

# Hydrogen-rich saline alleviates early brain injury through inhibition of necroptosis and neuroinflammation via the ROS/HO-1 signaling pathway after traumatic brain injury

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**Abstract.** Traumatic brain injury (TBI) has been recognized as a serious public health issue and a key contributor to disability and death, with a huge economic burden worldwide. Hydrogen, which is a slight and specific cytotoxic oxygen radical scavenger, has been demonstrated to ameliorate early brain injury (EBI) through reactive oxygen species (ROS), oxidative stress injury, apoptosis and necroptosis. Necroptosis refers to a type of programmed cell death process that has a vital function in neuronal cell death following TBI. The specific function of necroptosis in hydrogen-mediated neuroprotection after TBI, however, has yet to be determined. The present study aimed to examine the neuroprotective effects and possible molecular basis that underly hydrogen-rich saline in TBI-stimulated EBI by examining neural necroptosis in the C57BL/6 mouse model. The brain water content, neurological score, neuroinflammatory cytokines (NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ) and ROS were evaluated using flow cytometry. Malondialdehyde, superoxide dismutase (SOD) and glutathione (GSH) levels were evaluated using a biochemical kit. Receptor-interacting protein kinase (RIP)1, RIP3, Nrf2 and Heme oxygenase-1 (HO-1) were evaluated using western blotting. mRNA of Nrf2 and HO-1 were evaluated using quantitative PCR. Neuronal death was evaluated by TUNEL staining. The outcomes illustrated that hydrogen-rich saline treatment considerably enhanced the neurological score, increased neuronal survival, decreased the levels of serum MDA and brain ROS, increased the levels of serum GSH and SOD. In addition the protein expression levels of RIP1 and RIP3 and the cytokines NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were downregulated compared with the TBI

group, which demonstrated that hydrogen-rich saline-induced inhibition of necroptosis and neuroinflammation ameliorated neuronal death following TBI. The neuroprotective capacity of hydrogen-rich saline was demonstrated to be partly dependent on the ROS/heme oxygenase-1 signaling pathway. Taken together, the findings of the present study indicated that hydrogen-rich saline enhanced neurological outcomes in mice and minimized neuronal death by inducing protective effects against neural necroptosis as well as neuroinflammation.

## Introduction

Traumatic brain injury (TBI) has been recognized as a serious public health issue and a key contributor to disability and death, with a huge economic burden worldwide (1,2). High incidence (1/1,000 in China between 1983 and 1985) of TBI is predominantly reported in low- and middle-income countries as well as developing countries, including China and Iran (1-3). The prevalence of TBI has witnessed a rapidly growing trend due to the considerable increase in road accidents, such as motor vehicle collisions (2). Although an increasing number of randomized controlled trials have included intracranial pressure monitoring, therapeutic hypothermia, surgical methods and drug administration in recent years, long-term outcomes have not substantially improved, especially after drug intervention (2-8). Therefore, it is important to further clarify the pathophysiological processes of TBI and identify novel efficient pharmacological targets for TBI treatment. It is widely acknowledged that the pathophysiology of TBI encompasses several types of pathological and physiological changes, mainly involving primary brain injury and secondary brain injury, which leads to neuronal death, neurological deficits and mortality after TBI (9). Primary brain injury, which is a direct physical injury to the brain tissue, is difficult to prevent and cannot be usually reversed, and leads to brain tissue disorganization, intracerebral hemorrhage and blood-brain barrier (BBB) damage (1,10,11). Secondary brain injury includes calcium overload, oxidative stress, neuroinflammation, autophagy, lipid peroxidation, apoptosis and necroptosis, and can be reversed (10,11).

Necroptosis has recently been identified as a pathway of modulated necrosis and a mechanism of caspase-independent

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programmed cell death, requiring the proteins mixed lineage kinase domain-like (MLKL) and receptor-interacting protein kinase-3 (RIP3), and is triggered by death receptors (12). Accumulating evidence indicates that necroptosis performs an important function in central nervous system disorders, such as TBI (13-15), intracerebral hemorrhage (16,17), ischemic stroke (18), Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (19). The activation of a TNF ligand family member, such as RIP1 and MLKL, is the most upstream signaling activity necessary for necroptosis induction (20,21). The activation of RIP1 contributes to necroptosis via inducing RIP1-RIP3-MLKL complex (22). Necroptosis is common in early brain injury (EBI) and may be a mechanism of TBI.

In previous years, it has become widely acknowledged that hydrogen-rich saline or hydrogen gas can protect the human body from numerous diseases, such as neurodegenerative disorders, spontaneous subarachnoid hemorrhage, stroke and ischemia-reperfusion damage, by modulating neuronal apoptosis, inflammatory response and oxidative stress (23-26). A previous study has indicated that hydrogen may selectively minimize peroxynitrites and hydroxyl radicals, and subsequently have a crucial function exhibiting cytoprotective, anti-inflammatory, anti-apoptotic and antioxidant properties (27). However, the neuroprotective benefits of hydrogen-rich saline treatment on TBI are controversial. Heme oxygenase-1 (HO-1) is an essential element of the cellular defense system that is activated by oxidant-stimulated damage and acts against it (28,29). In the central nervous system (CNS), HO-1 exerts anti-necroptotic, anti-neuroinflammatory and neuroprotective functions (27,30). In our previous study, HO-1 was demonstrated to regulate neuronal death in acute CNS disease (31). Therefore, treatments that target HO-1 may be potentially effective at preventing necroptosis, oxidative stress and inflammation following TBI. Nonetheless, the specific mechanisms regarding the potential neuroprotective benefits of hydrogen-rich saline treatment are unknown. The present study examined whether neuroprotective benefits of hydrogen-rich saline treatment in a mouse model of TBI occur via effects on neuroinflammation and necroptosis, and whether neuroprotection is dependent on the reactive oxygen species (ROS)/HO-1 pathway.

## Materials and methods

**Animals.** All the animal experiments conducted in the present study were completed in compliance with guidelines for the appropriate handling of laboratory animals formulated by the National Institutes of Health (32). Approval of the experiments was provided by the Ethics Committee of Wuxi Clinical College of Anhui Medical University (Wuxi, China). A total of 36 healthy adult male C57BL/6J mice (age, 8-10 weeks; Anhui Medical University, Hefei, China) weighing between 22-25 g were used when conducting all the experiments for the current study. The mice were maintained in animal care facilities (temperature, 25±2°C; humidity, 55±5%) with a 12/12 h dark/light cycle and an unrestricted supply of water and food.

**Animal TBI model.** The Feeney weight-drop model of focal injury was strictly followed when developing the TBI

model (33,34). Briefly, anesthetization of the mice was performed using 1% sodium pentobarbital (40 mg/kg) injected intraperitoneally. The mice were subsequently placed in a brain stereotaxic apparatus. While being operated, a heating pad was utilized to ensure that the rectal temperature remained at 37±0.5°C. The coordinates utilized when making a burr hole in the left hemisphere were set as follows: 0.2 mm posterior, 1 mm lateral and 2.2 mm ventral of bregma's horizontal plane. To visualize the dura mater, the bone flap was removed. The dura was subsequently placed under weight-drop equipment that included an impact sensor pedal; (Anhui Zhenghua Biological Instrument Equipment Co., Ltd.). Metal with a tip diameter of 3-mm and weight of 240 g was dropped onto the dura mater from a distance of 1 cm above the dura via a catheter. The scalp was subsequently closed and the mice were removed from the stereotaxic apparatus. Subsequently, a medical bone wax was utilized to cover the hole. The animals in the sham cohort received comparable surgical treatments but without the weight-drop procedure. At 72 h following TBI, the mice were sacrificed with 100 mg/kg sodium pentobarbital via an intraperitoneal injection. Brain tissue samples were collected after the mice were sacrificed. Fresh specimens (cerebral cortex) and serum were stored in liquid nitrogen (-196°C) or hippocampus was stored in 4% formalin-fixed (4°C) for ≥48 h.

**Drug preparation and administration.** After the TBI model was established successfully, the mice received intraperitoneal injections every day for 72 h that contained either plain saline (control) or hydrogen-rich (5 ml/kg; experimental). Hydrogen-rich saline was prepared in accordance with the procedure described in previous studies (35,36). Briefly, purified hydrogen was dissolved in normal saline for 2 h at an elevated pressure of 0.4 MPa. The physiological concentration was maintained at 1.73 ml hydrogen per 100 ml saline (average, >6 mmol/l). Hydrogen-rich saline was stored at 4°C in an aluminum bag without dead volume under atmospheric pressure. Every week, fresh hydrogen-rich saline was synthesized to guarantee a consistent concentration. The content of hydrogen in saline was evaluated and detected by gas chromatography as reported in a previous study (37).

**Neurobehavioral assessment.** By employing a previously published neurological grading system (38,39), the degree of brain injury was assessed 72 h following TBI via the determination of neurological function. The scoring system consisted of reflex, sensory, balance and motor tests. The neurological scores ranged between 0 and 18 and were calculated by summing the individual scores (Table SI). A behavioral assessment was conducted on all mice in each cohort, with a higher score indicating impaired neurological function.

**Brain water-content measurement.** As reported previously, the degree of brain edema was examined by quantifying the brain water content utilizing the standard wet-dry technique (39-41). A total of 72 h following TBI the mice were euthanized, and their whole brain was extracted and divided into the contralateral and ipsilateral cortex, contralateral and ipsilateral basal ganglia and cerebellum (wet weight). The dry weight of each cohort's brain specimen was then determined after dehydrating each part at 105°C for 24 h. The proportion of

brain water content was equal to (wet weight-dry weight)/wet weight x100%.

**Cytokine measurements.** ELISA was utilized to measure cerebral cortex NF- $\kappa$ B (cat. no. ab176663), cerebral cortex TNF- $\alpha$  (cat. no. ab208348), cerebral cortex IL-6 (cat. no. ab222503) and cerebral cortex IL-1 $\beta$  (cat. no. ab197742; all from Abcam). The cerebral cortex was extracted from mice brain. The procedure was conducted in accordance with the manufacturer's protocols.

**Analysis of ROS.** The brain tissue intracellular ROS synthesis was evaluated utilizing the non-fluorescent diacetylated 2',7'-dichlorofluorescein diacetate (DCFH-DA) probe (Sigma-Aldrich; Merck KGaA) that becomes highly fluorescent upon oxidation and was used according to the guidelines provided by the manufacturer. Fresh cerebral cortex was used to form a single cell suspension. The cells were harvested at a concentration of  $2 \times 10^6$  cells/ml, then DCFH-DA ( $10 \mu\text{M}$  final concentration) was added and the mixture was incubated at  $37^\circ\text{C}$  for 15 min. The DCFH results were examined via flow cytometry (CytoFLEX; Beckman Coulter, Inc.). CytExpert (version 2.4; Beckman Coulter, Inc.) was used to analyze the data.

**Analysis of MDA, superoxide dismutase (SOD) and glutathione (GSH).** The malondialdehyde (MDA) Assay Kit (excitation/emission, 532/553 nm; cat. no. ab118970; Abcam) was utilized to detect serum MDA levels in compliance with the instructions provided by the manufacturer. The serum was extracted from mice venous blood. The SOD Assay Kit (cat. no. A001-3-2; Nanjing Jiancheng Bioengineering Institute) and GSH (42) Assay Kit (cat. no. A005-1-2; Nanjing Jiancheng Bioengineering Institute) were utilized to detect serum SOD and serum GSH levels in compliance with the manufacturer's instructions (43,44).

**TUNEL staining.** To evaluate neuronal death in the brain cortex, TUNEL staining was utilized. The procedure was performed with a TUNEL staining kit (cat. no. 11684817910; Roche Diagnostics GmbH) according to the protocols provided by the manufacturer. In each 4% formalin-fixed ( $4^\circ\text{C}$ ; duration,  $\geq 48$  h) for specimen, paraffin-embedded sections ( $10\text{-}\mu\text{m}$ ) were cut from formalin-fixed tissue. Subsequently,  $50 \mu\text{l}$  TUNEL reaction mixture was added. The negative control used did not include the TUNEL reaction mixture. Subsequently, the slides were subjected to incubation in a humidified dark chamber at a temperature of  $37^\circ\text{C}$  for 60 min. Then, a primary antibody against the neuronal marker NeuN (1:200; rabbit polyclonal; cat. no. ab128886; Abcam) diluted in PBS was added, followed by incubation overnight at  $4^\circ\text{C}$ . Subsequently, incubated with FITC goat anti-mouse IgG secondary antibodies (1:100; cat. no. BA1101; Boster Biological Technology Co., Ltd.) at temperature of  $37^\circ\text{C}$  for 1.5 h. Next, the slides were incubated at ambient temperature for 5 min in the dark with DAPI for staining of the nuclei, followed by imaging using a fluorescence microscope (magnification, x200). The validation of the cell count was performed using four high-power fields randomly selected, and the data obtained from each field were averaged.

**Western blot analysis.** Western blot analysis was conducted as indicated previously (40). Cerebral cortex specimens

were collected and extracted. Cerebral cortex samples were collected, homogenized and total protein was extracted using RIPA buffer (CoWin Biosciences). The protein concentrations were quantified utilizing a BCA Protein Assay kit (Beyotime Institute of Biotechnology) and proteins ( $40 \mu\text{g}/\text{lane}$ ) were separated using 10% SDS-PAGE. Then, the proteins were transferred onto Immobilon nitrocellulose membranes. Blocking of the membranes was performed with 5% non-fat milk for 1 h at room temperature. The membranes were then subjected to incubation using the following primary antibodies overnight at  $4^\circ\text{C}$ : Anti- $\beta$ -actin (1:1,000; rabbit polyclonal; cat. no. ab8227; Abcam), anti-RIP1 (1:1,000; rabbit polyclonal; cat. no. ab106393; Abcam), anti-nuclear factor erythroid 2-related factor 2 (Nrf2; 1:1,000; rabbit polyclonal; cat. no. ab31163; Abcam), anti-HO-1 (1:1,000; rabbit polyclonal; cat. no. ab13243; Abcam) and anti-RIP3 (1:1,000; rabbit polyclonal; cat. no. ab62344; Abcam). After washing the membranes using 0.5% TBS-Tween-20 three times, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies (1:2,000; cat. no. 7074s; Cell Signaling Technology, Inc.) at room temperature for 1.5 h. The signals were developed using an enhanced chemiluminescence reagent (MilliporeSigma) according to the manufacturer's instructions. A Bio-Rad imaging system (Bio-Rad Laboratories, Inc.) was utilized to identify protein bands that were then measured using ImageJ software (version 1.52; National Institutes of Health).

**RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).** TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.) was utilized to isolate total RNA from the cerebral cortex of brain specimens as per the guidelines provided by the manufacturer, which was then quantified utilizing a NanoDrop<sup>™</sup> 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The RevertAid First Strand cDNA Synthesis Kit (cat. no. K1622; Thermo Fisher Scientific Inc.) was subsequently utilized to perform reverse transcription of RNA to cDNA according to the manufacturer's protocol. Each specimen's mRNA levels were quantified via qPCR utilizing SYBR Green Master Mix (Toyobo Life Science). The expression levels of all genes were normalized to that of  $\beta$ -actin. The following qPCR thermocycling criteria were used:  $45^\circ\text{C}$  (2 min) and  $95^\circ\text{C}$  (10 min), followed by 40 cycles of denaturation at  $95^\circ\text{C}$  (15 sec), annealing at  $60^\circ\text{C}$  (1 min) and extension at  $72^\circ\text{C}$  (1 min). The analysis of all specimens was performed in triplicate. The primers used are listed as follows: Nrf2 forward, 5'-CAGTGCTCCTATGCGTGAA-3' and reverse, 5'-GCGGCTTGAATGTTTGTCT-3'; HO-1 forward, 5'-TGA CAGAAGAGGCTAAGACCG-3' and reverse, 5'-AGTGAG GACCCACTGGAGGA-3'; and GAPDH forward, 5'-ATG GGTGTGAACCACGAGA-3' and reverse, 5'-CAGGGATGAT GTTCTGGGCA-3' (45).

**Statistical analysis.** All experiments were performed with at least three experimental repeats, and the data are presented as the mean  $\pm$  SEM. Neurological scores are presented as the median and interquartile range. Statistical analyses were implemented using GraphPad Prism 6 (GraphPad Software, Inc.) and SPSS 14.0 (SPSS, Inc.). Differences between multiple groups were analyzed using one-way ANOVA followed by

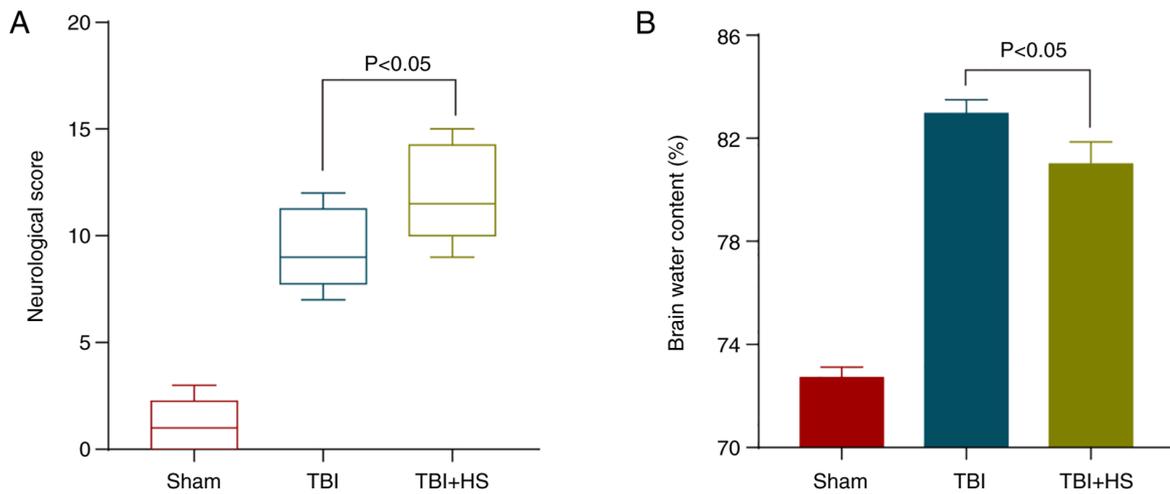


Figure 1. HS alleviates neurological impairment and brain edema after TBI. (A) Neurological scores of mice in the sham, TBI and TBI + HS cohorts 72 h after TBI (median and interquartile range;  $n=10$ ). (B) Brain water content in the three cohorts. Brain water content decreased significantly after HS treatment (mean  $\pm$  SEM;  $n=5$ ). TBI, traumatic brain injury; HS, hydrogen-rich saline.

Tukey's post hoc test. Neurological scores were analyzed using a non-parametric test (Kruskal-Wallis followed by Dunn's post hoc test).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Hydrogen-rich saline alleviates neurological deficits and brain edema after TBI.* Modified neurological severity scores were employed to assess neurological impairments to understand the neuroprotection of hydrogen-rich saline following TBI. To assess brain damage, the wet-dry technique was employed to measure brain water content 72 h after TBI. The findings illustrated that TBI was associated with a considerable increase in the brain water content, which was alleviated after hydrogen-rich saline treatment (Fig. 1B). Comparable findings in neurological scores indicated that the scores were considerably higher in animals suffering from hydrogen-rich saline treatment compared with the TBI group, and that hydrogen-rich saline treatment substantially improved the neurological function (Fig. 1A).

*Hydrogen-rich saline alleviates neuronal necroptosis after TBI.* Neuronal necroptosis is the main factor resulting in EBI after TBI (46). Therefore, a TUNEL assay was conducted to examine the degree of cell death in TBI mice treated or non-treated with hydrogen-rich saline 72 h following model establishment. Neuronal death in the hippocampus decreased upon hydrogen-rich saline treatment compared with the TBI group (Fig. 2A). The expression levels of necroptosis-related proteins were additionally detected via western blotting (Fig. 2B). The results of western blotting also demonstrated that hydrogen-rich saline significantly reduced the expression levels of the necroptosis-related proteins RIP1 and RIP3 compared with the TBI group (Fig. 2C and D). These findings indicated that hydrogen-rich saline exhibited neuroprotective benefits after TBI.

*Hydrogen-rich saline alleviates neuroinflammation after TBI.* Previous studies have demonstrated that neuroinflammation

exhibits a vital function in EBI after TBI and enhanced neuroinflammation can aggravate EBI (10,47-49). Activation of the inflammatory process can induce the release of inflammatory cytokines, which include NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (39,50). Therefore, ELISA was employed to examine the hippocampal levels of NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$ . The findings revealed that the inflammatory cytokines were increased substantially after TBI, while they were significantly decreased after hydrogen-rich saline treatment (Fig. 3A-D). Thus, these results suggested that hydrogen-rich saline exhibited a potent anti-inflammatory activity against TBI-induced neuroinflammation.

*Hydrogen-rich saline inhibits TBI-induced oxidative stress and decreases ROS levels.* To clarify whether oxidative stress performs a crucial function in TBI and whether hydrogen-rich saline can regulate oxidative stress, the oxidative stress-related biomarker levels of GSH, MDA and SOD were detected in each cohort. The findings illustrated that MDA increased following TBI, but considerably decreased following hydrogen-rich saline treatment (Fig. 4A). Additionally, SOD and GSH decreased after TBI but increased considerably following hydrogen-rich saline treatment (Fig. 4B and C). ROS are considered to be a biomarker of oxidative stress activation initiating programmed cell and neuronal death (31,51). ROS levels were detected using the DCF-DA probe. The results demonstrated that ROS levels were increased after TBI in the hippocampus, while decreased after hydrogen-rich saline administration (Figs. 4D and S1).

*Hydrogen-rich saline regulates necroptosis via the ROS/HO-1 signaling pathway after TBI.* It was explored whether necroptosis inhibition occurred through the ROS/HO-1 signaling pathway after hydrogen-rich saline treatment. The mRNA expression levels of HO-1 and Nrf2 were detected via RT-qPCR. The findings illustrated that the expression levels of HO-1 and Nrf2 were considerably reduced in the TBI cohort, and were elevated following hydrogen-rich saline administration (Fig. 5A and B). The protein expression levels of HO-1 and Nrf2 were also detected via western blotting (Fig. 5C).

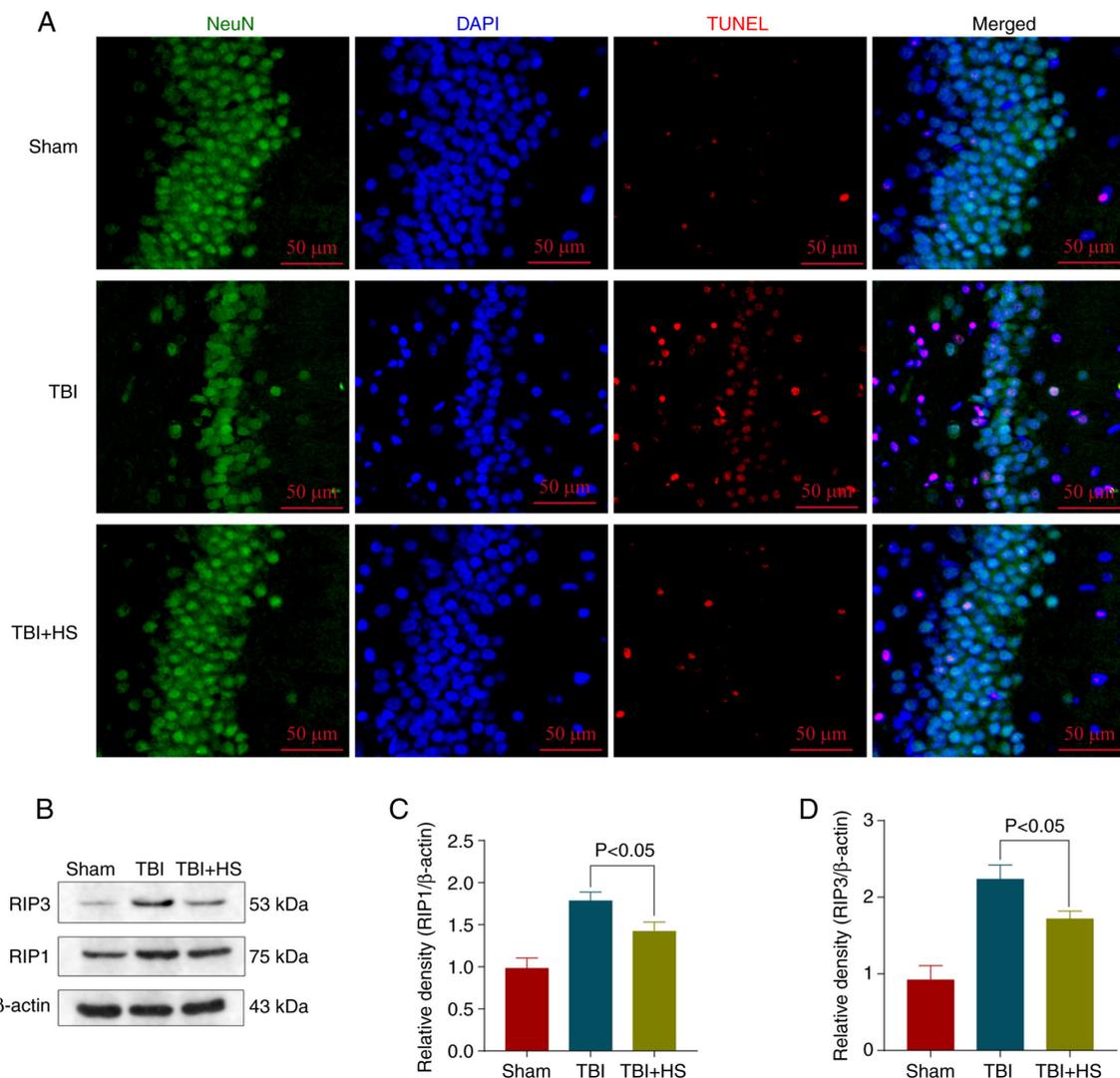


Figure 2. HS alleviates neuronal necroptosis after TBI. (A) TUNEL assay demonstrated that HS alleviated neuronal necroptosis. (B) Expression of RIP1 and RIP3 in the brain cortex of mice following TBI was evaluated via western blotting. Quantification of (C) RIP1 and (D) RIP3 protein levels in the brain cortex relative to  $\beta$ -actin loading control, indicating that HS suppressed RIP1 and RIP3 expression after TBI in mice (mean  $\pm$  SEM; n=6). TBI, traumatic brain injury; HS, hydrogen-rich saline; RIP, receptor-interacting protein kinase.

Quantification of the protein levels of HO-1 and Nrf2 in the brain cortex relatively to the  $\beta$ -actin loading control revealed that hydrogen-rich saline increased HO-1 and Nrf2 expression after TBI in mice (Fig. 5D and E). Thus, the present findings collectively demonstrated that hydrogen-rich saline may inhibit TBI-induced necroptosis by regulating the ROS/HO-1 signaling pathway.

## Discussion

The present study examined the therapeutic value of hydrogen-rich saline for ameliorating EBI in a TBI mouse model. The findings indicated that hydrogen-rich saline was a neuroprotective agent that can attenuate EBI following TBI. The results demonstrated that hydrogen-rich saline could achieve the following: i) Ameliorate neurological dysfunction following TBI; ii) relieve brain damage in a mouse TBI model; iii) relieve neuroinflammation after TBI and decrease brain inflammatory damage; and iv) prevent necroptosis after TBI and alleviate neuronal death. Furthermore,

the anti-neuroinflammatory and anti-necroptotic roles of hydrogen-rich saline may be associated with the ROS/HO-1 pathway.

Hydrogen-rich saline or hydrogen gas can easily penetrate the BBB via gaseous diffusion, which is acknowledged to achieve protective effects in several CNS disorders, such as TBI, neurodegenerative diseases, intracranial hemorrhage and ischemic stroke (23-26). Hydrogen gas or hydrogen-rich saline serves an important antioxidant role with high tissue transferability, and it has been demonstrated that  $H_2$  is safe for patients and animals (37). The anti-oxidative stress and anti-inflammatory effects of hydrogen-rich saline or hydrogen gas are mediated by selective suppression of toxic ROS, which include peroxynitrite and hydroxyl radical (25). Liu *et al* (52) also reported that  $H_2$  can markedly improve cognitive dysfunction and survival rates, decrease inflammatory response and oxidative stress, and increase antioxidant enzyme activities in the serum and hippocampus in a mouse model of sepsis. In the intracerebral hemorrhage model, it has also been demonstrated that hydrogen performs a neuroprotective function

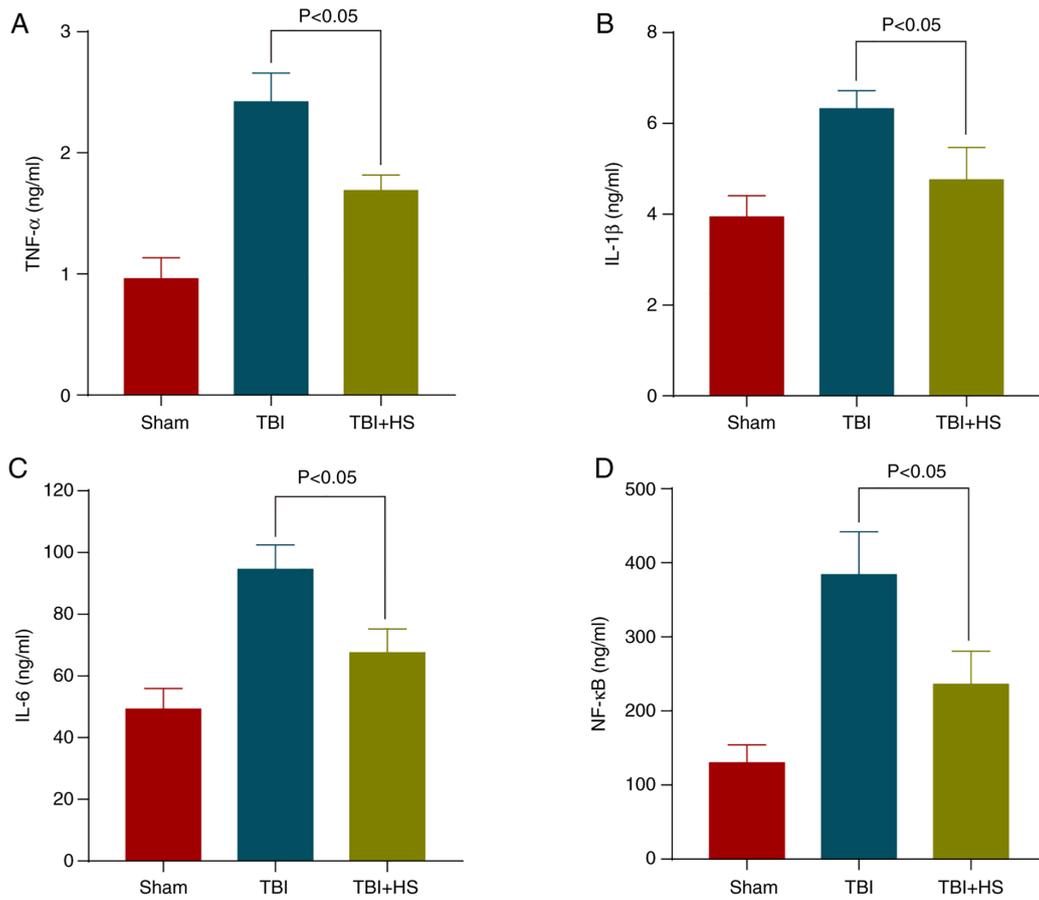


Figure 3. HS alleviates neuroinflammation after TBI. HS significantly decreased the levels of hippocampal (A) TNF- $\alpha$ , (B) IL-1 $\beta$ , (C) IL-6 and (D) NF- $\kappa$ B at 72 h after TBI (mean  $\pm$  SEM; n=6). TBI, traumatic brain injury; HS, hydrogen-rich saline.

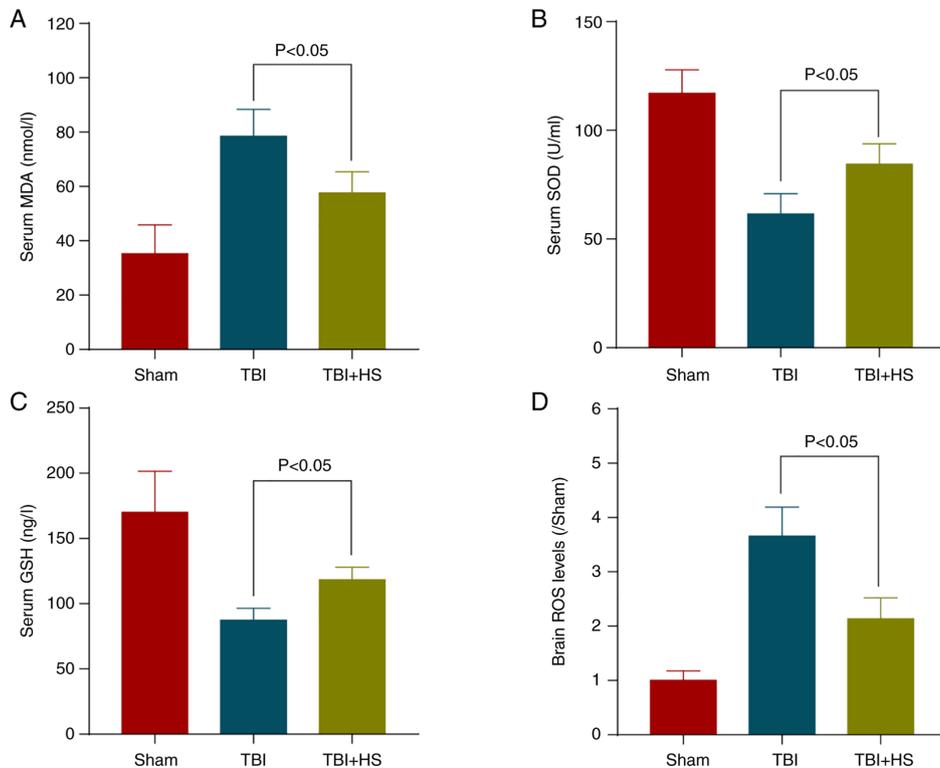


Figure 4. HS inhibits TBI-induced oxidative stress and decreases ROS levels. HS significantly (A) decreased the expression levels of serum MDA, increased the expression levels of (B) serum SOD and (C) serum GSH, and (D) decreased the levels of brain tissue ROS after TBI (mean  $\pm$  SEM; n=6). TBI, traumatic brain injury; HS, hydrogen-rich saline; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; ROS, reactive oxygen species.

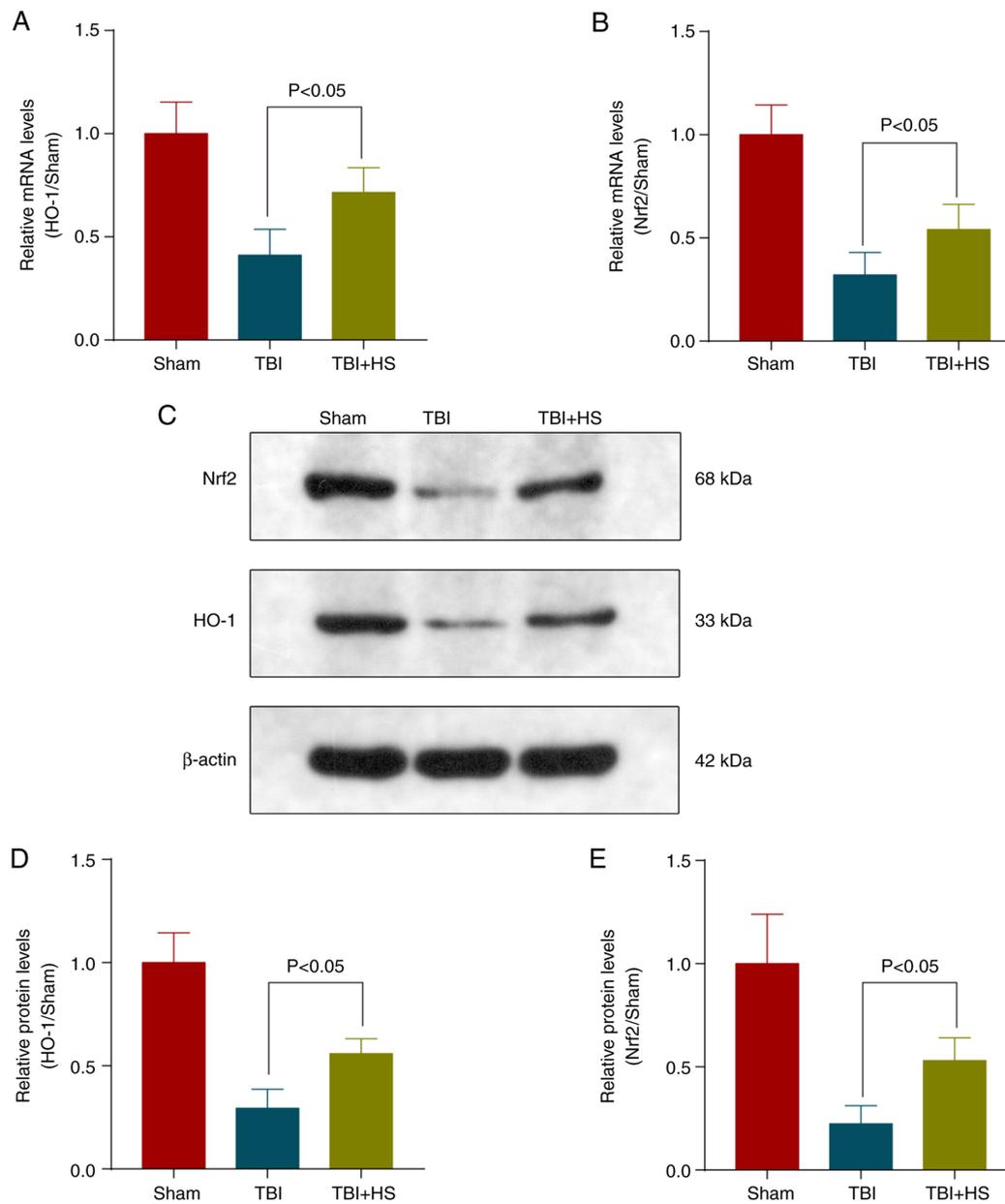


Figure 5. HS regulates necroptosis via the ROS/HO-1 signaling pathway after TBI. (A) HO-1 and (B) Nrf2 mRNA levels in the brain of TBI mice were quantified via reverse transcription-quantitative PCR. (C) Expression of HO-1 and Nrf2 in the brain cortex of mice following TBI was assessed via western blotting. Quantification of (D) HO-1 and (E) Nrf2 protein levels in the brain cortex relative to  $\beta$ -actin loading control. HS increased HO-1 and Nrf2 expression following TBI in mice. (mean  $\pm$  SEM; n=6). TBI, traumatic brain injury; HS, hydrogen-rich saline; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2.

against EBI following ICH, alleviating brain edema and neurologic deficits by regulating oxidative stress, neuroinflammation and apoptosis (53). In the hypoxic-ischemic brain injury neonatal rat model, H<sub>2</sub> inhalation could alleviate brain damage and improve early neurological outcomes through anti-inflammatory, anti-apoptotic and antioxidant responses via the MAPK/HO-1/peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  pathway (54). In the TBI model, molecular hydrogen water could also reverse the controlled cortical impact-induced brain edema through preserved or increased ATP levels (55). Tian *et al* (56) demonstrated that hydrogen-rich water could alleviate inflammation and decrease brain damage, exhibiting a neuroprotective function against TBI. Yuan *et al* (57) also discovered that hydrogen-rich

water can inhibit oxidative stress and activate the Nrf2 pathway, thereby improving TBI-induced brain damage. Both aforementioned studies explored the neuroprotective effect of hydrogen-rich water associated with inflammation and oxidative stress without investigating further molecular mechanisms. However, the current study also revealed that hydrogen-rich saline considerably enhanced neurological function, alleviated brain edema, increased neuronal survival and downregulated the protein expression of RIP3 and RIP1, as well as the cytokines NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6, following TBI.

Necroptosis is a cell death type of modulated necrosis, requiring the proteins MLKL and RIP3, and is stimulated by death receptors (12). Increasing evidence suggests that necroptosis performs an instrumental function in CNS diseases, such

as TBI (13-15). Our previous studies have also demonstrated that necroptosis has an integral function in the pathophysiology of neuronal death following *in vitro* traumatic neuronal injury, and that the potential regulatory mechanisms may be associated with the Akt/GSK-3 $\beta$  and the metabotropic glutamate receptor 1 signaling pathways (13,14). Therefore, necroptosis is a vital mechanism in TBI, and necroptosis inhibition via drugs may decrease neuronal death. Jia *et al* (58) reported that hydrogen can decrease the expression levels of necroptosis-related proteins in the hippocampus, including MLKL, phosphorylated MLKL and RIP3, thereby partly preventing neuronal and astrocyte necroptosis in the lithium-pilocarpine model of status epilepticus. Dong *et al* (59) also reported that high concentrations of hydrogen inhalation can alleviate skin ischemia/reperfusion injury by regulating necroptosis and the RIP-MLKL-serine/threonine-protein phosphatase PGAM5/dynamin-related protein 1 necrotic pathway. In the present study, the results confirmed that the mRNA and protein expression levels of RIP3 and RIP1 were elevated 72 h after TBI, while hydrogen-rich saline treatment could reduce the RIP3 and RIP1 expression levels.

The molecular mechanism of necroptosis and neuroinflammation is complex, and the specific mechanisms of the neuroprotective benefits of hydrogen-rich saline treatment are yet to be elucidated. Wang *et al* (60) reported that rehmapirogenin can improve adriamycin-induced nephropathy *in vitro* and *in vivo* by reducing ROS accumulation, thereby regulating the expression levels of Nrf2. Nrf2 is an important transcriptional regulation factor that can regulate the expression of >250 genes and is characterized by its binding site 'antioxidant response element'; the majority of regulated genes by Nrf2 are involved in oxidative stress and cell apoptosis, necroptosis, autophagy and ferroptosis (31). Yu *et al* (61) discovered that the inhalation of 2% molecular hydrogen gas may enhance the survival rates, reduce the lung edema and the lung injury score, and ameliorate the injuries induced by inflammation and oxidative stress in the septic mouse model, while Nrf2 knockout could reverse or weaken the protection of H<sub>2</sub> gas on lung damage, which was also dependent on HO-1 and high mobility group protein B1. Additionally, Chen *et al* (62) demonstrated that H<sub>2</sub> decreased endothelial inflammation and injury and increased the expression and activity of HO-1 *in vivo* and *in vitro*. Nrf2 knockout or HO-1 inhibition reversed the protection of H<sub>2</sub>, indicating that this was dependent on the activity of the Nrf2/HO-1 signaling pathway. However, the exact mechanism requires further elucidation.

In summary, the present findings demonstrated that necroptosis, which is mediated by the RIP1 and RIP3 proteins, is a key cellular regulatory mechanism that may lead to EBI following TBI. The present study, for the first time to the best of our knowledge, revealed that hydrogen-rich saline induced modulation of necroptosis and neuroinflammation via the ROS/HO-1 pathway, and also provided a novel insight into evaluating the biological impacts as well as the mechanisms that underly neuroprotection and inhibition of inflammation and necroptosis by hydrogen-rich saline.

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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

YH wrote the manuscript. YH, XF, YW, LS and JC performed the experiments and prepared the figures. YH and JC confirm the authenticity of all the raw data. YH and JC designed the study and revised the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee of Wuxi Clinical College of Anhui Medical University (approval no. YXLL-2020-012; Wuxi, China).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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