

Original Research

Hydrogen inhalation protects hypoxic–ischemic brain damage by attenuating inflammation and apoptosis in neonatal rats

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Impact statement

Oxidative stress is known to be involved in the main pathological progression of neonatal hypoxic–ischemic brain damage (HIBD). Hydrogen (H₂) is an antioxidant that can be used to treat HIBD; however, the mechanism by which hydrogen may be used as a promising treatment for neonates with HIBD is not very clear. This study demonstrated that inhaled H₂ is neuroprotective against HIBD in SpragueDawley rats by inhibiting the brain's inflammatory response and neuronal apoptosis or damage and protecting against spatial memory decline. Further, this study showed that inhaled H₂ has potential as a therapeutic approach for HIBD. This is relevant to clinical treatment protocols when hypoxia–ischemia is suspected in neonates.

Abstract

Hypoxic–ischemic brain damage (HIBD) is one of the leading causes of brain injury in infant with high risk of mortality and disability; therefore, it is important to explore more feasible and effective treatment strategies. Here, we assessed the neuroprotective effects of different hydrogen inhalation times for the treatment of HIBD. We induced hypoxia–ischemia in Sprague–Dawley rats (postnatal day 7, both sexes), followed by treatment with hydrogen inhalation for 30, 60, or 90 min. Morphological brain injury was assessed by Nissl and TUNEL staining. Acute inflammation was evaluated by examining the expression of interleukin-1 β (IL-1 β) and NF- κ B p65, as well as Iba-1 immunofluorescence in the brain. Neural apoptosis was evaluated by examining the expression of P-JNK and p53 as well as NeuN immunofluorescence. Neurobehavioral function of rats was evaluated by Morris water maze test at 36 days after surgery. The results showed that hypoxia–ischemia injury induced the inflammatory response of microglia; however, these changes were inhibited by hydrogen inhalation. The inhibitory effects became more apparent as the treatment duration increased ($P < 0.05$). Furthermore, hypoxia–ischemia induced neuronal damage and increased the

expression of the apoptotic factors, P-JNK, and p53, which were attenuated by hydrogen inhalation ($P < 0.05$). Hypoxia–ischemia caused long-term spatial memory deficits during brain maturation, which were ameliorated by hydrogen inhalation ($P < 0.01$). In conclusion, hypoxia–ischemia induced severe long-term damage to the brain, which could be alleviated by hydrogen inhalation in a time-dependent manner.

Keywords: Cerebral ischemia–hypoxia, hydrogen, spatial memory, inflammation, apoptosis

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Introduction

Hypoxic–ischemic brain damage (HIBD) refers to brain damage caused by asphyxia due to hypoxia and ischemia during the perinatal period, which is proved to be the main cause of mortality and disability in neonates.^{1,2} The global incidence of HIBD among live births is approximately 1–8‰, reaching up to 26‰ in developing countries and up to 60% among newborns with very low birth weight. The mortality rate for children with HIBD is

approximately 20–25% in the neonatal period, and approximately 25% of surviving children experience permanent neurological deficits, such as mental retardation, developmental retardation, motor or cognitive impairment, cerebral palsy, and epilepsy.^{2,3} Mild hypothermia therapy can alleviate the brain damage caused by hypoxia–ischemia (HI); however, some neonatal patients exhibit persistent neurological defects even after treatment.^{4–6} Therefore, it is essential to develop more feasible and

effective therapeutic strategies for reducing brain damage caused by HI in neonates.

Hydrogen (H_2) is a colorless, odorless, non-toxic antioxidant that is protective against diseases mediated by oxidative stress, including ischemia-reperfusion injury in the adult rat brain, heart, liver, kidney, small intestine, spinal cord, and other organs.⁷⁻¹² In 2008, Kajiya *et al.*¹³ investigated H_2 in clinical trials, observing that drinking H_2 -rich water could effectively improve the symptoms in patients with diabetes. At present, there are three primary forms of H_2 administration: H_2 inhalation, injection of H_2 -rich saline, and oral ingestion of H_2 -saturated water.^{14,15} One study reported that oral H_2 -rich water may result in harmful alterations to some biochemical indices.¹⁶ Oral ingestion or injection of H_2 -rich water or saline, respectively, can increase the allostatic load. In addition, it is difficult to control the concentration of H_2 using these methods. Moreover, the curative effect associated with these treatments are limited by short exposure times and other factors, and the complex protocols required are not suitable for newborns.^{17,18} In contrast, H_2 inhalation treatment is safe for the human body at concentrations $< 4\%$, is not associated with a risk of explosion when exposed to air, and the concentration is easy to control.^{19,20} H_2 has a low molecular weight and strong permeability, and it easily penetrates the biofilm and diffuses into the cytoplasm, mitochondria, and nucleus.¹² Thus, researchers have focused more heavily on this method of administration.

Cai *et al.*²¹ demonstrated that inhalation of H_2 has a neuroprotective effect in HIBD rats; however, Matchett *et al.*²² reported that H_2 inhalation does not protect against HIBD and aggravates brain damage in rats. To identify a safe and effective method, we examined the effect of 3% H_2 inhalation for different durations in HIBD rats. Furthermore, the potential mechanisms underlying the neuroprotective effect of H_2 inhalation will be assessed as well.

Materials and methods

Animals

Seven-day-old female and male Sprague-Dawley (SD) rats (body weight range from 9 to 13 g) were purchased from Slac Corporation (Changsha Slac Animal Corporation, Hunan, China). The rats' pups were housed in cages with their mothers. All experiments were approved by the Center for Medical Ethics of Central South University and were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (eight edition).

Hypoxia-ischemia model in neonatal rats

We induced HIBD in rats using the Rice-Vannucci model.²³ The pups were anesthetized with isoflurane, and the left common carotid artery (CCA) was carefully exposed. Afterwards, the CCA was double-ligated using a 6-0 surgical silk tie, which was cut between sutures. The skin incision was then sutured, and pups were permitted to recover for 2 h with their dam in their home cages. Following this, rats were placed in a glass anoxic chamber (constant

temperature of 37°C; 8% O_2 + 92% N_2) for 100 min to induce brain injury.

H_2 inhalation and experimental design

H_2 was generated via water electrolysis in a hydrogen gas supply device (MiZ Co. Ltd, Kanagawa, Japan). Following HI injury, the rat pups were treated with mixed gas (3% hydrogen and 97% air) in a device. The device was 30 × 13 × 12.5 cm in size, white, and transparent, allowing us to observe the activity of the rats. A total of 140 rats were divided randomly into seven groups. The four experimental groups were classified according to the length of H_2 inhalation. The rats divided in the first three groups listed below were interfered with H_2 inhalation immediately after HI, while the rats in the last group were started at 2 h after HI: (1) 30-min ($n = 22$; female: 12, male: 10); (2) 60 min ($n = 22$; female: 11, male: 11); (3) 90 min ($n = 22$; female: 10, male: 12); (4) 90 min (start at 2 h after HI, $n = 8$; female: 4, male: 4). The three control groups were classified as follows: (1) naive (no processing) ($n = 22$; female: 10, male: 12); (2) sham-surgery (left common carotid artery was separated without ligation then sutured, and no hypoxia) ($n = 22$; female: 13, male: 9); and (3) HIBD (carotid ligation and hypoxia) ($n = 22$; female: 11, male: 11). Rats were interfered with H_2 inhalation twice daily then euthanized at 24 h or 72 h after surgery for 2,3,5-triphenyltetrazolium chloride (TTC) staining or immunoblot and histological staining, respectively. Twelve rats per group were used for pathology and molecular biology experiments, and ten rats per group were used to evaluate neurobehavioral function.

Infarct volume

TTC (Sigma, St. Louis, MO, USA) staining was used to evaluate cerebral infarcted area in rats. At 24 h post-surgery, rats were sacrificed via decapitation, and brains were sliced into five sequential coronal sections (thickness, 2 mm), followed by immersed in the 1% TTC solution for 30 min at 37°C and turned every 5 min. The sections were sequentially photographed (Sony, Tokyo, Japan) and the cerebral infarction/non-infarction volume was calculated by Image J software. The calculation of the percentage of infarct volume was the same as Cai *et al.* described.²¹ The percentage of infarct volume (%) = the infarct volume/the total volume of the sections.

Nissl staining

Rats' pups were deeply anaesthetized and the brains were obtained following perfusion and fixed. Then, the fixed brain tissue was paraffin embedded, followed by serial sectioning into 5- μ m slices. After deparaffinization, the slices were immersed in 2% Nissl working solution for 5 min. Afterwards, the slices were rinsed in differentiation solution until the Nissl body was clarified (Wellbio, Beijing, China). The slices were photographed under a microscope (magnification 200×; Nikon, Tokyo, Japan).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

TUNEL staining assay kit (Roche, Basel, Switzerland) was applied to detect apoptosis. Briefly, the slices were incubated with TUNEL reaction solution for 1 h. Afterwards, washed the slices with PBS and counterstained the nucleus with DAPI for 10 min. The sections were visualized under a fluorescence microscope (Nikon) with 400 \times magnification. Four slide fields within the left frontal and parietal cortex and hippocampal CA1 were randomly examined and the results are expressed as the percentage of TUNEL-positive cells in selected field (TUNEL-positive cells/total number of nuclei).

Immunofluorescence

After deparaffinization, sections were under antigen retrieval process. Followed by blocking with 5% BSA for 1 h, the slices were immersed overnight at 4°C in primary antibodies. The antibodies examined in the experiment are listed below: IL-1 β (1:100; Proteintech, Rosemont, Maryland, USA), NF- κ B p65 (1:100; Abcam, Cambridge, CA, USA), P-JNK (1:100; Abcam), p53 (1:200; Proteintech), Iba-1 (1:500; Abcam) and NeuN (1:500; Millipore, Billerica, MA, USA). Next day, the sections were rinsed with PBST three times, and visualized with Alexa Fluor-labeled secondary antibodies (Alexa Fluor 594, 1:800; Thermo Scientific, Rockford, IL, USA; Alexa Fluor 488, 1:800; Thermo Scientific, Rockford, IL, USA) for 2 h. DAPI were used for counterstaining the nucleus. The micro- and macro-images from the frontal and parietal cortex region or hippocampal CA1 were acquired under a microscope (magnification 400 \times ; Nikon), and merged using Image J.

Western blot analysis

The tissue proteins of the frontal and parietal cortex region were extracted at 72 h after surgery and quantified with a BCA kit (CWBIO, Beijing, China). Protein samples were separated by using SDS-PAGE gels under different concentrations range from 8% to 12%, and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk in PBS for 2 h, membranes were incubated overnight at 4°C with the following primary antibodies: IL-1 β (1:500, Bioss, Beijing, China), NF- κ B p65 (1:500, Abcam, Cambridge, CA, USA), P-JNK (1:500, Abcam), p53 (1:1000, Proteintech, Rosemont, Maryland, USA), β -actin (1:1000, Proteintech), and GAPDH (1:1000, Proteintech). The next day, membranes were washed with PBST. Afterwards, membranes were immersed in the secondary antibody (IRDye 800CW, 1:10,000). Immunoblotting bands were automatically photographed by the Odyssey-CLX infrared imaging system (Li-COR®, USA). The relative intensities of the bands were analyzed using Image J.

Morris water maze

The MWM test was performed during adolescence (post-natal days 43–47) to assess neurobehavioral function of rats mainly related to the abilities including the spatial memory and learning.²⁴ The experiment was carried out in a

cylindrical pool (Panlab, Spain), and water temperature of 22 \pm 1°C. The pool included four equal quadrants: south-east, southwest, northeast, and northwest. A platform was hidden in the middle of the northeast quadrant. Visual reference objects were located around the pool for spatial localization, such as fixed experimental items, lights, and cards of different shapes, which were unchanged throughout the experiment.

The experiment included two parts: (1) Examination of spatial learning ability (location-navigation test). All rats underwent four trials per day, lasting four days. In a trial, animals were required to find the hidden platform in less than 1 min. An experimenter recorded the time to reach the platform, which was defined as the “escape latency.” If the rats could not reach the hidden platform in less than 1 min, the experimenter guided them to the hidden platform, and recorded the escape latency for 1 min. Each rat was required to stay on the hidden platform for 20 s and allowed a 20 min intertrial interval. The escape latency was automatically tracked and analyzed using tracking software (SMART 3.0 video-tracking system, Panlab, Spain). The average escape latency recorded for each of the four trials was taken as the learning achievement of the rats on the same day, and the trial was repeated for four days. (2) Examination of spatial memory (space exploration test). In probe trials, the platform was taken away, and each rat was placed in the same selected quadrant (opposite quadrant to the original hidden platform). The tracking software recorded the time (residence time) to stay in the probe quadrant and swimming path over 1 min. The residence time was used as a measure of spatial memory.

Statistical analysis

The quantified data were shown as means \pm SD. One-way analyses of variance (ANOVA) followed by Tukey’s *post hoc* tests were used for multiple comparisons of different groups. The univariate repeated measures ANOVA was used to analyze the MWM results for independent group comparisons. Statistical analysis was performed using SPSS 22.0 Software and the bar graphs were drawn by GraphPad Prism 6 software. Differences with $P < 0.05$ were defined as statistically significant. In the Nissl staining experiment, TTC staining, and immunofluorescence tests, no significantly differences were obtained between the naive group and the sham-surgery group. So, the results of the experiment in the naive group are not shown in the picture.

Results

H₂ inhalation improves neuronal injury and cerebral infarction volume

In the present study, Nissl staining was used to observe neuronal damage in the left frontal and parietal cortex and hippocampal CA1 of rats. In the sham-surgery group, neurons had a complete morphological structure, large cell bodies, and abundant cytoplasm and Nissl bodies; however, the HIBD group exhibited extensive neuronal damage, such as karyopyknosis, dark-blue staining of nuclei, neuronal structure and Nissl’s body disappeared.

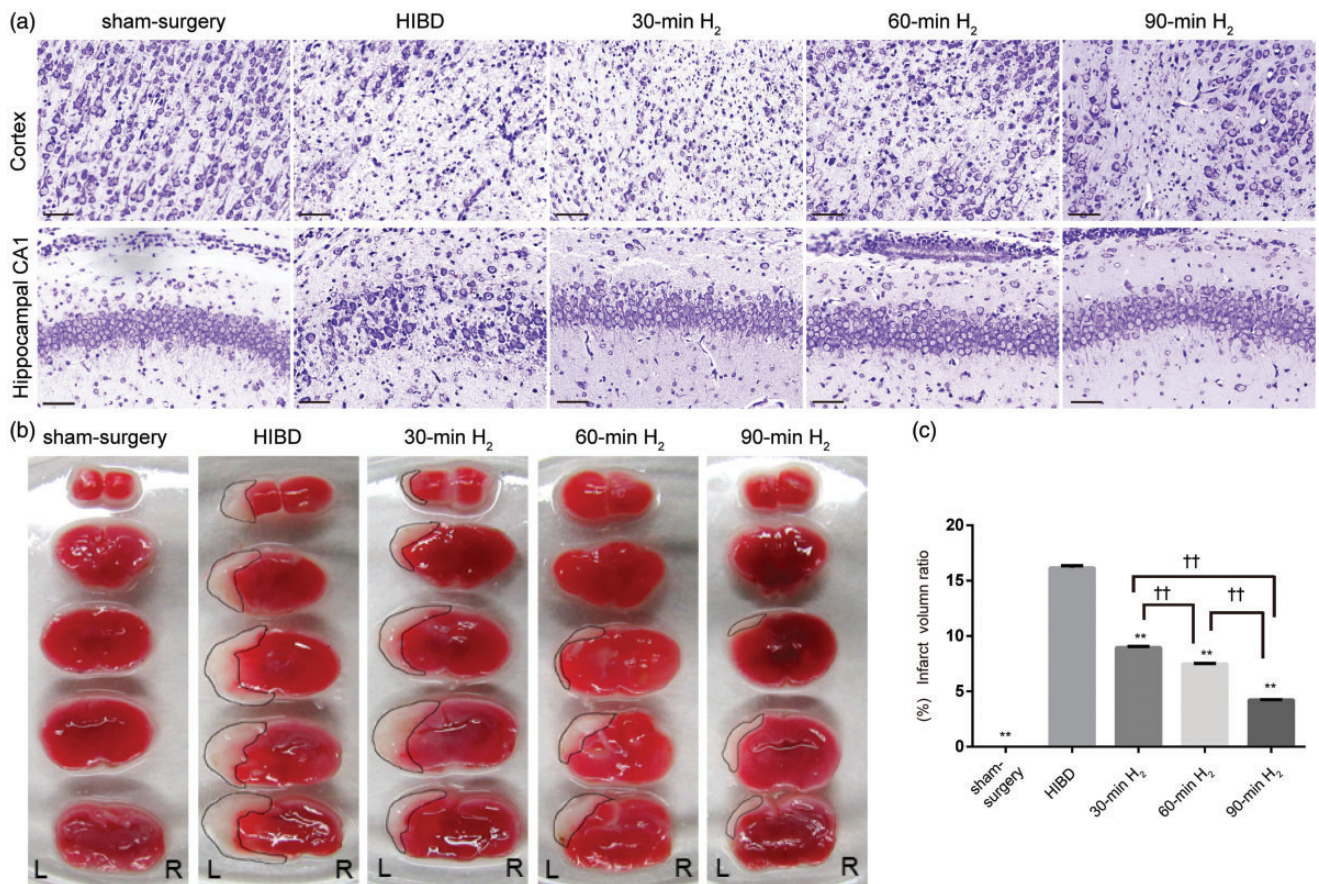


Figure 1. Nissl and TTC staining following H₂ inhalation in rats with HIBD. (a) Representative images of Nissl staining in the left frontal and parietal cortex and hippocampal CA1 region of the sham-surgery, HIBD, 30-min H₂, 60-min H₂, and 90-min H₂ groups at 72 h after surgery. Scale bar = 100 μ m. $n = 4$ per group. (b) Representative images of TTC staining on the first day after surgery from coronal brain sections of the sham-surgery, HIBD, 30-min H₂, 60-min H₂ and 90-min H₂ groups at 24 h after surgery. (c) The percentage of the cerebral infarct volume in the ipsilateral hemisphere of different groups. $n = 4$ per group. ** $P < 0.001$ vs. HIBD group, $^{++}P < 0.001$.

Interestingly, this neuronal damage was obviously alleviated after H₂ inhalation, especially in the 90-min H₂ group (Figure 1(a)).

In addition, TTC staining was used to analyze cerebral infarction volume. There was a dramatically higher cerebral infarction rate in HIBD rats than those in sham-surgery rats (Figure 1(a) and (b)). Infarction rates ($8.96 \pm 0.08\%$, $7.50 \pm 0.02\%$, and $4.22 \pm 0.04\%$, respectively) were significantly lower in the 30-min H₂, 60-min H₂, and 90-min H₂ groups than in the HIBD group ($16.16 \pm 0.16\%$, $P < 0.001$; Figure 1(c)). These results indicate that H₂ inhalation ameliorated neuronal injury and reduced cerebral infarction volume, which was most evident after 90-min of H₂ inhalation (30-min H₂ vs. 60-min H₂, $P < 0.001$; 60-min H₂ vs. 90-min H₂, $P < 0.001$). And we found that the effects of inhaling H₂ immediately after HI were better than that inhalation of H₂ at 2 h after HI ($P < 0.05$, Supplementary Figure 1).

H₂ inhalation inhibits microglial activation and inflammation

Microglia are immune cells. Iba-1 is a marker of resting and activated microglia. At 72 h after surgery, we examined Iba-1 fluorescence in the left frontal and parietal cortex and left

hippocampal CA1 regions to investigate microglial activation. In the sham-surgery group, microglia were dendritic, and few positive cells were observed, indicative of resting microglia. However, in the HIBD group, microglia were oval or amoeboid in shape, and the number of positive cells had increased significantly, indicative of activated microglia (Figure 2(a) and (b)). Immunofluorescence staining showed that H₂ inhalation remarkably reduced the activation of microglia after HI (Cortex, 30-min H₂ vs. HIBD, $P = 0.055$; 60-min H₂ vs. HIBD, $P < 0.001$; 90-min H₂ vs. HIBD, $P < 0.001$. Hippocampal CA1, 30-min H₂, 60-min H₂ or 90-min H₂ vs. HIBD, $P < 0.001$), and decreases in Iba-1 expression became more obvious as the duration of H₂ inhalation increased (Cortex, $P < 0.01$; Hippocampal CA1, $P < 0.001$) (Figure 2(a) and (b)). These findings indicate that H₂ inhalation significantly inhibited the activation and proliferation of microglia induced by hypoxia-ischemia, and this inhibition was most pronounced in the 90-min H₂ group.

Furthermore, to verify whether H₂ can inhibit the inflammatory response of microglia, we examined the expression of Iba-1, IL-1 β , and NF- κ B p65 in the left frontal and parietal cortex. There were dramatically increased Iba-1⁺/IL-1 β ⁺ and Iba-1⁺/NF- κ B p65⁺ cells at 72 h after surgery, which suggested that HI induced microglial

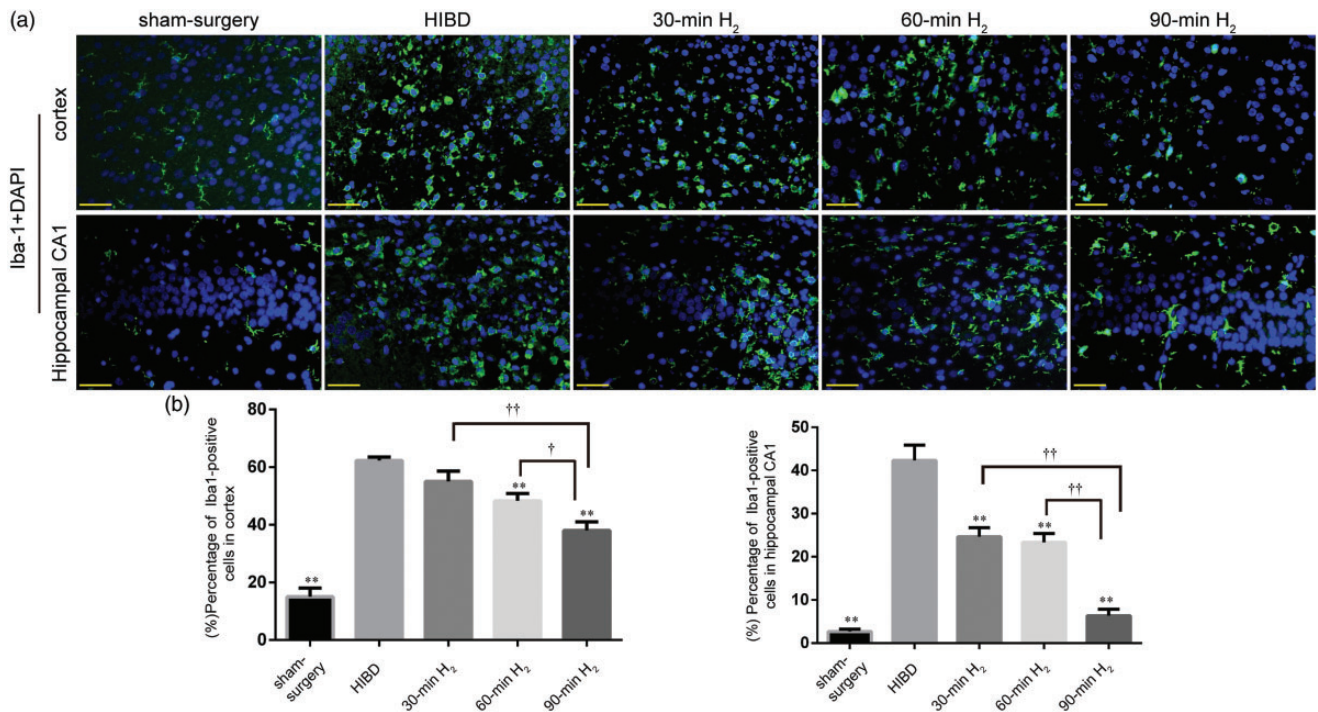


Figure 2. Microglial activation changes following H₂ inhalation in rats with HIBD. (a) Representative immunofluorescence staining Iba-1 (green, activated microglia) and DAPI (blue, all nuclei) in the left frontal and parietal cortex and hippocampal CA1 region of rats in sham-surgery, HIBD, 30-min H₂, 60-min H₂, and 90-min H₂ groups at 72 h after surgery. Scale bar = 50μm. (b) Differences in the percentage of Iba-1-positive cells in the cortex and hippocampal CA1 between groups. *n* = 4 per group. ***P* < 0.01 vs. HIBD group, ††*P* < 0.01, †*P* < 0.05.

inflammatory responses. However, immediate inhalation of H₂ after HI injury notably reversed these changes, and the effects were most pronounced in the 90-min H₂ group (*P* < 0.05, Figure 3(a) to (c)). The patterns of IL-1β and NF-κB p65 expression observed via Western blot analysis were consistent with those detected via fluorescence (Figure 3(d) and (e)). The expression of IL-1β and NF-κB p65 was dramatically increased after HI injury (*P* < 0.001), and significantly decreased by H₂ inhalation when compared with HIBD rats at 72 h post-surgery (IL-1β, 30-min H₂ vs. HIBD, *P* = 0.0026; 60-min H₂, 90-min H₂ vs. HIBD, *P* < 0.0001. NF-κB p65, *P* < 0.0001). Decreases became more obvious as the duration of H₂ inhalation increased (IL-1β, 30-min H₂ vs. 60-min H₂, *P* = 0.0019; 60-min H₂ vs. 90-min H₂, *P* < 0.001. NF-κB p65, 30-min H₂ vs. 60-min H₂, *P* = 0.9710; 60-min H₂ vs. 90-min H₂, *P* < 0.001).

H₂ inhalation inhibits neuronal apoptosis in HIBD rats

TUNEL staining assay was applied to examine the inhibition effect of apoptosis by H₂ inhalation. The results illustrated that numbers of TUNEL-positive cells were obviously raised after HI, while they were reversed by H₂ inhalation (*P* < 0.001, Figure 4(a) and (b)), especially in the 90-min H₂ group (Cortex, 30-min H₂ vs. 60-min H₂, *P* = 0.0258, 60-min H₂ vs. 90-min H₂, *P* = 0.0381. Hippocampal CA1, 30-min H₂ vs. 60-min H₂, *P* = 0.0058, 60-min H₂ vs. 90-min H₂, *P* = 0.0148). However, 30 min of H₂ inhalation did not reduce the number of TUNEL-positive cells in HI condition in the hippocampal CA1 region (30-min H₂ vs. HIBD, *P* = 0.1617), indicating that 30 min of H₂ inhalation had no obvious effect on neuronal

apoptosis induced by HI injury. Nonetheless, the inhibitory effects of H₂ inhalation on apoptosis were obvious in the 90-min H₂ group. And we also found that the effects of inhaling H₂ immediately after HI injury were better than that inhalation of H₂ started at 2 h after HI (*P* < 0.05, Supplementary Figure 2(a) and (b)).

H₂ inhalation inhibits upregulation of P-JNK/p53 signaling in HIBD rats

C-Jun N-terminal kinase (JNK) activates transcription factor p53 and induces apoptosis of the mitochondrial pathway. NeuN is neuronal-specific. In this study, we examined the double immunofluorescence of NeuN and P-JNK or p53 in the left frontal and parietal cortex. Double-immunofluorescence staining revealed that NeuN⁺/P-JNK⁺ and NeuN⁺/p53⁺ cells were notably raised in the HIBD rats, indicating that P-JNK and p53 expression in neurons increased after HI injury, thereby inducing apoptosis. However, hydrogen inhalation reversed this increase, and these effects were most pronounced in the 90-min H₂ group (*P* < 0.05, Figure 5(a) to (c)). Similar results were observed via immunoblot. The expression of P-JNK and p53 protein was remarkably increased by HI injury when compared with naive and sham-surgery rats at 72 h after surgery (*P* < 0.001; Figure 5(d) and (e)). However, H₂ inhalation attenuated these increases (P-JNK, 30-min H₂ vs. HIBD, *P* = 0.6215, 60-min H₂ vs. HIBD, *P* = 0.002, 90-min H₂ vs. HIBD, *P* < 0.001. p53, 30-min H₂, 60-min H₂ or 90-min H₂ vs. HIBD, *P* < 0.001), and such attenuation became more obvious as the duration of H₂ inhalation increased (*P* < 0.05). The result of this section illustrated that H₂ inhalation

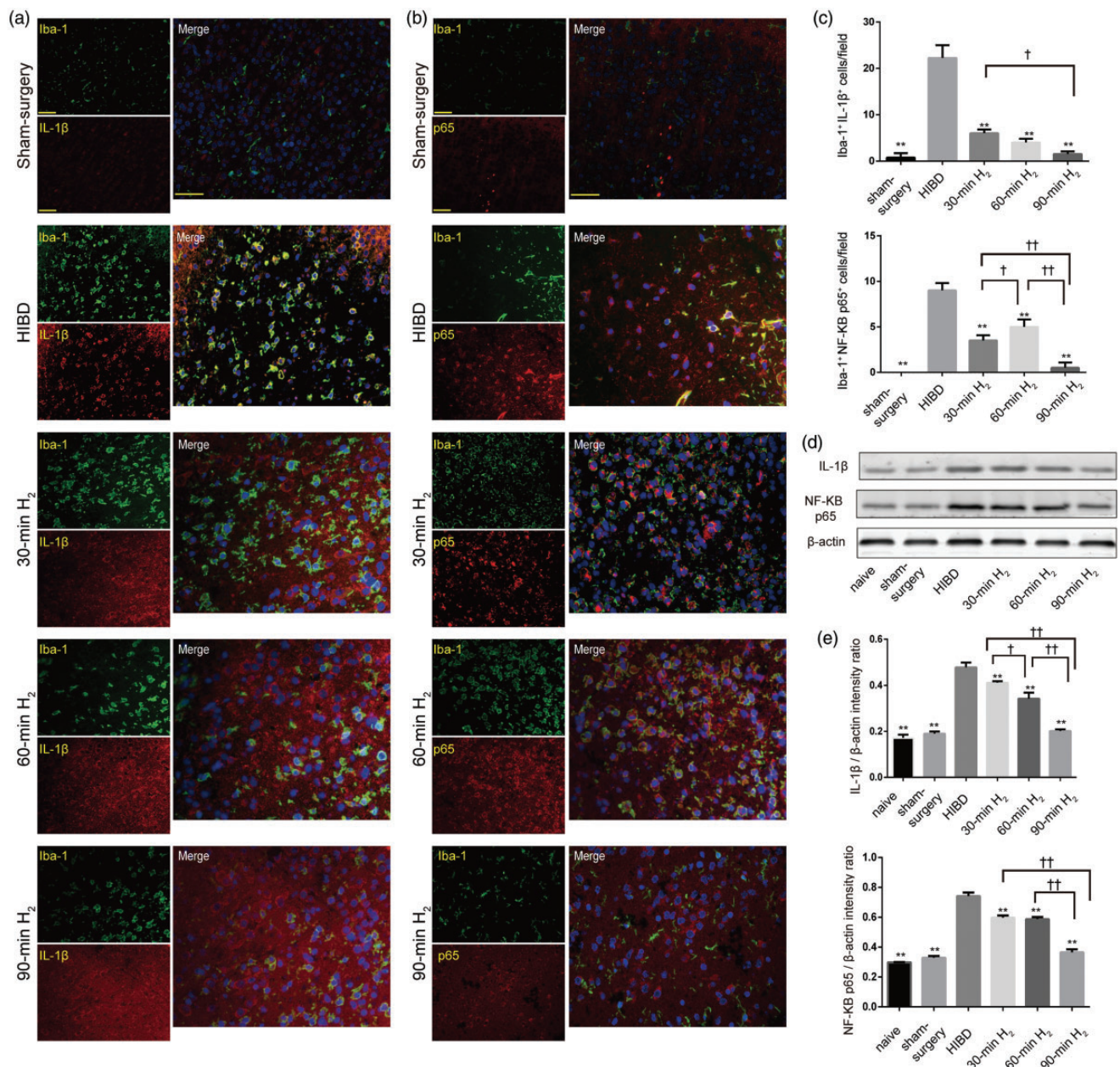


Figure 3. Inflammatory changes following H₂ inhalation in rats with HIBD. (a, b) Representative images of IL-1β (red) or NF-κB p65 (red) and Iba-1 (green) immunofluorescence staining, and merged immunofluorescence in the left frontal and parietal cortex of rats from sham-surgery, HIBD, 30-min H₂, 60-min H₂, and 90-min H₂ groups. Scale bar = 50 μm. (c) Quantitative analyses of Iba-1⁺/IL-1β⁺ and Iba-1⁺/NF-κB p65⁺ cells. *n* = 4 per group. (d) Representative western blot bands of IL-1β and NF-κB p65 in the cortex of rats from the naive, sham-surgery, HIBD, 30-min H₂, 60-min H₂, 90-min H₂ groups. β-actin protein levels were used as internal loading controls. (e) Data represent the mean ± SD of the normalized densitometry values of IL-1β and NF-κB p65. Quantitative protein levels were normalized with β-actin. *n* = 4 per group. ***P* < 0.01 vs. HIBD group, ††*P* < 0.01, †*P* < 0.05.

inhibited the P-JNK/p53 signaling in a time-dependent manner, thus, it inhibited neuronal apoptosis.

H₂ inhalation improves long-term neurobehavioral deficits in HIBD rats

To further assess the neuroprotective effects of H₂ inhalation, the long-term neurobehavioral function was examined by MWM experiment in HI rats during adolescence (P43–47). From the first to fourth day, the mean escape latency gradually decreased in all groups. On the first day, H₂ inhalation could not shorten the escape latency

after HI (*P* > 0.05). However, from days 2 to 4, H₂ inhalation significantly shortened the mean escape latency (main effects of group, *P* < 0.001; main effects of time, *P* < 0.001; the interaction between group and time, *P* < 0.001; days 2–4, *P* < 0.001) (Figure 6(b)), compared with the HIBD group. Furthermore, the mean escape latency was shortest in the 90-min H₂ group. These results indicated that all rats showed the abilities of learning and remembering the location of the platform; however, this learning ability was impaired because of hypoxia and ischemia. Furthermore, the findings suggest that H₂ inhalation rescued spatial learning in HIBD rats. On the fifth day, the swimming

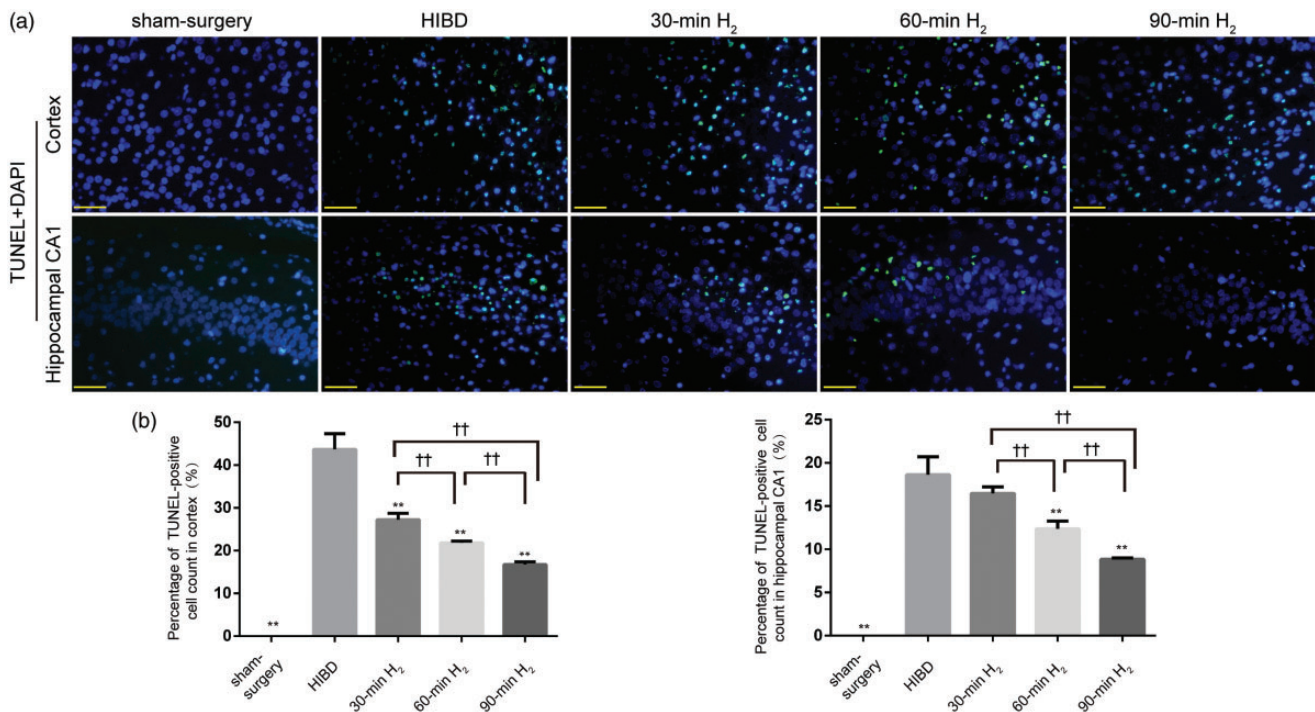


Figure 4. H₂ inhalation reduces neuronal apoptosis in the cortex and hippocampal CA1 of rats with HIBD. (a) Representative TUNEL staining in the left frontal and parietal cortex and hippocampal CA1 region of rats from the sham-surgery, HIBD, 30-min H₂, 60-min H₂, and 90-min H₂ groups at 72 h after surgery (green, TUNEL-positive cells; blue, cell nuclei). Scale bar = 50 μm. (b) The percentage of TUNEL-positive staining in each group. *n* = 4 per group. ***P* < 0.01 vs. HIBD group, ††*P* < 0.01.

path was located mainly around the initial quadrant (Figure 6(a)) in the HIBD group, which also exhibited the shortest time in the target quadrant (Figure 6(c)). These findings suggested that HI damage during the neonatal period resulted in memory impairment in adolescent rats. However, H₂ inhalation obviously improved the duration spent in the platform quadrant (30-min H₂ vs. HIBD, *P* = 0.0231, 60-min H₂ or 90-min H₂ vs. HIBD, *P* < 0.0001), which was longest in the 90-min H₂ group (Figure 6(c)). These results suggested that H₂ inhalation effectively improved memory impairments induced by HI.

Discussion

In summary, our present findings confirmed the neuroprotective effect of H₂ inhalation in HIBD rats. H₂ inhaling reduced the activation of microglia in HIBD rats, reduced the accumulation of IL-1β and NF-κB p65, inhibited the microglial inflammatory response, reduced the cerebral infarction rate, inhibited neuronal apoptosis by downregulating the expression of P-JNK and p53, reduced neuronal damage, and improved long-term neurobehavioral deficits induced by HI in a time-dependent manner.

The common clinical treatment methods for neonatal HIBD include mild hypothermia, hyperbaric oxygen, and stem cell transplantation^{26,26}; however, this effect is not consistent.^{4,27,28} Previous researches have indicated that oxidative stress, apoptosis, and inflammation are associated with neonatal HIBD.^{12,25} Oxidative stress is involved in the main pathological progression of HIBD. Hypoxia-ischemia injury induces the activation of microglia to produce oxygen free radicals, which then promote the

secretion of inflammatory mediators.²⁹ Both H₂ and hydrogen sulfide (H₂S) have shown neuroprotective effect by anti-oxidative stress and selectively scavenge oxygen free radicals.^{12,30} However, the discrepancy of H₂S after brain injury needs to be pointed. For example, Li *et al.*³¹ reported that 0.1 mmol/L protected against ischemic brain damage in rats; however, the absolutely opposite effect was obtained by Qu *et al.*³² when the concentration of H₂S reached 0.18 mmol/L in the same model. These results indicated that the effective window of H₂S is extremely small. Considering that H₂S is a poisonous gas having a pungent odor, it is not suitable for newborns. Moreover, neither is easy to control the therapeutic concentration, nor can instrument accurately measure the concentration of H₂S in cells, it is difficult to track and analyze the effects of H₂S.³³ Compared to H₂S, H₂ is a safe and stable molecular free radicals' scavenger odor, which can be measured easily and accurately. Therefore, H₂ was chosen for treatment in the present study.

We performed three different durations of H₂ inhalation, i.e. 30 min, 60 min, and 90 min. We compared the effects of three different H₂ inhalation durations on HIBD and found that inhaling H₂ for 90 min was the most effective. The therapeutic effect of inhaling H₂ continuously several times was more effective than single short-term inhalation of H₂.²² Simultaneously, we performed H₂ inhalation at two different time intervals after HI injury, i.e. immediately, and 2 h after HI injury. We found that the effects of inhaling H₂ immediately after HI were better than that inhalation of H₂ start at 2 h after HI. It seems to imply that the earlier H₂ intervention is performed, the better neuroprotective effect will be obtained.

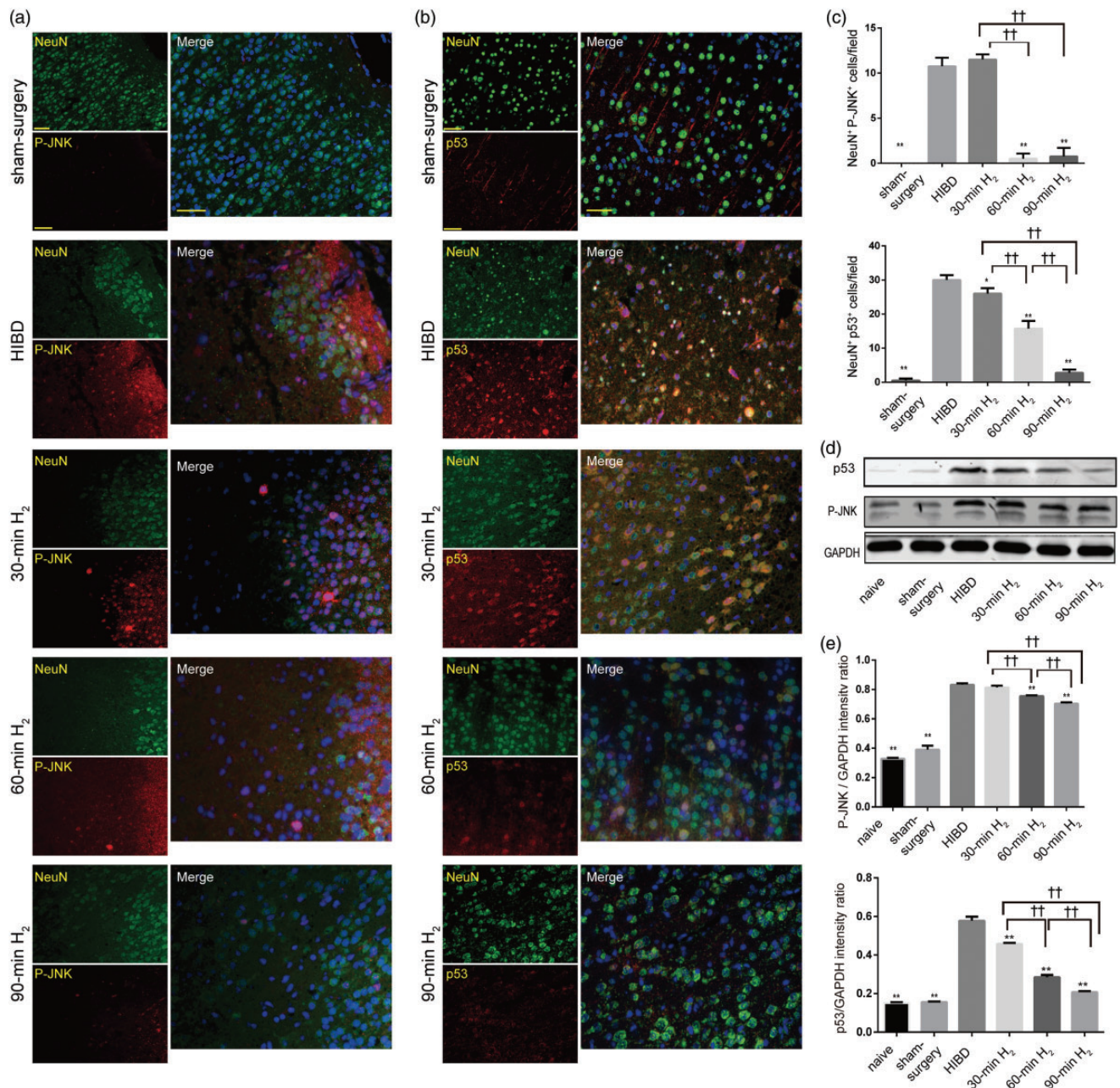


Figure 5. H₂ inhalation decreases the protein expression of P-JNK and p53 in the cortex of rats with HIBD. (a, b) Representative images of P-JNK (red) or p53 (red) and NeuN (green) staining, and merged immunofluorescence in the left frontal and parietal cortex of rats from the sham-surgery, HIBD, 30-min H₂, 60-min H₂, and 90-min H₂ groups at 72 h after surgery. Scale bar = 50 μm. (c) Quantitative analyses of NeuN⁺/P-JNK⁺ and NeuN⁺/p53⁺ cells. *n* = 4 per group. (d) Representative Western blot bands of P-JNK and p53 in the cortex of rats from the naive, sham-surgery, HIBD, 30-min H₂, 60-min H₂, and 90-min H₂ groups. GAPDH protein levels were used as internal loading controls. (e) Data represent the mean ± SD of the normalized densitometry values of P-JNK and p53. Quantitative protein levels were normalized with GAPDH. *n* = 4 per group. ***P* < 0.01 vs. HIBD group, ††*P* < 0.01.

Our MWM test results indicated that neonatal rats exposed to HI exhibit poor neurobehavioral outcomes during adolescence, suggesting that hippocampal and cortex injury during the stage of development impairs spatial memory and learning abilities. However, inhalation of H₂ immediately after HI injury significantly improved neurobehavioral function (Figure 6). The neuroprotective effect of H₂ on HIBD appears to be associated with the anti-inflammatory and anti-apoptotic effects after scavenging oxygen-free radicals, as H₂ treatment significantly

diminished the activation of microglia, in addition to IL-1β and NF-κB p65 protein expression. In addition, we observed that H₂ inhalation significantly increased neuronal survival, decreased the infarct ratio and level of apoptosis, and reduced P-JNK and p53 activity.

Microglia are derived from the hematopoietic system and constitute immune cells in the central nervous system, serving immune and self-defense functions.^{34,35} Microglia are first activated after cerebral ischemia and produce proinflammatory cytokines, which participate in the

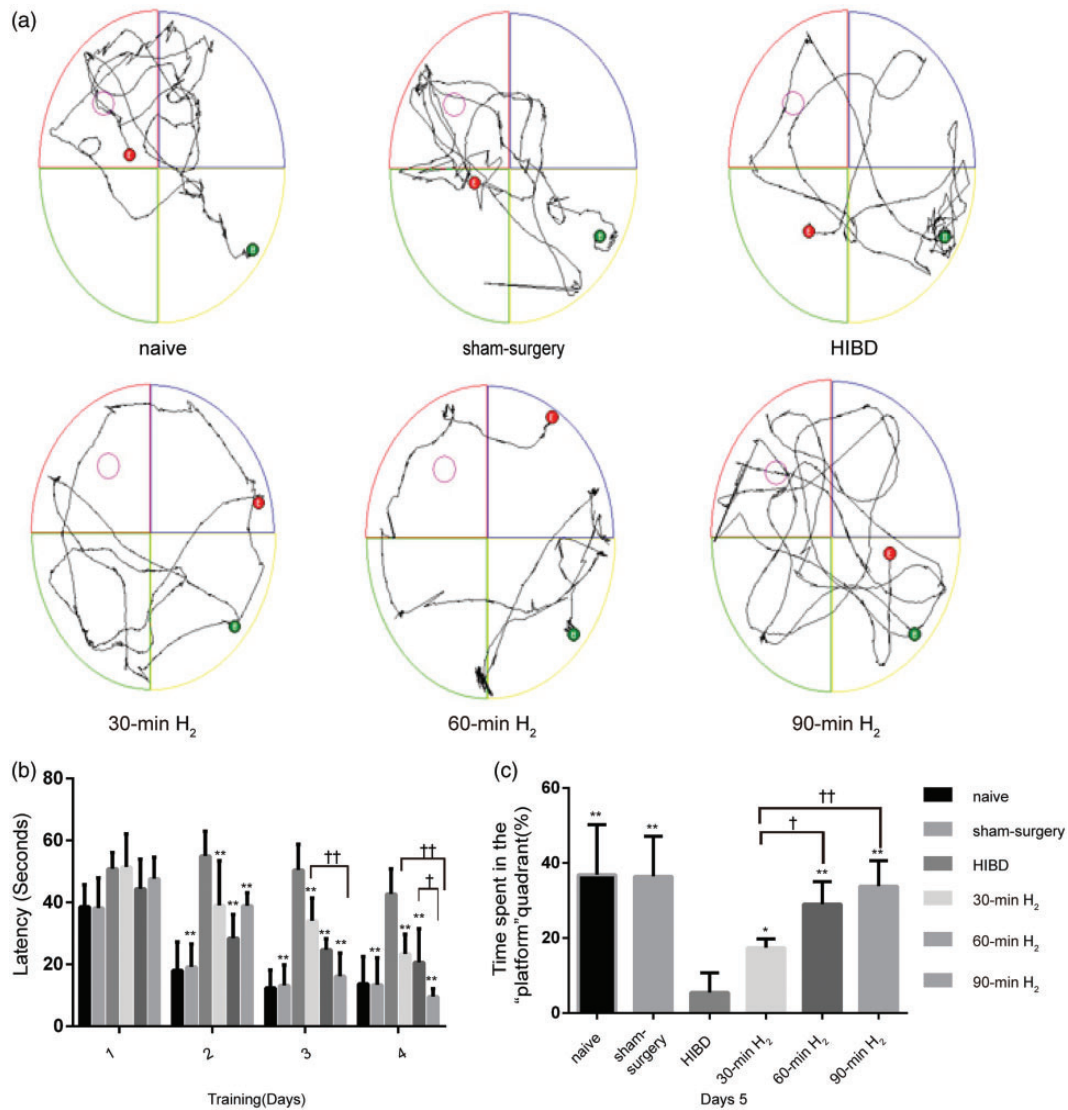


Figure 6. H₂ inhalation improves long-term spatial learning and memory in rats with HIBD. Rats were trained from postnatal days 43–47. Each rat underwent four trials per day for four consecutive days and a probe trial on the fifth day. (a) Representative swimming paths of rats on day 5 of testing in the naive, sham-surgery, HIBD, 30-min H₂, 60-min H₂, and 90-min H₂ groups of rats. (b) Latencies to reach the hidden platform for each group of rats. (c) The time spent (%) in the target quadrant on day 5 of testing for each group of rats. Data represent mean \pm SD from independent experiments. $n = 10$ per group. ** $P < 0.01$ vs. HIBD group, †† $P < 0.01$, † $P < 0.05$.

inflammatory response after ischemia injury.³⁶ Therefore, we investigated inflammatory responses based on microglial activation.

IL-1 β interacts with activated microglia, producing persistent inflammatory products that enhance inflammation and cause neuronal damage.^{37,38} NF- κ B upregulates the expression of inflammatory cytokines after activation under various extracellular stresses, thereby initiating the inflammatory response and apoptosis, which includes p65 and p50 subunits.^{39–41} The secretion of the proinflammatory cytokine IL-1 β activates NF- κ B, which in turn upregulates the expression of IL-1 β , thus enhancing the neuroinflammatory response. In the present study, we observed microglial activation, and a corresponding increase in IL-1 β and NF- κ B p65 expression, following HI injury, which were significantly attenuated following H₂ treatment in HIBD rats (Figures 2 and 3). Moreover, these inhibitory effects became more apparent as the treatment

duration increased. Furthermore, the therapeutic effects of H₂ were possibly mediated by the inhibition of IL-1 β and NF- κ B p65 activities.

Activated microglia produce proinflammatory cytokines that have been proven to be critical mediators of neurodevelopment.⁴² However, over-reactive microglia and inflammatory mediators may cause neuronal damage.³⁸ In the present study, the loss of Nissl bodies following HI injury was associated with vacuole formation, and there was a remarkable rise in infarction rates and apoptosis (Figures 1 and 4). These findings indicate that the increased inflammatory response results in neuronal damage; however, inhalation of H₂ after HI injury obviously attenuated neuronal damage. Taken together, these data further indicate that inhalation of H₂ attenuates neuronal damage induced by HI.

The JNK signaling pathway can be activated by proinflammatory cytokines, and associated with oxidative stress,

inflammation, and apoptosis.^{43,44} The JNK pathway mediates apoptosis via the death receptor, mitochondrial apoptosis, and endoplasmic reticulum stress apoptotic pathways. At the early stage of apoptosis, calcium ions in the cytoplasm continuously increase the activation of JNK [activated JNK (P-JNK)] in the nucleus, further activating transcription factors, such as c-Jun or p53, and regulating the activity of the Bcl-2 family of proteins in the cytoplasm.⁴⁵ This results in an increase in mitochondrial membrane permeability, which activates the mitochondrial apoptosis pathway. P53, a tumor suppressor protein, acts as a transcription factor to induce cell cycle arrest, DNA damage and repair, and cell senescence.⁴⁶ Therefore, we chose JNK as the major indicator, which was validated by the JNK/p53 pathway in our experiments. Our results indicate that HI injury activated the JNK signaling pathway and increased P-JNK expression, promoting the expression of the downstream pro-apoptotic protein p53, which induced apoptosis (Figure 5).

Our findings demonstrated that inhalation of 3% H₂ could reduce the activation of microglia. Furthermore, inhibition of the JNK/p53 pathway significantly improved learning and memory functions in HIBD rats. However, there are some limitations to our study that must be acknowledged. For example, we focused on microglia in this study. Astrocytes, other immune cells, and other brain regions should be assessed in future studies to further our understanding of the protective mechanism of H₂. We can further study the therapeutic effect of hydrogen on different sexes of rats.

Taken together, our study demonstrated that cerebral HI activates microglia, promotes the secretion of IL-1 β and NF- κ B p65, induces neuronal damage, upregulates P-JNK/p53 signal transduction, and induces long-term spatial memory defects. Inhalation of 3% H₂ immediately after HI injury reduced inflammation and inhibited cell apoptosis. In summary, H₂ may play a neuroprotective role and improve long-term spatial memory in HIBD rats by reducing inflammation and inhibiting apoptosis. Therefore, H₂ may be an effective method for the treatment of HIBD.

Authors' contributions: The designation of the experiments as well as the interpretation and analysis of the data was contributed by all authors. The manuscript was drafted by Wu and reviewed and approved by all authors; GW, ZC, PW, MZ, and WZ conducted the experiments; MF and SH supplied critical reagents.

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