Original Article
Ganoderma lucidum polysaccharides improves cerebral infarction by regulating AMPK/eNOS signaling

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Abstract: Recent studies have shown that *ganoderma lucidum* polysaccharides (GLP) are characterized by immune regulation and anti-tumor functions. However, little research has been conducted to evaluate their effects on cerebral ischemia. In the current study, we first showed that GLP treatment improved neurological deficits, cerebral infarct volume, and brain edema rate, indicating the protective role of GLP in middle cerebral artery occlusion (MCAO) in rats. Furthermore, GLP treatment can significantly increased the cerebral vascular density in the ischemic area as well as angiogenesis after cerebral ischemia. Moreover, after cerebral ischemia, Nestin positive cells in the subventricular zone (SVZ) area were significantly decreased, while the number of Nestin positive cells was significantly increased after administration of GLP. Western blot analysis showed that increased phosphorylation of Amp-activated protein (AMP) kinase (AMPK) and eNOS after cerebral ischemia, and GLP treatment can further enhanced the phosphorylation of AMPK and eNOS in rat brains. In summary, GLP improved cerebral infarction mainly by enhancing the activation of AMPK and eNOS, thereby prompting angiogenesis and neuron regeneration.

Keywords: *Ganoderma lucidum* polysaccharides, cerebral infarction, AMPK, eNOS

Introduction

Stroke is a the cerebral circulation disorder caused by blockage or rupture of cerebral vessels, which further leads to the damage of brain function or structure [1, 2]. Even after cure, sequele also affects the quality of life for many patients [3, 4]. Therefore, it is particularly important to find the proper drugs to enhance the repair of damaged brain cells.

Increasing evidence has indicated the protective role of Chinese herbal in the treatment of cerebral infarction [5, 6]. Among them, ganoderma lucidum is a kind of tonic medicinal herb in China, which has a long history [7]. Modern pharmacological research shows that *Ganoderma lucidum* polysaccharides (GLP) have many pharmacological effects, such as regulating immunity and possessing anti-tumor, anti radiation and anti-aging characteristics [8, 9]. Currently, the major studies on GLP have focused on their chemical characteristics, immune regulation and the corresponding mechanism [10, 11]. However, little research has been done to explore its effect on cerebral ischemia.

Amp-activated protein kinase (AMPK) is a serine/threonine protein kinase, that modulates the energy metabolism and stress signals in the development of cerebral infarction [12, 13]. It is composed of a catalytic α subunit and two regulatory subunits, β and γ. Different subunits have different functions and various tissue distribution [14]. Over the past decades, researchers have realized that AMPK plays an important role within the metabolism of the central nervous system and the peripheral nervous system [12, 15]. In peripheral tissues, including heart muscle, skeletal muscle, fat, pancreas and liver, the activation of AMPK can meet the needs of energy metabolism for these organs [16]. In addition, AMPK is shown to promote the phosphorylation of eNOS, and increase the activity of NO, thereby enhancing the regeneration of vessels [16-18]. In this study, we investigated the effects of GLP on the repair of ischemic stroke and its effects on the regeneration of nerve and vessels. Here, we first demonstrated that GLP enhanced the activation of AMPK and eNOS, thereby improving recovery from cerebral infarction.

Materials and methods

Drug administration in middle cerebral artery occlusion (MCAO) in rats

In brief, the rats were anesthetized with 10% chloral hydrate at the dose of 0.35 ml/100 g of rats and arterial blood samples obtained via a femoral catheter were collected to measure pO_2, pCO_2 and pH with an AVL 998 Blood Gas Analyzer (Roche Co., Basel, Switzerland). The rectal temperature was maintained at 37°C ± 0.5°C during MCAO via a temperature-regulated heating lamp. A fiber-optic probe was attached to the parietal bone overlying the middle cerebral artery territory 5-mm posterior and 5-mm lateral to the bregma and it was connected to a laser–Doppler flowmeter (PeriFlux System 5000, Stockholm, Sweden) for continuous monitoring of the cerebral blood flow (CBF). A 4-0nylon monofilament suture with a heat-blunted tip was introduced into the internal carotid artery. It was gently advanced for a distance of 18 mm from the common carotid artery bifurcation to block the origin of the middle cerebral artery for 90 min and then withdrawn to allow reperfusion. Only animals that exhibited a reduction in CBF of > 85% during right middle cerebral artery occlusion and a CBF recovery of > 80% after 10 min of reperfusion were included in the study. After the wound had been closed, the animals were allowed to recover from anesthesia before they were returned to their home cages. Sixty adult male Sprague Dawley (SD) rats were randomly divided into sham operation group (S), ischemia reperfusion group (I/R) and I/R+ GLP treated group. The rats were treated with 30 mg/kg GLP, or water (vehicle) by gavage once per day for 90 days followed.

All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Xi'an No.4 Hospital.

Assessment of neurological deficit score and analysis of survival rates

To assess the neurological deficit score, rats were sacrificed 24 h after reperfusion as described previously [19, 20]. Two examiners were kept unaware of the identity of the rat and the treatment protocol. The following neurological deficit scoring (NDS) system was used: 0, no motor deficits (normal); 1, forelimb weakness and torso turning to the ipsilateral side when held by tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, unable to bear weight on the affected side at rest (severe); and 4, no spontaneous locomotor activity or barrel rolling (critical). If no deficit was observed 2 h recovering from anesthesia, the animal was removed from further study.

Edema measurement

The ipsilateral and contralateral hemispheres were dissected and the wet weight of the tissue was determined. The tissues were dried at 120°C for 24 hours. The percent cerebral water was determined as (wet weights-dry weights)/ dry weights × 100.

Measurement of infarct volume

After reperfusion, the rats were deeply anesthetized with 3.5% chloral hydrate and then decapitated, after which the whole brains were rapidly removed. Coronal sections (n = 10 for each group) were cut into 2 mm slices and stained with standard 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37°C followed by overnight immersion in 4% formalin. Infarct volume, expressed as a percentage of whole-brain volume, was measured by an Image-Processing and analysis system (1.25 × objective, Q570IW; Leica, Wetzlar, Germany) and was calculated by integration of the infarct area on each brain section along the rostral caudal axis.

H&E staining

Rats were sacrificed 24 and 72 h after MCAO with an overdose of 3.5% chloral hydrate and transcardially perfused with 0.9% saline solution followed by 4% ice-cold phosphate-buffered paraformaldehyde (PFA). Brains were removed, postfixed overnight, and equilibrated in phosphate-buffered 30% sucrose. Coronal sections at 1.0 to 2.0 mm from the bregma were used for cutting on a cryostate (Leica CM3000, Leica) at a thickness of 25 μm and used for H&E staining.

Western blot analysis

The protein was extracted using RIPA lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1%
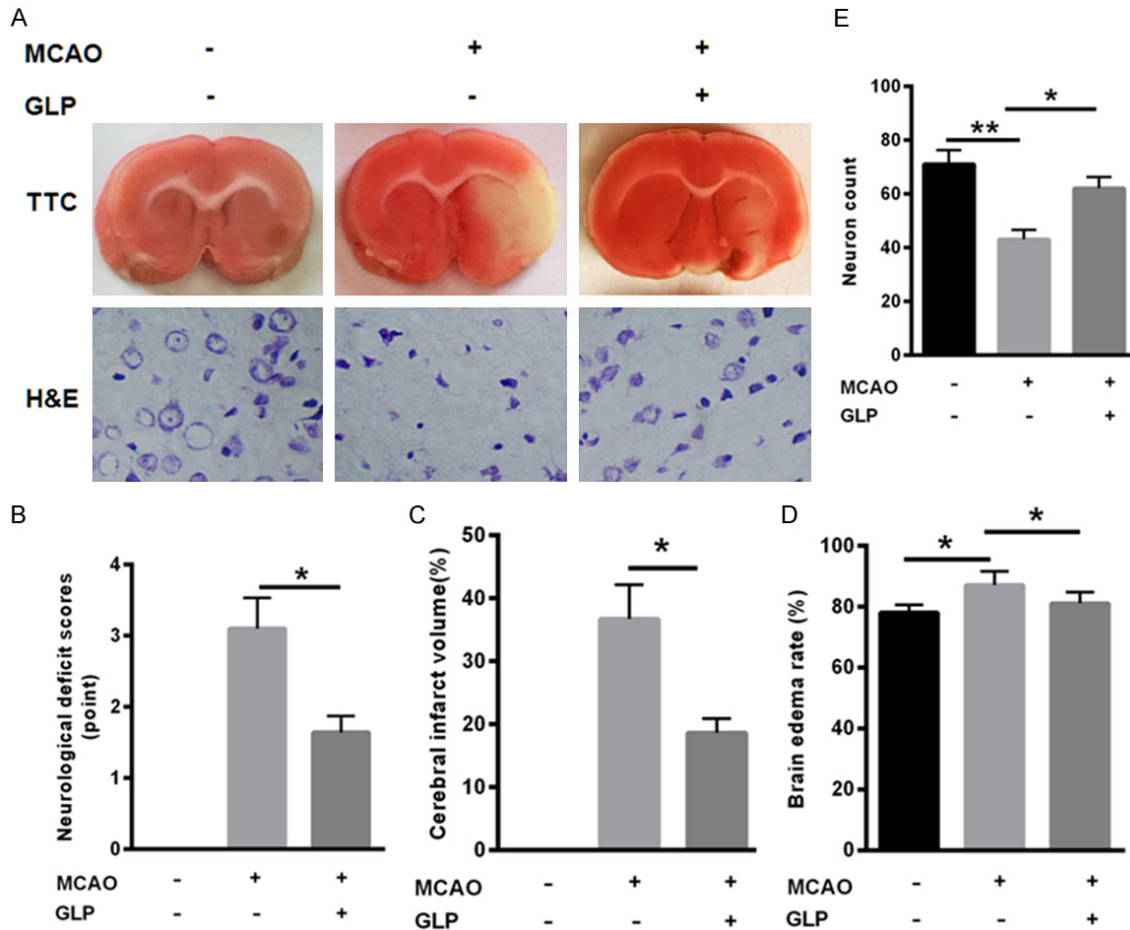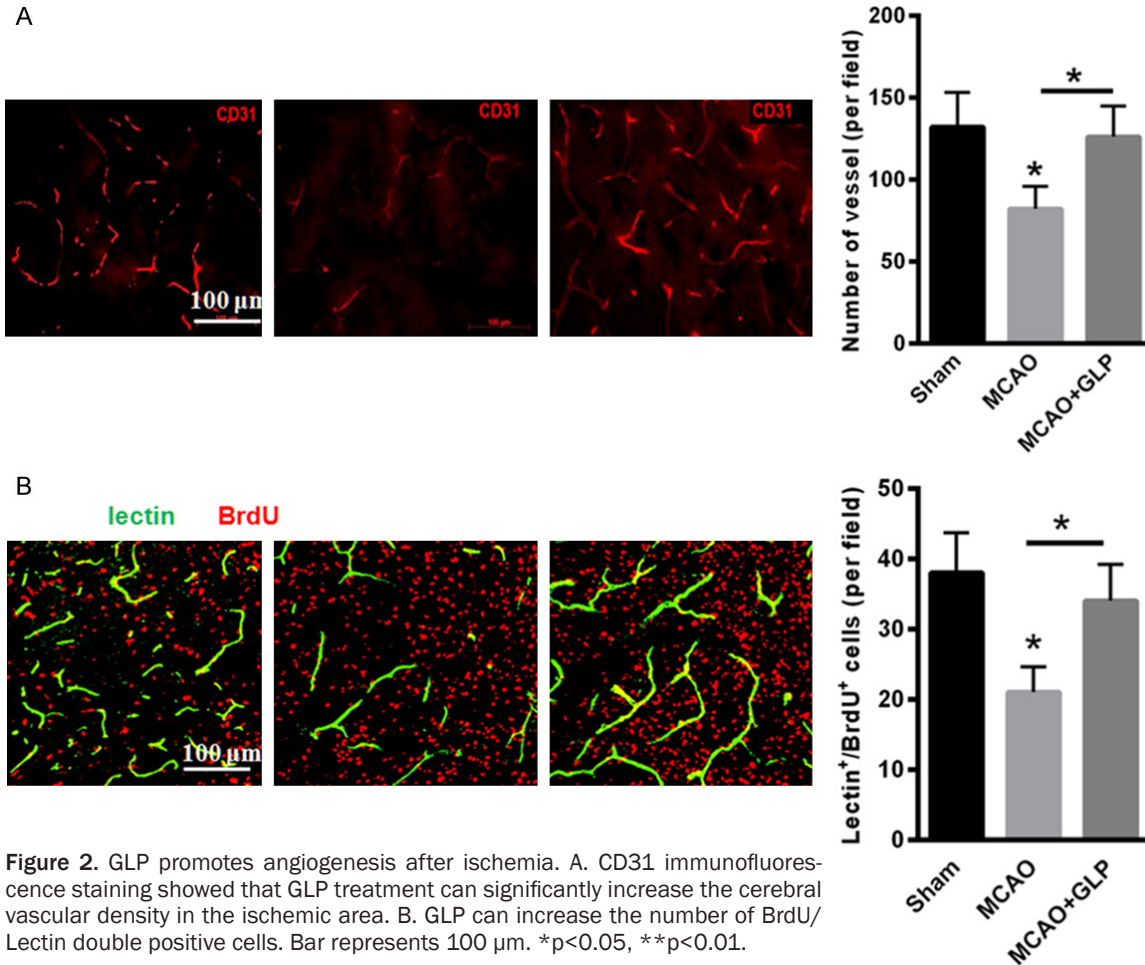
Figure 1. GLP improved cerebral infarction in rats. (A) TTC staining indicated that MCAO obviously induced cerebral infarction. The neurological deficits (B), cerebral infarct volume (C), brain edema rate (D) were markedly induced by MCAO treatment. (E) The neuron count was markedly reduced after MCAO treatment, but the neuron count was enhanced by GLP treatment. *p<0.05, **p<0.01.

Nonidet P40, and 0.1% sodium dodecyl sulfate) and a bicinchoninic acid (BCA) protein assay kit (Pierce) was applied to determine protein concentration. Then, the proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (10% SDS) and transferred to nitrocellulose membrane for 2 hours at 4°C. The blots were incubated overnight at 4°C with antibodies against p-AMPK, AMPK, p-eNOS, eNOS (1:1,000 dilution; CST), followed by incubation for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody (all 1:5,000; Zhongshan Gold Bridge Biological Technology Co., Beijing, China). Proteins were detected by chemiluminescence according to the manufacturer's recommendations (EMD Millipore, Billerica, MA, USA). β-actin was used as an internal control.

Immunostaining

The brain tissues collected were post-fixed with 4% PFA for 24 hr and allowed to settle in a 30% sucrose solution for 72 hr. Then, the brain tissues were sectioned at 30 μm thickness, and were processed for immunostaining. Coronal. After that, the coronal sections were permeabilized and blocked with PBS containing 10% donkey serum and 0.3% Triton X-100 for 45 min at room temperature. Afterward, samples were incubated with primary antibodies at 4°C overnight and then with appropriated fluorescent probe-conjugated secondary antibodies for 1 hr at room temperature. Nuclei were counterstained with DAPI. The slides were scanned using an Olympus FV100i confocal microscope to evaluate neurogenesis and proliferation. The

Figure 2. GLP promotes angiogenesis after ischemia. A. CD31 immunofluorescence staining showed that GLP treatment can significantly increase the cerebral vascular density in the ischemic area. B. GLP can increase the number of BrdU/Lectin double positive cells. Bar represents 100 μm. *p<0.05, **p<0.01.

number of single- or double-stained cells was counted using Image Pro-Plus software 6.0 (Media Cybernetics, Maryland, USA). For BrdU staining, sections were treated in 2 M HCl at 37°C for 20 min and rinsed in 0.1 M borate buffer (pH 8.5) before blocking. For CD31, lectin and Nestin staining (1:50 dilution; Cell Signaling Technology, Inc., Boston, MA, USA), antigens were retrieved with citrate buffer (10 mM [pH 6.5]) for 20 min at 95°C before blocking. Then, the slides were observed under a Zeiss Z1 microscope.

Statistical analysis

Statistically significant differences between groups were calculated using analysis of variance. The results from three different groups were evaluated using t-tests. The results are reported as the mean SEM. *P* values less than 0.05 were considered statistically significant. Data analysis was performed with SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

GLP improved cerebral infarction in MCAO rats

First, TTC staining indicated that MCAO obviously induced cerebral infarction (**Figure 1A**). In the sham-operated group, the cortex tissue remained intact and the neurons remained well-arranged, and the nuclei were centered with clear staining. However, in the MCAO group, a large number of neurons appeared shrunken, swollen, and karyopyknosis and interstitial edema were observed. In addition, neuron arrangement was disordered with loosened and vacuolar neural fibers. However, in the groups pre-treated with GLP, the extent of damage was significantly alleviated, and the number of normal neurons was also markedly increased (**Figure 1A**). Furthermore, neurologi-
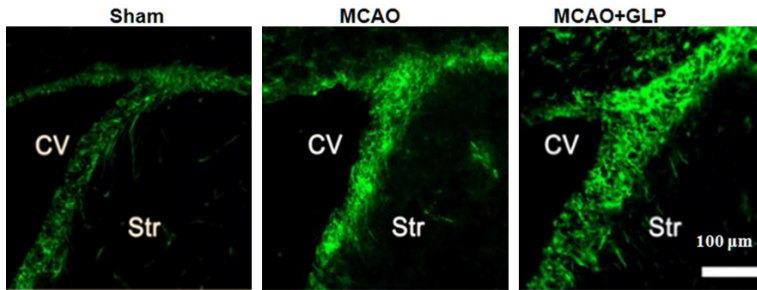
Figure 3. GLP increases the number of Nestin positive cells in subventricular zone (SVZ) region after cerebral ischemia. Bar represents 100 μm. *p<0.05, **p<0.01.
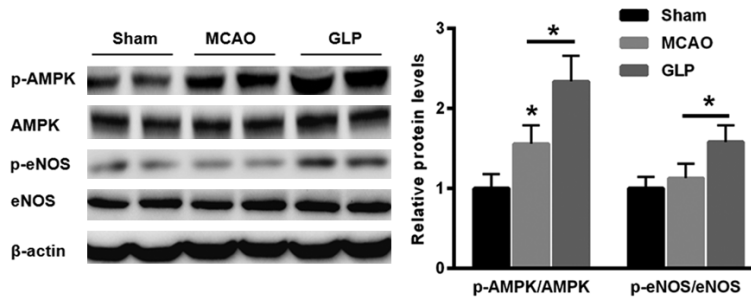

Figure 4. GLP promotes the phosphorylation of AMPK and eNOS after cerebral ischemia. *p<0.05, **p<0.01.

staining method to evaluate the effect of GLP on the angiogenesis effect. Our data showed that GLP can increase the number of BrdU/Lectin double positive cells (**Figure 2B**), showed that GLP promotes angiogenesis after cerebral ischemia.

GLP increases the number of Nestin positive cells in the subventricular zone (SVZ) region after cerebral ischemia

To study the effect of GLP on the nerve regeneration after ischemia, the Nestin positive cells were observed in the SVZ region after ischemia by Nestin staining, which are the markers of neural stem cells. Nestin-positive cells were sparse in the SVZ region of the sham-operated animals, but became more evident seven days after MCAO in the ischemic hemisphere (**Figure 3**), indicating cerebral infarction induced neural stem cell proliferation. GLP group demonstrated more numerous nestin-positive cells in the SVZ region than MCAO group (**Figure 3**), suggesting that GLP enhanced the proliferation of neural stem cells and might be helpful for the functional recovery of brains.

cal deficits, cerebral infarct volume, and brain edema rate were markedly induced by MCAO treatment, but such effects could be explained by improved neurological deficits, cerebral infarct volume, and brain edema rate (**Figure 1B-D**). In addition, the neuron count was markedly reduced after MCAO treatment, but the neuron count was enhanced by GLP treatment (**Figure 1E**). These data indicated the protective role of GLP in cerebral infarction of cerebral infarction rats.

GLP promotes angiogenesis after ischemia

Vascular regeneration after ischemia can promote the perfusion of brain tissue and enhance the repair of ischemic tissue. Therefore, CD31 immunofluorescence staining was applied to determine the density of blood vessels in the ischemic area. Our data showed that GLP treatment can significantly increase the cerebral vascular density in the ischemic area (**Figure 2A**). BrdU, a thymidine analogue incorporated into the DNA of dividing cells, was used to track proliferating cells. Immunostaining with vascular marker lectin showed the changes of vascular density. Thus, we used the BrdU/Lectin

GLP promotes the phosphorylation of AMPK and eNOS after cerebral ischemia

AMPK signaling pathway is suggested to be involved in cerebral ischemic preconditioning through alleviating the severe energy deficiency which is often secondary to ischemic brain injury [21]. Here, we found that AMPK was activated in ischemia, nevertheless conflicting results about its contribution to protection have become apparent. And this matter may indicate stress-mediated AMPK activation after ischemia (**Figure 4**). Interestingly, AMPK and eNOS activation by GLP has also been identified compared with that of MCAO group, indicating it may contribute to the amelioration of some pathologic features induced after stroke.

Discussion

GLP has anti radiation properties and can increase white blood cells, lowering blood sugar and enhance immunity [22, 23]. However, whether it is effective in protecting cerebral infarction has never been explored. In the present study, our results suggest that GLP could enhance the repair of ischemic brain tissue. Our data showed that GLP could not only enhance SVZ nerve regeneration after cerebral ischemia but also promote the angiogenesis after ischemia, thereby reducing the volume atrophy after ischemia.

AMPK activation is usually considered as an adaptive response to stress in ischemia injury, but it is still controversial for the consequences of stress-mediated AMPK activation [21]. During ischemic metabolic stress, AMPK activation is deleterious and its inhibition can lead to neuroprotection [24, 25]. However, other findings demonstrate that AMPK is an endogenous neuroprotective pathway under pathophysiological (stroke) conditions. For instance, overexpression of adiponectin in the brain of stroke mice can promote angiogenesis by activating AMPK [26]. Similarly, statins promote angiogenesis is in part by activating AMPK to enhance the regeneration of vessels, which suggests that the activation of AMPK may promote the angiogenesis after ischemia [27]. Moreover, the protective role of AMPK in peripheral vascular tissue is achieved by eNOS, which is an important enzyme that can promote the regeneration after stroke by activating brain-derived neurotrophic factor (BDNF) [28, 29]. In the chronic recovery phase, activation of AMPK can improve nerve regeneration, angiogenesis and brain function [30]. Thus, it will be important to identify the effects of GLP on the activation of AMPK under the circumstance of stroke. Here, we found that GLP can activate AMPK, and then promote the phosphorylation of eNOS. Thus, we concluded that the effect of GLP in promoting nerve vascular regeneration after ischemia might be through the activation of AMPK and eNOS.

In process of neurological function recovery after cerebral ischemia, it has been found that the subventricular zone (SVZ) and the dentate gyrus (SGZ) are areas of nerve regeneration in the adult mammalian brain [31, 32]. Under normal circumstances, the neurons in these areas migrate to the olfactory bulb and hippocampus. However, in the case of cerebral ischemia and hemorrhage, the nerve regeneration is significantly increased in these regions [32]. It is indicated that post-ischemic neurogenesis plays an essential role in functional recovery of cerebral infarction [33]. And the proliferation, migration, and differentiation of neural stem cells are suggested be amongst the most therapeutically effective strategies following stroke [34]. In the present study, we showed that GLP enhanced Nestin positive cells in SVZ area, indicating enhanced neuron repair after ischemia. In line with nerve regeneration and neuronal plasticity, there is a complex vascular remodeling during the recovery phase of ischemia. Angiogenesis is found in brain tissue after ischemia in both humans and rats. Now, we found that the nerve regeneration is closely linked to angiogenesis [35]. CD31 staining showed that GLP treatment increased the density of blood vessels was increased by GLP treatment in the ischemic area. Altogether, these data showed that GLP could enhance both nerve regeneration and angiogenesis in the ischemic rat brains.

In summary, we found that GLP exerts a protective effect on ischemic stroke. During the recovery period of ischemia, GLP can promote the recovery of nerve function by promoting nerve regeneration and angiogenesis. Furthermore, the phosphorylation of eNOS may be involved in the repair of nerve after ischemia. Thus, activation of AMPK by GLP may be a strategy to reduce stroke.

Disclosure of conflict of interest

None.

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