

## The protective effects of hydrogen on HO-1 expression in the brain after focal cerebral ischemia reperfusion in rats

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Received: 01.02.2015 • Accepted/Published Online: 28.11.2015 • Final Version: 17.11.2016

**Background/aim:** The aim of this study was to investigate whether a hydrogen administration can produce neuroprotective effects after brain ischemia reperfusion in rats.

**Materials and methods:** A brain ischemia reperfusion injury was induced by a 2-h left middle cerebral artery occlusion (MCAO) using an intraluminal filament, followed by 46 h of reperfusion. A hydrogen-rich saline (1 mL/kg body weight i.p.) was administered at the beginning of reperfusion. Saline (1 mL/kg)-treated animals were used as the control. Sham-operated animals were also used. Subsequently, 48 h after the MCAO, histological alternations, heme oxygenase-1 (HO-1) expression, and levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in the cerebral cortex and the hippocampus were examined.

**Results:** Hydrogen significantly alleviated brain tissue histological damage, promoted HO-1 expression, upregulated levels of SOD, and decreased the levels of MDA in brain tissue after the ischemia reperfusion injury.

**Conclusion:** The results suggest that the neuroprotective effects of hydrogen may be mediated by promoting HO-1 expression and attenuated the oxidative injury.

**Key words:** Hydrogen, heme oxygenase-1, brain, cerebral ischemia reperfusion, rats

### 1. Introduction

Strokes are a major cause of death and the most frequent cause of permanent disability in adults worldwide. According to the latest statistics, approximately 6.7 million people died from strokes around the world in 2012, and there are currently 33 million stroke survivors that need long-term follow-up and secondary preventive measures globally. (1). The risk of an ischemic stroke occurs when a cerebral blood vessel is ruptured or occluded, contributing to a variety of acute and chronic brain diseases (2,3). Therefore, a safe and effective therapy that can target multiple pathways for neuroprotection is urgently needed. Oxidative damage plays a detrimental role in the pathophysiology of cerebral ischemia and may represent a therapeutic target. Hydrogen, the most basic gas in nature, was recently reported as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals (4). However, there are no studies concerning the effects of hydrogen-rich saline on the expression of heme oxygenase-1 (HO-1)

in cerebral ischemia reperfusion in rats. The aim of this study was to demonstrate the antioxidative functions of hydrogen-rich saline on cerebral ischemia reperfusion and to discuss the mechanism of this process

### 2. Materials and methods

#### 2.1. Preparation and preservation of hydrogen-rich saline

The hydrogen-rich saline was purchased from the Second Military Medical University in Shanghai and was prepared according to the methods of a previous study (5). The hydrogen-rich saline was stored under atmospheric pressure at 4 °C in an aluminum bag without dead volume. It was freshly prepared on a weekly basis to ensure a constant concentration of >0.6 mmol/L (6).

#### 2.2. Cerebral ischemia reperfusion model and animal groups

Adult male Sprague-Dawley rats (weighing 280–320 g) obtained from the Experimental Animal Center of

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Shandong University, Jinan, China, were used in this study. The animals were anesthetized with a 3.5% chloral hydrate solution (1 mL/100 g, i.p.). Middle cerebral artery occlusion (MCAO) was induced by an intraluminal filament method as described previously (7). Briefly, the left common carotid artery (CCA) and the external carotid artery (ECA) were exposed. Following this, a 3-0 surgical monofilament nylon suture was carefully inserted from the external carotid artery into the internal carotid artery (ICA) and was advanced in a forward manner to occlude the origin of the left middle cerebral artery (MCA) until a light resistance was felt (18–20 mm from the CCA bifurcation). After 2 h of MCAO, the nylon suture was withdrawn, followed by 46 h of reperfusion. Twelve rats were treated with hydrogen-rich saline (1 mL/kg) after the start of the reperfusion. Twelve saline (1 mL/kg)-treated animals were used as the 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.

### 2.3. The measurement of superoxide dismutase (SOD) and malondialdehyde (MDA)

To determine the levels of SOD and MDA, the brain tissues were isolated 48 h after reperfusion. A 10% (w/v) homogenate was prepared in ice-cold saline. The homogenates were centrifuged at  $3000 \times g$  for 15 min at 4 °C. The supernatant was used for bioassays in line with the methods provided by the assay kits (Jiancheng, China). The assay results were normalized by a protein concentration in each sample. The activity of the SOD was determined using a detection kit following the manufacturer's protocol. Optical density was determined by spectrometer at 550 nm, and then the SOD activity was reported in U/mg protein. MDA was measured with a thiobarbituric acid (TBA) test. MDA reacts with TBA to form a red adduct (MDA-TBA). MDA-TBA has a maximum absorption at 532 nm; thus, it can be detected by a colorimetric method.

### 2.4. Histopathological investigation

The isolated ischemic cerebral cortex and hippocampus were fixed in 10% methanol, embedded with paraffin, and cut into 4- $\mu$ m-thick sections. Stained with a hematoxylin and eosin (H&E) staining method and observed under a light microscope (Olympus X71-F22PH, Japan), the percentage of injured neurons was calculated and analyzed.

### 2.5. Immunohistochemical staining

The ischemic cerebral cortex and hippocampus were fixed in 10% formalin for 48 h and embedded in paraffin. Tissues were sectioned at a thickness of 4  $\mu$ m, deparaffinized, and stained with an antirat HO-1 antibody (diluted 1:500), followed by a horseradish peroxidase-coupled antirabbit IgG antibody. Immunostaining was performed by 5 min of incubation with diaminobenzidine (DAB). PBS was used as the primary antibody in the negative control. The DAB

staining intensity was assessed with a microscopic image analysis system (GX51, Olympus, Japan).

### 2.6. Real-time reverse transcription polymerase chain reaction assay

Total RNA was isolated from the ischemic brain tissue using a TRIzol reagent and subsequently reverse-transcribed to cDNA using TaqMan Reverse Transcription Reagents. Real-time PCR was performed using SYBR Green PCR Master Mix reagent kits and the specific primers (Table). Data were analyzed using the comparative threshold cycle (Ct) method.

### 2.7. Statistical analysis

All of the data were analyzed using SPSS 16.0. Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. All of the values are expressed as mean  $\pm$  SEM. The results were considered significant at  $P < 0.05$ .

## 3. Results

### 3.1. Antioxidant effects of hydrogen

MCAO-induced antioxidant enzyme changes are shown in Figure 1. One-way ANOVA showed that at 48 h after reperfusion, SOD levels were much lower than that in the sham group ( $F = 4.358$ ,  $P = 0.026$ ), while MDA levels were higher ( $F = 3.488$ ,  $P = 0.034$ ). Treatments with hydrogen increased SOD levels ( $F = 4.760$ ,  $P = 0.013$ ) and decreased MDA levels ( $F = 4.865$ ,  $P = 0.015$ ) compared with the sham group, which shows that hydrogen saline injection significantly suppressed oxidative stress in ischemic brains.

### 3.2. Histopathological results of ischemic cerebral cortex and hippocampus

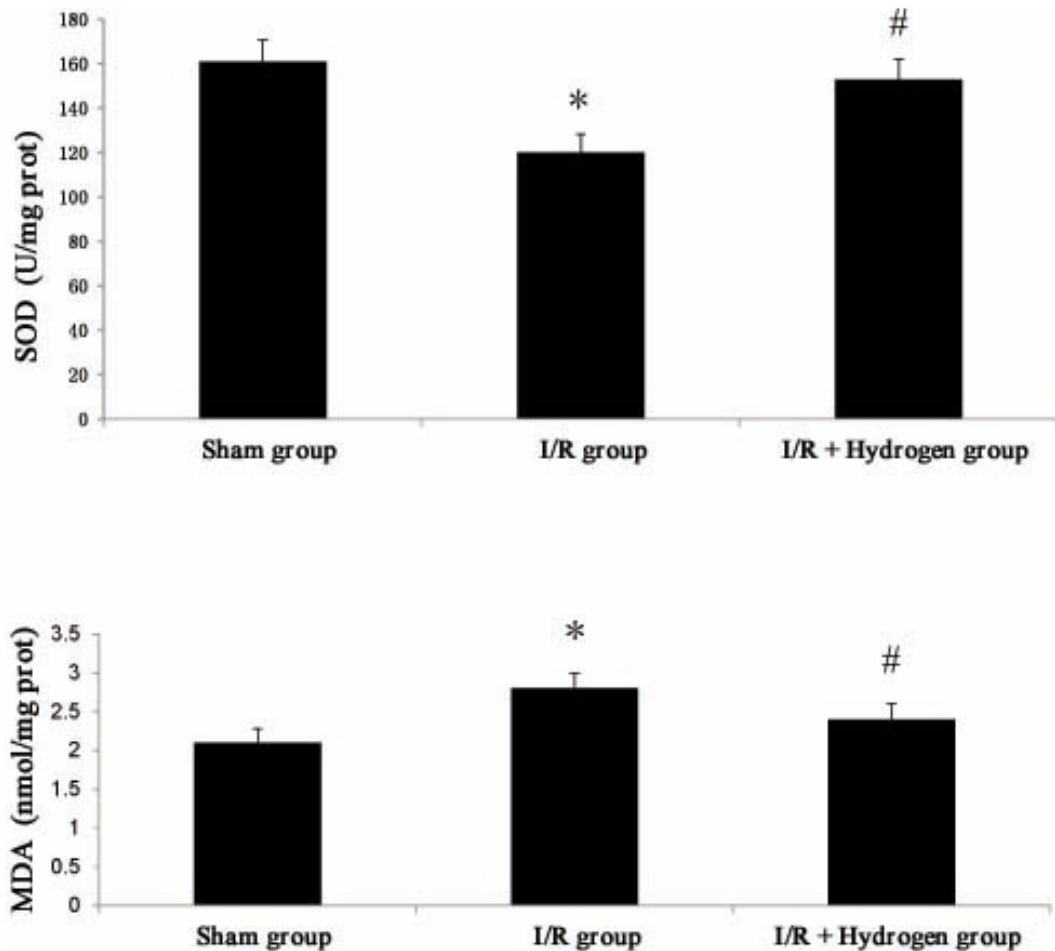
Micrographs showed variable degrees of neuron injury in the model group. The obvious changes appeared in the model group, in which neuron reduction with nuclei shrinkage, loss of Nissl bodies, and severe vacuolization had developed. The percentage of injured neurons in the hydrogen treatment group was significantly reduced ( $F = 3.759$ ,  $P = 0.028$ ) compared with the model group (Figure 2).

### 3.3. HO-1 expression by immunohistochemistry

HO-1 expression shows up as a brown staining in cells. One-way ANOVA showed that the HO-1 expressions in the model group increased more ( $F = 3.855$ ,  $P = 0.024$ ) than

**Table.** Primers of real-time PCR.

|                     | Gene primer pair (5–3)<br>F, forward; R, reverse |
|---------------------|--|
| Rat- $\beta$ -actin | F: AGAGGGAAATCGTGCGTGAC<br>R: CGATAGTGACCTGACCGT |
| Rat-HO-1            | F: TCCTCACACTCAGTTTCCT<br>R: GCATCTCCTTCCATTCCAG |



**Figure 1.** Antioxidant effects of hydrogen in MCAO rats. The levels of SOD decreased and MDA levels increased more in the model group than in the sham group. Treatment with hydrogen increased SOD levels and decreased MDA levels compared with those in the model group. \* $P < 0.05$  versus sham group, # $P < 0.05$  versus model group.

in the sham group. Moreover, they increased significantly higher ( $F = 4.722$ ,  $P = 0.013$ ) in the hydrogen group than in the model group, both in the ischemic cerebral cortex and the hippocampus (Figure 3).

#### 3.4. HO-1 mRNA expression by RT-PCR

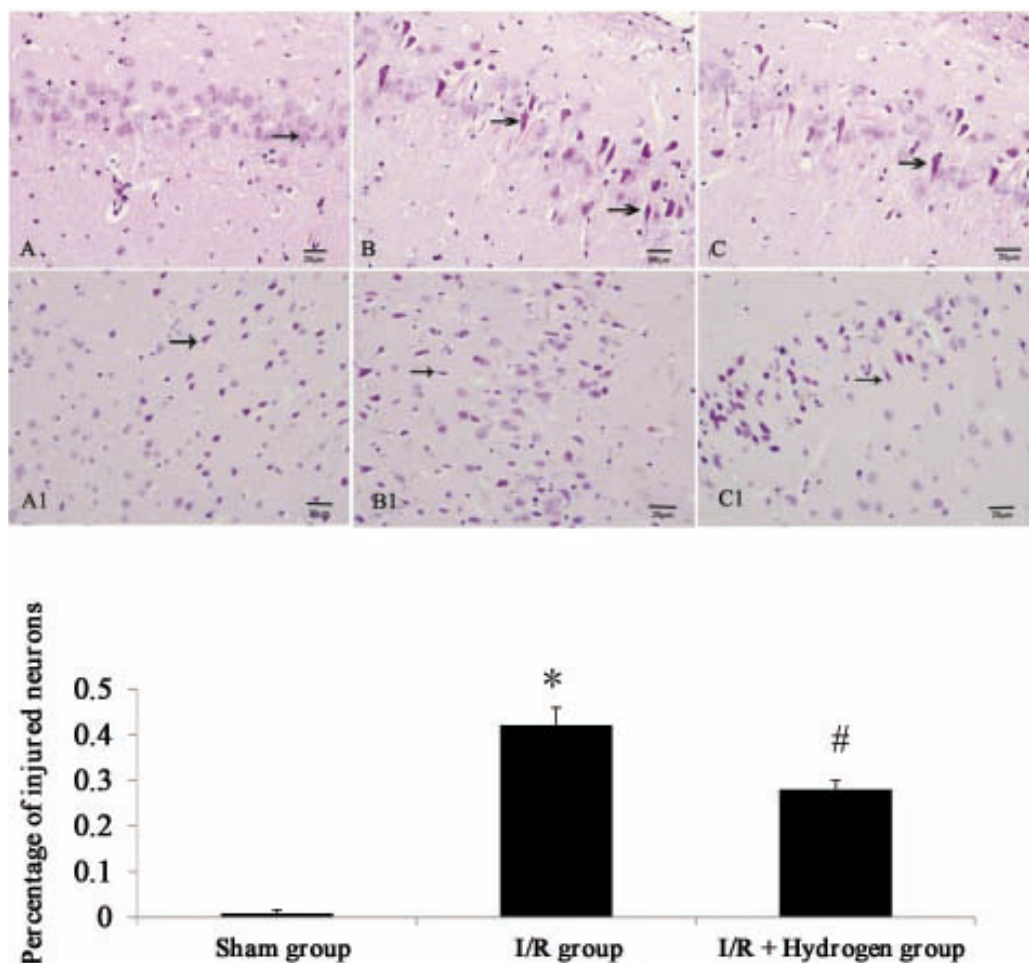
Real-time RT-PCR was performed to determine the relative levels of HO-1 mRNA transcriptions in the rat hippocampus tissue. As shown in Figure 4, the results showed weak HO-1 positive signals in the brain tissue in the sham group. In contrast, one-way ANOVA showed that a significant increase of the HO-1 mRNA transcriptions was found in the hydrogen group compared to the model group ( $F = 1.235$ ,  $P = 0.320$ ).

#### 4. Discussion

It is well known that oxidative metabolism is essential for the survival of brain neurons and, during these processes, large amounts of free radicals (reactive oxygen and nitrogen species, ROS) are produced (8). Normally,

there is a balance between the ROS generation and the endogenous antioxidant systems. Oxidative stress is a major cause of neuronal injuries induced by cerebral ischemia and reperfusion (9,10), which leads to ischemic cell death involving the formation of ROS through multiple injury mechanisms such as mitochondrial inhibition,  $Ca^{2+}$  overload, and inflammation (11,12). 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. In our results, increased MDA and decreased SOD in the model group (Figure 1) led to the injury of neurons both in the hippocampus and the cerebral cortex (Figure 2).

On the basis of this fact, antioxidative agents have been shown to be neuroprotective. Previous studies demonstrated that strategies used to reduce oxidative neuronal injury should have beneficial effects in improving the outcomes of ischemic stroke (13,14).



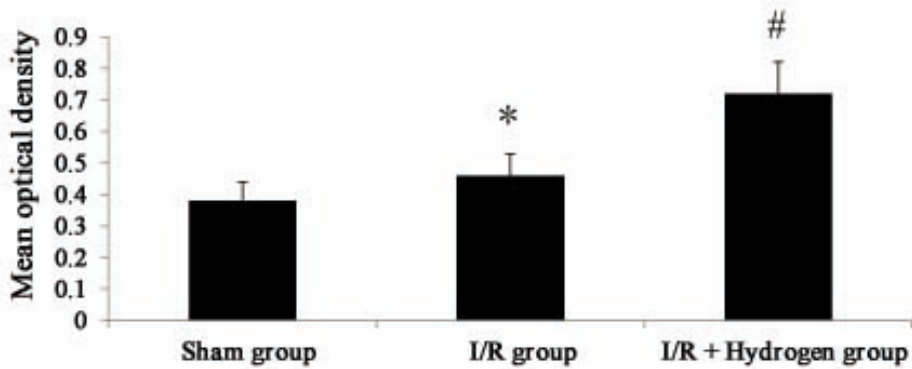
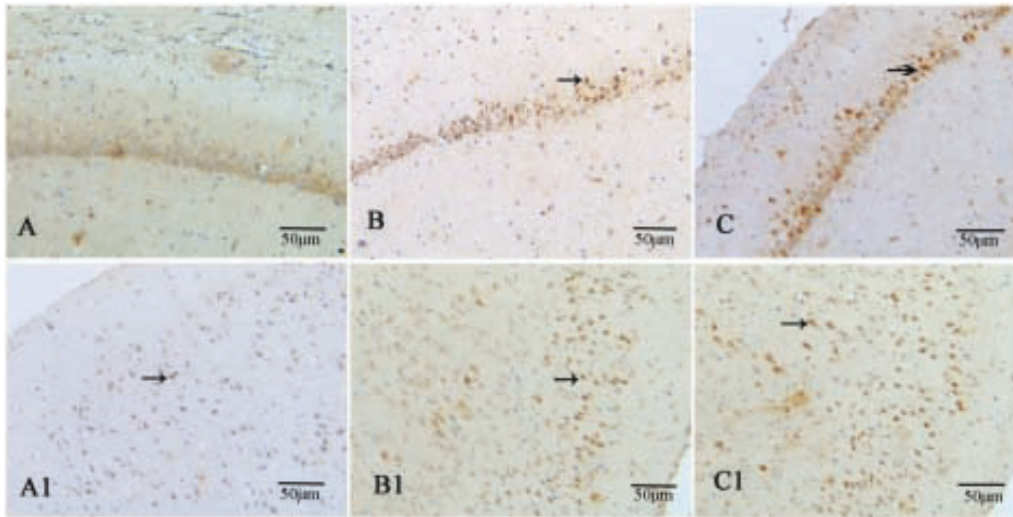
**Figure 2.** Histopathology staining in the ischemic brain area of rats. The hippocampus: A) sham group, B) I/R group, C) I/R group + hydrogen group. The cerebral cortex: A1) sham group, B1) I/R group, C1) I/R group + hydrogen group. Shrinkage of neurons with hyperchromatic nuclei and cytoplasm deeply dyed as the arrow denotes in the I/R group (B) and hydrogen treatment group (C) (400 $\times$ ). Normal neurons (A, A1) and damaged neurons (B, B1, C, C1) are shown with an arrow. Scale bars = 20  $\mu$ m. The percentage of injured neurons in the hydrogen treatment group was significantly reduced compared with the model group. \* $P < 0.05$  versus the sham group, # $P < 0.05$  versus the model group.

HO-1 is a phase II defense enzyme that possesses a potent antioxidative ability. This catalyzes the degradation of heme into carbon monoxide, biliverdin, and ferritin, and previous studies suggested a positive correlation between HO-1 expression and neuroprotection in ischemic strokes (15). The overexpression of HO-1 in the brain of mice produces neuroprotective effects against permanent MCAO, and the induction of HO-1 by pharmacological intervention protects the brain from ischemia-reperfusion injury (16). Some research has shown that the activation of the Nrf2/HO-1 antioxidant pathway contributes to the protective effects after ischemia reperfusion-induced damage (17). Furthermore, pharmacological induction of HO-1 has been shown to protect the retina from acute glaucoma-induced ischemia and reperfusion injury (18).

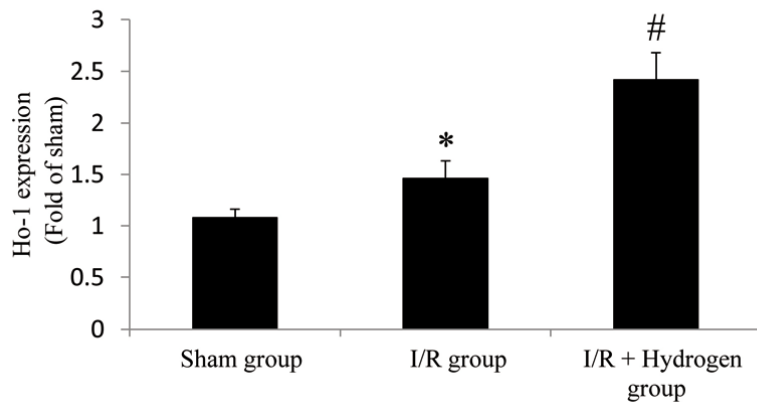
The production of both antioxidants by HO-1 may help increase the resistance of the ischemic brain to oxidative stress.

The close relationship between oxidative stress and cerebral ischemia has generated considerable interest in developing antioxidant therapies to combat ischemia-induced damage (19). It should be pointed out that HO-1 could be induced by some flavonoids such as 7,20-dihydroxy-8-hydroxyethyl-40-methoxyflavane-20-O-b-D-glucopyranoside (20) and hesperidin (21).

Hydrogen is considered to be a novel antioxidant as it inhibits inflammation, removes oxygen-derived free radicals, and reduces oxidative damage (6). We have demonstrated that hydrogen could play a significant antioxidative role by upregulating the levels of HO-1



**Figure 3.** Effects of hydrogen on the expression of HO-1 in brain and hippocampus. The hippocampus: A) sham group, B) I/R group, C) I/R + hydrogen group. The cerebral cortex: A1) sham group, B1) I/R group, C1) I/R + hydrogen group. Positive neurons are shown with an arrow. Scale bars = 50  $\mu$ m. Bar graphs represent the optical density of positive cells. \*: Significant difference when compared to the sham group ( $P < 0.05$ ), #: significant difference when compared to the I/R group ( $P < 0.05$ ).



**Figure 4.** Effects of hydrogen on the HO-1 mRNA transcriptions in brain tissue. The HO-1 mRNA transcriptions were increased in the I/R group and hydrogen treatment group. Meanwhile, compared with the I/R group, the signals were stronger in the hydrogen treatment group. \*: Significant difference when compared to the sham group ( $P < 0.05$ ), #: significant difference when compared to the I/R group ( $P < 0.05$ ).



mRNA and the expression of HO-1 (Figures 3 and 4). The mechanism of the antioxidative effect of hydrogen in hemorrhagic shock may be associated with its antiinflammatory effect. This hypothesis is supported by previous studies (22,23) and the results of the present study indicate that the neuron injury after cerebral ischemia and reperfusion was ameliorated because of the increased levels of HO-1 induced by hydrogen (Figure 2).

In summary, the present study suggests that MCAO-induced oxidative stress followed by upregulated MDA

levels and downregulated SOD levels lead to the injury of neurons in the hippocampus and cerebral cortex. However, hydrogen has an antioxidative role and neuron-protective effects by upregulating the levels of HO-1.

### Acknowledgments

This study was supported by the Natural Science Foundation of Shandong Province (No. ZR2011HQ048) and the Yantai Science and Technology Development Plan (No. 2011075).

### References

- Mendis S, Davis S, Norrving B. Organizational Update: The World Health Organization Global Status Report on Noncommunicable Diseases 2014; One more landmark step in the combat against stroke and vascular disease. *Stroke* 2015; 46: e121-e122.
- Hankey GJ. Potential new risk factors for ischemic stroke: what is their potential?. *Stroke* 2006; 37: 2181-2188.
- Brima T, Otáhal J, Mareš P. Increased susceptibility to pentetrazol-induced seizures in developing rats after cortical photothrombotic ischemic stroke at P7. *Brain Res* 2013; 1507:146-153.
- Zhai X, Chen X, Shigeo Ohta S, Sun X. Review and prospect of the biomedical effects of hydrogen. *Med Gas Res* 2014; 4: 19.
- Mao YF, Zheng XF, Cai JM, You XM, Deng XM, Zhang JH, Jiang L, Sun XJ. Hydrogen-rich saline reduces lung injury induced by intestinal ischemia/reperfusion in rats. *Biochem Biophys Res Commun* 2009; 381: 602-605.
- Du Z, Jia H, Liu J, Zhao X, Wang Y, Sun X. Protective effects of hydrogen-rich saline in uncontrolled hemorrhagic shock. *Exp Ther Med* 2014; 7: 1253-1258.
- Ford G, Xu Z, Gates A, Jiang J, Ford BD. Expression Analysis Systematic Explorer (EASE) analysis reveals differential gene expression in permanent and transient focal stroke rat models. *Brain Res* 2006; 1071: 226-236.
- Doyle KP, Simon RP, Stenzel-Poore MP. Mechanisms of ischemic brain damage. *Neuropharmacology* 2008; 55: 310-318.
- Allen CL, Bayraktutan U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. *Int J Stroke* 2009; 4: 461-470.
- Crack PJ, Taylor JM. Reactive oxygen species and the modulation of stroke. *Free Radic Biol Med* 2005; 38: 1433-1444.
- Coyle JT, Puttfarcken P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 1993; 262: 689-695.
- Ewen A, Matz P, Chan PH. Free radical pathways in CNS injury. *J Neurotrauma* 2000; 17: 871-890.
- Cheng CY, Ho TY, Lee EJ, Su SY, Tang NY, Hsieh CL. Ferulic acid reduces cerebral infarct through its antioxidative and anti-inflammatory effects following transient focal cerebral ischemia in rats. *Am J Chin Med* 2008; 36: 1105-1119.
- Ma S, Yin H, Chen L, Liu H, Zhao M, Zhang X. Neuroprotective effect of ginkgolide K against acute ischemic stroke on middle cerebral ischemia occlusion in rats. *J Nat Med* 2012; 66: 25-31.
- Siow RC, Sato H, Mann GE. Heme oxygenase-carbon monoxide signaling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? *Cardiovasc Res* 1999; 41: 385-394.
- Lin TN, Cheung WM, Wu JS, Chen JJ, Lin H, Chen JJ, Liou JY, Shyue SK, Wu KK. 15d-prostaglandin J2 protects brain from ischemia-reperfusion injury. *Arterioscler Thromb Vasc Biol* 2006; 26: 481-487.
- He M, Pan H, Chang RC, So KF, Brecha NC, Pu M. Activation of the Nrf2/HO-1 antioxidant pathway contributes to the protective effects of Lycium barbarum polysaccharides in the rodent retina after ischemia-reperfusion-induced damage. *PLoS One* 2014; 9: e84800.
- Sun MH, Pang JH, Chen SL, Han WH, Ho TC, Chen KJ, Kao LY, Lin KK, Tsao YP. Retinal protection from acute glaucoma-induced ischemia-reperfusion injury through pharmacologic induction of heme oxygenase-1. *Invest Ophthalmol Vis Sci* 2010; 51: 4798-4808.
- Simonyi A, Wang Q, Miller RL, Yusof M, Shelat PB, Sun AY, Sun GY. Polyphenols in cerebral ischemia: novel targets for neuroprotection. *Mol Neurobiol* 2005; 31: 135-147.
- Park JH, Lee DY, Yun P, Yeon SW, Ko JH, Kim YS, Baek NI. Flavonoids from silkworm droppings and their promotional activities on heme oxygenase-1. *J Asian Nat Prod Res* 2011; 13: 377-382.
- Chen MC, Ye YY, Ji G, Liu JW. Hesperidin upregulates heme oxygenase-1 to attenuate hydrogen peroxide-induced cell damage in hepatic L02 cells. *J Agric Food Chem* 2010; 58: 3330-3335.
- Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007; 13: 688-694.
- Qian L, Cao F, Cui J, Huang Y, Zhou X, Liu S, Cai J. Radio protective effect of hydrogen in cultured cells and mice. *Free Radic Res* 2010; 44: 275-282.