Salvianolic acid A attenuates ischemia reperfusion induced rat brain damage by protecting the blood brain barrier through MMP-9 inhibition and anti-inflammation

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[ABSTRACT] Salvianolic acid A (SAA) is a water-soluble component from the root of Salvia Miltiorrhiza Bge, a traditional Chinese medicine, which has been used for the treatment of cerebrovascular diseases for centuries. The present study aimed to determine the brain protective effects of SAA against cerebral ischemia reperfusion injury in rats, and to figure out whether SAA could protect the blood brain barrier (BBB) through matrix metallopeptidase 9 (MMP-9) inhibition. A focal cerebral ischemia reperfusion model was induced by middle cerebral artery occlusion (MCAO) for 1.5-h followed by 24-h reperfusion. SAA was administered intravenously at doses of 5, 10, and 20 mg·kg⁻¹. SAA significantly reduced the infarct volumes and neurological deficit scores. Immunohistochemical analyses showed that SAA treatments could also improve the morphology of neurons in hippocampus CA1 and CA3 regions and increase the number of neurons. Western blotting analyses showed that SAA downregulated the levels of MMP-9 and upregulated the levels of tissue inhibitor of metalloproteinase 1 (TIMP-1) to attenuate BBB injury. SAA treatment significantly prevented MMP-9-induced degradation of ZO-1, claudin-5 and occludin proteins. SAA also prevented cerebral NF-κB p65 activation and reduced inflammation response. Our results suggested that SAA could be a promising agent to attenuate cerebral ischemia reperfusion injury through MMP-9 inhibition and anti-inflammation activities.

[KEY WORDS] Salvianolic acid A; Ischemia; MCAO; Blood brain barrier; NF-κB; MMP-9

[Introduction] With the increasing pressure of life and work, the incidence of cerebrovascular diseases is increasing year by year. Among these diseases, stroke is a great threat to the human health and brings huge burden to society and families. Ischemic stroke is about 87% of the entire stroke cases [¹]. Up to date, recombinant tissue plasminogen activator (rt-PA) has been the most effective agent to improve the prognosis of acute ischemic stroke [²]. However, the clinical use of rt-PA is limited by its relatively narrow therapeutic time window and a serious risk of hemorrhagic complications [³-⁵]. The development of new drug for the treatment of brain ischemic stroke is an important task worldwide.

Matrix metalloproteinase 9 (MMP-9) is a member of the MMP family. Its main substrates are extracellular matrix components maintaining the integrity of blood-brain barrier [⁶]. Under normal physiological conditions, the expression of MMP-9 in brain is strictly controlled and remains at a low level [⁷]; while under cerebral ischemia and reperfusion (I/R)
conditions, the expression and activity of MMP-9 are increased significantly, which leads to the redistribution and degradation of tight junction proteins (ZO-1, claudin-5 and occludin) and causes severe blood-brain barrier injury with neuronal inflammation [8]. Tissue inhibitor of metalloproteinase 1 (TIMP-1) is the endogenous inhibitor of MMP-9 [9]. MMP-9 inhibition or maintaining the MMP-9/TIMP-1 balance may be a new target for the treatment of ischemic stroke.

Salvianolic acid A (SAA, Fig. 1) is one of the main active compounds extracted from *Salvia Miltiorrhiza Bge.*, a traditional Chinese medicine. It was first reported that the therapeutic effect of SAA on I/R might be related to the inhibition of brain lipid peroxidation and the scavenging of free radicals [10-11]. Then, other studies showed its protective mechanisms might include the inhibition of ICAM-1 (intercellular cell adhesion molecule-1), CD11b/CD18, soluble epoxide hydrolase and granulocyte adherence [12-17]. A most recent study showed SAA alleviated ischemic brain injury through the inhibition of inflammation and apoptosis and the promotion of neurogenesis [18], but the underlying mechanism remains to be further elucidated. It is reported that salvianolic acid B (SAB), a structurally similar polyphenol compound with SAA, significantly attenuates LPS-induced cell migration through the inactivation of MMP-2 and MMP-9 protein synthesis [19]. By the inhibition of MMP-9, SAA prevents cardiac remodeling in spontaneously hypertensive rats and attenuates aortic aneurysm formation in apolipoprotein E-deficient mice [20-21]. The above results suggest that the anti-ischemia effects of SAA may be due to the inhibition of MMP-9, which results in severe blood-brain barrier injury. In order to clarify the mechanism by which SAA exerts the brain protective effects against ischemia reperfusion injury, a middle cerebral artery occlusion and reperfusion (MCAO/R) rat model was used in the present study, and the MMP-9 inhibition and anti-inflammation effects of SAA were determined.

**Materials and Methods**

*Animals, agents, and chemicals*

Male Sprague-Dawley rats, weighing 240–260 g, were obtained from Beijing Vital River Experimental Animal Co., Ltd. (Beijing, China; certificate No. SCXK2012-0001). All the animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (No. 00000901, 2016. 03.15). Efforts were made to minimize the pain and discomfort of animals.

SAA with a purity of > 98% by HPLC analysis were provided by the Institute of Materia Medica (Beijing, China). Edaravone injection was purchased from Simcere (Nanjing, China) and was set as the positive control. The suture used in MCAO/R model was purchased from Cinontech Co., Ltd. (Beijing, China). Rat Interleukin 6 (IL-6), Interleukin 1β (IL-1β) and Tumor necrosis factor-α (TNF-α) ELISA Kit were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). MMP Zymography Assay Kit (for MMP-2 and MMP-9 analyses) was purchased from Applygen Technologies Inc. (Beijing, China). Evans Blue was purchased from Sigma Chemical Co., Ltd. (Shanghai, China). Total Protein Extraction Kit was purchased from Applygen Technologies, Inc. (Beijing, China). BCA Protein Assay Kit was purchased from Cwbio (Beijing, China). The antibodies against MMP-9, MMP-2, TIMP-1, phospho-IkBα (p-IκBα), IκBα, phospho-NF-κB p65 (p-NF-κB p65), NF-κB p65, ZO-1, and β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies against claudin-5, occludin, phospho-IKKα/β (p-IKKα/β), IKKα/β and Histone H1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). And the HRP conjugated goat anti-rabbit IgG and HRP conjugated goat anti-mouse IgG were both purchased from Cwbio (Beijing, China). All other chemicals used in the present study were of analytical reagent grade and commercially available.

**Preparation of rat middle cerebral artery occlusion (MCAO) model and SAA administration**

The rats were randomly divided into sham operation group (n = 35), I/R group (n = 40), I/R + SAA (5, 10, and 20 mg·kg⁻¹) groups (n = 40) and Edaravone (5 mg·kg⁻¹) group (n = 20). The SAA doses were selected based on our previous pharmacokinetic study [22-24]. Because SAA was rapidly eliminated from animal body, relatively high doses (5, 10, and 20 mg·kg⁻¹) were selected to maintain a high plasma and tissue concentration. SAA was diluted with normal saline. Focal cerebral I/R model was established by MCAO for 1.5-h followed by 24-h reperfusion as described before [25]. First, the rats were fasted overnight with free access to tap water. After anesthetized with 10% chloral hydrate (380 mg·kg⁻¹), i.p.), the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) of the rats were isolated. An 18-mm length of nylon suture (ϕ: 0.2 mm) was introduced into the ECA lumen and advanced into the ICA to block the origin of the middle cerebral artery. Occlusion was performed for 1.5-h, then the nylon suture was withdrawn for reperfusion. The sham operation rats received all surgical procedures but without the suture inserted. Right after the reperfusion, I/R + SAA groups were intravenously given dif-
different concentrations of SAA (5, 10, and 20 mg·kg⁻¹). The positive group received 5 mg·kg⁻¹ of Edaravone. The sham operation group and I/R group received equal volume of normal saline. After 12-h of the reperfusion, the above administration method was performed again to maintain relatively high plasma concentrations of SAA and Edaravone. During the operation procedure, the rectal temperature of the rats was kept between 36.5 and 37.5 °C. Rat body temperature was carefully monitored during the post-operation period. After the experiment, the animals were housed individually with free access to food and tap water.

**Evaluation of neurological deficit scores, motor deficits, infarct volumes and cerebral edema**

After 24-h of reperfusion, the neurological deficit scores and inclined plane test were carried out. According to Longa’s method, the scale of 0–4 scores was used: 0 (indicated no neurologic deficit), 1 (failure to extend left forepaw fully), 2 (circling to the left), 3 (falling to the left), and 4 (did not walk spontaneously and had a depressed level of consciousness) for each rat. The rats with a score of 0 were screened out of the further study, which indicated no neurologic deficit. The rats with a score of 4 indicating an extremely serious damage to brain, were also excluded from the further study. Inclined plane assessment was performed to investigate the motor function by placing the rats on the coarse surface of an inclined plane with an angle 60°. The duration of each rat stayed on the inclined plane was recorded. If a rat stayed on the inclined plane for more than 3 min, the score was recorded as 180 s.

For the quantification of infarct volumes, the rats were anesthetized with intraperitoneal injection of 10% chloral hydrate and sacrificed. After being removed and frozen at −40 °C for 15 min, the brains were dissected and sliced into 2-mm thick coronal sections and stained with a 2% (weight/volume) solution of TTC in saline at 37 °C for 15 min. The brain slices were then fixed with 4% paraformaldehyde before photographed. The infarct areas and hemispheric contours were outlined by an investigator blinded to the experimental groups using NIH Image J software (National Institutes of Health, Bethesda, MD, USA). Infarct volumes were calculated using the brain slice thickness and the calculated infarct areas. The results were expressed as a percentage of total hemisphere.

For cerebral edema evaluation, the rat brains were quickly removed and dissected into two halves along the sagittal suture. Ischemic hemispheres were weighed (wet weight) before dried overnight at 100 °C in a drying oven. After being dried, the hemispheres were weighed again (dry weight). Cerebral edema (%) was calculated as (wet weight−dry weight)/wet weight × 100.

**Hematoxylin & eosin staining**

Hematoxylin and eosin (H&E) staining was performed to determine the necrosis rate of brain neurons. After anesthetization with intraperitoneal injection of 10% chloral hydrate, the rats were perfused through the left ventricle with 0.9% saline followed by 4% paraformaldehyde. The rat brains were removed quickly, fixed with paraformaldehyde, embedded in paraffin, and sectioned into 5-µm thick slices serially. Then the slices were stained with H&E and the rat hippocampus CA1 and CA3 regions were captured.

**Nissl staining**

Nissl staining was performed to assess the ischemic damage. Briefly, after the rats were sacrificed, the brains were immediately perfused with saline, then followed by 4% paraformaldehyde phosphate buffer. Next, the prepared brains were embedded in paraffin and cut into 5-µm slices. These slices were then treated with xylene and different concentrations of alcohol following steps, and stained with 0.1% cresyl violet. After being dehydrated through alcohols, the brain slices were placed in xylene again and then examined under a light microscope.

**Measurement of cytokines**

The concentrations of IL-6 (CSB-E04640r), IL-1β (CSB-E08055r) and TNF-α (CSB-E11987r) in the brains were evaluated according to the ELISA kits instructions of Cusabio Biotech Co., Ltd. (Wuhan, China). Briefly, 0.1 g of cerebral cortex was carefully rinsed with 1 × PBS. After homogenized in 1 mL of 1 × PBS, the homogenates were kept overnight at −20 °C and followed by two freeze-thaw cycles. Then the homogenens were centrifuged (5 000 × g, 4 °C) for 5 min. The supernatants were immediately removed and assayed at 450 nm with SpectraMax M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The IL-6, IL-1β and TNF-α levels were expressed in pg·mg⁻¹ protein.

**Evans Blue extravasation test**

After 24-h reperfusion, the rats were anesthetized with 10% chloral hydrate (380 mg·kg⁻¹, i.p.). Then 4% Evans Blue in saline was intravenously administered (0.2 mL/100 g). Each rat was perfused with 20 mL of 10 U·mL⁻¹ heparinized saline to wash out blood. The brain was then isolated, weighed, and homogenized in 50% solution of trichloroacetic acid. After centrifugation at 400 × g for 20 min, the supernatant was collected and spectrophotometrically measured at 620 nm. Cerebral Evans Blue was quantified as nanograms per ischemic hemisphere.

**MMP zymography**

MMP-2 and MMP-9 activities in the brain tissue were determined by MMP Zymography Assay Kit according to the manufacturers’ instructions. Briefly, the proteins were extracted, and protein concentration was determined using the BCA Protein Assay Reagent Kit. Then the samples were diluted with 2 × SDS-PAGE non-reducing buffer, and the mixtures (40 µg sample per lane) were separated on 8% SDS polyacrylamide gels containing 0.1% gelatin. The gels were washed twice for 30 min in buffer A, incubated overnight in buffer B, stained for 2-h with 0.25% Coomassie brilliant blue, and destained in destaining buffer (10% acetic acid and 20% methanol) for 60 min before scanned using the Molecular Image ChemiDoc XRS Plus Gel Imagine System (Bio-Rad, Hercules, CA, USA).
**Western blotting analysis**

Total protein was extracted from the ischemic and control cortex using the Total Protein Extraction Kit following the manufacturers’ protocols. Protein concentration of the supernatant was determined using the BCA Protein Assay Reagent Kit with bovine serum albumin (BSA) as the standard. Equivalent amounts of protein samples were separated by 6% and 10% sodium dodecyl sulfate-polyacrylamide gels prior (SDS/PAGE) and transferred for 2 h onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). After blocking with 5% BSA in TBST for 2 h, the membranes were incubated overnight at 4 °C with the primary antibodies. After being washed thrice in TBST, the membranes were incubated with secondary antibodies against HRP conjugated rabbit or mouse (1:1000 dilution) IgG for 1.5 h at room temperature. An enhanced chemiluminescence (ECL) was used to visualize the immunoreactive bands, which were captured through ChemiDoc-it2 Imager (UVP, Upland, CA, USA). Optical density of the bands was analyzed by NIH Image J software.

**Statistical analysis**

All the data were expressed as means ± standard deviation (SD), and all statistical analyses were performed using the SPSS software (Version 16.0; SPSS, Chicago, IL, USA), and the differences among experimental groups were evaluated using a one-way analysis of variance (ANOVA) followed by a Tukey’s Multiple Comparison post-hoc test. \( P < 0.05 \) was considered statistically significant.

**Results**

**SAA improves neurological and motor deficits in I/R rats**

SAA (5, 10, and 20 mg·kg\(^{-1}\)) was injected intravenously to investigate its neuroprotective effects against I/R. Neurological deficit of the rats was examined and scored on the above mentioned five-point scale. Sham operation group didn’t have any abnormal symptom, and neurological deficit scores of these rats were marked as 0 point; the rest group rats showed more or less different levels of neurological deficits. The rats of model group subjected to MCAO and reperfusion showed significant motor behavioral deficits. While SAA could dose-dependently reduce the neurological deficit scores of the rats, compared with the model group (Fig. 2A).

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**Fig. 2** Neuroprotection effects of SAA on rat I/R injury. SAA (5, 10 and 20 mg·kg\(^{-1}\)) or vehicle was administered intravenously at 1.5 h after MCAO. (A) Neurological deficit scores at 24 h after MCAO/R. (B) Retention time on inclined plate. (C) Percent of cerebral water content or percent of cerebral edema. (D) Six consecutive TTC-stained coronal brain slices arranged in order. The white area represented infarcted tissue. (E) Infarct volumes calculated with the slices thickness and infract areas of TTC staining results. Data are expressed as means ± SD \((n = 8)\). * \( P < 0.05 \), ** \( P < 0.01 \) and *** \( P < 0.001 \) compared with model group, # \( P < 0.05 \) and ### \( P < 0.001 \) compared with sham operation group. Significance was determined by one-way analysis of ANOVA followed by Tukey’s test.
The improvement of coordinative motor function after SAA treatment could be observed on the inclined plane test. On this test, rat walked on an inclined plate and the time at which rats began to slip or fall was recorded. The rats of sham operation group stayed on the inclined plate for the longest time. While the rats of model group could hardly stay on the inclined plate. A significant beneficial effect of SAA was observed in the SAA treatment groups. SAA (20 mg·kg⁻¹) could significantly prolonged the retention time of rats on the (Fig. 2B, \( P < 0.05 \)).

SAA reduces infarct volumes and cerebral edema in I/R rats

After 24-h reperfusion, sham operation group didn’t show cerebral edema. While compared with sham operation group, the brain water content in model group significantly increased, which was attenuated significantly by SAA (10 and 20 mg·kg⁻¹) (Fig. 2C, \( P < 0.01 \) and \( P < 0.05 \), respectively).

Cerebral infarction volumes were analyzed and calculated at 24-h after reperfusion in rats with TTC staining. The whole brain tissues of sham operation group were deep red and there were no infarctions, while the rats of the model group showed extensive infarction in the cerebral cortical and subcortical areas. Compared with the model group, SAA (20 mg·kg⁻¹) reduced infarct volumes significantly (Fig. 2D and 2E, \( P < 0.001 \)).

SAA improves neuronal damage in I/R rats

In the sham operation group, the morphology and structures of nerve cells was normal, the cytoplasm was abundant, and the nucleus was round under a light microscope. In the model group, the structures of the nerve cells in the core infarct area showed disorder and loose. The chromatin condensation of nuclei was also observed. Extensively damaged cells were observed, and surviving neurons number was decreased significantly. Compared with the model group, SAA (5, 10, and 20 mg·kg⁻¹) could maintain the normal structures of neurons and increase neurons number (Fig. 3).

SAA attenuates I/R-induced BBB injury and tight junction degradation

The results of Evans Blue extravasation clearly showed that the BBB breakdown was significantly attenuated by SAA treatment (Figs. 4A and 4B). Tight junction proteins (ZO-1, claudin-5 and occludin) are essential components of BBB and involved in BBB integrity. These proteins are substrates of MMP-9. I/R markedly downregulated ZO-1, claudin-5 and occludin proteins levels. The downregulation of ZO-1 protein was significantly prevented by SAA treatment (Fig. 4C). And SAA (10 mg·kg⁻¹) significantly upregulated claudin-5 protein levels (Fig. 4D). SAA (20 mg·kg⁻¹) significantly increased occludin protein levels (Fig. 4E).

SAA down-regulates MMP-9 and up-regulates TIMP-1 to attenuate BBB injury

Under normal conditions, MMP-9 and MMP-2 were normally expressed at very low levels. I/R significantly induced MMP-9 and MMP-2 expression. SAA (20 mg·kg⁻¹) could significantly reduce I/R induced MMP-9 upregulation (\( P < 0.001 \), Fig. 5A). While the MMP-2 expression was not significantly affected by SAA (Fig. 5B). The results of the gelatin zymography assay also indicated that SAA could significantly downregulate the expression of MMP-9, but had almost no effects on MMP-2 (Fig. 5C). Tissue inhibitors of metalloproteinases (TIMPs) could inhibit the activity of MMPs through high affinity binding to MMPs catalytic domain. Our results showed that the expression of TIMP-1 was decreased significantly in the model group, while SAA treated group could dose-dependently inhibit the downregulation of TIMP-1 by I/R (Fig. 5D).

SAA prevents cerebral NF-κB activation and reduces inflammation response

Large amount of NF-κB p65, IκB-α and IKKα/β phosphorylation occurred in the model group, but not in the sham operation group (Fig. 6). Compared with the model group, the level of nucleus p-NF-κB p65 was decreased significantly in 20 mg·kg⁻¹ SAA-treated groups (\( P < 0.001 \), Fig. 6A). SAA treatment reduced the phosphorylation of...
IκB-α and IKKα/β (Figs. 6B and 6C). Furthermore, the levels of inflammatory cytokines IL-6, IL-1β and TNF-α in brain tissues of the model group were significantly higher than that in the control group (P < 0.001), while SAA treatment could reduce the production of these cytokines significantly (Figs. 6D, 6E, and 6F).

Discussion

Previous reports have shown that SAA might have good therapeutic effects in the treatment of cardiovascular and cerebrovascular diseases \[30-31\]. It could inhibit the endothelial dysfunction and cardiac remodeling in spontaneously hypertensive rats \[20, 32\]. SAA also has good anti-apoptotic effects during myocardial ischemic reperfusion injury by the activation of ERK1/2 and the inhibition of JNK \[33\].

In our present study, the protection effects of SAA on rats subjected to I/R were observed. Elevated activities and expression of MMP-9 occurred under I/R condition. The excessive activation and expression of MMP-9 induced BBB leakage and inflammation, and exerted damaging effects on the brain. SAA could suppress I/R-induced MMP-9 expression, and inhibit NF-κB activation in rats.

The BBB is a key regulator of brain homeostasis and a central target for neuroprotection \[34\]. It is mainly composed of microvascular endothelial cells closely surrounded by pericytes, neuron endings, astrocyte foot processes and tight junctions. Any of these components breakdown may result in BBB damage and dysfunction, which is an important feature of ischemic brain injury. Normally, the BBB could effectively protect brain from outside stimuli. After ischemic injury, the blood brain-barrier permeability is significantly increased, and lots of toxic and harmful substances move across the
BBB [35]. An abnormal BBB could lead to severe cerebral edema and infarction. In the present study, Evans Blue extravasation assay was used to assess the BBB permeability. The results clearly showed that SAA treatment could significantly attenuate the BBB components breakdown.

Matrix metalloproteinases (MMPs), especially gelatinases (MMP-2 and 9), are considered as new therapeutic strategy in ischemic stroke by protecting BBB [36-38]. MMP-9 upregulation occurs after ischemic stroke and is associated with BBB breakdown, cerebral edema and hemorrhagic transformation. Recently, salvianolic acids have been reported to be MMP-9 inhibitors [36-37, 20]. These studies well support our results that SAA could significantly reduce the I/R induced MMP-9 upregulation. The results of the present study demonstrated that SAA had good effects on attenuating BBB injury.

Tight junctions are particularly important structural and functional components of the BBB [39]. They are composed of cytoskeletal and tight junction proteins, including ZO-1, claudin-5 and occludin. The degradation of tight junction proteins contributes to the breakdown of BBB and the developments of cerebral edema [40]. In our studies, I/R markedly downregulated the levels of ZO-1, claudin-5 and occludin, while SAA treatment significantly inhibited the downregulation of ZO-1, claudin-5 and occludin proteins. Based on these results, we hypothesized that SAA could protect BBB integrity by inhibiting MMP-9 mediated tight junction degradation after I/R.
Fig. 6 Effects of SAA on inflammation related proteins and cytokines expression (n = 6). (A) Representative Western blots of p-NF-κB p65 and NF-κB p65, (B) Representative Western blots of p-IκB-α and IκB-α, (C) Representative Western blots of p-IKKα/β and IKKα/β, (D) The quantitative analysis of IL-6, (E) The quantitative analysis of IL-1β and (F) The quantitative analysis of TNF-α. *P < 0.05 and ***P < 0.001 vs model group, ###P < 0.001 vs sham operation group. Significance was determined by one-way analysis of ANOVA followed by Tukey’s test.

MMPs act on pro-inflammatory cytokines, chemokines, and other proteins to regulate a variety of inflammatory responses [41]. In the present study, the rats subjected to I/R produced severe inflammatory response, which played an important role in brain injury and resulted in extremely terrible final. SAA could attenuate NF-κB activation by the inhibition of IκBα phosphorylation and the nucleus translocation of NF-κB. SAA has been reported to own strong anti-inflammatory effects. It could modulate NF-κB-dependent inflammatory pathways through IKKβ inhibition in lipopolysaccharide-stimulated RAW264.7 cells [42-43]. SAB is also a compound isolated from Danshen, which has a similar chemical structure with SAA. SAB could also suppress LPS-induced expression of iNOS, TNF-α, and IL-1β mRNAs by inhibiting transcription factor NF-κB activation, which contributes to its anti-inflammatory effects [44]. What’s more, it is reported that SAB could improve motor function of rats subjected to cerebral ischemia [45], and also reduce the score of neurological deficits, increase serum SOD activities and decrease MDA concentrations of rats subjected to ischemia reperfusion [46]. The results of SAB have given a clue for us to study the anti-ischemia reperfusion effects of SAA in the present study.

In conclusion, our results from the present study suggested that SAA owned excellent neuroprotective effects against I/R injury, including BBB protection and anti-inflammation effects. These findings indicated a promising therapeutic potential for SAA as a leading compound towards the treatment of ischemic stroke.

References


