Combination of Paeoniflorin and Calycosin-7-glucoside Promotes Ischemic Stroke Recovery via the PI3K/AKT Signaling Pathway

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Research

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Abstract

Background

Paeoniflorin (PF) and calycosin-7-glucoside (CG) play a role in protecting against brain damage following cerebral ischemia. However, the mechanism of action of PF in combination with CG (PF + CG) against ischemia/reperfusion injury remains unclear.

Methods

The aim of this study was to investigate the protective role of PF + CG on ischemia/reperfusion injury *in vivo* and *in vitro*, as well as its potential mechanism of action indicating that PF + CG attenuates middle cerebral artery occlusion (MCAO) /oxygen-glucose deprivation reperfusion (OGD/R) injury via the PI3K/AKT pathway. MCAO rat model was prepared by modified suture method, and behavioral scoring, cerebral infarction area, brain tissue water content measurement, using PI3K, p-PI3K, AKT, p-AKT, Bcl-2, Bax, GSK-3 β protein expression as indicators, observe the effect of PI3K / AKT signaling pathway inhibitor LY294002 on the anti-ischemia-reperfusion effect of PF + CG. Oxygen deprivation method was used to prepare the OGD/R model, CCK-8 was used to determine the survival rate of HT22 cells, the contents of SOR, ROS, MDA, and LHD were determined, and apoptosis was detected by flow cytometry and mitochondrial membrane potential, using PI3K, p-PI3K, AKT, p-AKT, Bcl-2, Bax, GSK-3 β protein expression as indicators, observe the effect of PI3K/AKT signaling pathway inhibitor LY294002 on the anti-oxidative and glucose deprivation effect of PF + CG.

Results

The animal studies showed that PF + CG significantly decreased neurobehavioral deficits, cerebral infarct volume, and brain edema; ameliorated histopathological damage in model rats; increased levels of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2; and reduced BAX and GSK-3 β expression. After treatment with PF + CG, the morphology and number of cells in brain tissue were restored to normal, demonstrating a therapeutic effect in cerebral ischemia-reperfusion injury. Results of further studies revealed that, *in vitro*, PF + CG has a therapeutic effect to enhance cell vitality; elevate levels of superoxide dismutase (SOD); reduce levels of reactive oxygen species (ROS), lactate dehydrogenase (LDH), and malondialdehyde (MDA); decrease apoptosis rate; increase levels of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2; and reduce BAX and GSK-3 β expression.

Conclusion

These results demonstrate that PF + CG has a positive therapeutic effect on ischemia/reperfusion and OGD/R injury, and the mechanism is attributed to activation of the PI3K/AKT signaling pathway.

Background

Ischemic stroke (IS) is associated with high morbidity, high mortality, and high disability, which imposes a heavy burden on society and families, and its incidence has gradually increased in recent years ^{1,2}. When blood supply is blocked, brain tissue energy is depleted, which is the most direct cause of brain damage. Ischemia and hypoxia cause changes in the volume of adenosine triphosphate (ATP) in brain tissue ³, and K^+ , Ca^{2+} and Na^+ ions in the cells are dysregulated ^{4,5}. The intracellular pH changes, causing anaerobic metabolism of glucose in the cells to produce lactic acid. Excessive accumulation of lactate reduces lactate dehydrogenase (LDH) activity, causing cells to lose the ability to metabolize lactic acid, leading to cell death and brain damage ⁶.

Paeoniflorin (PF) is extracted from the *Astragalus propinquus*, which can inhibit prothrombin production and platelet aggregation, dilate veins and peripheral small blood vessels, improve blood microcirculation, resist cerebral ischemia-reperfusion injury, and can improve blood-brain barrier permeability ⁷⁻⁹. Calycosin-7-glucoside (CG) is extracted from the root of *Paeonia lactiflora* Schischkin, which can relax blood vessels and stimulate microvessels to improve hemodynamics and protect the integrity of the blood-brain barrier in experimental reperfusion cerebral ischemia ¹⁰. Paeoniflorin (PF) and calycosin-7-glucoside (CG) play a role in protecting against brain damage following cerebral ischemia.

The phosphatidylinositol 3-kinase (PI3K)/protein serine threonine kinase (AKT) pathway is an important signaling pathway in cells ¹¹⁻¹³. Activation of the signaling pathway can inhibit a variety of stimulation-induced apoptosis mechanisms and promote cell cycle progression, thereby promoting cell survival and proliferation while participating in angiogenesis ¹⁴. The current study found that activation of the PI3K/AKT pathway can significantly reduce the area of brain damage and the rate of apoptosis ¹⁵. Activated AKT can also protect rat hippocampal neurons and cortical neurons against hypoxia/reoxygenation-induced apoptosis ¹⁶⁻¹⁸. In this study, we hypothesized that PF + CG may confer protection from brain damage following cerebral ischemia via the PI3K/AKT pathway.

Methods

Chemicals and reagents

Paeoniflorin (Fig. 1A) (Lot: 56874114) and calycosin-7-glucoside (Fig. 1B) (Lot: 56411564) were purchased from Chroma Pharmaceuticals, Inc. LY294002 (Fig. 1C) (Lot: CSN11346-003) was purchased from SCN Pharmaceuticals, Inc. Annexin V-FITC/PI Apoptosis (Cat: 20190421) and JC-10 Apoptosis (Cat: 20181207) detection kits were purchased from Jiangsu Kaiji Biological Technology. SOD assay kit (Cat: 20190416), MDA assay kit (Cat: 20190408), ROS assay kit (Cat: 20190408), and LDH assay kit (Cat: 20190416) were provided by Nanjing Jiancheng Bioengineering Institute. SybrGreen qPCR Mastermix (Cat: DA5641) and qPCR RT Kit (Cat: DC9871) were purchased from DBI Bioscience. Anti-PI3K, anti-AKT,

anti-p-PI3K, anti-p-AKT, anti-BAX, anti-Bcl-2, anti-GSK-3 β , anti- β -actin, and goat anti-rabbit IgG (H + L) HRP were purchased from Affinity Biosciences.

Animal model and experiment groups

Sixty adult male Sprague–Dawley rats (Certificate No. SCXK2016-0002), weighing between 270 and 300 g, were provided by Hunan SJA Laboratory Animal Co., Ltd. All animal experimental procedures were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine according to the guidelines of the European Community and the National Institutes of Health of the USA.

All rats were provided standard rat chow and tap water ad libitum and housed at 25 ± 1 °C. After one week of acclimatization, rats were randomly divided into five groups with 12 rats per group: sham group (sham), model group (MCAO), administration group (PF + CG, 20 mg/kg PF + 40 mg/kg CG), LY294002 group (LY294002, 20 mg/kg LY294002), and administration + LY294002 group (PF + CG + LY294002, 20 mg/kg PF + 40 mg/kg CG + 20 mg/kg LY294002). The sham group and model group were orally administered normal saline at a dose of 1 mL/100 g. The complete experimental time was one week. Rats were given drugs 24 hours after modeling

Preparation of middle cerebral artery occlusion (MCAO) rat model

Replicating the middle cerebral artery occlusion model according to previous methods¹⁹. The rats were anesthetized with 20% urethane (intraperitoneal injection, 1.2 g/kg) and placed on a heating pad set at 37°C to maintain their body temperature throughout the experiment. A midline neck incision was made, and tissue and muscles were separated from the right common carotid artery, external carotid artery, and internal carotid artery. The common and external carotid arteries were tightly sutured for occlusion, and the internal carotid artery was loosely sutured for later ligation. A small incision was made on the common carotid artery 4 mm from the bifurcation, and a nylon fishing line with diameter 0.26 mm was inserted using forceps. The depth was approximately 18 mm from the vascular crotch. After occlusion of the right MCAO for 2 h, the suture was withdrawn to the external carotid artery, and the neck incision was stitched.

Evaluation of Neurologic Deficits

A neurological behavior assessment was made according to previous methods 24 h after reperfusion. By mNSS scoring standards²⁰, mild injury is scored 0 to 6 points, moderate injury 7 to 12 points, and severe injury 13 to 18 points. The mNSS score assessed was 18 points.

Measurement of cerebral infarction rate

Brains were cut into 2 mm sections, stained with 2% TTC and incubated at 37°C for 30 min. They were observed with Image J software, and the calculated volume of cerebral infarction is represented as a ratio of lesion volume to contralateral hemisphere volume. Lesion percent was calculated using the formula:

lesion percent (%) = (ipsilateral ischemic hemisphere volume – contralateral ischemic hemisphere volume) / contralateral ischemic hemisphere volume × 100).

Determination of brain edema

The brain was quickly removed, weighed (wet weight), dehydrated at 105 °C for 24 hours, and then weighed again (dry weight). Percentage of cerebral edema = (1 - dry weight / wet weight) × 100%.

Western blot analysis

Lysis buffer (with protease inhibitor and phosphatase inhibitor) was used to lyse brain cells on ice for 30 min. The supernatant was collected by centrifugation at 10,000 rpm for 10 min at 4 °C. Total protein concentration was measured using the BCA protein assay. Protein (20 µg) was separated by 10% SDS-PAGE gel electrophoresis and electro-transferred to PVDF membranes. The membranes were then blocked by 5% BSA solution and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-PI3K (1:1000 dilution), anti-AKT (1:1000), anti-p-PI3K (1:1000), anti-p-AKT (1:1000), anti-Bcl-2 (1:1000), anti-BAX (1:1000), anti-GSK-3β (1:1000), and anti-β-actin (1:1000). After three washes with TBST, membranes were incubated with biotinylated goat anti-rabbit IgG (H + L) (1:3000) in TBST for 1 h at room temperature. Imaging of each sample by chemiluminescence was analyzed with ImageJ software.

Cell culture and implementation of oxygen-glucose deprivation reperfusion (OGD/R) model

HT22 cells were purchased from Shanghai Kanglang Biological Technology Co., Ltd. The HT22 cell line is a mouse hippocampal neuronal cell line derived from HT4. HT22 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml penicillin, and 100 µg/ml streptomycin.

HT22 cells were passaged at least twice and then plated at 8×10^4 cells/mL in DMEM supplemented with 10% FBS for 24 h. After 24 h culture, HT22 cells were refreshed with glucose-free DMEM medium and placed in an anaerobic chamber fully flushed with 94% N₂ and 5% CO₂ at 37 °C for 8 h. Subsequent reoxygenation was carried out by exposing HT22 cells to normal medium and further incubating at 37 °C under humidified 5% CO₂ for 6 h.

Experiment groups

The cells were divided into five groups: control group (Control), model group (OGD/R), administration group (PF + CG, 40 mg/mL PF + 20 mg/mL CG), inhibitor group (LY294002, 20 mg/mL LY294002), administration + inhibitor group (PF + CG + LY294002, 40 mg/mL PF + 20 mg/mL CG + 20 mg/mL LY294002).

At the beginning of reoxygenation, the OGD/R group were exposed to normal medium; PF + CG, LY294002, and PF + CG + LY294002 groups were exposed to drug-containing medium for 6 h. The control group were cultured in normal medium and incubated for 14 h.

CCK-8, ROS, LDH, MDA, and SOD determination assay

Viability of five groups of cells was evaluated by CCK-8 assay according to previous methods ²¹.

ROS, LDH, MDA, and SOD release were detected using the ROS, LDH, MDA, and SOD assay kits (Jiancheng, Nanjing, China), respectively, according to the manufacturer's instructions to evaluate cell injury.

Apoptosis detection and mitochondrial membrane potential detection

Five groups of cells were digested with EDTA-free trypsin, resuspended in 200 mL staining buffer at a density of 1×10^6 /mL, and then loaded with Annexin V-FITC/PI at 4 °C for 30 min. The apoptotic frequency was detected by flow cytometry with FACSCanto II (Becton Dickinson), and the percentage of apoptotic cells was calculated by Flowjo software.

Mitochondrial membrane potential (MMP) is a prerequisite for maintaining oxidative phosphorylation of mitochondria and producing adenosine triphosphate. The stabilization of MMP is beneficial to maintain the normal physiological function of cells. MMP kit (Kaiji, Jiangsu, China) according to the manufacturer's instruction to evaluate cell injury. Observed under a laser confocal microscope.

Western blot analysis

Lysis buffer (with protease inhibitor and phosphatase inhibitor) was used to lyse HT22 cells on ice for 30 min. The supernatant was collected by centrifugation at 10,000 rpm for 10 min at 4 °C. Total protein concentration was measured using the BCA protein assay. Protein (20 µg) was separated by 10% SDS-PAGE gel electrophoresis and electro-transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked by 5% BSA solution and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-PI3K (1:1000 dilution), anti-AKT (1:1000), anti-p-PI3K (1:1000), anti-p-AKT (1:1000), anti-Bcl-2 (1:1000), anti-BAX (1:1000), anti-GSK-3β (1:1000), and anti-β-actin (1:1000). After three washes with TBST, membranes were incubated with biotinylated goat anti-rabbit IgG (H + L) (1:3000) in TBST for 1 h at room temperature. Imaging of each sample by chemiluminescence was analyzed with ImageJ software.

Statistical analysis

Each group of experiments was repeated three times, and statistical analysis was performed on each group of data using SPSS 19.0 statistical software. Measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm SD$). Significant differences between groups were analyzed by one-way analysis of variance, and pairwise comparisons were performed using LSD test. The test level was $\alpha = 0.05$, and $P < 0.05$ was considered statistically significant.

PF + CG improves behavioral outcomes in MCAO rats

Rats with reperfusion for 24 hours after MCAO were subjected to a seven-day neurological function assessment using mNSS scoring criteria. Evaluations of exercise, sensitivity, reflex ability, and balance beam detection are presented in Fig. 2A. Score results are shown in Figs. 2B and C. On day 1, the mNSS score was significantly higher for the MCAO group than for the Sham group ($P < 0.01$). On day 7, the mNSS score was significantly higher for the MCAO group than for the Sham group ($P < 0.01$). The mNSS score was significantly lower for PF + CG than for MCAO ($P < 0.01$), and the mNSS score for LY294002 was significantly increased ($P < 0.01$). The mNSS score was significantly lower for PF + CG + LY294002 than for LY294002 ($P < 0.01$). Compared with day 1, the mNSS score was significantly lower for PF + CG ($P < 0.01$) and significantly higher for LY294002 ($P < 0.05$) on day 7. These results show that intragastric administration of PF + CG during reperfusion of rat cerebral ischemic injury can significantly improve neurological function, and the administration of LY294002 can significantly aggravate symptoms of nerve injury. Thus, PF + CG has a protective effect on ischemia/reperfusion injury.

In order to further compare the effects of MCAO, PF + CG, LY294002, and PF + CG + LY294002 on the survival of postoperative rats, the number of surviving rats seven days after surgery was counted. Results are expressed in a survival curve, shown in Fig. 2D. The results show that PF + CG not only improved the exercise capacity of rats but also improved their survival rate; LY294002 had a deteriorative effect on exercise capacity and also reduced their survival rate.

PF + CG reduces cerebral infarct volume and brain edema in MCAO rats

Cerebral infarct volumes were evaluated in rat brains by TTC staining after ischemia reperfusion. Representative samples of TTC-stained brain sections are shown in Fig. 3A, with corresponding cerebral infarct volumes and statistical data shown in Fig. 3B. Cerebral infarct volumes were significantly increased in the MCAO and LY294002 groups ($P < 0.01$), whereas PF + CG reduced cerebral infarct volumes induced by MCAO ($P < 0.01$). Compared with MCAO, LY294002 significantly increased cerebral infarct volumes. Compared with LY294002 ($P < 0.01$), PF + CG + LY294002 significantly decreased cerebral infarct volumes ($P < 0.01$).

Figure 3C shows the brain edema results. Brain edema was significantly increased in the MCAO and LY294002 groups ($P < 0.01$), whereas PF + CG reduced brain edema induced by MCAO ($P < 0.01$). Compared with MCAO, LY294002 significantly increased brain edema ($P < 0.01$). Compared with LY294002, PF + CG + LY294002 significantly decreased brain edema ($P < 0.01$).

PF + CG treatment significantly increased levels of PI3K, AKT, p-PI3K, p-AKT and Bcl-2, and reduced BAX and GSK-3 β expression in MCAO rats

To understand the mechanism of action of PF + CG in cerebral protection, the expression of PI3K, AKT, p-PI3K, p-AKT, Bcl-2, BAX, and GSK-3 β in MCAO rat brain tissue was measured using Western blotting

(Fig. 4A-H).

The Western blot results showed that PF + CG treatment significantly increased the expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and reduced the expression of BAX and GSK-3 β in ischemic brains compared with MCAO ($P < 0.01$); LY294002 treatment significantly reduced expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and increased the expression of the BAX and GSK-3 β in ischemic brains compared with MCAO ($P < 0.01$); and PF + CG + LY294002 treatment significantly increased expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and reduced expression of BAX and GSK-3 β in ischemic brains compared with LY294002 ($P < 0.01$).

PF + CG enhances the vitality of OGD/R cells; elevates levels of SOD; and reduces levels of ROS, LDH, and MDA

CCK-8 can easily and accurately determine the activity of cells. Results of this analysis showed that OGD/R injury significantly decreased vitality of HT22 cells compared to the control group ($P < 0.01$). PF + CG enhanced the vitality of HT22 cells compared to the OGD/R group ($P < 0.01$). LY294002 reduced vitality of HT22 cells compared to the OGD/R group ($P < 0.01$). PF + CG + LY294002 enhanced the vitality of HT22 cells compared to LY294002 ($P < 0.05$) (Fig. 5).

SOD, LDH, ROS, and MDA levels were examined in order to assess the protective effect of PF + CG treatment on OGD/R cells. The results presented in Fig. 6 show that OGD/R injury significantly decreased SOD production and increased ROS, LDH, and MDA production compared to the control group ($P < 0.01$). Conversely, PF + CG treatment significantly increased SOD production and decreased ROS, LDH, and MDA production compared to the OGD/R group ($P < 0.01$); LY294002 treatment significantly decreased SOD production and increased ROS, LDH, and MDA production compared to the OGD/R group ($P < 0.01$); and PF + CG + LY294002 significantly increased SOD production and decreased ROS, LDH, and MDA production compared to LY294002 ($P < 0.05$, $P < 0.01$).

PF + CG has an anti-apoptotic effect on OGD/R cells

The apoptosis rate of HT22 cells was measured by flow cytometry (FCM). FCM sets up a four-point gate, as shown in Fig. 7. Q1 represents necrotic cells, Q2 late apoptotic cells, Q3 living cells, and Q4 early apoptotic cells. The results show that OGD/R injury significantly increased the apoptosis rate of cells compared to the Control ($P < 0.01$). Conversely, PF + CG treatment significantly decreased apoptosis compared to the OGD/R group ($P < 0.01$). The OGD/R that received LY294002 treatment had significantly increased apoptosis rates compared to the OGD/R group ($P < 0.01$), and PF + CG + LY294002 treatment significantly increased apoptosis rates compared to the LY294002 group ($P < 0.01$).

The mitochondrial membrane potential of HT22 cells was measured by laser confocal microscopy. As shown in Fig. 8, results show that PF + CG treatment ameliorated apoptosis rates in OGD/R cells, LY294002 treatment aggravated apoptosis rates in OGD/R cells, and PF + CG + LY294002 treatment ameliorated apoptosis rates compared to the LY294002 group.

PF + CG treatment significantly increased levels of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and reduced BAX and GSK-3 β expression in OGD/R cells

To understand the mechanism of action of PF + CG in neuronal protection, the expression of PI3K, AKT, p-PI3K, p-AKT, Bcl-2, BAX, and GSK-3 β in OGD/R cells was measured using Western blotting (Fig. 9A-H).

The Western blotting results showed that PF + CG treatment significantly increased expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and reduced the expression of BAX and GSK-3 β compared with the OGD/R group ($P < 0.01$); LY294002 treatment significantly reduced expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and increased expression of BAX and GSK-3 β compared to the OGD/R group ($P < 0.01$); PF + CG + LY294002 treatment significantly increased expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and reduced expression of BAX and GSK-3 β compared to LY294002 ($P < 0.01$).

Discussion

The pathogenesis of ischemic stroke is brain tissue ischemia, hypoxia, and lack of energy in secondary brain tissue, causing a pathological state of energy metabolism disorder, oxidative stress damage, apoptosis, and other effects²². In the present study, we used an MCAO rat model and OGD/R cell model to determine the effects of PF + CG following acute ischemic stroke through the activation of the PI3K/AKT pathway.

Our results show that neurobehavioral deficits, cerebral infarct volume, brain edema, and histopathological damage were decreased in MCAO rats, and OGD/R cell vitality was increased by PF + CG treatment. Additionally, the treated rats exhibited increased levels of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and reduced BAX and GSK-3 β expression compared to the MCAO and OGD/R groups. These results indicate that PF + CG could be a potential clinical cerebral ischemia/reperfusion injury therapy.

Evaluation of neurological behavioral impairment is important to determine the success of modeling and cerebral ischemia in animal experiments. There are many methods for evaluating cerebral ischemia/reperfusion injury. For example, the Narrow-alley Corner Test is used to evaluate asymmetric motor dysfunction²³, the Sticky Dot Test is used to evaluate fine motion function²⁴, and the Chimney Test is used to evaluate movement coordination²⁵. In our study, treatment with PF + CG improved neurobehavioral deficits in MCAO rats compared with the non-treated MCAO group. Additionally, PF + CG improved cerebral infarct volume and brain edema in MCAO rats compared with the non-treated MCAO group. These observations suggest that PF + CG has a protective effect on MCAO rats.

In oxidative stress, the rate of oxidation in the body exceeds the rate of anti-oxidation²⁶, which eliminates peroxide or high-reactive oxygen molecules in the body, leading to peroxidative damage of organs and tissues²⁷. Oxidative stress plays a key role in neuronal damage following cerebral ischemia. Free radical chain reaction is a core part of brain tissue ischemia-reperfusion pathology, and oxidative stress is one of the pathological mechanisms underlying ischemic brain damage^{28,29}. During cerebral

ischemia/reperfusion, a large number of oxygen free radicals are produced, which aggravates brain damage. SOD is a scavenger of oxygen free radicals, and a large number of oxygen free radicals inhibit the activity of SOD³⁰. During reperfusion, the ROS attack unsaturated fatty acids in the membrane of neuronal cells³¹, which produces a large amount of lipid peroxidation product MDA and destroys the integrity of the cell membrane, increasing permeability and causing apoptosis³². Our results showed that PF + CG elevates levels of SOD and reduces levels of ROS, LDH, and MDA in OGD/R cells.

The PI3K/AKT signaling pathway is a classical anti-apoptotic and pro-survival signal transduction pathway that plays an important role in the regulation of neuronal apoptosis after hypoxic-ischemic brain injury. AKT is an important molecule downstream of PI3K and synergizes with PI3K to promote cell proliferation and inhibit apoptosis^{33,34}. Bcl-2 and BAX are important components of the Bcl-2 family. Increasing Bcl-2 can inhibit apoptosis of nerve cells, while increasing BAX can promote apoptosis of nerve cells³⁵. GSK-3 β is mainly distributed in the nervous system and is a regulator of apoptosis³⁶. It is involved in cell differentiation, proliferation and apoptosis. During cerebral ischemia/reperfusion, a series of extracellular signals (growth factors, regulatory factors) activate PI3K and phosphorylate PI3K. Activated PI3K regulates and activates downstream AKT, and activated AKT and PDK1 combine with phosphorylated AKT to enhance its activity. Activated AKT depolymerizes the phosphorylation of BAD and Bcl-2, which becomes free Bcl-2 and causes anti-neuronal apoptosis. (BAX can bind to Bcl-2 to form Bcl-2-BAX to inhibit neuronal apoptosis, and it can also form BAX-BAX to induce neuronal apoptosis. The ratio of BAX/Bcl-2 represents the degree of neuronal apoptosis.) On the other hand, activated AKT can regulate the important downstream factor GSK-3 β , which can inhibit the activity of GSK-3 β and inhibit apoptosis of nerve cells. The results of our experiment suggest that PF + CG could attenuate ischemia/reperfusion and OGD/R injury.

Although we demonstrated the cerebral/neuronal protective effect of PF + CG in MCAO/R rats and OGD/R cells, we have yet to reveal the underlying mechanisms of action of PF + CG on PI3K/AKT signaling pathways. Thus, we further explored whether PF + CG protects cerebral/neuronal tissue by regulating PI3K/AKT signaling pathways. LY294002 is a protein kinase that specifically inhibits PI3K signaling and inhibits the PI3K/AKT signaling pathway. In our study, LY294002 aggravated neurobehavioral deficits, cerebral infarct volume, and brain edema in MCAO rats compared to the MCAO group; PF + CG + LY294002 improved neurobehavioral deficits, cerebral infarct volume, and brain edema in MCAO rats compared to the LY294002 group. LY294002 reduced the vitality of HT22 cells compared to the OGD/R group. LY294002 reduced cell vitality; decreased SOD production; increased ROS, LDH, and MDA production; and increased cell apoptosis rates compared to the OGD/R group. PF + CG + LY294002 enhanced cell vitality; increased SOD production; decreased ROS, LDH, and MDA production; and decreased cell apoptosis rates compared to the LY294002 group. LY294002 downgraded expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and upregulated expression of BAX and GSK-3 β compared with the MCAO/OGD/R groups. PF + CG + LY294002 upregulated expression of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and downgraded expression of BAX and GSK-3 β compared to the LY294002 group. These results suggest that PF + CG could attenuate ischemia/reperfusion injury via the PI3K/AKT pathway.

In conclusion, PF + CG exhibits protective effects against cerebral ischemia/reperfusion injury in MCAO rats, as indicated by the results of our experiments. The mechanism is attributed to activation of the PI3K/AKT signaling pathway.

Conclusion

In conclusion, our experiments suggest that PF + CG can alleviate ischemia/reperfusion injury in rats and alleviate OGD/R injury in cells. In animal experiments, treatment with PF + CG significantly decreased neurobehavioral deficits, cerebral infarct volume, and brain edema; ameliorated histopathological damage in MCAO rats; and increased levels of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2 and reduced BAX and GSK-3 β expression. In *in vitro* study, PF + CG enhanced cell vitality; elevated levels of SOD; reduced levels of ROS, LDH, and MDA; decreased cell apoptosis rates; increased levels of PI3K, AKT, p-PI3K, p-AKT, and Bcl-2; and reduced BAX and GSK-3 β expression. These results demonstrate that PF + CG has a positive therapeutic effect on ischemia/reperfusion and OGD/R injury, with the mechanism attributed to activation of the PI3K/AKT signaling pathway. These findings may provide a pharmacological foundation for the development of PF + CG as a treatment of IS. PF + CG might be a potential therapeutic agent for ischemic stroke in the clinic.

Abbreviations

Abbreviations	Full name
PI3K	Phosphatidylinositol 3-kinase
AKT	Protein serine threonine kinase
p-PI3K	Phosphorylation phosphatidylinositol 3-kinase
p-AKT	Phosphorylation protein serine threonine kinase
Bcl-2	B-cell lymphoma-2
BAX	Bcl-2-associated X protein
GSK-3 β	Serine/threonine kinase
PF	Paeoniflorin
CG	Calycosin-7-glucoside
NMDP	Nimodipine
MCAO	Middle cerebral artery occlusion
OGD/R	Oxygen-glucose deprivation/reperfusion
HT22	Hippocampal neuron cell line
CCK-8	Cell Counting Kit-8
MDA	Malondialdehyde
ROS	Reactive oxygen species
SOD	Superoxide dismutase
LDH	Lactate dehydrogenase

Declarations

Availability of data and materials

Not applicable.

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Contributions

WL designed the research. WS, YX and HY conducted the experiments. WS and WL wrote the manuscript. YX, HY, ZH, WP, SX, TY, revised the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

The study was established according to the ethical guidelines and approved by the Ethics Committee on Laboratory Animal Management of Guangzhou University of Chinese Medicine.

Consent for publication

We declare that the Publisher has the Author's permission to publish the relevant contribution.

Conflicts of interest

The authors declare no conflict of interest.

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Figures

Figure 1

Chemical structure. (A)PF, (B)CG, (C) LY294002.

Figure 2

mNSS standard score results. (A) Schematic diagram of neurological function,(B)7 days of changes in neurological function scores, (C). Statistical results of neurological function scores on the first and seventh days, (D) MCAO rat survival curve. Displayed value $\bar{x} \pm SD$, $n=3$, survival curve $n=12$, compared with the sham group on day 1, $\blacktriangledown\blacktriangledown p<0.01$; $**p<0.01$ compared with the sham group on day 7; compared with the 7th day MCAO group, $\#p<0.05$, $\#\#p<0.01$; Compared with the first day, $+p<0.05$, $++p<0.01$; compared with the 7th day LY294002 group, $\blacktriangle\blacktriangle p<0.01$.

Figure 3

Effect of PF+CG on cerebral infarct volume and brain edema in rats with MCAO. (A) TTC staining results, (B) Statistical results of cerebral infarction volume, (C) Statistical results of brain edema. Displayed as $\bar{x} \pm SD$, $n=3$, compared with the Sham group, $*p<0.05$, $**p<0.01$; compared with the MCAO group, $\# p<0.05$, $## p<0.01$; compared with the LY294002 group, $+ p<0.05$, $++p<0.01$.

Figure 4

PI3K, AKT, p-PI3K, p-AKT, Bcl-2, BAX and GSK-3β proteins expression in MCAO rats. (A) Expressions of PI3K, AKT, p-PI3K, p-AKT, Bcl-2, BAX and GSK-3β proteins were measured with Western blot, (B) Protein expressions of PI3K, (C) Protein expressions of AKT, (D) Protein expressions of p-PI3K, (E) Protein expressions of p-AKT, (F) Protein expressions of Bcl-2, (G) Protein expressions of BAX, (H) Protein expressions of GSK-3β. Displayed as $\bar{x} \pm SD$, $n=3$, compared with the Sham group, * $p<0.05$, ** $p<0.01$; compared with the MCAO group, # $p<0.05$, ## $p<0.01$; compared with the LY294002 group+ $p<0.05$, ++ $p<0.01$.

Figure 5

Effect of PF+CG on the viability of OGD/R cells. (A)CCK-8 measurement results, (B)Cell morphology, A~E: Control, OGD/R, PG+CG, LY294002, PF+CG+LY294002 (×300).Displayed as $\bar{x} \pm SD$, n=3, compared with the Sham group, *p<0.05, **p<0.01; compared with the MCAO group,# p<0.05, ## p<0.01; compared with the LY294002 group+ p<0.05, ++P <0.01.

Figure 6

Effect of PF+CG treatment on oxidative stress. (A)SOD, (B)MDA, (C)LDH, (D)ROS, (E)ROS cell fluorescence, A~E: Control, OGD/R, PG+CG, LY294002, PF+CG+LY294002 (×300).Displayed as $\bar{x} \pm SD$, n=3, compared with the Sham group, *p<0.05, **p<0.01; compared with the MCAO group,# p<0.05, ## p<0.01; compared with the LY294002 group+ p<0.05, ++P <0.01.

A

B

Figure 7

Effect of PF+CG treatment on apoptosis. (A)Flow cytometry results, (B)Apoptotic fluorescence(x20).Displayed as $\bar{x} \pm SD$, n=3, compared with the Sham group, *p<0.05, **p<0.01; compared with the MCAO group,# p<0.05, ## p<0.01; compared with the LY294002 group+ p<0.05, ++P <0.01.

Figure 8

Effect of PF+CG treatment on mitochondrial membrane potential($\times 20$)

Figure 9

PI3K, AKT, p-PI3K, p- AKT, Bcl-2, BAX and GSK-3 β proteins expression in OGD/R cells.(A)Expressions of PI3K, AKT, p-PI3K, p- AKT, Bcl-2, BAX and GSK-3 β proteins were measured with Western blot, (B)Protein expressions of PI3K, (C)Protein expressions of AKT,(D)Protein expressions ofp-PI3K,(E)Protein expressions ofp- AKT,(F)Protein expressions ofBcl-2,(G)Protein expressions ofBAX,(H)Protein expressions ofGSK-3 β .Displayed as $\bar{x} \pm SD$, n=3, compared with the Sham group, *p<0.05, **p<0.01; compared with the MCAO group,# p<0.05, ## p<0.01; compared with the LY294002 group+ p<0.05, ++P <0.01.