Biochanin A Provides Neuroprotection Against Cerebral Ischemia/Reperfusion Injury by Nrf2-Mediated Inhibition of Oxidative Stress and Inflammation Signaling Pathway in Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCDEF 1 Minmin Guo*
BCDEF 1,2 Huiling Lu*
CEFG 3 Jian Qin
BCDEF 1 Shengbiao Qu
CDEF 4 Wenbo Wang
BCEF 5 Yanhong Guo
CDF 5 Weiyong Liao
BDF 6 Mengwei Song
ACDEFG 1 Jian Chen
ABCDEFG 1,5 Yong Wang

1 Key Laboratory of Tumor Immunology and Microenvironmental Regulation, Guilin Medical University, Guilin, Guangxi, P.R. China
2 Department of Pathology and Physiopathology, Guilin Medical University, Guilin, Guangxi, P.R. China
3 Department of Radiation Oncology of Clinical Cancer Center, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi, P.R. China
4 Department of Neurosurgery, Affiliated Hospital of Guilin Medical University, Guilin, Guangxi, P.R. China
5 Department of Physiology, Guilin Medical University, Guilin, Guangxi, P.R. China
6 Functional Laboratory, Guilin Medical University, Guilin, Guangxi, P.R. China

Corresponding Authors:
Source of support:

* Minmin Guo and Huiling Lu contributed equally to this work
Jian Chen, e-mail: chenjian@glmc.edu.cn, Yong Wang, e-mail: wangyong1105@126.com
This work was supported by the National Natural Science Foundation of China (nos. 81560378 and 81860231), the Guangxi Natural Science Foundation (no. 2015GXNSFAA139197, 2016GXNSFAA380106, and 2017GXNSFDA198019), and the Independent Research Project of Guangxi Key Laboratory of Tumor Immunology and Microenvironmental Regulation (no. 203030301806)

Background:
Oxidative stress and neuroinflammation are 2 pivotal mechanisms in the progression of cerebral ischemia/reperfusion injury. Biochanin A, a natural phytoestrogen, has been reported to protect against ischemic brain injury in animal experiments, but the possible pharmacological mechanisms of its neuroprotection remain elusive. In this research, we sought to investigate the neuroprotective effects of biochanin A in experimental stroke rats and the probable mechanisms underlying oxidative stress and inflammation signaling pathways.

Material/Methods:
An ischemic stroke model was induced by inserting thread into the middle cerebral artery. Rats were pre-administered intraperitoneally with a vehicle solution or biochanin A (10, 20, or 40 mg·kg·d^{-1}) for 14 days prior to ischemic stroke. Neurological score, infarct volume, and cerebral edema were assessed after 2 h of ischemia and 24 h of reperfusion. The activities of SOD and GSH-Px and MDA content were measured. The expressions of Nrf2, HO-1, and NF-κB and the activity of phosphor-IκBα were detected by Western blotting.

Results:
Biochanin A pretreatment significantly improved neurological deficit and decreased infarct size and brain edema. Biochanin A also enhanced SOD and GSH-Px activities and suppressed the production of MDA. Additionally, biochanin A promoted Nrf2 nuclear translocation, promoted the expression of HO-1, and inhibited NF-κB activation in ischemic brain injury.

Conclusions:
The results indicated that biochanin A protected the brain against ischemic injury in rats by anti-oxidative and anti-inflammatory actions. The activation of the Nrf2 pathway and the inhibition of the NF-κB pathway may contribute to the neuroprotective effects of biochanin A.

MeSH Keywords:
NF-E2-Related Factor 2 • Oxidative Stress • Phytoestrogens • Stroke

Full-text PDF:
https://www.medscimonit.com/abstract/index/idArt/918665

📄 3209   ▦ —   📊 6   📑 42

Background

Stroke, including ischemic and hemorrhagic stroke, remains the main cause of death and disability in developed and developing countries [1]. Ischemic stroke is the most prevalent type of stroke and occurs due to decreased or blocked blood flow to brain tissues. Currently, thrombolytic agents and intravascular techniques are the 2 main treatment strategies for acute ischemic stroke [2]. However, there are limitations in the clinical use of thrombolytic agents, such as tissue plasminogen activator (tPA). Intravenous administration of tPA must be restricted to a strict 4.5-h therapeutic time window, and complications from bleeding and fatal edema are the main barriers [3]. Although restoration of blood supply is the goal of acute stroke treatment, it can further aggravate neuronal destruction due to ischemia/reperfusion (I/R) injury [4]. The pathophysiological injury mechanisms of cerebral I/R involve energy failure, the release of excitatory neurotransmitters, damage of the blood-brain barrier, intracellular Ca^{2+} accumulation, oxidative stress, inflammation, and apoptosis [5]. Therefore, developing neuroprotective agents as another promising approach may be an effective strategy for treatment after ischemic stroke [6].

Many recent studies have shown that phytoestrogens provide neuroprotective effects in animal models of cerebral I/R [7–10]. Because of its structural similarity to estrogen, phytoestrogens can selectively bind to estrogen receptors, thus regulating expression of related genes and producing estrogenic or anti-estrogenic effects [11]. Biochanin A, a natural O-methylated isoflavonoid phytoestrogen derived from red clover or chickpea, exhibits broad pharmacological functions such as anti-tumor, anti-oxidation, and hypoglycemic activity [12–15]. Our previous studies have also shown that biochanin A reduced the inflammatory injury of cerebral ischemia/reperfusion by inhibiting the P38MAPK signaling pathway [16].

A growing number of basic and clinical experiments have suggested that cerebral ischemia/reperfusion can cause cellular injury resulting from activation of multiple oxidative stress and inflammatory pathways [17, 18]. Nuclear factor E2-related factor-2 (Nrf2) is an important regulator of the antioxidant cell defense system. Once activated, Nrf2 translocates into the nucleus and activates its target genes through an antioxidant-response element (ARE) [19]. Among the target genes of Nrf2, heme oxygenase-1 (HO-1) is one of the anti-oxidative stress representatives. Currently, HO-1 is considered to protect tissues by repairing redox homeostasis and reducing inflammation. Research has shown that HO-1 expression is controlled by numerous signaling pathways and transcription factors, including Nrf2 and NF-κB [20]. Nevertheless, the detailed function of HO-1 and its role in cerebral I/R injury need to be further investigated.

In this research, we hypothesized that the administration of biochanin A would attenuate oxidative stress and neuroinflammation, and that the potential protective roles of biochanin A are mediated through the Nrf2-ARE pathway.

Material and Methods

Animals and reagents

Male Sprague-Dawley rats (45–50 days old, Grade II, 225–285 g) were purchased from the Experimental Animal Center, Guilin Medical University. All rats were raised in a 12-h light/dark cycle at 24±2°C and were given free access to water and food. The experimental protocols were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the Animal Ethics Committee of Guilin Medical University.

BiochaninA ($C_{16}H_{12}O_4$, molecular weight 284.27, purity more than 98%, and verified by high-performance liquid chromatography) was obtained from Sigma-Aldrich (Saint Louis, MO, USA). Biochanin A powder reagent was dissolved in dimethyl sulfoxide (DMSO) to prepare a mother solution of 200 mM and diluted with saline to a final concentration. The mother solution was stored at 4°C until further use.

Focal cerebral I/R model and neurological scoring

The rats used in this study were randomly assigned to the sham group (saline-treated), the I/R group (saline-treated), or the biochanin A pretreatment I/R groups. The biochanin A pretreatment I/R groups were injected intraperitoneally with biochanin A at low-dose (10 mg·kg·d^{-1}), middle-dose (20 mg·kg·d^{-1}), or high-dose (40 mg·kg·d^{-1}) for 14 days. A model of focal cerebral ischemia was established by middle cerebral artery occlusion (MCAO) as described in previous reports [21,22]. Briefly, the rats were anesthetized with 1.5% pentobarbital (30 mg/kg) at 1 h after the last dose. Then, the right common carotid artery was exposed over a midline neck incision. A monofilament coated with silicon tip (Jialing Biotechnology Co., Guangzhou, China) was inserted into the internal carotid artery through the common carotid artery until a slight resistance was felt, indicating it occluded the blood flow into the middle cerebral artery (MCA). After 2 h of ischemia, the monofilament was gently removed to induce MCA reperfusion, and the animals gradually recovered from anesthesia (Figure 1). In our experiment, the changes in cerebral blood flow during surgery were monitored using laser Doppler equipment (Periflux 5000, Sweden) with a probe positioned at the ipsilateral intact skull overlying the MCA area (2 mm posterior and 4 mm lateral to the bregma). The model rats whose cerebral blood flow was decreased by at least 70% by MCAO and who had quick restoration of blood
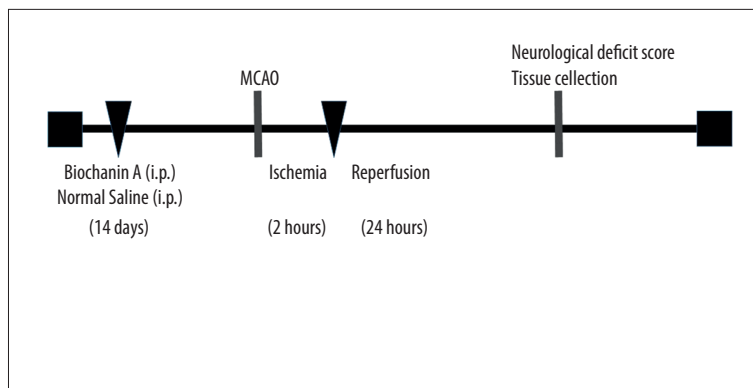
Figure 1. Schematic diagram of the experimental process. The rats were randomly assigned to a sham group (saline-treated), a I/R group (saline-treated), and a biochanin A pretreatment I/R groups at different doses. Normal saline or biochanin A (10 mg·kg·d^{-1}, 20 mg·kg·d^{-1} or 40 mg·kg·d^{-1}) were injected intraperitoneally for 14 days. After the last administration, a model of focal cerebral ischemia was established by middle cerebral artery occlusion (MCAO) for 2 h of ischemia. Subsequently, 24 h of reperfusion was induced by gently removing the monofilament thread. Neurological function was evaluated at 24 h after reperfusion. Then, the rats were sacrificed and brain samples were collected for TTC staining, assessment of brain edema, detection of oxidative stress, qRT-PCR, and Western blotting analysis.

flow after reperfusion were included in the follow-up experiment. The rats in the sham group were subjected to a similar surgical treatment, but monofilament thread was not inserted into the internal carotid artery. During the operation, the body temperature of the rats was maintained at 38±0.5°C with a heating pad.

Neurological function was evaluated at 24 h after reperfusion using the 5-point scoring system described by Longa et al. [23]. The specific scoring method was: 0=no neurologic deficit (normal walk), 1=a mild focal neurologic deficit (failure to extend opposite forepaw fully), 2=a moderate focal neurologic deficit (circling to the contralateral side), 3=a severe focal neurologic deficit (falling to the contralateral side), and 4=no spontaneous walking and a depressed consciousness level. Neurological deficit scoring was carried out by a researcher blinded to the experimental groups.

Infarct size analysis

We used 2,3,5-triphenyltetrazolium chloride (TTC) staining to assess the cerebral infarct volume. The rats were anesthetized and decapitated at 24 h after reperfusion, and the brain samples were removed quickly and kept in a freezer at −20°C for 10 min. Then, the frozen brains were cut into 2-mm-thick coronal sections with brain-cutting matrix, and stained in 2% TTC for 30 min at 37°C. The normal brain tissue was stained deep red, but the infracted area remained unstained. The stained brain slices were photographed, and the infracted sizes were quantified using Image analysis software (Image J, Version 1.50i, National Institutes of Health, Bethesda, MD, USA). The percentage of infarct volume was evaluated by the following formula, as previously described [24]: Percent of hemisphere infarct volume (I%)=[(V$_C$–V$_L$)/V$_C$]×100.

V$_C$ is the volume of normal structure in the control hemisphere and V$_L$ is the volume of normal structure in the lesioned hemisphere. The volume was estimated by summing each area times the distance between sections.

Assessment of the brain edema

Brain water content was determined by wet-dry method. After reperfusion for 24 h, the whole brain of each rat was taken out rapidly and the wet weight was obtained with an electronic analytical balance. The dry weight was obtained again after drying in an oven for 24 h at 100°C. Brain water content was calculated by the following formula: percentage of brain water=[(wet weight–dry weight)/wet weight] ×100%.

Detection of SOD, GSH-Px, and MDA

The brain tissues were washed, weighed, and homogenized with an external ice-cold saline bath. Then, the protein content was determined using a BCA (bicinchoninic acid) protein assay kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The levels of SOD, GSH-Px, and MDA in the ischemic boundary zone were detected with the corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the product manual. The test results were normalized to the protein content and are expressed as U or nmol/mg protein.

Quantitative real-time PCR (qRT-PCR) of HO-1

Total RNA was extracted from the ischemic penumbras zone of rat brain tissues using Trizol reagent (Invitrogen, NY, USA). The cDNA was subsequently synthesized from the total RNA with a Revert Aid First Strand cDNA Synthesis Kit (TIANGEN, Beijing, China) following the product manual. The expression levels of HO-1 mRNA were determined by qRT-PCR with the ABI PRISM 7500 Sequence Detector System (Applied Biosystems,

Carlsbad, CA, USA). The relative amount of OH-1 mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to an endogenous reference gene (β-actin) in all samples. The primer pair used for HO-1 detection was 5'-ACTCAGTTTCCTGTTGGCGA-3' (forward) and 5'-GGGGCCAACACTGCATTTAC-3' (reverse). The primer pair used for β-actin detection was 5'-GCAGGAGTACGATGAGTCCG-3' (forward) and 5'-ACGCAGCTCAGTAACAGTCC-3' (reverse).

Western blotting analysis

Rat brain tissue from the ischemic penumbras zone was collected and homogenized with RIPA lysis buffer. Protein extractions in the nuclear and cytosolic fractions were isolated by multiple centrifugations with a nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The lysate solution was centrifuged at 12 000 rpm for 10 min at 4°C. The protein samples (40 µg/lane) from different groups were separated with SDS-PAGE and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Then, the membranes were blocked with TBST (Tris-buffered solution plus 0.05% Tween 20) containing 5% non-fat milk for 2 h. After blocking, the membranes were incubated on a shaker at 4°C overnight with the following primary antibodies: anti-HO-1 (Santa Cruz Biotechnology, Dallas, TX, USA, 1: 200), anti-Nrf2 (Bioworld, Nanjing, China, 1: 500), anti-NF-κB p65 (Santa Cruz Biotechnology, Dallas, TX, USA, 1: 200), anti-phosphor-IκBα (Abcam, Cambridge, UK, 1: 10000), anti-IκBα (Abcam, Cambridge, UK, 1: 1000), anti-α-Tubulin (ZSGB-Bio, Beijing, China, 1: 200), anti-PCNA (Abbkine, Wuhan, China, 1: 2000), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 1: 2000). The membranes were then incubated with corresponding secondary antibodies coupled to horseradish peroxidase for 1 h, and were detected with an electrochemiluminescence (ECL) reagent kit (Beyotime, China). The density of the bands was evaluated by the ChemiDoc XRS+ system with Image Lab Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCNA, α-Tubulin, or GAPDH were used as a loading control in nuclear, cytosolic, or total protein samples, respectively. The phosphor-IκBα protein was normalized to total IκBα.

Statistical analyses

Data are presented as mean±SEM. SPSS 19.0 software (IBM, Armonk, NY, USA) was applied to statistical analysis. Comparisons between different groups were carried out by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. All experiments were performed at least in triplicate. $P<0.05$ indicates a statistically significant difference.

Results

Biochanin A improved neurological deficits, decreased infarct sizes, and alleviated brain edema in cerebral I/R rats

To determine whether biochanin A pretreatment improves neurological function after I/R injury, we observed the neurological deficit scores at 24 h after reperfusion in rats. As displayed in Figure 2A, no neurological deficits were discovered in the sham group, while severe neurological deficits were found in the I/R group, indicating that the MCAO model was successfully established. Biochanin A treatment markedly decreased the neurological deficit scores dose-dependently compared with that in the I/R group, suggesting that biochanin A improved brain function.

In addition, the infarct volume and water content of brain tissues were assessed in the different groups. TTC staining of brain slices showed that no infarct damage was observed in the sham group, but there was a significant rise of infarct volume in the I/R group. Compared to the I/R group, biochanin A pretreatment markedly reduced the infarct volume (Figure 2B). Accordingly, the brain water content was markedly increased in the I/R group, and was decreased dose-dependently by biochanin A treatment (Figure 2C). These results show that biochanin A had a neuroprotective effect in cerebral I/R rats.

Biochanin A improved antioxidant defenses and reduced lipid peroxidation in cerebral I/R rats

To reveal the mechanisms underlying the neuroprotective effect of biochanin A, we measured the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), as well as the contents of malondialdehyde (MDA), in brain tissues. Figure 3 shows that SOD and GSH-Px activity decreased and MDA level markedly increased in the I/R group. Compared to the I/R group, biochanin A pretreatment significantly increased SOD and GSH-Px activities and reduced MDA level in the brain tissues. These findings suggest that biochanin A suppressed brain oxidative stress after ischemia.

Biochanin A promoted Nrf2 nuclear translocation and increased the expression of HO-1 in cerebral I/R rats

To investigate the biochanin A-induced anti-oxidative effects, we next examined the expressions of Nrf2 and HO-1 in the Nrf2/ARE/HO-1 signaling pathway after I/R injury. Western blot analysis showed that Nrf2 expression was significantly increased in the nucleus but was markedly decreased in the cytoplasm with biochanin A treatment compared with that in the I/R group (Figure 4). The results demonstrated that biochanin A pretreatment induced Nrf2 nuclear localization. In addition,
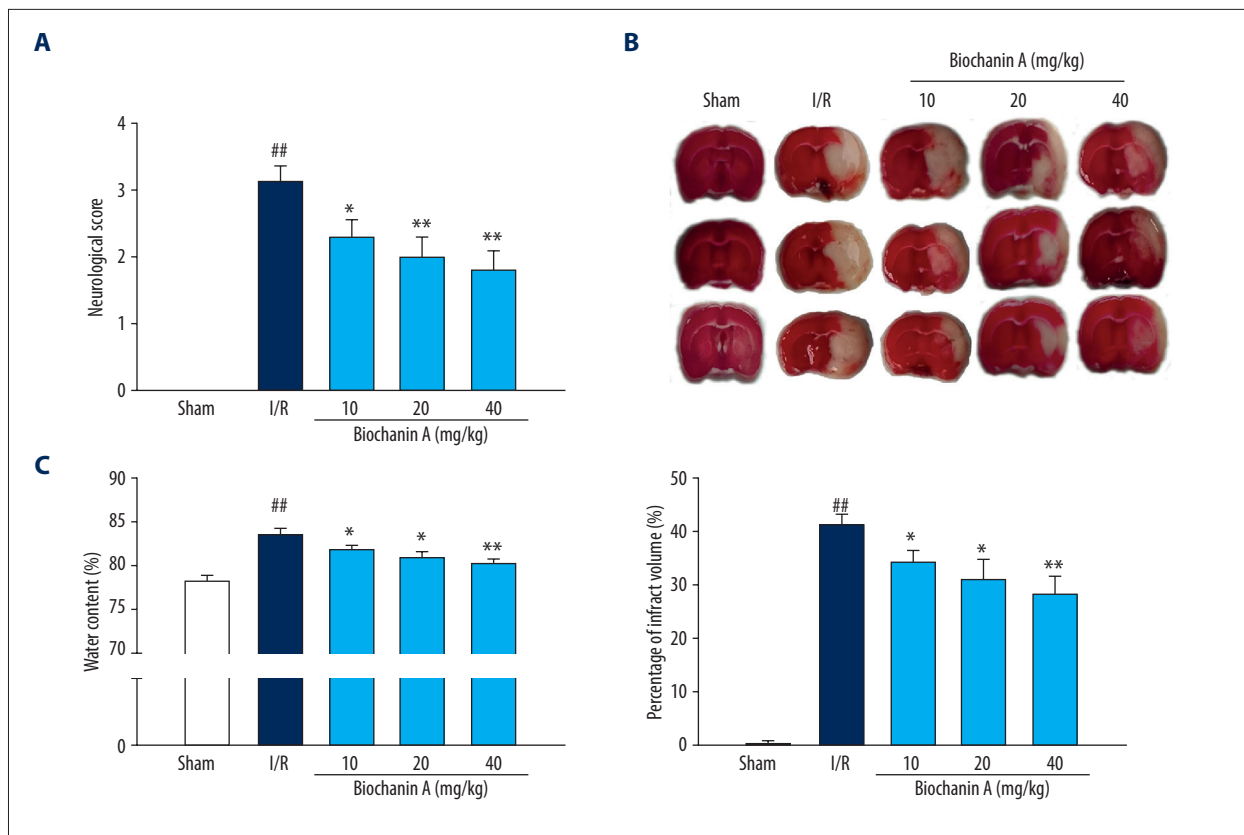
Figure 2. Biochanin A reduced neurological impairment in cerebral I/R rats. (**A**) Neurological deficit score was assessed using the 5-point scoring system after 2 h of ischemia and 24 h of reperfusion. (**B**) Infarct sizes were evaluated using TTC staining. (**C**) Brain edema was measured by standard wet-dry method. Neurological score, water content, and percentage of infarct volume were compared between the sham group and the I/R group (## P<0.01). Differences were significant when compared with the I/R group (* P<0.05, ** P<0.01), n=8.

HO-1, which is a key antioxidant enzyme, was evaluated in the ischemic penumbras zone of rat brain tissues. There was an increase in expression levels of HO-1 mRNA and protein following ischemia reperfusion, and biochanin A pretreatment augmented the MCAO-induced expression of HO-1 in a dose-dependent manner (Figure 5).

Biochanin A inhibited NF-κB activation in cerebral I/R rats

To further elucidate the potential mechanism of biochanin A in cerebral ischemia protection, we next investigated whether biochanin A affects the NF-κB signaling pathway in cerebral ischemia. The expression of NF-κB p65 and the activity of phosphor-IκBα were assessed by Western blotting. As shown in Figure 6A, phosphorylation of IκBα was significantly higher in the I/R group than in the sham group. In the biochanin A-treated groups, phosphorylation of IκBα was dose-dependently reduced compared with the I/R group. In addition, NF-κB p65 was mainly expressed in cytoplasm in the sham group and NF-κB p65 was predominately expressed in the nucleus in the I/R group, indicating the nuclear translocation of NF-κB p65 in

MCAO-induced ischemia. Biochanin A pretreatment significantly prevented nuclear translocation in a dose-dependent manner (Figure 6B). These results indicate that biochanin A inhibited NF-κB activation and translocation in ischemic brain tissues.

Discussion

Previous reports have demonstrated that estradiol and phytoestrogen mitigate ischemic stroke-induced damages by binding to the estrogen receptors [25–27]. However, the potential neuroprotective mechanisms of these compounds need to be studied further. In the present study, biochanin A, a typical phytoestrogen, was shown to improve neurological effect and to reduce infarct volume and brain edema in cerebral I/R rats. Moreover, biochanin A treatment suppressed brain oxidative stress levels, promoted Nrf2 nuclear translocation, increased the expression of HO-1, and inhibited activation of the NF-κB pathway in ischemic brain tissues. These findings suggest that biochanin A provides neuroprotection through regulation of oxidative stress and inflammation pathways.
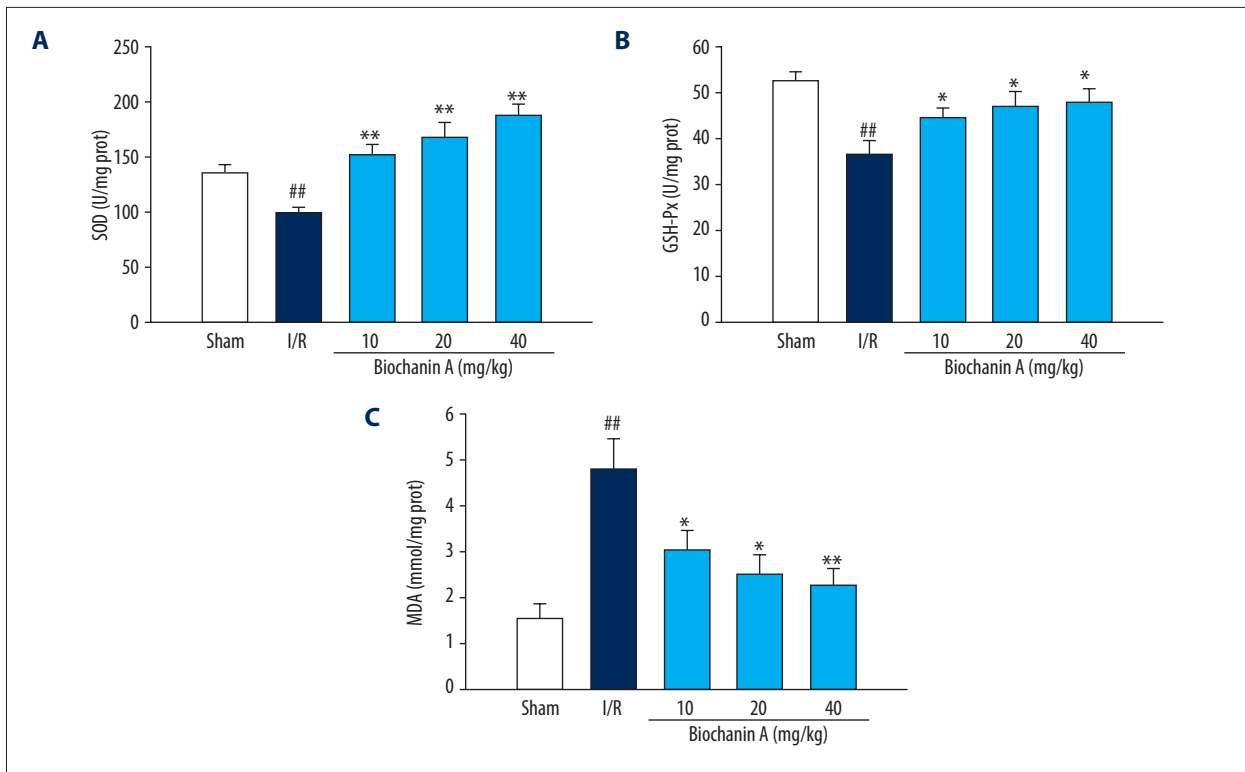
Figure 3. Biochanin A attenuated oxidative stress in cerebral I/R rats. After 2 h of ischemia and 24 h of reperfusion, the activities of SOD (**A**), GSH-Px (**B**), and MDA (**C**) contents in the ischemic boundary zone were measured. The levels of SOD, GSH-Px, and MDA were compared between the sham group and the I/R group (## P<0.01). Differences were significant when compared with the I/R group (* P<0.05, ** P<0.01), n=8.
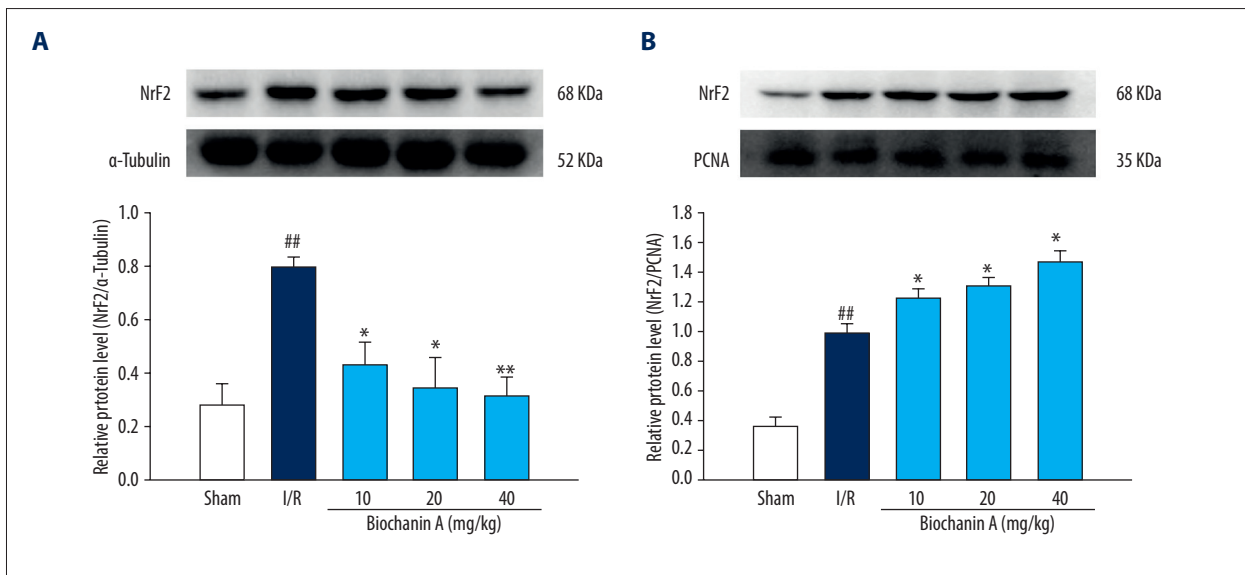
Figure 4. Biochanin A induced Nrf2 nuclear translocation in cerebral I/R rats. Protein extractions in the cytosolic and nuclear fractions from the ischemic penumbras zone of rat brain tissues were isolated, and the expression of Nrf2 was determined by Western blotting at 24 h after reperfusion. Representative Western blots and protein expression of Nrf2 in the cytosolic fractions (**A**) and nuclear fractions (**B**) were analyzed. α-Tubulin and PCNA were used as a loading control in cytosolic protein and in nuclear protein. The protein expression of Nrf2 were compared between the sham group and the I/R group (## P<0.01). Differences were significant when compared with the I/R group (* P<0.05, ** P<0.01), n =3.
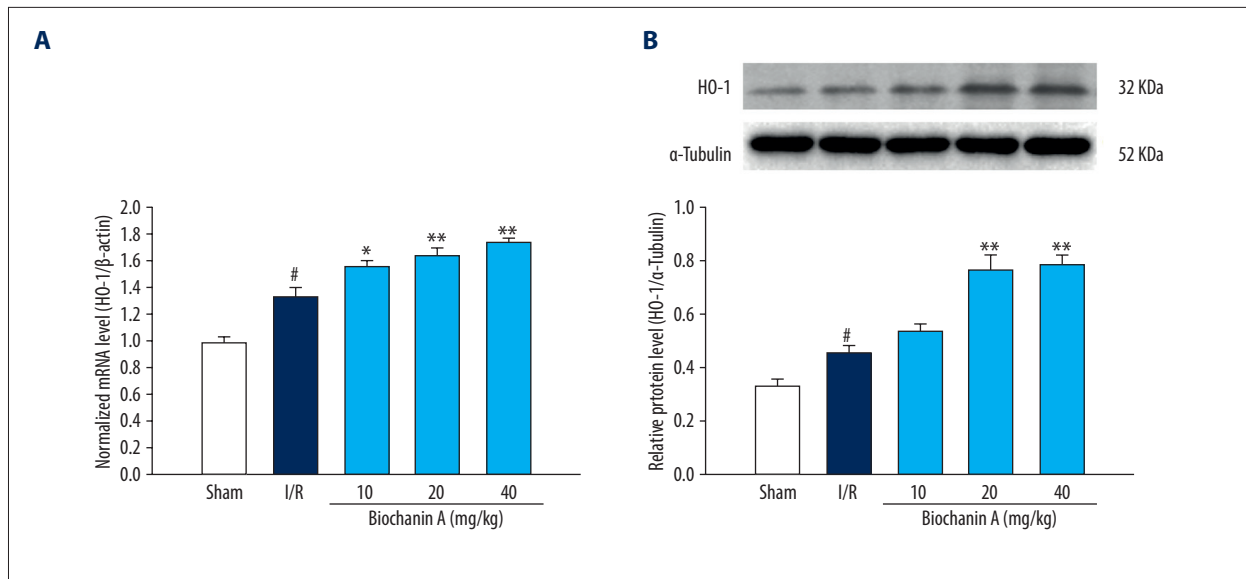
Figure 5. The effects of biochanin A on expression levels of HO-1 mRNA (**A**) and protein (**B**) in cerebral I/R rats. The mRNA and protein extracted from the ischemic penumbra cortex was analyzed by Western blotting at 24 h after reperfusion. The mRNA expression of HO-1 was normalized to β-actin, and the protein expression of HO-1 was normalized to α-Tubulin. The mRNA and protein expression of HO-1 were compared between the sham group and the I/R group (# P<0.05); Differences were significant when compared with the I/R group (* P<0.05, ** P<0.01), n=3.
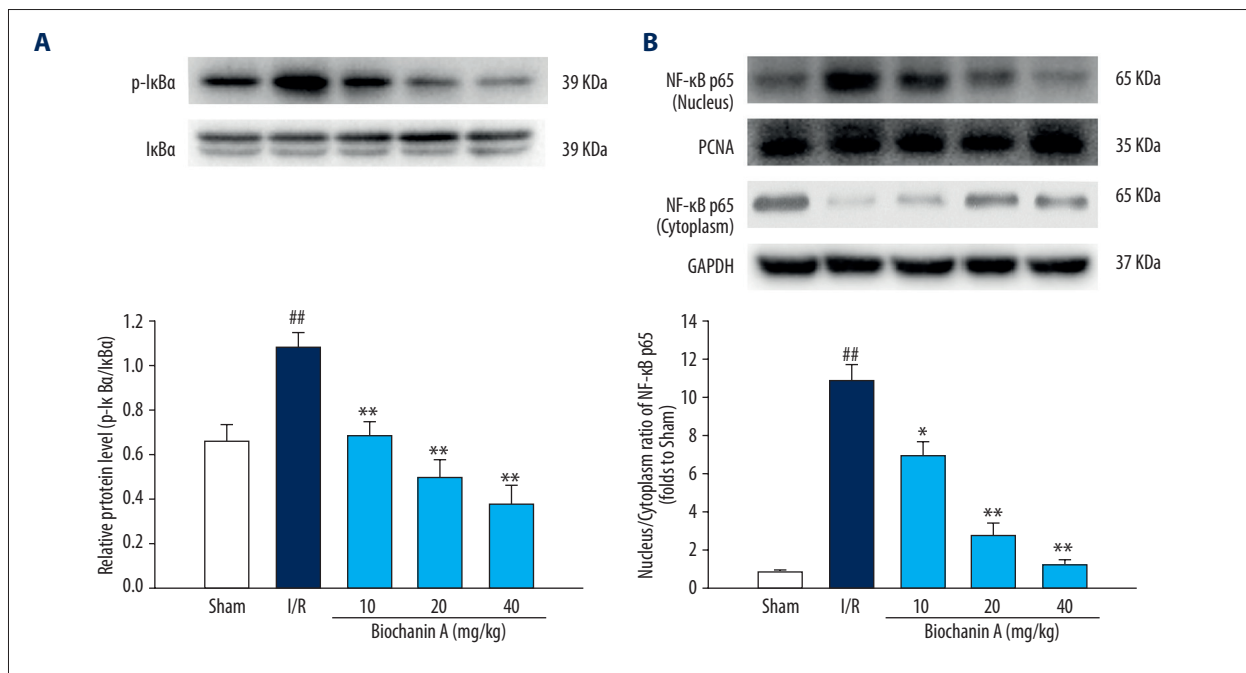
Figure 6. The effects of biochanin A on the activity of IκBα (**A**) and NF-κB p65 translocation (**B**) in I/R rats. Protein were extracted from the ischemic penumbra cortex at 24 h after reperfusion. The nucleus and cytoplasm fractions were isolated and used for examining the nuclear translocation of NF-κB p65. The activity of IκBα and the protein expression level of NF-κB p65 were determined by Western blotting. The ratio of phosphor-IκBα to total IκBα was used to evaluate protein activity, and nucleus/cytoplasm ratio of NF-κB p65 was used for quantitative analysis of nuclear translocation. PCNA was the internal control of nuclear fraction, and GAPDH was the internal control of cytoplasm fraction. The nucleus/cytoplasm ratio was normalized to their own internal control. The activity of IκBα and NF-κB p65 translocation were compared between the sham group and the I/R group (## P<0.01). Differences were significant when compared with the I/R group (* P<0.05, ** P<0.01), n=3.

Oxidative stress is mainly caused by excess production of re-active oxygen species (ROS) and is crucial in cerebral I/R in-jury. In organisms, antioxidant enzymes such as SOD, GSH-Px, and catalase can inhibit oxygen free radical production and protect brain tissue against ROS cytotoxicity [28]. Additionally, excessive ROS cause lipid peroxidation and produce active al-dehydes, including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein [29]. MDA, which is an important marker of oxidative stress, induces more serious oxidative stress dam-age by destroying lipids, enzymes, and nucleic acids in biolog-ical membranes or organelles. In the present study, we found that biochanin A significantly enhanced the activity of SOD and GSH-Px antioxidant enzymes, and reduced MDA levels. These findings revealed that biochanin A effectively prevents isch-emic brain injury caused by ROS-mediated oxidative damage.

Nrf2 is an important transcription factor inducing the expres-sion of antioxidant proteins such as SOD, catalase (CAT), and HO-1 by binding to antioxidant-response elements (ARE) [30]. It is known that the upregulation of these enzymes due to Nrf2 transcription results in the cellular defense against oxidative stress triggered by hypoxia, inflammation, and other injury. HO-1 is an enzyme that catalyzes the breakdown of pro-oxi-dant heme into the antioxidant biliverdin, carbon monoxide, and iron. The gene expression of HO-1 is also regulated by transcription factor Nrf2. Under normal circumstances, Nrf2 is mainly located in the cytoplasm and binds to Keap1 (Kelch-like ECH associated protein 1, also termed inhibitor of Nrf2). When oxidative stress occurs, Nrf2 is dissociated from Nrf2-Keap1 complex and is translocated to the nuclei, activating the expression of its target gene, HO-1 [31]. It has been reported that the neuroprotective effect of Nrf2 activators is weakened when HO-1 expression is inhibited, and gene knockout of Nrf2 abolishes the upregulation of HO-1 [32]. In addition, research-ers have reported that upregulation of HO-1 in brain tissue following ischemic preconditioning protects against cerebral ischemic injury [33], while the protective effects were weak-ened after treatment with the HO-1 inhibitor ZnPP [34]. Our data show that biochanin A pretreatment markedly increased Nrf2 nuclear translocation and upregulated expression of its downstream gene, HO-1. These results indicate that the acti-vation of the Nrf2/HO-1 pathway can increase the activity of SOD and GSH-Px antioxidant enzymes induced by biochanin A.

Given the interaction between oxidative stress and neuroin-flammation, the Nrf2/HO-1 signaling pathway may also regu-late anti-inflammatory gene expression and inhibit progression of inflammation [35]. NF-κB is a central regulator of inflamma-tory response, and activation of the NF-κB signaling pathway is required for transcriptional induction of many inflammatory mediators in neuroinflammation. The most common form of NF-κB is a heterodimer of the p50 and p65 (RelA) subunits. The canonical NF-κB signaling pathway is generally activated

in response to various stimuli, including cytokines, bacterial toxins, and oxygen-glucose deprivation. The initiation of this pathway depends on the phosphorylation of inhibitor of κB kinase (IKK) complex, which is composed of the kinases IKKα, IKKβ, and IKKγ. Activated IKK-complex phosphorylates the in-hibitory subunit IκBα (Inhibitor κBα), promoting IκBα degra-dation by the proteasome and inducing the transformation of p105 into its mature form, p50. Then, p65/p50 heterodimers are released from the inhibitor IκBα and translocated into the nu-cleus, where they bind to the DNA κB sites to induce transcrip-tion of NF-κB-targeted genes [36]. Recent studies have shown that many upstream molecules in cerebral ischemia reperfu-sion can regulate the NF-κB signaling pathway, including silent information regulator 1 (SIRT1), signal transducers, and acti-vators of transcription (STATs) [37,38]. In addition, there have been reports that the absence of Nrf2 can exacerbate NF-κB activity, leading to increased cytokine production [39,40], and NF-κB also can regulate Nrf2-mediated ARE expression, sug-gesting that the cross-talk between NF-κB activation and Nrf-2 inhibition results in neuroinflammation [41,42]. Therefore, the mutual promotion of neuroinflammation and oxidative stress can induce the further development of cerebral ischemia reper-fusion injury. In our study, we found that biochanin A markedly inhibited the activation of IκBα and the nuclear translocation of NF-κB in ischemic brain tissue. These results indicate that the ameliorative effect of biochanin A in neuroinflammation and oxidative stress may also be partly related to inhibition of the NF-κB signaling pathway. Our study, however, has po-tential limitations. First, our pretreatment strategy is intended for prevention, not therapy, and it would have been of greater clinical value if the biochanin A treatment had been adminis-tered after establishment of the MCAO model in the experi-ment. Second, we merely observed changes of the Nrf2/HO-1 and NF-κB signaling pathway in ischemia/reperfusion brain injury. Finally, the details of the relationship between oxida-tive stress and neuroinflammation should be explored further.

Conclusions

Our findings suggest that biochanin A is a therapeutic can-didate for ischemic stroke because of its anti-oxidative and anti-inflammatory properties. It provides promising targets for attenuating neurological impairment in stroke. However, whether biochanin A directly targets Nrf2 or NF-κB signaling molecules requires further investigation, and *in vivo* and *in vi-tro* experiments are needed to elucidate the specific mecha-nisms involved.

Conflict of interest

None.

References:

1. Pandian JD, Gall SL, Kate MP et al: Prevention of stroke: A global perspective. Lancet, 2018; 392: 1269–78

2. Khandelwal P, Yavagal DR, Sacco RL: Acute ischemic stroke intervention. J Am Coll Cardiol, 2016; 67: 2631–44

3. Prabhakaran S, Ruff I, Bernstein RA: Acute stroke intervention: A systematic review. JAMA, 2015; 313: 1451–62

4. Nour M, Scalzo F, Liebeskind DS: Ischemia-reperfusion injury in stroke. Interv Neurol, 2013; 1: 185–99

5. Durukan A, Tatlisumak T: Acute ischemic stroke: Overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav, 2007; 87: 179–97

6. Ginsberg MD: Neuroprotection for ischemic stroke: Past, present and future. Neuropharmacology, 2008; 55: 363–89

7. Wang S, Wei H, Cai M et al: Genistein attenuates brain damage induced by transient cerebral ischemia through up-regulation of erk activity in ovariectomized mice. Int J Biol Sci, 2014; 10: 457–65

8. Wang Y, Ren Q, Zhang X et al: Neuroprotective mechanisms of calycosin against focal cerebral ischemia and reperfusion injury in rats. Cell Physiol Biochem, 2018; 45: 537–46

9. Li Z, Wang Y, Zeng G et al: Increased mir-155 and heme oxygenase-1 expression is involved in the protective effects of formononetin in traumatic brain injury in rats. Am J Transl Res, 2017; 9: 5653–61

10. Khanna S, Stewart R, Gnyawali S et al: Phytoestrogen isoflavone intervention to engage the neuroprotective effect of glutamate oxaloacetate transaminase against stroke. FASEB J, 2017; 31: 4533–44

11. Lecomte S, Demay F, Ferriere F, Pakdel F: Phytochemicals targeting estrogen receptors: Beneficial rather than adverse effects? Int J Mol Sci, 2017; 18(7): pii: E1381

12. Youssef MM, Tolba MF, Badawy NN et al: Novel combination of sorafenib and biochanin-a synergistically enhances the anti-proliferative and pro-apoptotic effects on hepatocellular carcinoma cells. Sci Rep, 2016; 6: 30717

13. Wang J, He C, Wu WY et al: Biochanin a protects dopaminergic neurons against lipopolysaccharide-induced damage and oxidative stress in a rat model of Parkinson's disease. Pharmacol Biochem Behav, 2015; 138: 96–103

14. Harini R, Ezhumalai M, Pugalendi KV: Antihyperglycemic effect of biochanin a, a soy isoflavone, on streptozotocin-diabetic rats. Eur J Pharmacol, 2012; 676: 89–94

15. Moon YJ, Shin BS, An G, Morris ME: Biochanin a inhibits breast cancer tumor growth in a murine xenograft model. Pharm Res, 2008; 25: 2158–63

16. Wang W, Tang L, Li Y, Wang Y: Biochanin a protects against focal cerebral ischemia/reperfusion in rats via inhibition of p38-mediated inflammatory responses. J Neurol Sci, 2015; 348: 121–25

17. Manzanero S, Santro T, Arumugam TV: Neuronal oxidative stress in acute ischemic stroke: Sources and contribution to cell injury. Neurochem Int, 2013; 62: 712–18

18. Drieu A, Levard D, Vivien D, Rubio M: Anti-inflammatory treatments for stroke: From bench to bedside. Ther Adv Neurol Disord, 2018; 11: 1756286418789854

19. Kobayashi M, Yamamoto M: Molecular mechanisms activating the nrf2-keap1 pathway of antioxidant gene regulation. Antioxid Redox Signal, 2005; 7: 385–94

20. Ferrandiz ML, Devesa I: Inducers of heme oxygenase-1. Curr Pharm Des, 2008; 14: 473–86

21. Guo H, Li MJ, Liu QQ et al: Danhong injection attenuates ischemia/reperfusion-induced brain damage which is associating with nrf2 levels *in vivo* and *in vitro*. Neurochem Res, 2014; 39: 1817–24

22. Yang Y, Jiang S, Dong Y et al: Melatonin prevents cell death and mitochondrial dysfunction via a sirt1-dependent mechanism during ischemic-stroke in mice. J Pineal Res, 2015; 58: 61–70

23. Longa EZ, Weinstein PR, Carlson S, Cummins R: Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke, 1989; 20: 84–91

24. Swanson RA, Morton MT, Tsao-Wu G et al: A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab, 1990; 10: 290–93

25. Schreihofer DA, Redmond L: Soy phytoestrogens are neuroprotective against stroke-like injury *in vitro*. Neuroscience, 2009; 158: 602–9

26. Wang Y, Dong X, Li Z et al: Downregulated rasd1 and upregulated mir-375 are involved in protective effects of calycosin on cerebral ischemia/reperfusion rats. J Neurol Sci, 2014; 339: 144–48

27. Ma Y, Sullivan JC, Schreihofer DA: Dietary genistein and equol (4', 7 isoflavandiol) reduce oxidative stress and protect rats against focal cerebral ischemia. Am J Physiol Regul Integr Comp Physiol, 2010; 299: R871–77

28. Sun MS, Jin H, Sun X et al: Free radical damage in ischemia-reperfusion injury: An obstacle in acute ischemic stroke after revascularization therapy. Oxid Med Cell Longev, 2018; 2018: 3804979

29. Ayala A, Munoz MF, Arguelles S: Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev, 2014; 2014: 360438

30. Ishii T, Itoh K, Takahashi S et al: Transcription factor nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem, 2000; 275: 16023–29

31. Silva-Islas CA, Maldonado PD: Canonical and non-canonical mechanisms of nrf2 activation. Pharmacol Res, 2018; 134: 92–99

32. Shi H, Jing X, Wei X et al: S-allyl cysteine activates the nrf2-dependent antioxidant response and protects neurons against ischemic injury *in vitro* and *in vivo*. J Neurochem, 2015; 133: 298–308

33. Zeynalov E, Shah ZA, Li RC, Dore S: Heme oxygenase 1 is associated with ischemic preconditioning-induced protection against brain ischemia. Neurobiol Dis, 2009; 35: 264–69

34. Le LL, Li XY, Meng D et al: Heme oxygenase-1 mediated memorial and revivable protective effect of ischemic preconditioning on brain injury. CNS Neurosci Ther, 2013; 19: 963–68

35. Ahmed SM, Luo L, Namani A et al: Nrf2 signaling pathway: Pivotal roles in inflammation. Biochim Biophys Acta Mol Basis Dis, 2017; 1863: 585–97

36. Mincheva-Tasheva S, Soler RM: Nf-kappab signaling pathways: Role in nervous system physiology and pathology. Neuroscientist, 2013; 19: 175–94

37. Yang Y, Duan W, Li Y et al: New role of silent information regulator 1 in cerebral ischemia. Neurobiol Aging, 2013; 34: 2879–88

38. Liang Z, Wu G, Fan C et al: The emerging role of signal transducer and activator of transcription 3 in cerebral ischemic and hemorrhagic stroke. Prog Neurobiol, 2016; 137: 1–16

39. Li W, Khor TO, Xu C et al: Activation of nrf2-antioxidant signaling attenuates nfkappab-inflammatory response and elicits apoptosis. Biochem Pharmacol, 2008; 76: 1485–89

40. Khor TO, Huang MT, Kwon KH et al: Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis. Cancer Res, 2006; 66: 11580–84

41. Negi G, Kumar A, Sharma SS: Nrf2 and nf-kappab modulation by sulforaphane counteracts multiple manifestations of diabetic neuropathy in rats and high glucose-induced changes. Curr Neurovasc Res, 2011; 8: 294–304

42. Ganesh Yerra V, Negi G, Sharma SS, Kumar A: Potential therapeutic effects of the simultaneous targeting of the nrf2 and nf-kappab pathways in diabetic neuropathy. Redox Biol, 2013; 1: 394–97