Research Report

Senkyunolide I protects rat brain against focal cerebral ischemia–reperfusion injury by up-regulating p-Erk1/2, Nrf2/HO-1 and inhibiting caspase 3

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Abstract
Oxidative damage and apoptosis are critical factors contributing to neuronal death during a stroke. The aim of the present study was to evaluate the neuroprotective effects of senkyunolide I (SEI) on focal cerebral ischemia–reperfusion (I/R) injury in rats, and investigate the underlying mechanisms. Male Sprague-Dawley rats were subjected to transient middle cerebral artery occlusion (tMCAO) for 2 h, followed by 24 h reperfusion, and then randomly assigned into four groups: Sham (sham-operated), Vehicle (tMCAO + normal saline), SEI-L (tMCAO + SEI 36 mg/kg) and SEI-H (tMCAO + SEI 72 mg/kg) groups. SEI was administered intravenously, 15 min after occlusion. Neurological deficit, brain edema and infarct volume were detected after 24 h of reperfusion. Histological structures of cortices and hippocampus were observed by hematoxylin and eosin staining. Biochemical indexes in the cortex were assayed by colorimetry. The impact of SEI on the Nrf2-ARE-interaction was assayed using a luciferase reporter gene. Western blotting was performed to analysis the expressions of proteins related to anti-oxidation and apoptosis. SEI administration significantly ameliorated the neurological deficit, reduced the infarct volume and brain edema, reversed the cerebral morphologic damage, decreased the levels of MDA and increased the activities of superoxide dismutase. Furthermore, the high dose...
SEI could significantly activate the Nrf2/ARE pathway by up-regulating the phosphorylation of Erk1/2 and inducing Nrf2 nuclear translocation with enhanced HO-1 and NQO1 expressions. Additionally, treatment with SEI remarkably promoted the ratio of Bcl-2/Bax and inhibited the expressions of cleaved caspase 3 and caspase 9. These results suggest that the neuroprotective mechanisms of SEI are associated with its anti-oxidation and anti-apoptosis properties.

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1. Introduction

Stroke is one of the most serious cerebrovascular diseases, leading to significant mortality and morbidity worldwide (Donnan et al., 2008; Mandel et al., 2012). Despite the advanced intravascular procedures, the most commonly used treatment currently for ischemic stroke is the administration of chemical drugs, including thrombolytic agents, tissue-plasminogen activator and calcium ion antagonists (Ginsberg, 2008). However, the short time window for patients, as well as the frequent side effects of the drugs, seems to constraint their clinical efficacy, especially for long-term use (Goldstein and Rothwell, 2008).

Strokes are mostly caused by transient cerebral ischemia induced by obstruction of blood flow to the brain, causing the disturbance in the energy metabolism and leading to the excessive formation of reactive oxygen species (ROS), ultimately resulting in oxidative stress (Chan, 2004; Uzar et al., 2012). Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor, is considered as an important cytoprotective regulator against oxidative stress. The activation of Nrf2 can up-regulate the expression of several antioxidant enzymes and phase II detoxifying enzymes, such as hemeoxygenase-1 (HO-1), superoxide dismutase 1 (SOD1), glutathione S-transferase (GSTs) and quinone oxidoreductase 1 (NQO1), after binding to the antioxidant response element (ARE) (Liu et al., 2004; Shah et al., 2007). Furthermore, extracellular signal regulated kinase (ERK) activates Nrf2 under oxidative conditions (Zipper and Mulcahy, 2003). Accumulative evidence has revealed that ROS produced during ischemia not only contribute to oxidative damage, but also activate apoptotic cascades by disrupting mitochondrial function (Chan et al., 1998).

Senkyunolide I (SEI, Fig. 1A) is derived from the Chinese herb *Ligusticum chuanxiong* Hort., which has been widely used for the treatment of apoplexy and migraine in China (Jia et al., 2009). SEI could protect cultured PC12 cells and human liver HepG2 cells from oxidative damage induced by hydrogen peroxide (Qi et al., 2010; Tang et al., 2009). SEI also slightly reversed morphological changes of red blood cells induced by concanavalin A (Hong et al., 2003). Our previous research showed that SEI was a major constituent detected simultaneously in plasma and cerebrospinal fluid in the rat migraine model. Moreover, SEI was rapidly absorbed and penetrated through blood-brain barrier, as well as the blood-cerebrospinal fluid barrier (Yuan et al., 2010). Subsequently, a pharmacodynamic study of SEI demonstrated its anti-migraine effect (Wang et al., 2011), although the exact mechanism was unknown. Intriguingly, SEI is a primary metabolite of Z-ligustilide (Fig. 1B), which protects against cerebral ischemia-reperfusion injury in vivo and in vitro by activating the Nrf2/HO-1 pathway (Peng et al., 2013). Despite its better stability and bioavailability compared with Z-ligustilide (Yan et al., 2008), no information is available with respect to the possible neuroprotective effect of SEI on cerebral I/R injury.

Herein, the aim of present study was to evaluate the potential protective effect of SEI on focal cerebral I/R injury following 2 h of occlusion of the middle cerebral artery (MCA) and 24 h reperfusion in rats, and to investigate whether the therapeutic benefits of SEI were associated with anti-oxidation and anti-apoptosis.

2. Results

2.1. Senkyunolide I improved neurological deficits

Neurological deficit was examined and scored on a 6-point scale 24 h after reperfusion. As shown in Fig. 2A, rats in Vehicle group got higher scores than in Sham group. Compared with Vehicle group, the scores were significantly reduced in SEI-H group (P < 0.05). By contrast, there was also alleviation in motor function in SEI-L group, but without a significant level (P > 0.05).

2.2. Senkyunolide I attenuated brain edema

Brain water contents in ipsilateral and contralateral hemispheres were shown in Fig. 2B. In Sham group, brain water content in ipsilateral was 78.37% ± 0.37%, while it increased to 86.12% ± 1.67% after 24 h reperfusion following 2 h MCAO in Vehicle group. The two doses of SEI attenuated brain edema in ipsilateral hemisphere. The brain water content was markedly reduced in SEI-H group compared with Vehicle group (SEI-H: 82.30% ± 0.91%, P < 0.05). But no significant decrease was found in SEI-L group (SEI-L: 85.35% ± 1.29%, P > 0.05).

![Fig. 1 – The chemical structure of (A) senkyunolide I; (B) butylphthalide; (C) Z-ligustilide.](image-url)
2.3. Senkyunolide I reduced infarct volume

No infarction was observed in sham operated group, while extensive lesion was developed in striatum and lateral cortex in Vehicle group (Fig. 2C). Compared with Vehicle group, the infarct volume was significantly reduced in both SEI-L (42.20% \(\pm\) 8.62% vs. 35.70% \(\pm\) 5.35%) and SEI-H groups (42.20% \(\pm\) 8.62% vs. 27.31% \(\pm\) 2.40%) \((P<0.05)\) (Fig. 2D).

2.4. Senkyunolide I recovered neuronal morphologic damages

The histopathological changes in rats’ cerebral cortices and hippocampus were evaluated after 24 h reperfusion following 2 h MCAO with HE staining. As shown in Fig. 3, the pyramidal neurons in Sham group were tightly aligned with distinct nucleoli and moderate size. And those in Vehicle group exhibited obvious pathological abnormalities with loosed arranged neurons, pycnotic nucleus and loss or dark color staining. By contrast, these histopathological alterations was dramatically reduced by SEI at the high dose, while a slighter reduction in cortical neuron damage by SEI was observed at the low dose.

2.5. Senkyunolide I influenced the biochemical indexes in rats with I/R injury

As shown in Fig. 4A, the MDA level of Vehicle group rats was enhanced to 4.09 \(\pm\) 1.77 nmol/mg protein compared with that of Sham group 2.06 \(\pm\) 0.08 nmol/mg protein. In contrast to I/R group, the treatment with both low and high dose of SEI (36 mg/kg and 72 mg/kg) could diminish the MDA contents significantly \((P<0.05)\) vs. Vehicle group. Meanwhile, lower activities of SOD were detected in Vehicle group (74.66 \(\pm\) 14.51 U/mg protein) compared with that of Sham group (120.98 \(\pm\) 15.66 U/mg protein) from the data in Fig. 4B. Treatment with SEI (36 mg/kg and 72 mg/kg) could improve the enzymatic activities of SOD obviously \((P<0.05)\) vs. Vehicle group. There were no significant differences between these two SEI groups on MDA and SOD levels.

2.6. Senkyunolide I promoted p-Erk1/2/t-Erk1/2, c-Nrf2, n-Nrf2, HO-1 and NQO1 expressions

We utilized reporter gene technique to investigate the potential influence of SEI on Nrf2/ARE pathway by measuring the luciferase activity regulated by ARE in vitro. As shown in Fig. 5A, the positive compound (5 \(\mu\)M DL-sulforaphane) and various concentrations (25–100 \(\mu\)M) of SEI exerted no significant impact on cell viability and LDH leakage compared with Controls \((P>0.05)\). The fluorescence intensities of the cells were obviously enhanced in positive group (C) and SEI groups at the concentrations of 50 \(\mu\)M (E) and 100 \(\mu\)M (F) compared with that of control group (B). The data from Fig. 5G demonstrated that treatment with SEI could improve the luciferase activity in a concentration-dependent manner within a certain range. And a significant difference was observed between 100 \(\mu\)M SEI group and control group \((P<0.05)\).
Western blotting analysis was further conducted to investigate the possible effect of SEI on Erk/Nrf2/ARE signaling pathway. The results showed that the protein levels of c-Nrf2, n-Nrf2 and NQO1 were increased after I/R injury, while the expressions of p-Erk1/2/t-Erk1/2 and HO-1 were decreased. Meanwhile, the ratio of p-Erk1/2/t-Erk1/2 and the expressions of c-Nrf2, n-Nrf2, HO-1 and NQO1 were remarkably increased in both SEI-L and SEI-H groups (P<0.05), and SEI-H group got a higher level than SEI-L (P<0.05, Fig. 6A-C). The ratio of n-Nrf2/c-Nrf2 was promoted by both dose of SEI (P<0.05) and high dose raised it to a higher level (P<0.05) (Fig. 6F).

2.7. **Senkyunolide I regulated the expressions of Bcl-2, Bax and cleaved caspase 3, caspase 9**

In this study, the expression changes of Bcl-2 and Bax were detected. Compared with Sham group, I/R injury decreased the expression of Bcl-2 and increased the expression of Bax.
However, administration of SEI (36 mg/kg and 72 mg/kg) significantly reversed the down-regulation of Bcl-2 and the up-regulation of Bax (P<0.05, Fig. 6D). Moreover, treatment with high dosage of SEI more effectively down-regulated Bax protein level than that of SEI-L group (P<0.05). Thus, both SEI-L group and SEI-H group markedly elevated the ratio of Bcl-2/Bax (P<0.05, Fig. 6F).

In addition, the expressions of cleaved caspase-3 and caspase-9 were also examined. Fig. 6E showed that the protein levels of cleaved caspase-3 and caspase-9 were both promoted in Vehicle group compared with Sham group. Administration of SEI (36 mg/kg and 72 mg/kg) significantly down-regulated cleaved caspase-3 and caspase-9 protein expressions (P<0.05). However, there were no significant differences between SEI-L group and SEI-H group (P>0.05).

3. Discussion

Phthalides, a group of important components present in L. chuanxiong Hort., possess distinct biological activities, such as anti-migraine, anti-platelet aggregation and anti-thrombosis effects, and have actions on the central nervous system and cardiac function modulation, inhibition of smooth muscle cell proliferation and protection against cerebral ischemia. Among these phthalides, the cerebral protective effect and the underlying mechanism of butylphthalide and Z-ligustilide have been widely studied (Liu et al., 2007; Peng et al., 2013). SEI, a metabolite of Z-ligustilide (Yan et al., 2008) with better solubility and stability, higher bioavailability and faster penetration of blood-brain-barrier, has not been studied extensively concerning its actions on cerebral diseases, although some cellular studies have suggested SEI’s protective effects (Qi et al., 2010; Hong et al., 2003). The present investigation demonstrated the beneficial effects of SEI on I/R injury in rats induced by tMCAO for 2 h and reperfusion for 24 h, for the first time. The intravenous administration of SEI 15 min after MCAO could reduce neurological scores, lessen brain water content and infarct volume, and recover the neural structure.

Oxidative stress is the main pathogenesis involved in brain injury caused by reduced cerebral blood flow with subsequent reperfusion. In vivo, endogenous antioxidant enzymes like SOD, GSH-PX and GSH can scavenge normal amounts of ROS (Keller et al., 1998). Nevertheless, excess ROS damage lipids, generating cytotoxic MDA via lipid peroxidation, which sometimes induces cross-linked polymerization of macromolecules, such as proteins and nucleic acids (Schettler et al., 1999). Our results showed decreased formation of MDA as well as elevated activities of SOD in SEI-treated groups, suggesting that SEI could attenuate oxidative stress via simultaneous up-regulation of defense enzymes and reductions in lipid peroxidation.

Nrf2, a cap ‘n’ collar (CNC) transcription factor, is a vital endogenous regulator in neuronal anti-oxidative stress defense responses (Zhao et al., 2011). In normal non-activated conditions, Nrf2 interacts with Kelch like ECH-associated protein1 (Keap 1). Upon activation, Nrf2 separates from Keap 1, translocates into the nucleus and binds to a specific sequence in a promoter termed ARE, coordinately up-regulating the expressions of several antioxidant and detoxification genes, including HO-1, SOD, GST and NQO1 (Kensaler et al., 2007). Thus, the Nrf2/ARE pathway may represent a potential therapeutic target for neuroprotection. Based on the importance of the Nrf2/ARE pathway, we preliminarily employed a reporter gene technique to explore the relationship between SEI’s anti-oxidative effect and the Nrf2/ARE pathway in vitro. Briefly, a recombinant plasmid vector pGF1-ARE-Neo was constructed to express reporter gene luciferase regulated by the promoter ARE, which was then transfected into Hek293T cells (human embryonic kidney epithelial cells). The fluorescence produced, accompanied by oxidation of luciferin to oxyluciferin by luciferase, indicated the biological interaction of Nrf2 and ARE. Our findings showed that incubation with SEI at a certain concentration could enhance the fluorescence intensity, indicating that SEI exerts anti-oxidative stress effect by activation of Nrf2/ARE pathway. In accordance with the result above, administration of SEI significantly increased the expressions of cytoplasmic Nrf2 and nuclear Nrf2, and the ratio of n-Nrf2/c-Nrf2 was increased with the increasing of the dose. In addition, the expressions of downstream genes, including HO-1 and NQO1 were up-regulated as well. Here we suspected that SEI could up-regulate Nrf2’s expression and further promote its nuclear translocation in cerebral ischemic rats. Despite the molecular mechanism of activation of SEI on Keap1-Nrf2-ARE pathway was under investigation, these findings provided evidence that the protective effect of SEI against I/R injury was mediated by activation of the Nrf2/ARE pathway.
To date, many studies have demonstrated that activation/phosphorylation of Erk1/2 contributes to cellular proliferation and differentiation, morphology maintenance, inhibition of cell apoptosis and resistance to oxidative stress, despite a few conflicting views (Runden et al., 1998). With regard to oxidative stress induced lesions, the Erk1/2 signaling pathway is thought to exert a cytoprotective effect, especially in cerebral tissues (Li et al., 2013). Consistent with above observations, the present study showed that SEI apparently induced phosphorylation of Erk1/2. Given that p-Erk contributes to the activation of Nrf2 under conditions of oxidative stress and neurotoxic injury (Wang et al., 2012), we speculated that the Erk pathway might be, at least partially, involved in the antioxidant effects of SEI by activating Nrf2/ARE pathway. Although the exact mechanism underlying focal cerebral I/R injury is not clear, neuronal cell death mainly occurs by necrosis, and apoptosis is a noticeable feature of almost all neurodegenerative diseases (Yuan et al., 2003). Among many proteins involved in cell apoptosis, the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax both regulate the release of cytochrome C, which activates caspase 3 and the caspase cascades, ultimately causing apoptosis (Benchoua et al., 2001). Caspase activation is the final process of the death signaling pathway, in which pro-caspase 3 is activated into cleaved caspase 3 following the autoactivation of pro-caspase 9 to cleaved caspase 9 (Nagata, 1997). Here, we showed down-regulation of Bax, cleaved caspase 3 and caspase 9 and up-regulation of Bcl-2 in ischemic brains after SEI treatment, indicating an anti-apoptotic effect of SEI on I/R injury.

In conclusion, this study demonstrated the protective effects of SEI against focal cerebral I/R injury and provided further insight into its anti-oxidative and anti-apoptotic mechanisms. Further studies focusing on the establishment of cellular model simulating I/R injury in vivo and exploration of other potential involved signaling pathways are required.

4. Experimental procedures

4.1. Animals

Male Sprague-Dawley rats (250–300 g) were purchased from the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine. Procedures were performed according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Committee of the Experimental Animal Administration. Free access to forage and drinking water was allowed. Controlled conditions were implemented (12/12 h light/dark cycle with room temperature of 25±2°C and room relative humidity of 50±10%).

4.2. Cerebral ischemia–reperfusion model

Transient focal cerebral ischemia rats were prepared as previously described (Kuller, 1989). In brief, animals were anesthetized by intra-peritoneal injection of pentobarbital sodium (50 mg/kg). Rectal temperature was maintained at 37°C using a heating pad. After a median incision of the neck skin, the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed and isolated carefully. Occlusion of the right side MCA (MCAO) was achieved...
by inserting a monofilament nylon filament into the ICA through an incision in the ECA, and the tip of the filament was advanced to the origin of the MCA. The surgical wound was closed and the rat was allowed to recover from the anesthesia. Reperfusion was started after 2 h of MCAO by withdrawing the filament. Sham-operated rats received the same surgery without inserting the filament. During the experiments, MCA blood flow was monitored using a Laser Doppler Flowmetry (Moor Lab, Moor Instruments Ltd, UK). A drop in rate of cerebral blood flow (rCBF) was expressed as a percentage of the baseline value and rCBF more than 85% was excluded.

4.3. Groups and drug administration

Four groups of rats were included: the sham-operated group (Sham), in which rats received sham operation; the vehicle group (Vehicle), in which rats received tMCAO and an equal volume of saline; the SEI groups (SEI-L and SEI-H), in which rats received tMCAO and were given SEI (36 mg/kg and 72 mg/kg, respectively, dissolved in saline) intravenously 15 min after occlusion. SEI was separated and purified from L. chuanxiong Hort. 1H NMR, 13C NMR, MS identified the structure of SEI and the purity was above 98%.

Fig. 6 – Effect of senkyunolide I on the protein levels of p-Erk1/2, t-Erk1/2, c-Nrf2, n-Nrf2, HO-1, NQO1, Bcl-2, Bax, caspase 3 and caspase 9 in brains with MCAO-induced I/R injury. Representative photographs of Western blots and quantitative analysis of the proteins were presented. (A) The p-Erk1/2/t-Erk1/2 was significantly increased in SEI-L group and SEI-H group compared with Vehicle group (*) P<0.05, while the phosphorylation of Erk1/2 reached a higher level in SEI-H group (*) P<0.05. (B) The protein levels of c-Nrf2 and n-Nrf2 were apparently promoted in SEI-L and SEI-H groups compared with Vehicle group (*) P<0.05. SEI-H group further enhanced n-Nrf2 expression significantly (* P<0.05). (C) Compared with Vehicle group, significant differences on HO-1 and NQO1 protein levels were observed in both SEI-L and SEI-H groups (* P<0.05), and SEI-H group got higher levels than SEI-L group (* P<0.05). (D) The administration of SEI at two dosages could significantly reverse the down-regulation of Bcl-2 and up-regulation of Bax induced by I/R injury (*) P<0.05. High does group got a better effect on Bax expression (*) P<0.05. (E) The expressions of cleaved caspase 3 and caspase 9 were markedly reduced in SEI-L group and SEI-H group respectively (*) P<0.05. Between these two does groups, no significant differences were observed. (F) The ratios of n-Nrf2/c-Nrf2 and Bcl-2/Bax were elevated to higher levels in both SEI-L and SEI-H groups compared with Vehicle group (*) P<0.05, and high dose of SEI acted more effectively (*) P<0.05.
4.4. **Neurological function assessment**

A neurological test was performed after reperfusion for 24 h in a blinded fashion (n=10), based on a modified scoring system developed by Longa et al. (1989) as follows: 0 = no deficit; 1 = difficulty in fully extending the contralateral fore-limb; 2 = unable to extend the contralateral forelimb; 3 = mild circling to the contralateral side; 4 = severe circling; and 5 = falling to the contralateral side. The higher the neurological deficit score, the more severe impairment of motor motion injury.

4.5. **Measurement of brain water content**

Brain water content was measured after neurological function assessment, using the standard wet-dry method (Hatashita et al., 1988). Rats were reanesthetized and sacrificed by decapitation (n=6). The cerebral ipsilateral and contralateral hemispheres were removed quickly and packaged with pre-weighed tin foil, respectively. The packed hemispheres were immediately weighed to obtain wet weight and then dried for 24 h at 105 °C to obtain the dry weight. Brain water content was calculated as [(wet weight – dry weight)/wet weight] × 100%.

4.6. **Measurement of infarct volume**

Infarct volume was measured 24 h after the start of reperfusion. Rats were reanesthetized and the brains were removed quickly (n=6 per group). Coronal brain slices (about 2 mm thick) were stained with TTC at 37 °C for 20 min (Bederson et al., 1986), followed by fixation with 4% paraformaldehyde. The stained sections were photographed and analyzed using Image Pro-Plus 5.1 analysis software (Media Cybernetics Inc. Rockville, Maryland, US). To compensate for the effect of brain edema, the percentage of infarct volume was calculated as follows, [(total infarct volume – (the volume of intact ipsilateral hemisphere – the volume of intact contralateral hemisphere)/contralateral hemisphere volume) × 100%.

4.7. **Histopathology**

After 24 h of reperfusion following 2 h tMCAO, rats were reanesthetized and perfused with 100 mL precooled normal saline and subsequently with 250 mL precooled 4% paraformaldehyde. Brain cortices and hippocampus were removed quickly and then post-fixed for 24 h in the 4% paraformaldehyde (n=6). The tissues were dehydrated in graded ethanol, cleared in xylene, embedded in paraffin wax and cut coronally at 5 μm. The sections were then stained with hematoxylin and eosin (HE) for histopathological observation.

4.8. **Determination of MDA, and SOD levels**

Cortical tissues were collected after 24 h reperfusion and homogenized to obtain supernatant after centrifugation (n=6 per group). The contents of MDA and the enzymatic activities of SOD were measured using assay kits, according to the manufacturer’s instructions (Nanjing Jian Chen Bioengineering Institute).

4.9. **Detection of reporter gene activity**

The stably-transfected cells, Hek293T/pGF1-ARE, were generously provided by Dr. Ding (Shanghai Institute of Materia Medica, Chinese Academy of Sciences). Cells were cultured in Dulbecco’s modified Eagle’s media (DMEM, containing 4.5 g/L d-glucose and 2 mM l-glutamine), supplemented with 10% heat-inactivated fetal bovine serum (FBS). Prior to reporter gene activity detection, MTT assay and LDH leakage assay were conducted to evaluate cytotoxicities of positive compound (5 μM dl-sulforaphane) and various concentrations (25–100 μM) of SEI (n=6). Non-treated cells served as controls.

The cells were seeded onto 24-well plate at 5 × 10^5/well (n=4 per group) and compounds were added into each well, followed by overnight incubation, after which the microscopic photos were obtained with a laser scanning confocal microscope (Leica SP2, Leica Microsystems CMS GmbH, Mannheim, Germany). Subsequently, the cells were lysed, and the relative fluorescence intensity of the cell lysate were recorded according to previously described procedures (Brovko et al., 1994) and the total protein in each well was measured by BCA Protein Assay kit (Beyotime Institute of Biotechnology, China). The luciferase activity of cell lysate was expressed as relative fluorescence intensity per milligram total protein (RFI/mg protein).

4.10. **Western blot**

Following the neurological function assessment, rats were randomly chosen for decapitation under anesthesia (n=6 per group). Total protein extraction and cytoplasmic/nuclear protein extraction were obtained using ice-cold RIPA and Nuclear and Cytoplasmic Protein Extraction Kit containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Beyotime Institute of Biotechnology, China). The protein concentration of the supernatant was measured by BCA assay. Equal amounts of proteins per lane were separated by SDS/PAGE and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in TBST, membranes were incubated overnight at 4 °C with primary antibodies of p-Erk1/2 (1:2000, Cell Signaling Technology Inc, Beverly, MA, USA), Erk1/2 (1:1000, Cell Signaling Technology Inc), Nrf2 (1:500, Abcam Inc, Cambridge, UK), HO-1 (1:1000, Cell Signaling Technology Inc), NQO1 (1:500, Cell Signaling Technology Inc), Bcl-2 (1:500, Abcam Inc, Cambridge, UK), Bax (1:1000, Abcam Inc), caspase-3 (1:1000, Abcam Inc) and caspase-9 (1:1000, Abcam Inc). Antibodies against β-actin (1:5000, Cell Signaling Technology Inc) and Lamin B1 (1:10,000, Proteintech, US) were used as internal controls of cytoplasmic and nuclear protein, respectively. On the second day, membranes loaded with primary antibodies were washed and incubated with HRP conjugated secondary antibodies (goat anti-rabbit IgG, 1:10,000; goat anti-mouse IgG, 1:10,000, Abcam Inc) for 1 h at room temperature. The bands were scanned and the relative density of each band was analyzed by using the Image Pro-Plus 5.1 analysis software.

4.11. **Statistical analysis**

For neurological deficit scores, Mann–Whitney U test was used for comparisons between two groups. The other data
were analyzed by One-way ANOVA and followed by Student-Newman–Keuls test for intergroup comparisons. Data were expressed as mean ± SD. All statistical analysis was performed with SPSS 16.0 software and P<0.05 was considered statistically significant.

Conflict of interest

The authors declared that there are no conflicts of interest.

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