Brain Research
Manuscript Draft

Manuscript Number: BRES-D-10-02037

Title: Beneficial Effects of Hydrogen Gas against Spinal Cord Ischemia-Reperfusion Injury in Rabbits

Article Type: Research Report

Section/Category: Disease-related Neuroscience

Keywords: Spinal cord; Ischemia-reperfusion injury; Hydrogen gas; Oxidative stress; Inflammatory cytokines.

Manuscript Region of Origin: CHINA

Abstract: Recently, hydrogen gas (H2) is reported to be a new therapeutic agent in organ damage induced by ischemia-reperfusion (I/R). The present study was designed to investigate the beneficial effects of H2 against spinal cord I/R injury and its associated mechanisms. Spinal cord ischemia was induced by infrarenal aortic occlusion for 20 min in male New Zealand white rabbits. Treatment with 1%, 2% or 4% H2 inhalation was given from 10 min before reperfusion to 60 min after reperfusion (total 70 min). Here, we found that I/R-challenged animals showed significant spinal cord damage characterized by the decreased numbers of normal motor neurons and hind-limb motor dysfunction, which was significantly improved by 2% and 4 % H2 treatment. Furthermore, we found that the beneficial effects of H2 treatment against spinal cord I/R injury were associated with the decreased levels of oxidative products [8-iso-prostaglandin F2α (8-iso-PGF2α) and malondialdehyde (MDA)] and pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α) and high-mobility group box 1 (HMGB1)], as well as increased activities of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] in serum and spinal cord. In addition, H2 treatment reduced motor neuron apoptosis in the spinal cord of this model. Thus, H2 inhalation may be an effective therapeutic strategy for spinal cord I/R damage.

Research highlights:

1. 2% and 4 % H_2 treatment significantly attenuated spinal cord ischemia-reperfusion injury;
2. H_2 treatment significantly reduced the levels of oxidative products (8-iso-PGF2α and MDA) in serum and spinal cord;
3. H_2 treatment significantly upregulated the activities of antioxidant enzymes (SOD and CAT) in serum and spinal cord;
4. H_2 treatment significantly decreased the levels of pro-inflammatory cytokines (TNF-α and HMGB1) in serum and spinal cord;
5. H_2 treatment significantly reduced motor neuron apoptosis of spinal cord;
6. H_2 inhalation may be an effective therapeutic strategy for spinal cord I/R damage.

Beneficial Effects of Hydrogen Gas against Spinal Cord Ischemia-Reperfusion Injury in Rabbits

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Running Title: H_2 against Spinal Cord I/R Injury

Number of pages: 42

Number of figures: 6

Number of tables: 1

Number of supplementary figures: 0

Number of supplementary tables: 0

This work was supported by research grants from the National Natural Science Foundation of China (30725039 and 81071533).

Conflict of Interest Statement: The authors have declared that no conflict of interest exists.

ABBREVIATIONS----CAT, catalase; HMGB1, high-mobility group box 1; H_2, hydrogen gas; I/R, ischemia-reperfusion; 8-iso-PGF2α, 8-iso-prostaglandin F2α; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha, TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

ABSTRACT

Recently, hydrogen gas (H_2) is reported to be a new therapeutic agent in organ damage induced by ischemia-reperfusion (I/R). The present study was designed to investigate the beneficial effects of H_2 against spinal cord I/R injury and its associated mechanisms. Spinal cord ischemia was induced by infrarenal aortic occlusion for 20 min in male New Zealand white rabbits. Treatment with 1%, 2% or 4% H_2 inhalation was given from 10 min before reperfusion to 60 min after reperfusion (total 70 min). Here, we found that I/R-challenged animals showed significant spinal cord damage characterized by the decreased numbers of normal motor neurons and hind-limb motor dysfunction, which was significantly improved by 2% and 4 % H_2 treatment. Furthermore, we found that the beneficial effects of H_2 treatment against spinal cord I/R injury were associated with the decreased levels of oxidative products [8-iso-prostaglandin F2α (8-iso-PGF2α) and malondialdehyde (MDA)] and pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α) and high-mobility group box 1 (HMGB1)], as well as increased activities of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] in serum and spinal cord. In addition, H_2 treatment reduced motor neuron apoptosis in the spinal cord of this model. Thus, H_2 inhalation may be an effective therapeutic strategy for spinal cord I/R damage.

Keywords: Spinal cord; Ischemia-reperfusion injury; Hydrogen gas; Oxidative stress; Inflammatory cytokines.

1. Introduction

Paraplegia induced by spinal cord ischemia-reperfusion (I/R) injury remains a major devastating complication after surgical repair of thoracic and thoracoabdominal aneurysm (Kuniyoshi et al., 2003). It's reported that the incidence of residual permanent injury (acute and/or delayed paraplegia) is from 3% to 18% (MacArthur et al., 2005). Despite many strategies have been developed to increase the ischemic tolerance of spinal cord and minimize the incidence of neurological complications, there is still no clinical neuroprotective agent available for patients with paraplegia (Etz et al., 2008).

Recently, our and other studies have found that hydrogen gas (H_2) exerts a widely therapeutic role in cerebral injury, liver injury, lung injury, renal injury and myocardial injury induced by I/R via reducing oxidative stress and apoptosis (Cai et al., 2008; Fukuda et al., 2007; George and Agarwal, 2010; Hayashida et al., 2008; Ji et al., 2010; Ohsawa et al., 2007; Ohta, 2008; Xie et al., 2010a, b). 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 I/R (Cai et al., 2009; Mao et al., 2009; Sato et al., 2008; Zheng et al., 2009). In addition, our recent studies have shown that H_2 inhalation significantly improved the survival rate and multiple organ damage of septic mice in a concentration- and time-dependent manner (Xie et al., 2010a, b). Besides, we have found that the beneficial effects of H_2 treatment on sepsis were associated with the decreased levels of oxidative products and inflammatory cytokines as well as the increased activities of antioxidant enzymes in serum and

tissues (Xie et al., 2010a, b). These findings strongly indicate that H_2 treatment may be beneficial to spinal cord I/R injury.

So far, there are no studies attempting to describe the protective effect of H_2 on spinal cord ischemia. Therefore, this study, using a well-established model of the spinal cord I/R in rabbits induced by infrarenal aorta occlusion (Ding et al., 2009), was designed to investigate whether H_2 treatment could attenuate spinal cord I/R injury via reducing oxidative stress, inflammation and apoptosis.

2. Results

2.1. Physiological Parameters

In the present study, we firstly investigated the effects of H_2 inhalation on arterial pH, PaO_2, $PaCO_2$ and blood glucose in the animals with spinal cord I/R operation during the treatment. The detailed results are shown in **Table 1**. The animals were given with 1%, 2% or 4% H_2 inhalation from 10 min before reperfusion to 60 min after reperfusion (total 70 min), respectively. No significant differences in pH, PaO_2, $PaCO_2$ or blood glucose were observed among all groups during the treatment ($P > 0.05$, n=8 per group). The results demonstrate that H_2 inhalation at less than 4% concentration has no significant effects on arterial blood gas and blood glucose in this model during the treatment.

2.2. Neurological Outcomes

To observe the protective role of H_2 treatment, we investigated the effects of H_2 inhalation with different concentrations on the hind-limb motor function scores of spinal cord I/R rabbits at 24 h, 48 h and 72 h after reperfusion (**Fig. 1**). All animals survived until the final neurological behavior assessment at 72 h after reperfusion. The control animals developed complete paraplegia of hind-limbs at 48 h and 72 h after reperfusion. Treatment with 2% or 4% H_2 inhalation significantly improved the neurological outcome of spinal cord I/R animals at 24 h, 48 h and 72 h after reperfusion ($P < 0.05$ vs. Con group, n=8 per group, **Fig. 1**). However, the hind-limb motor function scores in the 1% H_2 treatment group did not significantly change when compared to that of control group at 24 h, 48 h, and 72 h after reperfusion ($P > 0.05$,

n=8 per group, **Fig. 1**). In addition, 4% H_2 treatment did not further improve the neurological scores of spinal cord I/R animals when compared to 2% H_2 treatment group ($P > 0.05$, n=8 per group, **Fig. 1**). The results indicate that spinal I/R damage can cause significant deficit in hind-limb motor function, which is improved markedly by 2% and 4% H_2 treatment.

2.3. Number of Normal Motor Neuron

The representative micrographs of hematoxylin and eosin staining in the ventral horn of L4 spinal cord segments at 72 h after reperfusion are shown in **Fig. 2A-D**. The number of normal motor neuron in the anterior horn of spinal cord at 72 h after reperfusion is shown in **Fig. 2E**. The numbers of normal motor neuron in the 2% H_2 and 4% H_2 treatment groups were more than that in control group ($P < 0.05$, n=8 per group), whereas the number of normal motor neuron in the 1% H_2 treatment group was similar to that in control group ($P > 0.05$, n=8 per group). Moreover, the number of normal motor neuron in the 4% H_2 treatment group did not significantly change when compared with that in the 2% H_2 treatment group ($P > 0.05$, n=8 per group). The above results indicate that spinal I/R injury can cause significant decrease of normal motor neuron in anterior horn, which is improved markedly by 2% and 4% H_2 treatment.

2.4. Oxidative Products

Based on the above results, we chose 2% H_2 treatment to further investigate the mechanisms of protective effects of H_2 inhalation against spinal cord I/R damage. First, we detected the levels of oxidative products [malondialdehyde (MDA) and

8-iso-prostaglandin F2α (8-iso-PGF2α)] in serum and spinal cord at different time points. The data showed that spinal cord I/R injury significantly led to the increase of MDA and 8-iso-PGF2α levels in serum and spinal cord ($P < 0.05$ vs. Sham group, n=6 per group, **Fig. 3**). When compared with control (Con) group, H_2 treatment significantly decreased the levels of MDA and 8-iso-PGF2α in serum and spinal cord ($P < 0.05$, n=6 per group, **Fig. 3**). The above results suggest that spinal cord I/R damage can lead to the increase of MDA and 8-iso-PGF2α levels in serum and spinal cord, which can be significantly attenuated by H_2 treatment.

2.5. Antioxidant Enzymes

Furthermore, we detected the activities of superoxide dismutase (SOD) and catalase (CAT) in serum and spinal cord at different time points, which is shown in **Fig. 4**. The results showed that spinal cord I/R injury significantly decreased the activities of SOD and CAT in serum and spinal cord ($P < 0.05$ vs. Sham group, n=6 per group). Compared with control (Con) group, however, H_2 treatment significantly increased the activities of SOD and CAT in serum and spinal cord ($P < 0.05$, n=6 per group). The above results suggest that spinal cord I/R damage can cause the decrease of SOD and CAT activities in serum and spinal cord, which can be significantly improved by H_2 treatment.

2.6. Inflammatory Cytokines

As shown in **Fig. 5**, we also measured the levels of early and late pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α) and high-mobility group box 1 (HMGB1)] in serum and spinal cord at different time points. Our results showed that

spinal cord I/R animals had a significant increase of TNF-α and HMGB1 levels in serum and spinal cord ($P < 0.05$ vs. Sham group, n=6 per group, **Fig. 5**). However, H_2 treatment significantly reduced the levels of TNF-α and HMGB1 in serum and spinal cord when compared with control (Con) group ($P < 0.05$, n=6 per group, **Fig. 5**). The results suggest that spinal cord I/R damage can lead to the increase of early and late pro-inflammatory cytokines in serum and spinal cord, which can be significantly attenuated by H_2 treatment.

2.7. Motor Neuron Apoptosis

In addition, we detected the motor neuron apoptosis at 72 h after reperfusion by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) staining and caspase-3 activity. The results are shown in **Fig. 6**. TUNEL staining identified no apoptotic cells in the spinal cord of sham-operated animals. In the spinal cord of control (Con) group, numerous motor neurons were strongly positive for TUNEL staining. In the samples of H_2 treatment group, however, a few of the motor neurons were positive for TUNEL staining. For quantitative measurement, the number of motor neurons that were positive for TUNEL staining was recorded in each specimen in a blind fashion. We found that spinal cord I/R animals showed a significant increase of the total number of apoptotic motor neurons ($P < 0.05$ vs. Sham group, n=6 per group, **Fig. 6A**). However, H_2 treatment significantly reduced the total number of apoptotic motor neurons compared with control (Con) group ($P < 0.05$, n=6 per group, **Fig. 6A**). Furthermore, we found that the caspase-3 activity was significantly increased in spinal cord I/R animals ($P < 0.05$ vs. Sham group, n=6 per

group, **Fig. 6B**), which was significantly reduced by 2% H_2 treatment ($P < 0.05$ vs. Con group, n=6 per group, **Fig. 6B**). The results suggest that spinal cord I/R damage can increase the motor neuron apoptosis of spinal cord, which can be significantly alleviated by H_2 treatment.

3. Discussion

In the present study, we found that 2% and 4% H_2 inhalation from 10 min before reperfusion to 60 min after reperfusion (total 70 min) significantly improved the histopathologic features and number of motor neurons in spinal cord I/R-challenged rabbits. Furthermore, H_2 treatment also significantly attenuated spinal cord I/R-induced neurological dysfunction. These results suggest that 2% and 4 % H_2 treatment can produce a beneficial effect against spinal cord I/R injury. In addition, we found that the beneficial effects of H_2 treatment against spinal cord I/R injury were associated with the decreased levels of oxidative products (8-iso-PGF2α and MDA) and pro-inflammatory cytokines (TNF-α and HMGB1), increased activities of antioxidant enzymes (SOD and CAT), as well as reduction of motor neuron apoptosis. The above results demonstrate that H_2 treatment is beneficial to spinal cord I/R damage via reducing oxidative stress, inflammatory response and apoptosis.

Spinal cord ischemia induced by infrarenal aortic occlusion in rabbits is a well-characterized model, which is used extensively to study histopathologic and neurological changes in spinal cord I/R damage (Ding et al., 2009; Dong et al., 2002). Accordingly, using this model, we investigated the protective effects of H_2 treatment and its associated mechanisms. In the present study, we found that spinal cord I/R-challenged rabbits showed significant spinal cord injury characterized by the decreased number of normal motor neuron and paraplegia of hind-limbs, which is similar to previous studies (Ding et al., 2009; Dong et al., 2002; Wang et al., 2009).

Spinal cord injury after a successful surgery of the thoracic aorta is still an

unpredictable and disastrous complication (MacArthur et al., 2005). The reported incidences of postoperative paraplegia vary from 3 to 18% (MacArthur et al., 2005). The main cause of acute spinal cord dysfunction is believed to be the result of spinal cord ischemia from hypoperfusion during aortic cross-clamping (Di Bartolomeo et al., 2009). In spite of the extensive efforts to understand the pathophysiological mechanisms underlying the primary and secondary lesions following spinal cord I/R, no clinical neuroprotective treatment is available now (Etz et al., 2008).

Interestingly, recent studies have demonstrated that H_2 inhalation can 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 I/R through reducing oxidative stress, suggesting that H_2 has a potential role in preventive and therapeutic applications for organ damage (Cai et al., 2008; Fukuda et al., 2007; George and Agarwal, 2010; Hayashida et al., 2008; Ohsawa et al., 2007; Ohta, 2008; xie et al., 2010a, b). 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 I/R (Cai et al., 2009; Mao et al., 2009; Sato et al., 2008; Zheng et al., 2009). Our recent studies have also shown that H_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., 2010a, b). Moreover, we have found that 2% H_2 treatment is beneficial to traumatic brain injury via reducing oxidative products and increasing antioxidant enzymatic activity (Ji et al., 2010). These findings strongly indicate that H_2 treatment

perhaps becomes a good measure for treating patients with spinal cord I/R injury.

Based on our and other studies, the present study was designed to investigate the effects of H_2 inhalation with different concentrations from 10 min before reperfusion to 60 min after reperfusion (total 70 min) on spinal cord I/R-challenged rabbits. Previous studies have proved the worsening of neurological function at 14-48 h after spinal cord I/R in the rabbit model (Moore and Hollier, 1991). Therefore, the final assessments for neurological and histological outcomes were performed at 72 h after reperfusion in our experiments. Here, we found that 2% and 4% H_2 treatment significantly attenuated the histopathologic damage and increased the number of normal motor neuron of spinal cord. Furthermore, 2% and 4% H_2 treatment significantly improve the hind-limb motor function in spinal cord I/R-challenged animals. However, 1% H_2 treatment did not significantly improve the histopathologic damage and hind-limb motor function in this model. The above results demonstrate that 2% and 4% H_2 treatment has a beneficial effect on spinal cord injury induced by I/R, while 1% H_2 treatment does not. Therefore, the protective effect of H_2 is different with different treatment concentrations. Many related investigations need to be further done.

Spinal cord I/R injury involves several mechanisms, including the initiation of acute oxidative stress, and the release of many immune mediators such as interleukins and chemotactic factors (Ege et al., 2004; Gonzalez et al., 2003; Hamann and Shi, 2009; Prow and Irani, 2008; Takenaga et al., 2006). Spinal cord ischemia results in cellular metabolic alterations and inflammatory responses, which lead to marked

increase in the production of reactive oxygen species (ROS) (Ege et al., 2004; Yune et al., 2004). More and more researchers have found that excessive production of ROS and reduction of antioxidant defense systems play an important role in the pathogenesis of spinal cord I/R injury (Ege et al., 2004; Genovese and Cuzzocrea, 2008; Xu et al., 2005; Yune et al., 2004). In excess, ROS and their by-products could exacerbate spinal cord damage and thus overall clinical outcome (Genovese and Cuzzocrea, 2008; Xu et al., 2005). To further investigate the possible mechanisms, therefore, we first investigate the effects of H_2 treatment on oxidative products and antioxidant enzymes in spinal cord I/R-challenged animals. 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). Thus, the detection of MDA and 8-iso-PGF2α has been widely used to estimate the overall status of oxidative stress (Basu et al., 2001; Sakamoto et al., 2002). Meanwhile, the detrimental effects of free oxygen radicals are countered by the antioxidant enzymes SOD and CAT (Satpute et al., 2009). Through detecting the levels of oxidative products (8-iso-PGF2α and MDA) and activities of antioxidant enzymes (SOD and CAT) in serum and spinal cord at different time points, we found that spinal cord I/R-challenged animals showed the significant increase of 8-iso-PGF2α and MDA levels as well as the decrease of SOD and CAT activities in serum and spinal cord, which were markedly improved by 2%

H_2 treatment. These results suggest that the decrease of oxidative damage and the increase of endogenous antioxidant enzymatic activities in serum and spinal cord may attribute to the protection of H_2 treatment, which is consistent with our previous studies (Ji et al., 2010; Xie et al., 2010a, b).

In addition, postischemic inflammation has been suggested to contribute to the late stages of reperfusion injury and to result in the worsening of neurologic outcome (Hasturk et al., 2009; Reece et al., 2006). There is a lot of evidence to suggest that the systemic inflammatory response associated with I/R injury contributes to the morbidity and mortality after surgical repair of thoracoabdominal aortic aneurysm (Hasturk et al., 2009; Reece et al., 2006). The inflammatory cytokines include early inflammatory cytokines such as pro-inflammatory cytokines TNF-α, IL-6 and anti-inflammatory cytokine IL-10, as well as the late inflammatory cytokine HMGB1 (Andersson et al., 2000; Sha et al., 2008; Wang et al., 1999). HMGB1 can activate inflammatory pathways when released from ischemic cells (Kim et al., 2006). Recently, our research has suggested that HMGB1 as a late cytokine-like mediator plays a critical role in the development of postischemic spinal cord damage through the amplification of inflammatory responses in serum and spinal cord (Wang et al., 2009). Meanwhile, the early and late pro-inflammatory cytokines can interact and facilitate the spinal cord injury in this model (Wang et al., 2009). In the present study, serum HMGB1 and TNF-α levels were increased up to 72 h after reperfusion in a time-dependent manner in this spinal cord I/R model. Moreover, we found that spinal cord HMGB1 and TNF-α contents were also significantly increased at 72 h after

reperfusion. However, 2% H_2 treatment significantly attenuated the increase of HMGB1 and TNF-α levels in serum and spinal cord. These results indicate that the decrease of early and late pro-inflammatory cytokines may also attribute to the protection of H_2 treatment, which is also similar to our previous studies (Ji et al., 2010; Xie et al., 2010a, b).

Besides, apoptosis has been demonstrated to be an important mode of neuron death in the ischemic spinal cord and play an important role in delayed paraplegia (Beattie et al., 2000; Hayashi et al., 1998). Apoptosis is a programmed cell death that is characterized by specific ultrastructural changes including cell shrinkage, nuclear condensation and DNA fragmentation (Elmore, 2007). TUNEL staining is a highly sensitive and specific means of identifying DNA fragmentation (Perry et al., 1997). In this study, apoptotic cells were detected on the basis of positive TUNEL staining, with the fluorescent nucleus in a granular pattern. As noted, numerous apoptotic motor neurons were observed in the spinal cord of control animals. The total number of TUNEL-positive motor neurons was significantly reduced after H_2 treatment. On the other hand, at the molecular level, apoptosis is activated by the aspartate-specific cysteineprotease (caspase) cascade, including caspase-12 and -3 (Sharifi et al., 2009). Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (Kaufmann et al., 2008). Capase-3 activates DNA fragmentation factor, which in turn activates endonucleases to cleave nuclear DNA and ultimately leads to cell death (Kaufmann et al., 2008). In this study, we also found that the spinal cord caspase-3 activity was significantly increased in the spinal

cord I/R model, which was markedly alleviated by H_2 treatment. Therefore, these results suggest that H_2 treatment significantly alleviated neuron apoptosis induced by spinal cord I/R.

Hydrogen is one of the most plentiful gases in the universe. The present and our previous studies have shown that less than 4% H_2 inhalation has no adverse effects on the saturation level of arterial oxygen (SpO_2) or hemodynamic parameters (Huang et al., 2010; Ohsawa et al., 2007; Xie et al.,2010a, b). Furthermore, H_2 is neither explosive nor dangerous at a concentration of less than 4.7% in air and 4.1% in pure oxygen, respectively (Huang et al., 2010; Ohsawa et al., 2007). In agreement with our recent observations (Xie et al., 2010a, b), the present study supports that H_2 inhalation may be an effective therapeutic agent in the conditions associated with spinal cord I/R damage.

4. Conclusion

In conclusion, to our best knowledge, our study demonstrates that 2% and 4 % H_2 treatment significantly attenuated spinal cord I/R injury. The protective effects of H_2 treatment were associated with the decreased levels of oxidative products and pro-inflammatory cytokines, as well as increased activities of antioxidant enzymes. Furthermore, the reduction of motor neuron apoptosis in spinal cord may contribute to the protection of H_2 treatment in this model. We conclude that H_2 inhalation may be an effective therapeutic strategy for the patients with spinal cord damage.

5. Experimental procedures

5.1. Subjects

Adult male New Zealand White rabbits weighing 2.2 to 2.5 kg were provided by the Laboratory Animal Center of Fourth Military Medical University, Xi'an, China. Animals were housed at 20 to 22 ℃ with a 12-h light/dark cycle. Standard animal chow and water were freely available. 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. All efforts were made to minimize animal suffering and the number of animals used for this study.

5.2. Surgical Preparation

According to our previous studies (Dong et al., 2002; Nie et al., 2006; Wang et al., 2009), the rabbits were anesthetized with sevoflurane (4% for induction, 1.5% for maintenance) in N_2/O_2 (70/30) gas (flow rate of 1 L/min) after an overnight fast with unrestricted access to water. The lactated Ringer's solution (4 mL/kg/h) was infused intravenously. Both 22-gauge catheters were implanted into the ear artery and left femoral artery to measure the proximal and distal blood pressure, respectively. Blood pressure and heart rate were monitored continuously by a calibrated pressure transducer connected to an invasive pressure monitor (Spacelabs Medical Inc., Redmond, WA). Rectal temperature was maintained at 38.5 ± 0.5 ℃ by a heating blanket and overhead lamps during the experiments.

5.3. Spinal Cord Ischemia-Reperfusion (I/R)

Spinal cord ischemia was induced by infrarenal aortic occlusion in rabbits as described in our previous studies (Dong et al., 2002; Nie et al., 2006; Wang et al., 2009). Briefly, animals were placed in supine position. After a 3- to 4-cm-long medial incision, abdominal aorta was exposed at the level of left renal artery. 400 units of heparin were administered 5 min before the aortic occlusion, and spinal cord ischemia was induced with the aorta clamped by a bulldog clamp just below renal artery. After the occlusion, distal blood pressure decreased immediately to less than 10 mmHg, and the pulsatility disappeared. The blood flow was obstructed for 20 min. Then the bulldog clamp was removed, and the abdominal wall was closed with a sterile 6-0 silk suture. Local anesthetic infiltration with 1% lidocaine hydrochloride was applied around the wound for postoperative analgesia. A prophylactic antibiotic (40, 000 IU gentamicin) was administered intramuscularly immediately after operation. After recovery from anesthesia, the animals were returned to their home cages. Bladders were voided manually twice a day until normal function returned. Over the course of postsurgery, all animals were monitored carefully for any distress, pain, and discomfort until they were alert, mobile, and could freely access to food and water.

5.4. Hydrogen (H_2) gas treatment

To administer H_2 gas to anesthetized animals, H_2 gas was supplied through a gas flowmeter, TF-1 (YUTAKA Engineering Corp., Tokyo, Japan) to the anesthetic gas (sevoflurane (1.5%) in N_2/O_2 (70/30) gas; flow rate of 1 L/min) from 10 min before reperfusion to 60 min after reperfusion (total 70 min). The concentration of H_2 gas in the anesthetic gas was continuously monitored with a commercially available detector

(Hy Alerta Handheld Detector Model 500, H2 Scan, Valencia, CA, USA) and maintained at the predetermined level during the treatment (Xie et al., 2010a, b).

5.5. Experimental Protocols

This study consisted of two experiments. Experiment 1 was designed to determine whether H_2 inhalation with different concentrations could attenuate spinal cord injury caused by I/R in rabbits. Experiment 2 was undertaken to elucidate whether the beneficial effects of H_2 treatment were associated with the reduction of apoptosis, oxidative stress and inflammation.

Experiment 1. 32 rabbits were randomly divided into 4 groups (n = 8 each group): control (Con), 1% H_2, 2% H_2 and 4% H_2 groups. The animals in all groups were exposed to spinal cord I/R operation. The animals in control group underwent only I/R surgery without H_2 treatment. The animals in the 1% H_2, 2% H_2 or 4% H_2 groups were exposed to H_2 inhalation with 1%, 2% or 4% concentration, respectively, from 10 min before reperfusion to 60 min after reperfusion (total 70 min). Arterial blood gases and blood glucose were measured using a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy) at preischemia, 10 min before reperfusion, and 60 min after reperfusion, respectively. The neurological assessment was taken at 24 h, 48 h and 72 h after reperfusion. After the last neurological assessment, we detected the histopathology of spinal cord.

Experiment 2. Based on the above experiment, 18 animals were randomly divided into 3 groups (n = 6 each group): Sham, control (Con), and H_2 groups. According to the results of Experiment 1, 2% H_2 treatment was chosen in this experiment. The

animals in sham group underwent the same procedure without spinal cord I/R. The detailed experimental protocols in control and H_2 groups were the same as described above. The levels of 8-iso-PGF2α and MDA, the activities of SOD and CAT, as well as the levels of TNF-α and HMGB1 in serum were measured at preischemia, as well as 6 h, 12 h, 24 h, 48 h and 72 h after reperfusion. At 72 h after reperfusion, in addition, we detected the same parameters (8-iso-PGF2α, MDA, SOD, CAT, TNF-α and HMGB1), TUNEL staining and caspase-3 activity in spinal cord.

5.6. Neurological Assessment

In experiment 1, the hind-limb motor function of animals was assessed at 24 h, 48 h and 72 h after reperfusion by an independent observer, who was unknown of the grouping and experimental protocols. The modified Tarlov criteria were used: 0, no voluntary hind-limb function; 1, only perceptible joint movement; 2, active movement but unable to stand; 3, able to stand but unable to walk; 4, complete normal hind-limb motor function (Jacobs et al., 1992; Wang et al., 2009).

5.7. Hematoxylin and Eosin Staining

In experiment 1, a histopathologic evaluation of spinal cord was performed at 72 h after reperfusion. After the last neurological assessment, all animals were anesthetized with 40 mg/kg pentobarbital sodium. Transcardiac perfusion and fixation was performed with 1000 mL heparinized saline followed by 500 mL of 10% buffered formalin. The lumbar spinal cord (L4-6 segments) was removed and refrigerated in 10% phosphate-buffered formalin for 48 h. After dehydration in the graded ethanol, the spinal cord was embedded in paraffin. Coronal sections of the spinal cord (L4

segment) were cut at a thickness of 6 μm and stained with hematoxylin and eosin. Neuronal injury was evaluated with a light microscope (Nikon Instruments Inc, Melville, NY, USA) by a pathologist who was unaware of the grouping. Ischemic features of motor neurons were identified by shrunken cellular bodies, disappearance of Nissl granules, an intensely eosinophilic cytoplasm, and triangular and pyknotic nuclei. The remaining normal neurons in the ischemic ventral spinal cord of each animal were counted in three sections selected randomly from the rostral, middle, and caudal levels of the L4 segment and then averaged. The number of normal motor neurons per section in the anterior spinal cord (anterior to an imaginary line drawn through the central canal perpendicular to the vertical axis) was compared among these groups.

5.8. Sample collection

In Experiment 2, blood was collected from auricular vein at preischemia, as well as 6 h, 12 h, 24 h, 48 h, 72 h after reperfusion. The serum was separated by centrifugation at 3, 000 g for 15 min at 4 ℃, aliquoted, and stored at -80℃ until assayed. At 72 h after reperfusion, in addition, the lumbar spinal cord (L4-6 segments) of all animals were removed under anesthesia. Spinal cord samples were frozen in liquid nitrogen immediately after harvest and then stored at -80 ℃ until the following analysis. The tissue protein concentration was determined using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

5.9. 8-iso-PGF2α and MDA Levels in Serum and Spinal Cord

The serum and spinal cord samples obtained above were used for detecting the

levels 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 also measured by a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA) (Ji et al., ; Xie et al., ; Xie et al.). All standards and samples were run in duplicate.

5.10. SOD and CAT Activities in Serum and Spinal Cord

The serum and spinal cord samples obtained above were also used for detecting the antioxidant enzymatic activities (SOD and CAT). The activities of SOD and CAT were measured using commercial kits purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All spectrophotometric readings were performed by using a spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA) (Ji et al., ; Xie et al., ; Xie et al.). All assays were conducted in triplicate.

5.11. Inflammatory cytokines in Serum and Spinal Cord

The serum and spinal cord samples obtained above were also used in this test. The levels of TNF-α and HMGB1 were detected by specific ELISA kits (TNF-α, R&D Systems Inc., Minneapolis, Minnesota, USA; HMGB1, IBL, Hamburg, Germany) with a microplate reader (CA 94089, Molecular Devices, Sunnyvale, Canada) (Ji et al., ; Xie et al., ; Xie et al.). All standards and samples were run in duplicate.

5.12. TUNEL Staining in Spinal Cord

To detect DNA fragmentation in the nuclei of cells, we applied a TUNEL kit (Roche Diagnostics GmbH, Mannheim, Germany) for quantification of apoptosis as

described in our previous study. To determine the number of motor neurons that underwent apoptosis, two independent and blinded pathologists counted the motor neurons that were positive or negative in the TUNEL staining (Wang et al., 2009).

5.13 Caspase-3 Activity in Spinal Cord

The activity of caspase-3 was measured with caspase-3/CPP32 Fluorometric Assay Kit (BIOVISION Research Products. 980 Linda Vista Avenue, Mountain View, CA 94043 USA) (Cai et al., 2008). The spinal cord samples obtained above were also used in this test. All assays were run in duplicate.

5.14. Statistical Analysis

SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) was used to conduct the statistical analyses. All values, except for neurological scores, are presented as mean ± SEM. The physiologic data and the serum levels of 8-iso-PGF2α, MDA, SOD, CAT, TNF-α and HMGB1 were analyzed using two-way repeated-measures (time and group) analysis of variance followed by the *post hoc* Student-Newman-Keuls test. The scores of hind-limb motor function were analyzed with Kruskal-Wallis test followed by the Mann-Whitney *U* test with Bonferroni correction. The rest data were analyzed by one-way analysis of variance, and between-group differences were performed with LSD-*t* test. A *P* value of less than 0.05 was considered to be statistically significant.

Acknowledgments

This work was supported by research grants from the National Natural Science Foundation of China (30725039 and 81071533).

REFERENCES

Andersson, U., Wang, H., Palmblad, K., Aveberger, A.C., Bloom, O., Erlandsson-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H., Tracey, K.J., 2000. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J. Exp. Med. 192, 565-570.

Basu, S., Hellberg, A., Ulus, A.T., Westman, J., Karacagil, S., 2001. Biomarkers of free radical injury during spinal cord ischemia. FEBS Lett. 508, 36-38.

Beattie, M.S., Farooqui, A.A., Bresnahan, J.C., 2000. Review of current evidence for apoptosis after spinal cord injury. J. Neurotrauma. 17, 915-925.

Cai, J., Kang, Z., Liu, W.W., Luo, X., Qiang, S., Zhang, J.H., Ohta, S., Sun, X., Xu, W., Tao, H., Li, R., 2008. Hydrogen therapy reduces apoptosis in neonatal hypoxia-ischemia rat model. Neurosci. Lett. 441, 167-172.

Cai, J., Kang, Z., Liu, K., Liu, W., Li, R., Zhang, J.H., Luo, X., Sun, X., 2009. Neuroprotective effects of hydrogen saline in neonatal hypoxia-ischemia rat model. Brain Res. 1256, 129-137.

Di Bartolomeo, R., Di Marco, L., Armaro, A., Marsilli, D., Leone, A., Pilato, E., Pacini, D., 2009. Treatment of complex disease of the thoracic aorta: the frozen elephant trunk technique with the E-vita open prosthesis. Eur. J. Cardiothorac Surg. 35, 671-676.

Ding, Q., Wang, Q., Deng, J., Gu, Q., Hu, S., Li, Y., Su, B., Zeng, Y., Xiong, L., 2009. Sevoflurane preconditioning induces rapid ischemic tolerance against spinal

cord ischemia/reperfusion through activation of extracellular signal-regulated kinase in rabbits. Anesth. Analg. 109, 1263-1272.

Dong, H., Xiong, L., Zhu, Z., Chen, S., Hou, L., Sakabe, T., 2002. Preconditioning with hyperbaric oxygen and hyperoxia induces tolerance against spinal cord ischemia in rabbits. Anesthesiology. 96, 907-912.

Ege, E., Ilhan, A., Gurel, A., Akyol, O., Ozen, S., 2004. Erdosteine ameliorates neurological outcome and oxidative stress due to ischemia/reperfusion injury in rabbit spinal cord. Eur. J. Vasc. Endovasc. Surg. 28, 379-386.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495-516.

Etz, C.D., Luehr, M., Kari, F.A., Bodian, C.A., Smego, D., Plestis, K.A., Griepp, R.B., 2008. Paraplegia after extensive thoracic and thoracoabdominal aortic aneurysm repair: does critical spinal cord ischemia occur postoperatively? J. Thorac. Cardiovasc. Surg. 135, 324-330.

Fukuda, K., Asoh, S., Ishikawa, M., Yamamoto, Y., Ohsawa, I., Ohta, S., 2007. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. Biochem. Biophys. Res. Commun. 361, 670-674.

Genovese, T., Cuzzocrea, S., 2008. Role of free radicals and poly (ADP-ribose) polymerase-1 in the development of spinal cord injury: new potential therapeutic targets. Curr. Med. Chem. 15, 477-487.

George, J.F., Agarwal, A., 2010. Hydrogen: another gas with therapeutic potential.

Kidney Int. 77, 85-87.

Gonzalez, R., Glaser, J., Liu, M.T., Lane, T.E., Keirstead, H.S., 2003. Reducing inflammation decreases secondary degeneration and functional deficit after spinal cord injury. Exp. Neurol. 184, 456-463.

Hamann, K., Shi, R., 2009. Acrolein scavenging: a potential novel mechanism of attenuating oxidative stress following spinal cord injury. J. Neurochem. 111, 1348-1356.

Hasturk, A., Atalay, B., Calisaneller, T., Ozdemir, O., Oruckaptan, H., Altinors, N., 2009. Analysis of serum pro-inflammatory cytokine levels after rat spinal cord ischemia/reperfusion injury and correlation with tissue damage. Turk. Neurosurg. 19, 353-359.

Hayashi, T., Sakurai, M., Abe, K., Sadahiro, M., Tabayashi, K., Itoyama, Y., 1998. Apoptosis of motor neurons with induction of caspases in the spinal cord after ischemia. Stroke. 29, 1007-1013.

Hayashida, K., Sano, M., Ohsawa, I., Shinmura, K., Tamaki, K., Kimura, K., Endo, J., Katayama, T., Kawamura, A., Kohsaka, S., Makino, S., Ohta, S., Ogawa, S., Fukuda, K., 2008. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. Biochem. Biophys. Res. Commun. 373, 30-35.

Huang, C.S., Kawamura, T., Toyoda, Y., Nakao, A., Recent advances in hydrogen research as a therapeutic medical gas. Free Radic. Res. 44, 971-982.

Jacobs, T.P., Kempski, O., McKinley, D., Dutka, A.J., Hallenbeck, J.M., Feuerstein, G.,

1992. Blood flow and vascular permeability during motor dysfunction in a rabbit model of spinal cord ischemia. Stroke. 23, 367-373.

Ji, X., Liu, W., Xie, K., Qu, Y., Chao, X., Chen, T., Zhou, J., Fei, Z., 2010. Beneficial effects of hydrogen gas in a rat model of traumatic brain injury via reducing oxidative stress. Brain Res. 1354, 196-205.

Kaufmann, S.H., Lee, S.H., Meng, X.W., Loegering, D.A., Kottke, T.J., Henzing, A.J., Ruchaud, S., Samejima, K., Earnshaw, W.C., 2008. Apoptosis-associated caspase activation assays. Methods. 44, 262-272.

Kim, J.B., Sig Choi, J., Yu, Y.M., Nam, K., Piao, C.S., Kim, S.W., Lee, M.H., Han, P.L., Park, J.S., Lee, J.K., 2006. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J. Neurosci. 26, 6413-6421.

Kumar, A., Panigrahi, I., Basu, S., Dash, D., 2008. Urinary malondialdehyde levels in newborns following delivery room resuscitation. Neonatology. 94, 96-99.

Kuniyoshi, Y., Koja, K., Miyagi, K., Shimoji, M., Uezu, T., Arakaki, K., Yamashiro, S., Mabuni, K., Senaha, S., Nakasone, Y., 2003. Prevention of postoperative paraplegia during thoracoabdominal aortic surgery. Ann. Thorac. Surg. 76, 1477-1484.

MacArthur, R.G., Carter, S.A., Coselli, J.S., LeMaire, S.A., 2005. Organ protection during thoracoabdominal aortic surgery: rationale for a multimodality approach. Semin. Cardiothorac. Vasc. Anesth. 9, 143-149.

Mao, Y.F., Zheng, X.F., Cai, J.M., You, X.M., Deng, X.M., Zhang, J.H., Jiang, L., Sun,

X.J., 2009. Hydrogen-rich saline reduces lung injury induced by intestinal ischemia/reperfusion in rats. Biochem. Biophys. Res. Commun. 381, 602-605.

Moore, W.M., Jr., Hollier, L.H., 1991. The influence of severity of spinal cord ischemia in the etiology of delayed-onset paraplegia. Ann. Surg. 213, 427-432.

Nie, H., Xiong, L., Lao, N., Chen, S., Xu, N., Zhu, Z., 2006. Hyperbaric oxygen preconditioning induces tolerance against spinal cord ischemia by upregulation of antioxidant enzymes in rabbits. J. Cereb. Blood Flow Metab. 26, 666-674.

Ohsawa, I., Ishikawa, M., Takahashi, K., Watanabe, M., Nishimaki, K., Yamagata, K., Katsura, K., Katayama, Y., Asoh, S., Ohta, S., 2007. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat. Med. 13, 688-694.

Ohta, S., 2008. Hydrogen gas and hydrogen water act as a therapeutic and preventive antioxidant with a novel concept. Nippon Ronen Igakkai Zasshi. 45, 355-362.

Perry, S.W., Epstein, L.G., Gelbard, H.A., 1997. Simultaneous in situ detection of apoptosis and necrosis in monolayer cultures by TUNEL and trypan blue staining. Biotechniques. 22, 1102-1106.

Prow, N.A., Irani, D.N., 2008. The inflammatory cytokine, interleukin-1 beta, mediates loss of astroglial glutamate transport and drives excitotoxic motor neuron injury in the spinal cord during acute viral encephalomyelitis. J. Neurochem. 105, 1276-1286.

Reece, T.B., Okonkwo, D.O., Ellman, P.I., Maxey, T.S., Tache-Leon, C., Warren, P.S.,

Laurent, J.J., Linden, J., Kron, I.L., Tribble, C.G., Kern, J.A., 2006. Comparison of systemic and retrograde delivery of adenosine A2A agonist for attenuation of spinal cord injury after thoracic aortic cross-clamping. Ann. Thorac. Surg. 81, 902-909.

Sakamoto, M., Takaki, E., Yamashita, K., Watanabe, K., Tabuchi, S., Watanabe, T., Satoh, K., 2002. Nonenzymatic derived lipid peroxide, 8-iso-PGF2 alpha, participates in the pathogenesis of delayed cerebral vasospasm in a canine SAH model. Neurol. Res. 24, 301-306.

Sato, Y., Kajiyama, S., Amano, A., Kondo, Y., Sasaki, T., Handa, S., Takahashi, R., Fukui, M., Hasegawa, G., Nakamura, N., Fujinawa, H., Mori, T., Ohta, M., Obayashi, H., Maruyama, N., Ishigami, A., 2008. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. Biochem. Biophys. Res. Commun. 375, 346-350.

Satpute, R.M., Kashyap, R.S., Deopujari, J.Y., Purohit, H.J., Taori, G.M., Daginawala, H.F., 2009. Protection of PC12 cells from chemical ischemia induced oxidative stress by Fagonia arabica. Food Chem. Toxicol. 47, 2689-2695.

Sha, Y., Zmijewski, J., Xu, Z., Abraham, E., 2008. HMGB1 develops enhanced proinflammatory activity by binding to cytokines. J. Immunol. 180, 2531-2537.

Sharifi, A.M., Eslami, H., Larijani, B., Davoodi, J., 2009. Involvement of caspase-8, -9, and -3 in high glucose-induced apoptosis in PC12 cells. Neurosci. Lett. 459,

47-51.

Takenaga, M., Ohta, Y., Tokura, Y., Hamaguchi, A., Nakamura, M., Okano, H., Igarashi, R., 2006. Lecithinized superoxide dismutase (PC-SOD) improved spinal cord injury-induced motor dysfunction through suppression of oxidative stress and enhancement of neurotrophic factor production. J. Control Release. 110, 283-289.

Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J.M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., Manogue, K.R., Faist, E., Abraham, E., Andersson, J., Andersson, U., Molina, P.E., Abumrad, N.N., Sama, A., Tracey, K.J., 1999. HMG-1 as a late mediator of endotoxin lethality in mice. Science. 285, 248-251.

Wang, Q., Ding, Q., Zhou, Y., Gou, X., Hou, L., Chen, S., Zhu, Z., Xiong, L., 2009. Ethyl pyruvate attenuates spinal cord ischemic injury with a wide therapeutic window through inhibiting high-mobility group box 1 release in rabbits. Anesthesiology. 110, 1279-1286.

Xie, K., Yu, Y., Pei, Y., Hou, L., Chen, S., Xiong, L., Wang, G., 2010a. Protective effects of hydrogen gas on murine polymicrobial sepsis via reducing oxidative stress and HMGB1 release. Shock. 34, 90-97.

Xie, K., Yu, Y., Zhang, Z., Liu, W., Pei, Y., Xiong, L., Hou, L., Wang, G., 2010b. Hydrogen gas improves survival rate and organ damage in zymosan-induced generalized inflammation model. Shock. 34, 495-501.

Xu, W., Chi, L., Xu, R., Ke, Y., Luo, C., Cai, J., Qiu, M., Gozal, D., Liu, R., 2005.

Increased production of reactive oxygen species contributes to motor neuron death in a compression mouse model of spinal cord injury. Spinal Cord. 43, 204-213.

Yune, T.Y., Lee, S.M., Kim, S.J., Park, H.K., Oh, Y.J., Kim, Y.C., Markelonis, G.J., Oh, T.H., 2004. Manganese superoxide dismutase induced by TNF-beta is regulated transcriptionally by NF-kappaB after spinal cord injury in rats. J. Neurotrauma. 21, 1778-1794.

Zheng, X., Mao, Y., Cai, J., Li, Y., Liu, W., Sun, P., Zhang, J.H., Sun, X., Yuan, H., 2009. Hydrogen-rich saline protects against intestinal ischemia/reperfusion injury in rats. Free Radic. Res. 43, 478-484.

FIGURE LEGENDS

Fig. 1. H_2 treatment improved the hind-limb motor function scores of rats at 24 h, 48 h, and 72 h after reperfusion. All animals were exposed to spinal cord I/R operation. The animals in the 1% H_2, 2% H_2 or 4% H_2 groups were exposed to H_2 inhalation with 1%, 2% or 4% concentration, respectively, from 10 min before reperfusion to 60 min after reperfusion (total 70 min). Neurological function scores were assessed at 24 h, 48 h and 72 h after reperfusion by an independent observer. Each symbol represents data for one rabbit (bar = median) (n = 8 per group). *P < 0.05 vs. Control (Con) group.

Fig. 2. H_2 treatment attenuated the histopathologic damage of spinal cord at 72 h after reperfusion. (A-D) Representative micrographs of hematoxylin and eosin staining in the ventral horn of spinal cord of L4 segments at 72 h after reperfusion (original magnification, ×200). (E) The quantitative analysis of number of normal motor neurons in the anterior horn of spinal cord of L4 segments. Data are expressed as means ± SEM (n = 8 per group). *P < 0.05 vs. Control (Con) group.

Fig. 3. H_2 treatment reduced the levels of MDA (A and B) and 8-iso-PGF2α (C and D) in serum and spinal cord. Based on the above results, 2% H_2 treatment was chosen in this experiment. The levels of MDA and 8-iso-PGF2α in serum were measured at preischemia (BL, baseline), as well as 6 h, 12 h, 24 h, 48 h and 72 h after reperfusion. The levels of MDA and 8-iso-PGF2α in spinal cord were detected at 72 h after reperfusion. Data are expressed as

means ± SEM (n = 6 per group). *P < 0.05 vs. Sham group; $^{\dagger}P$ < 0.05 vs. Control (Con) group.

Fig. 4. H_2 treatment upregulated the activities of SOD (A and B) and CAT (C and D) in serum and spinal cord. The activities of SOD and CAT in serum were measured at preischemia (BL, baseline), as well as 6 h, 12 h, 24 h, 48 h and 72 h after reperfusion. The activities of SOD and CAT in spinal cord were detected at 72 h after reperfusion. Data are expressed as means ± SEM (n = 6 per group). *P < 0.05 vs. Sham group; $^{\dagger}P$ < 0.05 vs. Control (Con) group.

Fig. 5. H_2 treatment reduced the levels of TNF-α (A and B) and HMGB1 (C and D) in serum and spinal cord. The levels of TNF-α and HMGB1 in serum were measured at preischemia (BL, baseline), as well as 6 h, 12 h, 24 h, 48 h and 72 h after reperfusion. The levels of TNF-α and HMGB1 in spinal cord were detected at 72 h after reperfusion. Data are expressed as means ± SEM (n = 6 per group). *P < 0.05 vs. Sham group; $^{\dagger}P$ < 0.05 vs. Control (Con) group.

Fig. 6. H_2 treatment reduced the motor neuron apoptosis of spinal cord at 72 h after reperfusion. The motor neuron apoptosis was detected by TUNEL staining (A) and caspase-3 activity (B). For quantitative measurement, the number of motor neurons that were positive for TUNEL staining was recorded in each specimen in a blind fashion. Data are expressed as means ± SEM (n = 6 per group). *P < 0.05 vs. Sham group; $^{\dagger}P$ < 0.05 vs. Control (Con) group. N.D. = not detected.

TABLE LEGENDS

Table 1. Physiologic parameters of spinal cord I/R-challenged rabbits with H_2 treatment. All animals were exposed to spinal cord I/R operation. The animals in the 1% H_2, 2% H_2 or 4% H_2 groups were exposed to H_2 inhalation with 1%, 2% or 4% concentration, respectively, from 10 min before reperfusion to 60 min after reperfusion (total 70 min). Arterial blood gases and blood glucose were measured using a GEM Premier 3000 gas analyzer at preischemia, 10 min before reperfusion and 60 min after reperfusion, respectively. Data are expressed as means ± SEM (n = 6 per group).

Figure 1

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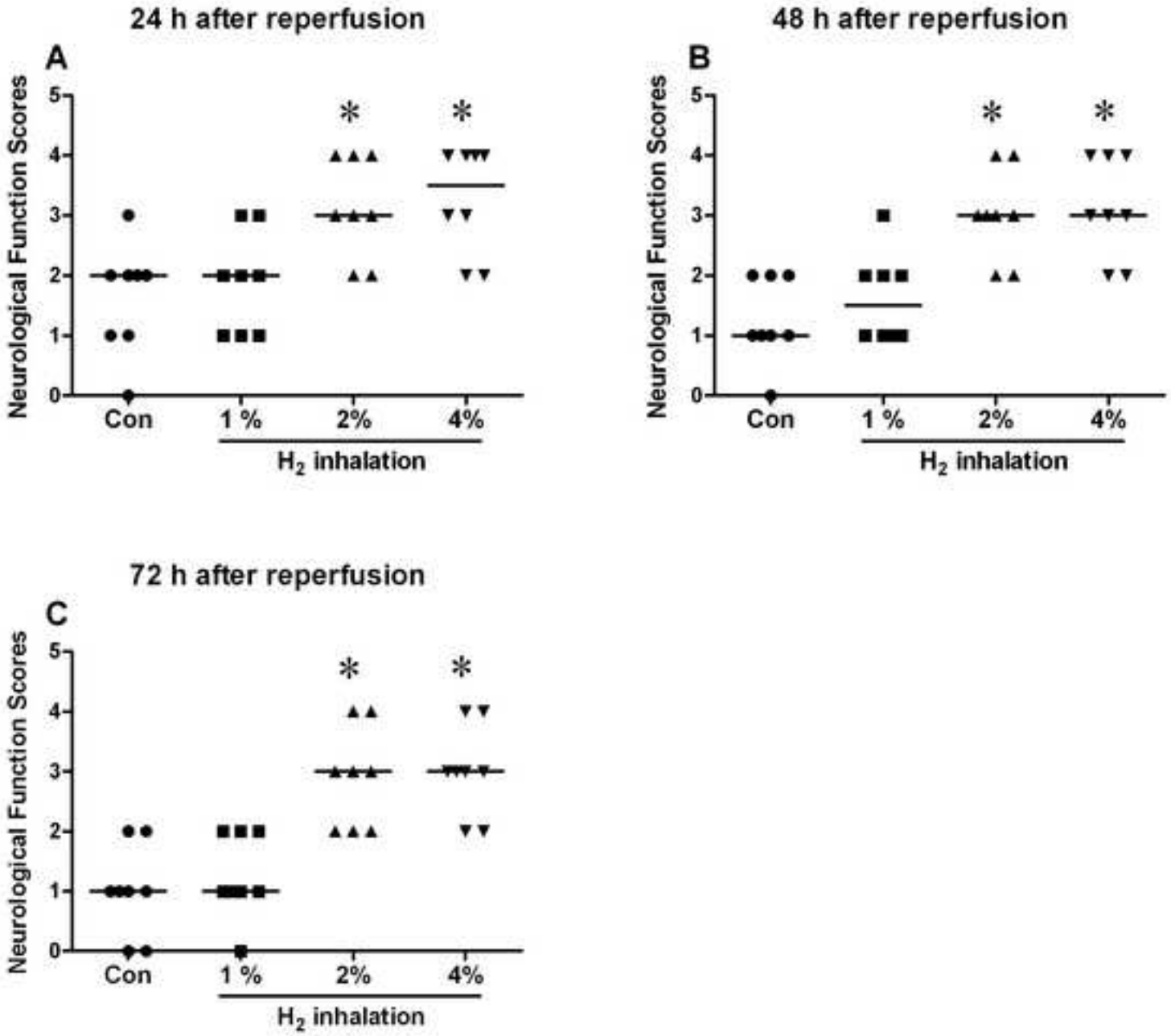

Figure 2

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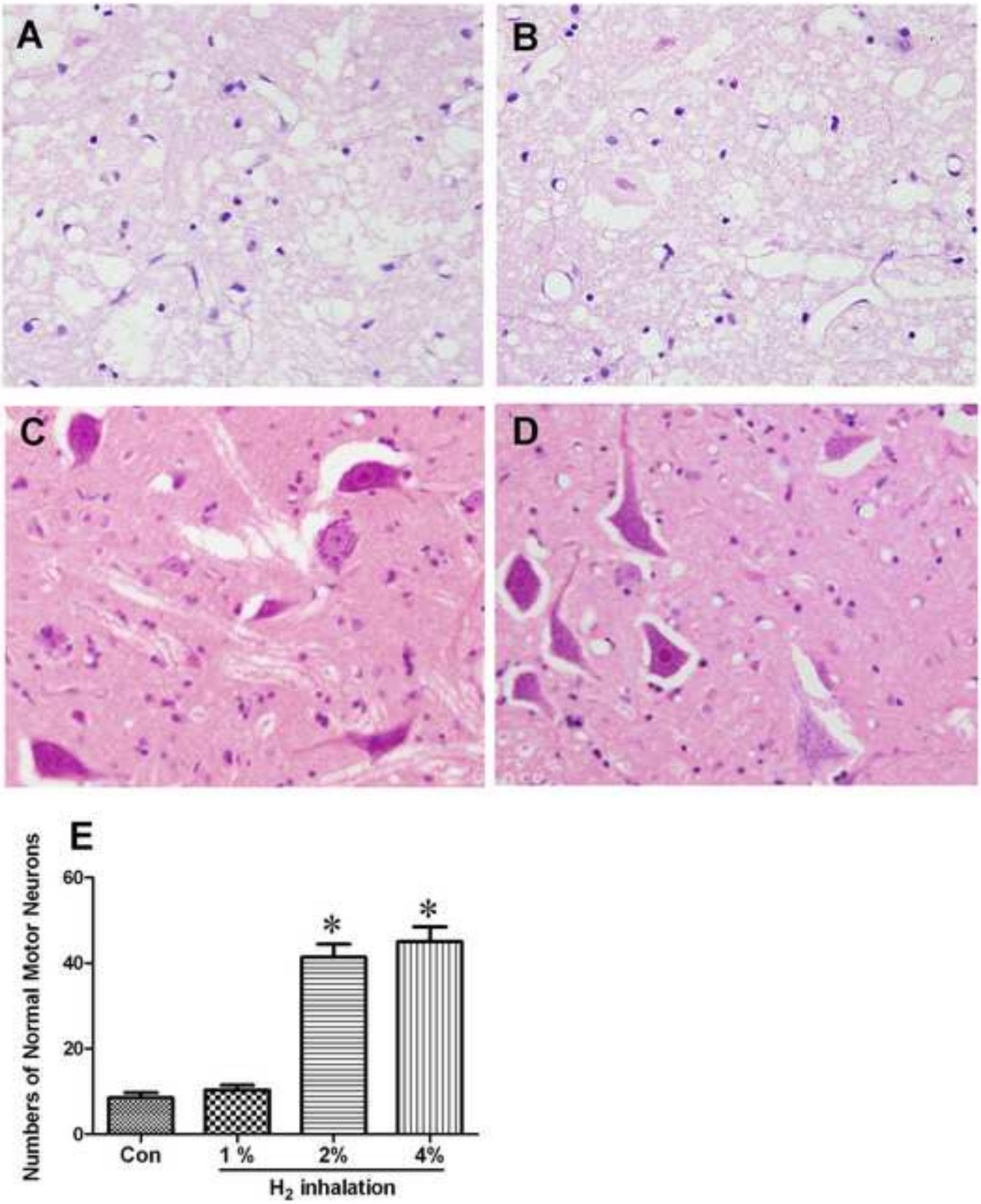

Figure 3

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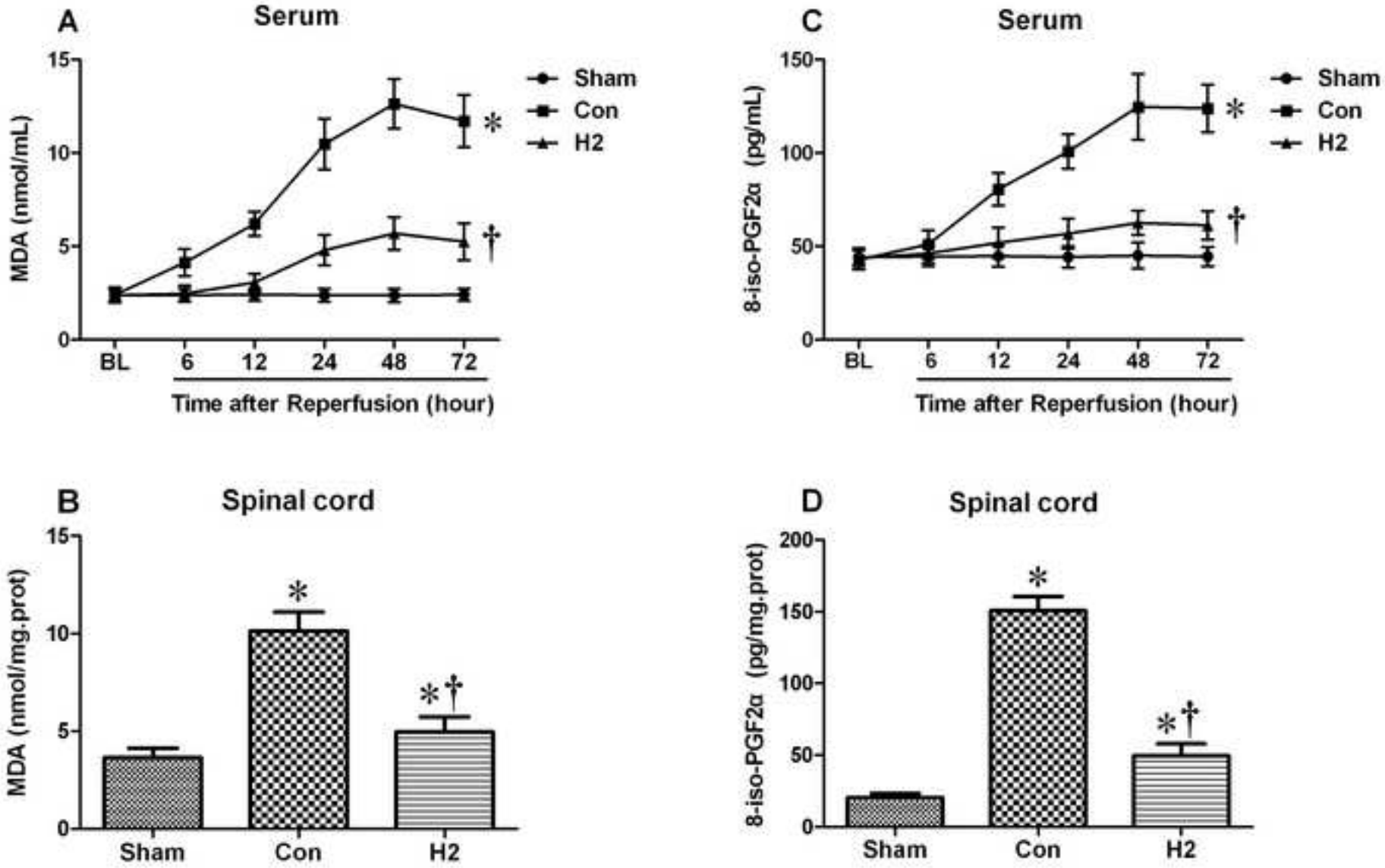

Figure 4

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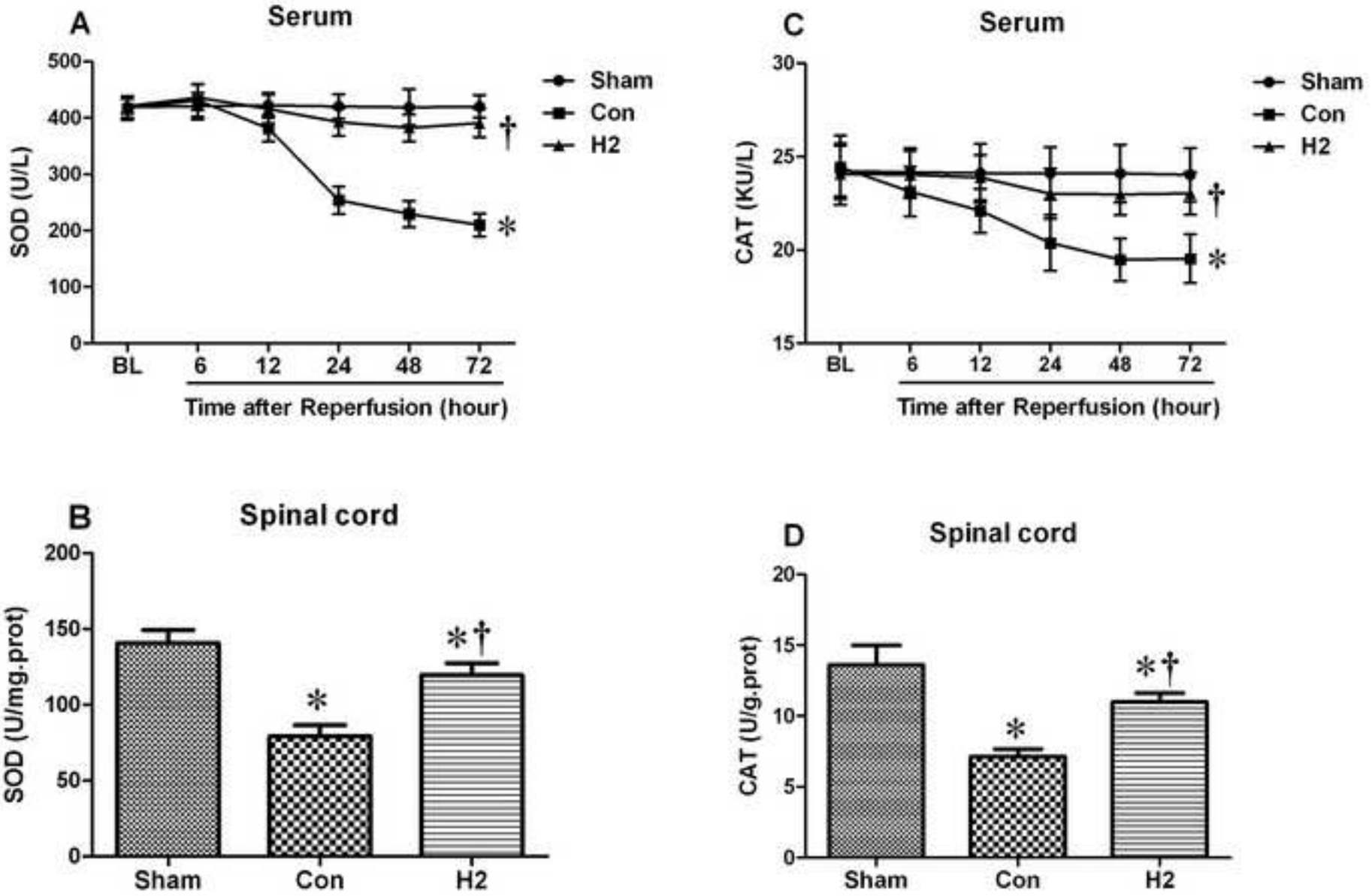

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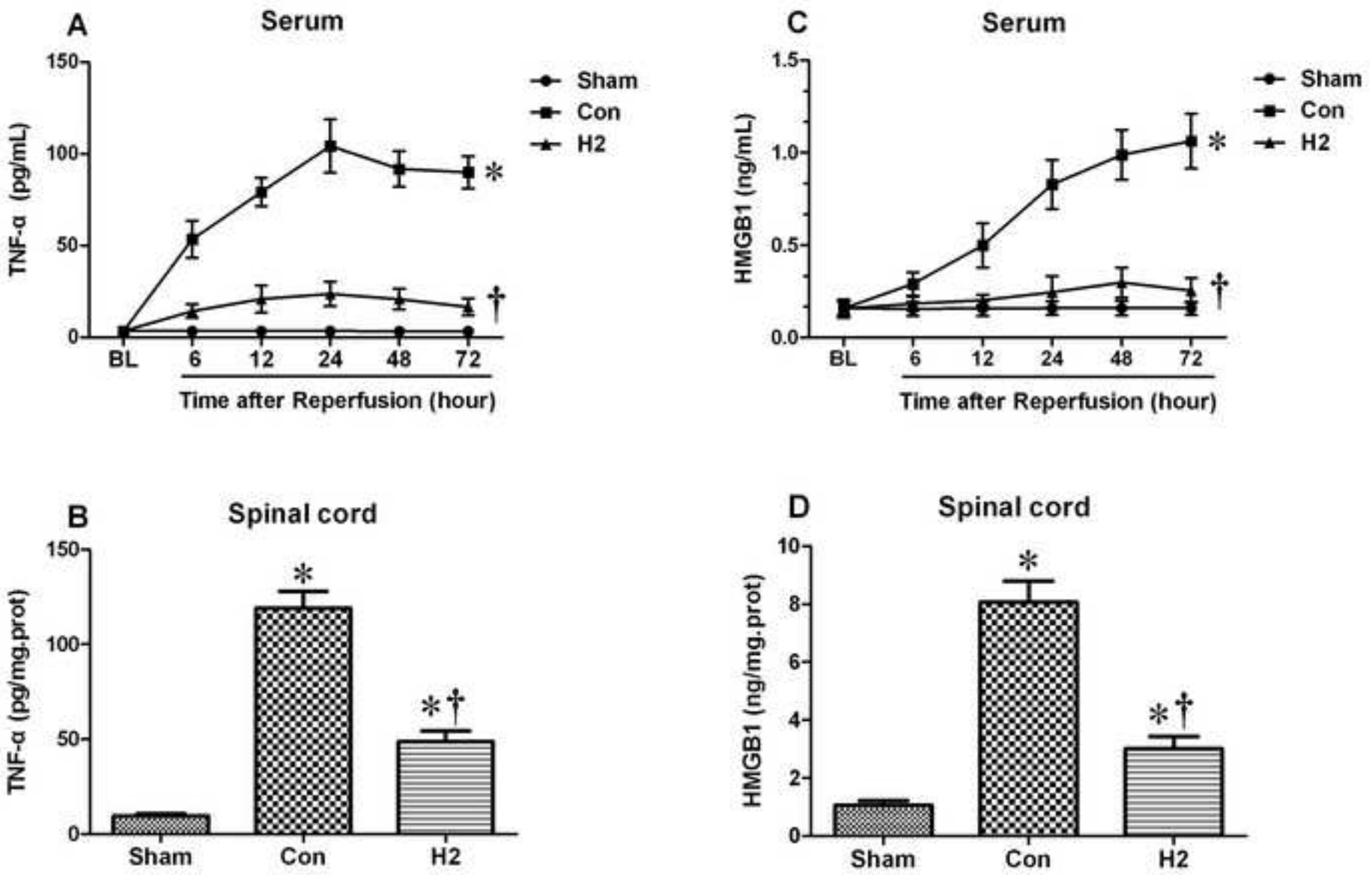

Figure 6

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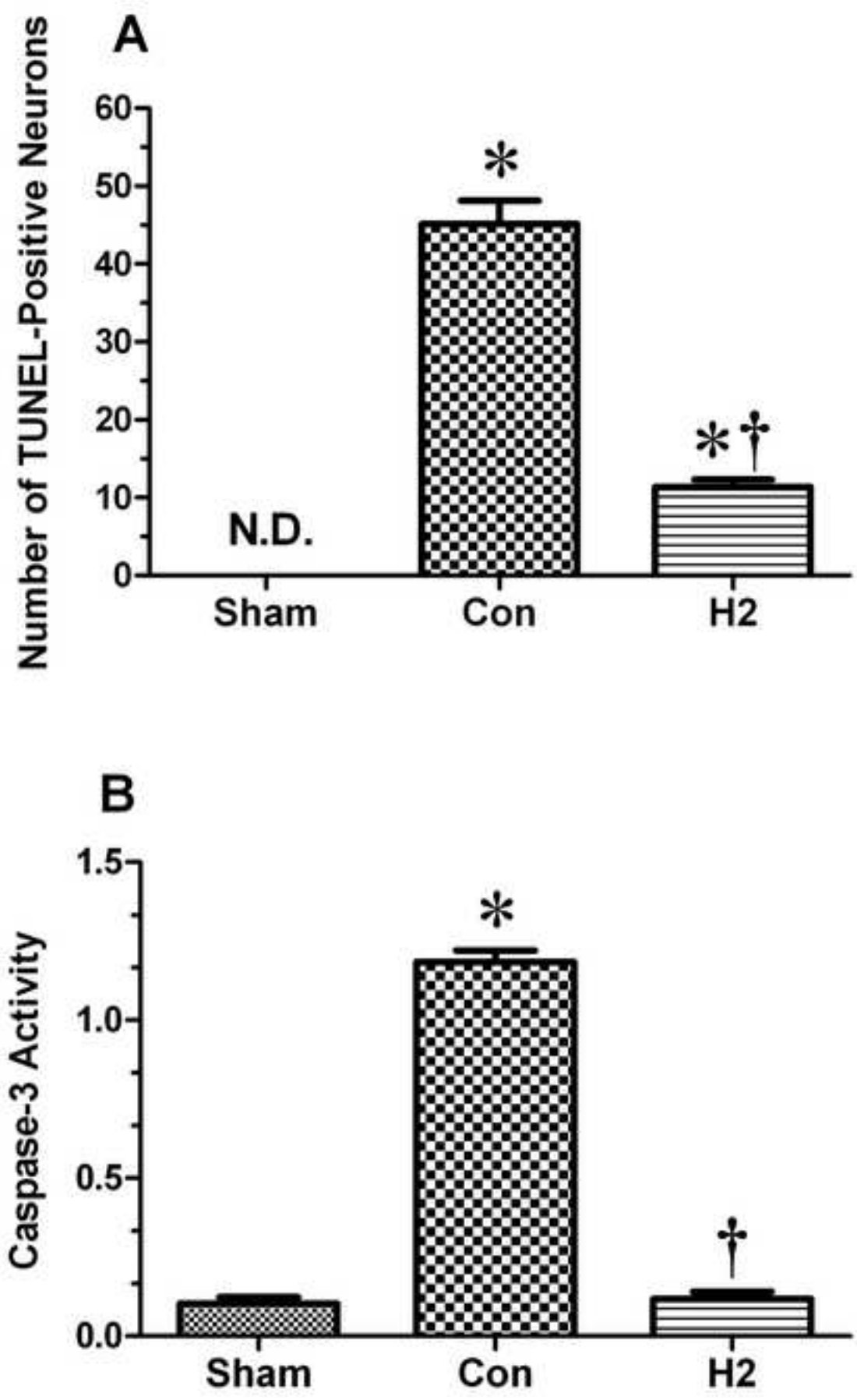

Table 1

Table 1. Physiologic parameters of spinal cord I/R-challenged rabbits with H_2 treatment

	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	Glu (mg/dl)
Preischemia				
Con	7.42±0.02	36.3±2.3	110.6±8.5	141.4±7.5
1% H_2	7.40±0.03	35.5±1.9	106.9±9.2	138.5±6.9
2% H_2	7.41±0.02	35.8±2.7	109.3±8.7	140.3±7.3
4% H_2	7.41±0.02	36.7±2.5	112.5±9.3	139.7±6.8
10 min before reperfusion				
Con	7.40±0.02	35.5±2.2	105.2±7.9	141.4±7.3
1% H_2	7.39±0.02	34.6±2.1	110.3±8.6	142.3±7.1
2% H_2	7.42±0.02	35.2±2.3	108.5±8.7	145.5±7.8
4% H_2	7.41±0.02	34.4±2.5	111.4±9.8	141.6±6.9
60 min after reperfusion				
Con	7.40±0.02	35.2±2.5	108.5±7.8	150.5±8.3
1% H_2	7.41±0.02	34.7±1.8	109.2±8.5	148.6±7.9
2% H_2	7.39±0.03	35.4±2.3	112.4±8.6	153.1±8.5
4% H_2	7.39±0.02	34.5±2.4	110.1±8.3	149.8±7.4