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Original Article

Osthole attenuates early brain injury following subarachnoid hemorrhage in rats

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Abstract: Osthole has been suggested to confer protective effects against cerebral ischemia; however, it is unclear whether osthole confers protection against early brain injury following subarachnoid hemorrhage. This study was undertaken to evaluate the effect of osthole on early brain injury in rats after SAH. Osthole was administered i.p. at 40 mg/kg 30 min and 6 h after the induction of SAH. Brain samples were extracted at 48 h after SAH. Neurological score, brain water content, blood-brain barrier (BBB) integrity were evaluated after SAH. The contents of malondialdehyde (MDA) and glutathione (GSH) after 48 h of SAH were assessed to investigate its anti-oxidative effect. In addition, neuronal apoptosis was also assessed using Western blot to show the expression of Bcl-2, Bax, and active caspase-3. Our results suggested that treatment with osthole markedly ameliorated neurological deficits, brain edema, BBB impairment, and neuronal apoptosis. In conclusion, post-SAH osthole administration may attenuate EBI. And anti-oxidative and anti-apoptotic effects of osthole may contribute to its beneficial effect against EBI after SAH.

Keywords: Subarachnoid hemorrhage, osthole, early brain injury, apoptosis

Introduction

Subarachnoid hemorrhage (SAH) is a severe neurological disease with high mortality and morbidity all around the world [1]. A study in China indicated that the annual incidence of SAH was about 6.2% for men and 8.2% for women [2], and aneurysm is one of the main causes of SAH [3]. In the past several years, cerebral vasospasm following SAH was regarded as the most critical cause of poor prognosis in SAH patients [4]. However, the outcome is not ameliorated when the vasospasm is removed [5]. Recently, early brain injury (EBI), including neuronal death, blood-brain barrier (BBB) disruption, and brain edema within 72 h of the induction of SAH, has been suggested to play an important role in the prognosis of SAH patients. Therefore, attenuating EBI effectively has been a potential therapeutic target in the treatment of SAH.

Osthole (Ost, chemical structure shown in **Figure 1**), a natural coumarin derivative, is extracted from various medicinal plants such as *Angelica pubescens*, *Cnidiummonnieri* [6]. Ost has been suggested to exhibit various pharmacological effects such as anti-inflammation, anti-apoptosis, anti-oxidative stress, and anti-tumor [6-9]. Recent studies have suggested that Ost protects against cerebral ischemia via an anti-apoptotic pathway [10]. In addition, Ost confers neuroprotection against traumatic brain injury in rats [11]. However, whether Ost attenuates EBI after SAH remains unclear. Therefore, this study aims to investigate the protective effects of Ost against EBI following SAH and its potential mechanisms.

Materials and methods

Animals

Sprague-Dawley (SD) rats (300-350 g) were purchased from the Experimental Animal Center of School of Medicine, Nanjing University, China. The rats were housed in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle and with free access to food and water. This study was performed

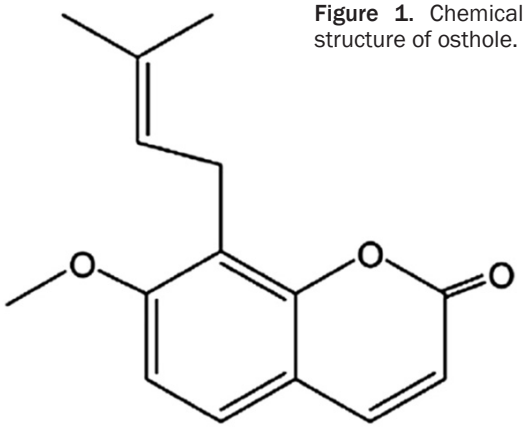

Figure 1. Chemical structure of osthole.

according to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996) and was approved by the Ethics Committee of Nanjing University.

Rat SAH model

Rat SAH model was produced according to the previous study [12]. Nonheparinized fresh autologous arterial blood (0.3 mL) was slowly injected into the prechiasmatic cistern over a 20-second period using a syringe pump and aseptic technique. Control animals were injected with 0.3 mL normal saline. The animals were allowed to recover 45 min after SAH. After the operation procedures, the rats were returned to the cages and the room temperature kept at 23±1°C. The rectal temperature was kept at 37±0.5°C throughout the experiment. Twenty milliliters of 0.9% NaCl was injected subcutaneously immediately after the operation to prevent dehydration. Twenty-four hours after SAH induction, the brains were removed and the inferior basal temporal lobes were collected.

Experimental protocol and drug administration

The rats were randomly assigned into 3 experimental groups: (1) the control group (n = 36), (2) the SAH + vehicle group (n = 36), (3) the SAH + Ost group (n = 36). Ost (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ost was dissolved in Tween 80 (less than 20%; Sigma, St. Louis, MO, USA). Rats of SAH + Ost group received 40 mg/kg Osti.p. at 30 min and 6 h after SAH induction. Rats of SAH + vehicle group received equal volumes of vehicle at the same time point. The dose of Ost was chosen according to previous studies [11, 13].

Evaluation of neurological score

Three behavioral activity examinations were performed at 48 h after SAH using the scoring system reported previously involving appetite, activity, and neurological deficits [14]. The behavior and activity scores were as follows: 1, appetite (finished meal 0; left meal unfinished 1; scarcely ate 2); 2, activity (walked and reach at least three corners of the cage 0; walked with some stimulation 1; almost always lying down 2); and 3, deficits (no deficits 0; unstable walk 1; impossible to walk 2). The minimum neurological score is 0 and the maximum is 6. A higher score represents more severe neurological deficits.

Evaluation of cerebral edema

Forty-eight hours after SAH, cerebral edema was determined by measuring the brain water content according to the wet-dry method [15]. These rats were killed and brains were immediately removed and placed on a frozen plate. Samples were immediately weighed to obtain wet weight. Then, samples were dried in a desiccating oven at 110°C for 48 h and weighed again to obtain the dry weight. Brain water content was calculated as follows: brain water content (%) = (wet weight - dry weight) × 100/wet weight.

Assessment of blood-brain barrier permeability

Blood-brain barrier permeability was determined by Evans blue (EB) extravasation at 48 h after SAH. Briefly, 2% EB was injected intravenously at a dose of 2 mL/kg. Animals were then re-anesthetized after 1 h with urethane (1000 mg/kg) and perfused using saline to remove intravascular EB dye. Animals were then decapitated, and the brains removed and homogenized in phosphate-buffered saline. Trichloroacetic acid was then added to precipitate protein, and the samples were cooled and centrifuged. The resulting supernatant was measured for absorbance of EB at 610 nm using a spectrophotometer.

ROS staining

ROS levels were analyzed using the ROS fluorescent probe dihydroethidium (DHE, Sigma-

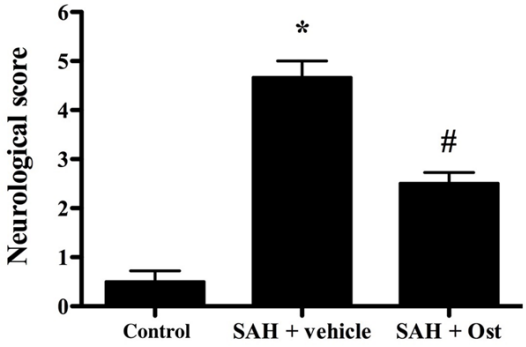

Figure 2. Effect of osthole on neurological score at 48 h after SAH. Osthole dramatically attenuated neurological deficit compared with that in the SAH + vehicle group. Data were expressed as mean ± S.E.M. (n = 6 for each group); $^{*}P < 0.05$ versus the Control group, $^{\#}P < 0.05$ versus the SAH + vehicle group.

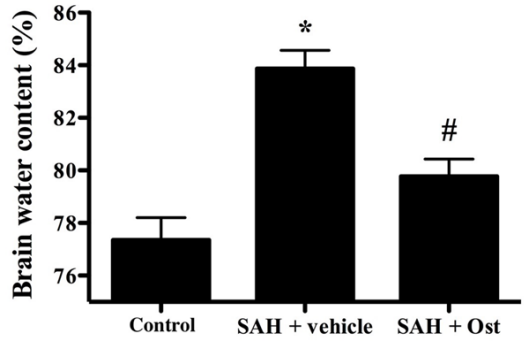

Figure 3. Effect of osthole on brain water content at 48 h after SAH. Data were expressed as mean ± S.E.M. (n = 6 for each group); $^{*}P < 0.05$ versus the Control group, $^{\#}P < 0.05$ versus the SAH + vehicle group.

Aldrich, St. Louis, MO, USA) 48 h after SAH. Rats were transcardially perfused with ice-cold 0.1 M phosphate-buffered saline (PBS; pH 7.4) after being anesthetized. The brains were quickly removed and frozen at -80°C for 20 min, and soon after, they were sliced into 15-μm-thick coronal sections by suing a freezing microtome. The sections were then mounted onto polylysine-coated slides. After a 30-min incubation in DHE (10 μmol/L), the slices was observed and photographed using a microscope. The DHE-positive cells were counted to reflect the tissues' ROS levels, as well as their oxidative stress levels [16].

TUNLEL staining

Apoptosis was detected using terminal deoxynucleotidyl transferased UTP nick end labeling

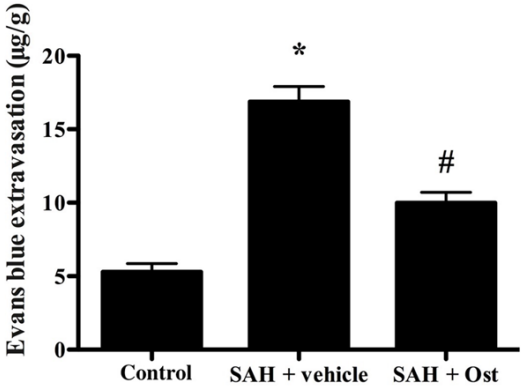

Figure 4. Effect of osthole on evans blue (EB) extravasation at 48 h after SAH. Data were expressed as mean ± S.E.M. (n = 6 for each group); $^{*}P < 0.05$ versus the Control group, $^{\#}P < 0.05$ versus the SAH + vehicle group.

(TUNEL) staining. Briefly, the brains were immediately removed and fixed in the paraformaldehyde solution at 4°C for 48 h. After dehydrating using sequential 24-h incubations in 10, 20, and 30% sucrose solutions, the tissues were sliced. The slices were put in a humidified box, treated with 0.3% hydrogen peroxide for 30 min at the room temperature and incubated with 0.25% proteinase K for 45 min at 37°C. The slices were then stained using the TUNEL reaction solution according to the manufacturer instructions in a humidified dark box for 60 min at 37°C. Finally, the slices were stained using DAPI for 10 min at 37°C, and observed with a fluorescence microscope. The apoptotic index was evaluated by calculating the ratio of TUNEL-positive cells to DAPI-staining cells.

Measurement of GSH level and MDA content

The rats were killed at 48 h after SAH and the brains were removed immediately. The tissues were homogenized with ice cold saline to 10% (w/v) homogenates. The oxidation-antioxidation status of the tissues was assessed by lipid peroxidation and GSH level. Lipid peroxidation was determined by detecting the level of MDA, a by-product of lipid peroxidation. The detailed procedures were followed the manufacturer's instructions (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

Western blot

The rats were sacrificed 48 h after SAH, and the brains were immediately removed. The tis-

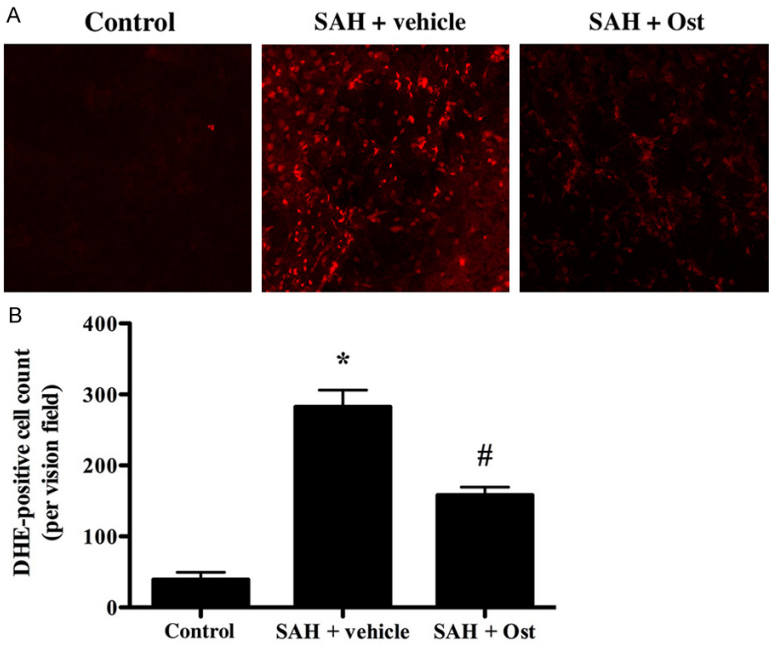

Figure 5. Effect of osthole on ROS production at 48 h after SAH. A. Representative confocal images of DHE staining are shown (400 ×). B. The histogram shows the percentages of DHE-positive cells. Data were expressed as mean ± S.E.M. (n = 6 for each group); $^{*}P < 0.05$ versus the Control group, $^{\#}P < 0.05$ versus the SAH + vehicle group.

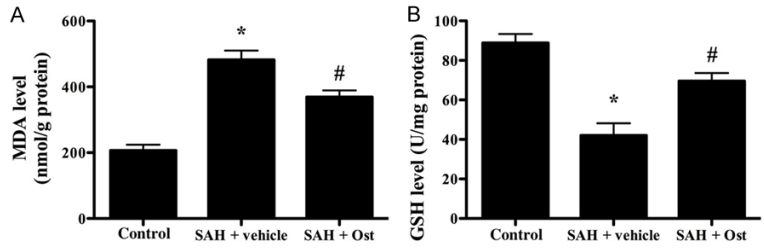

Figure 6. Effect of osthole on MDA and GSH levels at 48 h after SAH. A. MDA level. B. GSH level. Data were expressed as mean ± S.E.M. (n = 6 for each group); $^{*}P < 0.05$ versus the Control group, $^{\#}P < 0.05$ versus the SAH + vehicle group.

sue specimens were frozen and stored at -80°C. The samples were homogenized and quantified. The protein samples (40 μg/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% skimmed milk for 2 h at room temperature and incubated overnight at 4°C with preprimary antibody against Bcl-2 (1:1000, Cell Signaling Technology, Beverly, MA, USA), Bax (1:1000, Cell Signaling Technology, Beverly, MA, USA), active caspase-3 antibody (1:500, Abcam, Cambridge, UK), and β-actin (1:1000, Cell Signaling Technology, Beverly, MA, USA). After being washed, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody for 1 h at room temperature and processed for visualization by enzyme-linked chemiluminescence. Relative intensities of the bands were quantified by densitometric analysis.

Statistical analysis

Data are expressed as the means ± SEM. GraphPad Prism5 software (LaJolla, CA, USA) was used to analyze the data in the study. One-way ANOVA followed by Bonferroni multiple comparisons test was used for intergroup comparisons. A value of *P* less than 0.05 was considered statistically significant.

Results

Effect of osthole on neurological score

In comparison with the Control group, neurological deficits were observed in the SAH + vehicle group ($P < 0.05$). Neurological scores were significantly decreased in the SAH + Ost group compared to the SAH + vehicle group ($P < 0.05$) (**Figure 2**).

Effect of osthole on brain water content

In comparison with the Control group, the brain water content in the SAH + vehicle group significantly increased 48 h after SAH ($P < 0.05$). The brain water content significantly decreased in the SAH + Ost group compared with the SAH + vehicle group ($P < 0.05$) (**Figure 3**).

Effect of osthole on blood-brain barrier integrity

EB extravasation following SAH was shown in **Figure 4**. Rats in SAH + vehicle group indicated a significant increase in BBB permeability relative to rats of the Control group ($P < 0.05$). Administration of Ost significantly inhibited EB extravasation after SAH ($P < 0.05$). This result

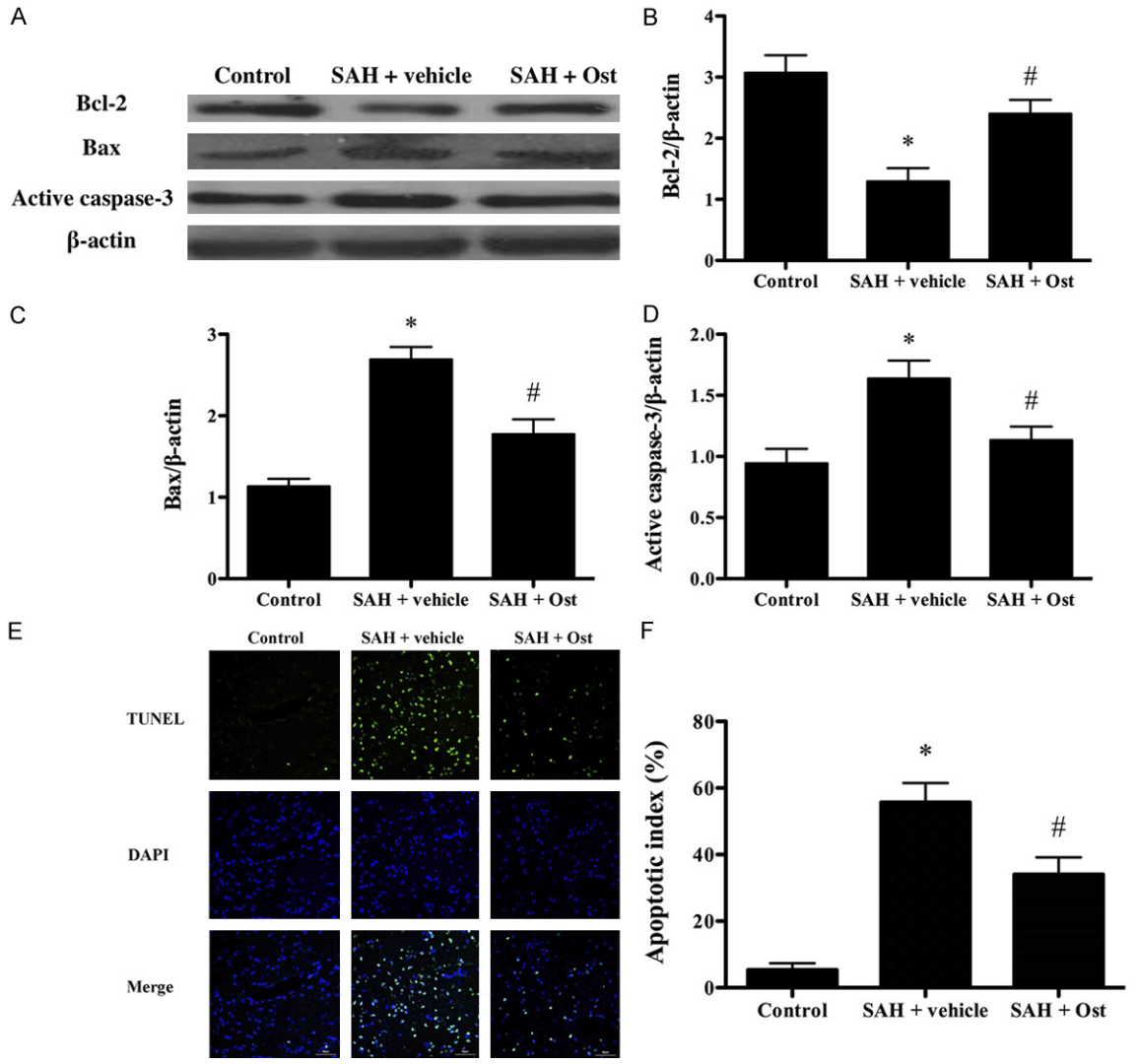

Figure 7. Effect of osthole on the expressions of Bcl-2, Bax and active caspase-3 detected by Western blot. A. Representative pictures of Western blot bands of Bcl-2, Bax and active caspase-3. B. Bcl-2/β-actin. C. Bax/β-actin. D. Active caspase-3/β-actin. E. Representative images of TUNEL staining. F. The quantification of TUNEL-positive cells. Data were expressed as mean ± S.E.M. (n = 6 for each group); $^{*}P < 0.05$ versus the Control group, $^{\#}P < 0.05$ versus the SAH + vehicle group.

suggested that Ost treatment could enhance the integrity of BBB.

Effect of osthole on ROS production

As shown in the **Figure 5**, the number of DHE-positive cells in the in the SAH + vehicle group significantly increased 48 h after SAH ($P < 0.05$). Ost treatment significantly decreased the number of DHE-positive cells compared with the SAH + vehicle group ($P < 0.05$).

Effect of osthole on MDA and GSH levels

As shown in **Figure 6**, the level of malondialdehyde (MDA) significantly increased, and the level of glutathione (GSH) significantly decreased in the SAH + vehicle group In comparison with the Control group ($P < 0.05$). Treatment with osthole significantly decreased MDA level, and increased GSH level compared with the SAH + vehicle group ($P < 0.05$).

Osthole treatment attenuates neuronal apoptosis following SAH

As shown in **Figure 7**, Bcl-2 expression significantly decreased in the SAH + vehicle group compared with that in the Control group ($P < 0.05$). However, osthole treatment increased Bcl-2 expression markedly ($P < 0.05$). On the

contrary, the Bax expression in the SAH + vehicle group increased significantly compared with that in the Control group ($P < 0.05$). However, Bax expression decreased in the SAH + Ost group ($P < 0.05$). In addition, the expression of active caspase-3 in the SAH + vehicle group increased dramatically compared with that in the Control group ($P < 0.05$). Meanwhile, active caspase-3 expression significantly decreased in the SAH + Ost group ($P < 0.05$). In addition, the results of TUNEL staining suggested that the apoptotic index significantly increased in the SAH + vehicle group compared with that in the Control group ($P < 0.05$). The administration of Ost dramatically decreased neuronal apoptosis (vs. the SAH + vehicle group, $P < 0.05$).

Discussion

The major findings of the current study are: (1) Ost treatment alleviates neurological deficits and brain edema after SAH. (2) Ost treatment enhances BBB integrity after SAH. (3) Ost treatment reduces oxidative stress and apoptosis following SAH. Ost treatment alleviates the animals' neurological deficits and brain edema 48 hours after SAH induction. Moreover, the numbers of DHE-positive cells, which represent reactive oxygen species production, were lower in SAH + Ost group than in the SAH + vehicle group. The reductions in MDA level and the elevation in GSH level also suggest Ost's persistent effects in attenuating oxidative stress following SAH. Ost can also decrease neuronal apoptosis.

EBI following SAH is regarded as the important cause of high mortality and morbidity [17]. And treatment of EBI is considered to be a major goal in the treatment of patients with SAH. The factors resulting in EBI include increased intracranial pressure (ICP), decreased cerebral blood flow (CBF), global cerebral ischemia, brain edema, BBB disruption, oxidative stress, and neuronal apoptosis [18]. Various mechanisms have been identified in the pathogenesis of EBI, such as inflammation, oxidative stress, and neuronal apoptosis. Meanwhile, these mechanisms also provide targets for the treatment of EBI. In the present study, our results suggest that Ost treatment significantly improves neurological deficits, attenuates brain water content, and alleviates BBB disruption. Then, we evaluate the possible mechanisms.

Oxidative stress has been indicated to play a pivotal role in the pathophysiological process of SAH [19]. Oxidative stress results from the excessive production of ROS, or free radicals. Plenty of ROS are produced after SAH include superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide, and peroxynitrate [20]. Oxidative stress leads to the peroxidation of biological membranes, thus changes the fluidity and permeability of the cell membrane, and ultimately destroys cell structure and function [16]. MDA, generated from the collapse of polyunsaturated fatty acids, is an indicator for lipid peroxidation. GSH, the most important intracellular nonprotein thiol, is a key ROS scavenger. In this experiment, the number of DHE-positive cells increased dramatically in the SAH + vehicle group. Ost treatment significantly decreased the number of DHE-positive cells. Moreover, the level of MDA was significantly increased and the level of GSH was significantly decreased in the SAH + vehicle group. Treatment with Ost significantly lowered the level of MDA, and increased GSH level after SAH. These results suggested that Ost may decrease the excessive production of ROS and enhance antioxidant ability against EBI induced by SAH in rats.

Additionally, it has been suggested that neuronal apoptosis is also a key process involved in SAH-induced EBI. Therefore, we evaluate the effects of Ost on apoptosis. The Bcl-2 family proteins play critical roles in regulating cell apoptosis [21]. The family can be divided into two categories: the members of anti-apoptosis group include Bcl-2, Bcl-XL, and Bcl-W, and the members of pro-apoptosis group includes Bax, Bak, and Bad [22]. Bcl-2 is located in the outer membrane of mitochondria, and it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins. Baxacts on the mitochondrial membrane to promote release of cytochrome c and ROS, leading to apoptosis cascade [23]. Bcl-2 can inhibit the release of cytochrome c (Cyt c), which may block the transport of electron in the respiratory chain. On the contrary, Baxinitiates apoptosis by forming a pore in the mitochondrial outer membrane that allows Cyt c to escape into the cytoplasm and activate the pro-apoptotic caspase cascade [23]. Caspase-3 is a critical caspase in the execution phase of cell apoptosis. In the present study, our results suggest that Ost treatment confers anti-apop-

totic effects via up-regulating Bcl-2 expression and down-regulating Bax and active caspase-3 expression.

In summary, the present study demonstrates that Ost alleviates EBI following SAH via the attenuation of oxidative stress and neuronal apoptosis. Our findings suggest the possible therapeutic application of Ost for the management of SAH. But some questions, such as whether Ost confers anti-inflammatory activity during SAH, need to be further investigated. And in our future study, we will evaluate whether Ost plays a neuroprotective role via its anti-inflammation activity and its potential mechanisms.

Disclosure of conflict of interest

None.

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