Osthole Attenuates Focal Inflammatory Reaction Following Permanent Middle Cerebral Artery Occlusion in Rats

Fei Li, a,b Qihai Gong, b Lina Wang, b and Jingshan Shi a,b

a School of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine; Shanghai 201203, China: and b Department of Pharmacology and The Key Laboratory of Basic Pharmacology of Guizhou Province, Zunyi Medical College; Zunyi 563000, China.

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Osthole, a main active constituent from Cnidium monnieri (L.) Cusson, has been considered therapeutic agent in the treatment of ischemic stroke. This study was designed to investigate the effect of osthole on permanent middle cerebral artery occlusion (MCAO) in rats. Osthole was administrated by gavage to the normal and the MCAO rats. Rats were assessed for neurological deficit after 24 h following MCAO, then their brains were evaluated to determine the infarct area, and the mRNA and protein levels of some inflammatory factors were detected. It was found that MCAO animals pre-treated with osthole for 7 d showed significant improvement in all neurological tests compared with vehicle-treated MCAO groups. In addition, there was a significant decrease in infarct volume 24 h after occlusion in animals pre-treated with osthole versus the vehicle-treated MCAO group. MCAO also dramatically caused some inflammatory factors increase. However, pretreatment with osthole restored the mRNA and protein levels of these factors, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) of ischemic penumbra cortices, suggesting that osthole possessed the function of preventing brain against ischemic damage, while no significant difference was found in any of normal groups with or without osthole. The present study demonstrated that osthole may be a novel neuroprotective therapy in the treatment of focal ischemic stroke.

Key words osthole; inflammation; neuroprotection; rat

Stroke is the third leading cause of death and the leading cause of long-term disability, of which ischemic stroke accounts for more than 80%.1) Inflammation was not only thought to be a reaction to tissue damage, but recognized as a key contributor to the pathophysiology of cerebrovascular diseases, especially stroke caused by arterial occlusion or ischemic stroke.2) Although there were some therapeutic drugs against cerebral ischemia clinically, the functional rehabilitation should be improved. During the pathophysiological process of the cerebral ischemia, a lot of investigators have been interested in a variety of interventions, especially in the interruptive mechanisms of active principles from medicinal herbs.

The fruit of Cnidium monnieri (L.) Cusson (Apiaceae), a Chinese herbal medicine which was known as Shechuangzi in China and collected in the Pharmacopoeia of the People’s Republic of China,3) has been used in the treatment of skin and gynecological conditions for many years. One of the major active components of this herb-osthole (7-methoxy-8-isopentenoxycoumarin, C13H10O5, 244.39 Da) is a natural coumarin derivative and is 69.52% by supercritical fluid extraction.4) Osthole possesses a broad spectrum of pharmacological activities, including anti-osteoporotic,5) anti-allergic,6) anti-diabetic7) and lipid-lowering effects,8) also exhibits anti-tumor,9) anti-seizure,10) anti-parasite.11) Recently, a volume of interesting studies have revealed that osthole owns beneficial effects on improving learning and memory functions in different experiment animals, such as ameliorating learning and memory impairment induced by Scopolamine12,13) or hydrocortisone acetate caused Kidney–Yang deficiency rats.14)

As previous data had demonstrated that osthole has neuroprotective effects, we conducted this study to further determine the protective effects of osthole on a rat model of middle cerebral artery occlusion (MCAO), including the infarct area and neurological deficit, then to investigate how osthole improves functional and physiological recovery following MCAO, whether the anti-inflammation of osthole is very important in this model.

MATERIALS AND METHODS

Drugs Osthole (purity>98% tested by HPLC, Fig. 1) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). Osthole was dissolved in 2% Tween-80 and ultra-sonicated 15 min. Every animal was administrated by gavage at a volume of 10 mL/kg.

Animals Adult male Sprague-Dawley rats weighing 280–330 g were used in this experiment. All efforts were made to minimize the number of animals used, and all experimental procedures were performed in accordance with the WHO guidance for Animal Use and Care and our College Animal Use and Care Guidelines. The rats were housed in groups of five per cage for one week prior to the study to allow the animals to acclimatize to room conditions. The animal room was maintained under controlled temperature (22±1°C) and a 12 h light–dark cycle (light on 6:00–18:00) conditions. Rats

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* To whom correspondence should be addressed. e-mail: shijs@zmc.edu.cn © 2012 The Pharmaceutical Society of Japan

Fig. 1. The Chemical Structure of Osthole
were fed pellet chow with access to water *ad libitum*.

**Experimental Design and Drug Administration** Rats were randomly divided into 4 groups: the sham-operation group (*n*=10), the sham-operation plus 30 mg/kg osthole-treated group (*n*=10), the ischemia-induction group (*n*=15), the ischemia-induction plus 30 mg/kg osthole-treated group (*n*=15). Rats in the osthole-treated groups pretreated with osthole orally once a day for 7 consecutive days, then received the surgery. The sham-operation group and ischemia-induction group received an equal amount of distilled water for the same duration.

**Surgical Procedure** MCAO was performed according to the method with minimal modification as described below.15) The rats were anesthetized with chloral hydrate (intraperitoneally (i.p.) 350 mg/kg, Sinopharm Chemical Reagent Co., Ltd.) and kept the body temperature at 37°C with a heating pad throughout the surgery. The right common carotid artery, right external carotid artery (ECA), and right internal carotid artery were exposed through a midline neck incision and carefully dissected from surrounding tissues. The right middle cerebral artery (MCA) was blocked by inserting a 4-0 nylon suture, with its tip coated by resin, 18–19 mm from the ECA into the origin of the MCA. The suture was left in place permanently. Sham-operated group was only subjected to anesthesia and a skin incision without MCAO.

**Neurological Severity Score** Two observers, who were blinded to this procedure, tested the animals for neurological deficits at 24 h following MCAO using the modified Bederson’s method.16) The criteria are: 0=no observable deficit; 1=forelimb flexion; 2=forelimb flexion plus decreased resistance to lateral push; 3=unidirectional circling; 4=unidirectional circling plus decreased level of consciousness.

**Morphological Examination and Measurement of Brain Infarct Volume** 2,3,5-Triphenyl tetrazolium chloride (TTC) staining was performed as previously described.17) After 24 h following onset of MCAO, the rats were sacrificed by decapitation. Brains were immediately isolated and sliced under the microscope into serial 1-2 mm-thick slices. The brain sections of each rat were stained with 1% TTC solution and photographed. The total area and the infarct area were determined using Image-Pro Plus software (Scion Image). The infarction volume was expressed as a percentage of the total brain.1

**Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** Twenty-four hours after MCAO, the brains were removed and the mRNA expressions of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) of ischemic penumbra cortices were assayed using real-time RT-PCR as we previously described.18) In brief, total RNA of ischemic penumbra cortices was isolated by Trizol reagent. Secondly, RNA was quantified by measuring the optical density at 260/280 nm, and adjusted RNA concentration of each sample to 50 ng/µL. Following reverse transcribed total RNA was according to the reverse transcription kit manuscripts. Then, the SYBR green DNA PCR Master Mix was used for the real-time PCR analysis. The nucleotide sequences of primers used in this experiment were designed according to the sequence searched on GeneBank (Table 1). The expression was calculated using cycle time (Ct) values, and the expressions of target genes were determined as relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level, setting the sham group as 100%.

**Measurement of the Proteins in Rat Ischemic Cortex by Western Blot** The ischemic penumbra brain tissue was homogenized in radio-immunoprecipitation (RIPA) lysis buffer in the presence of protease inhibitor and centrifuged at 20000×g for 15 min at 4°C. Supernatant was collected to quantify the protein using the bicinchoninic acid (BCA) protein assay. Then, separation 5% and 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, electrophoresis, transferring protein to the polyvinylidene difluoride (PVDF) membrane, followed by blocked with 5% nonfat dry milk and incubated with primary antibody (rabbit anti-rat IL-1β, COX-2, β-actin, 1:1000 in 5% bovine serum albumin (BSA)-Tris buffered saline (TBS)–0.05% Tween-20) at 4°C overnight. Subsequently, the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibodies for 2 h at room temperature with shaking. Then the membranes were developed using enhanced chemiluminescence reagent BeyoECL plus. The image was scanned, and band intensity was quantified using Quantity One software v4.52 (BioRad).

**Statistical Analysis** The data were presented as means±S.E.M. The differences in infarct volume and neurological scores were analyzed by t-test, the levels of mRNA and proteins were assessed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple-comparisons post-hoc test with SPSS 13.0 software. A p-value <0.05 was regarded as significant.

**RESULTS**

**Effect of Osthole on Mortality Rate of MCAO Rats** There was no significant difference for the number of animals that died in MCAO groups with or without osthole after 24 h of MCAO. There were 5 animals died in the MCAO group and 4 ones in the MCAO plus osthole group.

**Effect of Osthole on Neurological Deficit in MCAO Rats** Use Bederson’s score as criteria of evaluation of neurological deficit grading system in the present study. The higher neurological deficits score, the more severe impairment of motor motion. The rats of sham groups with or without osthole did not show any neurological deficits.

### Table 1. The Sequence of Genes in This Study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GeneBank</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>RA043092</td>
<td>TCAGTTCCATCGGCCAGAC</td>
<td>GTTGCTTTTGGAGTATGCATT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>RA011390</td>
<td>CGCTGGGCGAGCTACTATGTTG</td>
<td>AGGTCGCTATCCTCCAGAG</td>
</tr>
<tr>
<td>COX-2</td>
<td>RA031856</td>
<td>CGGCCGTGTACCTAGTAGG</td>
<td>CGAGCCATCGGACTATTAGC</td>
</tr>
<tr>
<td>iNOS</td>
<td>RA031856</td>
<td>CTCAGCTGTGGCTGTGTCACCTA</td>
<td>GGCTTCCTCGGGCTTCACGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>CAGTGCACGCTCGTCTCA</td>
<td>TAACCAGGGCCGCCATACCG</td>
</tr>
</tbody>
</table>
not have any neurological deficit, and all of their neurological score were zero. However, 24 h after MCAO of the right side, paresis of the left hind paw was observed. The mean neurological score of MCAO rats was 2.33 ± 0.33. In contrast, the MCAO rats pretreated with osthole for 7 d markedly improved the neurological impairment (1.43 ± 0.20, p < 0.05, Fig. 2).

Effect of Osthole on Brain Infarct Volume in MCAO Rats To investigate the protection of osthole against the local cerebral ischemia, this study assessed the infarct volume of the injured cerebrum. The rats from sham groups underwent the similar surgical procedure but without MCAO, so there was no infarct area observed in these animals. The other two groups’ animals subjected to ischemic insult by right MCAO, the infarct area was observed in the right cortex and striatum. Pretreated with osthole 30 mg/kg/d for 7 d, the infarct volume was reduced from 24.88 ± 0.84% (the MCAO group) to 16.11 ± 1.25% (MCAO plus osthole group, Fig. 3).

Effect of Osthole on the mRNA Expressions of TNF-α, IL-1β, COX-2, iNOS in Ischemic Cortex The mRNA levels of TNF-α, IL-1β, COX-2, iNOS in infarct penumbra showed a drastic rise 24 h subsequent to MACO compared with those of sham-operated rats (all ps < 0.05). In contrast, these values significantly lowered in MACO rats pretreated with osthole (all ps < 0.05). However, the difference between sham groups with or without osthole was no significant (Fig. 4).

Effect of Osthole on the Proteins of IL-1β, COX-2 in Ischemic Cortex To investigate the difference of the proteins of IL-1β, COX-2 in different groups, we utilized the Western blot. The results showed that the levels of COX-2 protein (6-folds) and IL-1β protein (4-folds) in MACO group were higher than sham group. Interestingly, pretreated with osthole (30 mg/kg) for consecutive 7 d, the levels of above mentioned proteins were dramatically decreased compared with MACO group (all ps < 0.05). However, no difference had been found between sham groups with or without osthole (Fig. 5).
DISCUSSION

Many experimental models have been developed in order to investigate the mechanisms of ischemic brain injury and test different neuroprotective strategies. Amongst all current animal models of stroke, the one used most frequently is a rat model of focal brain ischemia caused by a MCAO. Focal ischemia of brain immediately evokes a robust inflammatory response that begins within a few hours of onset and typifies the secondary or delayed response to ischemia and production of reactive oxygen species triggers the coagulation cascade, and lead to activation of complement, platelet and endothelial cells. The inflammatory response significantly contributes to neuronal cell death and the evolution of tissue injury.

This study has shown that osthole pre-treated to MCAO rats for 7 d could reduce the infarct volume and neurological damage, also restore the mRNA and protein levels of inflammatory factors in MCAO rat cerebral cortex ischemic penumbra. These results are assistant with other studies of osthole model of focal brain ischemia caused by a MCAO. Focal ischemic cascade need to be further researched. Remarkable, osthole has not affected these parameters in normal rats. Upon these data, we could speculate that osthole will be a useful protective agent against the damage by MCAO via anti-inflammation, which has been proved to be a wonderful target for development of novel therapies against ischemic brain injury.

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