

Total flavonoids in *Premna fulva* Craib alleviates brain neurological impairment and influences Nrf2 and HO-1 expressions in rats with ischemia-reperfusion

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ABSTRACT

Total flavonoids in *Premna fulva* Craib (TFPFC) are a kind of flavonoid compound synthesized via photo-synthesis extracted from *Premna fulva* Craib, which possess a strong anti-oxidative effect. Cerebral Ischemia-Reperfusion refers to the body's damage mainly caused by oxidative stress. This study aims to investigate the alleviating effect of TFPFC on brain neurological impairment and its influences on Nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1) expressions in rats with Ischemia-Reperfusion. The rat model of Ischemia-Reperfusion was established, and rats were treated with TFPFC or normal saline. At 24 h after reperfusion, the neurological score, volume of cerebral infarction and cerebral water content were analyzed in different groups. The influences of TFPFC treatment on the proliferative activity and apoptosis of oxygen and glucose deprivation/reoxygenation (OGD/R) neural stem cells were detected via methyl thiazolyl tetrazolium (MTT) assay and flow cytometry. Moreover, the malondialdehyde (MDA) level and superoxide dismutase (SOD) activity were measured to evaluate the oxidative stress effect. The influences of TFPFC treatment on the protein and messenger ribonucleic acid (mRNA) expressions of Nrf2 and HO-1 were analyzed using reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The TFPFC treatment alleviated the neurological impairment in rats after Ischemia-Reperfusion and reduced the volume of cerebral infarction and cerebral edema status in rats with Ischemia-Reperfusion. TFPFC increased the proliferative activity of OGD/R neural stem cells and decreased damage and apoptosis. In addition, the TFPFC treatment reduced the MDA level, improved the SOD activity, and up-regulated the protein and mRNA expressions of Nrf2 and HO-1. The TFPFC treatment may improve oxidative damage and protect the nervous system through the up-regulation of expressions of transcription factors Nrf2 and HO-1.

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Introduction

Stroke is one of the important causes of human death and disability in the world. Currently, there is still a lack of an effective therapeutic method for cerebral ischemia-induced stroke, and its pathophysiological mechanism remains unclear (1). A large number of studies have demonstrated that stroke is associated with cerebral ischemia (2, 3). The accumulation of free radicals and reactive oxygen species produced in cerebral ischemia can activate different signaling pathways and lead to damage. Oxidative stress is the major cause of neuronal damage in ischemic stroke (4). Oxidative stress injury plays an important role in the pathogenesis of cerebral Ischemia-Reperfusion (I/R). Therefore, studying the mechanism of an oxidative stress injury in cerebral I/R is of great significance.

Premna fulva Craib is a kind of authentic Chinese herbal medicine from the Guangxi region, whose active ingredient is flavonoids (5). The efficacy of total flavonoids in *Premna fulva* Craib (TFPFC) is mainly manifested in anti-oxidative stress. Its mechanism of action is as follows: Flavonols and metal in TFPFC form the chelate compound, and flavonols bind to free radicals to block the oxidative damage caused by free radical chain reaction (6).

Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that regulates various reactive oxygen species and antioxidant gene expression (7). The activation of Nrf2 can synergistically up-regulate the expressions of enzymes with an anti-oxidative stress effect, such as heme oxygenase 1 (HO-1) and superoxide dismutase 1 (SOD1) (8). HO-1 is a kind of ubiquitous inducible stress protein sensitive to redox, which is preferentially expressed in

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various anti-oxidative stress enzymes regulated by Nrf2. HO-1 exerts an effective indirect antioxidant function mainly through degrading heme into iron, biliverdin and CO (9).

Based on this, the influences of TFPFC on neurological impairment and expressions of Nrf2 and HO-1 in rats with Ischemia-Reperfusion (I/R) were studied in view of the anti-oxidative stress effect of TFPFC so as to clarify the effect of TFPFC on neurological impairment caused by cerebral I/R.

Materials and Methods

Main reagents and equipment

Premna fulva Craib was purchased from Shanghai Yansheng Biochemical Reagent Co., Ltd. Dulbecco's modified Eagle medium (DMEM)/F12 medium was purchased from Hyclon. B27 was purchased from Enge Biotech. Recombinant human epidermal growth factor was bought from Beijing T&L Biological Technology Co., Ltd. 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma, Shanghai. Neurobasal® medium was purchased from Thermo Fisher Scientific. Malondialdehyde (MDA) and SOD detection kits were bought from Nanjing Jiancheng Bioengineering Institute. Anti-Nrf2, anti-HO-1 and anti-β-actin antibodies were purchased from Shanghai Anyan Biology Co., Ltd.

Establishment of the rat model of cerebral I/R

A total of 80 male adult Sprague-Dawley (SD) rats (250 g) were purchased from XXX Animal Center, 60 of which were randomly selected and divided into 3 groups to study the I/R, including the Control group (n=20, sham-operation group), I/R group (n=20, I/R group) and I/R+TFPFC group (n=20, I/R+TFPFC treatment group, rats were treated with 20 mg/kg TFPFC). The remaining 20 rats were used for the establishment of the rat model of oxygen and glucose deprivation/reoxygenation (OGD/R) *in vitro*.

The model of cerebral ischemia was established via the focal cerebral ischemia induced by middle cerebral artery occlusion as follows: Rats were intraperitoneally anesthetized with chloral hydrate. Then the nylon monofilament wrapped with 0.01% polylysine was inserted into the right internal carotid artery through the external carotid artery and pushed forward at the carotid bifurcation for 18-20 mm until there was slight resistance. After 2 h, the nylon monofilament was removed, and rats were placed back into the cage and closely monitored for recovery from anesthesia.

Establishment of the rat model of OGD/R *in vitro*

The above 20 SD rats had free access to food for 1 week under the 12/12 h light/dark cycle and soaked in 75% ethanol. Then the rats were executed via decapitation, and the cerebral cortex was dissected. The meninges were washed with DMEM/F12 medium and digested with 0.125% trypsin at 37°C for 15 min. The cell density was adjusted to 1×10^6 /mL, and cells were transferred into the Neurobasal® medium containing 2% B27.

Establishment of OGD/R model: OGD/R of cortical neural stem cells was realized mainly according to the method of Chen et al. to simulate cerebral artery occlusion and reperfusion injury⁽¹⁰⁾. After neural stem cells were washed twice with phosphate-buffered saline (PBS), they

were incubated in glucose-free D-Hanks basal medium with 95% N₂ and 5% CO₂ for 150 min to realize cell hypoxia. Then neural stem cells were taken, cultured in the Neurobasal® medium and incubated in an incubator with 5% CO₂ at 37°C for another 24 h to produce reperfusion injury.

Neurological impairment score

At 24 h after reperfusion, the neurological impairment was scored by reviewers who had no idea about the grouping. The criteria are as follows: 0 points (no neurological impairment), 1 point (the left paw of rats cannot be fully stretched), 2 points (the rats rotate in one direction to the left), 3 points (the rats lean in one direction to the left), and 4 points (the rats cannot walk by themselves and lose consciousness).

Determination of the volume of cerebral infarction

After reperfusion, brain tissues were quickly removed from rats and cryopreserved. The brain tissues were prepared into 2 mm-thick coronal sections, stained with 2% TTC at room temperature for 0.5 h and soaked in 10% paraformaldehyde. The infarction volume of stained brain sections was quantitatively analyzed using the Image Pro-Plus 6.0 software.

Determination of cerebral water content

The cerebral water content was measured using the standard dry-wet weight method. The rats were anesthetized, and the brain was quickly removed at 24 h after reperfusion. Coronal brain sections (about 5 mm thick) at 3 mm away from the forehead were cut and divided into ipsilateral and contralateral hemispheres. The wet weight of brain samples was immediately measured, and then samples were dried in an oven at 110°C to measure the dry weight. Percentage of cerebral water content = (wet weight - dry weight)/wet weight × 100%.

Determination of MDA level

The MDA level was measured using the MDA kit. At 24 h after reperfusion, the rats were executed, the brain was quickly removed, and the right cortex sample was weighed. The content of lipid peroxide was measured using the thiobarbituric acid method to represent the MDA level.

Determination of SOD activity

At 24 h after reperfusion, the rats were executed, the brain was quickly removed, and the right cortex sample was weighed (n=3 in each group). The SOD activity was measured using a commercial kit: The superoxide radicals are produced by xanthine and xanthine oxidase and form the red formazan dye through the reaction with p-iodonitrotetrazolium. The optical density (OD) value was detected at 550 nm via spectrometry to reflect the SOD activity and indirectly reflect the oxygen radical-scavenging capacity.

Analysis of Nrf2 and HO-1 protein expressions via Western blotting

The brain tissues were taken from experimental rats, homogenized in the pre-cooled lysis buffer for 20 min and centrifuged, and the supernatant was collected. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

Table 1. Primers and their sequences in PCR.

Primer	Sequence
Nrf2 F:	5'-CCCAGCACATCCAGACAGAC-3'
Nrf2 R:	5'-TATCCAGGGCAAGCGACTC-3'
HO-1 F:	5'-GCTCTATCGTGCTCGCATGA-3'
HO-1 R:	5'-AATCCCACTGCCACGGTC-3'
β -actin F:	5'-TCAGGTCATCACTATCGGCAAT-3'
β -actin R:	5'-AAAGAAAGGGTGTAACGCA-3'

PAGE) was performed for the supernatant protein, and the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was sealed with 0.1% bovine serum albumin and reacted with the primary antibody at 4°C overnight. Then the secondary antibody was added for reaction, and the image was developed using the ECL enhancer. The relative expression levels of proteins were calculated via gray scale analysis.

Analysis of Nrf2 and HO-1 mRNA levels via RT-PCR

The total RNA was extracted from the right cortex using the TRIzol method. After PCR amplification and electrophoretic separation on agarose gel, the image of the band was developed using the gel imaging system SYSTEM GelDoc XR+, and the relative expression level of mRNA was calculated via gray scale analysis. Primers are shown in Table 1.

Determination of neural stem cell viability via MTT assay

The influence of TFPFC treatment on neural stem cell viability was determined via MTT assay. The experimental cells were divided into 3 groups: the Control group (neural stem cells cultured under normal conditions), OGD/R group (glucose-free hypoxia reperfusion) and OGD/R+TFPFC group (TFPFC treatment after hypoxia reperfusion). 1×10^6 neural stem cells were inoculated into a 96-well plate and treated with different methods in the 3 groups. MTT solution was added to each well, followed by incubation at 37°C for 24, 48 and 72 h. Finally, the medium was discarded, 200 μ L DMSO was added and the OD value was read at 570 nm using a microplate reader.

Analysis of neural stem cell apoptosis via flow cytometry

The influence of TFPFC treatment on neural stem cell apoptosis was determined via flow cytometry. The experimental cells were divided into 3 groups: Control group (neural stem cells cultured under normal conditions), OGD/R group (glucose-free hypoxia reperfusion) and OGD/R+TFPFC group (TFPFC treatment after hypoxia reperfusion). The cells cultured under different conditions were collected, fully digested and washed with PBS. After centrifugation, cells were resuspended with 0.5 μ L solution containing propidium iodide and RNase, and apoptosis was detected via flow cytometry.

Statistical analysis

The research data were statistically analyzed using GraphPad Prism 6.0 and Statistical Product and Service Solutions (SPSS) 18.0, and expressed as mean \pm standard deviation. Analysis of variance was used for the intergroup comparison of differences, and $p < 0.05$ suggested that the

difference was statistically significant.

Results

Effects of TFPFC treatment on neurological impairment, cerebral infarction volume and cerebral water content in rats after I/R

The neurological impairment was scored by experts who had no idea about the grouping using the 5-point scale. Results revealed that the neurological impairment score was significantly increased in I/R group [(2.95 \pm 0.45) points] compared with that in the Control group (0 points) ($p < 0.05$), while it was significantly decreased in the I/R+TFPFC group [(1.15 \pm 0.35) points] compared with that in I/R group ($p < 0.05$), indicating that TFPFC treatment alleviates the neurological impairment in rats after I/R.

The cerebral infarction volume is an important index indirectly reflecting the nerve injury. Results showed that there was no cerebral infarction in the Control group. The cerebral infarction volume was significantly increased in the I/R group [(36.75 \pm 3.87)%] compared with that in the Control group ($p < 0.05$), while it was significantly decreased in the I/R+TFPFC group [(22.31 \pm 2.11)%] compared with that in I/R group ($p < 0.05$), suggesting that TFPFC treatment reduces the cerebral infarction volume in rats after I/R.

In addition, the influence of I/R on cerebral edema in rats was also explored in this study. Results manifested that the percentage of cerebral water content was (76.14 \pm 2.75)% in the Control group, (86.04 \pm 3.22)% in the I/R group and (78.32 \pm 3.22)% in the I/R+TFPFC group, respectively. It can be seen that the cerebral water content in the I/R group was obviously increased compared with that in the Control group ($p < 0.05$), while it was obviously decreased in the I/R+TFPFC group compared with that in the I/R group ($p < 0.05$), indicating that TFPFC treatment significantly reduces the cerebral edema in rats after I/R. (Figure 1)

Influence of TFPFC treatment on proliferation of OGD/R neural stem cells in vitro

The influence of TFPFC treatment on the proliferation of neural stem cells in rats after I/R was investigated using

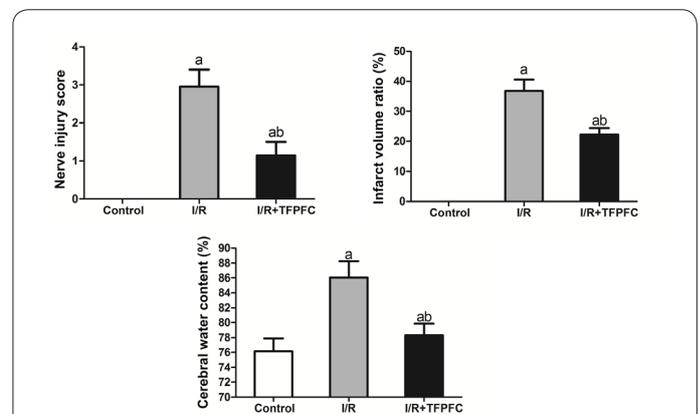


Figure 1. Influences of TFPFC treatment on neurological impairment, cerebral infarction volume and cerebral water content in rats after I/R. a. A significant difference in the I/R group compared with that in the Control group ($p < 0.05$), ab. a significant difference in the I/R+TFPFC group compared with that in the I/R group ($p < 0.05$, $n = 10$).

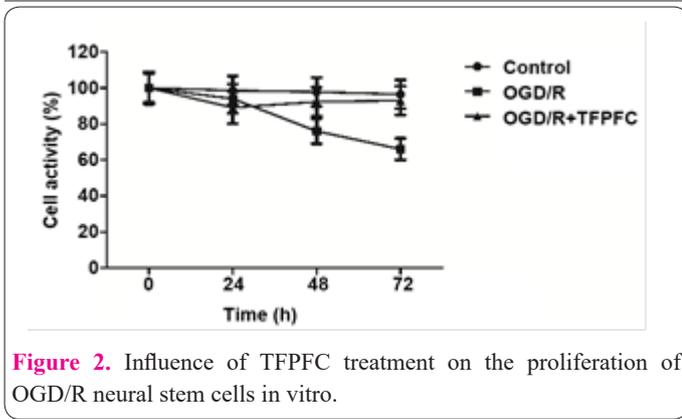


Figure 2. Influence of TFPFC treatment on the proliferation of OGD/R neural stem cells in vitro.

the in vitro model. First, the influence of TFPFC pretreatment on the proliferative activity of OGD/R neural stem cells in vitro was determined via MTT assay. Results manifested that the proliferative activity obviously declined in the OGD/R group compared with that in the Control group ($p < 0.05$), while it was obviously increased in the OGD/R+TFPFC group compared with that in the OGD/R group ($p < 0.05$), suggesting that TFPFC increases the proliferative activity of OGD/R neural stem cells and reduces the damage of neural stem cells induced by OGD/R. (Figure 2)

Impact of TFPFC treatment on apoptosis of OGD/R neural stem cells in vitro

To further verify the influence of TFPFC on the apoptosis of OGD/R neural stem cells in vitro, the apoptosis of neural stem cells in each group was detected via flow cytometry. As shown in figure 3, the apoptotic proportion was $(3.42 \pm 0.32)\%$ in the Control group, $(18.6 \pm 1.45)\%$ in the OGD/R group and $(10.2 \pm 1.04)\%$ in the OGD/R+TFPFC group, respectively. It can be seen that the apoptotic proportion of neural stem cells was remarkably increased in the OGD/R group compared with that in the Control group ($p < 0.05$), while it was remarkably decreased in the OGD/R+TFPFC group compared with that in the OGD/R group ($p < 0.05$), suggesting that TFPFC reduces the apoptosis of OGD/R neural stem cells in vitro.

Influences of TFPFC treatment on MDA level and SOD activity after I/R in rats

The MDA level and SOD activity were detected using the thiobarbituric acid method and Fridovich's method. As shown in Figure 4, the MDA level was significantly higher in I/R group than that in Control group ($p < 0.05$), while it significantly declined in I/R+TFPFC group compared with that in I/R group ($p < 0.05$), proving that the MDA level is increased after I/R injury in rats, and TFPFC treatment can effectively alleviate the overexpression of MDA level induced by I/R injury.

Contrary to MDA, the SOD activity was significantly lower in the I/R group than that in the Control group ($p < 0.05$), while it was significantly increased in the I/R+TFPFC group compared with that in the I/R group ($p < 0.05$), proving that the SOD activity declines after I/R injury in rats, and TFPFC treatment can effectively improve the decreased SOD activity induced by I/R injury.

Effects of TFPFC treatment on expressions of Nrf2 and HO-1 in brain tissues after I/R in rats

To study the influences of TFPFC treatment on expres-

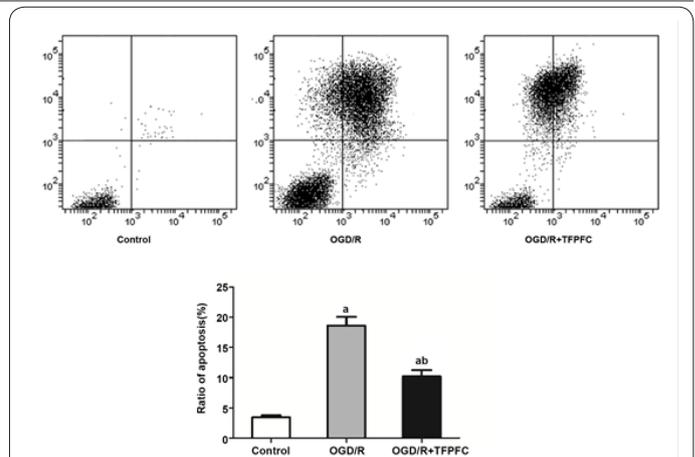


Figure 3. Influence of TFPFC treatment on the apoptosis of OGD/R neural stem cells in vitro. a. A significant difference in the OGD/R group compared with that in the Control group ($p < 0.05$), ab. a significant difference in the OGD/R+TFPFC group compared with that in the OGD/R group ($p < 0.05$, $n = 3$).

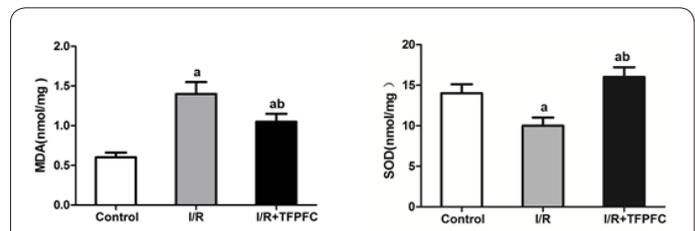


Figure 4. Influence of TFPFC treatment on the MDA level and SOD activity after I/R in rats. a. A significant difference in the I/R group compared with that in the Control group ($p < 0.05$), ab. a significant difference in the I/R+TFPFC group compared with that in the I/R group ($p < 0.05$, $n = 3$).

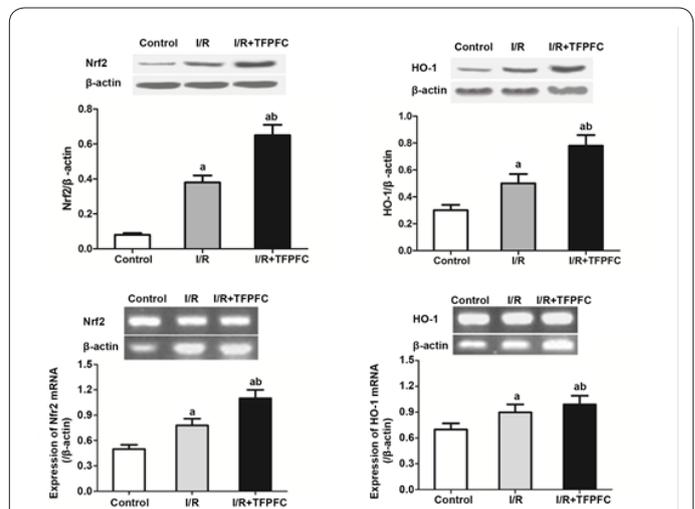


Figure 5. Influences of TFPFC treatment on expressions of Nrf2 and HO-1 in brain tissues after I/R in rats. a. A significant difference in the I/R group compared with that in the Control group ($p < 0.05$), ab. a significant difference in the I/R+TFPFC group compared with that in the I/R group ($p < 0.05$, $n = 3$).

sions of Nrf2 and HO-1 in brain tissues after I/R in rats, the protein and mRNA expression levels of Nrf2 and HO-1 in cortical tissues of rats in the Control group, I/R group and I/R+TFPFC group were detected via Western blotting and RT-PCR, respectively.

The consistent results were obtained in Western blotting and RT-PCR: The protein and mRNA expressions

of Nrf2 and HO-1 were obviously increased in I/R group compared with those in Control group ($p < 0.05$), and they were further increased in I/R+TFPFC group compared with those in I/R group ($p < 0.05$), proving that TFPFC promotes the protein and mRNA expressions of Nrf2 and HO-1 in brain tissues after I/R in rats. (Figure 5).

Discussion

TFPFC is a kind of flavonoid compound synthesized via photosynthesis extracted from *Premna fulva* Craib, which possesses a strong anti-oxidative effect. Results of this study demonstrated for the first time that TFPFC treatment significantly improved nerve injury and alleviated cerebral infarction and edema in I/R rats. The number of apoptotic cells after I/R injury determines the final area of infarction^(11, 12). In this study, TFPFC also reduced the apoptosis of neural stem cells induced by I/R injury.

A large number of studies have manifested that cerebral I/R injury is closely correlated with the oxidative stress produced by reactive oxygen species⁽¹³⁾. The excessive production of reactive oxygen species is a major feature during cerebral I/R injury, which usually leads to oxidative damage and cerebral dysfunction^(14, 15). MDA and SOD are closely related to oxidative stress⁽¹⁶⁻¹⁹⁾. In this study, the expression level of MDA was significantly increased after cerebral I/R, indicating that cerebral I/R injury in rats results in oxidative stress. TFPFC treatment obviously reduced the increased MDA level caused by I/R. At the same time, the SOD activity in the I/R group was significantly lower than that in the Control group, suggesting that the SOD activity declines after I/R. Moreover, the SOD activity was significantly up-regulated after TFPFC treatment, suggesting that TFPFC are able to promote the SOD activity.

In addition, previous studies have demonstrated that flavonols and metals in TFPFC form the chelate compound, and flavonols bind to free radicals to block the oxidative damage caused by free radical chain reaction. However, the neuroprotective effect of TFPFC on cerebral I/R injury has not been reported in the literature, which was revealed for the first time in this study. Nrf2 and HO-1 are important markers for neuronal damage response^(20, 21). This study showed that both Nrf2 and HO-1 were up-regulated after cerebral I/R in rats and further increased after TFPFC treatment, suggesting that the Nrf2/ARE signaling pathway is activated. The activation of Nrf2 by TFPFC leads to the up-regulation of antioxidant HO-1 and the decline in oxidative damage in cerebral ischemia, which also has a protective effect on cerebral I/R injury⁽²²⁾. In conclusion, the TFPFC treatment may improve oxidative damage and protect the nervous system through the up-regulation of transcription factors Nrf2 and HO-1.

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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 upon reasonable request.

Authors' contributions

HQ wrote the manuscript. HQ and YF were responsible for the establishment of the rat model of cerebral I/R. YJ, ZT and YZ established a rat model of OGD/R in vitro. WT and ML recorded and interpreted neurological impairment scores. GF and HW helped with the determination of the volume of cerebral infarction and MDA level. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of Rui-kang Hospital, affiliated to Guangxi University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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