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Original Article

Hydrogen has a neuroprotective effect via activation of Nrf-2/HO-1 pathway in ischemia reperfusion rat

Lianshuang Zhang[1*], Xifeng Wang[2*], Xinghan Tian[2], Wei Zhao[1], Tongshen Liu[1], Guoying Feng[1], Heng Cai[1], Jie Liu[3]

[1]*Department of Histology and Embryology, Bin Zhou Medical College, 346#, Guan Hai Road, Yan Tai 264000, Shandong, PR China;* [2]*Department of Critical Care Medicine, Yu Huang Ding Hospital, Qingdao University, 20#, Yu Huang Ding Dong Road, Yan Tai 264000, Shandong, PR China;* [3]*Department of Neurology, The Fourth Hospital in Jinan, Jinan 250031, Shandong, PR China.* [*]*Equal contributors.*

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Abstract: This study was designed to investigate whether hydrogen administration can produce neuroprotective effects and its related molecular mechanism after brain ischemia reperfusion in rats. 36 Sprague-Dawley rats were randomly divided into sham group, ischemia reperfusion group and ischemia reperfusion hydrogen therapy group. Brain ischemia reperfusion injury was induced by a 2-hour left middle cerebral artery occlusion (MCAO) using an intraluminal filament, followed by 24 hours of reperfusion. Subsequently, histological alternations, levels of malondialdehyde (MDA) and reactive oxygen species (ROS), apoptosis, inflammatory cytokines and the protein expression in brain were examined. The present results showed that the treatment of hydrogen could significantly attenuate brain tissue apoptosis, decreased the levels of ROS and MDA, activation the Nrf-2/HO-1 pathway in brain tissue after ischemia reperfusion injury. The results suggested that the neuroprotective effects of hydrogen maybe mediated via activation of Nrf-2/HO-1 pathway.

Keywords: Hydrogen, Nrf-2/HO-1 pathway, cerebral ischemia reperfusion, rats

Introduction

Stroke is the most frequent cause of permanent disability and the major cause of death in adults worldwide. The World Health Organization has reported that approximately 15 million people worldwide suffered from stroke annually [1]. The risk of ischemic stroke, occurs when a cerebral blood vessel is occluded or ruptured, contributing to a variety of acute and chronic diseases of the brain [2, 3]. Oxidative stress is well known to play a pivotal role in cerebral ischemia-reperfusion injury [4, 5]. And with the development of oxidative stress, endogenous antioxidant system of the body would be activated to inhibit oxidative stress. Nrf2 signaling is known to play a crucial role in the suppression of oxidative stress [6]. Nrf2 protects the cell against oxidative stress through ARE mediated induction of several phase 2 detoxifying and antioxidant enzymes, particularly the HO-1 [7-9]. The previous study reported Lipoxin A4 ameliorates cerebral ischaemia/reperfusion injury through upregulation of nuclear factor erythroid 2-related factor 2 [10].

Based the fact that oxidative damage plays a detrimental role in the pathophysiology of cerebral ischemia, it may be representing a therapeutic target. Therefore, a safe and effective therapy that can target oxidative stress for neuroprotection is urgently needed. Hydrogen, the simplest gas in nature, was recently reported as a therapeutic antioxidant through selectively reducing cytotoxic oxygen radicals [11].

However, the effects of hydrogen-rich saline in cerebral ischemia reperfusion and its mechanism in rat model has remain unknown. So the aims of this study were to investigate whether hydrogen contributes to protect brain and to evaluate the underlying molecular and cellular mechanisms.

Materials and methods

Preparation and preservation of hydrogen-rich saline

The hydrogen-rich saline was purchased from Second Military Medical University in Shanghai and was prepared according to the methods of a previous study [12]. Hydrogen-rich saline was stored under atmospheric pressure at 4°C in an aluminum bag without dead volume. Hydrogen-rich saline was freshly prepared on a weekly basis to ensure a constant concentration of > 0.6 mmol/l [13].

Experiment design

Adult male Sprague-Dawley Rats (weighing 280-320 g) used in this study were obtained from the Experimental Animal Center of Shandong University (Jinan, China) of China. The protocol involving animal experiments were approved by the Ethics Committee of the Bin Zhou Medical College and performed in accordance with the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

After a 1-week acclimation period, 36 rats were randomly divided into three groups of 12 rats each group. The animals were anesthetized with 3.5% chloral hydrate solution (1 ml/100 g, i.p.). Middle cerebral artery occlusion (MCAO) was induced by intraluminal filament method as described previously [14]. Briefly, the left common carotid artery (CCA) and the external carotid artery (ECA) were exposed. Then, a 3-0 surgical monofilament nylon suture was carefully inserted from the external carotid artery into the internal carotid artery (ICA) and was advanced towards to occlude the origin of the left middle cerebral artery (MCA) until a light resistance was felt (18-20 mm from CCA bifurcation). After 2 h of MCAO, the nylon suture was withdrawn, followed by 24 h of reperfusion. 12 rats were treated with hydrogen-rich saline (1 ml/kg) after the beginning of reperfusion. 12 (1 ml/kg) treated animals were used as control. Another 12 sham-operated animals were also used. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Binzhou Medical College, China.

The measurement of reactive oxygen species (ROS) and Malondialdehyde (MDA)

To determine the levels of ROS and MDA, the brain tissues were isolated at 24 h after reperfusion. A 10% (w/v) homogenate was prepared in ice-cold saline. The homogenates were centrifuged at 3,000 g for 15 min at 4°C. The supernatant was used for bioassays as the methods provided by the assay kits (Jiancheng, China). The assay results were normalized by protein concentration in each sample. The activity of ROS was determined using a detection kits as manual protocol. Optical density was determined by spectrometer at 550 nm and then the ROS activity reported as U/mg protein. The content of MDA was measured with a thiobarbituric acid (TBA) test. MDA could react with TBA to formation the red adduct (MDA-TBA). MDA-TBA has a maximum absorption at 532 nm, thus can be detected by a colorimetric method.

Histopathological investigation

Isolated ischemic cerebral cortex were fixed in 10% methanol, embedded with paraffin, and cut into 4-μm thick sections. Stained with hematoxylin and eosin (HE) staining method and observed under light microscope (OlympusX71-F22PH, Japan).

Determination of apoptotic cells

The ischemic cerebral cortex fixed in 10% formalin for 48 h and embedded in paraffin. Tissues were sectioned at a thickness of 4 μm, deparaffinized, TUNEL staining was completed by using an in situ cell death detection kit (Roche, Indianapolis, IN) according to the manuscript protocol. The extent of brain damage was evaluated by the apoptotic index, which represented the average percentage of TUNEL-positive cells in each region counted in 10 fields.

Determination of IL-1β and TNF-α levels

Total protein was determined using a bicinchoninic acid assay kit (Pierce Biochemicals). The levels of inflammatory cytokines of the brain tissue were quantified using Enzyme-linked immunosorbent assay kits specific for rats according to the manufacturer instructions

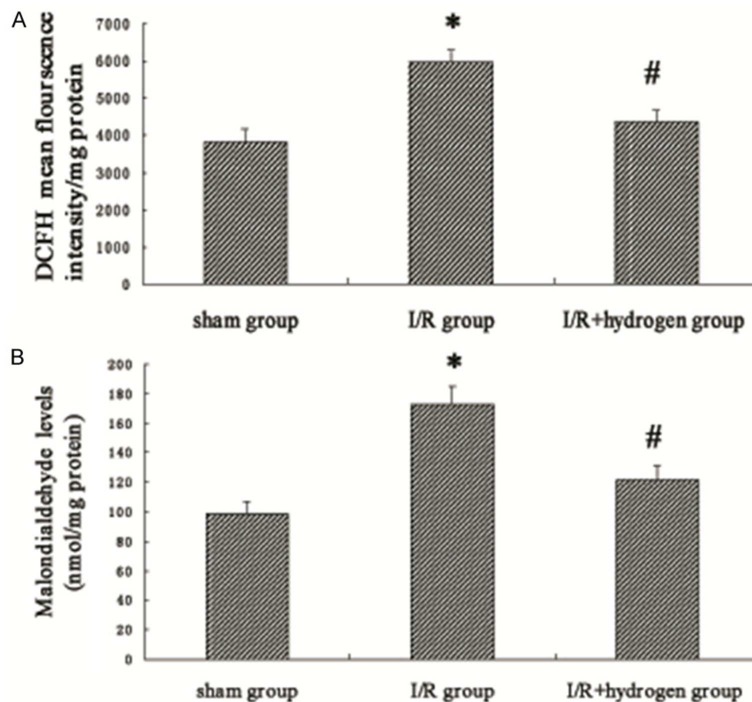

Figure 1. Antioxidant effects of hydrogen in MCAO rats. The levels of ROS and MDA increased in model group than that in sham group. Treatment with hydrogen decreased ROS and MDA levels compared with those in model group. Data are expressed as mean ± SD, **P* < 0.05, versus sham group, #*P* < 0.05, versus model group.

(TNF-α and IL-1β from Jiancheng Bioengineering institution, Nanjing). The inflammatory cytokines contents were expressed as picto-gram per milligram protein.

Western blotting

The protein extracted using special extraction kit (Keygen biotech, china). Equal amounts of lysate proteins (30 μg) were loaded onto SDS-polyacrylamide gels (15% separation gels) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% nonfat milk in TBST (tris-buffered saline containing 0.05% Tween-20) for 1 h at room temperature, the PVDF membrane was incubated with monoclonal antibodies against β-actin [1:1000, rabbit antibodies, Cell Signaling Technology (CST), USA], Nrf-2 [1:1000, rabbit antibodies, Cell Signaling Technology (CST), USA], HO-1 [1:1000, rabbit antibodies, Cell Signaling Technology (CST), USA] overnight at 4°C, respectively, washed three times with TBST for 10 min each time, and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody [1:5000, Immunology Consultants Laboratory (ICL), USA] for 1 h at room temperature. After washing three times with TBST for 10 min each time, the antibody-bound proteins were detected with the ECL chemiluminescence reagent (Pierce, USA). Protein levels were calculated relative to that of β-actin. The images were analyzed in a blind fashion using the Image J software.

Statistical analysis

Data were expressed as mean ± SEM. Statistical analyses were carried out using a one-way analysis of variance as well as the least significant difference test, and *P* < 0.05 was considered statistically significant.

Results

Changes of ROS and MDA levels

MCAO-induced oxidative stress changes were shown in **Figure 1**. At 24 h after reperfusion, ROS and MDA levels were much higher than that in the sham group. Treatments with hydrogen decreased ROS and MDA levels compared with the sham group, which implied that hydrogen saline injection significantly suppressed oxidative stress in ischemic brain.

Changes of histopathological structure

Micrographs showed variable degrees of neuron injury in model group. The obvious changes appeared in the model group in which the neurons reduction with nuclei shrinkage and Nissl body's loss, and severe vacuolization had developed. Rare injury cells were found in the sham group and injured cells detected in the I/R group was significant higher than that in the sham group. The percentage of injured cells has no obvious change after hydrogen treatment (**Figure 2**).

Changes of apoptosis

The terminal deoxynucleotidyl transferase-mediateduridine 50-triphosphate-biotin nicks

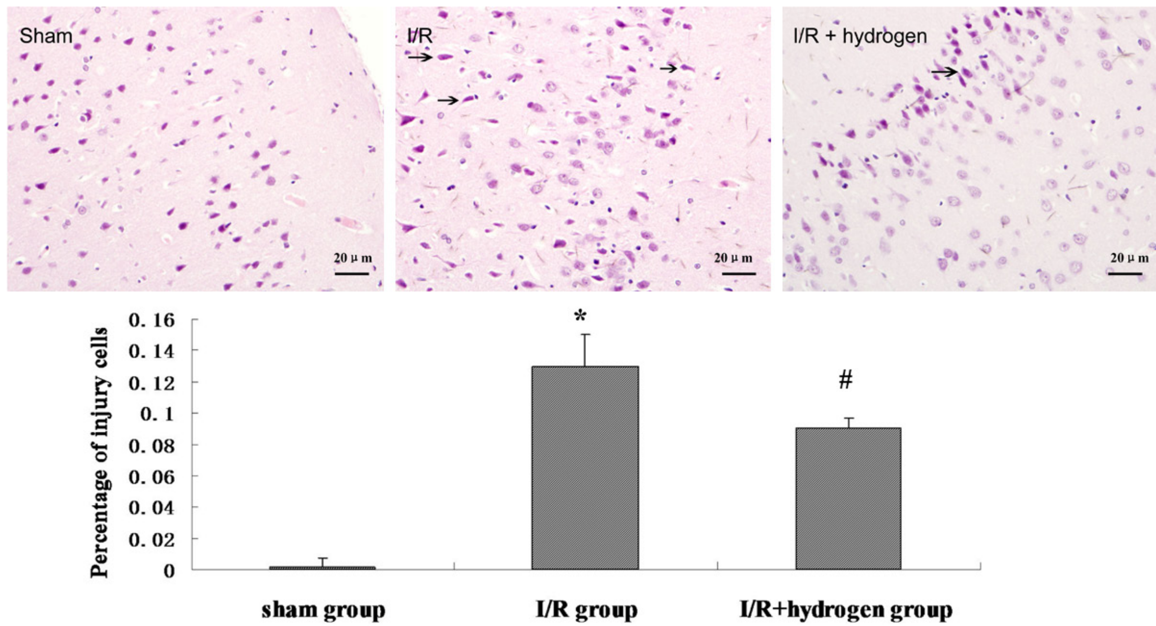

Figure 2. Histopathology staining in the ischemic brain area of rats. The shrinkage neurons with hyperchromatic nuclei and its cytoplasma deep dyed as the arrow denotes in I/R group and hydrogen treatment group (HE, 400×). Damaged neurons are shown with arrow. Scale bars = 20 μm. The number of injury cells was increased after MCAO. Data are expressed as mean ± SD, *represents significantly different when compared to sham group ($P < 0.05$).

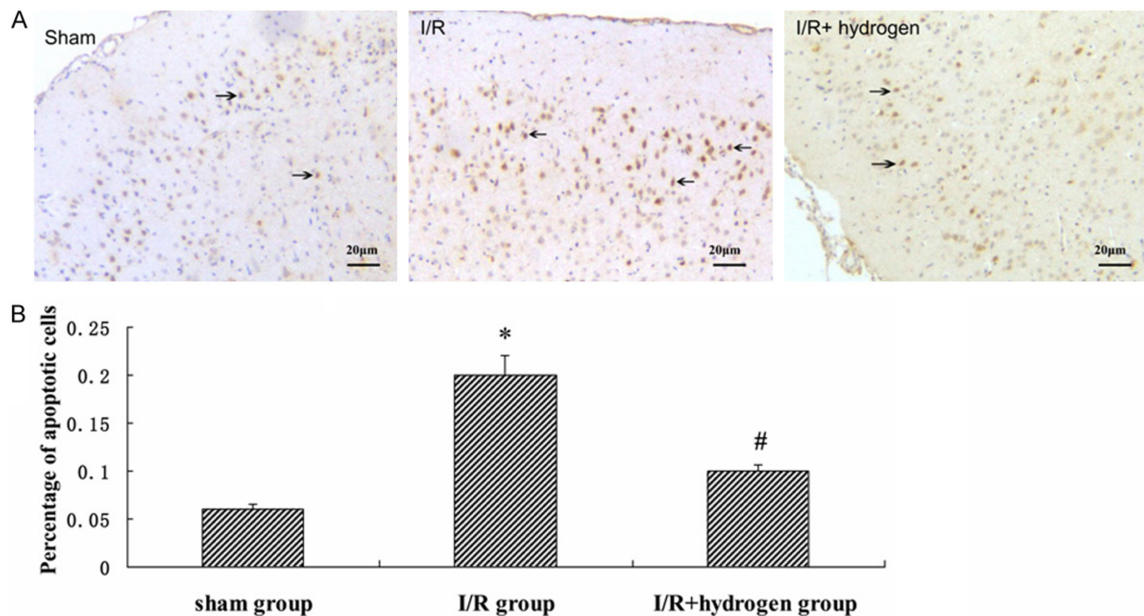

Figure 3. Representative photomicrographs of TUNEL in the cortex of rats at 24 h after MCAO. The number of positive TUNEL cells was increased after MCAO and reduced by hydrogen treatment. Data are expressed as mean ± SD, *represents significantly different when compared to sham group ($P < 0.05$), #represents significantly different when compared to I/R group ($P < 0.05$).

end-labeling (TUNEL) staining to investigate the potential effect of hydrogen on neural cell apoptosis in the cortex (**Figure 3**). Rare positive TUNEL cells were found in the sham group and

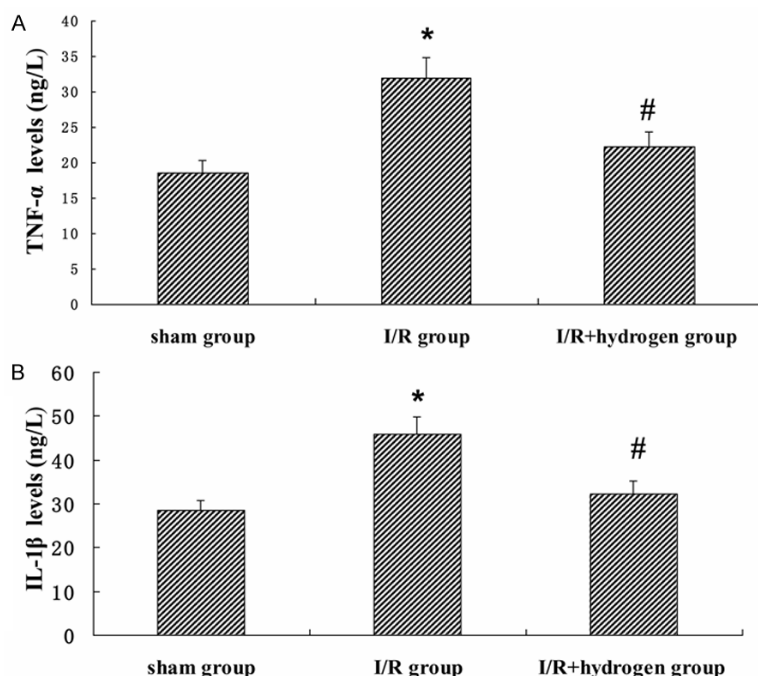

Figure 4. Effects of hydrogen on the levels of TNF-α and IL-β in the cortex of rats at 24 h after MCAO. The concentrations of TNF-α and IL-β were largely increased in modal group compared to that in the sham group and decreased after treating with hydrogen. Data are expressed as mean ± SD, *represents difference when compared to sham group ($P < 0.05$), #represents significantly different when compared to I/R group ($P < 0.05$).

apoptotic index detected in the I/R group was significant higher than that of rats in the sham group. The percentage of positive TUNEL cells was decreased after hydrogen treatment.

Changes of TNF-α and IL-1β levels

To investigate if hydrogen could inhibit inflammatory process after I/R, we used Enzyme-linked immunosorbent assay (ELISA) to measure the production of pro-inflammatory cytokines, such as TNF-α and IL-1β. As shown in **Figure 4**, we found the concentrations of TNF-α and IL-1β were largely increased after I/R compared to that in the sham group. However, they were dramatically decreased after treatment with hydrogen.

Changes of Nrf-2 and HO-1 expression

At 24 hours, the expression of Nrf-2 and HO-1 significantly increased in I/R group by comparing with sham group (**Figure 5**), and the hydrogen treatment further upregulated the protein level of Nrf-2 and HO-1 compared to I/R group.

Discussions

Many studies have investigated that ischemia reperfusion injury can lead to an extensive range of neurological impairments, including motor disabilities, autonomic dysfunction, and memory disorders [15, 16]. Although the exact mechanism of cerebral ischemia-reperfusion injury remains elusive, accumulating evidence has suggested that ROS produced by reperfusion after ischemia plays an important role in neuronal injury, oxidative stress which leads to ischemic cell death involves the formation of reactive oxygen species (ROS) through multiple injury mechanisms, such as mitochondrial inhibition and inflammation [17, 18]. On the basis of this, antioxidative agents have been demonstrated to be neuroprotective [19-21]. In this research, we showed that hydrogen could remarkably play a neuroprotective role through activation the Nrf-2/HO-1 pathway, attenuated oxidative stress, decreased inflammatory cytokines, reduced apoptotic cells and brain injuries.

Cells and tissues have highly developed endogenous antioxidant defense systems to counteract the oxidative stress generated in many diseases [22, 23]. In many cell types, numerous cellular responses to oxidative stress have been found to be involved in signaling proteins that act through the antioxidant response element, the nuclear factor erythroid 2-related factor 2 (Nrf2) [24], is a basic leucine zipper transcription factor, which transcriptionally regulates many genes including HO-1, c-glutamylcysteine synthase, and glutathione S-transferase [25]. HO-1 is a stress-responsive enzyme, responsible for the breakdown of heme to biliverdin, free iron and carbon monoxide [25, 26]. It is induced by a variety of cellular stresses, including heme, hyperoxia, hypoxia, and electrophiles [27].

Previous studies have suggested a positive correlation between HO-1 expression and neuro-

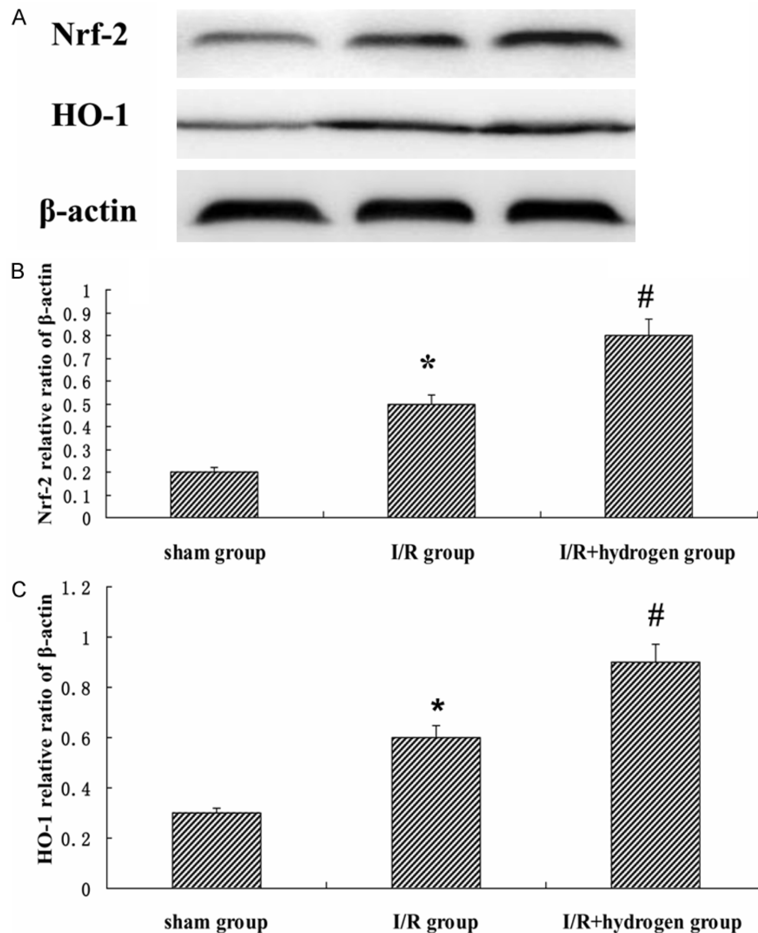

Figure 5. Western blot analysis of Nrf-2 and HO-1 in the cortex of rats at 24 h after MCAO. The protein levels were expressed as a ratio of the β-actin levels. The Nrf-2 and HO-1 protein levels were higher in modal group than that in the sham group. Treatment with hydrogen largely increased the protein level compared with that of rats in the modal group. Data are expressed as mean ± SD, *represents difference when compared to sham group ($P < 0.05$), #represents significantly different when compared to I/R group ($P < 0.05$).

protection in ischemic stroke. Some researchers have investigated that the activation of the Nrf2/HO-1 antioxidant pathway contributes to the protective effects after ischemia-reperfusion-indu-ced damage [28]. We analyzed expressions of Nrf2 and HO-1 in western blot analysis. The expressions of Nrf2 and HO-1 were increased significantly after ischemia-reperfusion injury.

The overexpression of HO-1 in the brain of mice confers neuroprotective effects against permanent middle cerebral artery occlusion (MCAO) and the induction of HO-1 by the pharmacological intervention protects the brain from ischemia-reperfusion injury [29]. Furthermore, pharmacological induction of HO-1 has been shown to protect the retina from acute glaucoma induced ischemia-reperfusion injury [30]. The production of both antioxidants by HO-1 may help increase the resistance of the ischemic brain to oxidative stress.

The close relationship between oxidative stress and cerebral ischemia has generated considerable interest in developing antioxidant therapies to combat ischemia induced damage [31]. On the basis of this evidence, strategies that are used to reduce oxidative neuronal injury should have beneficial effects in improving the outcomes of ischemic stroke. It should be pointed out that HO-1 can be induced by some flavonoids such as 7, 20-dihydroxy-8-hydroxyethyl-40-methoxyflavane-20-O-b-D-glucopyranoside [32] and hesperidin [33].

Hydrogen is considered to be a novel antioxidant as it inhibits inflammation, removes oxygen-derived free radicals and reduces oxidative damage [13]. In this study, we investigated that hydrogen could upregulate the expression of Nrf2 and HO-1, which means the activation of Nrf2/HO-1 pathway. The mechanism of the antioxidant effect of hydrogen in hemorrhagic shock may be associated with its anti-inflammatory effect. This hypothesis is supported by previous studies [34, 35] and the result of the present study that hydrogen decreased the levels of TNF-α and IL-1β.

In summary, this work demonstrated, for the first time, that hydrogen could protect neurons against brain ischemia followed by reperfusion through activation the Nrf2/HO-1 pathway.

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Disclosure of conflict of interest

None.

Address correspondence to: Jie Liu, Department of Neurology, The Fourth Hospital in Jinan, Jinan 250031, Shandong, PR China. E-mail: by-lszhang@hotmail.com

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