

Isoflurane reduces septic neuron injury by HO-1-mediated abatement of inflammation and apoptosis

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Abstract. Sepsis-associated encephalopathy (SAE) frequently occurs in critically ill patients with severe systemic infections. Subanesthetic isoflurane (0.7% ISO) possesses anti-inflammatory, antioxidant and anti-apoptotic properties against a number of human diseases, including brain injury. The activation of heme oxygenase-1 (HO-1) impedes inflammation, oxidation and apoptosis, thus alleviating sepsis-induced brain damage. However, whether 0.7% ISO affords protection against septic neuronal injury involving HO-1 activation is unclear. The present study aimed to investigate the neuroprotective effects of 0.7% ISO and its potential underlying mechanisms in SAE using a mouse model established by cecal ligation and puncture (CLP). The results indicated that the expression and activity of HO-1 in the mouse hippocampus were increased by CLP, and further enhanced by ISO. ISO reduced the death rate, brain water content and blood-brain barrier disruption, but improved the learning and memory functions of CLP-treated mice. ISO significantly decreased the production of pro-inflammatory cytokines and the levels of oxidative indicators in the serum and hippocampus, as well as the number of apoptotic neurons and the expression of pro-apoptotic proteins in the hippocampus. Inversely, anti-inflammatory factors, antioxidative enzymes and anti-apoptotic proteins were markedly increased by ISO administration. However, the neuroprotective effects of ISO were abolished by a HO-1 inhibitor. Overall, these findings suggested that 0.7% ISO alleviated SAE via its anti-inflammatory, antioxidative and anti-apoptotic properties, which involved the activated form of HO-1.

Introduction

Sepsis is a severe systemic inflammatory response syndrome caused by a dysregulated host response to infection (1), and is the leading cause of morbidity and mortality in critically ill patients worldwide (2). Multiple complications occur during sepsis, such as acute lung injury (3), kidney lesions (4), cardiac dysfunction (5), liver disorders (6) and brain damage (7). Among these manifestations, sepsis-associated encephalopathy (SAE) appears earlier and more frequently than other complications (8). Despite advancements in the treatment of patients in the intensive care unit (ICU), individuals with SAE exhibit longer ICU stays and higher 28-day mortality rates (9), with survivors experiencing a substantial cognitive deficit (10). Learning and memory impairments have also been observed in septic mammalian models (11,12). Thus, identifying the pathogenic mechanisms of SAE and developing more effective therapeutic strategies are critical.

Inflammatory lesions, oxidative damage and cell death are well associated with SAE (13,14). Inflammatory triggers, such as lipopolysaccharide (LPS), can bind to Toll-like receptor 4 (TLR4) and subsequent activate downstream signals of TLR4, such as mitogen-activated protein kinases (MAPKs) and nuclear factor- κ B (15,16), and untimely induce the production of adhesion molecules and proinflammatory factors, including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6 in vascular endothelial cells (17), which can result in endothelial abnormalities, leakage of the blood-brain barrier (BBB), immune cell migration, neuronal death, and ultimately brain damage (18,19). In addition, intracellular calcium is involved in LPS-stimulated cytokine production; calcium influx due to calcium store depletion is referred to as store-operated calcium entry (20). Calcium signaling involves activation of tyrosine kinases and leads to activation of MAPKs in endothelial cells (21). Reportedly, oxidative stress triggers neuronal injury and cerebral dysfunction during sepsis (22,23). Therefore, the alleviation of inflammation, oxidative stress and apoptosis would be an efficacious remedy for SAE. Heme oxygenases (HOs) are the rate-limiting enzymes that convert heme into biliverdin, ferrous iron and carbon monoxide. Heme oxygenase-1 (HO-1), the inducible isoform of HO, confers cytoprotection by exerting anti-inflammatory, antioxidative and anti-apoptotic functions (24-26). HO-1 upregulation

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and activation ameliorates sepsis-induced multiorgan injury, including lung, kidney, liver and brain damage (27-30). Therefore, HO-1-targeted treatments are a promising avenue for neuroprotection during sepsis.

Isoflurane (ISO) is a widely used inhalational anesthetic during surgery. Mounting evidence suggests that besides its use as an anesthetic, ISO possesses non-anesthetic physiologic properties against inflammation, oxidation and apoptosis (31,32). ISO affords neuroprotection against hypoxic ischemia-induced cerebral damage (33,34), and ISO post-treatment improves pulmonary vascular permeability in septic rats by upregulating HO-1 (27). ISO elicits its anti-inflammatory activity by activating the HO-1 pathway in LPS-stimulated macrophages (35). ISO also protects against oxygen glucose deprivation-induced neuron injury, which relies on the upregulation of HO-1 (36). However, 1.2-2.5% ISO has adverse effects for patients in the ICU who cannot tolerate its hemodynamic effects. ISO at <1% has a weak effect on hemodynamics, which is more beneficial for ICU patients (37). The previous literature revealed that 0.7% ISO inhalation for 1 h at 1 and 6 h post-zymosan insult reduced zymosan-induced inflammation and apoptosis *in vitro* and *in vivo* (38-40). Nevertheless, the roles of HO-1 in the neuroprotective effects of 0.7% ISO during sepsis remain largely unknown.

In the present study, the protective effects of 0.7% ISO were evaluated using an SAE mouse model created by cecal ligation and puncture (CLP), and the beneficial effects of 0.7% ISO were investigated for their potential association with HO-1 augmentation and/or activation.

Materials and methods

Animals and ethical guidelines. A total of 150 male C57BL/6 mice (age, 8 weeks; weight, 20-25 g) were purchased from the Animal Center of Xi'an Jiaotong University (Xi'an, China). The mice were maintained in specific pathogen-free conditions at 22±2°C with 60% relative humidity, 12-h dark/light cycles, and free access to food and water. This study was conducted in accordance with the ARRIVE guidelines for the Care and Use of Laboratory Animals (41), and the experimental procedures were approved by the Ethics Committee of Xi'an Jiaotong University (approval no. XAJU.No20180730A10120). All efforts were made to minimize suffering. The mice were anaesthetized with 50 mg/kg of pentobarbital sodium through intraperitoneal injection. At the end of the experiments, the mice were administrated with 30% volume displacement rate of CO₂ for euthanasia. No heartbeat and mydriasis were used to confirm animal death.

CLP-induced SAE mouse model. A CLP-induced SAE mouse model was established as previously described with slight modifications (42). After anesthesia with pentobarbital sodium (50 mg/kg), the mice were immobilized on an aseptic operating table and received a 1-cm abdominal midline incision to expose the cecum. The distal 20% of the cecum (below the ileocecal valve and 1 cm from the tip) was ligated with a 6-0 suture and punctured twice with a 20-gauge needle. The cecum was gently squeezed to extrude a small amount of feces from the perforation sites, and then returned to the abdomen. The incision was subsequently sutured with sterile 6-0 silk. In the

sham group, the mice underwent a similar procedure without ligation and puncture of the cecum. All animals received fluid resuscitation by subcutaneous injection of 1 ml pre-warmed 0.9% saline solution using a 25-gauge needle.

ISO administration. As previously reported, 0.7% ISO inhalation for 1 h was conducted at 1 and 6 h post-challenge in mice (40,43). The mice were placed in an air-tight plastic chamber (Billups-Rothenberg, Inc.) with an inflow and an outflow. ISO (Sigma-Aldrich; Merck KGaA) was delivered into the chamber by air at 2 l/min through a tube, and Baralyme (Allied Healthcare Products, Inc.) was used to remove CO₂ from the chamber gases. The concentration (0.7%) of ISO in the outflow hose was continuously monitored using an anesthetic gas analyzer (Smart Anesthesia Multi-gas Module; Cytiva), and the temperature of the room and chamber was maintained at 20-24°C.

Experimental groups. A total of 150 mice were randomly assigned to the following five groups (n=30 per group): i) Control group, mice received sham surgery and no drug treatments; ii) ISO group, mice received sham surgery and ISO exposure; iii) CLP group, mice received CLP surgery and no drug treatments; iv) CLP + ISO group, mice received CLP surgery and ISO exposure; and v) CLP + ISO + zinc protoporphyrin IX (ZnPP; Sigma-Aldrich; Merck KGaA) group, mice received CLP surgery and both ISO and ZnPP treatments. 0.7% ISO inhalation for 1 h was conducted at 1 and 6 h post-CLP surgery, and ZnPP (10 mg/kg) was peritoneally injected into the mice 1 h before CLP surgery.

Evaluation of survival rate. Following CLP or sham treatment, 10 mice from each group were returned to their cages and the survival rate was calculated up to 7 days post-surgery.

Morris water maze (MWM) test. To evaluate the spatial learning and memory abilities of the mice, the MWM test was performed as previously described (44). The apparatus consisted of a circular pool (diameter, 150 cm; height, 60 cm) filled with water (22±2°C) containing food-grade titanium dioxide (Shanghai Jianghu Industrial Co., Ltd.). A removable platform (diameter, 10 cm) was placed in one quadrant of the pool and submerged 1 cm below the surface of the water. The pool was divided into four quadrants and the swimming paths of the mice were captured using a video camera above the center of the pool. From 4-7 days post-treatment, CLP or sham-treated mice (n=10) were individually placed into the pool at each of the four quadrants (facing the sidewalls) and allowed to circumnavigate in search of the platform for four trials per day (60 sec per trial). If a mouse failed to find the platform within 60 sec, it was guided by the investigator and permitted to search for an extra 10 sec. The escape latency, swimming distance and time spent in the target quadrant were recorded at each trial. The probe test comprised a single trial with the platform removed. The mice were released into the water and allowed to locate the original platform for 90 sec, and the time spent in the target quadrant was recorded. On the 7th day, the mice were subjected to the probe trial with the platform removed, 2 h after the last training trial. Each mouse was monitored for 60 sec to observe the swimming path, time

spent in the target quadrant of the platform, and the number of times that the platform was crossed.

Brain water content. Brain water content was measured using the standard wet-dry method as previously reported (45). The mice were anesthetized and sacrificed 48 h post-CLP or sham surgery, and the brains were immediately removed and weighed to obtain the wet weight. Subsequently, the brain samples were dried in an oven at 100°C for 48 h, and the dry weight was acquired. Brain water content was calculated as follows: (Wet weight-dry weight/wet weight) x100%.

BBB permeability assay. BBB permeability was evaluated by extravasation of Evans blue (EB) as previously reported (30). The mice were injected with 2% EB (3 ml/kg; Sigma-Aldrich; Merck KGaA) via the tail vein at 48 h post-CLP or sham treatment. After 2 h, the mice were anesthetized and transcardially perfused with normal saline. The brain samples were harvested, weighted, homogenized in formamide (10 ml/g), and incubated at 37°C for 48 h. The supernatants were collected after centrifugation at 10,000 x g for 30 min at 4°C, and the optical density (OD) at 625 nm was determined using a microplate reader (Molecular Devices, LLC). Standard formamide was used as the blank control, and the results were expressed as the relative amount of EB ($\mu\text{g/g}$ wet weight).

Blood collection and hippocampus tissue preparation. At 48 h after CLP or sham surgery, the mice were anesthetized and blood samples were collected from the cervical artery, which were centrifuged at 1,000 x g (4°C) for 30 min. The mice were then euthanized, and the hippocampus tissues were immediately harvested. The tissues were homogenized and centrifuged at 10,000 x g and 4°C for 10 min. The sera and supernatants were stored at -80°C for the measurement of inflammatory mediators and oxidative parameters.

Enzyme-linked immunosorbent assay (ELISA). The levels of TNF- α (cat. no. RTA00), IL-1 β (cat. no. RLB00), IL-6 (cat. no. R6000B) and IL-10 (cat. no. R1000) in the serum and hippocampus supernatants were determined using commercially available ELISA kits (R&D Systems, Inc.), according to the manufacturer's instructions. The serum and hippocampus supernatants were collected and the OD at 450 nm was measured on an ELISA plate reader (Molecular Devices, LLC) and the results are expressed as pg/ml serum or pg/mg protein.

Reactive oxygen species (ROS) assay. ROS production was assessed using a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) kit (Nanjing Jiancheng Bioengineering Institute) as per the manufacturer's protocol. Briefly, 50 μl serum or hippocampal supernatant were added to a 96-well plate and incubated with 100 μM DCFH-DA solution (200 μl) for 30 min (37°C) in the dark. DCFH-DA can be hydrolyzed by cellular esterases to form DCFH, which is oxidized by ROS to produce the fluorescent compound dichlorofluorescein (DCF). DCF oxidation was fluorometrically measured using a fluorescence microplate reader (Molecular Devices, LLC) at 485 nm excitation and 525 nm emission wavelengths. The data are represented as nmol/ml serum or nmol/mg protein.

Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) production. The levels of MDA (cat. no. A003-1-2), SOD (cat. no. A001-3-2) and CAT (cat. no. A007-1-1) in the serum and hippocampal supernatants were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's protocols. Briefly, the serum and supernatants were collected and the absorbance at 530, 450 and 405 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.) to determine MDA content and SOD and CAT levels, respectively. The MDA content is displayed as nmol/ml serum or nmol/mg protein, and the SOD and CAT activities were calculated as U/ml serum or U/mg protein.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay. TUNEL assay was carried out to detect neuronal apoptosis using the Cell Death Detection kit (Roche Diagnostics) as per the manufacturer's protocol. Hippocampal tissues were homogenized and centrifuged at 500 x g (4°C) for 5 min. The supernatants were discarded and the cell pellets were resuspended in PBS; 1×10^5 cells were seeded into 6-well plates. Cells were fixed with 4% paraformaldehyde for 1 h at 25°C. The TdT Enzyme (EMD Millipore) was added to the wells, which were then incubated for 1 h at 37°C in a dark humidified chamber. Stop/Wash buffer (EMD Millipore) was added for 10 min at room temperature, and the wells were rinsed with PBS before anti-digoxigenin fluorescein (EMD Millipore) was added. The cells were then incubated for 30 min at room temperature in a dark humidified chamber. In the negative control group, TdT was omitted. The cells were then incubated with DAPI for 15 min at 37°C in the dark. The slides were mounted with neutral balsam (Sangon Biotech Co., Ltd.). Stained cells were observed in five randomly selected fields of view using a fluorescence microscope (Olympus Corporation). The apoptotic index is expressed as the ratio of TUNEL⁺ (green) to DAPI⁺ cells (blue) x100%.

Flow cytometric assessment of apoptosis. Hippocampal neuron apoptosis was detected via flow cytometry using the propidium iodide (PI)-Annexin V-FITC Apoptosis Detection kit (BD Biosciences). Hippocampal tissues were homogenized and centrifuged at 500 x g (4°C) for 5 min; the supernatants were discarded and the cell pellets were resuspended in 500 μl 1X Annexin V Binding Buffer. Subsequently, 5 μl Annexin V-FITC and 5 μl PI were added and the cells were incubated for 10 min in the dark at room temperature. The cells were immediately analyzed using a FACSCalibur™ flow cytometer (BD Biosciences) and the percentage of apoptotic cells (early + late apoptosis) was calculated by FlowJo software (version 7.6.2; FlowJo LLC).

Caspase-3 activity assay. Caspase-3 activity was measured using the Caspase-3/CPP32 Colorimetric Assay kit (BioVision, Inc.). Hippocampal tissues were homogenized and centrifuged at 500 x g (4°C) for 5 min. The supernatants were discarded and the cell pellets were resuspended in PBS; 1×10^5 cells were incubated on ice for 10 min with 50 μl chilled lysis buffer. The supernatant was then centrifuged at 12,000 x g for 5 min at 4°C, and 100 μg protein (in a total volume of 40 μl) was added to 40 μl 2X reaction buffer containing 5 μl N-Acetyl-Asp-Glu-Val-Asp-pNA substrate

(150 μ M final concentration). After incubation at 37°C for 2 h, N-Acetyl-Asp-Glu-Val-Asp-pNA cleavage was monitored by detecting the enzyme-catalyzed release of pNA using a microplate reader (BioTek Instruments, Inc.) at 405 nm.

Reverse transcription-quantitative (RT-q) PCR analysis. Total RNA was isolated from the hippocampal tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as per the manufacturer's instructions. cDNA was synthesized using the PrimeScript™ Reverse Transcription Reagent kit (Takara Biotechnology Co., Ltd.) with the following conditions: 37°C for 15 min, 85°C for 5 sec and maintained at 4°C. The qPCR assay was performed using the SYBR® Advantage® qPCR Premix (Takara Biotechnology Co., Ltd.) and conducted on a 7500 Fast Real-Time System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 95°C for 10 min; followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec. Relative mRNA levels were calculated using the $2^{-\Delta\Delta C_q}$ method (46). The primers sequences were as follows: HO-1 forward, 5'-TTCAGAAGGGTCAGG TCC-3' and reverse, 5'-CAGTGAGGCCCATACCAGAA-3'; and GAPDH forward, 5'-ACCCAGAAGACTGTGGATGG-3' and reverse, 5'-CACATTGGGGGTAGGAACAC-3'.

Western blot analysis. Hippocampal tissues were homogenized in RIPA lysis buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Total protein was quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein sample (30 μ g/lane) were separated via 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride membranes (BD Biosciences). After blocking with 10% non-fat milk in Tris-buffered saline containing Tween-20 (50 mM Tris, pH 7.4, 250 mM NaCl and 0.1% Tween-20) at room temperature for 1 h, the membranes were incubated with primary antibodies against HO-1 (1:1,000; cat. no. ab189491; Abcam), Bax (1:1,000; cat. no. 5023s; Cell Signaling Technology, Inc.), Bcl-2 (1:1,000; cat. no. 3498s; Cell Signaling Technology, Inc.), caspase-3 (1:1,000; cat. no. 14220s; Cell Signaling Technology, Inc.), cleaved (cl)-caspase-3 (1:1,000; cat. no. 9664s; Cell Signaling Technology, Inc.) and β -actin (1:1,000; cat. no. 4967s; Cell Signaling Technology, Inc.) overnight at 4°C. Following treatment with the appropriate horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000; cat. no. 7074s; Cell Signaling Technology, Inc.), the protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham; Cytiva) and Kodak radiography film (FUJIFILM Wako Pure Chemical Corporation). The Western blot results were normalized to those of the internal control β -actin. The proteins were visualized using Image Quant™ LAS 4000 (GE Healthcare) and semi-quantified using ImageJ Software (version 1.46; National Institutes of Health).

Statistical analysis. All data are expressed as the mean \pm standard deviation (SD). Survival rates were assessed using the Kaplan-Meier method and compared using the log-rank test. The latency, distance and time during water maze training were analyzed using two-way ANOVA followed by Bonferroni's post hoc test. Other comparisons among multiple groups were made using one-way ANOVA followed by Tukey's multiple

comparisons test. The statistical analyses were performed using SPSS 18.0 software (SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

0.7% ISO increases the expression and activity of HO-1 in the hippocampus of CLP-induced septic mice. RT-qPCR and western blot assays were performed to investigate the expression of HO-1 in the hippocampus of CLP-challenged mice. As shown in Fig. 1A-C, CLP resulted in an increase in HO-1 mRNA and protein expression in the hippocampus, which was further enhanced by 0.7% ISO. However, the elevation of HO-1 was attenuated by treatment with the HO-1 inhibitor ZnPP. Similarly, HO-1 activity increased substantially in the hippocampus of mice subjected to CLP, compared with those in the control group. 0.7% ISO exposure resulted in further elevation of HO-1 activity compared with the CLP group, while ZnPP treatment reduced this augmented HO-1 activity (Fig. 1D). These results suggested that 0.7% ISO increases the expression and activity of HO-1 in the hippocampus of CLP-induced septic mice.

0.7% ISO reduces the mortality, brain water content and BBB disruption in CLP-induced septic mice. Next, the effects of 0.7% ISO on the survival rate of CLP-treated mice were investigated. During the 7-day observation period, no mortalities were observed in the control mice or those treated with ISO alone. However, the survival rate significantly decreased following CLP surgery, compared with that of the control group. 0.7% ISO significantly prolonged the survival time of CLP-challenged mice, which was subsequently abolished by ZnPP administration (Fig. 2A). As depicted in Fig. 2B and C, mice in the CLP group exhibited an increase in brain water content and a decrease in BBB integrity. 0.7% ISO exposure attenuated these effects in the CLP + ISO group compared with the CLP group. However, these beneficial effects were reversed by ZnPP treatment. These findings indicated that 0.7% ISO could decrease mortality rates and brain edema, and increase BBB integrity in CLP-induced mice, which partly depends on HO-1 activation.

0.7% ISO improves the memory and learning capacity of CLP-induced septic mice. A MWM training assay was conducted to investigate the effects of 0.7% ISO on the learning abilities of CLP-induced mice. Compared with the control group, CLP-treated mice showed markedly prolonged escape latency from the 5th day post-surgery. CLP-challenged mice exposed to 0.7% ISO displayed considerably shorter escape latency compared with mice that underwent CLP surgery only. Furthermore, ZnPP administration attenuated the effects of 0.7% ISO on escape latency (Fig. 3A). Of note, there were no significant differences in the distance and time spent in the target quadrant among all five groups (Fig. 3B and C). Following water maze training, the platform was removed to analyze the role of 0.7% ISO on the memory of CLP-challenged mice, including the distance, time spent in the target quadrant, and the frequency at which the platform was crossed. As depicted in Fig. 3D, the travelled distance was similar among the five groups. However, the CLP-treated mice spent a significantly shorter time in the target quadrant than the control mice, which was significantly prolonged by 0.7% ISO;

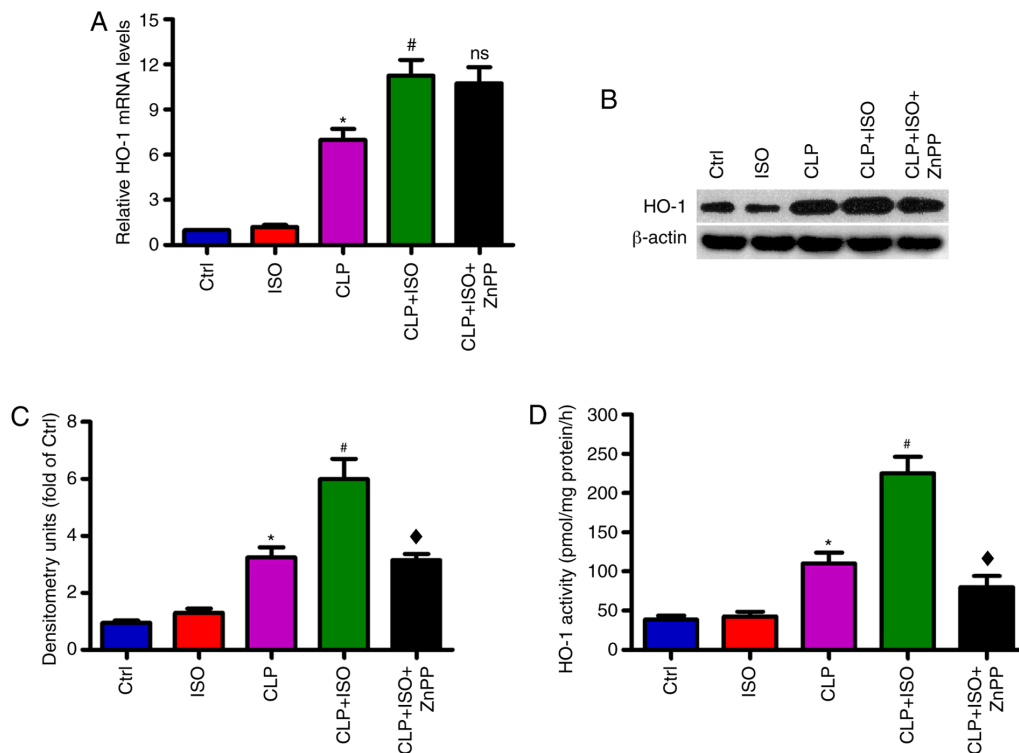


Figure 1. 0.7% ISO enhances HO-1 expression and activity in the hippocampus of the CLP-induced mice. Mice were treated with or without 0.7% ISO by inhalation for 1 h, starting at 1 and 6 h after sham or CLP surgery, respectively, in the presence or absence of ZnPP (10 mg/kg), peritoneally administered 1 h before sham or CLP surgery. At 48 h post-surgery, the mice were anesthetized and the hippocampi were isolated. (A) Reverse transcription-quantitative PCR and (B) western blotting assays were performed to determine the mRNA and protein levels of HO-1. GAPDH and β -actin were used as the endogenous controls. (C) HO-1 protein levels were calculated and (D) HO-1 activity was detected. Representative data are expressed as the mean \pm SD from three independent experiments. * $P < 0.05$ vs. Ctrl or ISO group; # $P < 0.05$ vs. CLP group; ♦ $P < 0.05$ vs. CLP + ISO group. HO-1, heme oxygenase-1; ISO, isoflurane; CLP, cecal ligation and puncture; ZnPP, Zinc protoporphyrin IX; Ctrl, control; ns, not significant.

the time spent at the quadrant was subsequently decreased by ZnPP treatment (Fig. 3E). Similarly, the frequency of platform crossing was significantly reduced in the CLP group compared with the control mice, which was significantly increased by 0.7% ISO, and decreased by ZnPP administration (Fig. 3F). These data demonstrated that 0.7% ISO ameliorates the learning and memory functions of CLP-induced septic mice, which is indicated to involve activated HO-1.

0.7% ISO inhibits CLP-induced inflammation in the serum and hippocampus of mice. To identify the effects of 0.7% ISO on inflammatory responses in CLP-induced septic mice, ELISA assays were conducted to evaluate the levels of pro- and anti-inflammatory factors in the serum and hippocampus. CLP was found to significantly increase the levels of pro-inflammatory cytokines, including TNF- α (Fig. 4A), IL-1 β (Fig. 4B) and IL-6 (Fig. 4C), and the anti-inflammatory cytokine IL-10 (Fig. 4D) in the serum. 0.7% ISO resulted in a significant reduction of TNF- α , IL-1 β and IL-6, and a further elevation of IL-10 in the sera of CLP-treated mice. Whereas, ZnPP reversed the changes in TNF- α , IL-1 β , IL-6 and IL-10 expression (Fig. 4A-D). Consistently, 0.7% ISO exposure significantly reduced the CLP-induced increase in the levels of TNF- α , IL-1 β and IL-6, and further enhanced the production of IL-10 in the hippocampus, which were significantly counteracted by ZnPP (Fig. 4E-H). These results indicated that HO-1 is highly important to the anti-inflammatory effects of 0.7% ISO in CLP-induced septic mice.

0.7% ISO represses CLP-induced oxidative stress in the serum and hippocampus of mice. To further elucidate the effects of 0.7% ISO on sepsis-induced oxidative stress, the ROS and MDA contents, and anti-oxidative SOD and CAT activities were determined in the serum and hippocampal tissues of the mice. Compared with the control group, the production of ROS (Fig. 5A) and MDA (Fig. 5B) significantly increased, and the SOD (Fig. 5C) and CAT (Fig. 5D) activities significantly decreased in the sera of CLP-challenged mice. 0.7% ISO exposure significantly reduced the levels of ROS and MDA, and enhanced the activities of SOD and CAT, which were reversed by ZnPP administration (Fig. 5A-D). Similarly, 0.7% ISO reduced CLP-induced ROS and MDA, and enhanced CLP-reduced SOD and CAT activity in the hippocampus, and ZnPP reversed these alterations (Fig. 5E-H). These findings illustrated that the antioxidative effects of 0.7% ISO on CLP-induced septic mice are reliant on the activation of HO-1.

0.7% ISO suppresses CLP-induced neuronal apoptosis in the mouse hippocampus. Next, a TUNEL assay was performed to assess the impacts of 0.7% ISO on CLP-induced neuronal apoptosis. As shown in Fig. 6A and B, an increased number of apoptotic neurons were observed in the hippocampus of CLP-challenged mice than those of the control group. 0.7% ISO significantly decreased the number of TUNEL⁺ cells in the hippocampus, while ZnPP abrogated these protective effects. The flow cytometry results also demonstrated that 0.7% ISO decreased CLP-induced neuronal apoptosis

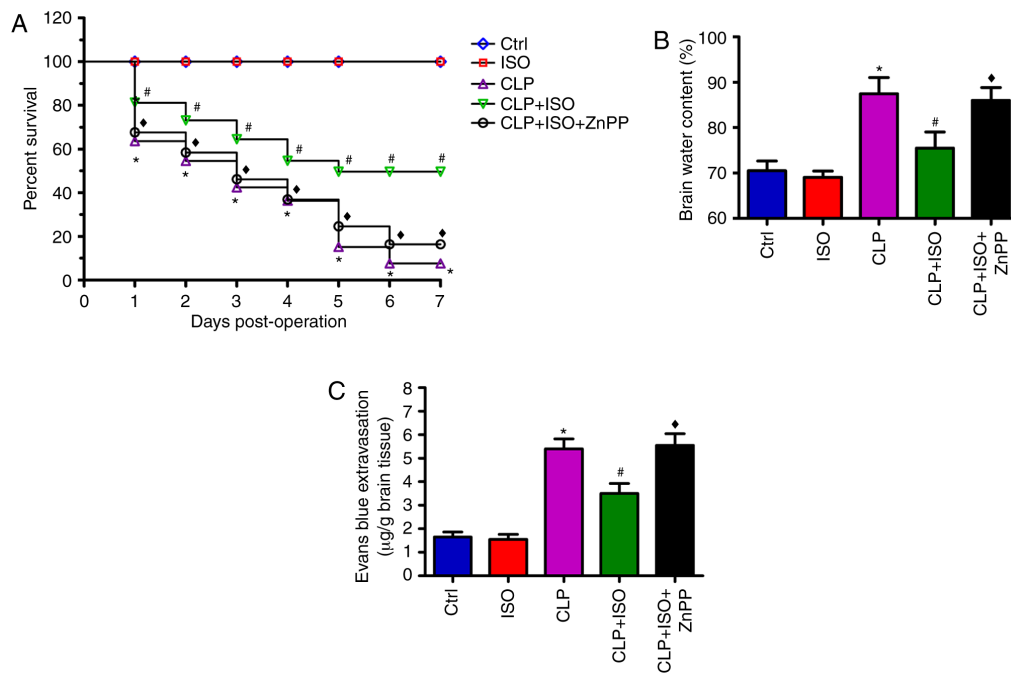


Figure 2. 0.7% ISO improves the survival rate and reduces brain water content and BBB disruption in CLP-treated mice. Mice were treated with or without 0.7% ISO by inhalation for 1 h, starting at 1 and 6 h after sham or CLP treatment, respectively, in the presence or absence of ZnPP (10 mg/kg), peritoneally administered 1 h before sham or CLP surgery. (A) A 7-day survival curve was plotted using the Kaplan-Meier method and compared with the log-rank test. (B) Brain water content and (C) blood brain barrier integrity were assessed at 48 h post-sham or CLP surgery. Representative data are expressed as the mean \pm SD from three independent experiments. * $P < 0.05$ vs. Ctrl or ISO group; # $P < 0.05$ vs. CLP group; * $P < 0.05$ vs. CLP + ISO group. ISO, isoflurane; CLP, cecal ligation and puncture; ZnPP, zinc protoporphyrin IX; Ctrl, control.

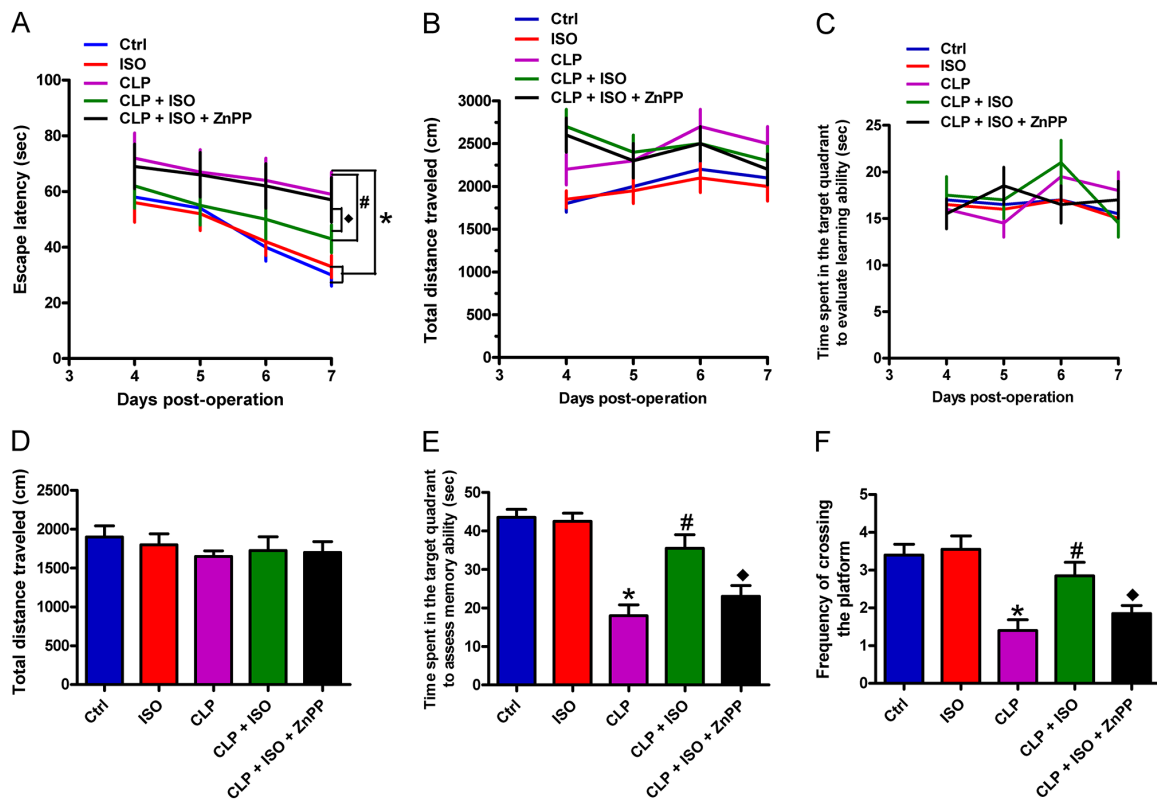


Figure 3. 0.7% ISO ameliorates the learning and memory abilities of CLP-induced mice. Mice were treated with or without 0.7% ISO by inhalation for 1 h, starting at 1 and 6 h after sham or CLP treatment, respectively, in the presence or absence of ZnPP (10 mg/kg), peritoneally administered 1 h before sham or CLP surgery. Morris water maze training and hidden platform assays were conducted from day 4 to 7 post-surgery. (A) Escape latency, (B) total distance travelled and (C) time spent in the target quadrant were recorded to evaluate the learning ability. (D) Total distance travelled, (E) time spent in the target quadrant and (F) the frequency of platform crossing were calculated to assess memory ability. Representative data are expressed as the mean \pm SD from three independent experiments. * $P < 0.05$ vs. Ctrl group; # $P < 0.05$ vs. CLP or ISO group; * $P < 0.05$ vs. CLP + ISO group. ISO, isoflurane; CLP, cecal ligation and puncture; ZnPP, zinc protoporphyrin IX; Ctrl, control.

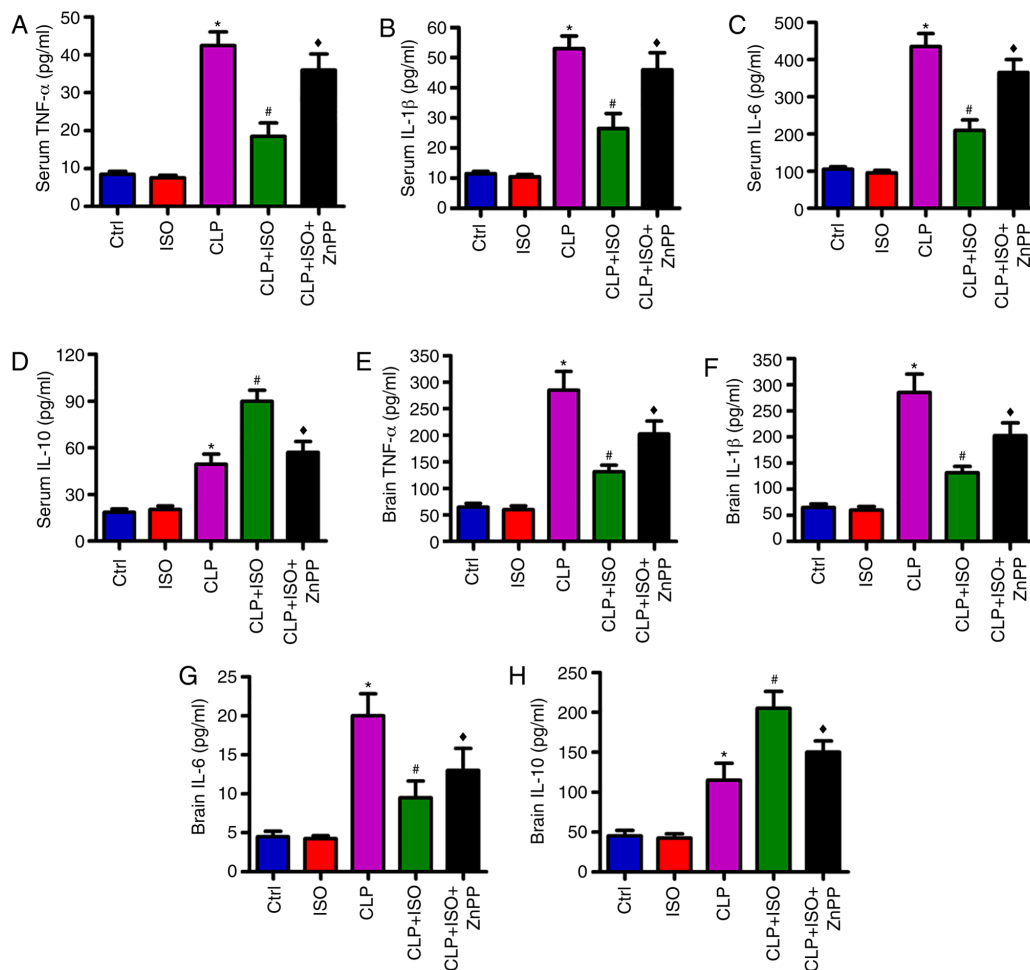


Figure 4. 0.7% ISO reduces the levels of pro-inflammatory cytokines in the serum and hippocampal tissues of CLP-treated mice. Mice were treated with or without 0.7% ISO by inhalation for 1 h, starting at 1 and 6 h after sham or CLP treatment, respectively, in the presence or absence of ZnPP (10 mg/kg), peritoneally administered 1 h before sham or CLP surgery. At 48 h post-surgery, the mice were anesthetized and the serum and hippocampi were collected. The levels of (A) TNF- α , (B) IL-1 β , (C) IL-6 and (D) IL-10 in the serum, and the production of (E) TNF- α , (F) IL-1 β , (G) IL-6 and (H) IL-10 in hippocampus were determined by an enzyme-linked immunosorbent assay. Representative data are expressed as the mean \pm SD from three independent experiments. * P <0.05 vs. Ctrl group; # P <0.05 vs. CLP or ISO group; * P <0.05 vs. CLP + ISO group. ISO, isoflurane; CLP, cecal ligation and puncture; ZnPP, zinc protoporphyrin IX; Ctrl, control; TNF- α , tumor necrosis factor- α ; IL-, interleukin.

(Fig. 6C and D) in the hippocampus, which was reversed by ZnPP administration. Furthermore, 0.7% ISO decreased CLP-induced caspase-3 activation (Fig. 6E) in the hippocampus, whereas ZnPP administration significantly reversed this effect. On a molecular level, 0.7% ISO resulted in a significant upregulation in anti-apoptotic Bcl-2 and downregulation in pro-apoptotic Bax and cl-caspase-3 in the hippocampus of CLP-treated mice. ZnPP reversed the expression changes in these proteins (Fig. 6F-I). There were no significance differences in the expression of caspase-3 among the five groups (Fig. 6F and J). These data indicated that HO-1 is implicated in the anti-apoptotic functions of 0.7% ISO in CLP-induced septic mice.

Discussion

The present study demonstrated that 0.7% ISO exerted neuro-protective effects on CLP-induced septic mice, which involved HO-1 signaling. The following key findings were observed in septic mice: i) ISO enhanced the expression and activity of HO-1 in the hippocampus; ii) ISO improved the survival rate

and reduced brain water content and BBB disruption; iii) ISO ameliorated the memory and learning ability; iv) ISO reduced the production of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and increased the release of the anti-inflammatory cytokine IL-10 in the serum and hippocampus; v) ISO resulted in decreased oxidative products (ROS and MDA) and a simultaneous increase in antioxidant enzyme (SOD and CAT) activity in the serum and hippocampus; vi) ISO inhibited neuronal apoptosis by upregulating Bcl-2 and downregulating Bax and cl-caspase-3 in the hippocampus; and vii) the neuro-protective effects of ISO were mediated by HO-1 activation. Overall, 0.7% ISO prevented CLP-associated neuronal injury by reducing inflammation, oxidative stress and apoptosis, which was dependent on HO-1 activation.

A previous study reported that the use of ISO during surgery is considered optimal for neuroprotection (47). ISO post-conditioning notably decreases the infarct volume and improves the neurobehavioral performance of rats subjected to middle cerebral artery occlusion, thereby providing increased ischemic tolerance in the brain (48). ISO increases the weight ratio and neuronal density of neonatal rats, and ameliorates

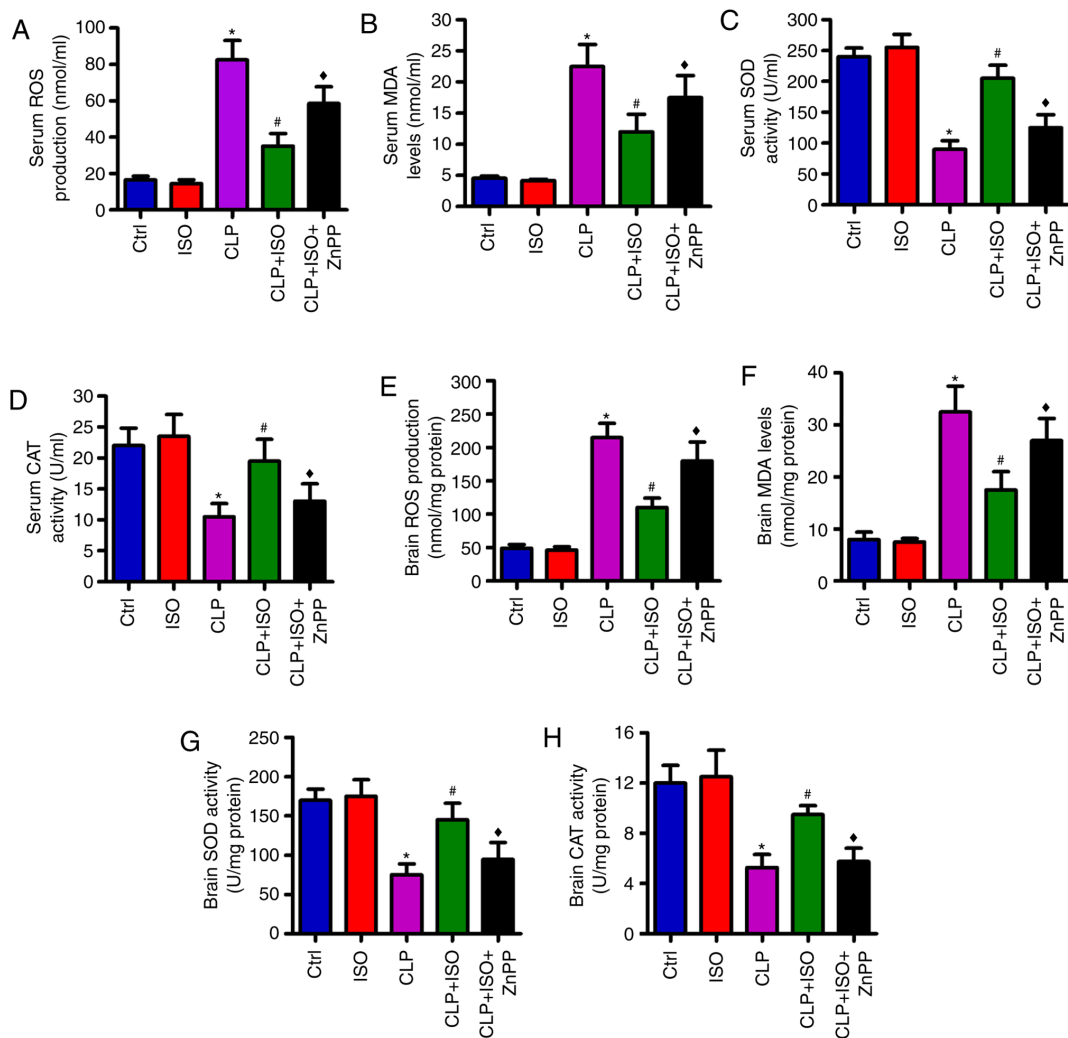


Figure 5. 0.7% ISO inhibits oxidation and increases antioxidative processes in the serum and hippocampi of CLP-injured mice. Mice were treated with or without 0.7% ISO by inhalation for 1 h, starting at 1 and 6 h after sham or CLP treatment, respectively, in the presence or absence of ZnPP (10 mg/kg), peritoneally administered 1 h before sham or CLP surgery. At 48 h post-surgery, the mice were anesthetized and the serum and hippocampus were collected. (A) ROS and (B) MDA content, as well as (C) SOD and (D) CAT activity in the serum, the production of (E) ROS and (F) MDA, and the activities of (G) SOD and (H) CAT in the hippocampus were determined using commercial kits. Representative data are expressed as mean \pm SD from three independent experiments. * P <0.05 vs. Ctrl group; # P <0.05 vs. CLP or ISO group; * P <0.05 vs. CLP + ISO group. ISO, isoflurane; CLP, cecal ligation and puncture; ZnPP, zinc protoporphyrin IX; Ctrl, control; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase.

the memory and learning ability of adolescent rats following brain hypoxia and ischemia (49). ISO exerts a protective effect on hypoxia-induced neuronal injury in the hippocampus by suppressing apoptosis (50). The present study highlighted that ISO exerted neuroprotection against CLP-induced SAE in mice by increasing overall survival, and reducing brain water content and BBB disruption, as well as increasing the memory capacity and learning ability.

Systemic and neuroinflammation can induce endothelial cell damage, microglial activation and neuronal death (51). As a critical structure of learning and memory, the hippocampus is vulnerable to systemic inflammatory factors (52,53), and pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, can readily promote endothelial dysfunction, BBB disruption and brain edema (54,55). IL-10 is a potent anti-inflammatory cytokine that alleviates such injuries (56). Reportedly, ISO represses neuroinflammation, coinciding with a decrease in TNF- α and IL-1 β in the mouse brain following subarachnoid hemorrhage (57). In the current study, 0.7% ISO decreased the

production of TNF- α , IL-1 β and IL-6, and increased IL-10 generation in the serum and hippocampi of CLP-induced septic mice. Previous studies have suggested that ISO induces HO-1 expression in Hep3B cells (58) and hippocampal neurons (36). HO-1 signaling mediates the anti-inflammatory effects of ISO in CLP-induced lung injury (27) and ischemia/reperfusion-induced liver injury (59), as well as in LPS-challenged macrophages (35). Consistently, the present study revealed that 0.7% ISO induced the expression and activity of HO-1 in the hippocampi of CLP-treated mice, and that ZnPP reversed the anti-inflammatory abilities of 0.7% ISO, suggesting that the protective effects of 0.7% ISO against CLP-induced sepsis partially rely on activated HO-1 signaling.

Oxidative stress is one of the primary causes of SAE during sepsis (60). Excessive ROS generation induces lipid peroxidation, disrupts cellular and mitochondrial membranes, and ultimately results in the apoptosis and necrosis of cells (61). MDA is a direct product of lipid peroxidation and is recognized as an indicator of ROS-mediated injury (62).

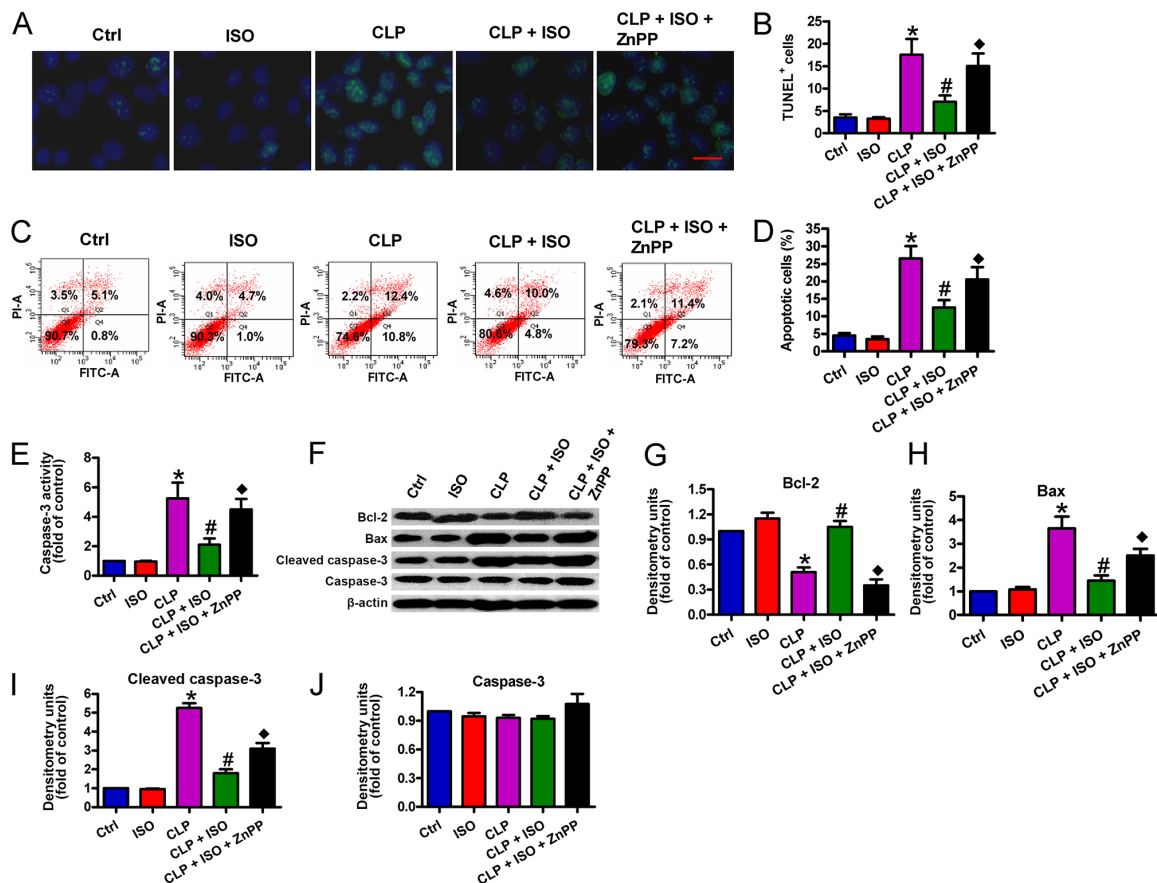


Figure 6. 0.7% ISO suppresses neuronal apoptosis in the hippocampi of CLP-treated mice. Mice were treated with or without 0.7% ISO by inhalation for 1 h, starting at 1 and 6 h after sham or CLP surgery respectively, in the presence or absence of peritoneal ZnPP administration (10 mg/kg) at 1 h before sham or CLP surgery. At 48 h post-surgery, the mice were anesthetized and the hippocampi were collected. (A and B) TUNEL assays were conducted and the number of TUNEL⁺ cells was calculated (scale bar, 10 μ m). (C and D) Neuronal apoptosis was analyzed by flow cytometry and the percentage of apoptotic neurons was determined. (E) Neuronal caspase-3 activity was detected. (F) Representative western blotting results for Bcl-2, Bax, cl-caspase-3 and caspase-3 detection; β -actin was used as the endogenous control. Protein levels of (G) Bcl-2, (H) Bax, (I) cl-caspase-3 and (J) caspase-3 were calculated. Representative data are expressed as the mean \pm SD from three independent experiments. * P <0.05 vs. Ctrl group; # P <0.05 vs. CLP or ISO group; * P <0.05 vs. CLP + ISO group. ISO, isoflurane; CLP, cecal ligation and puncture; ZnPP, zinc protoporphyrin IX; cl-, cleaved; Ctrl, control.

Antioxidant enzymes, such as SOD and CAT, are key ROS scavengers that specifically eliminate superoxide radicals and prevent ROS-induced brain damage during sepsis (24). A published review summarizes the anti-oxidative aspects of ISO in acute brain injury (63). HO-1 is a key participant in sepsis (27-29), and has been reported to hamper oxidative stress during sepsis-induced acute lung injury (64). ISO enhanced HO-1 expression in the lungs of CLP-treated rats (27). Herein, the present findings demonstrated that 0.7% ISO decreased the ROS and MDA content, and enhanced SOD and CAT activity in the serum and hippocampi of CLP-induced septic mice. Moreover, ZnPP administration reversed these effects, indicating that HO-1 signaling is implicated in the anti-oxidative effects of 0.7% ISO in septic mice.

Due to its close association with cognitive dysfunction, neuronal apoptosis in the hippocampus is a major causative factor of SAE (65). The balance between anti- and pro-apoptotic Bcl-2 family proteins dominates the progression of neuron apoptosis (66). Pro-apoptotic Bax translocates to the outer mitochondrial membrane, disrupting mitochondrial transmembrane potential and ultimately promoting cell death. On the contrary, anti-apoptotic Bcl-2 inhibits Bax activation (67). Caspase-3, the final apoptotic executor,

plays a crucial role in neuronal apoptosis (14). ISO has been confirmed to increase Bcl-2 expression and reduce cytochrome c release from mitochondria in the ischemic penumbra of the rat brain (68). Bedirli *et al* (69) reported that ISO upregulates Bcl-2 and downregulates Bax in the rat brain following focal cerebral ischemia. ISO reduces oxygen and glucose deprivation-induced hippocampal neuron apoptosis by inactivating caspase-3 (50). HO-1 signaling mediates the inhibitory effects of 0.7% ISO on zymosan-induced pulmonary cell apoptosis (70). In line with previous findings, it was confirmed in the present study that 0.7% ISO repressed neuronal apoptosis in the hippocampus of CLP-induced septic mice, which was accompanied by reduced expression of Bax and cl-caspase-3, and increased Bcl-2 expression. Treatment with ZnPP, a HO-1 inhibitor, neutralized the anti-apoptotic effects of 0.7% ISO, implying that HO-1 signaling is involved in the 0.7% ISO-reduced neuronal apoptosis in CLP-induced septic mice.

In conclusion, the present study demonstrated that 0.7% ISO plays crucial neuroprotective roles in a CLP-established SAE mouse model, largely involving the activation of HO-1. The upregulation and activation of HO-1 mediates the anti-inflammatory, antioxidative and anti-apoptotic effects of 0.7% ISO

on CLP-induced neuronal damage in the brains of septic mice. These findings provided strong evidence for the potential application of 0.7% ISO for the treatment of patients with SAE.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZW contributed to the conception and design of the present study. LZ and XZ conducted all the experiments. ZW and LZ drafted the manuscript and revised it critically for important intellectual content. TW and XP interpreted and analyzed the data. ZW provided final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental protocols were approved by the Animal Care and Use Committee of Xi'an Jiaotong University (approval no. XAJU.No20180730A10120) and were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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