

Ginkgo biloba extract protects diabetic rats against cerebral ischemia-reperfusion injury by suppressing oxidative stress and upregulating the expression of glutamate transporter 1

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Abstract. The current study aimed to evaluate the neuroprotective effect of *Ginkgo biloba* extract (*GbE*) on the progression of acute cerebral ischemia-reperfusion injury in diabetic rats, and to determine the molecular mechanism associated with this effect. Streptozotocin (STZ) induced diabetic rats were pretreated with *GbE* (50, 100 and 200 mg/kg/day; intragastric) for 3 weeks. During this period, body weight changes and fasting blood glucose levels were assessed each week. Following pretreatment, rats were subjected to suture occlusion of the middle cerebral artery for 30 min, which was followed by 24 h of reperfusion. Neurological deficits were subsequently evaluated at 2 and 24 h following reperfusion. Rats were sacrificed after 24 h reperfusion, and infarct volume and S100B content were measured to evaluate the neuroprotective effect of *GbE*. The results of the present study demonstrated that *GbE* pretreatment improved neurological scores, and reduced cerebral infarct volume and S100B content. Oxidative stress markers, including glutathione (GSH) and superoxide dismutase (SOD) were increased, and malondialdehyde (MDA) contents were reduced following *GbE* treatment. The levels of p-Akt, p-mTOR and glutamate transporter 1 (GLT1) were observed to be increased in *GbE*-pretreated rats. These results indicated that *GbE* pretreatment may serve a protective role against cerebral ischemia-reperfusion injury in diabetic rats by inhibiting oxidative stress reaction, upregulating the expression of Akt/mTOR and promoting GLT1 expression. In conclusion, the current study revealed the protective role and

molecular mechanisms of *GbE* in diabetic rats with cerebral ischemia-reperfusion injury, and may provide novel insight into the future clinical treatment of this condition.

Introduction

Diabetes mellitus is a complex metabolic syndrome, which significantly affects systemic and cerebral vasculature (1). Chronic and uncontrolled diabetes mellitus is characterized by a persistent elevation in blood glucose, which is in association with a number of long-term complications, including ischemic stroke (1). Diabetes is an independent risk factor for ischemic stroke (2). Diabetes exacerbates cerebral ischemia injury in experimental and clinical stroke subjects by accelerating neuronal damage and increasing infarct volume (3-5). Patients with diabetes exhibit double the risk of ischemic stroke compared with people without diabetes, after correction for other risks, and these individuals are predicted to exhibit increased morbidity and mortality (6,7). However, the cellular and molecular mechanisms by which hyperglycemia is associated with ischemic brain damage have not been fully determined.

Oxidative stress serves a pivotal role in the development of microvascular and macrovascular diabetes complications (8). The overproduction of reactive oxygen species (ROS), which is induced by hyperglycemia, is a mediator of tissue damage that occurs during diabetes, and can lead to cerebral dysfunction (8,9). The brain is vulnerable to radical-mediated attack due to its limited antioxidant defenses (10). When the redox balance is impaired, free radicals and oxidative stress-associated mechanisms can cause cell injury and necrosis (11). During periods of oxidative stress, Akt/mTOR pathways are closely integrated and have been revealed to directly determine cell fate (12-14). Research has demonstrated that the Akt/mTOR signaling cascade serves an important role in the onset and progression of cerebral ischemia injury (13). A number of agents that increase the phosphorylation levels of Akt and mTOR have been demonstrated to reduce brain injury in stroke models (15,16). A previous study has also indicated that lentiviral-mediated overexpression of cAkt can protect against stroke-induced neuronal injury *in vivo* and *in vitro*, and

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mTOR inhibition with rapamycin can block these protective effects (17).

Excitotoxicity, which is induced by the overactivation of glutamate, has been identified as a key factor in the pathogenesis of cerebral ischemia (18). GLT1, which is predominantly located on astrocytes, is responsible for up to 90% of glutamate clearance to maintain glutamate homeostasis in adult brain tissue (19). However, the downregulation or dysfunction of GLT1 following ischemia leads to the accumulation of extracellular glutamate and neuronal death (20). It has been demonstrated that GLT1 knockdown exacerbates the neuronal death and neurological deficit in rats with middle cerebral artery occlusion (MCAO) (21). Recent evidence has demonstrated that mTOR is a downstream target of the PI3K/Akt pathway, which regulates GLT1 expression (22). mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2) are associated with GLT1 expression (23), and oxidative stress and excitotoxic mechanisms have been suggested to operate in tight conjunction to induce irreversible damage of brain tissue (24). Chen *et al.* (25) proposed that glutamate-mediated excitotoxicity with oxidative stress fulfill the 'two-hit' hypothesis that accelerates neurodegeneration. Therefore, the current study hypothesized that oxidative stress causes the downregulation of Akt/mTOR signaling, and mTOR participates in the downregulation of GLT1, which can lead to further excitotoxicity, and eventually exacerbate diabetic ischemic stroke.

Ginkgo biloba extract (*GbE*) is a standardized mixture that is extracted from *Ginkgo biloba* leaves, containing 22-27% *Ginkgo* flavone glycosides (myricetin, quercetin, kaempferol and isorhamnetin) and 5-7% terpene lactones (ginkgolide A, B, C and bilobalides) (26). *GbE* has been used as a therapeutic agent for a number of cardiovascular and neurological diseases (27,28). Although the exact mechanism is unclear, an accumulation of evidence has demonstrated that *GbE* exhibit a number of benefits, including improving hemodynamics, inhibiting the platelet-activating factor, scavenging ROS and relaxing vascular smooth muscles (29). These results demonstrate the pharmacological use of *GbE* for the treatment of diabetic ischemic stroke. Recent studies have demonstrated that *GbE* protects against a number of diabetic complications, including diabetic cataract (30), diabetic nephropathy (31) and diabetic cardiomyopathy (32). However, the effect of *GbE* on diabetic ischemic stroke is yet to be determined. Therefore, the present study was designed to evaluate the protective effect and its possible mechanism of action in diabetic rats with cerebral ischemia-reperfusion injury.

Materials and methods

Animals. Adult male Sprague-Dawley rats (8-10 weeks old; 180-220 g) were obtained from the Laboratory Animal Center of Xuzhou Medical University, (license no. SCXK2007-2005; Xuzhou, China), where an SPF level laboratory was founded, as authorized by the Jiangsu province government. All animals were maintained at a constant temperature of 25±2°C under a 12/12 h light/dark cycle. Rats were allowed free access to food and water *ad libitum*. Animal experiments were conducted in accordance to the principles provided by National Institute of Health (NIH) Guideline for the Care and use of Laboratory Animals. The approval to proceed with this experiment was

issued by the Animal Ethics Committee of Xuzhou Medical University which also conforms to the Guidelines for Ethical Conduct in the Care and Use of Animals. All efforts were made to prevent unavoidable pain and distress when the approved endpoint is reached, no animal death occurred during this study. Euthanasia should result in rapid loss of consciousness, followed by respiratory and cardiac arrest and ultimate loss of all brain function. Death was confirmed after euthanasia and prior to disposal of the animal. Each rat was weighed weekly, while the measurement of blood glucose was performed using a glucometer via the tail vein (Nanjing Jianqiao Medical Device Co. Ltd.).

Drugs. *GbE* was used in the current study and is an extract of dried *Ginkgo biloba* leaves. *GbE* was obtained from Shaanxi Huike Botanical Development Co., Ltd. (Purity, >98%; cat. no. HK20121201). For administration *in vivo*, *GbE* was dissolved in 1% CMC-Na at concentrations of 10, 20 and 40 mg/ml. Streptozotocin (STZ; cat. no. 0130) was purchased from Sigma-Aldrich; Merck KGaA. Nimodipine (cat. no. 120554), which was used as a positive control, was purchased from Yabao Pharmaceutical Group Co., Ltd. and suspended in 1% CMC-Na solution.

Diabetic model. The rats that were fasted overnight were subjected to a single intraperitoneal injection of 60 mg/kg STZ that was freshly dissolved in 0.1 mol/l cold citrate buffer at pH 4.3. Age-matched normal rats were injected with an equal volume of citrate buffer alone. Blood glucose was measured a period of one week after STZ injection. Rats with fasting blood glucose of ≥13.88 mmol/l were considered diabetic and included in the present study (30).

Focal cerebral ischemic model and grouping. Normal and diabetic rats were placed in the supine position on a heated pad and had a body temperature of 36.5-37.5°C, which was monitored using a rectal thermometer. After being anesthetized with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg) (33,34), rats were subjected to 30 min of middle cerebral artery (MCA) occlusion followed by 24 h of reperfusion. No signs of pain and peritonitis were observed following administration of 10% chloral hydrate. Sham operation and transient middle cerebral artery occlusion were performed as previously described (35). The right common carotid artery (CCA), external carotid artery, and internal carotid artery (ICA) were isolated. A nylon filament, which was purchased from Beijing Sunbio Biotech Co., Ltd., was subsequently introduced into the CCA lumen and gently advanced to the ICA until a slight resistance was felt.

A total of eighty rats were randomly divided into eight groups: The Con + sham group were nondiabetic and sham-operated rats (Con + sham group; n=10); Con + ischemia-reperfusion (I/R) rats were nondiabetic and received I/R injury (Con + I/R group; n=10); diabetic and sham-operated rats were in the STZ + sham group (STZ + sham group, n=10); diabetic rats treated with ischemia-reperfusion injury were the STZ + I/R group (STZ + I/R group; n=10). These aforementioned groups were administered the same volume of 1% CMC solution for a period of three weeks. Before ischemia-reperfusion injury, the diabetic rats were administrated intragastrically with

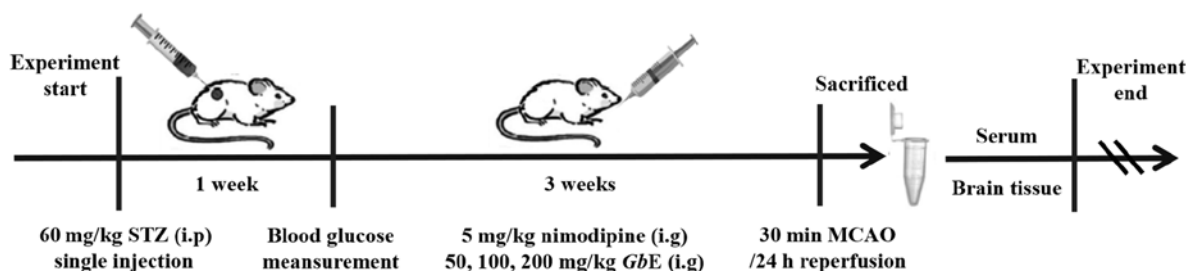


Figure 1. Schematic representation of the experimental protocol used. STZ, streptozotocin; GbE, *Ginkgo biloba* extract; MCAO, middle cerebral artery occlusion.

50, 100 and 200 mg/kg GbE for the GL group (low dose; n=10), the GM group (moderate dose; n=10) and the GH group (high dose; n=10), respectively. Diabetic rats that received nimodipine prior to ischemia-reperfusion injury were the positive control group (Nimo group; n=10; 5 mg/kg/day; intragastrically). All groups were administered with the corresponding agents for three weeks before induction of ischemia. All rats were anesthetized with pentobarbital (45 mg/kg; intraperitoneal injection) and sacrificed using cervical dislocation. Rat brains and blood samples were collected. The experimental protocol is presented in Fig. 1.

Neurological deficit evaluation. Neurological function was evaluated 2 and 24 h after reperfusion by an investigator blinded to the study groups: 0, no deficit; 1, failure to extend right forelimb while the tail was pulled; 2, spontaneous circling or walking to the contralateral side; 3, stumble only when stimulated with a depressed level of consciousness; 4, unresponsive to stimulation.

Infarct volume measurement. 2,3,5-Triphenyltetrazolium chloride (TTC) staining was performed according to previous descriptions (36) for the evaluation of the infarct volume in experimental ischemic stroke. TTC stained the normal cerebral areas deep red without any effect on the infarct tissue, which enables identification of the healthy regions from the infarcted areas. A total of 5 rats from each group were used for infarct volume measurement (n=5 per group). Rats were euthanized and brains were removed immediately. Brain samples were placed in a brain matrix and sliced into 2 mm sections. The slices were incubated in a 2% solution of TTC (cat. no. 129K1867V; Sigma-Aldrich; Merck KGaA) at 37°C for 30 min, then fixed in a 4% buffered paraformaldehyde solution and scanned using a scanner (EPSON Perfection V33). The infarct area and the hemisphere area of each section were traced and quantified using ImageJ software (National Institutes of Health) and expressed as the percent of infarct area in the whole brain.

S100B measurement. The amount of serum S100B protein was detected using a commercially available ELISA kit (cat. no. 1302271; Shanghai Bio-Tech Co., Ltd.), according to the manufacturer's protocol, and expressed as ng/ml.

Measurement of malondialdehyde level. The right striatum (50 mg) was homogenized with 450 μ l 0.9% NaCl and centrifuged at 4°C at 12,000 x g for 15 min. A total of 20 μ l 6 mol/l

NaOH was then added to 100 μ l supernatant in an Eppendorf tube and the sample was incubated in a water bath at 60°C for 30 min. The hydrolyzed sample was acidified with 50 μ l 35% (v/v) perchloric acid. The resulting suspension was then mixed on a vortex for 30 sec and centrifuged at 12,000 x g for 10 min. A total of 200 μ l top clear supernatant was transferred to a 1.5 ml Eppendorf tube. The resultant supernatant was mixed with 20 μ l 2,4-dinitrophenylhydrazine solution (5 mmol/l in 2 mol/l HCl; pH=0.09) and incubated at room temperature for 30 min. After derivatization, samples were filtered through a 0.22 μ m filter. Aliquots of 50 μ l were injected into a HPLC system (37), in which an Agilent Zorbax SB-C18 column (250x4.6 mm; Agilent Technologies, Inc.) was used. The mobile phase was acetonitrile-distilled water (38:62, v/v) containing 0.2% (v/v) acetic acid at a flow rate of 1.0 ml/min, and the wavelength of the UV detector was set at 310 nm. The level of striatum malondialdehyde (MDA) was expressed as μ mol/g protein, and protein concentration was determined using a bicinchoninic acid (BCA) assay.

Measurement of glutathione (GSH) content. The level of GSH was measured as previously described by Liu *et al* (38). The compound 3-carboxy-4-nitrophenyl disulfide can react with sulfhydryl compounds (including GSH) and form a yellow compound with a strong absorption at 420 nm. The measurement of GSH was performed using a commercial kit (cat. no. A006-1; Nanjing Jiancheng Bioengineering Institute), and the level of striatum GSH was expressed as mg/g protein.

Superoxide dismutase (SOD) activity assay. SOD is an important antioxidative enzyme, and, in the current study, its activity was determined according to the method of Sun *et al* (39). This method uses the inhibition of nitroblue tetrazolium reduction by the xanthine and xanthine oxidase system as a superoxide generator. SOD activity was subsequently measured at 550 nm by the degree of inhibition using a commercial kit (cat. no. A001-1; Nanjing Jiancheng Bioengineering Institute). A total of one unit of enzyme was defined as the amount of enzyme required at an inhibitory rate of 50%. The activity of SOD was expressed as units/mg protein.

Western blot analysis. A total of 5 rats from each group were used for western blot analysis (n=5 per group). After weighing the rats, the right hippocampus was dissected and homogenized using a sonicator with six-fold volumes (w/v) of 50 mmol/l Tris buffer (pH=7.4) containing 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 mmol/l Na₃VO₄, 2 mmol/l

DTT and 50 mmol/l NaF, in an ice-cold bath. Samples were left at 4°C for at least 30 min, the homogenates were then centrifuged at 4°C at 10,000 x g for 15 min and the supernatant was collected and denatured in SDS. The protein concentration in the supernatant was determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc.).

The same amount of protein (80 µg) was electrophoresed on 8% SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated overnight at 4°C with primary antibodies, including Akt Rabbit monoclonal antibody (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.), p-Akt (Ser473) Rabbit monoclonal antibody (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), mTOR Rabbit monoclonal antibody (1:1,000; cat. no. 2983; Cell Signaling Technology, Inc.), p-mTOR (Ser2448) Rabbit monoclonal antibody (1:1,000; cat. no. 5536; Cell Signaling Technology, Inc.), GLUT1 Rabbit polyclonal antibody (1:1,000; cat. no. ab106289; Abcam), and β-actin Rabbit polyclonal antibody (1:1,000; cat. no. AP0060; Bioworld Technology, Inc.), respectively. The membranes were washed and incubated with alkaline phosphatase-conjugated IgG (1:10,000; cat. no. E030220-02; EarthOx Life Sciences) at room temperature for 2 h before being exposed to BCIP/NBT alkaline phosphatase color developing reagent (cat. no. C3206; Beyotime Institute of Biotechnology) for 15 min. Western blot density was measured using Image J software (Rawak Software, Inc.) and normalized using β-actin as an internal control.

Statistical analysis. All data in the different experimental groups were expressed as the mean ± SD. Data were analyzed using GraphPad Prism (version 5.0; GraphPad Software, Inc.). A comparison between groups was conducted using one-way ANOVA, followed Tukey's multiple comparisons tests. P<0.05 and P<0.01 were considered to indicate a statistically significant difference.

Results

Effect of GbE on body weights and fasting blood glucose. The body weights of rats were measured after STZ injection (day 0) and on days 7, 14, 21 and 28. As presented in Fig. 2, the original body weights of eight groups were ~200 g. After STZ injection, the body weights of diabetic rats (STZ + sham group and STZ + I/R group) remained unchanged, whereas a marked increase was observed in nondiabetic rats (Con + sham group and Con + I/R group) during the 4 consecutive weeks (P<0.01). GbE (50, 100 and 200 mg/kg/day) was administered intragastrically once per day from day 7 to day 28, and there were no significant differences among the groups at each time point.

Fasting blood glucose levels were measured on days 7 (after a week of STZ injection) and 28 (after 3 weeks of consecutive administration). On days 7 and 28, the fasting blood glucose levels in diabetic rats was significantly increased compared with nondiabetic rats (P<0.01; Table I). No significant differences in fasting blood glucose levels were observed in the three GbE groups and the Nimo group.

Effect of GbE on neurological deficits. Neurological deficits were evaluated 2 and 24 h after reperfusion. Compared with the 2 h time point after reperfusion, I/R injury in nondiabetic

Table I. Effect of GbE on the fasting blood glucose of STZ-induced diabetic rats subjected to 30 min MCAO/24 h reperfusion.

Groups	Fasting blood glucose (mmol/l)	
	Day 7	Days 28
Con + sham	5.75±1.28	4.99±0.49
Con + I/R	5.89±1.48	5.16±0.59
STZ + sham	22.38±2.21 ^a	26.90±3.44 ^a
STZ + I/R	22.51±4.32 ^b	27.63±3.46 ^b
Nimo	19.42±1.87	24.44±6.81
GL	21.03±4.42	26.64±3.90
GM	21.22±4.74	27.85±5.39
GH	21.33±5.41	24.41±5.42

Data are expressed as mean ± SD (n=10 per time point). ^aP<0.01 vs. Con + sham group; ^bP<0.01 vs. Con + I/R group. GbE, *Ginkgo biloba* extract; STZ, streptozotocin; MCAO, middle cerebral artery occlusion; Con, control; I/R, ischemia-reperfusion; Nimo, nimodipine group; GL, GbE low dose group; GM, GbE moderate dose group; GH, GbE high dose group.

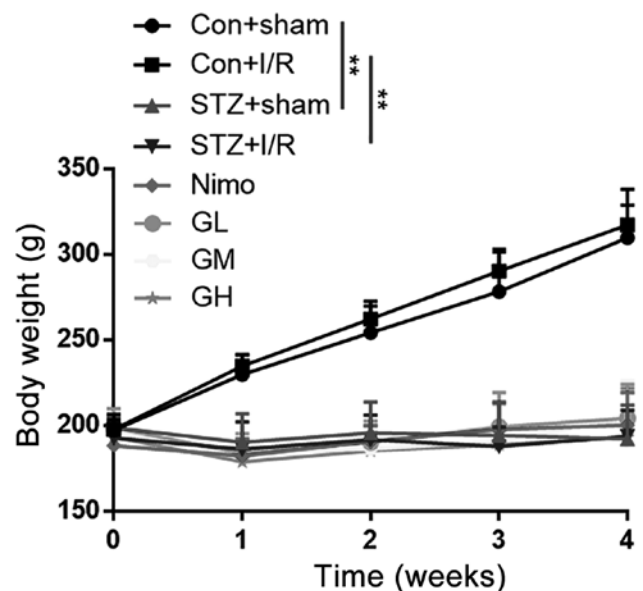


Figure 2. Effect of GbE on the body weight of STZ-induced diabetic rats subjected to 30 min MCAO/24 h reperfusion. Data are expressed as mean ± standard deviation (n=10 per time point). **P<0.01. GbE, *Ginkgo biloba* extract; STZ, streptozotocin; MCAO, middle cerebral artery occlusion; Con, control; I/R, ischemia-reperfusion; Nimo, nimodipine group; GL, GbE low dose group; GM, GbE moderate dose group; GH, GbE high dose group.

rats (Con + I/R group) had severe to mild neurological deficits (P<0.01), whereas I/R injury in diabetic rats (STZ + I/R group) resulted in severe to very severe neurological deficits 24 h after reperfusion (P<0.01), and sham-operated animals did not exhibit any deficits (Table II). The Nimo and three GbE dose groups had significantly improved neurological scores at 24 h of reperfusion (P<0.01; Table II).

Table II. Effect of *GbE* on the behavioral scores of neurological function of rats 2 and 24 h after reperfusion.

Groups	Neurological scores (mean \pm SD)		n	Neurological deficits
	2 h after reperfusion	24 h after reperfusion		
Con + sham	0	0	10	None
Con + I/R	1.92 \pm 0.57	0.30 \pm 0.67 ^a	10	Severe \rightarrow mild
STZ + sham	0	0	10	None
STZ+I/R	2.00 \pm 0.67	2.90 \pm 0.57 ^a	10	Severe \rightarrow very severe
Nimo	2.10 \pm 0.57	1.00 \pm 0.47 ^a	10	Severe \rightarrow mild
GL	2.00 \pm 0.47	1.10 \pm 0.57 ^a	10	Severe \rightarrow mild
GM	2.10 \pm 0.74	0.80 \pm 0.63 ^a	10	Severe \rightarrow mild
GH	2.00 \pm 0.67	0.70 \pm 0.48 ^a	10	Severe \rightarrow mild

Data are expressed as mean \pm standard deviation (n=10 for each group). ^aP<0.01 vs. 2 h time point after reperfusion of each group. *GbE*, *Ginkgo biloba* extract; STZ, streptozotocin; MCAO, middle cerebral artery occlusion; Con, control; I/R, ischemia-reperfusion; Nimo, nimodipine group; GL, *GbE* low dose group; GM, *GbE* moderate dose group; GH, *GbE* high dose group.

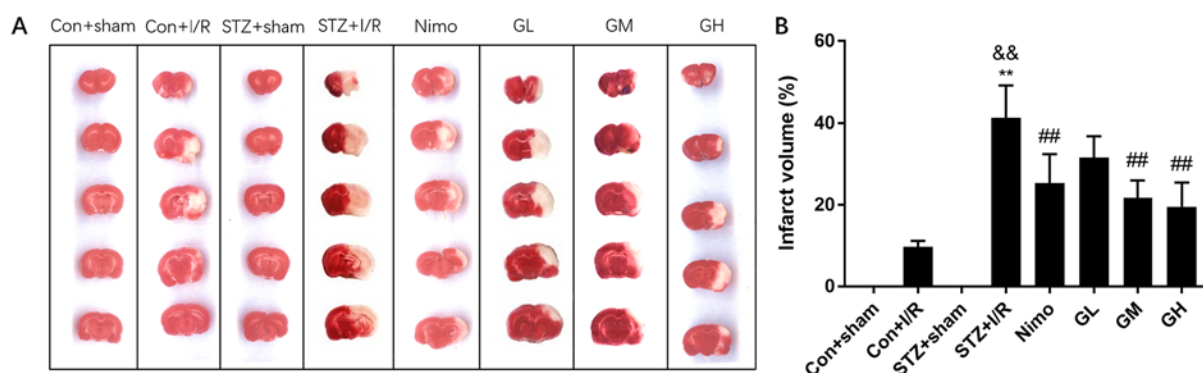


Figure 3. Effect of *GbE* on the infarct volume of STZ-induced diabetic rats subjected to 30 min MCAO/24 h reperfusion. TTC stained brain slices of rats in different groups. (A) The normal brain areas are red and the infarct areas are white. (B) Infarct volume of rats in different groups. Data are expressed as mean \pm standard deviation (n=5). ^{**}P<0.01 vs. STZ + sham group; ^{&&}P<0.01 vs. Con + I/R group; ^{##}P<0.01 vs. STZ + I/R group. *GbE*, *Ginkgo biloba* extract; STZ, streptozotocin; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-Triphenyltetrazolium chloride; Con, control; I/R, ischemia-reperfusion; Nimo, nimodipine group; GL, *GbE* low dose group; GM, *GbE* moderate dose group; GH, *GbE* high dose group.

Effect of *GbE* on cerebral infarct volume. The effects of *GbE* on rat infarct volume were investigated using TTC staining. No lesion was observed in sham-operated groups. Con + I/R rats that were subjected to 30 min MCAO followed by 24 h reperfusion presented smaller infarct volumes of 9.80 \pm 1.48%. The STZ + I/R group exhibited markedly increased infarct volume percentages (41.34 \pm 7.88%) compared with the Con + I/R group (P<0.01; Fig. 3B). Intermediate and high doses of *GbE*, and nimodipine markedly reduced the infarct volume (P<0.01; Fig. 3B). However, the low dose of *GbE* had little effect on it.

Effect of *GbE* on S100B in serum of rats. S100 calcium-binding protein B (S100B), which is a biomarker of traumatic brain injury, is primarily expressed in the central nervous system by astrocytes (40). Ischemia is associated with the increased expression of S100B, which may be released from damaged astrocytes (41). The level of serum S100B is an indicator of brain injury following stroke (42). Therefore, the concentration of S100B was examined using an ELISA to investigate the neuroprotective effect of *GbE*. As presented in Fig. 4,

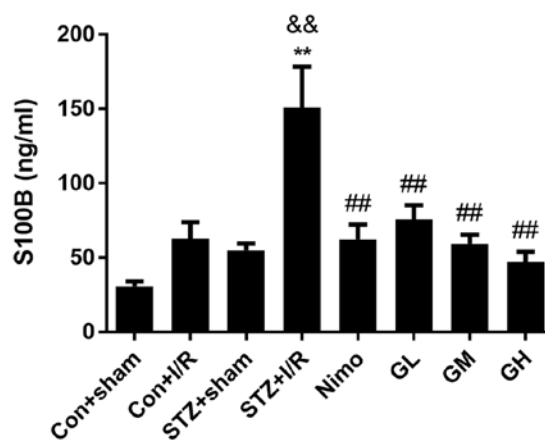


Figure 4. Effect of *GbE* on the content of serum S100B in diabetic rats subjected to 30 min MCAO/24 h reperfusion. Data are expressed as mean \pm standard deviation (n=10). ^{**}P<0.01 vs. STZ + sham group; ^{&&}P<0.01 vs. Con + I/R group. ^{##}P<0.01 vs. STZ + I/R group. *GbE*, *Ginkgo biloba* extract; STZ, streptozotocin; MCAO, middle cerebral artery occlusion; Con, control; I/R, ischemia-reperfusion; Nimo, nimodipine group; GL, *GbE* low dose group; GM, *GbE* moderate dose group; GH, *GbE* high dose group.

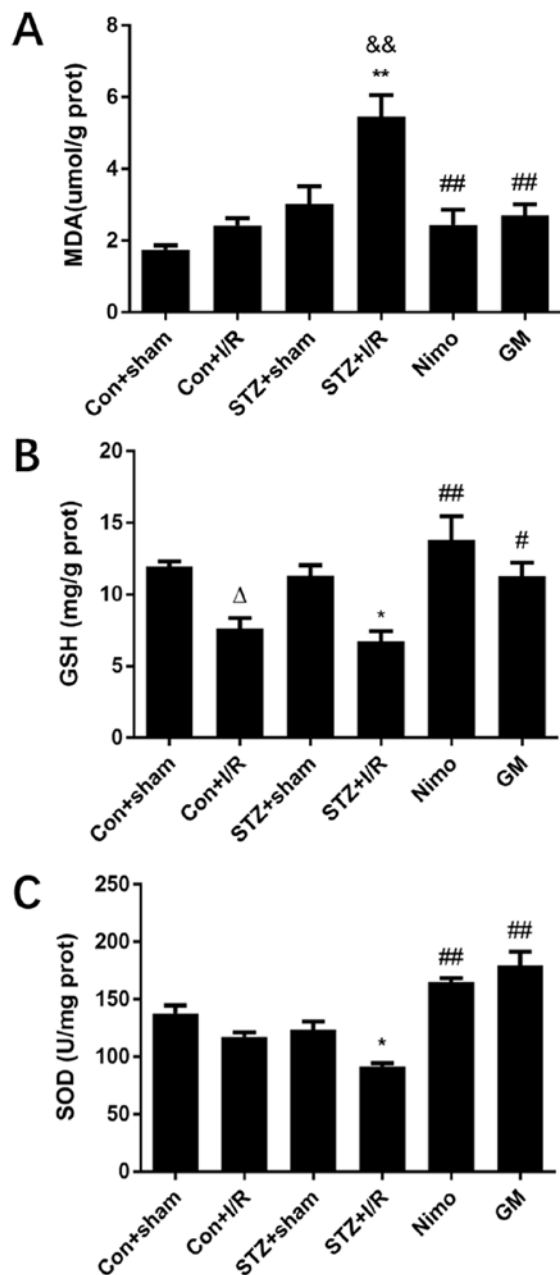


Figure 5. Effect of *GbE* on oxidative stress in the striatum of rats. (A) MDA, (B) GSH and (C) SOD were determined. Data are expressed as mean \pm standard deviation ($n=5$). $^{\Delta}P<0.05$ vs. Con + sham group; $^{*}P<0.05$ or $^{**}P<0.01$ vs. STZ + sham group; $^{\& \&}P<0.01$ vs. Con + I/R group; $^{#}P<0.05$ or $^{##}P<0.01$ vs. STZ + I/R group. *GbE*, *Ginkgo biloba* extract; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; STZ, streptozotocin; Con, control; I/R, ischemia-reperfusion; Nimo, nimodipine group; GM, *GbE* moderate dose group.

in diabetic rats, I/R injury significantly increased the level of serum S100B ($P<0.01$). STZ-induced diabetic increased S100B level ($P<0.01$) in STZ + I/R group compared with the nondiabetic rats with I/R injury (Con + I/R group). However, pretreatment with nimodipine and *GbE* significantly decreased S100B compared with the STZ + I/R group ($P<0.01$).

Increasing doses of *GbE* resulted in a stepped decrease of infarct volume and S100B level. The moderate dose of *GbE* (100 mg/kg, GM) was subsequently used for the following experiments.

Effect of GbE on oxidative stress in the rat striatum. One potential mechanism for diabetes and its complications is oxidative stress (43). To investigate whether the neuroprotective effect of *GbE* was associated with the decrease in oxidative stress levels, three associated molecules were examined, including MDA, GSH and SOD. As presented in Fig. 5, in nondiabetic rats, compared with the Con + sham group, I/R injury significantly decreased GSH level ($P<0.05$). In diabetic rats, I/R injury significantly caused oxidative stress damage, and this was indicated by increased MDA level ($P<0.01$), and decreased GSH ($P<0.05$) and decreased SOD activity ($P<0.05$). Compared with nondiabetic rats with I/R injury (Con + I/R group), STZ-induced diabetic rats exhibited increased MDA level ($P<0.01$). However, *GbE* pretreatment significantly suppressed MDA level ($P<0.01$), and inhibited the decrease of GSH ($P<0.05$) and SOD ($P<0.01$).

Effect of GbE on the quantities of p-Akt and p-mTOR in the hippocampus of rats. It was previously revealed that *GbE* pretreatment improved neurological deficits, reduced the infarct volume and relieved oxidative stress following cerebral I/R injury in diabetic rats. To elucidate the mechanism by which *GbE* ameliorated neuronal damage, western blot analysis was used to identify associated protein expression. The results indicated that in diabetic rats, I/R injury decreased the expression of p-Akt ($P<0.01$; Fig. 6A). Compared with nondiabetic rats with I/R injury (Con + I/R group), STZ-induced diabetic decreased p-Akt/Akt ratio ($P<0.01$) in the STZ + I/R group. However, in the nimodipine and *GbE*-pretreated groups, the ratio of p-Akt/Akt was significantly increased compared with the STZ + I/R group ($P<0.01$; Fig. 6A). Variation in the p-mTOR/mTOR ratio was consistent with the change in the p-Akt/Akt ratio (Fig. 6B).

GbE inhibits ischemia-induced downregulation of GLUT1 in diabetic rats subjected to 30 min MCAO/24 h reperfusion. A recent study has revealed that glutamate uptake exhibits a protective function in hippocampal astrocytes (44). Therefore, in the current study, the hippocampus was collected to elucidate the possible mechanism underlying the neuroprotective effect of *GbE* against injury. The results indicated that whether in nondiabetic or diabetic rats, I/R injury decreased the expression of GLUT1 ($P<0.01$ or $P<0.05$; Fig. 6C). Compared with nondiabetic rats with I/R injury (Con + I/R group), STZ-induced diabetic rats exhibited decreased GLUT1 expression ($P<0.01$) in the STZ + I/R group. However, in the nimodipine and *GbE*-pretreated groups, the expression of GLUT1 was significantly increased compared with the STZ + I/R group ($P<0.01$; Fig. 6C).

Discussion

Ischemic stroke generally occurs in diabetic patients with poor glycemic control (1). It has been well established that patients with hyperglycemia are four to five times more likely to suffer from a stroke compared with patients with normoglycemia, with worse neurologic outcomes (5,45). The present work demonstrated *in vivo* evidence of the protective effect of *GbE* against cerebral ischemic injury in diabetic rats. These results indicated that cerebral injury of 30 min MCAO/24 h

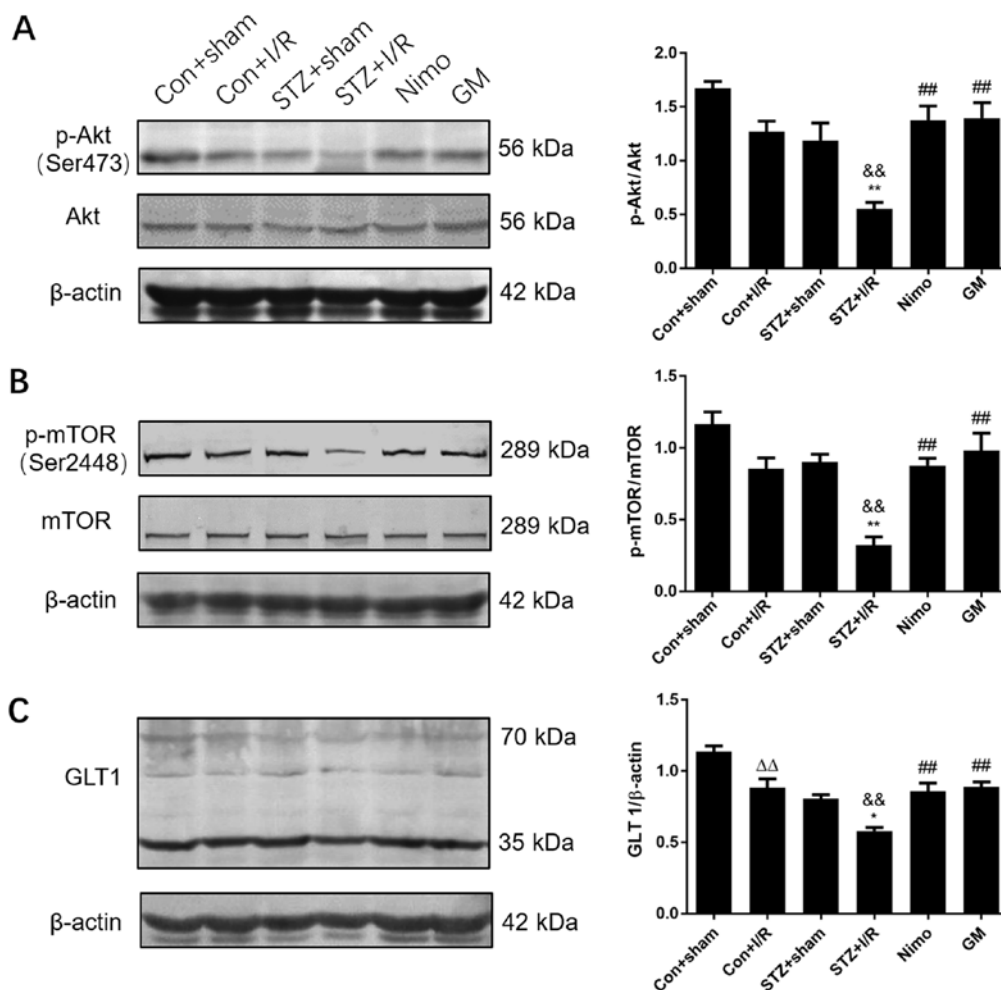


Figure 6. Effect of *GbE* on the protein expression of (A) p-Akt, (B) p-mTOR and (C) GLT1 in the hippocampus of rats. Data are expressed as mean \pm standard deviation (n=5). $\Delta\Delta$ P<0.01 vs. Con + sham group; *P<0.05 or **P<0.01 vs. STZ + sham group; &&P<0.01 vs. Con + I/R group; ##P<0.01 vs. STZ+I/R group. *GbE*, *Ginkgo biloba* extract; GLT1, glutamate transporter 1; STZ, streptozotocin; Con, control; I/R, ischemia-reperfusion; Nimo, nimodipine group; GM, *GbE* moderate dose group.

reperfusion in STZ induced diabetic rats causes more damage compared with nondiabetic rats. However, pretreatment with *GbE* protected against cerebral I/R injury in diabetic rats, which may be associated with the inhibition of oxidative stress, the activation of Akt/mTOR signaling cascade and the upregulation of GLT1 expression.

The current study indicated that a single injection of STZ significantly prevented weight gain and increased fasting blood glucose level compared with normal control rats. However, *GbE* exhibited no effect on body weight or fasting blood glucose level. Furthermore, it was demonstrated that *GbE* exhibited a protective effect on cerebral ischemic injury in diabetic rats, which was assessed by measuring the neurological scores, infarct volume and serum S100B level. The current study revealed that whether in nondiabetic or diabetic rats, deficits in performance due to I/R injury, significantly reduced the neurological deficit following 2 h of reperfusion. However, long term reperfusion ameliorated injury of nondiabetic rats and aggravated diabetic rats, which may be due to high glucose levels preventing the repair of ischemic penumbra and resultant brain tissue damage (46). *GbE* pretreatment exhibited a markedly decreased neurological deficit. The cerebral infarct volume of 24 h following reperfusion was consistent with the

results of neurological deficits. Furthermore, it was revealed that the dose-dependent administration of *GbE* significantly decreased serum S100B level. The mechanism by which *GbE* protects against cerebral ischemic injury in diabetic rats was subsequently assessed.

A large number of ROS are produced during cerebral ischemia and excessively consume the endogenous antioxidative proteins, leading to the changes in the expression and activity of SOD and GSH (47). The decreased activity of antioxidant enzymes reduces the ability of brain tissue to scavenge ROS (48). MDA is the final product of lipid peroxidation and indirectly reflects changes in ROS content (49). The results of the current study indicated that *GbE* enhanced antioxidant enzyme activities and reduced lipid peroxidation.

The hippocampus is an essential brain region which plays roles in memory forming, organizing, and storing. Brain ischemia leads to movement, visual, sensory, and behavioral disorders, especially aphasia and impaired spatial learning (50,51). Neurons in the hippocampal CA1 regions are very sensitive, and they quickly react to brain I/R (52,53). Studies have demonstrated that hippocampal areas of the mouse brain are more vulnerable to neuron death following an ischemic insult (54). Several researchers

chose the hippocampus to study Akt pathway in rat models of ischemic brain damage (54-56). The phosphatidylinositol-3 (PI3) kinase/Akt signal pathway enhances cell survival, proliferation and differentiation (57). It has been demonstrated that ROS regulates the PI3K/Akt pathway, which leads to changes in a number of downstream signaling proteins and induces a variety of pathophysiological responses (58,59). A study performed by Wang *et al.* (60) demonstrated that ROS overproduction, which was induced by high glucose in astrocytes, can lead to decreased cell viability and apoptosis. A number of studies have indicated that oxidative stress-induced neuronal damage and death following cerebral ischemia can be inhibited by activation of the PI3K/Akt/mTOR signaling pathway (16). In the present study, the results demonstrated that I/R injury significantly decreased the ratio of p-Akt/Akt and p-mTOR/mTOR, and this was exacerbated by diabetes. However, *GbE* pretreatment markedly reversed this decreased expression by upregulating the ratio of p-Akt/Akt and p-mTOR/mTOR. This result suggested that *GbE*, which is a free radical scavenger, could attenuate I/R injury in diabetic rats by activating the Akt/mTOR signal cascade.

Glutamate is the most abundant neurotransmitter in the cerebral neural system (61). GLT1, which is also known as EAAT2, is the predominant subtype that performs the majority of glutamate reuptake (19). Extracellular glutamate concentration is mediated primarily by the astrocytic glutamate transporter GLT1. However, disruption of glutamate transporter activity and expression can lead to excitotoxicity and is implicated in ischemic events (20,21). It has been demonstrated that Akt is a key regulator of GLT1 expression (23). Additionally, mTOR, which is a downstream target of the PI3K/Akt pathway, is associated with the regulation of GLT1 (22). Previous reports have demonstrated that GLT1 knock-down exacerbates neuronal damage in ischemia rats (21), whereas GLT1 upregulation reduces cerebral ischemic injury in hyperglycemic rats (34). In the current study, I/R injury was demonstrated to down-regulate GLT1 expression in diabetic rats, and *GbE* resulted in the upregulation of GLT1 expression, suggesting that *GbE* might serve an important role in protecting against I/R injury in diabetic rats via resistance to glutamate excitotoxicity. The results of the present study are supported by a study performed by Mdzinarishvili *et al.* (62) that demonstrated that EGb761 reduces the release of glutamate in the brain of ischemic mice by monitoring extracellular glutamate concentration, however the underlying mechanism for this was not determined.

Previous studies have identified that the neuron-dependent regulation of GLT 1 transcription requires NF- κ B and κ B motif-binding phosphoprotein (23,63). Therefore, the downstream transcription factor(s) require identification in future studies. Additionally, nimodipine, which is a positive control drug used in the present study, is a Ca²⁺ channel blocker. The protective effect of nimodipine is positive, which indicated that the experimental design of the current study is feasible. Nimodipine worked as well as *GbE* in the present study, however nimodipine can lead to hypotension (64), while the incidence of adverse reactions by *GbE* is low. Additionally, *GbE* may also serve a role in lowering blood glucose (65). As an effective adjuvant drug, *GbE* exhibits a glycemic control value in the clinical treatment of patients with T2DM (66).

In conclusion, the results of the current study demonstrated that *GbE* pretreatment ameliorated neurological deficit, reduced infarct volume, decreased S100B level, inhibited oxidative stress, and upregulated the expression of Akt/mTOR and GLT1 in diabetic rats with cerebral ischemia-reperfusion injury. However, further studies using cell models are required to determine the *in vitro* protective effect and to clarify the accurate mechanism of the effect of *GbE* in cerebral ischemia-reperfusion injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ML and SG designed the study. MY, ML, ZS, TM and XM performed the experiments. MY and ML collected and analyzed the experimental data. MY drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and patient consent to participate

All experimental and surgical procedures for animals were strictly performed in accordance with the Guiding Principles for Care and Use of Laboratory Animals of Xuzhou Medical University. The present study was approved by the Ethics Committee of Xuzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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