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ORIGINAL PAPER

Gastrodin Alleviates Cerebral Ischemic Damage in Mice by Improving Anti-oxidant and Anti-inflammation Activities and Inhibiting Apoptosis Pathway

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Abstract Gastrodin (GAS), an active constituent of the Chinese herbal medicine Tianma, has anti-oxidant and anti-inflammation activities but its protective effect to the prevention of neurotoxicity induced by ischemic stroke is unclear. In the present study, middle cerebral artery occlusion (MCAO) was used to establish a mice ischemic stroke model. Infarct volume ratio and neurobehavioral score were evaluated, Nissl staining was performed and the expression of cleaved Caspase 3, Bax and B cell lymphoma 2 (Bcl-2) were assessed at 24 h or 7 days after reperfusion. In addition, the total superoxide dismutase (SOD) activity and malondialdehyde (MDA) content, as well as the expression of Nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), SOD1, phospho-Akt and total Akt and TNF-α and IL-1β in the ischemic hemispheres were also observed at 6 h after reperfusion to assess oxidative stress and inflammatory changes after GAS treatment. It was found that GAS, especially at high dose (100 mg/kg) reduced tested neuronal injury and neurobehavioral deficient in MCAO mice. Enhanced expression of cleaved Caspase 3 and Bax and decreased expression of Bcl-2 by MCAO were also reversed by GAS. Moreover, GAS treatment decreased the MDA content and the expression of TNF-α and IL-1β, and increased amount of SOD activity and the expression of HO-1 and SOD1 in GAS-treated ischemic brain. Furthermore, GAS significantly increased Akt phosphorylation and Nrf2 expression. These results support the neuroprotective effects of GAS, and the activation of Akt/Nrf2 pathway may play a critical role in the pharmacological action of GAS.

Keywords Gastrodin · Cerebral ischemia/reperfusion · Akt · Nrf2

Abbreviations

GAS	Gastrodin
Nrf2	Nuclear factor erythroid 2-related factor 2
TNF-α	Tumor necrosis factor-α
IL-1β	Interleukin-1β
ROS	Reactive oxygen species
CAT	Catalase
SOD	Superoxide dismutase
MCAO	Middle cerebral artery occlusion
PI3-K	Phosphatidylinositol 3-kinase
HO-1	Heme oxygenase-1
MDA	Malondialdehyde

Background

Stroke is one of the primary causes of severe acquired disability worldwide [1]. The sudden blockage of blood

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flow from a thrombus or embolism causes about 80 % of all stroke victims experienced an ischemic event [2, 3]. Administration of the thrombolytic agent tissue plasminogen activator (tPA) is an efficient treatment and it is still the only FDA approved therapy for acute ischemic stroke [4, 5]. However, tPA has many shortcomings including the potential risk of hemorrhagic transformation, limited therapeutic window and efficacy [6, 7]. Thus, cerebral ischemia is still a major medical problem and novel and effective therapeutic approaches are still urgently required.

The exact mechanisms responsible for the brain damage suffering from ischemic insult are still not fully understood; there are increasing evidence suggesting that post-ischemia oxidative stress and inflammation are significant contributing factors to the pathogenic process [8–11]. Reactive oxygen species (ROS) are found greatly elevated which could exceed the antioxidant capacity of the ischemic brain, thus activate diverse signaling pathways and result in oxidative stress [12, 13], and ROS could initiate the expression of inflammatory cytokines [14], including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). Meanwhile, studies reported that systemic administration of antioxidants or anti-inflammatory agents could improve neurological deficit, reduce brain edema and infarct sizes and regulate cytokines expression in the cortex [15–17]. It suggests that agents with anti-oxidative or anti-inflammatory effects might be benefit for the treatment of cerebral ischemia.

Gastrodia elata Blume, known as tianma in Chinese, is a plant of the orchidaceae family, has been used for thousands of years in China. Pharmacological studies indicate that tianma has analgesic, nootropic, and anti-inflammatory effects, and improves microcirculation and general circulatory functions [18, 19]. The phenolic glucoside gastrodin (GAS) is a main phenolic compound derived from Gastrodia elata Blume root [20]. Studies indicate that GAS has a neuroprotective action against hypoxia in cultured cortical neuron [21], and GAS could inhibit the expression of inducible NO synthase (iNOS), cyclooxygenase-2 and pro-inflammatory cytokines in cultured LPS-stimulated microglia [22]. It may also improve learning ability and facilitate memory consolidation and retrieval [23]. Our previous study also showed that GAS could reduce the levels of IL-6 and IL-1β, down-regulate the expression of iNOS and inhibite p38 MAPK phosphorylation in the hippocampus of posttraumatic stress disorder model [24], and GAS could also reverse the expression of phospho-inhibitor of kappa B (p-iκB), nuclear factor-kappa B (NF-κB), and IL-1β of hippocampus in chronic unpredictable stress rats and protect hippocampal derived neural stem cell from IL-1β-induced damage [25], suggesting that GAS may suppress specific signaling pathways associated with the inflammatory response. Moreover, recent studies showed that the beneficial effects of gastrodin might result from its antioxidant properties [26]. GAS could protect primary cultured rat hippocampal neurons against amyloid-beta peptide-induced neurotoxicity and attenuate the reduction of catalase (CAT) and superoxide dismutase (SOD) expression via the activation of ERK1/2-Nrf2 pathway [27]. These observations lead to the hypothesis that GAS may provide neuroprotection against ischemic brain injury. To test this possibility, we investigated the potential neuroprotective efficacy of GAS in a mouse middle cerebral artery occlusion (MCAO) model. We also explored its anti-oxidative effects by assessing oxidative-related factors against stroke damage induced by MCAO in mice.

Materials and Methods

Experimental Animals and Drug Injection

The C57BL/6 J mice in this study were obtained from the Experimental Animal Center at the Fourth Military Medical University, Xi'an, China. The animals were housed at 21 ± 2 °C, with 60–70 % humidity for at least 1 weeks before surgery. They were also under a fixed 12-h light/dark cycle and had free access to food and water. Procedures for animals were reviewed and approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University.

GAS (purity >99.2 %) was supplied by Kunming Pharmaceutical Corporation (Kunming, China). In the present study, GAS was dissolved in saline and administered intraperitoneally to all GAS groups at the same time. The doses were chosen according to previous pharmacological studies of GAS in rats [28, 29] and adjusted into dose for mice according to our preliminary tests. After the adaptive phase, mice were randomly divided into five groups: sham (only received saline), MCAO + vehicle (saline), MCAO + GAS (L) (low-dose, L = 10 mg/kg), MCAO + GAS (M) (medium dose, M = 50 mg/kg), MCAO + GAS (H) (high-dose, H = 100 mg/kg). Saline or GAS were immediately injected intraperitoneally at the onset of cerebral reperfusion.

Induction of Transient Focal Cerebral Ischemia

The focal cerebral ischemia was induced by MCAO with an intraluminal filament as described previously [30, 31]. Briefly, mice were anesthetized with 2 % isoflurane carried by 2 L/min oxygen through a face mask. Six-0 monofilament nylon suture with a rounded tip (Beijing, shadong) was inserted through a small incision on the right common carotid artery and forwarded into the internal carotid artery

until a small resistance was felt. And the filament was fixed with silk suture knot on the common carotid artery. After 1 h, the filament was slowly withdrawn to allow reperfusion. Regional cerebral blood (rCBF) flow was monitored using laser Doppler flowmetry (Perimed AB, PeriFlux System 5000, Stockholm, Sweden) in the ipsilateral cortex. MCAO was considered successful if rCBF sharply decreased below 30 % of baseline; successful reperfusion was considered if rCBF was recovered up to 80 % of baseline. Otherwise, animals were excluded from analysis and sacrificed by anesthesia death. The mice in sham group were not experienced middle cerebral artery occlusion; they just anesthetized with 2 % isoflurane and exposed their carotid artery. The insufficient ischemia rate is 15 %, and the premature death is 10 %.

Neurological Deficit Evaluation and Infarct Assessment

Neurological deficits were monitored 24 h or 7 days after vascular occlusion using the 6-point scoring system [32] by a blinded observer. Mice were scored as follows: zero, no apparent deficits; one, failure to extend left forepaw fully if pulled by the tail; two: left contralateral circling if pulled by the tail; three: circling or walking to the left; four: walking only if stimulated; five: unresponsiveness to stimulation and with depressed level of consciousness. Immediately after neurological deficit evaluation, mice were decapitated and brains were dissected into six equal thickness coronal slices (1 mm per slice) and immediately stained with 2 % 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, St Louis, MO, USA, T8877) at 37 °C for 10 min. The infarct volumes were evaluated with the following formula: (total contralateral hemispheric volume − total ipsilateral hemispheric stained volume)/total contralateral hemispheric volume × 100.

Nissl Staining

At 24 h or 7 days after reperfusion, mice were subjected to deep anesthesia with 300 mg/kg i.p. chloral hydrate and perfused with saline followed by ice cold 4 % paraformaldehyde phosphate buffer through transcardiac perfusion. Brain tissues were then extracted and post-fixed in 4 % paraformaldehyde for 2 h at 4 °C. After successively infiltrated with 0.1 M PBS containing 20 and 25 % sucrose overnight, 10 μm coronal sections were cut with a cryostat (Leica, CM 1950) and stored at −20 °C until use. Nissl staining kit (Beyotime Institute of Biotechnology, China) was used to detect the numbers of morphologically normal neurons and neurons showing the features of ischemic cell change (shrunken cell bodies, triangulated, pyknotic nuclei, and eosinophilic cytoplasm) by two blinded investigators using ImagePro Plus 5.1 software (Media Cybernetics, Inc., Bethesda, MD). Neurons were counted in 10 different fields in each region in the cortex (primary somatosensory cortex, S1) and CA1 of hippocampus. The percentage of ischemic neurons was calculated through dividing the sum of ischemic neuron number by the total number of neurons in the 10 defined areas [33].

Measurement of Cerebral MDA and SOD Levels

Cerebral content of MDA, the product of lipid peroxidation, was measured according to the method by a detection kit purchased from Beyotime Institution of China. The absorbance of the supernatant was measured by spectrophotometry at 532 nm. Total cerebral SOD activity was determined using detection kits from Jiancheng Bio Institution, Nanjing, China, and the absorbance of the supernatant were measured by spectrophotometry at 450 nm.

Western Blotting

The ischemic hemispheres were lysed with SDS-PAGE sample buffer composed of 62.5 mM Tris–HCI, 2 % w/v SDS, 10 % glycerol, 50 mM DTT, and 0.1 % w/v bromphenol blue. After homogenization, extracts were clarified via centrifugation at 12,000*g* for 10 min. The supernatant was collected as the total cellular protein extract. Nuclear extracts were prepared using a NEPER Nuclear Extraction Kit (Pierce) according to manufacturer instructions. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). Aliquots of the lysates with loading buffer (Beyotime Institution of China) were boiled for 10 min. and then cooled to room temperature. For western blot analysis, equal amounts of protein (20–40 mg) were separated with SDS polyacrylamide gel electrophoresis and blotted onto PVDF membranes (Millipore). Membrane was blocked with 5 % skim milk in Tris buffered saline (TBS) and then incubated overnight at 4 °C with the primary antibodies as follow: rabbit anti-Nrf2 antibody (1:200, Santa Cruz Biotechnology), rabbit anti-HO-1 antibody (1:500, Enzo Life Sciences), mouse anti-phospho-Akt antibody (1:1,000, Cell Signaling), rabbit anti-total Akt antibody (1:1,000, Cell Signaling), rabbit anti-cleaved caspase-3 antibody (1:1,000; Cell Signaling), rabbit anti-Bcl-2 antibody (1:2,000, Abcam) or rabbit anti-Bax antibody (1:2,500, Abcam), rabbit anti-IL-1β antibody (1:400, Abcam), goat anti-TNF-α antibody (Santa Cruz biotechnology, 1:500) and mouse anti-β-actin antibody. After washing, membranes were incubated with a horseradish peroxidase (HRP)-conjugated IgG secondary antibody in accordance with the origin of the primary antibody for 1 h at room temperature. Then the antibody-reactive bands were visualized on X-ray film using super ECL plus detection reagent (Thermo, USA, 34077). The protein

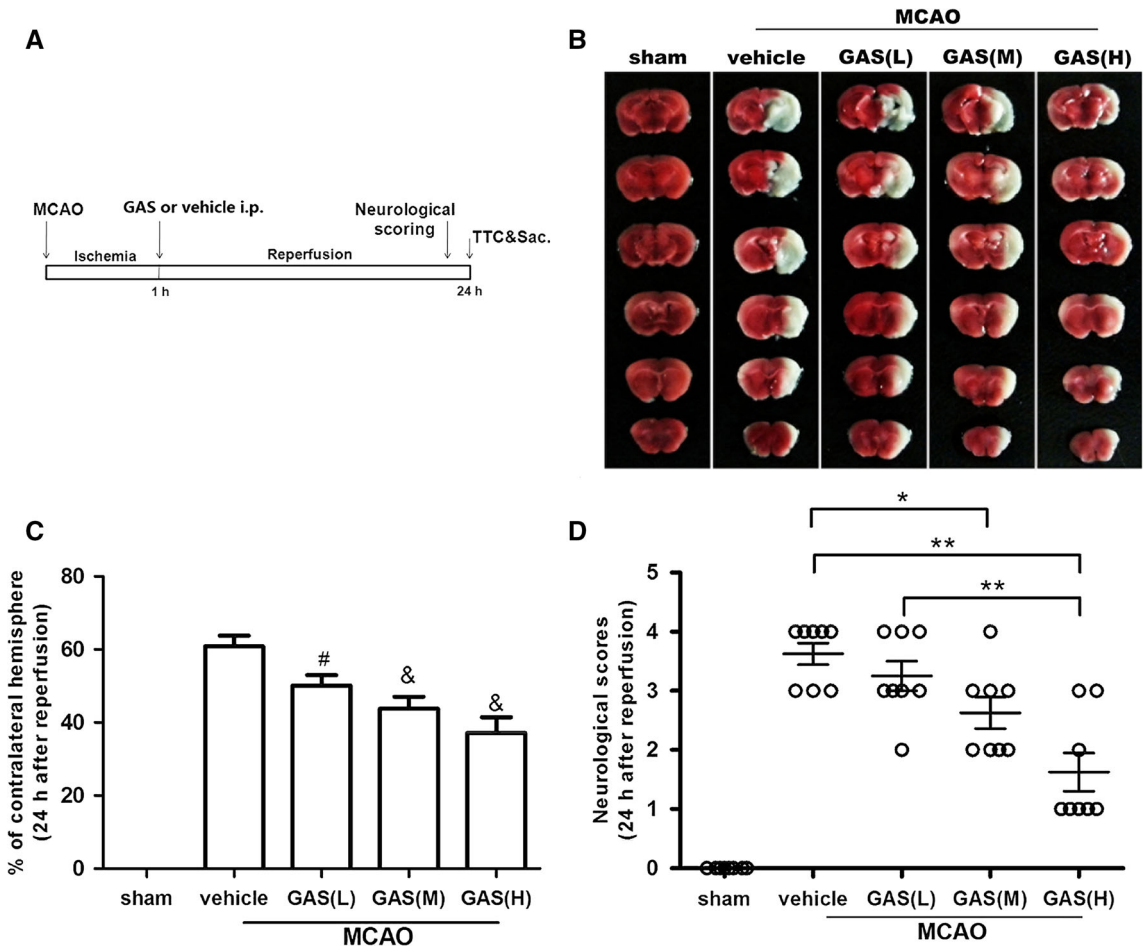


Fig. 1 Gastrodin improved neurological scores and reduced infarct volumes 24 h after reperfusion injury. **a** A diagram of the experimental design for drug treatment and assessment of the ischemic injury. **b** Representative TTC-stained thick brain sections and quantitative evaluation of the infarction volume **c** and neurobehavioral score **d** for each group (n = 8/group). GAS significantly reduced infarct volumes ($F_{3,\ 24} = 8.847$, $P < 0.01$) and improved neurological scores at 24 h after reperfusion. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\#}P < 0.05$ vs. MCAO + GAS (H) group, $^{\&}P < 0.05$vs.. MCAO + vehicle group

quantifications were adjusted for the corresponding to β-actin level, which was not consistently changed by the different treatment conditions.

Statistical Analysis

The software SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to conduct statistical analyses. All values, except for neurological scores, are presented as mean ± SEM and analyzed by one-way analysis of variance, and between-group differences were detected with the post hoc Student–Newman–Keuls test. The neurological deficit scores were expressed as median (range) and were analyzed with the Kruskal–Wallis test followed by the Mann–Whitney *U* test with the Bonferroni correction. Differences were considered significant when $P < 0.05$.

Results

GAS Reduced the Infarct Volume and Improved the Neurobehavioral Score Induced by Cerebral Ischemia

To detect the neuroprotective effect of GAS, mice were subjected to MCAO followed by GAS or vehicle treatment. As shown in Fig. 1, at 24 h after reperfusion, significant differences were found among groups in infarct volume ratio and neurobehavioral score ($P < 0.01$). Multiple comparisons further showed that MCAO induced definite infarct volume ratio and neurologic deficiency compared to sham group. These changes were alleviated by GAS at medium or high dose ($P < 0.05$ compared to MCAO + Vehicle). Additionally, high dose of GAS was

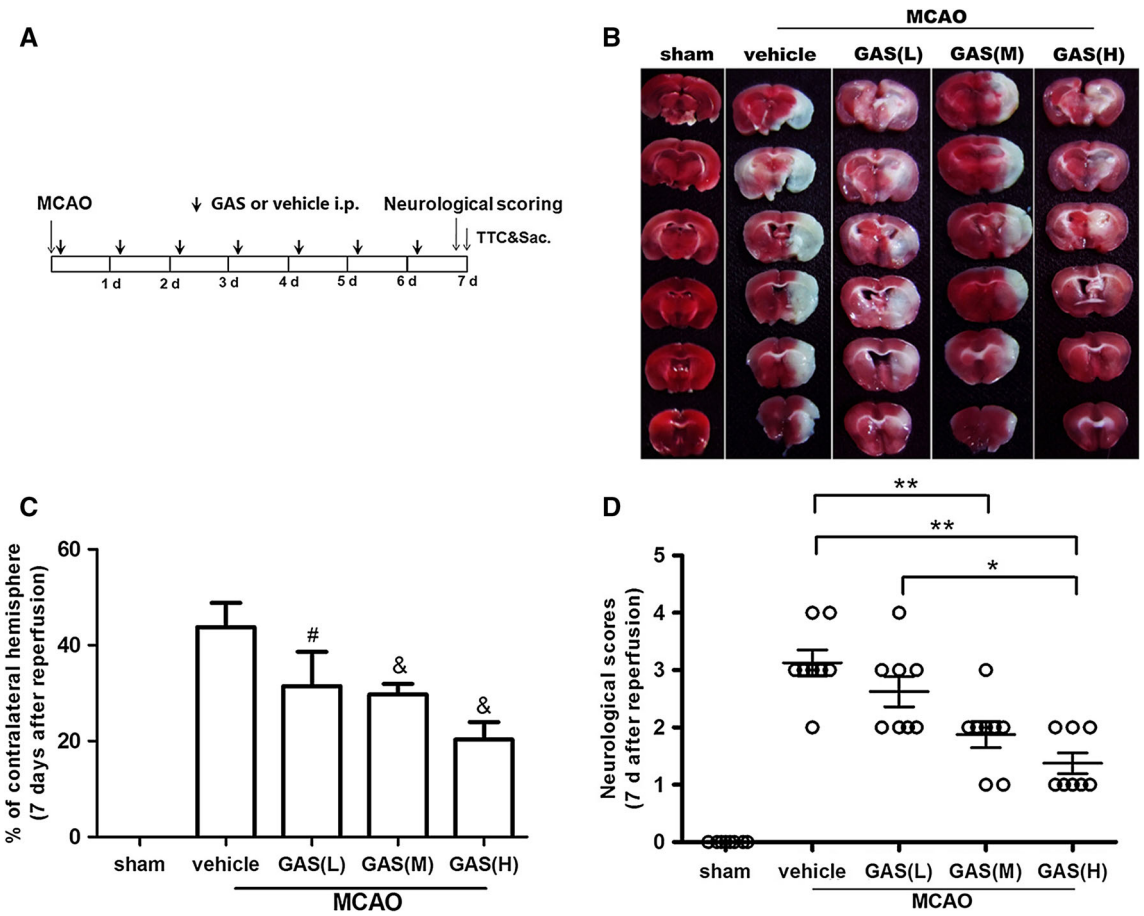


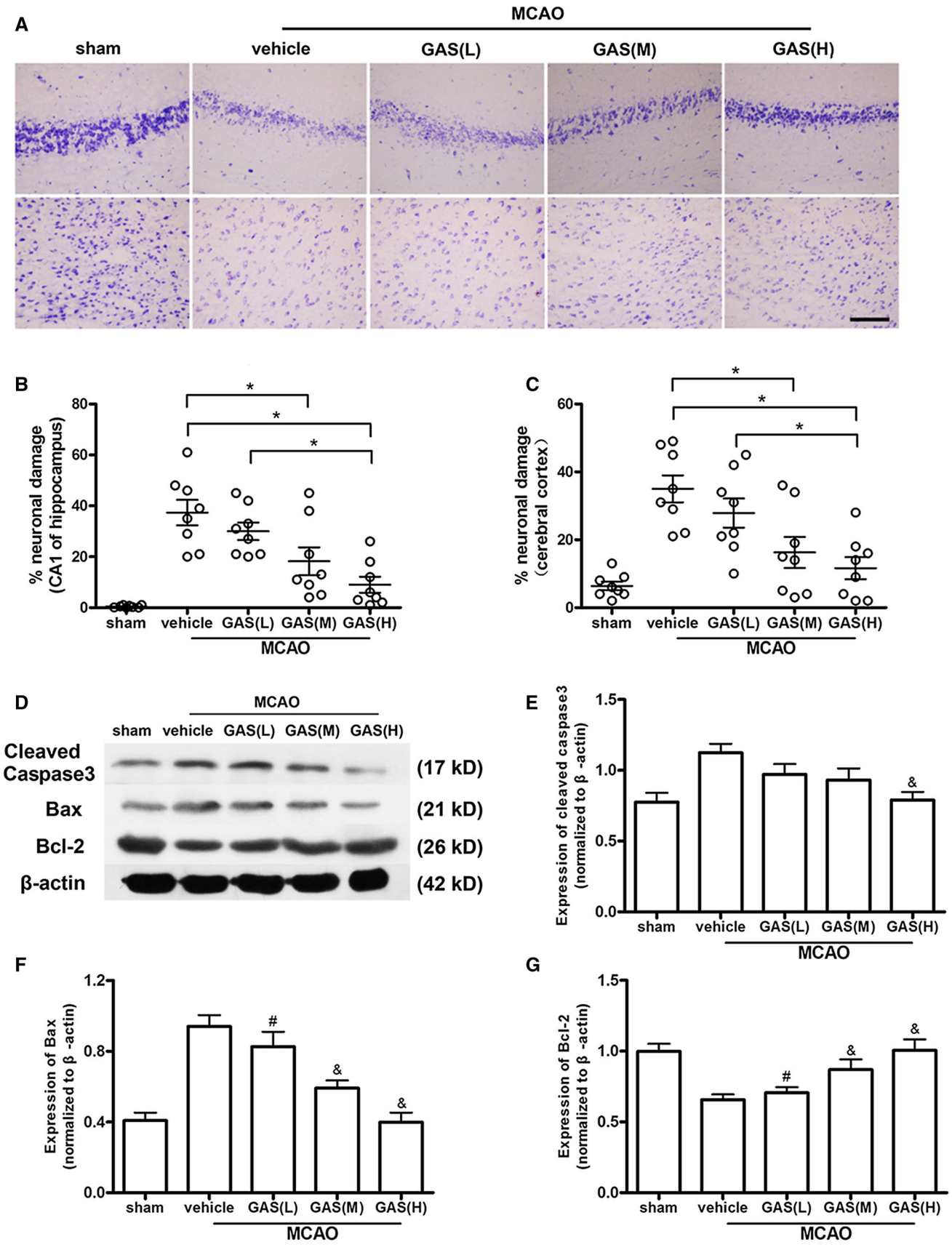
Fig. 2 GAS improved neurological scores and reduced infarct volumes 7 days after reperfusion injury. **a** The experimental design, **b** Representative TTC-stained thick brain sections, **c** quantitative evaluation of the infarction volume and **d** neurobehavioral score for each group (n = 8/group). GAS significantly reduced infarct volumes ($F_{3,\ 23} = 5.629$, $P < 0.01$) and improved neurological scores 7 days after reperfusion. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\#}P < 0.05$ vs. MCAO + -GAS (H) group, $^{\&}P < 0.05$ vs. MCAO + vehicle group

more effective in alleviating ischemic brain injury for that compared to MCAO + GAS (L) group.

To detect if the neuroprotective effect of GAS was long lasting but not transient, mice were subjected to MCAO and then received GAS or vehicle once daily for 7 consecutive days. Infarct volume ratio and neurobehavioral score was detected at 7 days after reperfusion. As shown in Fig. 2, at 7 days after reperfusion, significant differences among groups were found for both infarct volume and neurobehavioral score ($P < 0.01$). Multiple comparisons showed that GAS at medium dose or high-dose alleviated the neuronal damage induced by MCAO, high dose of GAS was more potent in reducing cerebral damage induced by ischemia [$P < 0.05$ vs. MCAO + GAS (L)]. These results indicated that GAS dose-dependently reduced cerebral ischemic injury in mice.

GAS Improved Morphology Damage and Inhibited Apoptosis Pathway in the Ischemic Hemispheres

As shown in Fig. 3, at 24 h after reperfusion, there were significant differences between each group in the percentage of neuronal damage in the cortex and CA1 of hippocampus, as well as the protein expression of cleaved Caspase 3, Bax and Bcl-2. Post hoc comparisons further showed that MCAO induced significant neuron damage both in the cortex and CA1 hippocampus, increased the protein expression of cleaved Caspase 3 and Bax, but decreased the expression of Bcl-2 ($P < 0.05$). These changes were alleviated by GAS at medium dose or high-dose ($P < 0.05$). High dose of GAS further reduced the increase of Bax expression and inhibited the reduction of Bcl-2 expression caused by MCAO compared to MCAO + GAS (L) group.


A
MCAO
sham
vehicle
GAS(L)
GAS(M)
GAS(H)
B
% neuronal damage (CA1 of hippocampus)
C
% neuronal damage (cerebral cortex)
D
Cleaved Caspase3
Bax
Bcl-2
β-actin
(17 kD)
(21 kD)
(26 kD)
(42 kD)
E
Expression of cleaved caspase3 (normalized to β -actin)
F
Expression of Bax (normalized to β -actin)
G
Expression of Bcl-2 (normalized to β -actin)

◀**Fig. 3** GAS improved morphology damage and inhibited apoptosis pathway 24 h after reperfusion injury. Representative microphotographs **a** and quantitative evaluation showing Nissl staining to determine the cell damage by ischemia/reperfusion (I/R) in ischemic CA1 of hippocampus **b** and primary somatosensory cortex **c** of brain at 24 h after reperfusion. Scale bars = 100 μm. **d** Representative bands for each group. **e**, **f**, **g** Densitometric analysis for cleaved Caspase3, Bax and B-cell lymphoma 2 (Bcl-2), respectively. GAS relieved MCAO induced neuron damage both in the primary somatosensory cortex ($F_{4,35} = 10.330$, $P < 0.01$) and CA1 of hippocampus ($F_{4,35} = 14.836$, $P < 0.01$), and inhibited the increased expression of cleaved Caspase 3 ($F_{4,20} = 6.796$, $P < 0.01$), Bax ($F_{4,20} = 16.676$, $P < 0.01$) and Bcl-2 ($F_{4,20} = 7.681$, $P < 0.01$) 24 h after reperfusion. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\#}P < 0.05$ vs. MCAO + -GAS **h** group, $^{\&}P < 0.05$ vs. MCAO + vehicle group

As shown in Fig. 4, at 7 days after reperfusion, there were significant differences among groups for the percentage of injured neurons in the cortex and CA1 of hippocampus, as well as the protein expression of cleaved Caspase 3, Bax and Bcl-2. In accordance with the tendency of 24 h, Post hoc comparisons showed that medium dose or high-dose of GAS alleviated the neuronal damage induced by MCAO. Moreover, GAS at medium dose or high-dose inhibited the expression of cleaved Caspase 3 and Bax and elevated the expression of Bcl-2. Compared to MCAO + GAS (L) group, protein expression changes were more obvious in MCAO + GAS (H) group ($P < 0.05$), indicating that a larger dose of GAS was more effective in inhibiting pro-apoptotic protein expression and preserving anti-apoptotic protein expression.

GAS Reversed MCAO-Induced Oxidative Damage in the Ischemic Hemispheres

To evaluate the protective effect of GAS on the oxidative damage in mice brain induced by MCAO, we determined the protein expression of nuclear factor erythroid 2-related factor 2 (Nrf2), Heme oxygenase-1 (HO-1), SOD1, phospho-Akt, total Akt as well as the content of MDA and total cerebral SOD activity in the ischemic hemisphere at 6 h after reperfusion. As shown in Fig. 5, there were significant differences among groups in the expression of Nrf2, HO-1, SOD1 and phosphorylation level of Akt. The expression of SOD1 and phosphorylation level of Akt were significantly decreased after MCAO, which was dose dependently reversed by the administration of GAS. In addition, the expression of Nrf2 and HO-1 were significantly also dose dependently increased by the administration of GAS.

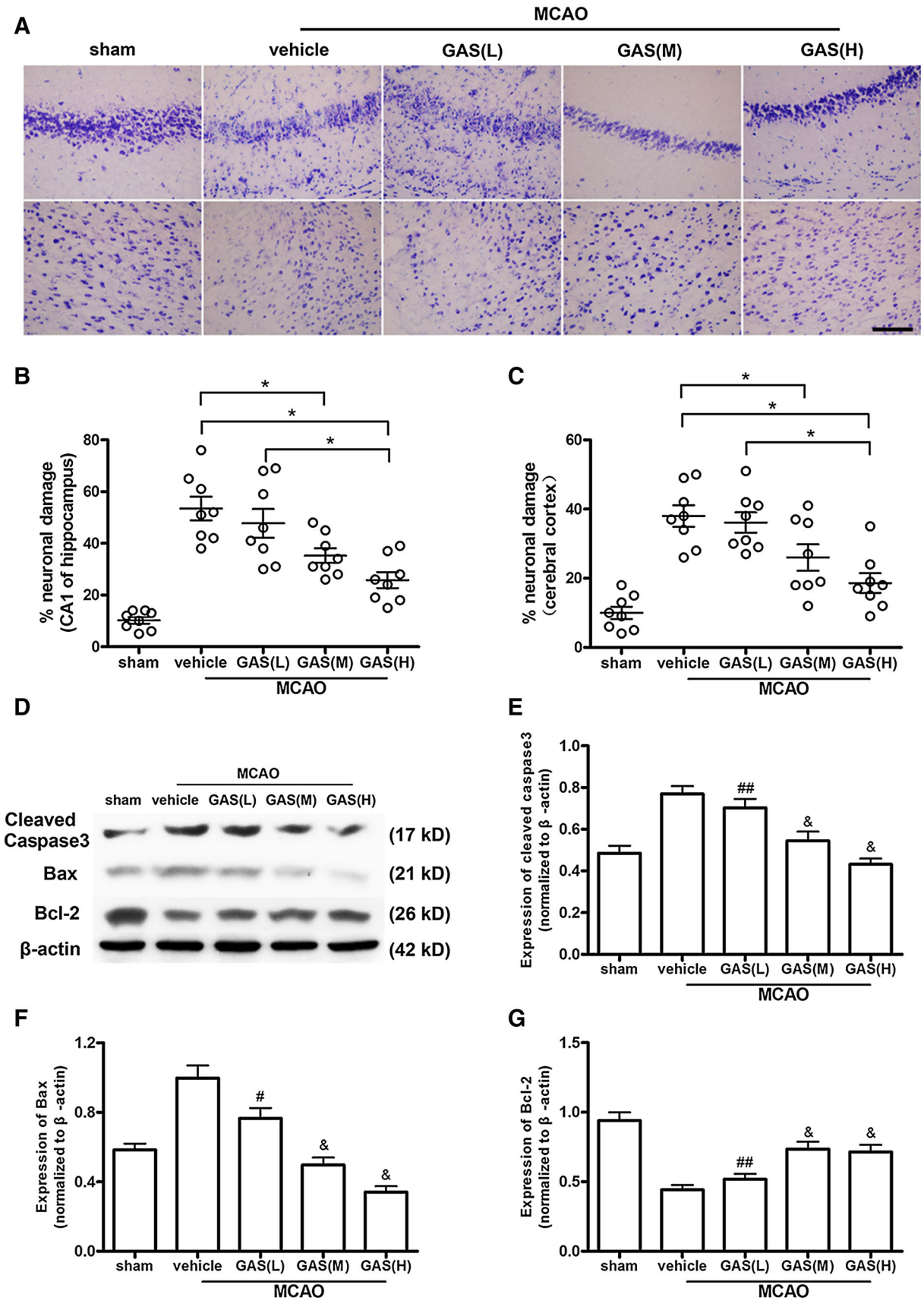
There were also significant differences between each group in the concentrations of malondialdehyde (MDA) and activity of SOD. MCAO significantly increased the concentrations of MDA (Fig. 5g), which was product of lipid peroxidation, and the concentrations of MDA were dose-dependently reduced by the administration of GAS. In contrast, MCAO markedly decreased the activity of SOD compared with that of sham group. Different doses of GAS showed certain effect of increasing the activity of SOD (Fig. 5h).

GAS Inhibited the Expression of Inflammatory Cytokines in the Ischemic Hemispheres

To evaluate the anti-inflammatory effect of GAS in mice brain after MCAO, we determined the protein expression of TNF-α and IL-1β in the ischemic hemispheres at 6 h after reperfusion. As shown in Fig. 6, there were significant differences among groups in the expression of TNF-α and IL-1β. The expression of TNF-α and IL-1β were significantly increased in MCAO + Vehicle group, but this increase was inhibited by medium and/or high dose of GAS treatment.

Discussion

Ischemic pathogenesis is rapidly initiated within minutes after the onset of MCAO, which involved a series of subsequent biochemical events including oxidative stress, inflammatory responses, programmed cell death, etc. [3, 34]. These events eventually lead to irreversible damage of proteins, nucleic acids and cell organelles that cause cellular dysfunction and neuronal death. The structure of GAS is a phenol 4-Hydroxybenzyl alcohol 4-O-beta-D-glucopyranoside (C13H18O7) and its molecular weight is 286.28. It was found that GAS could bind with human fibrinogen at the atomic level [35], and the entry of GAS into the brain was rapid via the femoral vein administration [36]; Additionally, GAS could penetrate through the blood–brain barrier into brain [37], we believe that it could allow for peritoneal injection. Previous study indicated that pretreatment of GAS could decrease the glutamate/GABA ratio during ischemia and reperfusion [38], and it could markedly decreased the infarct volume and edema volume in MCAO rats at 24 h after reperfusion [28, 39]. But whether this effect of GAS in neuroprotection is transient or permanent is still unknown. Also the mechanism was not identified yet. As described in the preciously studies, the definite infarct and iNOS expression in microglia were not detected until 24 h after reperfusion [40], and the long lasting neuroprotective effect could be observed 7 days after reperfusion [41, 42]. In the present study, we showed that GAS reduced the infarct volume ratio from ischemia-induced injury when administered one hour after MCAO. Besides, GAS treatment daily for 7 consecutive days showed long lasting

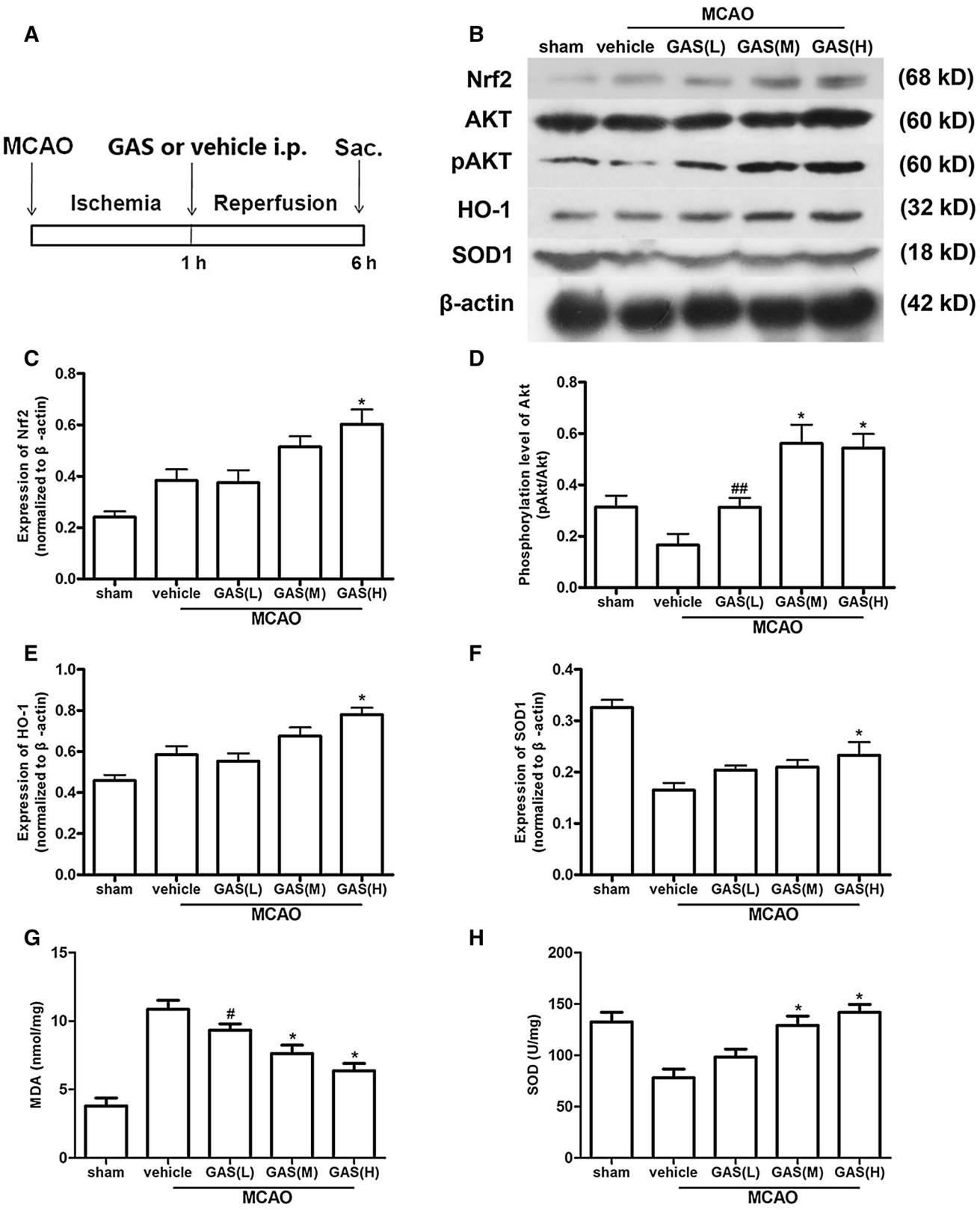

A
MCAO
sham
vehicle
GAS(L)
GAS(M)
GAS(H)
B
% neuronal damage (CA1 of hippocampus)
C
% neuronal damage (cerebral cortex)
D
Cleaved Caspase3
Bax
Bcl-2
β-actin
(17 kD)
(21 kD)
(26 kD)
(42 kD)
E
Expression of cleaved caspase3 (normalized to β -actin)
F
Expression of Bax (normalized to β -actin)
G
Expression of Bcl-2 (normalized to β -actin)

◀**Fig. 4** GAS improved morphology damage and inhibited apoptosis pathway 7 days after reperfusion injury. Representative microphotographs **a** and quantitative evaluation showing Nissl staining to determine the cell damage by ischemia/reperfusion (I/R) in ischemic CA1 of hippocampus **b** and primary somatosensory cortex **c** of brain 7 days after reperfusion. Scale bars = 100 μm. **d** Representative bands for each group. **e**, **f**, **g** Densitometric analysis for cleaved Caspase3, Bax and Bcl-2, respectively. GAS relieved MCAO induced neuron damage both in the cortex ($F_{4,35} = 15.508$, $P < 0.01$) and CA1 of hippocampus ($F_{4,35} = 21.126$, $P < 0.01$), and inhibited the increased expression of cleaved Caspase 3 ($F_{4,20} = 14.405$, $P < 0.01$), Bax ($F_{4,20} = 24.153$, $P < 0.01$) and Bcl-2 ($F_{4,20} = 16.623$, $P < 0.01$) after reperfusion for 7 days. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\#}P < 0.05$ vs. MCAO + GAS(H) group, $^{\&}P < 0.05$ vs. MCAO + vehicle group

neuroprotective effects even at 7 days after reperfusion. Since cerebral ischemia can induced both necrotic and apoptotic cell death [43], the present study investigated the cerebral damage in the primary somatosensory cortex and CA1 of hippocampus, which were related to the sensorimotor and memory deficits in the brain ischemia [44–46], and the expression of cleaved Caspase 3, Bax and Bcl-2 in the ischemic hemispheres as well. Caspase-3 can enzymatically digest specific substrates and inhibit DNA repair enzymes, thus leading to breakage of the chromosome into small fragments and eventual apoptosis. Moreover, proteins in the Bcl-2 family are central to the intrinsic apoptosis pathway, it is known that the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 can migrate from the cytoplasm to mitochondria, which are distributed in a manner that is consistent with mitochondrial release of cytochrome C and caspase [47]. The results showed that GAS treatment presented a significant functional recovery and morphology change and preserved the expression of Bcl-2 protein and suppressed expression of cleaved Caspase 3 and Bax induced by cerebral ischemia at 24 h and 7 days after reperfusion. These data provide direct evidence that GAS has a significant neuroprotective effect in cerebral ischemia.

We chose a transient cerebral ischemia model (1 h MCAO) and didn't test whether GAS has a neuroprotective effect against permanent cerebral ischemic injury in the current study, mainly because GAS was reported to have an anti-oxidative and anti-inflammation property, which are two of the major characteristics for ischemic-reperfusion injury in the brain. It has been reported that several neuroprotective agents or strategies like dextromethorphan, riluzole, pioglitazone or HBO preconditioning are selectively effective only in transient ischemic injury models but not permanent ischemic injury models [48–50]. What's more, the basic pathophysiological processes are in ways different between transient and permanent cerebral ischemia including the pattern of neurotoxic excitatory amino acid-glutamate release [51]. And the inflammatory response were more intense in permanent ischemia models as described in previous report that leukocyte infiltration was elevated at 24 h after reperfusion but did not further increase in transient ischemia model while permanent ischemia caused much more infiltration T cells in the brain at 5 days after ischemia [52]. In the current study, GAS significantly inhibited inflammatory cytokine expression in the ischemic brain within 24 h after ischemia, which effectively inhibited the acute inflammatory response in transient cerebral ischemia and provided effective neuroprotection. As a potent anti-inflammatory agent, repeated injection of GAS after cerebral ischemia might provide neuroprotective effect for permanent cerebral ischemic injury in animal models. But this effect and optimal dosage of GAS administration needs to be explored.

The phosphatidylinositol 3-kinase (PI3-K)/Akt pathway has been shown to play critical roles in the regulation of cell growth, proliferation, differentiation, survival, and intracellular trafficking. In addition, this pathway negatively regulates LPS-induced acute inflammatory responses [53, 54]. Furthermore, PI3-K/Akt signaling was required for the activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) [55], which is a major regulator for several cytoprotective factors such as anti-oxidative enzymes, anti-inflammatory factors and transcriptional factors [56, 57]. Activation of the Nrf2 plays a pivotal role in enhancing the endogenous defense mechanism by which the brain protects itself against progressive ischemic damage and recovers from stroke [58, 59]. Moreover, the activation of Nrf2 can coordinately up-regulate the expression of several anti-oxidative enzymes such as heme oxygenase-1 (HO-1) and superoxide dismutase1 (SOD1) [60, 61]. All of them were recognized to play important roles in combating oxidative stress. Therefore, therapies targeting the Akt/Nrf2 pathway have provided a promising target for stroke treatment [62]. Previous studies showed that GAS could inhibit NF-κB activation, MAPKs phosphorylation and pro-inflammatory mediators and pro-inflammatory cytokines production by activating PI3-K/Akt pathway [63]. GAS could also up-regulate gene expression of Nrf2 to protect primary cultured rat hippocampal neurons against amyloid-beta peptide-induced neurotoxicity [27]. In addition, Studies have revealed a therapeutic window of approximately 6 h between the onset of ischemia and irreversible neuronal death [64, 65]. Therefore, it is desirable that neuroprotective interventions should be attempted before stroke occurs or very soon afterward.


A
MCAO
GAS or vehicle i.p.
Sac.
Ischemia
Reperfusion
1 h
6 h
B
MCAO
sham
vehicle
GAS(L)
GAS(M)
GAS(H)
Nrf2
AKT
pAKT
HO-1
SOD1
β-actin
(68 kD)
(60 kD)
(60 kD)
(32 kD)
(18 kD)
(42 kD)
C
Expression of Nrf2 (normalized to β -actin)
D
Phosphorylation level of Akt (pAkt/Akt)
E
Expression of HO-1 (normalized to β -actin)
F
Expression of SOD1 (normalized to β -actin)
G
MDA (nmol/mg)
H
SOD (U/mg)

◀**Fig. 5** Modulation of Akt phosphorylation, Nrf2, HO-1 and SOD1 expression and MDA concentration and activity of SOD by GAS at 6 h after reperfusion injury. **a** A diagram of the experimental design. **b** Representative western blot bands and quantitative evaluation of Akt phosphorylation **c**, Nrf2 **d**, HO-1 **e** and SOD1 **f**. Histograms showed the concentration of MDA **g** and the activity of SOD1 **h**. GAS dependently reversed the increased expression of Nrf2 ($F_{4,20} = 9.974$, $P < 0.01$), HO-1 ($F_{4,20} = 10.903$, $P < 0.01$), SOD1 ($F_{4,20} = 13.479$, $P < 0.01$) and phosphorylation level of Akt ($F_{4,20} = 10.672$, $P < 0.01$), as well as the concentrations of malondialdehyde (MDA) ($F_{4,15} = 22.166$, $P < 0.01$) and activity of SOD ($F_{4,15} = 9.636$, $P < 0.01$) at 6 h after reperfusion. $^{\#\#}P < 0.05$ vs. MCAO + GAS (H) and MCAO + GAS (M) group, $^{\#}P < 0.05$ vs. MCAO + GAS **h** group, $^{*}P < 0.05$ vs. MCAO + vehicle group

To further elucidate the mechanism of GAS on alleviating MCAO-induced brain injury, the present study investigated the effects of GAS on the amount of SOD and MDA production, which is one of the classic oxidative stress markers to directly reflect the rate and extent of lipid peroxidation. In addition, the protein expression of phosphorylation level of Akt and Nrf2, as well as anti-oxidative enzymes (HO-1/SOD1) and pro-inflammatory cytokines (TNF-α/IL-1β) to reveal the role of Akt and Nrf2 signaling in the anti-oxidative and anti-inflammatory effect of GAS after cerebral ischemic injury at 6 h after reperfusion, which is based on the therapeutic window that there are approximately 6 h between the onset of ischemia and irreversible neuronal death [64, 65]. Our findings showed that MDA production was decreased after GAS treatment, as well as the expression of TNF-α and IL-1β. However, the production and expression of HO-1 and SOD1 in the ischemic brain was increased by GAS treatment. Furthermore, GAS significantly increased the phosphorylation Akt and Nrf2 expression. These results suggest that GAS regulates Akt phosphorylation and Nrf2 expression, thereby enhancing the protective defense mechanisms through anti-oxidative and anti-inflammatory pathway.

In conclusion, our findings suggest that GAS had neuroprotective effects against cerebral ischemic injury in a mice MCAO model. Notably, we also found that neuroprotective effect of GAS was associated with the preservation of anti-oxidative enzymes and inhibition of pro-inflammatory cytokines. This effect of GAS may be intermediated by the activation of Akt/Nrf2 pathway. These findings underscore GAS as a potential therapy for the treatment of ischemic brain injury, and it would be a useful agent when co-application to other drugs (such as tPA). However, the involvement of Akt/Nrf2 in the neuroprotective effects of GAS and the mechanism of how GAS affects the phosphorylation status of Akt and the expression of Nrf2 needs to be further investigated. Further studies are needed to explore the detailed neurobiological and cellular mechanisms underlying GAS treatment.

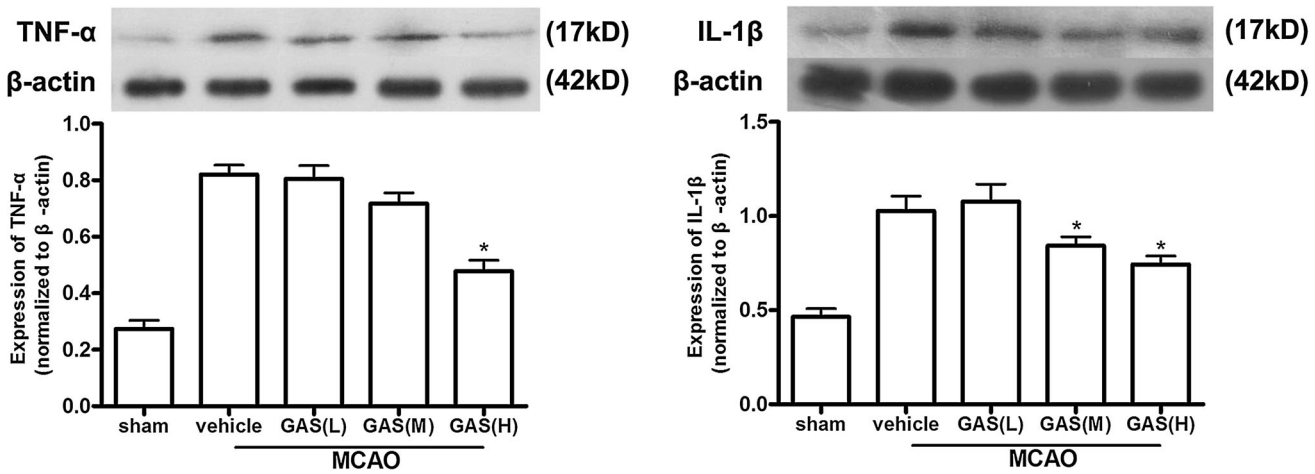


Fig. 6 Representative western blot bands and quantitative evaluation of TNF-α (*left*) and IL-1β (*right*) in the ischemic hemispheres. GAS inhibited the expression of TNF-α ($F_{4,20} = 31.66$, $P < 0.01$) and IL-1β ($F_{4,20} = 19.03$, $P < 0.01$) in the ischemic hemispheres. $^{*}P < 0.05$ vs. MCAO + vehicle group

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Conflict of interest The authors declared that there are no conflicts of interest.

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