Original Paper

Inhalation of Hydrogen of Different Concentrations Ameliorates Spinal Cord Injury in Mice by Protecting Spinal Cord Neurons from Apoptosis, Oxidative Injury and Mitochondrial Structure Damages

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Key Words

Hydrogen • Spinal cord injury • Neuron • Mitochondrial • Oxidative stress • Apoptosis

Abstract:

Background/Aims: Hydrogen selectively neutralizes reactive oxygen species (ROS) and ameliorates various ROS-induced injuries. Spinal cord injury (SCI) is a serious injury to the central nervous system, and secondary SCI is closely related to excessive ROS generation. We hypothesized that hydrogen inhalation ameliorates SCI, and the mechanism of action may be related to the protective effects of hydrogen against oxidative stress, apoptosis, and mitochondrial damage. **Methods:** Mechanically injured spinal cord neurons were incubated with different concentrations of hydrogen *in vitro*. Immunofluorescence staining and transmission electron microscopy were used to confirm the protective effects of hydrogen. ROS and related proteins were detected with dihydroethidium fluorescence staining, enzyme-linked immunosorbent assays, and western blotting. Terminal deoxynucleotidyl transferase dUTP nick end labeling assays, flow cytometry, and western blotting were used to detect neuronal apoptosis. ATP concentrations, Janus Green B staining, and mitochondrial permeability transition pore (mPTP) status were assessed to investigate mitochondrial damage. RNA sequencing was performed to screen potential target genes of hydrogen application. Hydrogen was administered to mice after spinal cord contusion injury was established for 42 days. The Basso Mouse Scale (BMS) and footprint analyses were used to assess locomotor functions, and immunofluorescence staining of the injured spinal cord segments was performed to detect oxidative stress status. **Results:** Spinal cord neurons were preserved by hydrogen administration after mechanical injury in a dose-dependent manner. ROS generation, oxidative stress injury-related markers,

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and the number of apoptotic neurons were significantly reduced after hydrogen treatment. The ATP production and mPTP function in injured neurons were preserved by hydrogen incubation. The expression levels of Cox8b, Cox6a2, Cox7a1, Hspb7, and Atp2a1 were inhibited by hydrogen treatment. BMS scores and the footprint assessment of mice with SCI were improved by hydrogen inhalation. **Conclusions:** Hydrogen inhalation (75%) ameliorated SCI *in vivo* and attenuated neuronal mechanical injuries *in vitro*, and its protective effect on spinal cord neurons was exerted in a dose-dependent manner. The underlying mechanisms included reducing ROS generation and oxidative stress, inhibiting neuronal apoptosis, and restoring mitochondrial construction and function. Cox8b, Cox6a2, Cox7a1, Hspb7, and Atp2a1 were identified as potential target genes of hydrogen treatment.

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Introduction

Hydrogen selectively neutralizes toxic reactive oxygen species (ROS) and ameliorates oxidative stress-related injuries [1]. Studies have demonstrated its potential applications in various diseases, such as stroke and myocardial infarction [2].

Spinal cord injury (SCI) leads to severe and irreversible loss of function and remains a serious health issue worldwide. After a primary injury occurs in the central nervous system (CNS), secondary injuries follow. Oxidative stress due to excessive ROS generation is a hallmark of the pathophysiology of SCI [3]. Alleviating oxidative stress may be a potential treatment option for many neurological disorders, including SCI [4, 5].

Hydrogen is reportedly effective in preventing neural injuries. Hydrogen-rich saline protected against SCI in rats [6] and against spinal cord ischemia/reperfusion (I/R) injury in rabbits [7]. Subarachnoid cavity injection promoted recovery [8]. Theoretically, higher concentrations of hydrogen produce better effects. We previously demonstrated that hydrogen inhalation ameliorated cerebral I/R injuries [9]. However, the effects of high-concentration hydrogen on SCI have not yet been reported.

The mechanism underlying the effective amelioration of neural injuries by hydrogen remains unclear. We hypothesized that hydrogen impacts small signaling molecules and signal transduction, consequently regulating gene expression and inhibiting oxidative stress, apoptosis, and mitochondrial dysfunction.

Materials and Methods

Gas production

The mixed gas consisting of H₂ and O₂ was produced by the AMS-H-01 hydrogen nebulizer (Asclepius, Shanghai, China), which was designed to produce hydrogen by electrolyzing water. Thermal trace GC ultra-gas chromatography (Thermo Fisher, MA, USA) was used to monitor the concentration of hydrogen gas in the box.

Primary spinal cord neuron culture

Primary spinal cord neuron was isolated and cultured as previously reported [10] with minor modifications. In brief, female mice 4 days after birth were sacrificed and spinal cords were harvested. After the meninges and dorsal root ganglia were digested with 2mg/ml papain at 37°C for 30 min. Fetal bovine serum was added to stop the digestion and cell suspension was collected for culture. Cells were cultured with neuron culture medium and culture medium was replaced with fresh medium every 2 days until they were then used after 1-week culture. Cells were divided into groups as follows: Control group, model group, 50% H₂ group, 65% H₂ group and 75% H₂ group.

Mechanical injury and hydrogen application

Mechanical neuron injury was caused by “#” shaped scratches with 0.5cm intervals by a plastic tip on the cultured neurons. After the injury, hydrogen was introduced to cultured cells for 4 hours.

The gas was stored in gas cylinders before experiment. Then hydrogen, oxygen and nitrogen were mixed and the hydrogen gas concentration (50%, 65%, 75%, (vol/vol)) was adjusted through a three-way connection and measured with Thermal trace GC ultra-gas chromatography (Thermo Fisher, MA, USA).

Microscopic observation and immunofluorescent staining

At 4h after injury, neurons were stained with fixed with 4% paraformaldehyde and then rinsed with PBS 3 times for 5 min. Then NSE (Neuron specific enolase), tubulin and phalloidin antibodies were added and incubated overnight. FITC and CY3 marked secondary antibodies were then added and incubated for 30 min. Neurons were observed under a fluorescent microscope and dendrites' length was measured. A confocal microscope was used to collect confocal images of spinal cord neurons.

At the 0h and 24h after the co-culture with hydrogen, a light microscope was used to observe the shape and activity of single cells.

Transmission electron microscopic (TEM) observation

After 24h of treatment, cells were collected (1×10^6) with 0.5% trypsin-EDTA and washed with PBS. After centrifugation, cells were fixed with 4% paraformaldehyde at room temperature for 30 min. Osmium tetroxide was added and incubated for 1h at room temperature. After several washes and gradient centrifugation, propylene oxide was added. Then pure Epon was added and incubated for several times. Thick sections were obtained with ultracut using diamond knife. Once cells were found on the thick section, the thickness of ultracut was changed to get thin sections (65-70 nm). The sections were stained with uranyl acetate and lead citrate for 3 min sequentially. Images were obtained from digital camera on TEM with identical magnification.

Reactive oxygen species (ROS) generation measurement

Cell concentration was adjusted to 5×10^5 cells/ml and inoculated to 96-well plates with 100 μ l in each well. Twenty-four hours after injury, 10 μ M DHE (Dihydroethidium) fluorescent probe was added and incubated for 20 min. Cells were observed under fluorescent microscopy.

Measurement of lipid peroxidation and protein oxidative

The 8-OHdG (8-hydroxy-2-deoxyguanosine) and MDA (malondialdehyde) were determined with enzyme-linked immunosorbent assay (ELISA) kits specific for murine 8-OHdG and MDA.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Primary neuron culture or spinal cord sections were labeled with DeadEnd™ Fluorometric TUNEL System following the manufacturer's manual. Then cells and spinal cord sections were observed under a fluorescent microscope.

Apoptosis measured by flow cytometry

Cell concentration was adjusted to 10^5 cells/ml and inoculated on 6-well plates. Twenty-four hours after injury and hydrogen incubation, cells were collected and prepared to cell suspension. Annexin V-FITC 5 μ l was added and incubated for 15 min. Then PI 10 μ l was added. Flow cytometry was performed and results were analyzed with CELL Quest software.

ATP assay

Cells (1×10^4) during log phase were collected and ATP concentrations in cell homogenates were measured by using the ATP determination kit from Invitrogen Life Technologies. The assay is based on luciferase's requirement for ATP in producing light (emission maximum 560 nm at pH 7.8). Luminescence was read on a Synergy 4 microplate reader and values were calculated based on an ATP standard curve.

Mitochondrial viability evaluation with Janus Green B (JG-B) assay

Cell concentration was adjusted to 5×10^4 cells/ml and inoculated to 24-well plates with 500 μ l in each well. Twenty-four hours after injury, the specialized mitochondrial staining, JG-B was used for mitochondrial viability assay [11]. JG-B (0.5%) was added for 1 min, and then neurons were washed with PBS. Cells were observed with an inverted microscope.

Assessment of Mitochondrial permeability transition pore (mPTP) opening by calcein loading/Co2+ Quenching

A well-characterized calcein-AM loading /Co2+ quenching technique [12] was used to measure the activity of mPTP, which was monitored directly in intact cells. Then the cells were observed with a confocal laser microscope.

Western blotting analysis

Protein levels of all samples were determined by the Bradford assay according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Crude supernatants or concentrated conditioned media were placed in each well. The following primary antibodies were used: anti-CAT (ab52477 1:1000); anti-CuZnSOD (ab13498 1:5000); anti-GST (ab19256 1:5000); anti-MnSOD (ab13533 1:5000); anti-Caspase-3 (ab90437 1:1000); anti-Bcl-2 (ab32124 1:1000); anti-Bax (ab32503 1:1000-1:10000); anti-cytochrome C (ab110325 1 µg/ml); anti-Prx3 (ab73349 1:1000); anti-Trx2 (ab185544 1:10000-1:50000); anti-Nox2 (ab80508 1:500); anti-Nox4 (ab133303 1:1000-1:5000); anti-GAPDH (1:5, 000; Abcam Inc.) antibody was used as protein loading control. Horseradish peroxidase-conjugated secondary antibodies (polyclonal antibody 1:10, 000 and monoclonal antibody 1: 5, 000) (Abcam) were used for the assay.

RNA-Sequencing (RNA-Seq)

RNeasy Micro Kit (Qiagen) was used to extract RNA from spinal cord neuron cells following manufacturer's instructions [13]. All libraries were sequenced as 36-base-length reads using the Genome Analyzer (GA II X; Illumina).

HTSeq v0.6.1 was used to count the read numbers mapped of each gene. And then RPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene [14].

The high-throughput sequencing data from this study have been submitted to the NCBI Sequence Read Archive (SRA) under accession number GSE94925.

Real-time PCR (RT-PCR) analysis

TRIzol reagent (Invitrogen Carlsbad, CA) was used to isolate total RNA for RT-PCR analysis. The mouse primer sequences for the experiment are shown as follows: Cox8b (Forward: AGTGGTCAGAGGACGTGCAG; Reverse: GTCGACTCCTTCCTTGGC), mt-Co3: (Forward: ACCTACCAAGGCCACCACAC; Reverse: AGGTCAGCAGCCTCCTAGATCA), Cox7a1: (Forward: AAAGTGCTGCACGTCCTTGG; Reverse: GACCCGTAGGCCCTCATTT), Nox4 (Forward: CTCACCCTCCTGGCTGCATT; Reverse: AACCTCGAGGCAAAGATCCA), Cox6a2 (Forward: AGAGCCTCTCGACTGGGTGA; Reverse: AGCCAGCACAAAGGTCAGGA), Ndufa412 (Forward: CTCCCTGCTCCTCCTGACTG; Reverse: CACGTCGGACACGCTCAAC), mt-Co2 (Forward: GGCACCAATGATACTGAAGCTACG; Reverse: TGGCAGAACGACTCGGTTATCA), Hspa1a (Forward: GACGAGGTCTCAAGGGCAA; Reverse: TGGCACTTGTCAGCACCTT), Hspa1b (Forward: GACGAGGTCTCAAGGGCAA; Reverse: TGGCACTTGTCAGCACCTT), Hspb7 (Forward: GGGACCTGTCTGCTCTCACC; Reverse: CCATTGAAGCCGCCACTCTG), Atp2a1 (Forward: AGCGAGACCACAGGCCTTAC; Reverse: TCAGCAGGGAGCTCATTGGG). The 2^{-ΔΔCT} was used to analyze data, and all values were normalized to the level of the house keeping gene, GAPDH.

Animals and compressive spinal cord injury model

Adult male C57BL/6 mice were employed in this study. The animal experiments were performed in accordance with the laboratory animal guidelines and were approved by the institutional animal care and use committee of the second military medical university. Animals were divided into three groups: sham group, model group and 75 % H2 group. The gas was inhaled immediately after surgery for 1h per day. Spinal cord injury was caused by a pair of specially modified forceps, which is the similar to those by Faulkner et al. [15]. Metal epoxy putty spacers were used to alter the distance between two blades to be 0.4mm when completely closed. The surgery procedure was as follows: In brief, after anesthesia with inhalant isoflurane mixed with carboxygen (95% O2/5% CO₂), midline skin was incised and the T9-T11 spinous processes were exposed. Laminectomy was performed, and spinal cord was exposed. Then the modified forceps were used to laterally compress the spinal cord to a thickness of 0.4 mm for 30s.

Neurological evaluation

The nine-point Basso Mouse Scale (BMS) [16] was used to evaluate the post-injury locomotor recovery at 1d, 3d, 5d, 7d, 14d, 21d, 35d, and 42d after surgery. Two independent assessors performed the BMS analysis of hindlimb movements and coordination. During the whole procedure, the assessors had no idea of the animal group information. After the assessment, the consensus score was taken.

Footprint Analysis

At 7d, 28d, and 42d after injury, gait patterns of the mice were examined with a white platform (5.2 cm wide and 60 cm long), which is surrounded with opaque, black walls. Paint the forelimbs blue and hind limbs red with colored ink before the mice were required to traverse the platform, so that the red or blue footprint will be recorded on the white platform. Stride length was defined as the average of the distance between ipsilateral hindprints. And the distance between bilateral feet (DBF) was also measured to evaluate the holding power of hind limbs.

Nissl and NSE, 8-OHdG, MPO immunohistochemical staining

After 3d, 7d, 14d, 28d, and 42d of hydrogen inhalation procedure, the spinal cord samples were collected for pathological examination. The mice were perfused with physiologically saline and 4% paraformaldehyde before the spinal cord samples were taken out of the animals' body. Then the spinal cord samples were immersed in 4% paraformaldehyde for 48 hours before the paraffin block procedure. The injured segments were taken to prepare 5- μ m sections for Nissl and immunohistochemical staining with 1% toluidine blue and anti-NSE, anti-MPO, anti-8-OHdG respectively according to the protocol of the manufacturer.

Statistics

All quantitative data are expressed as mean $\pm$ SD. The software SPSS Statistics 22.0 (SPSS, Chicago, IL) was used to perform the statistical analysis. One-way ANOVA (analysis of variance) was used to verify the differences between groups. $P \leq 0.05$ was regarded as statistically significant.

Results

Hydrogen protects spinal cord neurons from mechanical injury in vitro

To confirm the protective effects of hydrogen incubation on spinal cord neurons, cells were observed after FITC and CY3 fluorescence staining. Based on fluorescence microscopy observations (Fig. 1A), the number of neurons in the model group significantly decreased after mechanical injury. Hydrogen incubation promoted neuronal migration and increased the number of neurons after mechanical injury. These

Fig. 1. Hydrogen protect spinal cord neurons in vitro. A. Spinal cord injury cell were cultured with 50%, 65%, and 75% hydrogen for 4h after mechanical injury. FITC (green) and CY3 (red) fluorescent staining was used to reflect microfilament and microtubules, which served to observe the number of neurons. B. FITC fluorescent staining and confocal microscope were used to observe the shape and structure of neurons. C. Light microscope image of single neurons at 0h and 24h after injury. D. TEM was used to observe the mitochondrial structure. Arrows point to mitochondria. * $P < 0.05$, ** $P < 0.01$ vs hydrogen-untreated controls and # $P < 0.05$, ## $P < 0.01$ between model group and hydrogen-treated group.

effects of hydrogen incubation grew more significant at higher concentrations, and the length of dendrites also increased; therefore, we chose a 75% hydrogen concentration as the treatment dose for further research. Confocal microscopy images (Fig. 1B) showed that the control group neurons had normal dendrites and clear, intact microtubule structures. Mechanical injury decreased the number of neurons in the model group and damaged the normal structure of the neurons, whereas administration of 75% hydrogen protected against these effects in the H₂ group. Single neurons in the control group died and lost their synaptic structure after 24 h; however, with hydrogen treatment, single neurons in the 75% H₂ group survived and maintained their synaptic structure after 24 h (Fig. 1C). Transmission electron microscopy was used to observe neuronal mitochondria after mechanical injury (Fig. 1D). Neurons in the control group had normal, clear mitochondrial structures, whereas mechanical injury damaged the mitochondrial structures of neurons in the model group. These negative impacts of mechanical injury were rescued by hydrogen treatment in the 75% H₂ group.

Fig. 2. Hydrogen inhibits oxidative stress of neuron mechanical injury. A. ROS generation was measured with the ROS fluorescent probe-DHE. B. Ratio of DHE fluorescence positive neurons number compared to control group. C and D. Elisa results of 8-OHdG and MDA concentration. E-M. Western-blot and optical density analysis of expression of Nox2, Nox4, PrxIII, Catlase, SOD2, SOD1, and Trx2. *P<0.05, **P<0.01 vs hydrogen-untreated controls and #P<0.05, ##P<0.01 between model group and hydrogen-treated group.

Hydrogen reduces oxidative stress of neurons

The ROS fluorescent probe dihydroethidium (DHE) was used to measure ROS generation (Fig. 2A). The ratios of DHE fluorescence-positive cells were calculated and compared between the treatment groups and the control group (Fig. 2B). The ratio was significantly lower in the hydrogen treatment group than in the model group. The concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) were measured with enzyme-linked immunosorbent assay (ELISA) (Figs. 2C, 2D). Hydrogen treatment significantly decreased the concentrations of 8-OHdG and MDA in the H₂ group compared to the model group. Western blotting was used to measure the concentrations of NADPH oxidase (NOX)2, NOX4, peroxiredoxin III (PrxIII), catalase, superoxide dismutase (SOD)2, SOD1, and thioredoxin 2 (Trx2) (Figs. 2E, 2H). According to the western blot results, NOX2 and NOX4 expression levels were significantly lower in the H₂ treatment group than in the model group (Figs. 2F, 2G), and PrxIII, catalase, SOD2, SOD1, and Trx2 levels were significantly higher in the H₂ treatment group than in the model group (Figs. 2I-M).

Hydrogen prevents spinal cord neuron apoptosis after mechanical injury

To confirm the effects of hydrogen in preventing spinal cord neuron apoptosis after mechanical injury, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays (Fig. 3A), western blotting (Fig. 3C), and flow cytometry (Fig. 3G) were performed. There was a significant increase in TUNEL-positive neurons after mechanical injury, but H_2 treatment decreased the number of apoptotic neurons (Fig. 3B). Based on the western blotting results, there were no significant differences in the expression levels of caspase-3, Bax, and Bcl-2 between neurons in the model group and the H_2 group 4 h after injury. However, H_2 treatment decreased the expression of caspase-3 and Bax, which were elevated after mechanical injury to spinal cord neurons. At the same time, Bcl-2 was preserved by hydrogen treatment after injury (Figs. 3D-F). The results of apoptosis cytometry indicated that the rate of neuronal apoptosis significantly decreased with H_2 treatment (Fig. 3H).

Fig. 3. Hydrogen prevents spinal cord neurons apoptosis after mechanical injury. A. TUNEL staining of spinal cord neurons. B. TUNEL positive nuclei ratio (vs total nuclei number). C. Western-blot of Caspase-3, Bcl-2, and Bax at 4h and 12h after injury. D, E, and F. Western-blot optical density analysis of expression of Caspase-3, Bcl-2, and Bax at 12h. G. Results of apoptosis neurons flow cytometry. H. The apoptosis rate counting results. * $P < 0.05$, ** $P < 0.01$ vs hydrogen-untreated controls and # $P < 0.05$, ## $P < 0.01$ between model group and hydrogen-treated group.

Hydrogen protects the mitochondrial function of spinal cord neurons after mechanical injury

Fig. 4A shows ATP concentration levels among the treatment groups. Cellular ATP levels were significantly lower in neurons in the model group compared to the control group; however, ATP levels increased after H_2 treatment. The results indicated that mechanical injury damaged mitochondrial function, leading to ATP loss in spinal cord neurons; however, H_2 treatment prevented the damage and ATP loss. We further investigated the opening status of the mPTP, which is one of the main mitochondrial regulators during cell death [17]. As shown in Fig. 4B, the distinct punctate fluorescence indicated that the neurons in the control group maintained the mPTP in a closed configuration, and the massive loss of fluorescence in the mitochondria indicated mPTP opening in the model group, which was blocked by hydrogen treatment. These results were supported by the Janus Green B staining results (Fig. 4C). Single mitochondria were clearly observed in the neurons in the control group, whereas mechanical injury damaged the mitochondrial structures in the model group, and hydrogen treatment protected neurons against the effects of mechanical injury in the H_2 group.

Fig. 4. Hydrogen protects mitochondrial of spinal cord neurons after mechanical injury. A. Cellular ATP concentration of neurons after injury with or without hydrogen treatment. B. Fluorescent images to observe the mPTP opening status. C. Results of JG-B staining of mitochondria. * $P < 0.05$, ** $P < 0.01$ vs hydrogen-untreated controls and # $P < 0.05$, ## $P < 0.01$ between model group and hydrogen-treated group.

Fig. 5. RNA sequencing (RNA-Seq) for spinal cord neurons of normal group (NG), mechanical injured group (IG), and treated group (TG). A. Biological replicates of RNA-Seq for neurons of 3 groups. B. The number of overlapping transcripts detected in each group was shown in Venn diagram. C. Principal component analysis score plot of PC1 versus PC2. D. Heatmap showing differences in gene expression of neurons in each group. E. Gene expression of secretory molecules with RT-PCR.

RNA sequencing (RNA-seq) of spinal cord neurons of different treatment groups after mechanical injury

Sequencing libraries were constructed from spinal cord neurons of the normal group (NG), injured group (IG), and treated group (TG) and sequenced on an Illumina Genome Analyzer IIX system. Mechanical injury was administered to IG neurons and TG neurons, and TG neurons were treated with 75% hydrogen as previously described 4 days before RNA-Seq. Very strong correlations were observed (Fig. 5A) in biological replicates between current samples and previous samples [13], which confirmed the high biological reproducibility of our experimental procedure. The overlapping transcripts among the three groups are shown in

a Venn diagram (Fig. 5B). To further investigate the effects of mechanical injury and hydrogen incubation on spinal cord neurons, we performed a principal component analysis (PCA). For principal component (PC) 1, the values of IG neurons and TG neurons were both higher than those of NG neurons (Fig. 5C). For PC2, the value of IG neurons was higher than that of NG neurons, whereas the value of TG neurons was lower. The heatmap revealed that mechanical injury significantly influenced the expression of oxidative stress-related genes, and hydrogen incubation ameliorated this regulation (Fig. 5D). Reverse transcription polymerase chain reaction (RT-PCR) analysis confirmed the RNA-Seq results (Fig. 5E), indicating that the expression levels of cytochrome c oxidase subunit VIIIb (Cox8b), Cox6a2, Cox7a1, heat shock protein family B member 7 (Hspb7), and sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (Atp2a1) were significantly upregulated after mechanical injury, and this up-regulation was ameliorated by hydrogen incubation. The series entry (GSE94925, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94925>) provides access to all data.

Hydrogen protects against spinal cord compression injury in mice

Morphological images of the mice in the three treatment groups 7 days after surgery are shown in Fig. 6A. Mice in the model group were curled up, with stiffened bodies, but hydrogen treatment relieved these SCI characters. Spinal cord segments were collected and observed (Fig. 6B), showing that the spinal cords of mice in the model group were swollen at the compressed spot, but the injury was relieved by hydrogen treatment. As shown in Fig. 6C, the Basso Mouse Scale (BMS) scores of mice in the model and hydrogen groups were significantly lower than those of mice in the sham group. The BMS scores increased over time, and the BMS scores of mice in the hydrogen group were significantly higher than those in the model group from 7 days after injury. The results of footprint analysis (Figs. 6D–F) corroborated these BMS results. The distance between hind limbs and the stride length both decreased after surgery, but the injuries recovered over time, and hydrogen treatment accelerated recovery.

Hydrogen protects mice against SCI by ameliorating oxidative stress

Nissl staining revealed the morphology of spinal cord neurons (Fig. 7A). The neurons of mice in the sham group had abundant Nissl bodies with normal, clear, intact structure and abundant cytoplasm. SCI damaged the normal structure of the neurons and reduced the number of Nissl bodies, as observed in the model group, but hydrogen treatment protected neurons against these effects, as observed in the H₂ group. Neuron-specific enolase (NSE) immunohistochemistry was used to detect neurons (Fig. 7B), indicating that neurons in the model group were damaged by SCI, whereas hydrogen treatment protected the neurons in the H₂ group. The 8-OHdG and MPO activities were measured with immunohistochemical staining (Figs. 7C, D). SCI increased the 8-OHdG and MPO activities in the model group,

Fig. 6. Hydrogen protects spinal cord compression injury in mice. Morphology pictures of mice in sham, model, and hydrogen group. B. Fresh spinal cord of mice in sham, model, and hydrogen group. C. BMS score of mice. D. Results of footprints. E and F. Distance between hind limbs and stride length. *P<0.05 between model group and hydrogen-treated group.

whereas hydrogen treatment inhibited this change.

Discussion

We confirmed that inhalation of different concentrations of hydrogen ameliorated neuron mechanical injuries *in vitro* and protected against SCI *in vivo*. For the first time, we confirmed that hydrogen application ameliorated SCI mechanical injury in a dose-dependent manner. The mechanism of action of hydrogen involved in reducing ROS generation and oxidative stress injury, inhibiting spinal cord neuronal apoptosis, and restoring mitochondrial construction and function (Fig. 8). We performed RNA-seq and identified potential target genes of hydrogen, Cox8b, Cox6a2, Cox7a1, Hspb7, and Atp2a1 (Fig. 8).

Currently, although modern medicine has made tremendous advances, SCI remains an unsolved problem. Countless laboratory studies have demonstrated potential therapeutic options, including traditional Chinese herbs [18, 19], but none has shown clinical efficacy [20]. Even the most promising candidate, prednisone, remains controversial [21-23].

SCI involves a primary mechanical injury and subsequent secondary injury due to inflammation and apoptosis [24]. The primary SCI is confined to a localized area, but the secondary injury caused by inflammation, oxidative stress, neuronal apoptosis, and mitochondrial injury may lead to further destruction of adjacent cells and expand the damaged area. Apoptosis occurs 4 h after injury and lasts for 3 weeks after SCI [25]. Oxidative stress resulting from excessive ROS generation plays an important role in secondary SCI. SCI triggers the activation of the NOX enzyme and induces excessive ROS generation [26]. When the antioxidant capacity of cells is exceeded, apoptosis occurs. NOX2-derived ROS in spinal cord microglia contribute to neuropathic pain [27]. Inhibiting NOX2 with specific inhibitor gp91ds-tat significantly reduced oxidative stress markers and improved functional recovery after SCI in mice [26]. In this study, we found that mechanical injury to neurons increased NOX2 and NOX4 expression and ROS production. The lipid peroxidation and DNA oxidation markers, MDA and 8-OHdG, were significantly increased. Caspase-3 was significantly elevated and activated 4 h post-injury. The

Fig. 7. Hydrogen protects mice spinal cord injury by inhibit oxidative stress. A, Nissl staining of spinal cord of mice in sham, model, and hydrogen group (Single-narrow pointed to Nissl body, double-narrow pointed to normal neuron). B, Representative immunohistochemistry of NSE (Single-narrow pointed to oxidative stress damaged neuron, double-narrow pointed to normal neuron). C, and D. Representative immunohistochemistry of 8-OHdG, and MPO of spinal cord (Narrows pointed to 8-OHdG- or MPO-positive neurons).

Fig. 8. A schematic diagram of the mechanism by which hydrogen ameliorates spinal cord injury. Hydrogen application inhibits oxidative stress, neurons apoptosis, and mitochondrial dysfunction, subsequently preserves spinal cord neurons, and ameliorates spinal cord injury. The possible targeting gene are Cox8b, Cox6a2, Cox7a1, Hspb7, and Atp2a1.

above results confirmed that a neuronal mechanical injury model is appropriate for simulating SCI in most situations.

In 2007, Ohta et al. [1] reported that hydrogen selectively neutralized toxic oxygen radicals, including hydroxyl radicals and peroxynitrite, and protected cells from oxidative stress damage, ushering in a new era of the study of the biological effects of hydrogen. Over 3,000 studies have demonstrated the antioxidant effects of hydrogen in various models [2], including intestinal I/R injury [28], lung I/R injury [29], myocardium I/R injury [30, 31], type 2 diabetes [32], hepatic encephalopathy [33], cerebral injury [9, 34–36], and aplastic anemia [37]. Based on this evidence, we hypothesized that hydrogen would offer a promising therapeutic option in SCI. Hydrogen-rich saline protects against SCI in rats [6] and spinal cord I/R injury in rabbits [7]. Subarachnoid cavity injection promotes recovery [8]. From the perspective of antioxidant activity, the higher the hydrogen concentration, the greater the effects. However, no previous studies have demonstrated the biological effects of high-concentration hydrogen. Thus, after demonstrating safety and feasibility in a previous study [9], we designed this study to test the effects of hydrogen on SCI at much higher concentrations than previously reported.

In this study, we confirmed the protective effects of hydrogen on spinal cord neuron mechanical injury and observed that the effects occurred in a dose-dependent manner. This is the first report of the biological effects of high-concentration hydrogen *in vitro*. Previous studies primarily employed 2–4% hydrogen inhalation or hydrogen-rich saline administration. Few studies have examined the biological effects of hydrogen at higher concentrations, which is more feasible for application. In this study, we found that the neuroprotective effects of hydrogen were exerted in a concentration-dependent manner. At a 75% concentration, hydrogen showed the most significant effects in that both the structure and the function of spinal cord neurons were protected by hydrogen incubation after mechanical injury.

In able-bodied individuals, the activity of oxidative species (including free radicals and ROS) is balanced by cellular antioxidants. After SCI, oxidative stress pathways commence, causing membrane and cellular damage constituting secondary SCI [38]. Previous studies have indicated that activated leukocytes and parenchyma were the main sources of ROS [39]. We found that 75% hydrogen treatment significantly inhibited superoxide anion generation, as demonstrated by DHE fluorescence staining, which contradicted a previous report [1]. Ohta et al. reported that 0.6 mM hydrogen in culture medium did not alter superoxide anion generation. However, we demonstrated that hydrogen acted in a different manner in this study, challenging the hypothesis of selective antioxidant activity. In their analysis, Ohta et al. first used antimycin A to induce superoxide anion generation, which is not a normal pathophysiological process. We used mechanical damage *in vitro*, which better simulates real-life situations. Second, a higher concentration of hydrogen may act in a different manner, namely, producing complete, rather than selective, antioxidation, which requires further clarification.

During the process of SCI, the principal components of ROS are the superoxides ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$), hydrogen peroxide (H_2O_2), nitric oxide ($\cdot NO$), and peroxynitrites ($ONOO^-$) [40]. The above principal ROS components are synthesized by NOX in both the microglia and leukocytes. MDA and 8-OHdG are markers of lipid peroxidation and DNA oxidation. To further explore the molecular mechanisms of hydrogen antioxidant activity, we performed a series of *in vitro* and *in vivo* experiments. The concentrations of MDA and 8-OHdG were significantly reduced after hydrogen incubation, and the expression levels of NOX-2 and NOX-4 were also reduced. The expression levels of antioxidant enzymes PrxIII, catase, SOD2, SOD1, and Trx2 were concomitantly elevated. These results confirmed our hypothesis that 75% hydrogen incubation prevented oxidative stress injury via mechanisms in addition to selectively mediating superoxide anions.

The SCI procedure is related to complicated pathophysiological mechanisms, among which apoptosis is the major event of secondary injury after SCI [41, 42]. Apoptosis is primarily regulated by the upstream Bcl-2 family and the downstream caspase family,

among which anti-apoptotic (Bcl-2) or pro-apoptotic (Bax) molecules are the most common markers of programmed cell death [41, 43]. In this study, we demonstrated that 75% hydrogen incubation prevented neuronal apoptosis. TUNEL staining and flow cytometry results revealed that the number of apoptotic neurons was significantly decreased by hydrogen incubation after mechanical injury, and the expression levels of apoptosis-related genes, such as caspase-3 and Bax, were inhibited.

As demonstrated in a previous study, mitochondrial function is closely related to neuronal apoptosis and ROS production [44]. Mitochondria are the special organelles in most cells that generate ATP, for which mitochondria are sometimes called “cellular power factories” [45]. As the mitochondria supply cellular energy, mitochondrial dysfunction may play an important role in a variety of pathological conditions, including SCI [45]. Studies [46-48] have demonstrated that preserving mitochondrial function improved functional recovery after SCI. In this study, our experiments revealed that 75% hydrogen incubation restored ATP levels after mechanical injury, and mPTP opening was also inhibited by hydrogen incubation. Taken together, these results indicated that the protective effects of hydrogen against SCI were at least partly due to improving mitochondrial function.

As described in previous studies, there were close relationships among oxidative stress, apoptosis, and mitochondria dysfunction during the process of second spinal cord injury. After the considerable ROS production in central neural system injury, the pathological stimulation of NMDA receptors would lead to an increased flux of Ca^{2+} into the cytoplasm [49]. In an apparent attempt to prevent these potentially lethal events, Ca^{2+} is sequestered by mitochondria [50]. The accumulation of high levels of Ca^{2+} and formation of ROS that induce oxidative stress can destabilize mitochondria. The destabilized mitochondria consequently cause opening of mPTP, and release cytochrome c and apoptosis-inducing factor (AIF), which can act as pro-apoptotic proteins [51]. In brief, the oxidative stress after SCI can cause mitochondria-dependent apoptosis.

We next performed RNA-Seq on spinal cord neurons. Biological replicates confirmed the high biological reproducibility of our experimental procedure and established a reliable foundation. PCA indicated that hydrogen incubation altered the effects of mechanical injury on spinal cord neurons. Oxidative stress-related genes were screened as possible target genes of hydrogen treatment. To investigate the exact genes related to the mechanism underlying the effects of hydrogen, we performed RNA-seq and RT-PCR, revealing that hydrogen incubation prevented oxidative stress injury by regulating the expression of related genes. The results indicated that the potential target genes of hydrogen treatment included Cox8b, Cox6a2, Cox7a1, Hspb7, and Atp2a1. There have been no previous reports on hydrogen regulating the expression of certain genes. We hypothesized that the regulatory effects of hydrogen resulted from its influence on the generation of certain small molecules (e.g., NO), which also act as signaling messengers in the human body.

This study has several limitations. First, although we used mouse SCI to evaluate the protective effects of hydrogen *in vivo*, the mechanism was studied using an *in vitro* model of neuronal mechanical injury, which cannot ideally simulate *in vivo* situations. Second, our study focused on spinal cord neurons, but glial cells are also important in SCI conditions, as was observed in the mPTP results, and this will be explored further in our future studies.

In conclusion, our study revealed that inhalation of high-concentration hydrogen protected mice against SCI. We also confirmed for the first time that the protective effects of hydrogen on *in vitro* neuronal mechanical injury was dose-dependent. The mechanism of hydrogen protection against SCI includes inhibiting oxidative stress injury, preventing neuronal apoptosis, and maintaining mitochondrial construction and function. Additionally, we identified Cox8b, Cox6a2, Cox7a1, Hspb7, and Atp2a1 as potential target genes of hydrogen. Further research is needed to investigate the mechanism by which high-concentration hydrogen exerts protective effects on SCI *in vivo*. High-concentration hydrogen may be a promising clinical therapy for SCI.

Abbreviations

ROS (reactive oxygen species); SCI (spinal cord injury); CNS (central nervous system); NSE (neuron specific enolase); TEM (transmission electron microscopic); TUNEL (Terminal deoxynucleotidyl transferase dUTP nick-end labeling); JG-B (Janus Green B); mPTP (Mitochondrial permeability transition pore); RNA-Seq (RNA-Sequencing); RT-PCR (Real-time PCR); BMS (Basso Mouse Scale); DBF (distance between bilateral feet); ANOVA (analysis of variance); ELISA (enzyme-linked immunosorbent assay); DHE (Dihydroethidium); 8-OHdG (8-hydroxy-2-deoxyguanosine); MDA (malondialdehyde).

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Disclosure Statement

No conflict of interests exists.

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