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Beneficial effects of hydrogen gas in a rat model of traumatic brain injury via reducing oxidative stress

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**ABBREVIATIONS**----BBB, blood-brain barrier; CAT, catalase; CCI, controlled cortical impact; EB, Evans blue; H\(_2\), hydrogen gas; H\(_2\)O\(_2\), hydrogen peroxide; 8-iso-PGF\(_{2\alpha}\), 8-iso-prostaglandin F\(_{2\alpha}\); MDA, malondialdehyde; •OH: hydroxyl radicals; ROS, reactive oxygen species; SOD, superoxide dismutase; TBI, traumatic brain injury.
ABSTRACT

Traumatic brain injury (TBI) is a leading cause of mortality and disability among the young population. It has been shown that hydrogen gas (H₂) exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radical (•OH, the most cytotoxic ROS). Recently, we have found that H₂ inhalation significantly improved the survival rate and organ damage of septic mice. In the present study, we investigated the effectiveness of H₂ therapy on brain edema, blood-brain barrier (BBB) breakdown, neurological dysfunction and injury volume in TBI-challenged rats. In addition, we investigated the effects of H₂ treatment on the changes of oxidative products and antioxidant enzymes in brain tissue of TBI-challenged rats. Hydrogen treatment was given by exposure to 2% H₂ from 5 minutes to 5 hours after sham or TBI operation, respectively. Here, we found that TBI-challenged rats showed significant brain injuries characterized by the increase of BBB permeability, brain edema and lesion volume as well as neurological dysfunction, which was significantly attenuated by 2% H₂ treatment. In addition, we found that the decrease of oxidative products and the increase of endogenous antioxidant enzymatic activities in brain tissue may be associated with the protective effects of H₂ treatment in TBI-challenged rats. The present study supports that H₂ inhalation may be a more effective therapeutic strategy for patients with TBI.

Research highlights:

1. H₂ inhalation has a beneficial effect on TBI in a concentration-dependent manner.

3. 2% H₂ inhalation improves neurological dysfunction of TBI rats.

4. The protective effects are associated with the decrease of oxidative products.

**Keywords:** Traumatic brain injury (TBI); reactive oxygen species (ROS); oxidative stress; hydrogen gas
1. Introduction

Traumatic brain injury (TBI) is a leading cause of mortality and disability all over the world, particularly among the young population (Elliott et al., 2009; Langlois et al., 2006). TBI results in an estimated 1.4 million deaths, hospitalizations, and emergency department visits annually in the United States with an estimated annual cost of $60 billion (Langlois et al., 2006). More importantly, it may lead to permanent functional deficits due to both primary and secondary damages (Davis, 2000). Currently, approximately 5.3 million Americans are living with disabilities related to TBI (Narayan et al., 2002). In spite of extensive improvements in medical interventions, unfortunately, there are still no clinical neuroprotective agents available for patients with TBI (McAllister, 2009).

Numerous human and animal studies have shown that excessive production of reactive oxygen species (ROS) and reduction of antioxidant defense systems play an important role in the pathogenesis of TBI (Homsi et al., 2009; Marklund et al., 2001a; Marklund et al., 2001b). Recently, more and more researchers have found that H₂ exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radical (•OH, the most cytotoxic ROS) and effectively protects against organ damage such as transient cerebral ischemia, neonatal cerebral hypoxia-ischemia, liver injury, lung injury, renal injury and myocardial injury induced by ischemia/reperfusion (Cai et al., 2008; Fukuda et al., 2007; George and Agarwal, 2010; Hayashida et al., 2008; Ohsawa et al., 2007; Ohta, 2008). Furthermore, some studies have found that hydrogen-rich saline or hydrogen-rich water also can reduce lung injury, intestinal
injury and brain injury induced by ischemia/reperfusion through reducing oxidative stress (Cai et al., 2009; Mao et al., 2009; Sato et al., 2008; Zheng et al., 2009). Our recent studies have shown that H\textsubscript{2} inhalation significantly improved the survival rate and multiple organ damage of septic mice in a concentration- and time-dependent manner (Xie et al., 2010; Xie et al., 2010). Furthermore, we have found that the beneficial effects of H\textsubscript{2} treatment on sepsis and sepsis-associated organ damage were associated with the decreased levels of oxidative products and the increased activities of antioxidant enzymes in serum and tissues (Xie et al., 2010; Xie et al., 2010). These findings strongly indicate that H\textsubscript{2} treatment may be beneficial to TBI.

To the best of our knowledge, no studies have investigated the potential effects of H\textsubscript{2} on TBI. Therefore, we hypothesized that H\textsubscript{2} treatment might attenuate TBI by reducing oxidative stress. The purpose of this study was to investigate the effectiveness of H\textsubscript{2} therapy on brain edema, blood-brain barrier (BBB) breakdown, neurological dysfunction and lesion volume after controlled cortical impact (CCI), a well-characterized model of focal TBI resembling many aspects of TBI in patients (Hall et al., 2008; Longhi et al., 2001; Xiong et al., 2007). In addition, we investigated the effects of H\textsubscript{2} treatment on changes of oxidative products and antioxidant enzymes in animals with TBI.
2. Results

2.1 Physiological parameters

Based on our previous studies (Xie et al., 2010; Xie et al., 2010) and the result of Supplementary Fig. 1, the present study was designed to investigate the effects of 2% H₂ inhalation on TBI-challenged rats. In the present study, we firstly investigated the effects of H₂ inhalation on arterial pH, PaO₂, PaCO₂ and blood glucose in rats with TBI or sham operation during the treatment. Hydrogen treatment was given by exposure to 2% H₂ from 5 minutes to 5 hours after sham or TBI operation, respectively. The arterial blood gas and blood glucose were conducted at pre-operation state, 60 minutes after operation as well as 300 minutes after operation, respectively. No significant differences in pH, PaO₂, PaCO₂ or blood glucose were observed among all groups during the treatment (Table 1). The results demonstrate that H₂ inhalation at a 2% concentration has no significant effects on arterial blood gas and blood glucose in rats with or without TBI during the treatment.

2.2 BBB integrity

BBB integrity was determined by Evans blue (EB) extravasation at 6 h after TBI operation. It has been previously shown that this is the point of maximum BBB permeability in this model of TBI (Barzo et al., 1996; Beaumont et al., 2000). TBI caused a significant increase of EB extravasation in the injured ipsilateral cortex (P < 0.05 vs. Sham group, n = 6 per group, Fig. 1), indicating that TBI can cause damage to BBB integrity and increase the BBB permeability. However, H₂ treatment significantly reduced EB extravasation in the injured ipsilateral cortex (P < 0.05 vs.
TBI group, n = 6 per group, Fig. 1). The results demonstrate that TBI can cause damage to BBB integrity, which was significantly attenuated by H\(_2\) treatment.

2.3 Brain edema

Brain water content as an indicator of brain edema was measured at 24 h after injury (Dogan et al., 1997). TBI caused a significant increase in the percentage of water content in the injured ipsilateral cortex \((P < 0.05\) vs. Sham group, n = 6 per group, Fig. 2). Treatment with H\(_2\) inhalation significantly reduced the percentage of water content in the injured ipsilateral cortex \((79.18 \pm 1.56\%\) and \(83.02 \pm 1.61\%,\) respectively, n = 6 per group, \(P < 0.05,\) Fig. 2). The results suggest that H\(_2\) treatment can significantly attenuate the development of TBI-induced brain edema.

2.4 Motor performance

2.4.1 Beam-balance

Beam-balance task was utilized to assess gross motor function. All groups were able to balance on the beam for the designated 60 seconds on each of three trials prior to surgery (Fig. 3). The animals in the TBI group showed significantly worse performance on the first day after operation when compared to pre-surgery baseline or Sham group, but improved over time and recovered by the end of the behavioral testing regimen (Fig. 3). Bonferroni post hoc analysis revealed significant differences between Sham group and TBI group \((P < 0.05,\) n = 6 per group, Fig. 3). Additionally, treatment with H\(_2\) inhalation significantly improved the bad performance of animals with TBI \((P < 0.05,\) n = 6 per group, Fig. 3). The results indicate that TBI can cause significant deficit in gross motor function, which is improved markedly by H\(_2\).
2.4.2 Beam-walk

Beam-walk task was utilized to assess fine motor function. There were no significant differences in time to traverse the beam among groups prior to operation ($P > 0.05$). As depicted in Fig. 3, TBI animals showed greater traversal time (i.e., more impairment) during the behavioral testing regimen when compared with Sham animals ($P < 0.05$, $n = 6$ per group, Fig. 3). However, 2% H$_2$ treatment significantly decreased the time to traverse the beam in animals with TBI ($P < 0.05$, $n = 6$ per group, Fig. 3). The results indicate that TBI can cause significant deficit in fine motor function, which is improved markedly by H$_2$ treatment.

2.5 Lesion volume

Cerebral lesion volume was determined using cresyl violet after the last behavioral test (i.e., on the 5$^{th}$ day after operation). TBI caused a significant increase in the percentage of lesion volume when compared with Sham group ($P < 0.05$, $n = 6$ per group, Fig. 4). However, treatment with H$_2$ inhalation significantly reduced the percentage of lesion volume in animals with TBI ($P < 0.05$, $n = 6$ per group, Fig. 4). Furthermore, we also investigated the effects of 2% H$_2$ inhalation on mild and severe TBI rats. H$_2$ treatment significantly decreased the lesion volume of mild TBI rats ($P < 0.05$, $n = 6$ per group, Supplementary Fig. 2). However, 2% H$_2$ treatment decreased the lesion volume of severe TBI animals with no statistically significant differences ($P > 0.05$, $n = 6$ per group, Supplementary Fig. 2). The results suggest that H$_2$ treatment can significantly attenuate the mild and moderate TBI-induced cerebral...
lesion volume.

2.6 Antioxidant enzymes

We detected the activities of superoxide dismutase (SOD) and catalase (CAT) in the injured ipsilateral cortex at pre-operation state as well as at 6 h, 24 h, 48 h and 72 h after TBI or sham operation, respectively. The results showed that the activities of SOD and CAT were slightly elevated at 6 h after TBI operation ($P > 0.05$, TBI group vs. Sham group), but decreased significantly from 24 h to 72 h after TBI operation ($P < 0.05$, TBI group vs. Sham group, $n = 6$ per group, Fig. 5). Compared with TBI group, $H_2$ treatment significantly increased the activities of SOD and CAT from 6 h to 72 h after TBI operation ($P < 0.05$, $n = 6$ per group, Fig. 5). The above results suggest that TBI can cause the decrease of SOD and CAT activities in the injured ipsilateral cortex, which can be significantly improved by $H_2$ treatment.

2.7 Oxidative products

We also detected the levels of malondialdehyde (MDA) and 8-iso-prostaglandin F2α (8-iso-PGF2α) in the injured ipsilateral cortex at pre-operation state as well as at 6 h, 24 h, 48 h and 72 h after TBI or sham operation, respectively. The data showed that the level of MDA were significantly elevated from 24 h to 72 h after TBI operation ($P < 0.05$, TBI group vs. Sham group, $n = 6$ per group, Fig. 6). However, the level of 8-iso-PGF2α were significantly elevated from 6 h to 72 h after TBI operation ($P < 0.05$, TBI group vs. Sham group, $n = 6$ per group, Fig. 6). Compared with TBI group, $H_2$ treatment significantly attenuated the increase of MDA and 8-iso-PGF2α levels in animals with TBI ($P < 0.05$, $n = 6$ per group, Fig. 6). The above results suggest that
TBI can cause the increase of MDA and 8-iso-PGF2α levels in the injured ipsilateral cortex, which can be significantly attenuated by H₂ treatment.

3. Discussion

In the present study, we found that 2% H₂ inhalation from 5 minutes to 5 hours after TBI operation significantly attenuated the increase of BBB permeability, brain edema and lesion volume in TBI-challenged rats. Furthermore, 2% H₂ treatment also significantly improved TBI-induced neurological dysfunction. These results suggest that 2% H₂ treatment can produce a beneficial effect on TBI in rats. In addition, we found that the beneficial effects of H₂ treatment on TBI were associated with the decreased levels of oxidative products (8-iso-PGF2α and MDA) and increased activities of antioxidant enzymes (SOD and CAT) in brain tissues.

A controlled cortical impact (CCI) model as a well-characterized model of focal TBI is used extensively to study cellular and molecular changes in injured tissue during and after trauma in mice and rats (Hall et al., 2008; Longhi et al., 2001; Xiong et al., 2007). Accordingly, using a rat model of CCI-induced TBI, we investigated the protective effects of 2% H₂ treatment and its associated mechanisms. In the present study, we found that TBI-challenged rats showed significant brain injuries characterized by the increase of BBB permeability, brain edema and lesion volume as well as neurological dysfunction, which is similar to previous studies (Elliott et al., 2009; Yu et al., 2009; Zhang et al., 2009).

TBI is a leading cause of mortality and disability in the young population. In spite of the extensive efforts to understand the pathophysiological mechanisms
underlying the primary and secondary lesions following TBI, no clinical neuroprotective treatment is still available (McAllister, 2009). A growing number of studies have found that excessive production of ROS and reduction of antioxidant defense systems play an important role in the pathogenesis of TBI (Homsi et al., 2009; Marklund et al., 2001a; Marklund et al., 2001b). In excess, ROS and their by-products could exacerbate brain damage and thus overall clinical outcome (Liu et al., 2008). It is well known that ROS include many types such as superoxide anion, hydroxyl radicals (•OH), hydrogen peroxide (H$_2$O$_2$), and so on (Ohsawa et al., 2007).

Despite their cytotoxic effects, superoxide anion and H$_2$O$_2$ play an important physiological role at low concentration: they function as regulatory signaling molecules that are involved in numerous signal transduction cascades and regulate biological processes such as apoptosis, cell proliferation and differentiation (Ohsawa et al., 2007; Sauer et al., 2001). At higher concentrations, H$_2$O$_2$ is converted into hypochlorous acid by myeloperoxidase; hypochlorous acid defends against bacterial invasion (Winterbourn, 2002). In addition, some endogenous antioxidant enzymes can scavenge H$_2$O$_2$ and superoxide anion in vivo (Xie et al., 2010). However, •OH is the strongest of the oxidant species and reacts indiscriminately with nucleic acids, lipids and proteins (Ohsawa et al., 2007). More importantly, there is no known detoxification system for •OH in vivo (Ohsawa et al., 2007). Therefore, scavenging •OH is a critical antioxidant process, which may be a good and critical measure for treating TBI.

Interestingly, recent studies demonstrate that H$_2$ exerts a therapeutic antioxidant
activity by selectively reducing hydroxyl radicals (•OH, the most cytotoxic ROS) and effectively protected against organ damage such as transient cerebral ischemia, neonatal cerebral hypoxia-ischemia, renal injury, liver injury, lung injury and myocardial injury induced by ischemia/reperfusion, suggesting that H\textsubscript{2} has a potential role as an antioxidant for preventive and therapeutic applications (Cai et al., 2008; Fukuda et al., 2007; George and Agarwal, 2010; Hayashida et al., 2008; Ohsawa et al., 2007; Ohta, 2008). Furthermore, some studies have found that hydrogen-rich saline or hydrogen-rich water also can reduce lung injury, intestinal injury and brain injury induced by ischemia/reperfusion through reducing oxidative stress (Cai et al., 2009; Mao et al., 2009; Sato et al., 2008; Zheng et al., 2009). Our recent studies has also shown that H\textsubscript{2} treatment significantly improves the long-term survival rate and multiple organ damage of moderately or severely septic mice in a concentration- and time-dependent manner (Xie et al., 2010; Xie et al., 2010). These findings strongly indicate that H\textsubscript{2} treatment maybe become a good measure for treating patients with TBI.

Based on our previous studies (Xie et al., 2010; Xie et al., 2010) and preliminary study (Supplementary Fig. 1), the present study was designed to investigate the effects of 2% H\textsubscript{2} inhalation from 5 minutes to 5 hours after TBI operation on TBI-challenged rats. Here, we found that 2% H\textsubscript{2} inhalation from 5 minutes to 5 hours after TBI operation significantly attenuated the increase of BBB permeability, brain edema and lesion volume of TBI-challenged rats. Furthermore, 2% H\textsubscript{2} treatment also significantly improved TBI-induced neurological dysfunction including gross and fine
motor function. However, we also investigated the effects of 2% H\textsubscript{2} inhalation on the lesion volume of mild and severe TBI rats. H\textsubscript{2} treatment significantly decreased the lesion volume of mild TBI rats. However, 2% H\textsubscript{2} treatment did not significantly decrease the lesion volume of severe TBI animals (Supplementary Fig. 2). The above results demonstrate that H\textsubscript{2} treatment has a beneficial effect on brain injury in the CCI-induced mild and moderate TBI models. Matchett et al (Matchett et al., 2009) has found that 2% H\textsubscript{2} is ineffective in moderate and severe neonatal hypoxia-ischemia rat models. However, we previously have found that 2% H\textsubscript{2} also has a protective effect on severe septic mice (Xie et al., 2010). Therefore, the protective effects of H\textsubscript{2} maybe differentiate with different animal models. Many related investigations need to be further done.

Trauma to the brain causes tissue damage by primary and secondary injuries to the neural tissue. Primary injury due to initial mechanical trauma results in loss of tissue, edema, and the tearing and/or shearing of axons within the brain, including cells composing the BBB (Lenzlinger et al., 2001). The secondary injury involves several mechanisms, including the initiation of an acute oxidative stress, and the release of many immune mediators such as interleukins and chemotactic factors (Lenzlinger et al., 2001). TBI results in cellular metabolic alterations and inflammatory responses, which lead to marked increases in the production of ROS (Lewen et al., 2000; Tyurin et al., 2000). To further investigate the possible mechanism, we studied the effects of H\textsubscript{2} treatment on oxidant and antioxidant system in TBI-challenged rats. MDA is a commonly measured end point of free radical-
induced lipid peroxidation, and MDA level correlates with the extent of free radical-induced damage (Kumar et al., 2008). In addition, measurement of 8-iso-PGF2α, free radical-catalysed products of arachidonic acid, can offer a reliable approach for quantitative measurement of oxidative stress status in vivo (Sakamoto et al., 2002). The detection of MDA and 8-iso-PGF2α has been widely used to estimate the overall status of oxidative stress (Sakamoto et al., 2002). The detrimental effects of free oxygen radicals are countered by the antioxidant enzymes SOD and CAT (Satpute et al., 2009). In the present study, we found that the decreased activities of SOD and CAT as well as the increased levels of MDA and 8-iso-PGF2α in brain tissue during the early and late stages, indicating that TBI sets up an environment favorable for oxidative stress. We further showed that 2% H2 treatment significantly increased the activities of SOD and CAT as well as decreased the levels of MDA and 8-iso-PGF2α. These results suggest that the decrease of oxidative damage and the increase of endogenous antioxidant enzymatic activities in brain tissue may attribute to the protection of H2 treatment, which is similar with our previous study (Xie et al., 2010; Xie et al., 2010). About the reasons why H2 could induce the expression of CAT and SOD, we think that there are two main causes. Firstly, because H2 directly reduces ROS in vivo, the consumptions of CAT and SOD were decreased, and then the amounts and actitivities of CAT and SOD were relatively increased. Secondly, H2 as a signaling molecule may directly induce the expression of CAT and SOD. To conclude, the detailed mechanism needs further investigation.

The present and our previous studies have shown that 2% H2 inhalation has no
adverse effects on the saturation level of arterial oxygen (SpO$_2$) or hemodynamic parameters (Ohsawa et al., 2007; Xie et al., 2010; Xie et al., 2010). Furthermore, H$_2$ is neither explosive nor dangerous at a concentration of less than 4.7% in air, which has been proved by 17-year long studies on cells, mice, monkeys and deep-sea divers (COMEX HYDRA program, Marseille). Moreover, H$_2$ as a potential antioxidant has certain unique properties (Ohsawa et al., 2007): 1) unlike most known antioxidants, H$_2$ is permeable to cell membranes and can target organelles, including the cytosol, mitochondria and nuclei. 2) Its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals. 3) H$_2$ is mild enough not to disturb metabolic oxidation-reduction reactions or to disrupt ROS involved in cell signaling (unlike some antioxidant supplements with strong reductive reactivity, which can affect essential defensive mechanisms). Ohsawa et al. (Ohsawa et al., 2007) found that H$_2$ directly reacted with free radical species such as •OH in vitro. However, the detailed mechanisms are unclear in vivo. Further studies will reveal the mechanisms by which H$_2$ protects cells and tissues against oxidative stress in vivo.

In conclusion, our findings in a model of CCI-induced TBI support, in agreement with our recent observations (Xie et al., 2010; Xie et al., 2010), the potential use of H$_2$ as a therapeutic agent in the therapy of conditions associated with mild and moderate TBI. We propose that H$_2$, one of the most well-known molecules, could be widely used in medical applications as a safe and effective neuroprotective drug with minimal side effects.

4. Conclusion
To conclude, we found that TBI-challenged rats showed significant brain injuries characterized by the increase of BBB permeability, brain edema and lesion volume as well as neurological dysfunction, which was significantly attenuated by 2% \( \text{H}_2 \) inhalation from 5 minutes to 5 hours after TBI operation. In addition, we found that the decrease of oxidative products and the increase of endogenous antioxidant enzymatic activities in brain tissue may be associated with the protection of \( \text{H}_2 \) treatment in TBI-challenged rats. The present study supports that \( \text{H}_2 \) inhalation may be a more effective therapeutic strategy for patients with mild and moderate TBI.

5. Experimental procedures

5.1 Subjects

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Fourth Military Medical University, and performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the use of experimental animals. Adult male Sprague-Dawley rats weighing 250-300 g, provided by the Laboratory Animal Center of Fourth Military Medical University, were used in all experiments. Animals were housed in a temperature- and humidity-controlled room that was maintained on 12 h light/dark cycles for at least 1 week before surgery. Standard animal chow and water were freely available. All efforts were made to minimize animal suffering in this study.

5.2 Traumatic brain injury

Traumatic brain injury (TBI) was produced using a well-known model, controlled cortical impact (CCI) injury described previously (Hall et al., 2008; Longhi
et al., 2001; Xiong et al., 2007; Yu et al., 2009). After an overnight fast with unrestricted access to water, the animals were initially anesthetized with isoflurane (4.0% for induction, 2.0% for maintenance, in 100% O\textsubscript{2}). After loss of corneal and pedal reflexes, the scalp and scapular regions were shaved, the animal was secured in a stereotaxic head frame, and the scalp was cleansed with ethanol and betadine. Rectal temperature was monitored and maintained between 37.0 and 38.0 °C with a thermostatically controlled heating pad (Braintree Scientific Inc., Braintree, MA, USA). A 6-mm craniectomy was performed using a high-speed drill (Champ-Air Dental Drill, Benco Dental, Wilkes-Barre, PA) over the right sensorimotor cortex, midway between lambda and bregma sutures. A pneumatic piston impactor device (Biomedical Engineering Facility, Virginia Commonwealth University, Richmond, VA) with a 5-mm-diameter, rounded tip was used to impact the brain at a velocity of 4.0 m/s reaching a depth of 1.0 mm, 2.0 mm or 3.0 mm for mild, moderate and severe TBI model, respectively, and remained in the brain for 130 minutes. Following injury, the bone flap was replaced and sealed with bone wax, and the skin was sutured. Sham animals underwent an identical surgery with the exception of TBI. Arterial blood was sampled at pre-operation state, 60 minutes after operation as well as 300 minutes after operation, respectively, for the determination of arterial oxygen tension (PaO\textsubscript{2}), arterial carbon dioxide tension (PaCO\textsubscript{2}), pH, and blood glucose. Arterial blood gases were measured by means of a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy).

5.3 Hydrogen gas (H\textsubscript{2}) treatment
The animals were put in a sealed plexiglas chamber with inflow and outflow outlets. H₂ was supplied through a gas flowmeter, TF-1 (YUTAKA Engineering Corp., Tokyo, Japan), and delivered by air into the chamber through a tube at a rate of 4 L/min. The concentration of H₂ in the chamber was continuously monitored with a commercially available detector (Hy Alerta Handheld Detector Model 500, H₂ Scan, Valencia, CA, USA) and maintained at 2% during the treatment. The concentration of oxygen in the chamber was maintained at 21% by using supplemental oxygen and continuously monitored with a gas analyzer (Medical Gas Analyzer LB-2, Model 40 M, Beckman, USA). Carbon dioxide was removed from the chamber gases with baralyme. The animals without H₂ treatment were exposed to room air in the chamber. The room and chamber temperature was maintained at 22 to 24 °C. Food and water were available ad libitum during the treatment (Xie et al., 2010; Xie et al., 2010).

5.4 Experimental design

Experiment 1: Effects of H₂ inhalation on moderate TBI in rats. Seventy-two male rats were randomly divided into 4 groups: Sham group, Sham+H₂ group, TBI group and TBI+H₂ group. The animals in the Sham+H₂ and TBI+H₂ groups were exposed to 2% H₂ from 5 minutes to 5 hours after sham or TBI operation, respectively. As a control, the animals from the Sham and TBI groups were exposed to room air at the same time points. Twenty-four animals were used for evaluation of Blood-Brain Barrier (BBB) integrity at 6 h after TBI or sham operation (n=6 per group). Furthermore, another twenty-four animals were used for evaluation of brain edema at 24 h after TBI or sham operation (n=6 per group). The rest twenty-four
animals were used for assessment of behavior and lesion volume (n=6 per group).

**Experiment 2**: Effects of H$_2$ treatment on oxidant and antioxidant system in the brain tissues of moderate TBI-challenged rats. Additional one hundred and twenty animals were used in this experiment and were assigned to 4 groups (n = 6 per group). The grouping method and experimental protocols were the same as Experiment 1. The antioxidant enzymes (superoxide dismutase [SOD] and catalase [CAT]) and oxidative product (malondialdehyde [MDA] and 8-iso-prostaglandin F2α [8-iso-PGF2α]) in the injured ipsilateral cortex were measured at pre-operation state as well as at 6 h, 24 h, 48 h and 72 h after TBI or sham operation.

### 5.5 Evaluation of BBB integrity

BBB integrity was determined by Evans blue (EB) extravasation at 6 h after TBI (n =6 per group). It has been previously shown that this is the point of maximum BBB permeability in this model of injury (Barzo et al., 1996; Beaumont et al., 2000). Briefly, at 5 h after injury, 2% Evans blue was injected intravenously at a dose of 2 ml/kg. Animals were then re-anesthetized at 6 h and perfused with saline to remove intravascular EB dye. Animals were then decapitated, the ipsilateral cortical tissues were disected. Each tissue sample was weighed, homogenized in 2 ml of 50% trichloroacetic acid (w/v), and centrifuged at 10, 000 rpm for 20 minutes. The supernatant was then diluted with solvent (one part 50% trichloroacetic acid to three parts ethanol). Tissue levels of Evans blue dye were quantitated using a spectrofluorometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. Sample values were compared with those of Evans blue dye.
standards mixed with the solvent (100-1000 ng/ml).

5.6 Evaluation of brain edema

Brain water content, an indicator of brain edema, was measured with the wet-dry method 24 h after injury (Dogan et al., 1997). After the animals were killed by decapitation under anesthesia, their brains were removed and the ipsilateral cortical tissues were dissected and weighed immediately to get wet weight. After drying in a desiccating oven for 48 hours at 100 °C, the tissues were reweighed to yield dry weight. The percentage of water in the tissues was calculated according to the formula: % brain water = [(wet weight - dry weight)/wet weight]×100.

5.7 Behavioral assessments

Beam-balance and beam-walk tasks were utilized to assess gross and fine motor function, respectively. The beam-balance task consists of placing the animal on an elevated (90 cm) narrow wooden beam (1.5 cm wide) and recording the duration. It remains on for a maximum of 60 s. The beam-walk task, originally devised by Feeney et al. (Feeney and Westerberg, 1990), allows for the assessment of refined locomotor activity. Briefly, the task consists of training/assessing animals using a negative-reinforcement paradigm to escape ambient light and high decibel white noise (Lafayette Instruments, Inc., Lafayette, IN) by traversing an elevated (90 cm) narrow wooden beam (2.5×100 cm) and entering a darkened goal box at the opposite end. Performance was assessed by the time to traverse the beam. Animals were pre-trained on both motor tasks one day prior to surgery and assessed on the day of surgery to
determine baseline performance. Post-operative testing occurred on days 1 to 5 and consisted of providing three trials (60 s allotted time) per day on each task. The average daily scores for each subject were used in the statistical analysis.

5.8 Quantification of lesion volume

After the last behavioral assessment, animals were euthanized under anesthesia, and then perfused transcardially with 200 ml normal saline followed by 200 ml ice-cold 4% paraformaldehyde (Sigma) for fixation. Brains were stored in 4% paraformaldehyde overnight at 4 °C, then transferred to a 30% sucrose solution for 3 days for cryoprotection. Brains were then sectioned coronally (40 μm) on a freezing microtome with approximately 500 μm distance between slices. Sections were mounted onto gelatin coated slides, dried overnight and stained with 0.2% cresyl violet (Sigma Chemical, St. Louis, MO) for measurement of brain tissue loss. The stained sections were viewed using light microscopy for gross morphology (Olympus BX5) and photographed using a Nikon Coolpix 950 digital camera.

5.9 Measurement of antioxidant enzymatic activity

At pre-determined time points, animals were euthanized under anesthesia, and then perfused transcardially with 200 ml chilled PBS (0.1 M, pH 7.4). The ipsilateral cortical tissues were dissected and homogenized in chilled PBS (0.1 M, pH 7.4), and then centrifuged at 10,000 g at 4 °C for 10 minutes. The supernatants were collected, aliquoted, stored at -80 °C until the following analysis.

The activities of SOD and CAT were measured using commercial kits purchased from Cayman Chemical Company (Ann Arbor, MI, USA). According to the
manufacturer’s instructions, total SOD activity was assayed by detecting superoxide radicals generated by xanthine oxidase and hypoxanthine. The reaction was monitored at 450 nm and one unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The CAT activity was assayed by measuring the reduction of hydrogen peroxide at 540 nm and one unit was defined as the amount of enzyme that would cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. All spectrophotometric readings were performed by using a spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA). All assays were conducted in triplicates. The tissue protein concentration was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

5.10 Measurement of oxidative product level

The homogenates obtained above were also used for detecting the level of oxidative products (8-iso-PGF2α and MDA). The level of 8-iso-PGF2α was detected by specific enzyme-linked immunosorbent assay (ELISA) kits (8-iso-PGF2α, Ann Arbor, MI, USA) using a microplate reader (CA 94089, Molecular Devices, Sunnyvale, Canada). MDA content was measured by commercial kits (Cayman Chemical Company, Ann Arbor, MI, USA). All standards and samples were run in duplicate. The tissue protein concentration was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

5.11 Statistical analysis

All data are expressed as mean ± SEM. The behavioral data were analyzed by repeated measures analysis of variance (ANOVA) with Bonferroni post hoc test.
utilized to determine specific group differences. The inter-group differences of the rest data were tested by one-way ANOVA followed by LSD-\( t \) Test for multiple comparisons. The statistical analysis was performed with SPSS 16.0 software. In all tests, a \( P \) value of less than 0.05 was considered statistically significant.

**ACKNOWLEDGMENTS**

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FIGURE LEGENDS

**Fig. 1.** Hydrogen treatment improved the blood-brain barrier (BBB) integrity in rats with moderate TBI. Hydrogen treatment was given by exposure to 2% H₂ from 5 minutes to 5 hours after sham or TBI operation, respectively. BBB integrity in the injured ipsilateral cortex was determined by Evans blue (EB) extravasation at 6 h after TBI operation. The values are expressed as mean ± SEM (n = 6 per group). *P < 0.05 vs. Sham group; †P < 0.05 vs. TBI group.

**Fig. 2.** Hydrogen treatment attenuated the brain edema in rats with moderate TBI. Hydrogen treatment was given by exposure to 2% H₂ from 5 minutes to 5 hours after sham or TBI operation, respectively. Brain water content as an indicator of brain edema was measured at 24 h after injury. The values are expressed as mean ± SEM (n = 6 per group). *P < 0.05 vs. Sham group; †P < 0.05 vs. TBI group.

**Fig. 3.** Hydrogen treatment improved the motor performance in rats with moderate TBI. Hydrogen treatment was given by exposure to 2% H₂ from 5 minutes to 5 hours after sham or TBI operation, respectively. The gross and fine motor function was assessed by beam-balance and beam-walk tasks, respectively. (A): Beam-balance test: time to maintain balance on an elevated narrow beam before and after TBI or Sham operation. (B): Beam-walk test: time to traverse an elevated narrow beam before and after TBI or Sham operation. The values are expressed as mean ± SEM (n = 6 per group). *P < 0.05 vs. Sham group; †P < 0.05 vs. TBI group. s: second; d: day.
Fig. 4. Hydrogen treatment reduced the cerebral lesion volume in rats with moderate TBI. Hydrogen treatment was given by exposure to 2% H\textsubscript{2} from 5 minutes to 5 hours after sham or TBI operation, respectively. Cerebral lesion volume was determined using cresyl violet after the last behavior test (i.e., on the 5\textsuperscript{th} day after operation). The values are expressed as mean ± SEM (n = 6 per group). *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. TBI group.

Fig. 5. Hydrogen treatment upregulated the activities of SOD (A) and CAT (B) in rats with moderate TBI. Hydrogen treatment was given by exposure to 2% H\textsubscript{2} from 5 minutes to 5 hours after sham or TBI operation, respectively. The activities of SOD and CAT in the injured ipsilateral cortex were detected at pre-operation state as well as at 6 h, 24 h, 48 h and 72 h after TBI or sham operation. The values are expressed as mean ± SEM (n = 6 per group). *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. TBI group.

Fig. 6. Hydrogen treatment reduced the levels of MDA (A) and 8-iso-PGF2α (B) in rats with moderate TBI. Hydrogen treatment was given by exposure to 2% H\textsubscript{2} from 5 minutes to 5 hours after sham or TBI operation, respectively. The levels of MDA and 8-iso-PGF2α in the injured ipsilateral cortex were detected at pre-operation state as well as at 6 h, 24 h, 48 h and 72 h after TBI or sham operation. The values are expressed as mean ± SEM (n = 6 per group). *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. TBI group.
TABLE LEGENDS

Table 1. Physiologic parameters of moderate TBI or Sham rats before and after operation. Hydrogen treatment was given by exposure to 2% H₂ from 5 minutes to 5 hours after sham or TBI operation, respectively. Arterial blood was sampled at pre-operation state, 60 minutes and 300 minutes after operation, respectively, for the determination of pH, PaCO₂, PaO₂ and glucose using a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy).
SUPPLEMENTARY FIGURE LEGENDS

**Supplementary Fig. 1.** Hydrogen treatment reduced the cerebral lesion volume in rats with moderate TBI in a concentration-dependent manner. Hydrogen treatment was given by exposure to 2% H\textsubscript{2} from 5 minutes to 5 hours after sham or TBI operation, respectively. Cerebral lesion volume was determined using cresyl violet on the 5\textsuperscript{th} day after operation. The values are expressed as mean ± SEM (n = 6 per group). *P < 0.05 vs. TBI group.

**Supplementary Fig. 2.** Effects of hydrogen treatment on the cerebral lesion volume in rats with mild and severe TBI. Hydrogen treatment was given by exposure to 2% H\textsubscript{2} from 5 minutes to 5 hours after sham or TBI operation, respectively. Cerebral lesion volume was determined using cresyl violet on the 5\textsuperscript{th} day after operation. The values are expressed as mean ± SEM (n = 6 per group). *P < 0.05 vs. Air group.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5

A  

SOD activity (U/mg.prot)

- Sham
- Sham+H₂
- TBI
- TBI+H₂

B  

CAT activity (U/g.prot)

- Sham
- Sham+H₂
- TBI
- TBI+H₂

Fig. 5
Fig. 6
Table 1. Physiologic parameters of TBI or Sham rats before and after operation

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