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***Cover Letter**

Dear Editors:

We are submitting a paper entitled "*Hydrogen-rich saline protects against spinal cord injury in rats* " to be considered for publication in *neurochemical research.* The authors are Chengwen Chen, Qianbo Chen, Yanfei Mao, Shengming Xu, Chunyan Xia, Xueyin Shi, John H. Zhang, Hongbin Yuan, Xuejun Sun. I am the corresponding author and can be reached at: Tel +86-2181885822 or Fax +86-63610109. My E-mail address is jfjczyy@yahoo.cn. Currently, I am working as a professor in the Department of Anesthesiology, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai 200003, People's Republic of China. My current mailing address is Changzheng hospital, 415 Fengyang Road, Shanghai 200003, P.R. China.

In this paper, the standardized model of contuses spinal cord injury described by Allen was used with revision. Levels of oxidative stress, inflammation, neural cell death, BDNF and locomotor motor function recovery were studied after injury. We found that hydrogen-rich saline reduced acute spinal cord contusion injury, possibly by reduction of oxidative stress and elevation of BDNF.

None of the materials in this manuscript has been published or is under consideration for publication elsewhere at this time. All the authors agree with and are responsible for the data presented and deny any potential conflicts of interest. We hope you will find it suitable for publishing in *neurochemical research*. The manuscript is prepared according to the instruction. We would like to be most happy to provide any additional information required by you and the reviewers.

Thank you for your consideration and look forward to hearing from you soon.

Sincerely,

Hongbin Yuan, MD

Professor of Anesthesiology

**Complete title:** Hydrogen-rich saline protects against spinal cord injury in rats

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**Running title:** Hydrogen attenuates spinal cord injury.

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**Abstract**

In the present study, we examined the mechanisms of hydrogen-rich saline, a reported therapeutic antioxidant, in the treatment of acute spinal cord contusion injury. Male Sprague-Dawley rats were used to produce a standardized model of contuses spinal cord injury (125 kdyn force). Hydrogen-rich saline was injected intraperitoneally (5ml/kg) immediately, and at 24 and 48 hours after injury. All rats were sacrificed at 72 hours after spinal cord injury (SCI). Apoptotic cell death, oxidative stress, inflammation, level of Brain derived neurotrophic factor (BDNF) were evaluated. In addition, locomotor behavior was assessed using the Basso, Beattice and Bresnahan (BBB) scale. We observed that administration of hydrogen-rich saline decreased the number of apoptotic cells, suppressed oxidative stress, and improved locomotor functions. Hydrogen-rich saline increased the release of BDNF. In conclusion, hydrogen-rich saline reduced acute spinal cord contusion injury, possibly by reduction of oxidative stress and elevation of BDNF.

**Keywords:** hydrogen-rich saline; oxidative free radicals; neuroprotection; BDNF; spinal cord injury

## Introduction

Traumatic spinal cord injury (SCI) has two phases, a primary injury and a secondary injury phases. The secondary injury may lead to further chronic neurodegeneration. Reactive oxygen species (ROS), including superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($^{\cdot}OH$), are believed to play an important role in the secondary injury. At high concentration, reactive oxygen species cause cytotoxicity by damaging proteins, nucleic acids and lipids, attacking the double bonds of unsaturated fatty acids, causing peroxidation of lipids and fragmentation of protein [1]. In particular, neuronal tissues are especially vulnerable to oxidative injury because of the over abundance of polyunsaturated fatty acids [2]. Therefore antioxidant treatment protocols are being developed for treatment of SCI [1, 3, 4]

Hydrogen gas has been found to be a safe and effective antioxidant with minimal side effect in decompression sickness in divers [5] and in animal models of brain injuries [6,7,8]. Hydrogen gas can penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus, and can protect cells and tissues against oxidative stress by scavenging hydroxyl radicals [7]. However, hydrogen gas is difficult to use and special hydrogen gas tanks are needed. In the present study, we examined the neruroprotective effects of hydrogen-rich saline in a spinal cord injury rat model. This translational study will take the advantage of hydrogen-rich saline and test the therapeutic effect on oxidative stress and functional improvement in spinal cord injury.

## Experimental Procedure

### Animals

Male Sprague-Daley rats weighing 250-300 g were used and the animal protocols were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University in Shanghai, China. All animals were given free access to normal rat diet and tap water and were maintained on a 12:12-h light/dark cycle (lights on at 06:00h).

### Hydrogen-rich Saline Production

Hydrogen gas was dissolved in physiological saline for 6 hr under high pressure (0.4MPa) to a supersaturated level using hydrogen-rich saline-producing apparatus produced by the Diving Department in the Second Military Medial University. The saturated hydrogen saline was stored under atmospheric pressure at 4℃ in an aluminum bag with no dead volume. Hydrogen-rich saline was sterilized by gamma radiation. Hydrogen-rich saline was freshly prepared every week, which ensured that a concentration of 0.6 nmol/L was maintained. Gas chromatography was used to confirm the content of hydrogen in saline by the method described by Ohsawa et al [7].

### Experimental Protocols

Animals were divided into three groups: (1) sham-operated plus vehicle physiological saline treatment (n=11); (2) spinal cord injury plus vehicle physiological saline treatment (n=11); (3) spinal cord injury plus hydrogen-rich saline treatment (n=11). A standardized model of contuse rat spinal cord injury

described by Allen was used with revision. Briefly, rats were anaesthetized with chloral hydrate (10%, 0.3ml/100g) intraperitoneally. The spinous process and the vertebrae lamina were removed to expose a circular region of dura matter at the T7-T9 spinal level. Under stereotaxic control, a 10g stainless-steel cylinder with a flat tip of 2mm diameter was dropped from a height of 12.5mm, through a guide tube onto the exposed dura mater. Sham-operated rats only had their spinous process and vertebrae lamina removed, and their muscle and skin were sutured. All animals received preventive benzylpenicillin sodium antibiotic (20 IU once a day). Bladder evacuation was accomplished twice daily. The rats were sacrificed at 72 hours after spinal cord injury.

**Tissue Processing**

At 72 hours after injury, 6 sham, 6 vehicle-treated and 6 hydrogen-rich saline-treated rats were perfused through a cannula inserted in the ascending aorta with 50 ml isotonic ice-cold saline followed by 150 ml fixation fluid (4℃) over 6 min after deep anesthesia. The fixative consisted of 4% paraformaldehyde (w/v) in 0.1M phosphate buffer (PH 6.9). A 10 mm spinal cord segment containing the T9 impact epicenter was removed, kept in the fixative solution at 4℃. For the other animals, after deep anesthesia, a 10 mm section of the spinal cord section at the injury epicenter was dissected. The samples were cleaned thoroughly of blood, and the meninges were removed carefully. The samples were then immediately frozen and stored at -80℃until the analyses were performed.

**Biochemical Analysis**

Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction which was defined previously [9]. This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to TBA with MDA at 532nm.

Protein carbonyl as an indicator protein oxidation was also measured. Briefly, the spinal cord homogenate was mixed with steptomycin followed by centrifugation and then it was incubated with DNPH for 1h followed by precipitation with TCA. The pellet was washed with ethanol-ethyl acetate to remove excess DNPH and resupended in guanidine hydrochloride. The solution was centrifuged and incubated at 37℃ in a water bath for 10min. Finally, the absorbance was measured at 366 nm wavelength and the results were expressed as nmol/mg protein.

The extent of neutrophils infiltration was determined by measuring the tissue myeloperoxidase (MPO) activity. For this purpose, the spinal cord was homogenized in 10 volume of ice-cold 20 mmol/L pltassium phosphate buffer (PH 7.4) containing 30 mmol/L KCL. The homogenate was centrifuged at 12000 rpm for 10 min at 4℃. The pellet was then rehomogenized with an equivalent volume of 50 mmol/L acetic acid containing 5% hexadecyltrimethylammonium bromide (HETAB). MPO activity was assessed by measuring the $H_2O_2$ dependent oxidation of 0-dianizidine 2 HCL. One unit of enzyme activity was defined as the amount of the MPO present that caused

a change in absorbance of 1.0/min at 460 nm and 37℃.

**Histology and Immunocytochemistry**

For histological evaluation, segments at the lesion epicenter were obtained, embedded in paraffin, and sectioned at 5 μm thick sagittal sections. The sections were stained with hematoxylin and eosin (H&E) for histopathologic changes. TUNEL, caspase-3 and caspase-12 staining were performed to estimate neuron apoptosis.

Briefly, TUNEL staining was performed on 5μm paraffin-embedded sections by using the in situ cell death detection kit (cat # 4828-30-DK, Trevigen, USA). According to standard protocols, sections were dewaxed in xylene, rehydrated in graded alcohols and placed in $dH_2O$.Then these sections were incubated in a 20μg/ml proteinase K working solution for 15 min at room temperature. The slides were rinsed three times with PBS before they were incubated in TUNEL reaction mixture for 1h at 37℃. After rinsing with PBS (5 min, 3 times), sections were incubated with HRP-streptavidin reagent (1:200) in PBS for 30 minutes at room temperature. Sections were rinsed in PBS for 3x5min and then incubated with 0.04% DAB and 0.03% $H_2$ solution for 10 minutes. After rinsing with PBS for 3x5min, sections were counterstained with hematoxylin. Then rinse sections in distilled water 2x5min and coverslip with mounting medium.

For caspase-3 and caspase-12 staining, 5μm parafin-embedded tissue sections were investigated using polyclonal antibodies (ab44976, ab62484,

ABCAM, USA ) by the avidin–biotinperox-Idase complex (ABC) method. The technique protocol was standardized following the manufacturer's instructions. Briefly, sections were dewaxed in xylene, rehydrated in graded alcohols and placed in $dH_2O$.Antigen retrieval was done by immersion of sections in 0.01M citrate buffer, pH6.0, in a steam bath (at 98℃) for 25 min, followed by rapid cooling (20min). Subsequently, endogenous peroxidase was inactivated by incubation in 3% $H_2O_2$ in $dH_2O$for 30 min at RT. After rinsed with 0.1MTris-buffered saline (TBS), pH7.4, sections were treated in 2% Bovine serum albumin (BSA) in TBS (30min, RT). After incubation (4℃, overnight) with the primary antibody diluted (1/50inTBS) with 2% BSA, sections were washed and incubated with abiotinylated goat anti-rabbit antibody (1/200inTBS) for 60min at RT and subsequently treated with the avidin–biotin Peroxidase complex (1/100 in TBS) for 1 h at RT. Finally, sections were incubated in 0.05 % DAB plus 3% $H_2O_2$ in TBS for 10 min, rinsed in $dH_2O$, counterstained with hematoxylin, dehydrated, and coversliped with mounting medium.

**Cell Counting**

To estimate the number of TUNEL positive cells in the spinal cord after injury, photos of immunostained slides were taken using a digital camera (PANASONIC) connected to microscope at ×200 magnification. More than five pictures per well were taken from random fields, taking care to avoid the margin of the well and to keep a balanced selection of different areas of the well (center, upper tight, lower right, upper left, lower left). Each photo captured

an area corresponding to ~0.1mm$^2$, and cells in photographs were counted manually. Fot each experiment, at least 10 photos were examined for each group; results presented are based on three independent experiments.

**Quantitative Real Time RT-PCR (qRT-PCR)**

Levels of BDNF were determined by two-step RT-PCR following the manufacture's instruction. Briefly, in the first step, MMLV Reverse Transcriptase was used to synthesize a cDNA copy of input RNA. Then the resulting cDNA was subjected to qPCR that was performed with a Light Cycler using a SYBR Green Two-Step qPCR Kit. The amplified product was detected by the presence of an SYBR green fluorescent signal. The standard curve was designed with designed with β-actin cDNA. The resulted amplicon was quantified with the standard curve. The primers and qPCR conditions included: oligo, forward: 5' CGTGGGGAGCTGAGCGTGTG 3'; reverse: 5 ' GCCCCTGCAGCCTTCCTTCG 3'.

**Functional Test**

Hind limb neurological function was evaluated at 24, 48 and 72 hours after surgery, using the Basso, Beattie, Bresnahan (BBB) scale in an open field [10,11]. Each rat was placed in the open field and observed for 5 min. All behavioral assessments were done by two observers who were blind to the subject's experimental treatment.

**Statistical Analysis**

Statistical analysis was performed by spss 13.0 software ( Shanghai, SMMU ) .

Data are expressed as means ± Standard Deviation (x±S) for each group. The differences among experimental groups were detected by one-way analysis of variance. Between groups, variance was determined using the student-Newman-Keuls test. Repeated-measures two-way ANOVA was used to compare matched data at multiple time points. A p-value of less than 0.05 was considered to be statically significant.

## Results

### Hydrogen-rich Saline Attenuates Cell Death

Spinal cord injury produced distinct cell death, hemorrhage and inflammatory cell infiltration which were reduced by hydrogen-rich saline (Figure 1). In the TUNEL staining conducted at 72 hrs after the spinal cord injury, more positive stained cells were observed when compared with sham operated animals (38.30±6.10 % vs. 24.50±2.98 %, $p < 0.01$). Hydrogen-rich saline decreased the number of tunnel-positive cells (28.61±3.49 % vs. 38.30±6.10 % , $p < 0.01$) (Figure 2). Only a few of tunnel-positive cells were found in the samples of sham-operated animals.

### Hydrogen-rich Saline Decreased Caspases

Marked activation of caspase-3 and caspase-12 in cytoplasm were observed in the contused spinal cord after spinal cord injury. Hydrogen-rich saline reduced caspased-3 and caspase-12 activation (Figure 3).

### MDA and Protein Carbonyl

The content of MDA and protein carbonyl was detected at 72 hours after spinal cord injury. Spinal cord injury increased MDA when compared with sham-operated group (3.05±0.60 vs. 0.30±0.05, $p < 0.01$). Hydrogen-rich saline decreased MDA levels when compared with the vehicle treatment group (1.60±0.34 vs. 3.05±0.60, $p<0.01$) (Fig 4).

The content of protein carbonyl was significantly elevated in the contused

spinal cord when compared with sham-operated rats (8.73±1.83 vs. 1.06±0.44, $p<0.01$) (Figure 5). Hydrogen-rich saline suppressed the production of protein carbonyl compared with the vehicle group (3.32±1.15 vs. 8.73±1.83, $p<0.01$).

**Activity of MPO**

The activity of MPO was measured at 72 hours after spinal cord injury. Fig 6 illustrated that MPO activity was dramatically increased in the contused spinal cord when compared with sham-operated group (11.60±1.28 vs 0.94±0.27, $p<0.01$) and Hydrogen-rich saline significantly suppressed the activity of MPO when compared with the vehicle group (1.38±0.34 vs11.60±1.28, $p<0.01$).

**Release of BDNF**

The gene transcription of BDNF was detected at 72 hours after spinal cord injury. As shown by Fig 7, hydrogen-rich saline group did significantly increase the gene transcription of BDNF (0.49±0.25% vs 0.07±0.01%, $p<0.01$), while physiological saline treatment did not (0.18%±0.04 vs 0.07%±0.01, $p>0.05$).

**Hind Limb Motor Function (BBB Score)**

BBB locomotor scores demonstrated that spinal cord injury affected the hind limb motor functions of the rats (Fig 8). Animals that received hydrogen-rich saline treatment exhibited higher BBB scores at 48 hours and 72 hours after injury. Repeated-measures two-way ANOVA revealed a significant treatment effect over time ($p<0.01$). one-way ANOVA followed by SNK post hoc test indicated significantly improved locomotor function in the hydrogen group compared with vehicle group at 48 hours and 72 hours (5.32±2.19 vs

7.50±2.29, p<0.01; 4.23±2.56 vs 9.95±4.30, p<0.01).

**Discussion**

Hydrogen gas has been used for injuries in the brain, heart and liver [12, 13, 14], but we do not aware of a hydrogen study in spinal cord injury previously. We applied hydrogen-rich saline instead of hydrogen gas to take advantages of the safe, easy and higher concentration of hydrogen-rich saline in this study. We observed that hydrogen-rich saline decreased oxidative stress, inflammation, apoptotic cell death, and improved the locomotor function after spinal cord injury.

Oxidative stress and lipid peroxidation play an important role in the progression of secondary damages after a spinal cord injury [1]. Under normal conditions, a dynamic equilibrium exists between the oxidative damage potential and the natural antioxidant defense system. When production of ROS, such as $O_2^-$, $H_2O_2$ and $^{\cdot}OH$, exceeds the natural antioxidant capacity, ROS can attack, modify and damage functional and structural molecules leading to tissue injury. Excess production of $O_2^-$, $H_2O_2$ and $^{\cdot}OH$ in the extracellular space has been reported following SCI. In addition, $O_2^-$ may react with NO to produce highly reactive and cytotoxic products, namely, peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH). After SCI, reactive oxygen species (ROS) and reactive nitrogen species (RNS) appear to play a critical role in cell death. Spine like other neuronal tissues is especially vulnerable to oxidative injury because of the overabundance of polyunsaturated fatty acids in the spinal cord

tissues [2]. Therefore, the ability of hydrogen to reduce or eliminate $^{\cdot}OH$ and $ONOO^{-}$ may be responsible for the neuroprotective effect observed in this study.

Hydrogen, a safe and effective antioxidant with minimal side effect, could effectively neutralize hydroxyl radicals [7]. Therefore, hydrogen was reported to markedly decrease oxidative stress and protected cells and tissues in several animal models [6, 8, 14]. The primary action of hydrogen-rich saline in this study may be related to the reduction of oxidative stress, as presented by the suppression of MDA, the end product of membrane lipid peroxidation. Similarly, protein carbonyl as an indicator of protein oxidation was also decreased by hydrogen-rich saline treatment. Myeloperoxidase (MPO) is a well-known oxidative enzyme expressed by neutrophils and generates hypochlorous acid that damages nearby tissue. MPO activity is commonly used to measure the extent of neutrophils infiltration. In the present study, SCI caused an elevation in tissue MPO activity, indicating the presence of enhanced neutrophils recruitment in the spinal cord injury. We found that hydrogen-rich saline decreased neutrophils infiltration after spinal cord injury. Hydrogen-rich saline also reduced caspase-3 and caspase-12 and TUNEL positive cells. These results demonstrated that hydrogen protected the spinal cord tissue and cells by reducing inflammation and oxidative stress.

This study also investigated another potential action of hydrogen-rich saline treatment after spinal cord injury. BDNF is a neurotrophic factor derived from

microglial cells and plays an important role in supporting the survival of neurons, enhancing the remyelination of injured axons and decreasing necrosis in SCI [15,16,17]. Increased production of BDNF from activated microglia/macrophages in the injured spinal cord leads to functional recovery as reported by others [18, 19]. Our data for the first time demonstrated that hydrogen-rich saline increased the gene transcription of BDNF after spinal cord injury. BDNF may contribute to the functional recovery after spinal cord injury as represented by the improved BBB scores after hydrogen-rich saline treatment.

In conclusion, hydrogen-rich saline protected the spinal cord from contusion injury by reducing oxidative stress, inflammation and cell death. In addition, the level of BDNF in the contused spinal cord seems enhanced by hydrogen-rich saline treatment. All of these actions of hydrogen-rich saline may be translated into improved neurological functional recovery.

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**Fig.1** Hydrogen-rich saline treatment attenuated hemorrhage and inflammatory cell infiltration at the lesion site. Representative photomicrographs of the sections stained by the hematoxylin and eosin in sham-operated animals, vehicle-treated animals and hydrogen-treated animals, at 72 hrs after SCI. (A) sham: histological features of normal spinal cord tissue were observed (Magnification=200 × ). (B) vehicle-treated: hemorrhage, liquefaction and inflammatory cells infiltration were observed in the middle of the lesion (Magnification=100×). (C) hydrogen-treated: local hemorrhage and a few inflammatory cells infiltration were observed (Magnification=200×). n=5 per group.

**Fig. 2** TUNEL staining of the spinal cord sections and TUNEL-positive cell counting at 72 h after injury in sham-operated animals (Magnification=100×), vehicle-treated animals (Magnification=200×) and hydrogen-treated animals (Magnification=200 × ). (A) representative slides of TUNEL staining. The TUNEL-positive material was localized in the nuclei of the neurons. In samples collected from the vehicle groups, the damaged cells were characterized by a round and shrunken morphology. The processed disappeared and the neuronal body became rounded with strong TUNEL staining in the nucleus (white arrow). (B) Cell counting. Vehicle groups had higher proportion of TUNEL-positive cells than that of $H_2$-treated groups. $H_2$ saline significantly reduced the number of TUNEL-positive cells. *p<0.01 vs sham; #p<0.01 vs vehicle group. Magnification=200×. n=5 per group.

**Fig.3** Representative slides of caspases-3 (A, B, C) and caspase-12 (D, E, F) immunostaining at 72 h after SCI in sham-operated animals (Magnification=100 × ), vehicle-treated animals (Magnification=200 × ) and hydrogen-treated animals (Magnification=200×). Strong immunohistochemical reactivity for caspase-3 and caspase-12 were found in the neurons. $H_2$ treatment resulted in reduction staining for caspase-3 and caspase-12 immunoreactivity. n=5 per group.

**Fig.4** MDA in spinal cord samples (Mean±SD) of the experimental groups at 72 h after SCI, nmol/mg wet tissue (n=6 per group).*p<0.01 vs sham; #p<0.01 vs vehicle group.

**Fig.5** protein carbonyl levels in spinal cord samples (Mean±SD) of the experimental groups at 72 h after SCI, nmol/mg wet tissue (n=6 per group).*p<0.01 vs sham; #p<0.01 vs vehicle group.

**Fig.6** MPO in spinal cord samples (Mean±SD) of the experimental groups at 72 h after SCI, u/g (n=6 per group).*p<0.01 vs sham; #p<0.01 vs vehicle group.

**Fig.7** BDNF levels in spinal cord samples (Mean±SD) of the experimental groups at 72 h after SCI, (n=6 per group).*p>0.05 vs sham; #p<0.01 vs vehicle group.

**Fig 8** the BBB scores assessing hind leg function at 24, 48, 72 after spinal cord injury in experimental groups (n=11 per group).*p>0.05, # p<0.01, **p<0.01 between vehicle and hydrogen groups.

**Figure**

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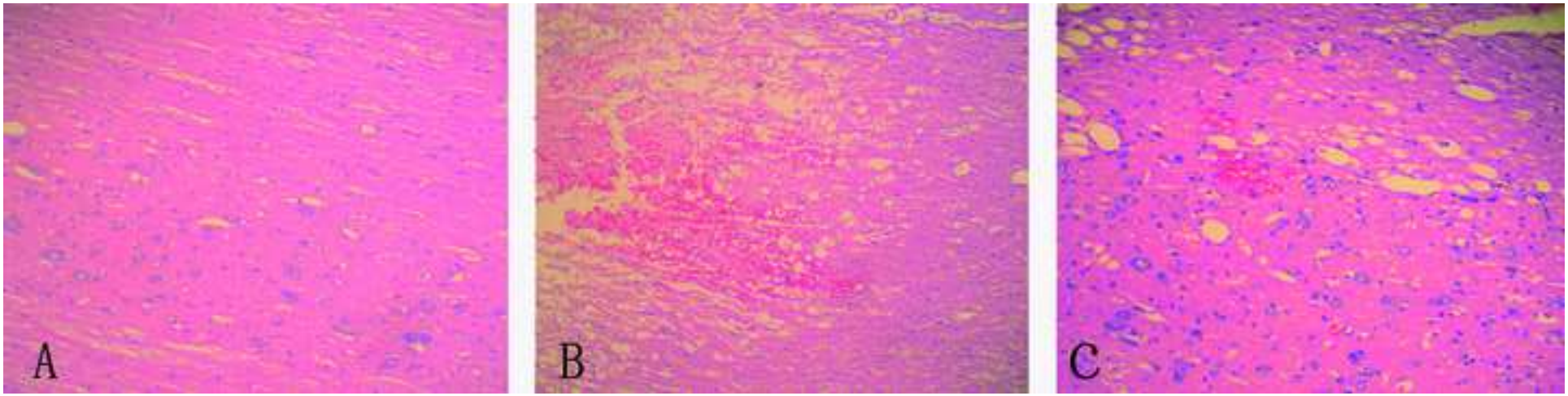

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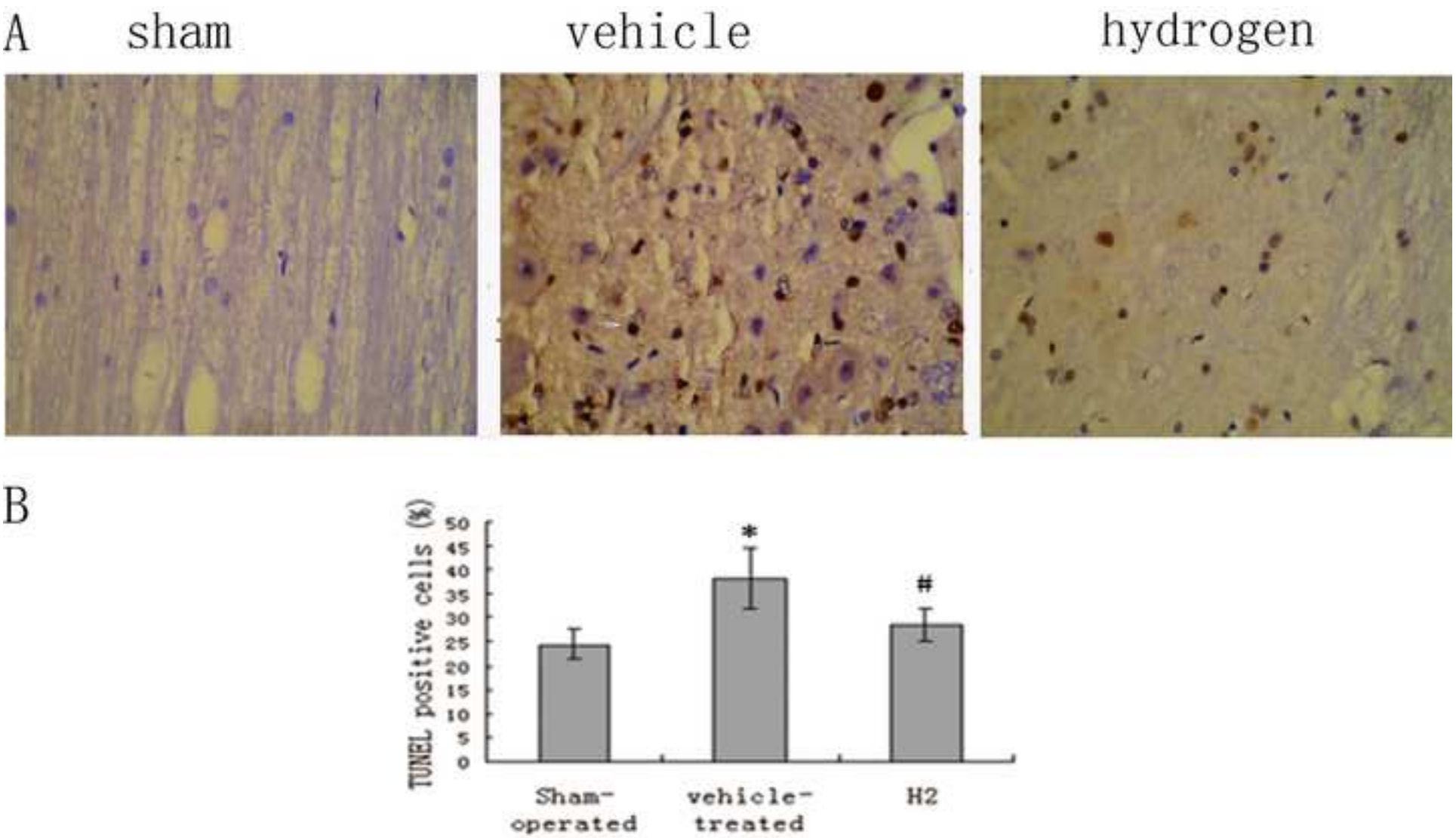

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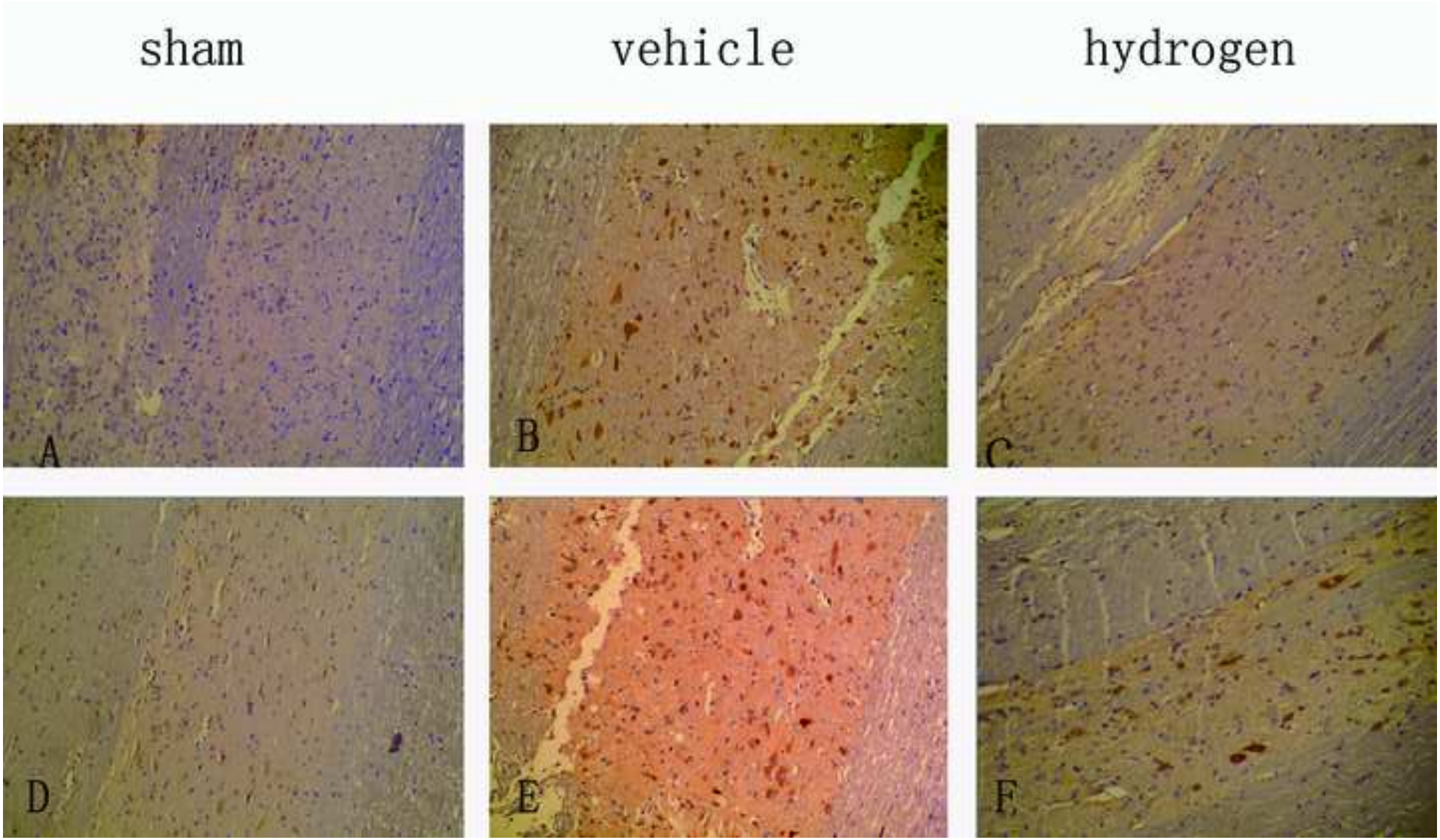

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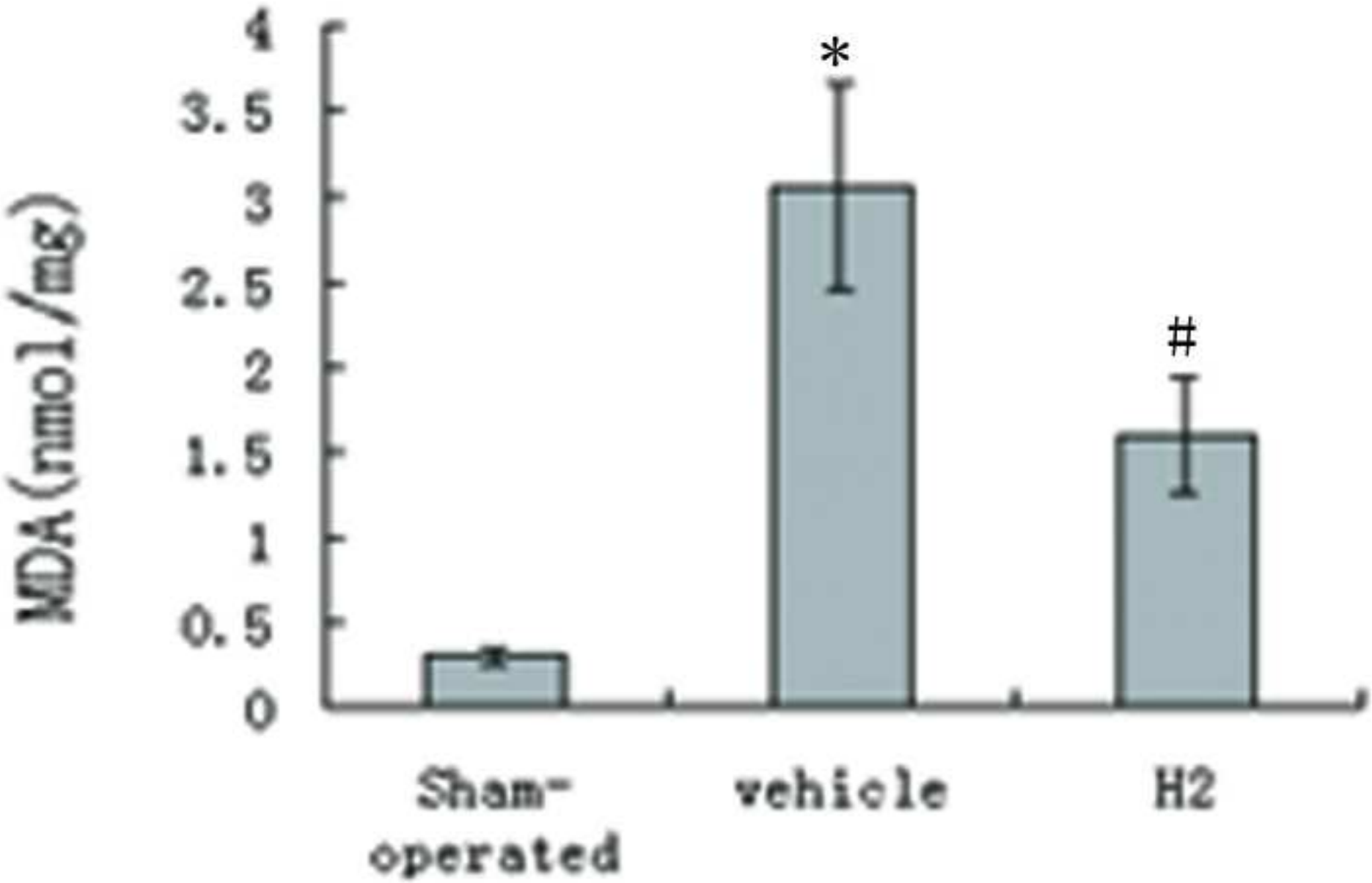

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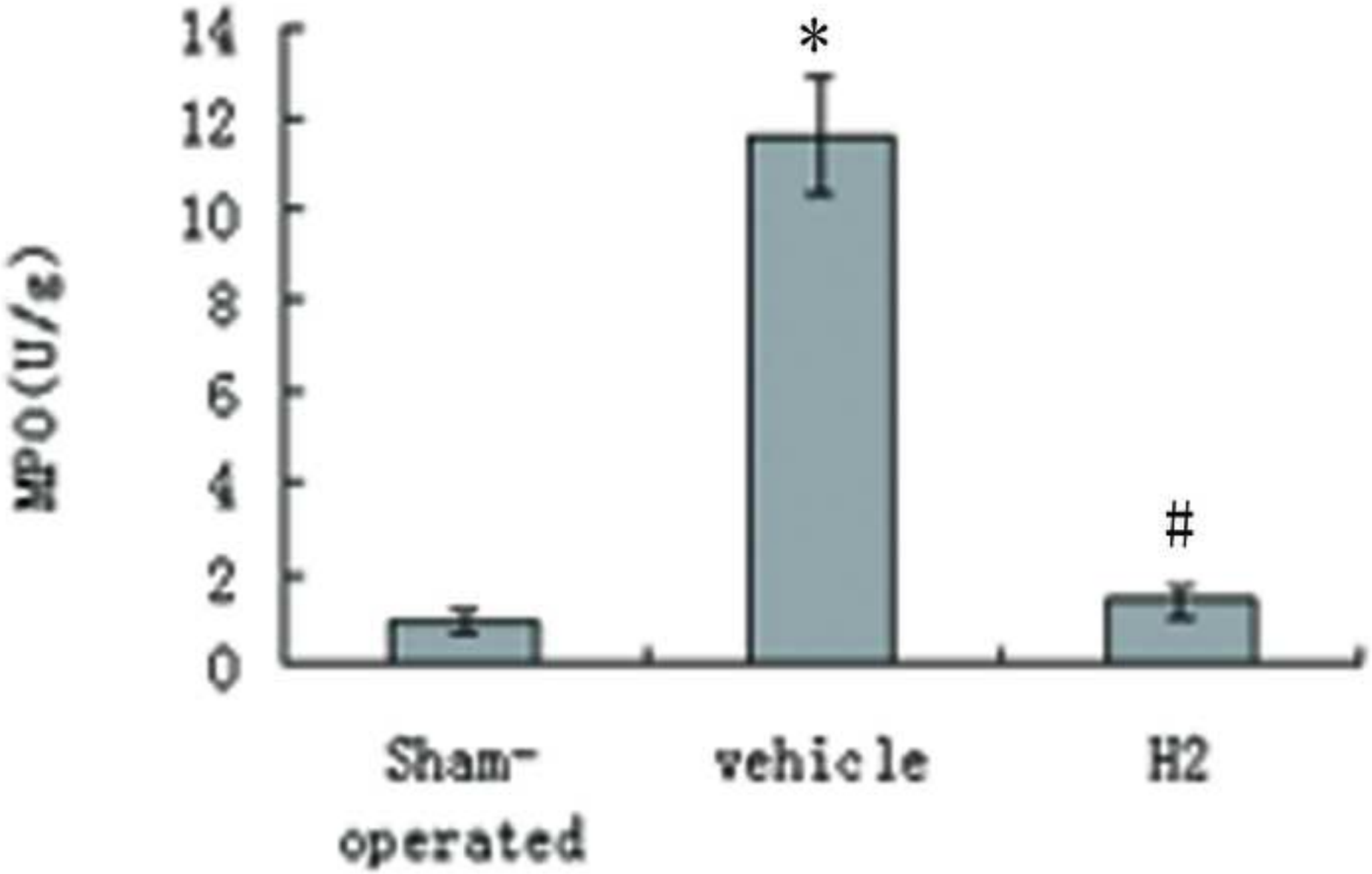

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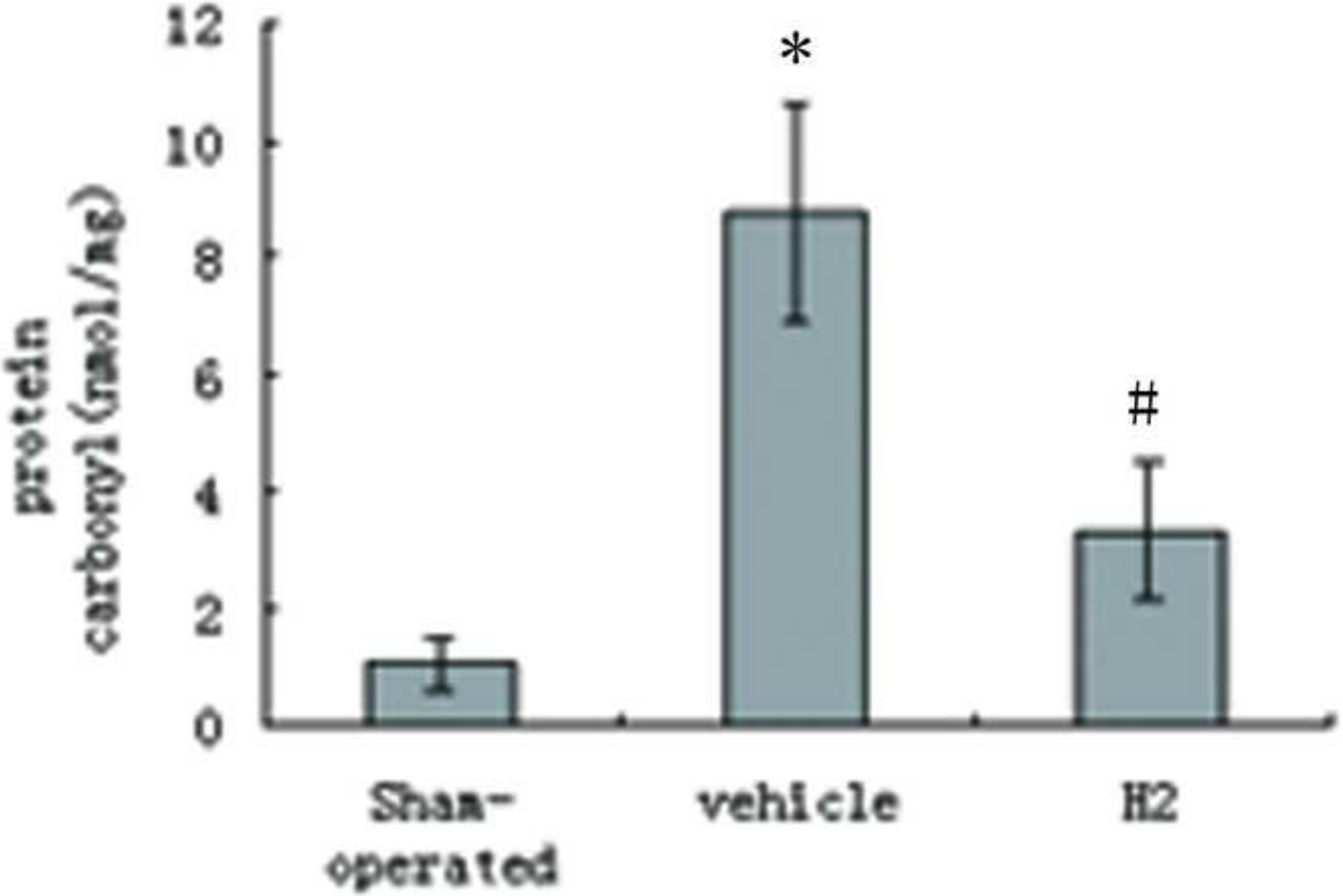

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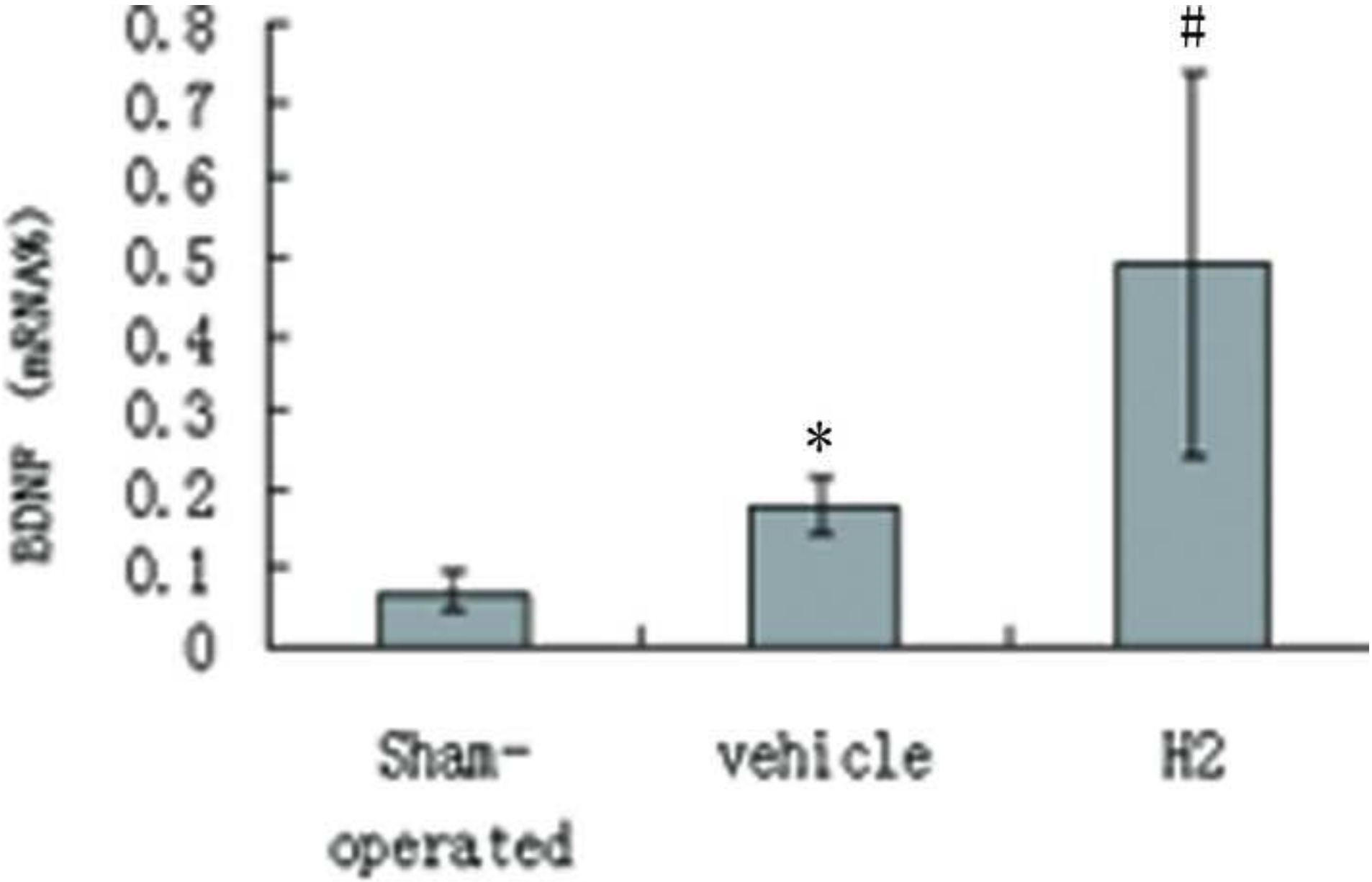

Figure

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