Neuroprotection of Osthole against Cerebral Ischemia/Reperfusion Injury through an Anti-apoptotic Pathway in Rats

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Cerebral ischemia/reperfusion (I/R) injury is a major cause of acute brain injury. The pathogenetic mechanisms underlying I/R injury involve apoptosis, inflammation and oxidative stress. Osthole—a plant coumarin compound—has been reported to protect against focal cerebral I/R-induced injury in rats. However, the mechanism remains unknown. Here we hypothesize that osthole acts through inhibition of apoptosis during focal cerebral I/R injury in rats. We induced cerebral I/R injury by middle cerebral artery occlusion (MCAO) for 2 h followed by reperfusion. We randomly assigned 60 rats to three groups (20 rats per group): sham-operated, vehicle-treated I/R, and osthole-treated I/R. We treated rats intraperitoneally with osthole (40 mg/kg) or vehicle 30 min before cerebral ischemia. We harvested the brains for infarct volume, brain water content, histological changes and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining as well as cleaved caspase-3, bax, and bcl-2 levels 24 h after reperfusion. Osthole treatment significantly attenuated cerebral dysfunction and histologic damage induced by I/R injury. Moreover, osthole-treated rats had a dramatic decrease in apoptotic neuronal cells along with a decrease in bax and cleaved caspase-3. The bcl-2 levels increased. Osthole treatment protects the brain from cerebral I/R injury by suppressing cell apoptosis. Thus, osthole may represent a novel practical strategy to prevent cerebral I/R injury.

Key words  osthole; cerebral; ischemia; reperfusion; apoptosis

Stroke is the second most common cause of death and a major cause of long-term disability worldwide. Of the 15 million people who suffer a stroke each year worldwide, more than 5 million die and a further 5 million are left permanently disabled. The best treatment is to restore blood supply to the ischemic brain tissue as soon as possible. After focal cerebral ischemia and reperfusion (I/R), most of the cells in the ischemic core undergo necrosis; cell death in the ischemic penumbra is an active process that leads to apoptosis. There is overwhelming evidence that in addition to necrosis, apoptosis contributes significantly to cell death both in the ischemic core and in the surrounding penumbra region subsequent to cerebral I/R. Previous study has selected the penumbra as a target tissue and found that anti-apoptotic therapy offers significant therapeutic benefit.

Osthole (7-methoxy-8-isopentenoxy-coumarin; Fig. 1) is a natural coumarin derivative isolated from several plants including *Cnidium monnieri*, *Angelica pubescens*, and *Picea densata ostruthium*. Researchers have reported that osthole can prevent anti-Fas antibody-induced hepatitis by affecting the caspase-3-mediated apoptotic pathway. Recent studies found that pretreatment with osthole on PC12 cells significantly reduced the loss of cell viability, the release of lactate dehydrogenase, and the activity of caspase-3 and cytochrome c. The increase in bax/bcl-2 ratio and generation of intracellular reactive oxygen species (ROS) induced by 1-methyl-4-phenylpyridinium ion (MPP+) were also reduced. Previous studies have demonstrated that osthole can protect the brain from I/R injury. However, whether osthole has a neuroprotective effect against apoptosis in focal cerebral I/R injury remains unclear. Therefore, we studied the neuroprotection of osthole and its potential mechanism.

MATERIALS AND METHODS

Chemicals  Osthole (99% purity; purchased from Xi’an Green Spring Technology Co., Ltd., Xi’an, China) was dissolved in a 1:9 (v/v) mixture of Tween 80 and 0.9% sodium chloride. 2,3,5-Triphenyltetrazodium chloride (TTC) was purchased from Sigma-Aldrich Co. (MO, U.S.A.).

Animals  Adult male Sprague-Dawleys rats weighing 250–280 g were purchased from Xi’an Jiaotong University. All animal experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council for the Care and Use of Animals for Experimental Purposes in China. All rats were allowed free access to food and water before surgery under optimal conditions (12/12 h light/dark with humidity 60±5%, 22±3°C).

Model of Middle Cerebral Artery Occlusion (MCAO)

![Fig. 1. Chemical Structure of Osthole](image)
Transient cerebral ischemia was induced by MCAO in rats as described previously. Rats were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally). The common left carotid artery was exposed and isolated, while the middle cerebral artery was occluded by inserting a nylon filament (diameter 0.24–0.28 mm) into the internal carotid artery. This was advanced 18–20 mm past the carotid bifurcation until a slight resistance was felt. After 2 h of ischemia, the nylon filament was slowly removed for reperfusion. Throughout the procedure, the body temperature was maintained at 36.5 to 37.5°C with a thermostatically controlled infrared lamp. Rats with no neurological deficits after 24 h of reperfusion (neurological score <1) were excluded from the study. The total elimination rate was 3/60. The sham-operated animals were treated identically, except that the middle cerebral arteries were not occluded after the neck incision.

Experimental Protocol In this experiment, 60 rats were randomly divided into 3 groups (n=20 for each group): a sham-operated group (sham), vehicle-treated group (vehicle), and 40 mg/kg osthole-treated group (osthole). The rats in osthole group were injected intraperitoneally 30 min before MCAO. This dose of osthole was chosen on the basis of the previous study and our preliminary experiment. The rats in the sham group and the vehicle group received equal volumes of vehicle.

Evaluation of Neurological Deficits Neurological deficit testing in the vehicle-treated group and osthole-treated group was carried out by an investigator blinded to the experimental groups after 24 h of reperfusion according to the method of Longa et al. Neurological findings were scored on a five-point scale: no neurological deficits=0; failure to extend right forepaw fully=1; circling to the right=2; falling to the right=3; and inability to walk spontaneously and depressed levels of consciousness=4.

Measurement of Brain Water Content Twenty-four hours after MCAO, cerebral edema was determined by measuring the brain water content according to the wet–dry method. Rats from all three groups (n=4–5 for each group) were killed with chloral hydrate anesthesia, and the brain was immediately removed and dissected. Brain samples from the ischemic hemisphere were rapidly weighed on an electronic balance to obtain wet weight and then dried in an oven at 100°C for 24 h to obtain the dry weight. The brain water content was calculated as follows: [(wet weight–dry weight)/wet weight]×100%.

Measurement of Infarct Volume Samples from all three groups (n=5 for each group) were used for further analysis. After neurological deficit testing, the rats were killed and their brains quickly removed and frozen at −20°C for 10 min. Coronal brain sections (2 mm thick) were cut starting at the bregma. The third slices were stained with 2% TTC in the dark for 20 min at 37°C followed by fixation with 4% paraformaldehyde. Images were recorded using a digital camera (Canon EOS M2) and quantitated with a Mias-2000 image analysis system (Institute of Image & Graphics, Sichuan University, China). Edema correction was performed with the equation: Percentage hemisphere lesion volume (%HLV)={[(total infarct volume–(left hemisphere volume–right hemisphere volume)]/right hemisphere volume]}×100%. The infarct volume measurements were carried out by one examiner blinded to the treatment groups.

Histological Examination Animals were humanely killed 24 h after reperfusion (n=4–5 for each group). The brains were perfused transcardially with 0.9% sodium chloride followed by 4% paraformaldehyde. Following decapsulation, the brains were removed and embedded in paraffin. Coronal sections were prepared starting 2.8 to 4.4 mm from the bregma. Four micrometers of the coronal sections were cut and stained with hematoxylin–eosin (H&E staining) or 0.1% cresyl violet (Nissl staining).

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling (TUNEL) Staining The histology samples were also tested with TUNEL staining. The TUNEL assay was performed according to the manufacturer’s instructions (Roche Molecular Biochemicals, Inc., Mannheim, Germany). First, the tissue sections were treated with Proteinase K (20 μg/mL) and 0.3% H2O2. Next, the sections were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 1 h and further incubated with peroxidase-conjugated antibody for half an hour at 37°C. Then the 3,3′-diaminobenzidine (DAB) was used for the coloration of apoptotic cells. TUNEL-positive cells in the penumbra of the brain were counted in ten high power fields with a light microscope (<400).

Western Immunoblot Analysis Other left brain hemisphere samples from the three groups (n=5 for each group) were collected and lysed with a lysis buffer (Tris-hydroxymethyl)-aminomethane 50 mmol/L, pH 7.5, NaCl 150 mmol/L, 1% Triton X-100, edetic acid 1 mmol/L, phenylmethylsulfonyl fluoride 1 mmol/L, and aprotinin 5 mg/L). The whole lysates were centrifuged at 14000×g at 4°C for 20 min, and the supernatant was collected as total cellular proteins. Equal amounts of lysate protein (40 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) and incubated with primary antibody (anti-bax, 1:500, Santa Cruz; anti-bcl-2, 1:500, Santa Cruz; anti-cleaved caspase-3, 1:500, Cell Signaling Technology; and anti-β-actin, 1:1000, Santa Cruz) overnight at 4°C. After five washes with TBS, the membranes were incubated with the secondary antibodies (goat anti-rabbit, 1:1000) for 2 h at room temperature. Gels were scanned by imaging densitometer (BIO-RAD) and quantitated by Quantity One analysis. The amount was normalized with β-actin values in the same lane.

Statistical Analysis The data were expressed as the mean±standard error of the mean (S.E.M.). The changes between various groups were analyzed by one-way ANOVA followed by multiple comparison tests as a post hoc comparison. The neurological deficit scores were analyzed by a Mann–Whitney U-test. The statistical software package SPSS13.0 was used throughout with a level of p<0.05 considered statistically significant.

RESULTS

Effects of Osthole on Neurological Deficit and Brain Water Content To determine the neuroprotective effects of osthole against I/R injury, we measured the neurological deficit score and the brain water content with and without administration of osthole. The neurological scores were assessed
24 h after the MCAO in the sham, vehicle, and osthole group (Fig. 2A). There were no significant neurological deficits in the sham group. However, severe deficits were observed in the vehicle group. This indicates that the I/R injury model was successful. The scores of the osthole group are markedly lower than that of the vehicle group ($p<0.05$). As shown in Fig. 2B, relative to the vehicle group, the brain water content of the ipsilateral hemispheres were obviously decreased after treatment with osthole ($p<0.05$). No differences were found across the contralateral hemispheres ($p>0.05$).

**Effects of Osthole on Infarction** To evaluate whether treatment with osthole decreases infarct volume following cerebral I/R, adult rats were subjected to cerebral ischemia (2h) followed by reperfusion up to 24h. The infarct volumes were then evaluated. No infarction was observed in the sham group. As shown in Figs. 3A and B, infarct volumes in the osthole group were reduced by approximately 20.9% ($30.41\pm8.96$, $p<0.01$) relative to the vehicle group ($51.36\pm12.88$). This suggests that osthole obviously protects against cerebral I/R injury.

**Effects of Osthole on Morphology and Nissl Staining** We used H&E and Nissl staining to investigate the morphological changes in the infarct core. Figure 4 shows H&E and Nissl-staining of the ipsilateral brain 24 h after reperfusion. There were many atrophic neurons with shrunken cytoplasm and damaged nuclei in the vehicle group. There were no morphological changes to the sham group. Administration of osthole reduced the number of degenerated neurons and markedly increased the number of intact neurons ($p<0.05$).

**Effects of Osthole on in Situ Labeling of DNA Fragments** To evaluate the effect of osthole on apoptosis, we examined apoptotic cell death using the TUNEL assay (Figs. 5A, B). The TUNEL-positive cells containing apoptotic bodies and darkly stained cells were considered apoptotic. The sham group did not show any obviously TUNEL-positive cells, and the cells treated with osthole 30 min after MCAO showed a significantly reduced number of TUNEL-positive cells in the penumbra of the brain compared with the vehicle group ($p<0.05$).

**Effects of Osthole on Expression of Bax, Bcl-2 and Activated Caspase-3** To explore the mechanisms by which osthole elicits its anti-apoptotic effects, the expression levels of bax, bcl-2 and activated caspase-3 in the ischemic cortex were examined by Western blot analysis (Fig. 6). As shown in Fig. 6B, the expression level of bcl-2 was markedly increased in the osthole-treated group in the brain sample 24 h after reperfusion. There were many atrophic neurons with shrunken cytoplasm and damaged nuclei in the vehicle group. There were no morphological changes to the sham group. Administration of osthole reduced the number of degenerated neurons and markedly increased the number of intact neurons ($p<0.05$).
Fig. 4. Effects of Osthole on Morphology (A) and Nissl Staining (B) 24h after Focal Cerebral I/R Injury in Rats

Normal morphological features of neurons are present in the sham group (black arrow showing the normal neuron). Most neurons in the vehicle group have a reduced nucleus and cytoplasm and enlarged intercellular space (gray arrow showing the damaged cell). Osthole clearly prevents cell loss and preserved the structure of most neurons (magnification ×400). (C) Intact neurons were counted in ten high power fields under light microscopy (×400). Osthole clearly increased the number of intact neurons (mean±S.E.M., n=4–5 for each group, *p<0.01 vs. sham; **p<0.05 vs. vehicle).

Fig. 5. Effects of Osthole on in Situ Labeling of DNA Fragmentation 24h after I/R Injury

(A) No TUNEL-positive cells were found in the sham group; many TUNEL-positive cells were found in the vehicle group; fewer TUNEL-positive cells were found in the osthole group than in the vehicle group (magnification ×200). (B) Quantification of TUNEL-positive cells. Only densely labeled cells were considered as positive apoptotic cells. The number of TUNEL-positive cells in the three groups were counted (×400), and the values are expressed as the mean±S.E.M. The data were analyzed by one-way ANOVA (n=4–5 for each group; *p<0.01 vs. sham; **p<0.05 vs. vehicle.
focal cerebral ischemia relative to the vehicle group \( (p<0.05) \).
The expression levels of bax (Fig. 6A) and activated caspase-3 (Fig. 6C) were increased in the vehicle group after 24 h, but remarkably decreased in the osthole group relative to the vehicle group \( (p<0.05) \).

DISCUSSION

MCAO is a widely accepted model of cerebral ischemia.\(^{17,19}\) In this research, we demonstrated that pretreatment with osthole can reduce brain infarct volume and water content while improving neurological outcomes after focal cerebral I/R injury. We also found that osthole can inhibit the expression of activated caspase-3 and bax, while increasing bcl-2 activity induced by I/R injury. Osthole provides neuronal protection partly through its anti-apoptotic effects.

Recent studies have demonstrated that osthole reduces cerebral I/R injury. Mao et al. reported that, osthole down-regulates MMP-9 protein level/activity in the I/R brain.\(^{13}\) Chao et al. showed that osthole treatment could significantly attenuate the formation of brain edema and infarct area 24 h after ischemic injury; 40mg/kg osthole was most significant.\(^{16}\) Zheng et al. found that 40mg/kg osthole in renal ischemia is the most effective concentration.\(^{10}\) All of these studies administer osthole 30 min before ischemia and showed that this was the most effective time point. Zhou et al. reported that, after oral administration, the osthole plasma level could be detected after 5 min in both the normal and the cerebral ischemia hypoperfusion model rats, with half-lift (T1/2) of 4.94 h in the normal animals and 8.57 h in the model animals.\(^{20}\) Their results indicate that osthole may be also effective to protect neurons if it is treated after the beginning of cerebral ischemia. These reports are consistent with our preliminary experiment (data not show). Thus, we here used 40mg/kg osthole 30 min before ischemia. Our results demonstrate that osthole can improve neurological deficits and decrease brain infarct volume and water content. We further found that administration of osthole reduce the number of degenerated neurons and markedly increase the number of intact neurons.

Focal cerebral ischemia injury produces core infarct tissue that is severely compromised and may not be repairable. The ischemic penumbra, \textit{i.e.}, the peri-infarct region, will have intermediate perfusion where cells depolarize intermittently. Without treatment, the penumbra often progresses to infarction. Reperfusion of the occluded vessels as soon as possible is the standard treatment for acute ischemic stroke.\(^{21}\) How-

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**Fig. 6.** Expression of Bax, Bcl-2 and Activated Caspase-3 of the Cerebral Cortex

The protein expressions of bax (A), bcl-2 (B) and activated caspase-3 (C) in the ipsilateral brain tissues were detected by Western blot analysis \( (n=5 \text{ for each group}) \). After osthole treatment, the expression of bcl-2 was markedly increased compared with the vehicle group, while the expression of bax and activated caspase-3 in the injured brain tissues decreased. *\( p<0.05 \) vs. sham; **\( p<0.05 \) vs. vehicle.
ever, reperfusion may still subject the surrounding tissue to further damage through activation of apoptosis and neurodegenerative cascades.\textsuperscript{22,23}\ This phenomenon is called cerebral I/R injury.\textsuperscript{24} Experimental studies have aimed to decrease or slow down this apoptotic process to gain time and ensure that some functions are regained with reperfusion.

Researchers have reported that osthole can prevent anti-Fas antibody-induced hepatitis through an anti-apoptotic pathway.\textsuperscript{11} Liu et al. showed that pretreatment with osthole on PC12 cells significantly reduced the loss of cell viability, the release of lactate dehydrogenase, and the activity of caspase-3 and cytochrome c. The increase in bax/bcl-2 ratio and generation of intracellular ROS induced by MPP+ were also reduced.\textsuperscript{12} Recent studies have demonstrated that osthole has protective effects against focal cerebral I/R injury.\textsuperscript{13,16}

Apoptosis during I/R injury plays a major role in brain injury associated with stroke.\textsuperscript{25,26} However, whether osthole has a neuroprotective effect against apoptosis remains unidentified. This study investigated the neuroprotective effects of osthole. Our results showed a smaller number of TUNEL-positive cells in the penumbra of the treated group—a values significantly less than in the vehicle group.

The bcl-2 family plays a critical role in the mitochondrial apoptotic pathway.\textsuperscript{27} Data have shown that the bcl-2 protein families include both positive and negative regulation of apoptotic cell death. Both bax and bcl are positive regulators, which promote cell death. By contrast, bcl-2 and bcl-xl are negative regulators and prevent cells from undergoing apoptosis induced by various stimuli. The bcl-2 or bax may facilitate the activation of a caspase cascade and result in apoptosis.\textsuperscript{28,29}

Studies have also demonstrated that the caspase family is the promoter and implementer of apoptosis in mammalian cells. Of these, caspase-3 is a key downstream apoptosis factor in rats.\textsuperscript{30} To clarify the actual mechanisms by which osthole regulate neuronal apoptosis, we measured the expression of important apoptosis-related molecules and found that activated caspase-3 and bax were increased in the vehicle group. Bcl-2, however, was significantly increased in the osthole group. Bcl-2, however, was significantly increased in the osthole group—a values significantly less than in the vehicle group.

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Conflict of Interest The authors declare no conflict of interest.

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