

Neuroprotective effect of hydrogen sulfide against glutamate-induced oxidative stress is mediated via the p53/glutaminase 2 pathway after traumatic brain injury

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ABSTRACT

Several reports suggest that hydrogen sulfide (H₂S) exerts multiple biological and physiological effects on the pathogenesis of traumatic brain injury (TBI). However, the exact molecular mechanism involved in this effect is not yet fully known. In this study, we found that H₂S alleviated TBI-induced motor and spatial memory deficits, brain pathology, and brain edema. Moreover, sodium hydrosulfide (NaHS), an H₂S donor, treatment markedly increased the expression of Bcl-2, while inhibited the expression of Bax and Cleaved caspase-3 in TBI-challenged rats. Tunnel staining also demonstrated these results. Treatment with NaHS significantly reduced the glutamate and glutaminase 2 (GLS-2) protein levels, and glutamate-mediated oxidative stress in TBI-challenged rats. Furthermore, we demonstrated that H₂S treatment inhibited glutamate-mediated oxidative stress through the p53/GLS-2 pathway. Therefore, our results suggested that H₂S protects brain injury induced by TBI through modulation of the glutamate-mediated oxidative stress in the p53/GLS-2 pathway-dependent manner.

INTRODUCTION

Traumatic brain injury (TBI), which contributes to subsequent damage of associated neurons, has imposed a significant burden on family and society in the world [1, 2]. Although great improvements have been made in medical intervention, many approaches or neuroprotective agents for TBI failed during clinical trials [3–5].

Glutamate has been reported to play an important role in the excitement of nerve activity during TBI [6, 7]. Garzón et al showed that NeuroEPO protected cortical neurons from glutamate-induced apoptosis via the upregulation of Bcl-2 and inhibit glutamate-induced activation of caspase-3 [8]. Glutathione depletion, perhaps triggered by early glutamate-mediated excitotoxicity, led to late post-repetitive mild TBI loss of parvalbumin-positive

interneuron-dependent cortical inhibitory tone [9]. Also, zolpidem prevented glutamate-induced toxicity in differentiated P19 neurons via the PI3K/Akt pathway [10]. Therefore, searching for neuroprotective agents, which can inhibit glutamate-induced toxicity, may be a promising therapeutic strategy for TBI treatment.

As an important gasotransmitter and endogenous neuromodulator, hydrogen sulfide (H₂S) has been reported to exert multiple biological and physiological effects on the pathogenesis of numerous diseases, such as stroke, Alzheimer's disease, and TBI [11–13]. Sodium hydrosulfide (NaHS), an H₂S donor, improved spatial memory impairment of rats with TBI [14]. H₂S could prevent scratch-induced cellular injury, alteration of mitochondrial membrane potential, intracellular accumulation of reactive oxygen species, and cell death

in PC12 cells through modulation of the PI3K/Akt/Nrf2 pathway [15]. Besides, NaHS treatment increased endogenous antioxidant enzymatic activities and decreased oxidative product levels in the brain tissue of TBI-challenged rats [16]. However, the biological role of H₂S in regulating glutamate-induced apoptosis and oxidative stress during TBI remains unclear. In the present study, we used the TBI rat model to determine the protective effects of H₂S on the motor and spatial memory deficits and brain edema. Furthermore, we explored its potential neuroprotective mechanism through glutamate-induced apoptosis and oxidative stress.

RESULTS

H₂S alleviated TBI-induced motor and spatial memory deficits and brain edema

To determine the effect of H₂S on TBI-induced motor deficits, a wire-grip test and a rota-rod test were performed. Compared with the sham group, TBI led to a significant decline in motor performance at 0 to 7 days post-injury. NaHS, an H₂S donor, treatment

significantly improve the motor function on days 0 to 7 after TBI compared to TBI and TBI+ vehicle groups (Figure 1A, 1B).

We then performed a Morris water maze test to analyze spatial memory ability on days 8 to 15. Compared with the sham group, rats from TBI and TBI+ vehicle groups showed increased latencies to find the hidden platform. We observed a significant decrease in the latencies in the TBI+ NaHS group (Figure 1C). The data of a spatial probe test also showed that H₂S improved the spatial memory ability of TBI rats (Figure 1D).

To determine the effect of H₂S on TBI-induced cerebral injury, we analyzed brain pathology and brain edema. As shown in Figure 1E, compared with the sham group on day 7, rats from TBI and TBI+ vehicle groups showed serious pathological changes. Treatment with NaHS significantly improved the pathological changes. In addition, TBI led to a significant increase in the percentage of brain water content compared to the sham group (Figure 1F). Treatment with NaHS markedly reduced the percentage of brain water content.

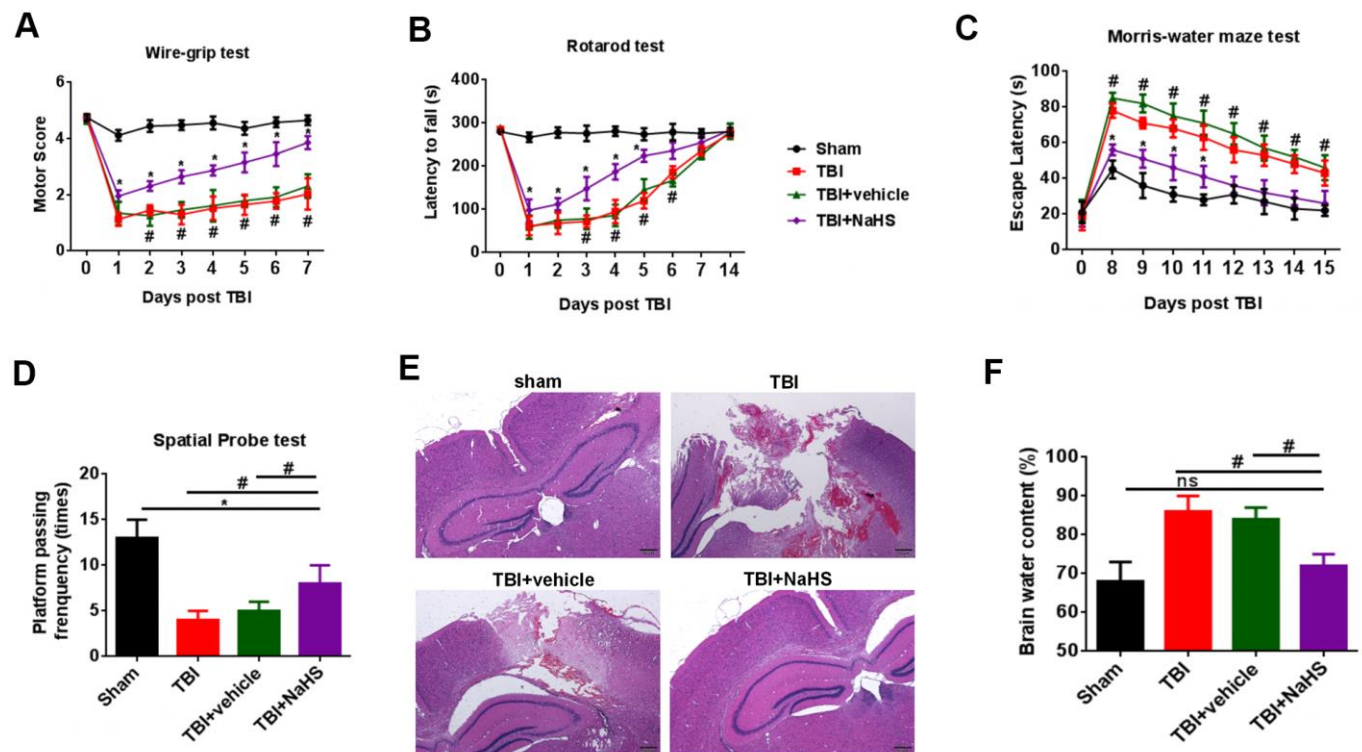


Figure 1. H₂S alleviated TBI-induced motor and spatial memory deficits and brain edema. (A) A wire-grip test was performed to analyze the motor function at 0 to 7d after TBI (n = 5). (B) A Rota-rod test was performed to analyze the motor function at 0 to 7d and 14d after TBI (n = 5). (C) A Morris water maze test was performed to test spatial memory ability on days 8 to 15 (n = 5). (D) A spatial probe test was performed to test spatial memory ability on days 16 (n = 5). (E) The pathological changes was analyzed by H&E staining. (F) The brain water content was measured at 24 h after TBI (n = 5). #P<0.05 vs. sham group.* P<0.05, vs. TBI and TBI+ vehicle groups. #P<0.05 vs. sham group.

H₂S inhibited apoptosis after TBI

To investigate the effect of H₂S on apoptosis after TBI, western blot was performed to detect the expression of the apoptosis-associated protein, such as Bcl-2, Bax, and Cleaved caspase-3. As shown in Figure 2A, 2B, the Bcl-2 expression was significantly down-regulated after TBI compared to the sham group. Treatment with NaHS markedly increased the expression of Bcl-2 after TBI. By contrast, treatment with NaHS inhibited the

expression of Bax and Cleaved caspase-3, which was increased in TBI-challenged rats (Figure 2A, 2C, 2D). Moreover, Tunnel staining also showed that treatment with NaHS reduced the apoptosis rate of cerebral cortex in TBI rats (Figure 2E).

H₂S inhibited the levels of glutamate after TBI

We then investigated the effect of H₂S on glutamate levels after TBI. As shown in Figure 3A, the glutamate

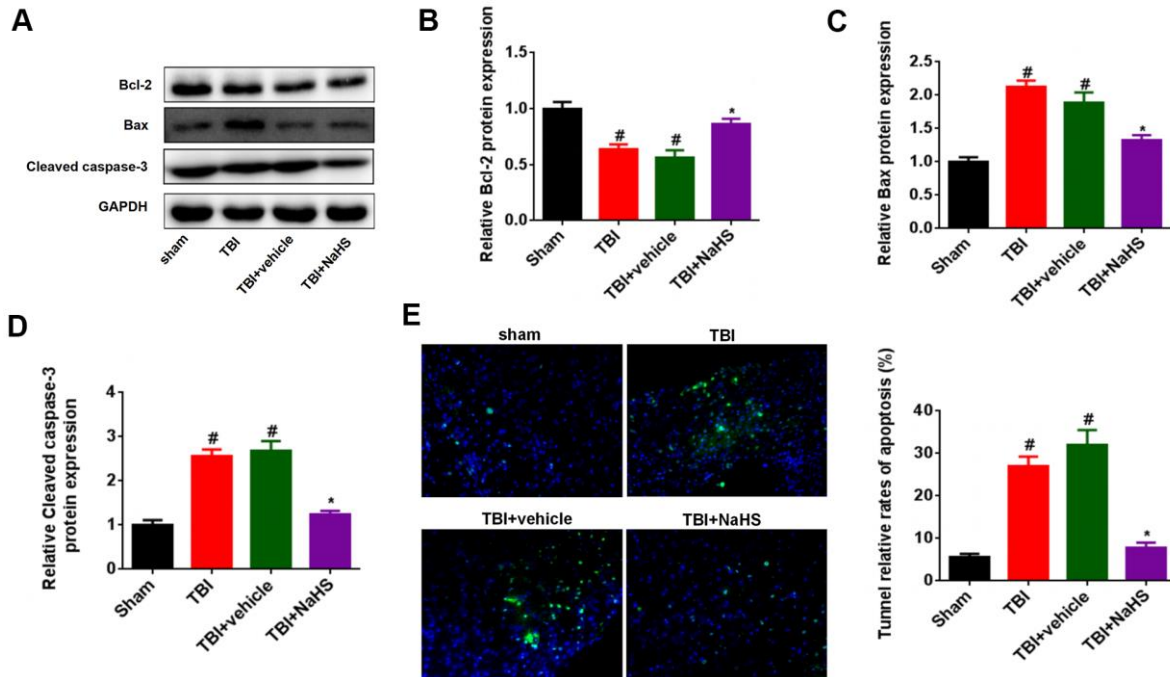


Figure 2. Treatment with H₂S inhibited TBI-induced apoptosis. (A) Western blot was performed to analyze the protein level of Bcl-2, Bax, and Cleaved caspase-3 in TBI-challenged rats after treatment with NaHS. (B–D) The band density of Bcl-2 (B), Bax (C), and Cleaved caspase-3 (D) was analyzed using Image J. (E) The apoptosis rate of cerebral cortex in TBI rats was analyzed by Tunnel staining. * P<0.05, vs. TBI and TBI+ vehicle groups. #P<0.05 vs. sham group.

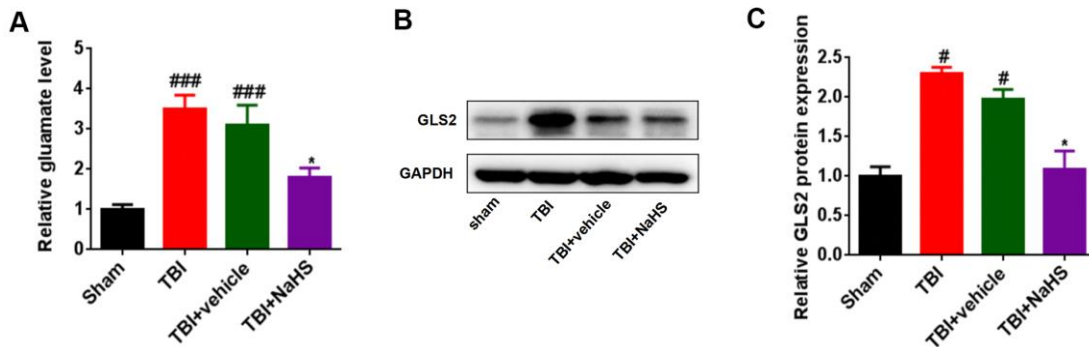


Figure 3. Treatment with H₂S inhibited TBI-induced glutamate. (A) The level of glutamate in TBI-challenged rats after treatment with NaHS. (B) Western blot was performed to analyze the protein level of GLS-2 in TBI-challenged rats after treatment with NaHS. (C) The band density of GLS-2 was analyzed using Image J. * P<0.05, vs. TBI and TBI+ vehicle groups. #P<0.05, ###P<0.001 vs. sham group.

level was significantly increased after TBI. Treatment with NaHS markedly reduced the glutamate level in TBI-challenged rats. Given that the GLS2 level was regulated by glutaminase, we also determined the effect of H₂S on GLS2 expression after TBI. The protein expression of GLS2 in TBI-challenged rats was higher than that in the sham group rats. Moreover, the GLS2 protein level was significantly decreased in the TBI+NaHS group compared to TBI and TBI+ vehicle groups (Figure 3B, 3C).

H₂S inhibited brain oxidative stress after TBI

To explore the effect of H₂S on brain oxidative stress after TBI, malondialdehyde (MDA) content, superoxide dismutases (SOD) activities, and glutathione peroxidase (GPx) activities were assessed, respectively. Compared to the sham group, TBI lead to an increase in the level of MDA and a decrease in the activities of SOD and GPx (Figure 4A–4C). Treatment with NaHS reversed the effect of TBI on MDA content, SOD activities, and GPx activities. Besides, we further analyzed the expression of HO-1, an oxidative stress gene, using RT-

qPCR and western blot analysis. The mRNA and protein expression of HO-1 in TBI-challenged rats was higher than that in sham group rats (Figure 4D–4F). Treatment with NaHS increased the mRNA and protein expression of HO-1 in TBI-challenged rats.

H₂S inhibited glutamate-mediated oxidative stress via the p53/GLS-2 pathway

Since H₂S can inhibit glutamate and oxidative stress, we hypothesized that the inhibited glutamate and oxidative stress effect was induced by the p53/GLS-2 signaling pathways. As shown in Figure 5A, 5B, treatment with NaHS inhibited the protein expression of p53 in TBI-challenged rats, while pifithrin- α treatment abolished the inhibitory effect of H₂S on p53 expression in TBI-challenged rats. Furthermore, pifithrin- α treatment reversed the effect of H₂S on GLS2 and glutamate expression in rats after TBI (Figure 5A, 5C, 5D). More importantly, pifithrin- α treatment abolished the effect of H₂S on MDA content, SOD activities, and GPx activities in TBI-challenged rats (Figure 5E–5G).

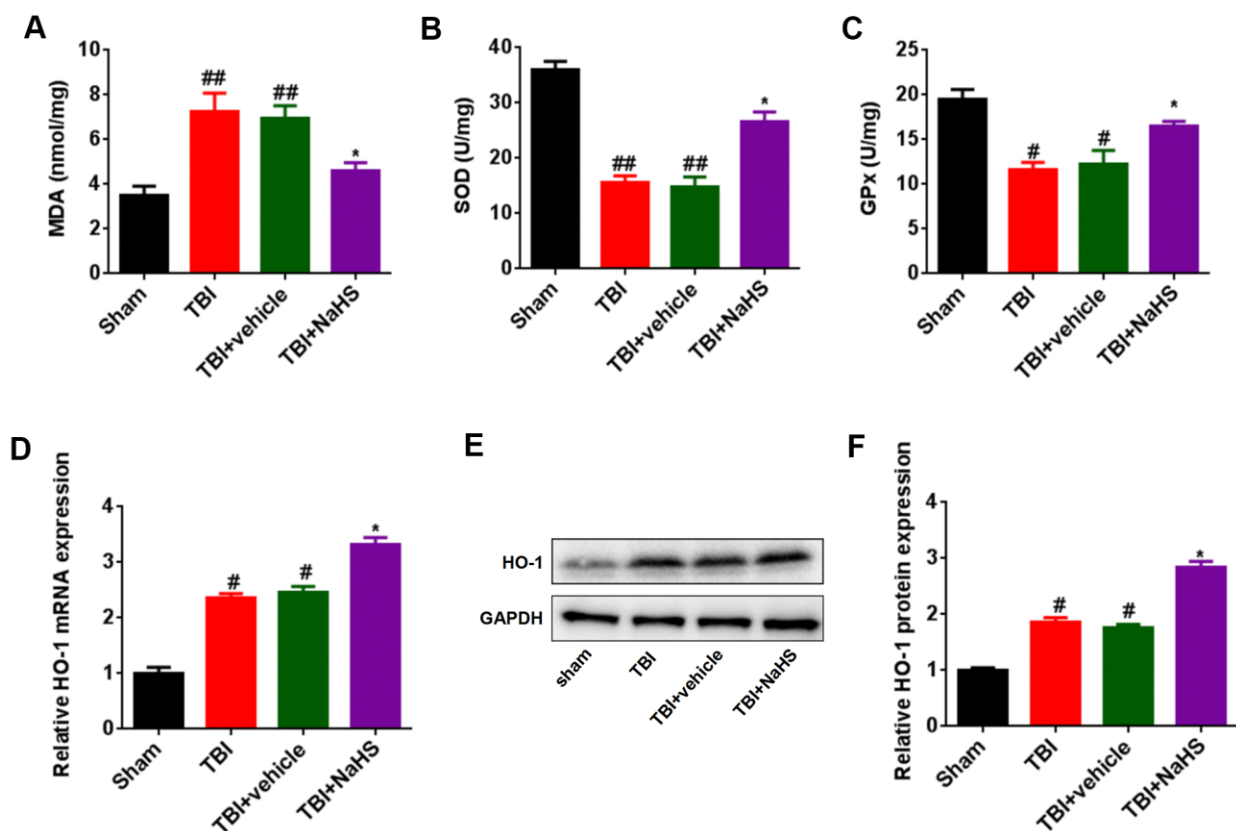


Figure 4. Treatment with H₂S inhibited TBI-induced oxidative stress and HO-1 expression. (A) The level of MDA in TBI-challenged rats after treatment with NaHS. (B, C) The activities of SOD (B) and GPx (C) in TBI-challenged rats after treatment with NaHS. (D) The mRNA level of HO-1 in TBI-challenged rats after treatment with NaHS. (E) The protein level of HO-1 in TBI-challenged rats after treatment with NaHS. (F) The band density of HO-1 was analyzed using Image J. * P<0.05, vs. TBI and TBI+ vehicle groups. #P<0.05, ##P<0.01 vs. sham group.

DISCUSSION

Previous studies have shown the neuroprotective roles of H₂S on TBI [13, 15]. Mingyang et al. reported that H₂S pretreatment had reduced brain edema, improved motor performance, and ameliorated performance in the Morris water maze test after TBI [17]. Karimi et al. showed that NaHS has a neuroprotective effect on TBI-induced memory impairment in rats [14]. Also, NaHS treatment ameliorated brain injuries, characterized by an increase of blood-brain barrier permeability, brain edema, and lesion volume, as well as neurologic dysfunction in TBI-challenged rats [16]. In this study, our data also demonstrated that H₂S alleviated TBI-induced motor and spatial memory deficits, brain pathology, and brain edema. Moreover, we also found that NaHS treatment markedly increased the expression of Bcl-2, while inhibited the expression of Bax and Cleaved caspase-3 in TBI-challenged rats. Tunnel staining also demonstrated this result. These results were similar to the results of previous studies [17, 18], and suggested that H₂S played the protective effect against TBI by regulating apoptosis.

It has been well known that glutamate-induced excitotoxicity, which leads to neuronal damage and functional impairments, is implicated in TBI [19]. The metal chaperone, PBT2, currently in clinical trials for Huntington's disease, could protect against glutamate-induced excitotoxicity thought to underlie both acute and chronic neurodegenerative diseases [20]. Moreover, zolpidem exerted the neuroprotective effect in differentiated P19 neurons by inhibiting the glutamate-induced toxicity [10]. Herein, we hypothesized that H₂S-mediated neuroprotective effects on TBI may be associated with glutamate-induced toxicity. We found that treatment with NaHS markedly reduced the glutamate and glutaminase protein levels in TBI-challenged rats. These results showed that H₂S played neuroprotective effects on TBI through inhibiting glutamate-induced toxicity. Jiang et al. found that exogenous H₂S administered could exert a protective effect against TBI via activation of mitoK(ATP) channels and reduction of oxidative stress [16]. In addition, the protective effect of H₂S against TBI was associated with regulating apoptosis and autophagy [17]. Therefore, we inferred that H₂S played neuroprotective effects

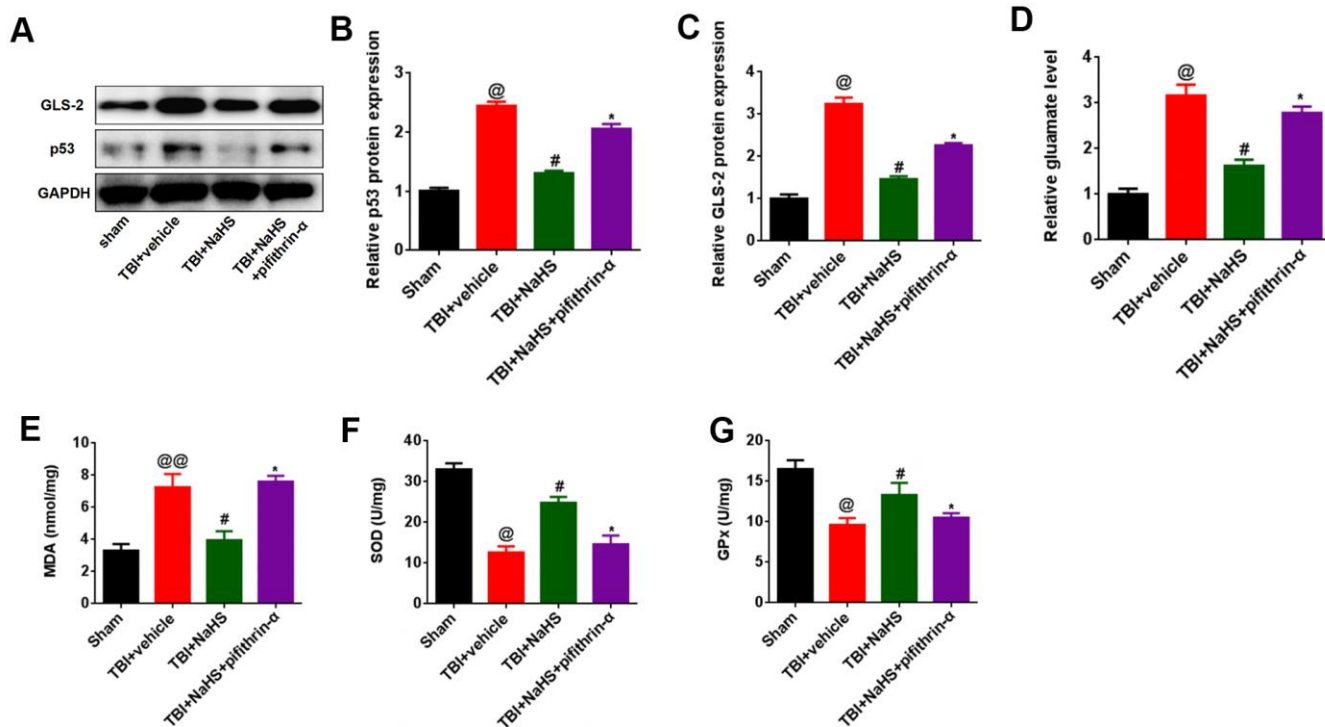


Figure 5. p53 inhibition reversed the effect of H₂S on glutamate and glutamate-mediated oxidative stress after TBI. (A) The protein level of GLS-2 and p53 in TBI-challenged rats after co-treatment with NaHS and pifithrin- α . (B, C) The band density of GLS-2 (B) and p53 (C) was analyzed using Image J. (D) The level of glutamate in TBI-challenged rats after co-treatment with NaHS and pifithrin- α . (E) The level of MDA in TBI-challenged rats after co-treatment with NaHS and pifithrin- α . (F, G) The activities of SOD (F) and GPx (G) in TBI-challenged rats after co-treatment with NaHS and pifithrin- α . * P<0.05, vs. TBI+NaHS+ pifithrin- α group. #P<0.05, vs. TBI+ vehicle group. @P<0.05, @@<0.01, vs. sham group.

on TBI via multiple mechanisms such as inhibiting glutamate-induced toxicity, activating of mitoK(ATP) channels, and regulating apoptosis and autophagy.

Oxidative stress and their byproducts could cause brain damage and overall clinical outcome [21]. Nrf2 is an important protective factor against TBI-induced injuries by inhibiting oxidative stress after TBI [22]. PDIA3 provided significant improvements in cognitive impairments and contusion volume induced by TBI through attenuating oxidative stress [23]. Given that glutamate-mediated oxidative stress was associated with multiplication, differentiation, inflammation, survival, and apoptosis of cells [24], we also explored the effect of H₂S on brain oxidative stress after TBI. We found that treatment with NaHS reversed the effect of TBI on MDA content, SOD activities, and GPx activities, three biomarkers of oxidative stress. Moreover, NaHS treatment inhibited the mRNA and protein expression of HO-1 in TBI-challenged rats. These results suggested that the neuroprotective effects of H₂S on TBI may be associated with glutamate-mediated oxidative stress.

In addition, we investigated the molecular mechanisms that H₂S regulated the glutamate-mediated oxidative stress. A previous study showed that the p53 pathway was involved in the progression of TBI. Hong et al. reported that JNK-mediated p53 expression could regulate neuron autophagy following TBI in rats [25]. The p53 inactivator pifithrin- α oxygen analogue could inhibit glutamate-induced excitotoxicity and improve histological and functional outcomes after experimental TBI [26]. Since GLS-2 was a p53 target gene [27], we hypothesized that the inhibited glutamate and oxidative stress effect was induced by the p53/GLS-2 signaling pathways. Our results showed that pifithrin- α treatment reversed the inhibitory effect of H₂S on p53, GLS-2, and glutamate expression in TBI-challenged rats. Moreover, pifithrin- α treatment abolished the effect of H₂S on MDA content, SOD activities, and GPx activities in TBI-challenged rats. These results suggested that H₂S inhibited glutamate-mediated oxidative stress via the p53/GLS-2 pathway.

In conclusion, the present study showed that H₂S treatment alleviated TBI-induced motor and spatial memory deficits and brain edema, as well as inhibited apoptosis, glutamate-mediated oxidative stress through the p53/GLS-2 pathway.

MATERIALS AND METHODS

Mouse TBI model

Sprague-Dawley (SD) rats, weighing 250-300 g, were obtained from the Animal Center of the Hebei Medical

University (Shijiazhuang, China). All mouse experiments were approved by the Ethics Committee of The Second Hospital of Hebei Medical University. The mouse TBI model was established as previously described [28]. Briefly, the rats were anesthetized with 4% chloral hydrate. The following surgery was performed under aseptic conditions and mounted in a stereotaxic system (David Kopf Instruments, Tujunga, California). A midline incision was made to expose the skull, and the bone flap was carefully removed by a manual trephine (Roboz Surgical Instrument Co., Gaithersburg, MD). We then used a weight-drop device to perform the TBI in the left part of the brain as described previously [29, 30]. For the sham operation group, rats underwent the same procedure as TBI rats except for the impact.

To examine the effect of H₂S on TBI, all rats were randomly allocated into sham, TBI, TBI+vehicle, and TBI+NaHS groups. The rats in the TBI+NaHS group were i.p. injected with NaHS (1mmol/kg, Sigma-Aldrich, St. Louis, MO), and the rats in the TBI+ vehicle group were i.p. injected with saline 30 min before TBI.

Wire-grip test

We used a wire-grip test to evaluate the motor function as described previously [28, 29]. Briefly, we placed rats on the metal wire, which was suspended 45 cm above a foam pad. We then measured and recorded the latency that a mouse remained on the wire within a 60 s' interval. The scores of wire-grip were calculated as described previously [31].

Rota-rod test

We also used a rota-rod test to evaluate the motor function as described previously [32]. Before surgery, all rats were pre-trained for balancing on an automated rota-rod (Ugo Basile, Comerio, Italy) at a constant speed of 40 rpm. The average latency to fall from the rod was recorded, and the maximum cutoff time was 180 s.

Morris water maze test

We performed a Morris water maze test to evaluate spatial learning and memory performance as described previously [28, 33]. Briefly, the water was added into an experimental apparatus, and colored by white non-toxic food pigment. A clear plexiglass goal platform was placed 0.5 cm under the water surface. We allowed rats to find the submerged platform at a maximum of 90 s on days 8 to 15, and remain on the platform for an additional 10 s if the mouse reached the submerged platform. Then, we used a video/compute system to recorded and analyzed the escape latency that a mouse

reached the visible platform. For the spatial probe test, we removed the submerged platform and allowed rats to explore the pool within 90 s on day 16. A video tracking system was used to monitor the frequency of passing through the target quadrant.

Pathological analysis of the brain tissues

For H&E staining, the 5 µm slides from brains of rats in sham, TBI, TBI+vehicle, or TBI+NaHS group were stained by H&E staining kit (Solarbio, China) according to the manufacturer's instructions. Briefly, the slides were deparaffinized using xylene and ethanol. After permeabilized, the slides were stained with hematoxylin and eosin. Finally, images were obtained from an optical microscope.

Brain water content

A wet-dry weight method was used to measure the brain water content as described previously [28, 33]. Briefly, the whole brains were removed from anesthetized rats and immediately weighed to determine wet weight. The brain tissues were completely dried in an oven at 100° C. After 24h, the dry weight was measured. Brain water content was calculated as (wet weight–dry weight)/wet weight×100%.

Malondialdehyde (MDA), superoxide dismutases (SOD), and glutathione peroxidase (GPx) assay

The activities of MDA, SOD, and GPx in brain tissue were analyzed using commercial kits (Jiangcheng, Nanjing, China, #A003-1-2 for MDA, #A001-4-1 for SOD; #A005-1-2 for GPx) according to the manufacturer's instructions.

Western blot

The total protein from cortical was lysed in RIPA lysis with PMSF and protease inhibitor (Thermo Fisher Scientific, USA). The protein concentration was detected using a BCA assay kit (Beyotime, Shanghai China). Equal proteins (30 µg) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). The membranes were with 5% BSA and were incubated with primary antibodies to anti-Bcl-2 (Abcam), anti-Bax (Abcam), anti-Cleaved caspase-3 (CST), anti-GLS2 (Abcam), anti-HO-1 (CST), anti-p-AKT (CST), anti-t-AKT (CST) and anti-GAPDH (Abcam) overnight at 4° C. Then the membranes were incubated with secondary antibody and visualized using an enhanced chemiluminescence system (Pierce Biotech, IL, USA). The densitometry of each band was quantified by ImageJ software.

Tunnel apoptosis staining

For Tunnel apoptosis staining, the 5 µm slides from brains of rats in sham, TBI, TBI+vehicle, or TBI+NaHS group were stained by the Tunnel staining kit (Roche, USA) according to the manufacturer's instructions. Briefly, the slides from brains of rats were dewaxed and rehydrated. Then, the slides were incubated with TUNEL reaction mixture at 37° C for 1 h. The nuclei were stained with DAPI.

RNA isolation and quantitative real-time PCR (RT-qPCR) assays

Total RNA was extracted from cortical using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The method used to detect HO-1 mRNA expression was based on a previous report [22]. Briefly, cDNA was constructed using a commercial kit (GeneCopia, Rockville, USA). Then, RT-PCR was performed on an Applied Biosystems 7500 Sequence Detection system using a SYBR PrimeScript RT-qPCR Kit (Takara). The primers used in this study were as follows: HO-1 Forward Primer 5'-CTGTGCCACCTGGAACTGAC-3', Reverse Primer 5'-TCTTGTGGGTCTTGAGCTGTT-3'; β-Actin Forward Primer 5'-GTTGA GAACCGTGTACCATGT-3', Reverse Primer 5'-TTCC CACAATTTGGCAAGAGC-3'. β-Actin was used as an internal control.

Statistical analysis

All data were present as mean±SD. Student t-test was used to determine statistically significant differences between two groups, and one-way ANOVA was used for > 2 groups. P<0.05 was considered to be statistically significant.

AUTHOR CONTRIBUTIONS

Feng Wang conceived and designed the experiments. Xiaoyu Gu gave an experimental guidance in the lab. Hailong Du collected the data. Gengshen Zhang and Jianliang Wu performed the experiments. Xiaoyu Li analyzed the data. Jianping Sun wrote the paper.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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