Eupafolin alleviates cerebral ischemia/reperfusion injury in rats via blocking the TLR4/NF-κB signaling pathway

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Abstract. *Eupatorium perfoliatum L. (E. perfoliatium)* has been used traditionally for treating fever, malaria and inflammation-associated diseases. Eupafolin, the extract of *E. perfoliatium*, was also reported to suppress inflammation. The present study aimed to investigate the protective effects of eupafolin on cerebral ischemia/reperfusion (I/R) injury in rats and its possible underlying mechanisms. Cerebral I/R injury was induced in rats by middle cerebral artery occlusion (MCAO) for 1.5 h, followed by reperfusion. The rats were randomly assigned into six groups: Control, model, 10 mg/kg eupafolin, 20 mg/kg eupafolin, 50 mg/kg eupafolin and 20 mg/kg nimodipine. Eupafolin and nimodipine were intragastrically administrated to the rats 1 week before MCAO induction. Following reperfusion for 24 h, the neurological deficit was scored, and brain samples were harvested for evaluating encephaledema**,** infarct volume, oxidative stress, apoptosis, inflammation and the expression of TLR4/NF-κB signaling. The results revealed that eupafolin decreased the neurological score, relieved encephaledema and decreased infarct volume. Eupafolin also attenuated oxidative stress, neuronal apoptosis and inflammation, with decreases in lactate dehydrogenase, malondialdehyde, TUNEL-positive cells, Bax and caspase-3, along with TNF-α, IL-1β and IL-6, but increases in superoxide dismutase and Bcl-2 levels. Furthermore, eupafolin may decrease the expression of TLR4 downstream proteins and proteins involved in the NF-κB pathway. Treatment with TLR4 agonist-LPS significantly blunted the protective effect of eupafolin on encephaledema and cerebral infarct. Meanwhile, 20 mg/kg eupafolin showed nearly equivalent effects to the positive-control drug nimodipine. In conclusion, eupafolin protected against cerebral I/R injury in rats and the underlying mechanism may be associated with the suppression of apoptosis and inflammation via inhibiting the TLR4/NF-κB signaling pathway.

Introduction

Ischemic stroke remains a leading cause of mortality or long-term disability. The goal of clinical treatment is to restore the blood supply as soon as possible, allowing timely supply of oxygen to ischemic brain tissue (1). However, blood reperfusion that occurs after a long period of ischemia is likely to result in a higher infarction volume and to, in turn, aggravate the initial injury, which is the main cause of cerebral ischemia reperfusion (I/R) injury (2). The mechanisms involved in cerebral I/R is complicated and require comprehensive understanding. To date, inflammatory response, free radical damage, cytotoxicity, increased mitochondrial permeability and intracellular calcium overload have been implicated to participate in the occurrence or progression of cerebral I/R injury (3). Cerebral I/R injury is pathologically characterized by the damage of brain tissues, infiltration of leukocyte cells into the brain, influx of inflammatory cells, excessive production of reactive oxygen species (ROS), degradation of cytoskeletal protein and collapse of the blood-brain barrier (4). Increased ROS production following reperfusion will increase hemorrhagic infarction, cerebral edema and infarct size (5). Inhibition of oxidative stress has been demonstrated to protect against cerebral I/R injury (6,7). Therefore, the use of safe and effective therapeutic agents with antioxidant properties to interfere with oxidative damage provides an encouraging treatment strategy.

Eupafolin is an active flavonoid component of *Eupatorium perfoliatum L.*, which is a traditional herbal medicine from China and India, and has been widely used for centuries to treat fever, malaria, infections and inflammation-associated diseases (8). In recent years, eupafolin was reported to exhibit anti-inflammatory, anti-oxidant and anti-tumor cell proliferation effects (9). For example, Zhang *et al* (10) indicated that eupafolin improved acute renal injury and exhibited effective anti-oxidant and anti-inflammatory activities via inhibiting reactive stress and inactivating NF-κB, MAPK, ERK 1/2 and c-JNK signaling pathways (10). Eupafolin ameliorated cardiomyocyte autophagy via activation of the PI3K/AKT/mTOR signaling pathway (11). Eupafolin also protected against TNF-α-induced lung inflammation via inhibiting NF-κB/p65

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activation and also resulted in nuclear translocation (12). In addition, the suppressive effects of eupafolin on various tumor types, including esophageal cancer, hepatocellular carcinoma, renal carcinoma and prostate cancer has been extensively studied (13-16). These data suggested the potential value of eupafolin in treating cancer and inflammation-related diseases. However, whether eupafolin may protect the brain against I/R injury remains to be elucidated.

Toll-like receptor (TLR) is a type of transmembrane protein that converts extracellular antigen information into cells and triggers an inflammatory response. Toll-like receptor 4 (TLR4) is the first discovered member of TLR, which serves a role in immune defense and immune regulation by recognizing and binding multiple endogenous and exogenous ligands. TLR4 transduces these signals via the membrane and subsequently regulates the expression inflammation mediators and cytokines (17). A previous study demonstrated that TLR4 serves a role in I/R inflammation injury of the liver, lung and heart, and induces apoptosis during cerebral I/R (18). NF-κB is a transcription factor that may specifically bind promoters and enhancers of numerous genes and thus participate in a variety of cellular functions, including inflammation, cell proliferation and apoptosis. NF-κB is typically inactivated in the cytoplasm due to the interaction of p65/p52 with the inhibitory protein IκB. The subsequent activation of p65/p52 may be stimulated by pro-inflammatory cytokines, cellular stress, DNA damaging agents and phosphorylation of κBs by the IκB kinase complex (19). NF-κB also serves a crucial role in the activation of I/R injury (20).

The present study investigated the protective effects of eupafolin against cerebral I/R injury in rats and investigated whether its action was dependent on blocking the TLR4/NF-κB signaling pathway.

Materials and methods

Animals. Adult male Sprague-Dawley rats (7-8 week-old; n=48) weighing 250-280 g were supplied by Nanjing Better Biotechnology Co., Ltd, and were acclimatized for 1 week before experiments at room temperature under a controlled 12/12 h light/dark cycle. All rats received food and water *ad libitum*. The experimental protocols involving rats were approved by the Animal Studies Ethics Committee of the Shenzhen Hospital of Integrated Traditional Chinese and Western Medicine.

The animals were randomly assigned to six groups (n=8 for each group): Control, model, 10 mg/kg eupafolin (purity >98%; Shanghai Rongbia Biological Technology Co., Ltd.), 20 mg/kg eupafolin, 50 mg/kg eupafolin and 20 mg/kg nimodipine (MedChemExpress). The control group underwent sham surgery. Eupafolin and nimodipine were intragastrically (i.g.) administered into the rats once a day for 7 consecutive days. The control and model groups were administered 200 μl normal saline i.g. For the eupafolin + lipopolysaccharide (LPS) group, the animals were intraperitoneally injected with 50 μg/kg LPS (21) at the same time that they received eupafolin 20 mg/kg for the last time.

Establishment of a cerebral I/R model. One hour after the last administration, the focal cerebral I/R rat model was prepared using the middle cerebral artery occlusion (MCAO) method, as previously reported with slight modifications (22). In brief, following weighing, the rats were anesthetized with 1% pentobarbital (50 mg/kg; i.p) and fixed in the supine position on a heated operating table with the body temperature maintained at 37±0.5˚C. Following skin incision, the left common carotid artery, external carotid artery and internal carotid artery were carefully exposed and dissected away from the adjacent nerve. The left middle cerebral artery was occluded by inserting a 3.5-mm monofilament suture through the internal carotid artery from the external carotid artery. Following ischemia for 1.5 h, the suture was gently withdrawn allowing reperfusion. At 24 h post-reperfusion, various indexes were measured (23). The animals in the sham-operated group were anesthetized with 1% pentobarbital (50 mg/kg; i.p) prior to being subjected to the same surgical procedure as the model group but without occlusion of the middle cerebral artery (24).

Neurological score. The neurological deficit was scored in each mouse 24 h after reperfusion in a blinded manner by two independent investigators according to the 3-point scoring system of Bederson *et al* (25): No neurological symptoms=0; forelimb flexion and no other abnormality=1; decreased resistance to lateral push (and forelimb flexion) without circling=2; same behavior as grade 2, with circling toward the paretic side=3.

Measurement of brain edema. To evaluate brain edema, the brain water content was measured using the standard wet-dry method. After 24 h of reperfusion, the rats were decapitated under deep anesthesia and the brains were carefully removed. The wet weight was obtained immediately by weighing the ischemic hemispheres, and the tissues were dried at 100˚C for 24 h to determine the dry weight. The degree of brain edema was calculated using the following equation: Water content=(wet weight-dry weight)/wet weight x100%; brain index=wet weight/body weight x100%.

Measurement of infract volume. Brain infarction size was evaluated by the 2,3,5-triphenyltetrazolium chloride (TTC) staining method (26) 24 h after I/R. Brains were carefully removed and maintained at -20˚C for 10 min. Brain tissues were then sliced into consecutive 2-mm-thick coronal sections and immersed in 2% TTC solution (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. TTC stains non-infarcted regions with a deep red pigment, while the infarcted brain area appears white. The infarct area of each section was photographed and image analysis software (NIH Image version 1.63; National Institutes of Health) was applied to measure the infarcted area.

Measurement of oxidative stress. The fresh brain tissues of rats at 24 h after cerebral I/R were collected and the superoxide dismutase (SOD; cat. no. ab65354), malondialdehyde levels (MDA; cat. no. ab118970) and lactate dehydrogenase (LDH; cat. no. ab102526) activities in the brain tissues were determined using the commercial kits (Abcam). The brain tissues were harvested, washed with PBS and homogenized using RIPA lysis buffer supplemented with PMSF protease inhibitor (both from Abcam). After being centrifuged at 13,000 x g and 4˚C for 10 min to remove insoluble material, the supernatants were collected and incubated with corresponding reaction

mix according to the manufacturer's protocols. The optical density was measured (OD of 450 nm for SOD and LDH; OD of 532 nm for MDA) to calculate the relative level of SOD, MDA and LDH. The relative MDA levels, as well as SOD and LDH activities was expressed as the value dividing by the OD of the control group after normalization to the standard curve.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of TNF-α, IL-1β and IL-6 in the serum of rats at 24 h after cerebral I/R were measured in strict accordance with the manufacturer's protocols provided by the ELISA kits (Abcam) for TNF-α (cat. no. ab236712), IL-1β (cat. no. ab255730) and IL-6 (cat. no. ab234570). In brief, blood samples were collected into a serum separator tube. Following clot formation, samples were centrifuged at 2,000 x g and 4°C for 10 min to collect serum. Samples were incubated with antibodies (included in the kits) targeting TNF-α, IL-1β and IL-6 at room temperature for 1 h. Following washing with the supplied wash buffer, TMB development solution was added and incubated at room temperature for 10 min. The stop solution was added and the absorbance at a wavelength of 450 nm was detected using a microplate reader (Thermo Fisher Scientific, Inc.).

TUNEL staining. A TUNEL assay (Beyotime Institute of Biotechnology) was used according to the manufacturer's protocols to assess neuronal apoptosis in brain tissues. In brief, isolated brains were fixed in 4% paraformaldehyde at room temperature for 10 min and cut into sections of 20-μm thickness, followed by paraffin embedding. Following dewaxing with xylene, sections were incubated with protease K for 30 min and subsequently washed with phosphate-buffered saline (PBS). Subsequently, 50 μl TUNEL reaction mixture was added and incubated for 1 h at 37°C. The sections were then washed with PBS and incubated for 30 min following the addition of 50 μl confining liquid. The nuclei were stained with DAPI at room temperature for 5 min and TUNEL-positive cells were observed under a DMi8 fluorescence microscope (Leica Microsystems GmbH). Three fields of view were examined (magnification, x200).

Western blotting. The ischemic side of the cerebral cortex was dissected to extract total protein using RIPA buffer (Applygen Technologies Inc.) and quantified using a BCA assay. Following quantification, equal amount of proteins (8 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore). Following blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with the following primary antibodies at 4°C overnight (all Abcam): Bcl-2 (cat. no. ab32124; dilution, 1:1,000), Bax (cat. no. ab32503; dilution, 1:5,000), pro-caspase 3 (cat. no. ab32499; dilution, 1:10,000), cleaved-caspase 3 (cat. no. ab32024; dilution, 1:500), myeloid differentiation factor 88 (MyD88; cat. no. ab133739; dilution, 1:10,000), TNF receptor-associated factor 6 (TRAF6; cat. no. ab33915; dilution, 1:10,000), TGF-β-activated kinase 1 (TAK1; cat. no. ab109526; dilution, 1:1,000), IKKα (cat. no. ab32041; dilution, 1:10,000), p65 (cat. no. ab16502; dilution, 1:5,000), phosphorylated (p)-IKKα (cat. no. ab38515; dilution, 1:1,000), p-p65 (cat. no. ab183559; dilution, 1:1,000) and GAPDH (cat. no. ab8245; dilution, 1: 10,000). The antibodies

Table I. Effect of eupafolin on the neurological deficit score.

Group	Neurological deficit scores (range, 0-3)
Control	0.00±0.00
Model	2.63±0.51[a]
Eupafolin (10 mg/kg)	2.13±0.31[b]
Eupafolin (20 mg/kg)	1.10±0.43[c]
Eupafolin (50 mg/kg)	0.85±0.17[c]
Nimodipine	1.13±0.24[c]

The neurological deficit scores of rats among the Control, Model, 10 mg/kg eupafolin, 20 mg/kg eupafolin, 50 mg/kg eupafolin and nimodipine groups were measured at 24 h after cerebral I/R (n=8). Data are presented the mean ± standard deviation. [a]$P<0.001$ vs. Control group; [b]$P<0.05$ and [c]$P<0.001$ vs. Model group.

were detected using horseradish peroxidase-conjugated IgG (Abcam; goat anti-rabbit, cat. no. ab7090; dilution, 1:10,000) at room temperature for 2 h and visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS (version 22.0, IBM Corp.) was used to perform the paired T test between two groups and one-way analysis of variance, followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of eupafolin on the neurological defect score, brain edema and infract volume in rats subjected to cerebral I/R. The alteration in neurological functions and phenotypes was first investigated. The effects of eupafolin on the neurological defect scores at 24 h post-reperfusion in the control, model, eupafolin (10, 20 and 50 mg/kg) and nimodipine (20 mg/kg) groups are presented in Table I. Rats in the control group did not exhibit neurological deficits, with scores of 0. However, in the model group, there were clear signs of neurological deficits, including forelimb flexion, decreased resistance to lateral push and circling toward the paretic side, with significantly higher neurological defect scores compared with the control group, indicating the occurrence of cerebral I/R injury. Eupafolin at any dose significantly decreased the neurological defect scores compared with the model group, similar to that in the nimodipine group.

The results of the water content and brain index studies were consistent with the trend observed for the neurological defect scores. As shown in Table II, the brain water content and brain index of the rats were increased in the model group, while those in the low, moderate and high-dose eupafolin and nimodipine groups were significantly lower those in the model group.

The cerebral infarct volumes were assessed using TTC staining. As shown in Fig. 1, no infarction (white staining) was found in the control group. Compared with the control group, cerebral I/R injury induced significant infarction in the model group. However, treatment with eupafolin at 10,

Table II. Effect of eupafolin on brain edema.

Group	Brain water content, %	Brain index, %
Control	82.58±0.44	0.52±0.02
Model	84.10±0.51[a]	0.72±0.04[a]
Eupafolin (10 mg/kg)	83.41±0.41[b]	0.68±0.02[b]
Eupafolin (20 mg/kg)	82.90±0.43[c]	0.60±0.03[d]
Eupafolin (50 mg/kg)	82.60±0.38[c]	0.57±0.01[d]
Nimodipine	82.94±0.34[c]	0.63±0.03[d]

The brain samples of each group were harvested at 24 h after cerebral I/R, and then the brain water content and brain index were measured (n=8). Data are presented as the mean ± standard deviation. [a]$P<0.001$ vs. Control group; [b]$P<0.05$, [c]$P<0.01$ and [d]$P<0.001$ vs. Model group.
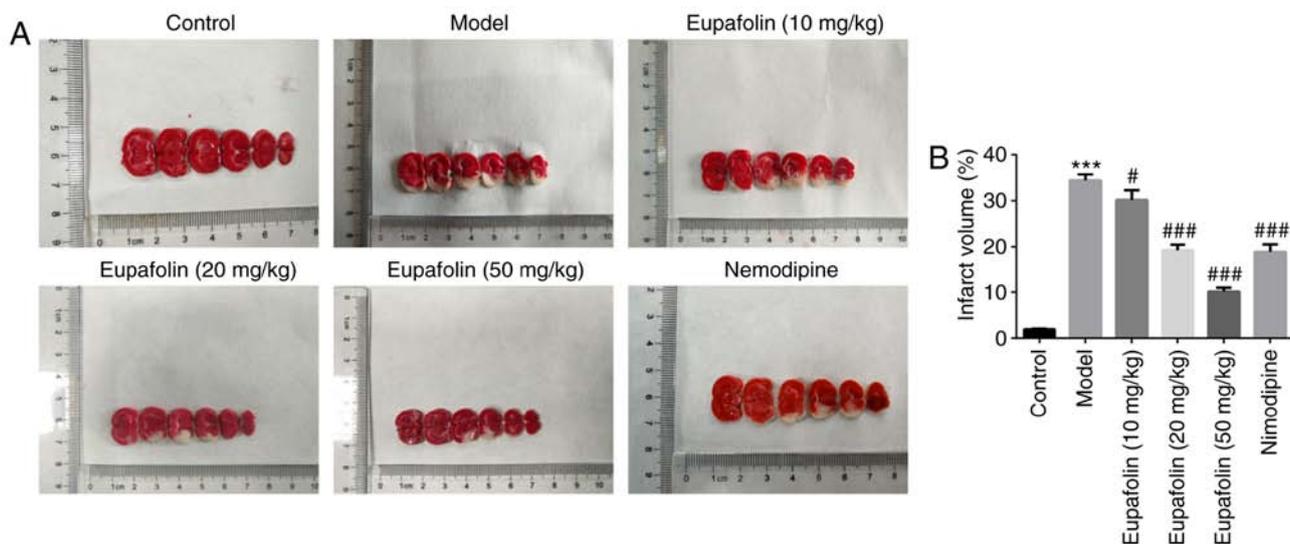
Figure 1. Cerebral infarction volume of rats from control, model, 10 mg/kg eupafolin, 20 mg/kg eupafolin, 50 mg/kg eupafolin and nimodipine groups. (A) TCC staining images of brain tissues in rats at 24 h after cerebral I/R. (B) Histogram of cerebral infarction volume in different groups. [***]$P<0.001$ vs. control. [#]$P<0.05$ and [###]$P<0.001$ vs. model. I/R, ischemia/reperfusion; TTC, 2,3,5-triphenyltetrazolium chloride.

20 and 50 mg/kg markedly decreased the infarct volume of brain tissues. Nimodipine exerted similar effects in terms of decreasing the infarct volumes.

Eupafolin decreases oxidative stress and inflammation in brain tissues and serum of rats subjected to cerebral I/R. Subsequently, oxidative stress was examined in ischemic brain tissues, which is considered the initial step of cerebral I/R injury. SOD is an important antioxidant enzyme, while MDA and LDH activities reflect oxidative damage. Therefore, SOD, MDA and LDH contents were investigated (Fig. 2A-C). Compared with the control group, SOD activity was significantly decreased, while MDA and LDH content was increased in the model group. Treatment with eupafolin effectively increased SOD activity and decreased MDA and LDH content, which was similar to the results in the nimodipine group.

To investigate the effects of eupafolin on inflammation, the generation of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 were assessed (Fig. 2D-F). Cerebral I/R injury significantly increased the concentration of TNF-α, IL-1β and IL-6 in the serum of model rats. However, this increase was

reversed by eupafolin or nimodipine treatment. The aforementioned findings suggested that, similar to nimodipine, eupafolin attenuated oxidative stress and inflammation induced by cerebral I/R injury.

Eupafolin prevents brain cell apoptosis in rats subjected to cerebral I/R. Next, the effects of eupafolin on cell apoptosis were observed by TUNEL staining and western blotting. As shown in Fig. 3, the number of TUNEL-positive cells in the brain tissues of the model group was significantly increased, compared with the control group. These effects were significantly impaired by eupafolin or nimodipine treatment. Furthermore, western blotting was utilized to detect the expression of proteins associated with apoptosis. The results revealed that cerebral I/R lead to an enhancement in Bax and caspase-3 expression, but a decline in the anti-apoptotic protein Bcl-2 expression (Fig. 4). However, eupafolin and nimodipine partially reversed the levels of these proteins, compared with the model group. Consistent with TUNEL staining, these results demonstrated that eupafolin may prevent cerebral I/R-induced cell apoptosis in the brain tissues of rats.
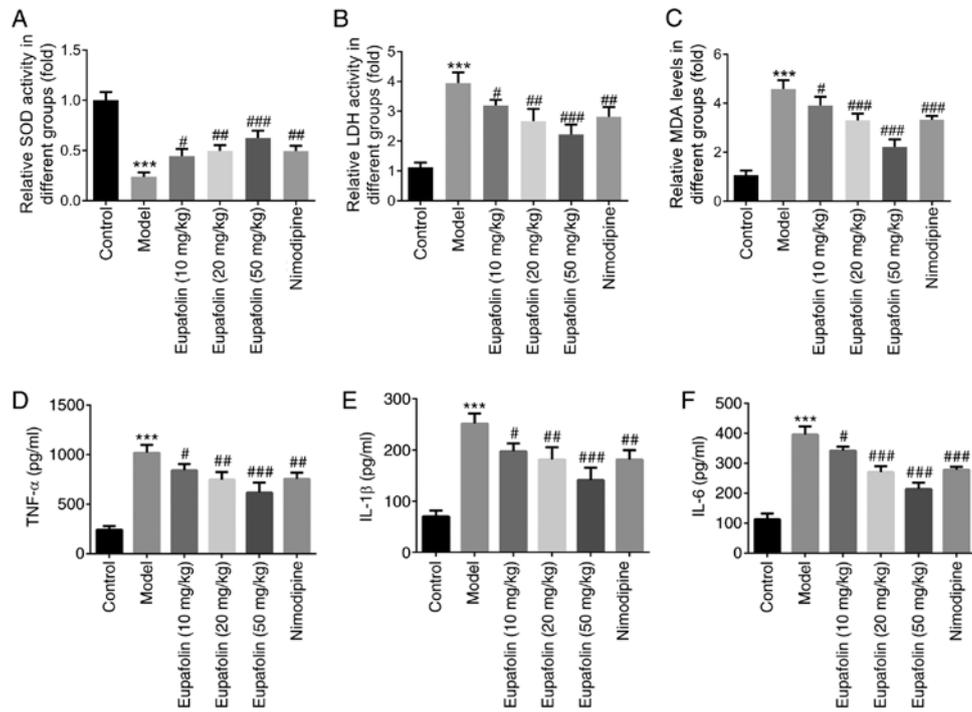
Figure 2. Levels of oxidative stress and inflammation in rats from control, model, 10 mg/kg eupafolin, 20 mg/kg eupafolin, 50 mg/kg eupafolin and nimodipine groups. The activities of (A) SOD, (B) MDA and (C) LDH in the brain tissues of rats at 24 h after cerebral I/R. The concentrations of (D) TNF-α, (E) IL-1β and (F) IL-6 in the serum of rats at 24 h after cerebral I/R. ***$P<0.001$ vs. control. #$P<0.05$, ##$P<0.01$ and ###$P<0.001$ vs. model. SOD, superoxide dismutase; MDA, malondialdehyde; LDH, lactate dehydrogenase; I/R, ischemia/reperfusion.
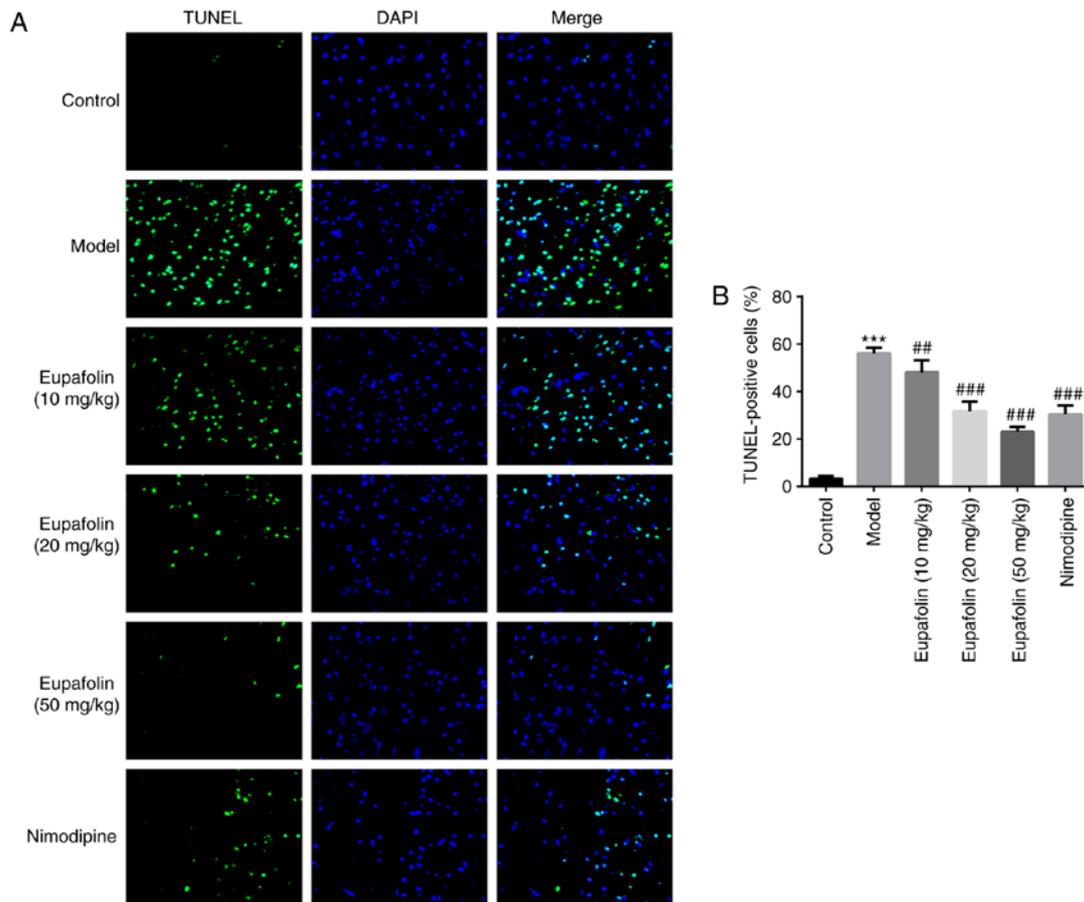
Figure 3. Percentage of apoptotic cells in rats from control, model, 10 mg/kg eupafolin, 20 mg/kg eupafolin, 50 mg/kg eupafolin and nimodipine groups. (A) Representative TUNEL staining images to determine cell apoptosis in brain tissues of rats at 24 h after cerebral I/R. (B) Percentage of TUNEL-positive cells in different groups. ***$P<0.001$ vs. control. ##$P<0.05$ and ###$P<0.001$ vs. model. I/R, ischemia/reperfusion.
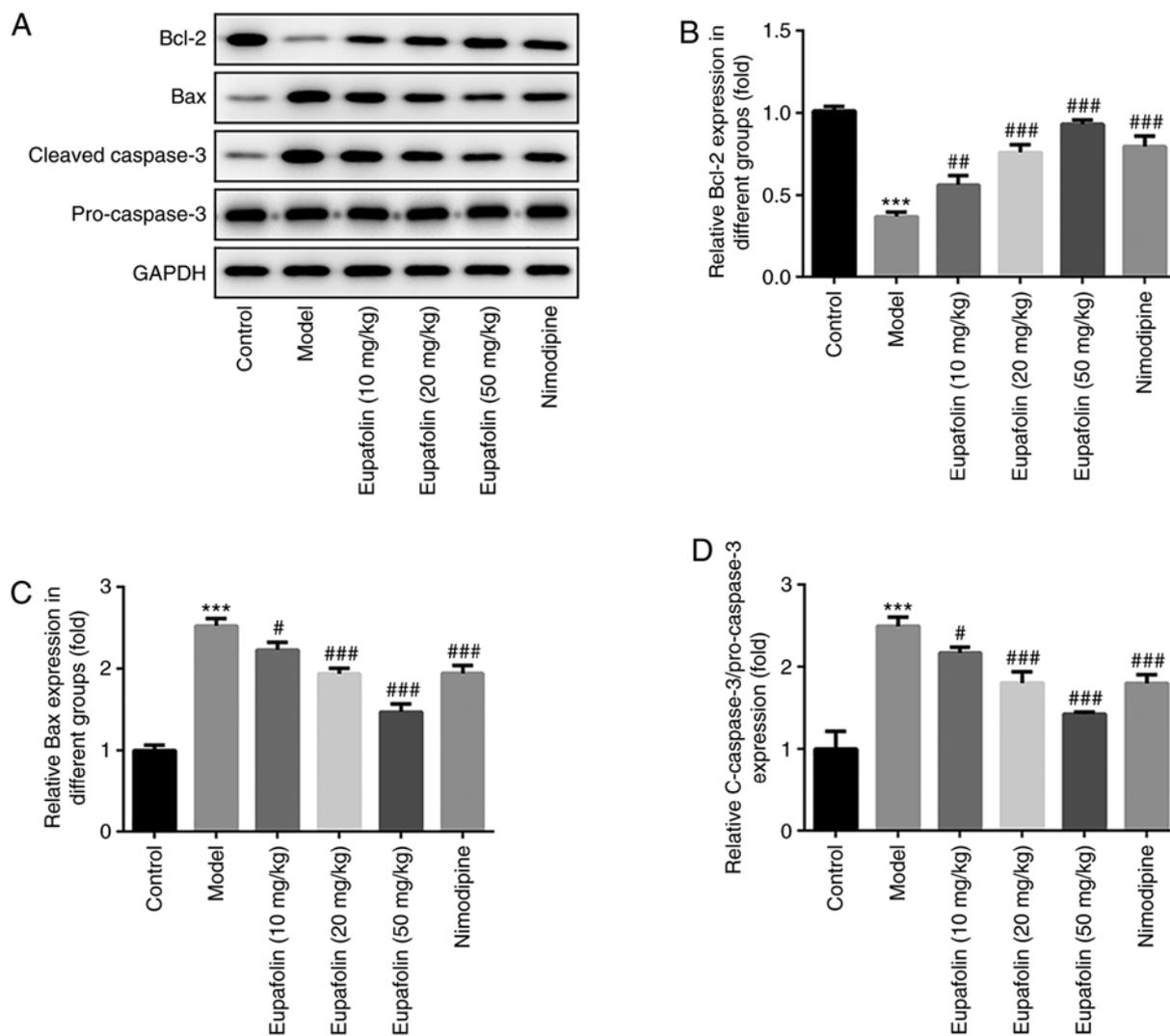
Figure 4. Expression of proteins involved in cell apoptosis in brain tissues of rats from control, model, 10 mg/kg eupafolin, 20 mg/kg eupafolin, 50 mg/kg eupafolin and nimodipine groups. (A) Representative western blot bands for detecting Bcl-2, Bax, pro-caspase 3 and cleaved-caspase 3. GAPDH was used as the loading control. Densitometric quantification of (B) Bcl-2, (C) Bax and (D) cleaved-caspase 3/pro-caspase 3 expression. $^{***}P<0.001$ vs. control. $^{#}P<0.05$, $^{##}P<0.01$ and $^{###}P<0.001$ vs. model.

Eupafolin inhibits the activation of the TLR4/NF-κB signaling pathway. Finally, to underline the possible mechanism of eupafolin protection against cerebral I/R injury, the expression of proteins involved in TLR4/NF-κB signaling, including MyD88, TRAF6, TAK1, IKKα and p65, in brain tissues were examined. As shown in Fig. 5, in the model group, the expression of MyD88, TRAF6, TAK1, p-IKKα and p65 was significantly higher than that in the control group, suggesting the activation of TLR4 signaling and nuclear translocation of the NF-κB complex. However, eupafolin (10, 20 and 50 mg/kg) or nimodipine (20 mg/kg) significantly inhibited the expression of these proteins.

To verify whether the protective effects of eupafolin rely on blocking TLR4 signaling, animals that were treated with 20 mg/kg eupafolin were injected with LPS, which is the agonist of TLR4 (21). Table III and Fig. 6 demonstrate that, compared with the eupafolin groups, following MCAO and reperfusion, animals in the eupafolin + LPS group exhibited markedly higher neurological deficit scores, brain water content and brain indexes (Table III), as well as larger infract volume (Fig. 6).

Discussion

In the present study, treatment of rats subjected to cerebral I/R with eupafolin not only decreased the neurological deficit score, brain edema and cerebral infarct size, but also weakened oxidative stress, inflammation and cell apoptosis. This protection by eupafolin against cerebral I/R injury was accompanied by the downregulation of MyD88, TRAF6, TAK1, p-IKKα and p-p65. These results indicated that TLR4/NF-κB pathways are inactivated in this process, implying that the TLR4/NF-κB pathways are involved in the neuroprotective effects of eupafolin.

Previous studies have shown that inflammation, oxidative stress and apoptosis are the three dominating mechanisms underlying the detrimental process of cerebral I/R injury, which was proven to be the second most common lethal factor and the leading cause of adult neurological disabilities worldwide (3,4,27,28). In addition, inflammation is one of the central preventive mechanisms against cerebral I/R injury (3,4). Therefore, inhibiting neuronal inflammation is a pivotal target
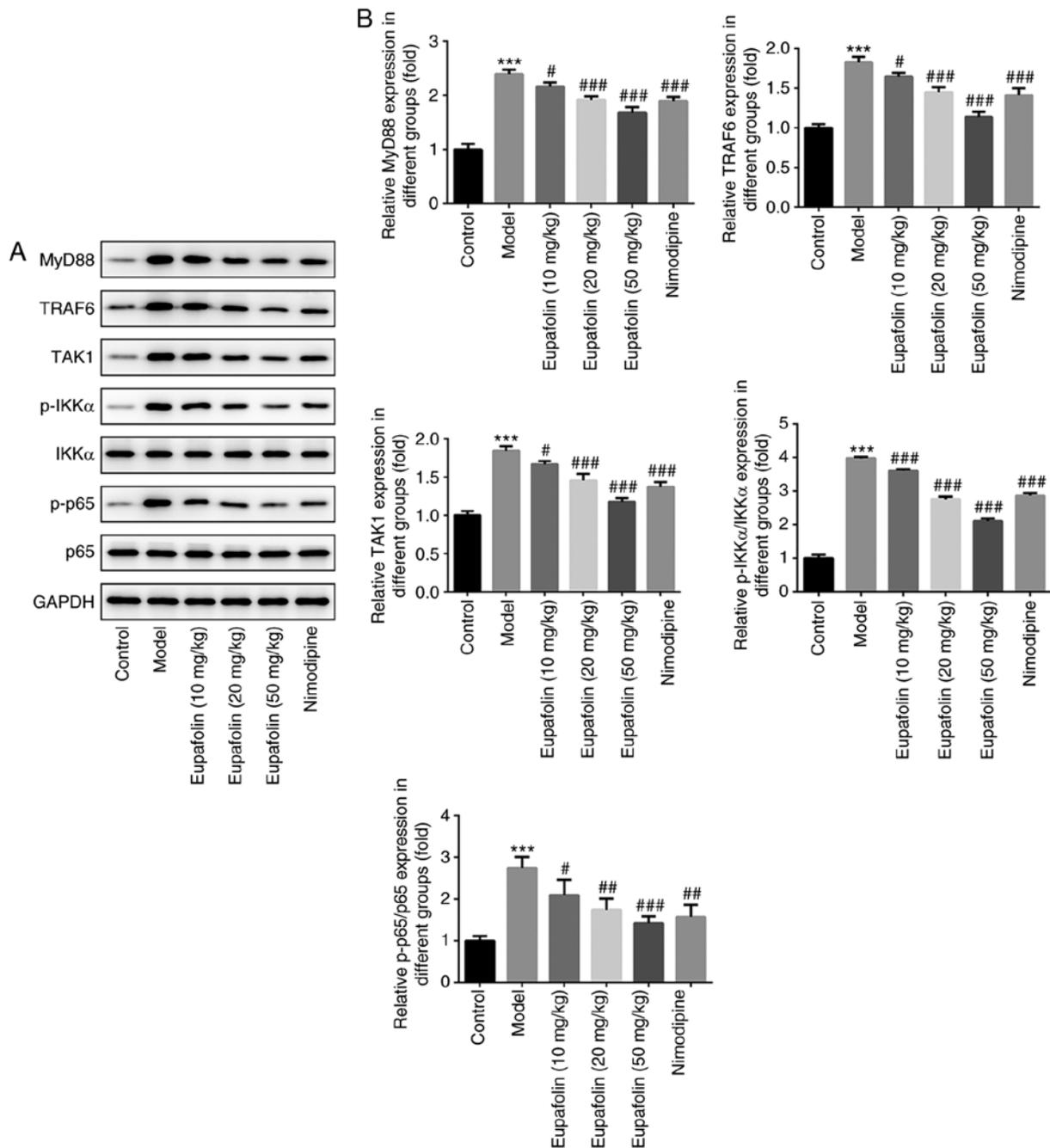
Figure 5. Expression of proteins involved in TLR4/NF-κB in brain tissues of rats from control, model, 10 mg/kg eupafolin, 20 mg/kg eupafolin, 50 mg/kg eupafolin and nimodipine groups. (A) Representative western blot bands for detecting MyD88, TRAF6, TAK1, p-IKKα/IKKα and p-p65/p65. GAPDH was used as loading control. (B) Densitometric quantification of protein expression. ***$P<0.001$ vs. control. #$P<0.05$, ##$P<0.01$ and ###$P<0.001$ vs. model. MyD88, myeloid differentiation factor 88; TRAF6, TNF receptor associated factor 6; TAK1, TGF-activated kinase 1; p, phosphorylated.

for the treatment of ischemic stroke. To date, in preclinical animal studies, >700 drugs have shown beneficial effects in cerebral ischemia, but the results are far from satisfactory (29). Eupafolin is a potent anti-inflammatory, antioxidant and anti-tumor agent, extracted from the traditional herb *E. perfoliatum*. Although eupafolin has been used as a traditional medicine for treating inflammatory-related diseases in China and India, the pharmacological research on it has only begun in the past ten years. Published reports have mainly focused on *in vitro* studies, and are lacking evidence from *in vivo* studies for clinical therapy (30). Jiang *et al* (14) demonstrated that eupafolin at 60 mg/kg significantly inhibited tumor growth and

tumor angiogenesis in a hepatocellular carcinoma xenograft model. The present study investigated the protective effects of eupafolin on cerebral I/R injury in rats and revealed that eupafolin at a dose of 10, 20 and 50 mg/kg all exhibited significant protective effects on cerebral I/R via inhibiting inflammation, oxidative stress and apoptosis. Concurrently, 20 mg/kg eupafolin showed nearly equivalent effects to nimodipine, which is used to improve blood circulation in the recovery period of acute cerebrovascular disease and has been proven to exert anti-inflammation and anti-apoptosis effects (31-33). These results provided *in vivo* data for the therapeutic effect of low-dose eupafolin in treating cerebral I/R injury.

Table III. The neurological deficit score and brain edema evaluation in the absence or presence of TLR4 agonist-LPS.

Group	Neurological deficit scores (range, 0-3)	Brain water content, %	Brain index, %
Eupafolin (20 mg/kg)	1.15±0.31	83.04±0.53	0.62±0.02
Eupafolin + LPS	2.09±0.47[b]	83.84±0.66[a]	0.69±0.04[b]

The neurological deficit scores of rats among the eupafolin 20 mg/kg and eupafolin 20 mg/kg + LPS groups were measured at 24 h after cerebral I/R (n=8). The brain samples of each group were harvested 24 h after cerebral I/R, and the brain water content and brain index were then measured (n=8). Data are presented as the mean ± standard deviation. [a]$P<0.05$ and [b]$P<0.001$ vs. eupafolin 20 mg/kg group. TLR, toll-like receptor; LPS, lipopolysaccharide; I/R, ischemia/reperfusion.
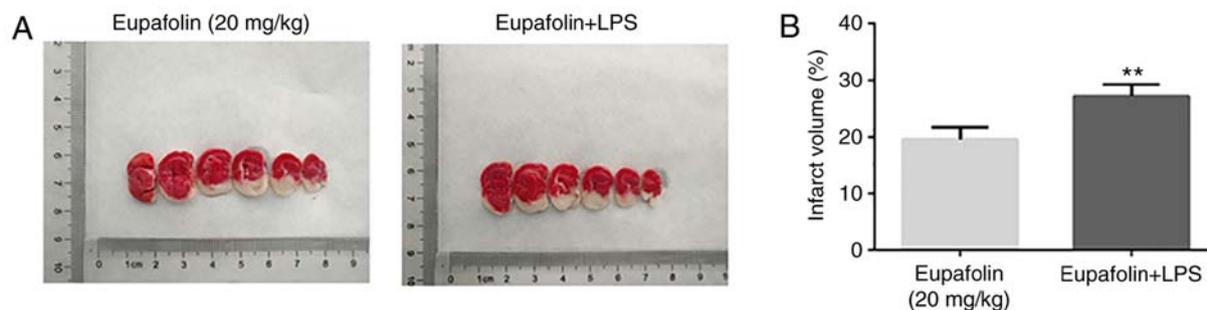
Figure 6. The presence of TLR4 agonist-LPS blunts the effect of eupafolin. (A) TCC staining images of brain tissues in rats at 24 h after cerebral I/R. (B) Histogram of cerebral infarction volume in different groups. [**]$P<0.01$ vs. eupafolin 20 mg/kg. I/R, ischemia/reperfusion; TTC, 2,3,5-triphenyltetrazolium chloride; LPS, lipopolysaccharide.

Eupafolin may exert its actions via targeting multiple pathways, including NF-κB, PI3K/AKT and MAPK (11,34,35). It is widely implicated that TLR4-MyD88 association may activate NF-κB, which may activate neurons to secrete numerous pro-inflammatory mediators, including TNF-α, IL-1β and IL-6, ultimately resulting in ischemic injury (36). The results of the present study demonstrated that cerebral I/R injury may significantly increase MyD88, TRAF6, TAK1, p-IKKα and p-p65 expression, and TNF-α, IL-1β and IL-6 levels. Following ligand binding, the toll/interleukin-1 receptor domain of TLR4 interacts with MyD88, thereby binding to TRAF6, resulting in the activation of TAK1. Activated TAK1 continues to signal via MAPK or NF-κB (37). The present data indicated that eupafolin may prevent the activation of TLR4/NF-κB signaling. In addition, the neurological deficit score, brain edema and cerebral infarct size were decreased compared with the model group, indicating that the blockade of the TLR4/NK-κB signaling pathway in cerebral I/R may have a protective effect. Furthermore, the presence of LPS, which is publicly considered as the ligand of TLR4 and activates TLR4 signaling (38), markedly blunted the protective effect of 20 mg/kg eupafolin on neurological functions, brain edema and infarct volume of rats that underwent cerebral I/R injury, implicating that the actions of eupafolin may be at least partially dependent on the blocking of the TLR4/NF-κB signaling pathway.

However, the activation of TLR4 signaling is not only associated with the NF-κB pathway but is also associated with other processes, including the MAPK pathway. Therefore, the protective effects of eupafolin against cerebral I/R injury may not only be associated with the activation of the NF-κB pathway, but also with other mechanisms, which require further investigation. In addition, whether the inhibitory effect of eupafolin on inflammation, oxidative stress and apoptosis during cerebral I/R were dependent on blocking TLR4 signaling remain to be investigated. Meanwhile, the employment of TLR4-knockout or -knockdown mice is necessary to further confirm the results of the present study and to identify the underlying molecular mechanisms, and this will be performed in future experiments.

In conclusion, the results of the present study provided novel evidence that eupafolin exerted protective effects against cerebral I/R injury in rats exposed to MCAO followed by reperfusion. This may be associated with inhibiting the TLR4/NF-κB signaling pathway.

Acknowledgements

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL and XC contributed toward study conception and design; XC, ZY, XP and LW contributed toward acquisition of data; HW and YO contributed toward analysis and interpretation of data; XC drafted the initial manuscript and JL revised it

critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments involving animals were approved by the Animal Studies Ethics Committees of the Shenzhen Hospital of Integrated Traditional Chinese and Western Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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