Lou Gui Zhi decotion (GLGZD) is a traditional Chinese medicine that consists of the following extracts: Radix Trichosanthis, Radix Paeoniae Alba, Ramulus Cinnamomi, Rhizoma Zingiberis Recens, Radix Glycyrrhizae and Fructus Ziziphi Jujubae. Clinical trials have found that GLGZD improves limb muscle tension, benefiting locomotive function and quality of life in patients with stroke (12-14). However, the effect of GLGZD on the release of EAAs in the brain during I/R and the underlying mechanisms have yet to be elucidated.

Middle cerebral artery occlusion (MCAO) followed by reperfusion is a model of focal ischemia in rats, which resembles human ischemic stroke (15). The present study aimed to investigate the levels of EAAs and their receptors within the brain and whether GLGZD had a protective effect against I/R through modulating the release of EAAs and the expression of EEA receptors. Infarct volumes and neurological deficits were measured and analyzed in five groups.

## Materials and methods

*Chemicals, reagents and animals*. Specific pathogen-free male Sprague-Dawley rats (weight, 180-220 g) were obtained from the Laboratory Animal Center of Fujian University of Traditional Chinese Medicine (Fuzhou, China). The principles of laboratory animal care were followed and the study was approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine.

Dried drugs were purchased from Tongchun Drugstore (Fuzhou, China) and were identified by Professor Yang (College of Pharmacy, Fujian University of Traditional Chinese Medicine). Voucher specimens were deposited at the College of Pharmacy of Fujian University of Traditional Chinese Medicine. Peoniflorin, liquiritigenin, liquiritin, cinnamaldehyde, cinnamic acid and glycyrrhizin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were of high performance liquid chromatography (HPLC)-grade and purchased from Merck KGaA (Darmstadt, Germany). Phosphoric acid, hydrochloric acid, petroleum, chloroform, acetoacetate and N-butanol were used as analytical reagents and were purchased from Aladdin Reagents Co., Ltd. (Shanghai, China). Other reagents were all of analytical grade. The deionized water used throughout the experiments was generated using a Millipore water purification system (Billerica, MA, USA). A CX31 microscope was purchased from Olympus Corporation (Tokyo, Japan).

*Preparation of GLGZD*. According to 'Jinkui Yaolue' (16), GLGZD consists of Radix Trichosanthis, Radix Paeoniae Alba, Ramulus Cinnamomi, Rhizoma Zingiberis Recens, Radix Glycyrrhizae and Fructus Ziziphi Jujubae. In order to obtain the GLGZD water extract, volatile oils were extracted from Ramulus Cinnamomi and Rhizoma Zingiberis Recens. Other medical materials were decocted through boiling in distilled water twice for 1 h. The solution was then dried under vacuum to obtain a final concentration of 1.08 g/ml. GLGZD was then stored for further analysis.

*HPLC analysis of GLGZD*. The GLGZD extract was subjected to HPLC analysis. Sample solutions were injected into the HPLC system (Shimadzu Corporation, Kyoto, Japan) for analysis in triplicate. The HPLC system was equipped with a LC-20A pump system, SPD-M20A photodiode array detector and Diamonsil® $C_{18}$ reversed-phase column (I.D. 4.6x250 mm, 5 $\mu$m). Separation was achieved using a linear gradient program for mobile phase A (acetonitrile) and mobile phase B (water containing 0.1% phosphoric acid). The elution was initiated with a gradient of 95% B for 45 min, followed by 68% for 15 min and 52% for 5 min. Flow rate and injection volume were 1.0 ml/min and 10 $\mu$l, respectively.

*Focal cerebral I/R model*. An intraluminal suture method was used for the induction of focal cerebral ischemia. Rats were anesthetized using 10% chloral hydrate solution (0.3 ml/100 g body weight; intraperitoneal injection). MCAO was induced using an intraluminal suture method as described previously, but with certain modifications (15). In brief, the left common carotid artery (CCA) and the external carotid artery (ECA) were exposed. A 3-0 surgical monofilament nylon suture was then inserted from the ECA into the internal carotid artery (ICA) and was used to occlude the origin of the left MCA until light resistance was felt (18-20 mm from the CCA bifurcation). After 2 h of MCAO, the nylon suture was withdrawn, followed by 2 h of reperfusion.

*Experimental grouping and treatment*. Sixty rats were divided into the following five experimental groups and underwent the following treatments: Sham-operated group (n=12), the rats were subjected to surgical procedure, but MCAO was not induced, except for exposure of the right ICA and the right ECA; MCAO model group (n=12), rats received normal saline and underwent MCAO surgery; positive control group (n=12), rats received piracetam (6 g/kg body weight) and underwent MCAO surgery; GLGZD low-dose group (n=12), rats received GLGZD (3.6 g/kg body weight) and underwent MCAO surgery; and GLGZD high-dose group (n=12), rats received GLGZD (14.2 g/kg body weight) and underwent MCAO surgery. In the GLGZD treatment groups, GLGZD was administered once a day for seven days.

*Scoring neurological defects*. Rats were scored based on a five-point scale (17). The scale ratings were as follows: 0, no neurological symptoms; 1, unable to completely extend the front jaw on the other side; 2, rotating while crawling and falling to the contralateral side; 3, unable to walk without assistance; and 4, unconsciousness. Rats with a score of 1-3 were considered successful models and were included in the study. A sample of the ipsilateral cortex was taken at the indicated time and used for sample preparation.

Behavioral tests were performed on the rats after 60 min of ischemia followed by seven days of exercise. Each rat was scored based on a five-point scale as described previously (18).

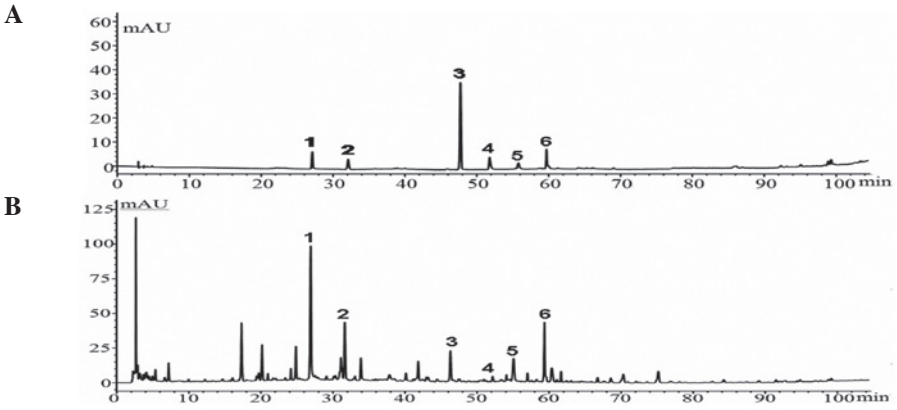


Figure 1. Liquid chromatograms of (A) a standard and (B) GLGZD. 1, peoniflorin; 2, liquiritigenin; 3, liquiritin; 4, cinnamic acid; 5, cinnamaldehyde; 6, glycyrrhizic acid. mAU, milligram Absorbance unit; GLGZD, Gua Lou Gui Zhi decotion; AU, absorbance unit.

*Screen tests*. In order to investigate motor function recovery in the rats subjected to ischemia, motor performance was measured prior to surgery and on days one and eight following surgery. In order to measure the muscle strength of the forelimbs, a net screen was used (19). The trial commenced subsequent to placing the rat on the horizontal screen on the ground. The screen was turned over 90˚ within 2 sec by raising one side gradually. The screen was maintained in this position for 5 sec.

The duration of time for which rats held on to the net screen was recorded in seconds. The scoring criterion was as follows: 5, holding on to the screen and climbing upward; 4, holding on to the screen with forelimbs and not falling down; 3, holding on to the screen temporally, but slipping a certain distance; 2, falling down to the ground within the test period; 1, falling down to the ground as soon as the screen was at 90˚.

*2,3,5-triphenyltetrazolium chloride (TTC) staining and measurement of brain ischemic infarct volume*. The six rats in each group were sacrificed by chloral hydrate and decapitated in order to remove the brain to measure the infarct volume following I/R. The brain was placed at 20˚C for ~10 min, then cut into six coronal slices continuously from front to back using a blade. The brain tissues were immersed into 2% TTC solution (T8877; Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS; pH 7.4) and stained at 37˚C for 1 h and turned over several times. Subsequent to staining, the viable cerebral tissue was stained red while the infarcted cerebral tissue remained pale. Images were captured using a high-resolution digital camera (IXUS130; Canon, Tokyo, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD, USA). Infarct volume was quantified using the Motic Med 6.0 Digital Medical Image Analysis system (Motic Instruments Inc., Richmond, Canada), The infarct size was calculated as a percentage of the viable cerebral tissue of the whole brain (20).

*Analysis of Glu, Asp and glycine (Gly) levels in the cerebrospinal fluid*. Cerebrospinal fluid was collected and the Glu, Asp and Gly levels were analyzed using a Hitachi automatic amino acid analyzer (21).

*Cerebral histopathology*. Brain samples were collected from six rats in each group for cerebral histopathological analysis. The brain samples were paraffin-embedded, sliced and stained with hematoxylin and eosin. Histopathological changes were observed using a light microscope.

*Immunohistochemistry*. Paraffin-embedded brain tissue samples (0.5x0.5x0.1 cm) were used for immunohistochemical analysis of N-methyl-D-aspartic acid receptor (NMDAR) and glutamate receptor (GluR) 1/2/3/4 expression. Briefly, the paraffin sections were dewaxed, repaired using citric acid, incubated with 3% $H_2O_2$, washed using PBS and blocked with normal goat serum. Sections were then incubated with primary antibodies (polyclonal rabbit Anti-NMDA-NR and Anti-GluR1/2/3/4; Beijing Biosynthesis Co., Ltd., Beijing, China) at room temperature for 2 h, washed using PBS and incubated with biotinylated secondary antibodies. Sections were then washed, incubated with horseradish peroxidase-labeled streptavidin and stained with 3,3'-diaminobenzidine. PBS was used to replace the primary antibody as a negative control. Five high-power fields (magnification, x400) were randomly selected in each slide.

*Statistical analysis*. Data are presented as the mean ± standard deviation. Analysis of variance was performed to determine significant differences between the groups. SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*HPLC of GLGZD*. Various mobile phase systems were used to resolve the active constituents of GLGZD. The proposed method of analysis gave simultaneous quantification of six compounds. The individual constituents were identified by comparing their peaks, UV spectra and retention times against their corresponding reference standards (Fig. 1). The percentage content of each compound was estimated using a calibration curve. The compounds present in the GLGZD extract were peoniflorin (74.2 mg/g), liquiritigenin (0.92 mg/g), liquiritin (0.09 mg/g), cinnamic acid (0.20 mg/g), cinnamaldehyde (2.13 mg/g) and glycyrrhizic acid (1.14 mg/g).

*Effect of GLGZD on infarct volume*. As shown in Fig. 2, the infarct volume in the MCAO model group was found to be significantly higher than that in the sham-operated group.

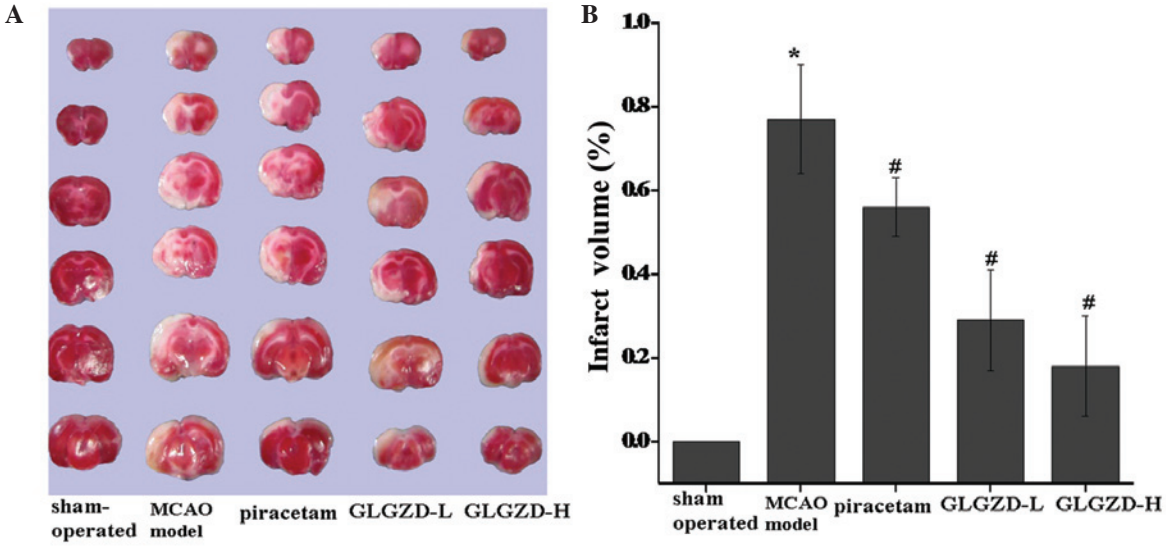


Figure 2. Effect of GLGZD on cerebral infarction in focal cerebral ischemia-reperfusion injury rats. (A) Following the treatment conditions, cerebral tissues from rats from each group were coronally sectioned into 2-mm thick sections and stained using 2,3,5-triphenyl tetrazolium chloride. Images were captured using a high-resolution digital camera. (B) Infarct volume was quantified using the Motic Med 6.0 Digital Medical Image Analysis system, which was represented as a percentage of the total brain volume. Data are presented as the mean ± standard error from five individual rats in each group. *P<0.05, vs. sham-operated group and #P<0.05, vs. MCAO model group. GLGZD, Gua Lou Gui Zhi decotion; MCAO, middle cerebral artery occlusion; H, high-dose; L, low-dose.

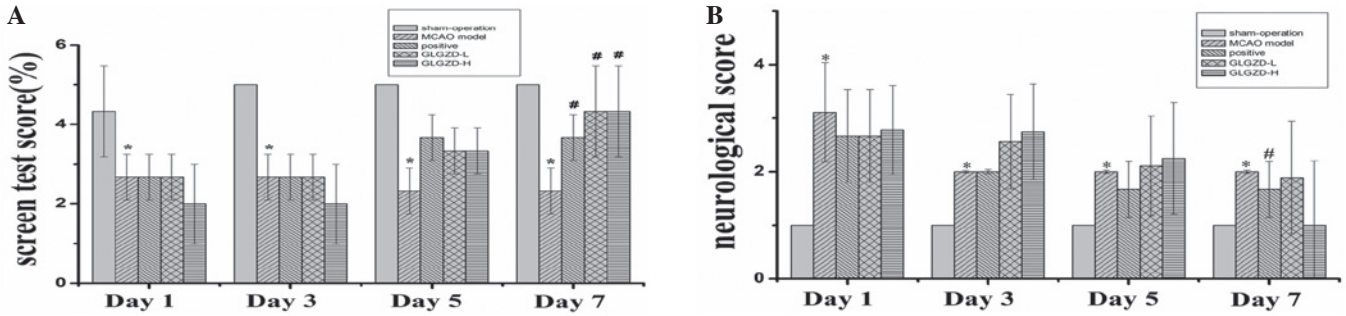


Figure 3. Effect of GLGZD on (A) motor performance detected using a screen test and (B) neurological behavior in focal cerebral ischemia-reperfusion injury rats. GLGZD, Gua Lou Gui Zhi decotion; MCAO, middle cerebral artery occlusion.

Furthermore, seven days following ischemia, infarct volumes were observed to be decreased in the GLGZD groups compared with the MCAO model group. The infarct volumes in the MCAO model group vs. the GLGZD groups were as follows: 0.77±0.13 vs. 0.29±0.12% for the GLGZD low-dose group and 0.77±0.13 vs. 0.18±0.12% (P<0.05) for the GLGZD high-dose group. Infarct volume was more greatly reduced in the GLGZD high-dose group than the GLGZD low-dose group. The infarct volumes in the MCAO model group vs. the piracetum group was 0.77±0.13 vs. 0.56±0.07% (P<0.05), showing that the results were significantly different between the two groups.

*Effect of GLGZD on neurological behavior and motor performance*. The effect of GLGZD on neurological and motor function were assessed by measuring neurological and motor performance. The induction of MCAO for 60 min followed by reperfusion for 2 h caused marked changes in rat behavior. The neurological behavior of the rats that were subjected to MCAO was measured using the scoring method described by Longa *et al* (15). Motor performance was measured using a screen test scoring method in order to investigate the motor function recovery of the rats subjected to MCAO.

As shown in Fig. 3A and B, the rats in the MCAO model group that were subjected to I/R injury, exhibited severe neurological deficit (score: 2) and motor disorder, and showed circling towards the contralateral side with a reduced mobility compared with the rats in the sham-operated group (score: 0). Rats in the GLGZD groups showed significant improvements in behavior between days 1 and 7, particularly those in the high-dose group. Rats in the piracetum group showed improvements in behavior between days 5 and 7, while no changes in neurological function or motor performance were observed in the rats in the sham-operated group.

*Effect of GLGZD on levels of the EAAs Glu, Asp and Gly in the cerebrospinal fluid*. The excessive release of EAAs in the brain following I/R injury is closely associated with post-apoplectic limb spasm. As shown in Table I, MCAO induced significant increases in the levels of the excitatory neurotansmitters Glu, Asp and Gly. The levels of Glu, Asp and Gly in the low- and

Table I. Levels of the excitatory amino acids Glu, Asp and Gly in the cerebrospinal fluid.

| Group | Asp | Glu | Gly |
|---|---|---|---|
| Sham-operation | 3.00±0.12. | 4.27±0.72. | 1.02±0.04. |
| MCAO model | 4.84±0.23[a] | 6.34±0.66[a] | 1.65±0.02[a] |
| Piracetam | 3.25±0.15[b] | 4.27±0.68[b] | 0.85±0.06[b] |
| GLGZD-L | 3.36±0.26. | 4.64±0.81[b] | 0.96±0.04. |
| GLGZD-H | 3.34±0.11[b] | 4.43±0.75[b] | 0.72±0.03[b] |

[a]P<0.05, vs. the sham-operation group; [b]P<0.05, vs. the MCAO model group. Glu, glutamic acid; ASP, aspartic acid; Gly, glycine; GLGZD, Gua Lou Gui Zhi decotion; L, low-dose; H, high-dose; MCAO, middle cerebral artery occlusion.

high-dose GLGZD groups were lower than those in the MCAO model group, but higher than those in the sham-operated group. Similarly, compared with the MCAO model group, the levels of Glu, Asp and Gly in the piracetum group decreased and were significantly different (P<0.05). These findings show that GLGZD had a modulatory effect on EAA levels.

*Cerebral histopathology*. As shown in Fig. 4, the cortical neural cells in the sham-operated group were observed to be arranged orderly and to exhibit normal cell morphology, with clearly visible structures and integrity. The cell membranes and nuclei were normal, as was the tissue interspace, which exhibited no edema or inflammatory cell infiltration. The cortical region of the ischemic side of the brain in the rats in the MCAO model group exhibited visible disorder and high levels of cell necrosis, with cells showing nucleolar shrinkage and breakdown, as well as vacuolar degeneration. Furthermore, the mesenchymal cells demonstrated high levels of edema and inflammatory cell infiltration compared with those in the MCAO model group. The cortical neural cells in the rats in the GLGZD groups showed decreased pathological changes compared with those in the MCAO model group. Neuronal degeneration and necrosis, as well as disorderly cell arrangement, tissue edema, nuclear dissolution and nucleolar shrinkage were observed in the cells in the GLGZD treatment groups; however, the quantity and extent was decreased compared with that in the MCAO model group. Similarly, compared with the MCAO model group, the cortical neural cells in the rats in the piracetum groups exhibited reduced pathological changes

*Immunohistochemistry*. Semi-quantitative scoring was performed according to the ratio of positively stained cells and the staining intensity (22,23). Immunohistochemical scoring revealed a significant increase in NMDAR and GluR1/3/4, and a significant decrease in GluR2 in the hippocampus of the MCAO model rats compared with the sham rats (Fig. 5; Table II). After seven days of high-dose GLGZD administration, the scores were further reduced to 4.4±0.6 for NMDAR (P<0.05), 4.4±0.7 for GluR1 (P<0.05), 3.6±0.3 for GluR3 (P<0.01) and 3.6±0.7 for GluR4, while they were upregulated to 3.6±0.3 for GluR2 (P<0.01). For the piracetum groups, the scores were reduced to 4.0±0.1 for NMDAR (P<0.05), 4.2±0.6 for GluR1 (P<0.05), 3.6±0.4 for GluR3 (P<0.01) and 2.8±0.5 for GluR4, while they were upregulated to 4.0±0.0 for GluR2 (P<0.01). Alteration in the expression of NMDAR and GluR1/2/3/4 was neutralized with GLGZD treatment.

## Discussion

Stroke often causes motor impairment, which may be defined as a velocity-dependent increase in resistance to passive stretch or movement, resulting in abnormal signaling between the CNS and the muscles, leading to muscle excitability (24). In the CNS, EAAs and their receptors have been shown to have a crucial role in motor impairment following cerebral I/R injury. Glu is the predominant excitatory neurotransmitter in the brain, and the subsequent activation of its receptors induces an increase in intracellular calcium and the generation of free radicals, leading to cell death, necrosis or apoptosis (25). Asp is present in high levels in the CNS within the hypothalamus and cerebellum, and is an excitatory neurotransmitter of intermediate neurons, which causes neuron depolarization and generates excitatory postsynaptic potential with slow and persistent function (26). It is well established that Gly has an important role in inhibitory synaptic transmission in the spinal cord and the brain stem. Furthermore, it has been reported that Gly is an important facilitator of glumate function in the cerebrum, through an allosteric site on the NMDA receptor (27). Therefore, Gly may have excitatory and inhibitory roles in the CNS.

Ionotropic glutamate receptors consist of three subclasses: NMDA, AMPA and kainate receptors, termed based on their characteristic agonists. AMPA and kainate receptors are often known as non-NMDA receptors. NMDA and non-NMDA glutamate receptors have a role in mediating motor impairment (28). NMDAR toxicity is dependent on extracellular $Ca^{2+}$ and occurs as a consequence of high $Ca^{2+}$ influx directly through the receptor-gated ion channel in the receptor (29). However, the majority of AMPA receptor (AMPAR) channels have poor $Ca^{2+}$ permeability (30). AMPA-type GluRs are heteromeric channels comprised of a combination of GluR1-4 subunits (31), with changes in the expression of GluR1-4 being associated with development, aging, epilepsy and alterations in synaptic efficacy. The presence of the GluR2 subunit determines the permeability of the AMPAR to $Ca^{2+}$ and other divalent cations. The majority of neurons exhibit high levels of GluR2 expression, which renders these cells impermeable to calcium influx thorugh AMPARs (32). Thus, AMPAR $Ca^{2+}$ permeability is considered to be determined by the relative abundance of GluR2 subunits in the heteromeric AMPA receptors. It has been reported that ischemic conditions may alter the subunit composition of AMPA receptors towards a more $Ca^{2+}$-permeable combination, which may promote neuronal degeneration (33).

GLGZD has been used for the treatment of post-apoplectic limb spasm. Yang *et al* (14) showed that GLGZD had a significant therapeutic effect on post-apoplectic limb spasm. The present study aimed to investigate the effect of GLGZD on focal I/R insult-induced motor impairment and behavioral disturbance, by inducing cerebral I/R injury in rats. GLGZD was found to improve neurological and motor performance. Furthermore, the present study identified a large infarct volume mainly in the cortex and striatum (or in the transient focal cerebral ischaemic brains) of the ipsilateral hemisphere

Table II. Immunohistochemical scores of NMDAR and GluR1/2/3/4 expression in brain tissue.

| Group | NMDAR | GluR1 | GluR2 | GluR3 | GluR4 |
|---|---|---|---|---|---|
| Sham-operation | 1.8±0.4. | 2.0±0.6. | 5.6±0.4. | 4.8±0.5 | 3.2±0.5 |
| MCAO model | 5.6±0.2[a] | 5.2±0.7[a] | 1.6±0.6[a] | 5.6±0.4 | 4.0±0.9 |
| Piracetam | 4.0±0.1[b] | 4.2±0.6[b] | 4.0±0.0[c] | 3.6±0.4[c] | 2.8±0.5 |
| GLGZD-L | 4.8±0.5. | 4.6±0.1. | 3.2±0.4[c] | 4.4±0.7 | 4.4±0.7 |
| GLGZD-H | 4.4±0.6[b] | 4.4±0.7[b] | 3.6±0.3[c] | 3.6±0.7 | 3.6±0.7 |

[a]P<0.01, vs. the sham-operation group; [b]P<0.05 and [c]P<0.01, vs. the MCAO model group. GluR, glutamate receptor; NMDAR, N-methyl-D-aspartic acid receptor; GLGZD, Gua Lou Gui Zhi decotion; L, low-dose; H, high-dose; MCAO, middle cerebral artery occlusion.

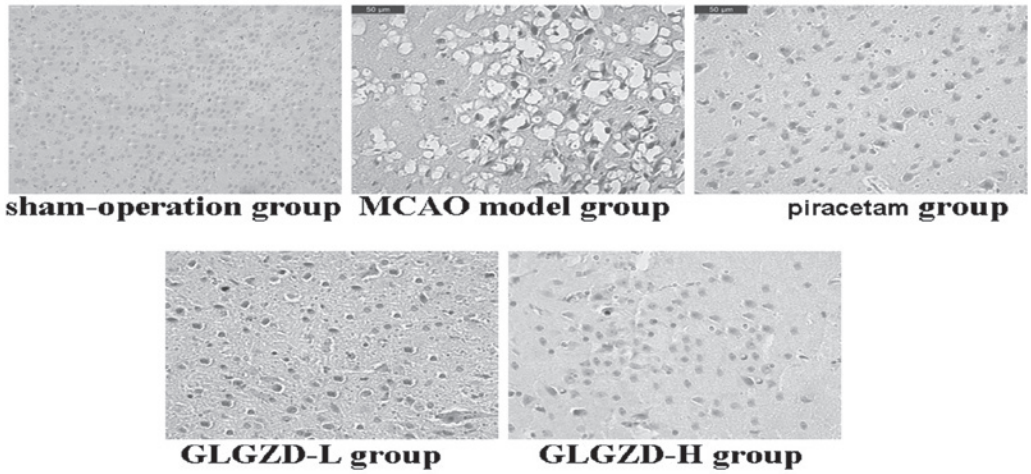


Figure 4. Effect of GLGZD on histopathological changes in the brain tissue of focal cerebral ischemia-reperfusion injury rats. Hematoxylin and eosin staining; magnification, x200. GLGZD, Gua Lou Gui Zhi decotion; L, low-dose; H, high-dose; MCAO, middle cerebral artery occlusion.

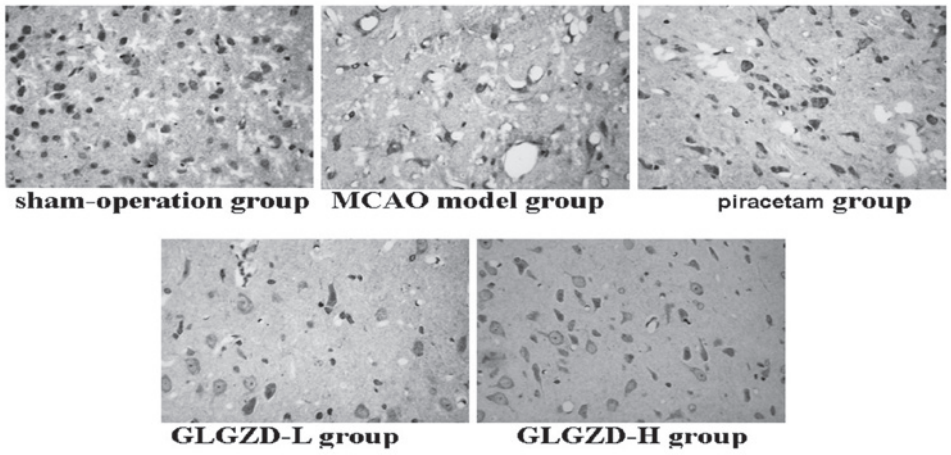


Figure 5. N-methyl-D-aspartic acid receptor 1 expression detected using immunohistochemistry (magnification, x100). GLGZD, Gua Lou Gui Zhi decotion; L, low-dose; H, high-dose; MCAO, middle cerebral artery occlusion.

of the brain in the ischemic rats. Pretreatment with GLGZD at high- and low-doses was observed to reduce the infarct volume compared with the sham-operated group. This finding suggests that oral administration of GLGZD extracts significantly inhibits reperfusion-induced neuronal cell death. In the present study, behavioral tests were used to assess the success of MCAO. Screen tests were used to assess behavioral dyskinesia caused by cerebral infarction. The findings suggest that GLGZD improves stroke-induced neurobehavioral abnormalities in rats.

In the present study, the quantity of Glu and Asp were found to increase markedly in the brain tissue of the rats in the MCAO model group, which indicates that the cytotoxicity of EAAs is involved in the establishment of ischemic brain injury

and delayed nerve damage. The level of Gly in the brain tissue was observed to increase significantly in the MCAO model group, which may have a role in cerebral ischemia injury and a synergistic toxic effect with Glu. GLGZD treatment was found to inhibit the release of Glu and Asp during I/R.

Immunohistochemistry of the NMDAR revealed that the mice in the low- and high-dose GLGZD groups exhibited decreased positive NMDA expression, which may reduce excessive activation of the NMDA receptor, as well as reduce nerve cell cytotoxicity, necrosis and apoptosis. Immunohistochemistry of AMPAR subunits revealed that the the mice in the low- and high-dose GLGZD groups exhibited increased GluR2 expression, but decreased GluR1, 3 and 4 expression, which may prevent $Ca^{2+}$ influx and have a protective effect against nerve injury. Thus, the neuroprotective effects of GLGZD may be partially due to its capacity to attenuate excitotoxic Glu and Asp accumulation. The effect of Gly and its receptors on cerebral I/R injury requires further investigation.

In conclusion, the present study established a HPLC method to qualitatively and quantitatively analyze the effect of GLGZD on EEAs and their receptors in the brains of MCAO model rats. The findings provides important insight into GLGZD as a neuroprotective agent.

## Acknowledgements

This research was done in state key laboratory of Chinese pharmacies of Fujian Provincial Department of Science and Technology, Collaborative Innovation Center for Rehabilitation Technology and TCM Rehabilitation Research Center of SATCM. It was funded by the Important Subject of Fujian Province Science and technology hall of China (2012Y0041) and the important subject of Fujian province Education Hall of China (JA12176).

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