Neuroendocrinology Letters Volume 45 No. 4 2024
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

ORIGINAL ARTICLE

Hydrogen sulfide improved learning and memory deficits by reversing the inhibition of methylmercuric chloride on BDNF/TrkB signaling pathway.

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Submitted: 2024-03-12 Accepted: 2024-10-06 Published online: 2024-10-23

Key words: **Learning and memory impairment; MMC; H2S; BDNF/TrkB**

Neuroendocrinol Lett 2024; **45**(4):281–288 **PMID:** 39607357 45042404

Abstract

OBJECTIVE: Methylmercuric chloride (MMC) has neurotoxicity, while hydrogen sulfide (H_2S) has shown inhibitory properties against nerve damage induced by various factors. The study aimed to investigate the impact of H_2S on MMC-induced learning and memory impairment in mice and to explore the underlying mechanisms.

METHODS: A mouse model of learning and memory impairment was established by MMC gavage, and sodium hydrosulfide (NaHS) was used as an H_2S donor for intervention. Cell viability and live/dead cell ratio in HT22 neuronal cells were assessed by CCK-8 assay and Calcein/PI staining, respectively. The Morris water maze test was performed to evaluate the learning and memory abilities of mice. Western blotting was utilized to determine protein expressions of BDNF and TrkB. The effects of H_2S on MMC-induced learning and memory impairment were investigated based on the BDNF/TrkB pathways.

RESULTS: (1) MMC treatment decreased cell viability and reduced the ratio of live cells in HT22 cells, while H_2S reversed these changes. (2) MMC prolonged escape latency, decreased platform crossing frequency, and reduced quadrant distance percentage of the platform in the Morris water maze test, while H_2S reversed the above changes. (3)MMC downregulated BDNF and TrkB expression levels, while H_2S suppressed these changes induced by MMC. (4)Treatment with 7, 8-DHF (a TrkB agonist) significantly attenuated MMC-induced prolonged escape latency and reduced platform crossing frequency.

CONCLUSIONS: Our findings demonstrated that H_2S ameliorated learning memory deficits in mice by reversing the inhibitory effects of MMC on BDNF/TrkB signaling pathway.

Abbreviations:

MMC	- Methylmercuric chloride
BDNF	- Brain-derived neurotrophic factor
NaHS	- Sodium hydrosulfide
TrkB	- Tyrosine Kinase receptor B
AD	- Alzheimer's disease
7, 8-DHF	- 7,8-Dihydroxyflavone

INTRODUCTION

Methylmercuric chloride (MMC) is a widespread and highly toxic environmental contaminant characterized by its lipophilic nature that readily crosses the blood-brain barrier, endowing it with potent neurotoxic properties (Wu *et al.* 2023). Exposure to MMC can induce loss of neuronal viability, reduction in the number of cortical neurons, disruption of cell organization, alterations in mitochondrial function, and inhibition of proliferation and differentiation of hippocampal neural stem cells, thereby impairing hippocampal-dependent spatial learning and memory (Tian *et al.* 2016; Krishna *et al.* 2019). Although these toxic effects have been extensively demonstrated in animal models (Augustyniak *et al.* 2023; Jackson, *et al.* 2018), the underlying mechanisms remain incompletely understood.

The brain-derived neurotrophic factor (BDNF)/tyrosine receptor kinase B (TrkB) signaling pathway has been established as a pivotal player in learning and memory processes (Zhang *et al.* 2022; Saral *et al.* 2023). BDNF has been shown to accelerate the establishment of axon-dendrite polarity in hippocampal neurons (Feng *et al.* 2022), with TrkB, the receptor for BDNF, being widely expressed in areas such as the cerebral cortex and hippocampus. TrkB is the primary regulator of dendritic growth within the central nervous system (Moya-Alvarado *et al.* 2023). Dysfunction in the BDNF/TrkB pathway is closely linked to neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, affecting synaptic plasticity, synaptogenesis, and consequently, learning and memory (Chiu *et al.* 2023; Hacioglu *et al.* 2023; Zulkifli *et al.* 2023; Sgritta *et al.* 2022). While Methylmercury exposure has been shown to downregulate BDNF expression and promote neuronal apoptosis (Guida *et al.* 2018), it remains unclear whether MMC affects learning and memory through the BDNF/TrkB signaling pathway.

Studies have shown hydrogen sulfide (H_2S) to ameliorate learning and memory deficits from various causes, exerting remarkable neuroprotective effects (Li *et al.* 2023; Salehpour *et al.* 2023). Our previous work has highlighted the neuroprotective functions of H_2S, involving enhancement of mitochondrial function, inhibition of reactive oxygen species-activated caspase-3 signaling, and reduction of apoptosis in hippocampal neurons induced by oxygen-glucose deprivation and reoxygenation (Luo *et al.* 2013). Further investigations revealed that H_2S inhibits hippocampal endoplasmic reticulum stress and produces antidepressant effects by upregulating the BDNF/TrkB signaling pathway (Wei *et al.* 2018). However, whether H_2S can ameliorate cognitive impairment caused by MMC exposure through regulation of the BDNF/TrkB pathway remains unclear.

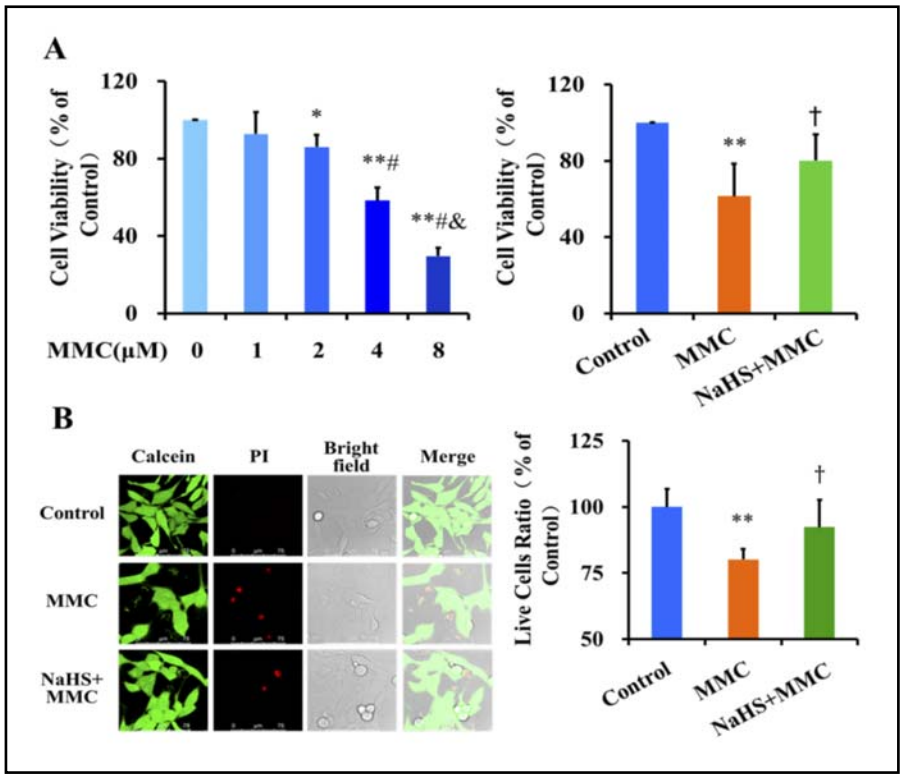

Fig. 1. Effect of H_2S on MMC-induced neuronal cell damage. (A) Effect of MMC on the viability of HT22 cells. HT22 cells were seeded in 96-well plates and cultured for 24 h. The cells were pretreated with 300 μM NaHS for 30 min, then exposed to 4 μM MMC for 24 h. Cell viability was detected using the CCK-8 kit. *: $p < 0.05$, **: $p < 0.01$, vs. 0 μM MMC; #: $p < 0.01$, vs. 2 μM MMC; &: $p < 0.01$, vs. 4 μM MMC; †: $p < 0.05$, vs. MMC n = 6. (B) Effect of H_2S on MMC-induced neuronal cytotoxicity. HT22 cells were seeded on confocal dishes and cultured until the confluency was 70-80%. Cells were pre-treated with 300 μM NaHS for 30 min, following which they were exposed to 4 μM MMC for 24h. Calcein AM and PI dye were added and incubated for 30 min at 37°C away from light. HT22 cells were observed under a confocal microscope, and three fields of view were randomly selected and imaged. **: $p < 0.01$, vs. control; †: $p < 0.05$, vs. MMC, n = 6.

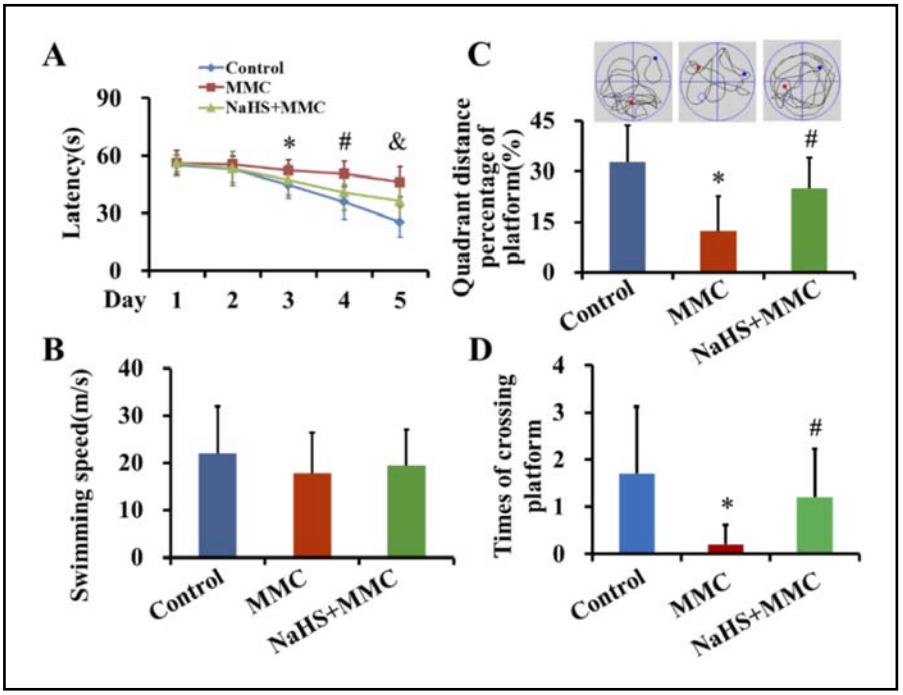

Fig. 2. The effect of H_2S on MMC-induced learning and memory impairment in mice. On the 22nd to 26th day of intragastric administration, Morris water maze was used to train mice to find the platform, and on the 27th day, Orientation navigation experiment was carried out, and then the platform was withdrawn for space exploration experiment. (A) Escape latency, *: $p < 0.05$, MMC vs. Control; #: $p < 0.01$, &: $p < 0.01$, MMC vs. Control or NaHS+MMC, n = 10. (B) Swimming speed, MMC vs. Control or NaHS+MMC, $p > 0.05$, n = 10. (C) Quadrant distance percentage of platform, *: $p < 0.01$, vs control, #: $p < 0.05$, vs MMC, n=10. (D) Platform crossing frequency, *: $p < 0.05$, vs. Control, #: $p < 0.05$, vs. MMC, n = 10.

This study was designed to investigate the effects and mechanisms of H_2S treatment on MMC-induced learning and memory impairment in mice. Specifically, a rodent model of cognitive deficits was established through oral gavage administration of MMC, followed by the administration of NaHS as an H_2S donor. Spatial learning and memory abilities were assessed through the Morris water maze test. Additionally, Western blot analysis was performed on mouse hippocampal tissue to evaluate the activities of the BDNF/TrkB pathways. Through this combined behavioral and biomolecular approach, our study aimed to provide novel insights into the potential neuroprotective role of H_2S against MMC-induced neurotoxicity.

MATERIALS AND METHODS

Main reagents

MMC and NaHS were obtained from Aladdin (Shanghai, China). The TrkB agonist, 7,8-Dihydroxyflavone (7,8-DHF), was purchased from BIOFOUNT (Beijing, China), while antibodies against BDNF, TrkB, were sourced from Proteintech (Wuhan Sanying, China). The CCK-8 kit was obtained from GlpBio (Shanghai, China) and the Calcein/PI kit from Beyotime (Shanghai, China).

Animals and Materials

Kunming mice (male, 10 months old, weighing 40 ± 5 g) were procured from Hunan SJA Laboratory Animal Co., Ltd. The mice were used for experiments after being kept for one week in a clean animal room at 25°C with *ad libitum* food and water. The animal study protocol was approved by the Medical Ethics Committee of Jinggangshan University.

Cell culture and animal treatment

HT22 cells were cultured in 89% high-sugar DMEM supplemented with 10% FBS and 1% double antibody. Cells with good growth conditions and 70%-80% confluence were taken for experiments. HT22 cells were exposed to MMC, with NaHS serving as an H_2S donor for intervention.

Based on previous literature (Mou *et al.* 1995), MMC was dissolved in a 10% ethanol solution by volume, and NaHS was dissolved in double-distilled water. The MMC group received oral gavage of 0.5 mg/kg/d MMC. The NaHS intervention group was treated with 5 mg/kg/d NaHS via gavage, followed by MMC gavage after 1 h. The control group received an equivalent volume of 10% ethanol via gavage. Gavage volume was 0.02 mL/g body weight. The treatment duration was one month.

Cell viability assay

HT22 cells were seeded on a 96-well plate, allowed to grow for 24 h, and then subjected to experimental conditions. To measure the cell viability, the CCK-8 solution and culture medium were mixed in a ratio of 1:9. After removing the culture medium from each well, 100 mL of the mixed solution was slowly added. The 96-well plate was incubated for 1 h, following which the absorbance was measured at 450 nm using a microplate reader. The formula used for calculating cell viability was:

Cell viability (%) = (OD_{450} treated group – OD_{450} blank group) / (OD_{450} control group – OD_{450} blank group) × 100%.

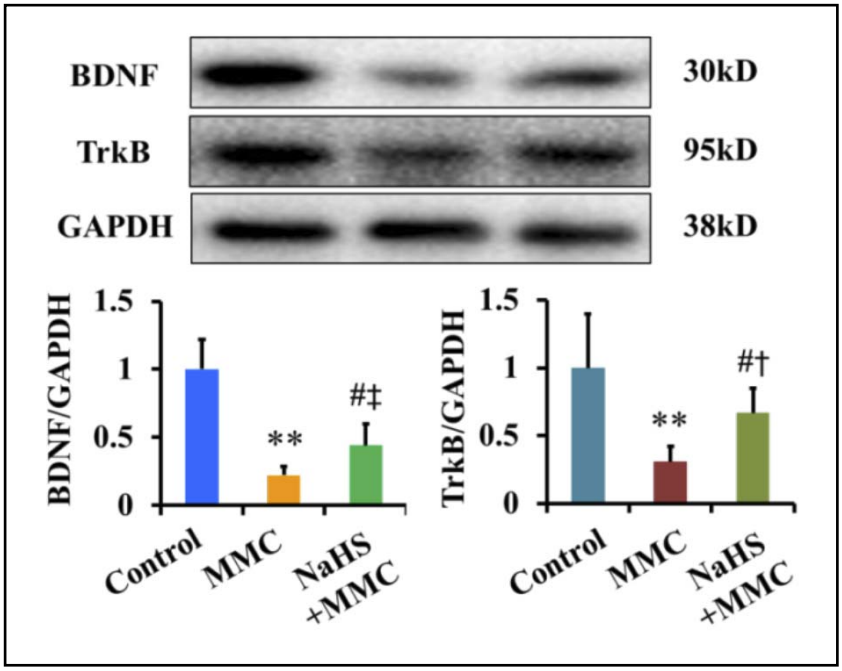

Fig. 3. Effect of H_2S on MMC-induced changes in BDNF/TrkB pathway activity. Following the exploration experiment of Kunming mice, cervical dislocation was executed, the brain was extracted, hippocampal tissue was isolated, the protein was extracted, and the expression of BDNF, TrkB proteins was detected using immunoblotting. **: $p < 0.01$, vs. Control, #: $p < 0.05$, vs. MMC, †: $p < 0.05$, ‡: $p < 0.01$, vs. Control, n = 6.

Viable cell ratio detection

The culture medium was aspirated after HT22 cell treatment. Cells were washed twice with PBS and stained with Calcein AM and PI dyes according to the manufacturer's instructions. Subsequently, the cells were incubated for 30 min at 37°C in the absence of light, aspirated, washed twice with PBS, and were observed under a confocal microscope and randomly selected to image three fields of view (Calcein, Ex/Em = 494/517nm, PI, Ex/Em = 535/617nm).

Live cell ratio (%) = number of green positive cells / (number of green positive cells + number of red positive cells) × 100%.

Orientation navigation and space exploration experiments

A water maze experiment was conducted during the last week of MMC gavage in mice. The platform was placed in the third quadrant, 2 cm below the water surface. The mice were gently introduced to the water facing the pool wall for training. The total time for mice to swim in the pool was 60 sec. If a mouse successfully located the hidden platform within this time frame and remained on it for at least 2 sec, the duration from placing the mouse in the water to finding the platform is recorded as the escape latency. When the mouse failed to locate the platform within 60 sec, the escape latency was noted as 60 sec. Mice were trained twice a day for 5 consecutive days. On day 6, following the escape latency measurement, the platform was removed for space exploration experiments. The mice were allowed to search the platform for 60 sec, and the number of times the mice crossed the platform area, the percentage of trips in the third quadrant, and the swimming speed were recorded.

Immunoblotting

After the space exploration experiment was completed, mice were sacrificed by cervical dislocation, and the brain tissue was quickly extracted. Bilateral hippocampal tissues were rapidly isolated on ice, and protein lysate was added at a ratio of 1:10 (i.e., 1 mg weight to 10 μL volume). The hippocampus was made into protein homogenate using a 1 mL homogenizer in iced water, followed by centrifugation at 12,000 rpm for 10 minutes. The resulting supernatant was extracted, and a portion of the protein samples were used for protein concentration measurement using the BCA method. The remaining protein samples were added to the sample buffer, immediately boiled for 10 min, and subjected to electrophoresis, membrane transfer, and subsequent blocking. The primary antibody was incubated overnight at 4°C, rinsing with TBST, then followed by incubation with the second antibody at room temperature for 1 h. After rinsing with TBST, enhanced chemiluminescence (ECL) was employed for signal development, and images were captured using the BIO-RAD ChemiDoc XRS + imaging system. Image analysis was performed using Image J software.

Data processing

Data were presented as mean±standard deviation ($\overline{x} \pm s$) and analyzed by SPSS 16.0 software. The significance of the differences between groups was determined by one-way analysis of variance, and the LSD-t (Homogeneity of variance) or Tamhane's T2 (Heterogeneity of variance) test was used for comparison between groups. In all experiments, $p < 0.05$ was considered statistically significant.

RESULTS

H_2S reverses MMC-induced nerve injury

Previous studies have shown the neurotoxic effects of MMC, while H_2S has shown inhibitory properties against nerve damage induced by various factors. We aimed to elucidate whether H_2S can serve a protective role in MMC-induced nerve cell damage. Employing concentrations of 2 μM, 4 μM, and 8 μM MMC for 24 h, the viability of HT22 cells was decreased to (85.97 ± 6.17)%, (58.31 ± 6.63)%, and (29.60 ± 4.28)% of the control group, respectively. Notably, pretreatment with 300 μM NaHS increased the viability of HT22 cells from (61.41 ± 17.03)% in the MMC group to (80.15 ± 13.60)%. These results indicate that MMC decreased the neuronal cell viability, which was effectively reversed by H_2S (Figure 1A).

Treatment of HT22 cells with 4 μM MMC for 24 h decreased the ratio of living cells to (80.07 ± 4.08) % of the control, whereas pretreatment with 300 μM NaHS increased the ratio of living cells to (92.37 ± 10.37) % of the control. The ratio of living cells in NaHS+MMC group was significantly higher than that in MMC group, indicating that H_2S inhibited the MMC-induced neuronal cytotoxicity (Figure 1B).

H_2S inhibits MMC-induced learning memory impairment in mice

Male Kunming mice were used as the experimental subjects. Following a 1-hour gavage with 5 mg/kg/d NaHS, treatment with 0.5 mg/kg/d MMC was administered for one month. Spatial learning and memory abilities were assessed using a water maze, measuring escape latency, swimming speed, quadrant distance percentage of platform, and platform crossing frequency.

Results revealed that on days 3, 4, and 5 of training, the escape latency in the MMC group was significantly prolonged compared to the control group. Notably, the NaHS+MMC group exhibited significantly shorter escape latency than the MMC group but still longer than the control group (Figure 2A). The space exploration experiment revealed that the quadrant distance percentage of platform and platform crossing frequency in the MMC group was lower than in the control group. In contrast, the quadrant distance percentage of platform and platform crossing frequency in the NaHS+MMC group was significantly higher than in the MMC group (Figure 2C and 2D). Notably, no significant differences were observed in swimming speed among the groups (Figure 2B). These results indicate that MMC induces learning and memory impairment in mice, while H_2S reverses this effect.

Effect of H_2S on MMC-induced changes in BDNF/TrkB signaling pathway activity

Following a month of MMC gavage, the expression of BDNF and TrkB in the mouse hippocampus was lower than that in the control group. However, with NaHS intervention, the expression of BDNF and TrkB increased compared to the MMC group yet remained lower than that in the control group. These results indicate that MMC downregulated the expression of BDNF and TrkB in mouse hippocampal tissue, while H_2S inhibited this effect (Figure 3).

BDNF/TrkB mediates MMC-induced learning memory impairment in mice

Our findings above indicated that MMC inhibits the BDNF/TrkB signaling pathway, a critical player in learning and memory processes. However, whether the BDNF/TrkB signaling pathway acts as a mediator in MMC-induced learning and memory impairment in mice remained unclear. To address this, the inhibitory impact of MMC on the BDNF/TrkB signaling pathway was antagonized with 7, 8-DHF, a specific agonist of TrkB, and the Morris water maze was used to assess the learning and memory abilities in mice. Results showed that, compared with the MMC group, the escape latency in the 7, 8-DHF+MMC group was significantly reduced (Figure 4A), while platform crossing frequency increased (Figure 4B). These findings suggest that the BDNF/TrkB signaling pathway mediated MMC-induced learning and memory impairment in mice.

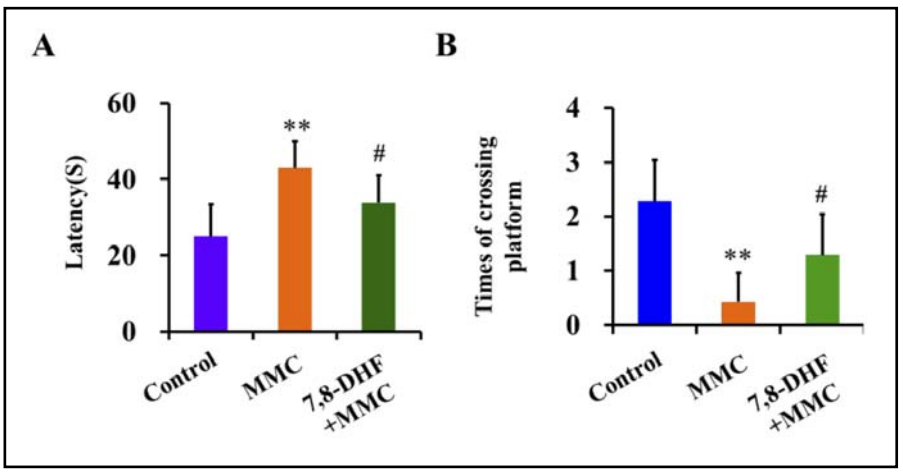

Fig. 4. Role of BDNF/TrkB pathway in MMC-induced learning memory deficits in mice. Kunming mice were given 0.5 mg/kg/d MMC by gavage for 1 month, while 2 mg/kg/d 7, 8-DHF was injected intraperitoneally. Orientation navigation and spatial exploration experiments were performed during the last week of treatment. (A) Latency. **: MMC vs. Control, $p < 0.01$, #: 7, 8-DHF+MMC vs. MMC, $p < 0.05$, n = 7. (B) Times of crossing platform. **: MMC vs. Control, $p < 0.01$, #: 7, 8-DHF+MMC vs. MMC, $p < 0.05$, n = 7.

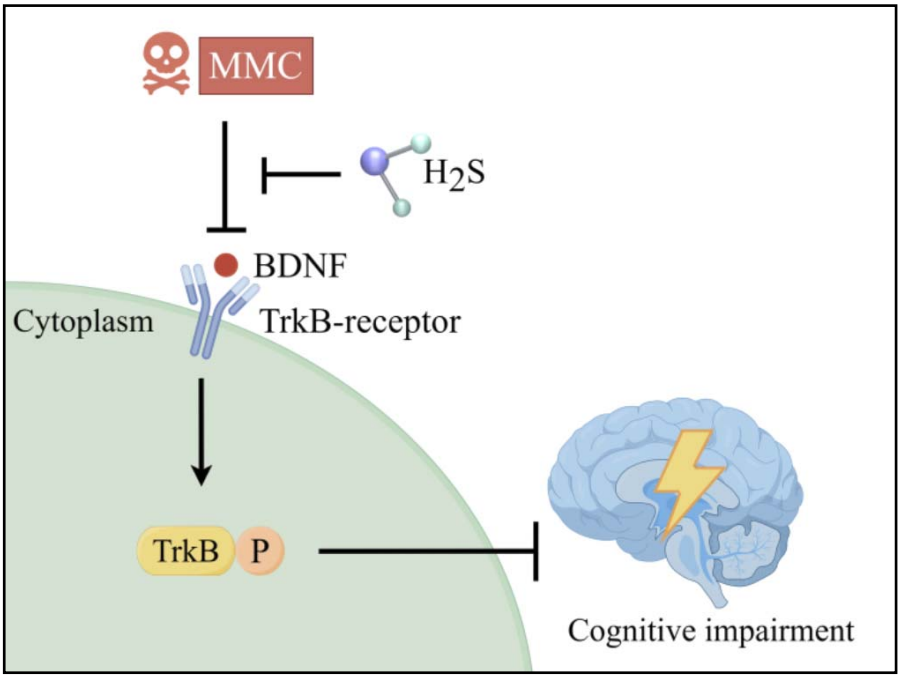

Fig. 5. Schematic diagram of the mechanism of hydrogen sulfide reducing MMC-induced learning and memory impairment in mice. MMC downregulates the expression of BDNF and TrKB, inhibiting the BDNF/TrKB signaling pathway, thereby inducing learning and memory impairment in mice. Hydrogen sulfide improves learning and memory impairment in mice by reducing the inhibitory effect of MMC on BDNF/TrKB signaling pathway .

DISCUSSION

MMC categorized as an organic mercury compound, primarily targets the nervous system due to its lipophilic nature. Studies have demonstrated that exposure to MMC results in neuronal toxicity, manifested as changes in cell morphology, structural damage, increased reactive oxygen species (ROS) production, a significant decrease in cell viability and survival rates (Liu *et al.* 2017; Xia *et al.* 2021). Our study found that MMC reduced both the viability and survival of HT22 cells (Figure 1). Notably, previous studies have shown that NaHS, a H_2S donor, exhibits neuroprotective effects by reducing neuronal apoptosis induced by inflammation in hepatic encephalopathy (Zhuge *et al.* 2020). In line with these findings, our study demonstrated that NaHS pretreatment reversed the MMC-induced decrease in cell viability and the live cell ratio, indicating the neuroprotective effect of NaHS against nerve cell damage induced by MMC exposure. The potential interaction between H_2S and methylmercury was explored, revealing that in SH-SY5Y cells, H_2S facilitated the conversion of methylmercury into dimethyl mercury sulfide, thereby reducing the toxicity of methylmercury (Yoshida *et al.* 2011). This interaction may be attributed to the role of H_2S molecules as thiol equivalents, with methylmercury serving as an electron acceptor and having a strong affinity for sulfur-containing groups (Oliveira *et al.* 2017). Additionally, compounds containing mercury are also recognized as inhibitors of cystathionine-γ-lyase (Bridges *et al.* 2012), possibly contributing to a decrease in cellular H_2S levels after mercury exposure.

Rats exposed to MMC have reported neurotoxicity and cognitive changes (Toyama *et al.* 2021) and even exhibited motor coordination dysfunction, hearing impairment, and memory impairment (Al-Mazroua *et al.* 2022). Prenatal and postpartum exposure to methylmercury has been associated with motor and cognitive impairments in rats (Fagundes *et al.* 2022). Our study showed that the escape latency with MMC treatment is significantly prolonged, while the platform crossing frequency was reduced, indicating that MMC induced learning and memory disorders. H_2S has been shown to improve propofol-induced learning and memory impairment by inhibiting apoptosis and pyroptosis in hippocampal neurons (Li *et al.* 2023). Hydrogen sulfide has been demonstrated to improve spatial memory impairment after early alcohol exposure by increasing BDNF expression and promoting hippocampal neurogenesis (Mohseni *et al.* 2020). Additionally, it has shown efficacy in mitigating spatial memory deficits induced by lipopolysaccharide in mice by inhibiting cell apoptosis, oxidative stress, and inflammation (Kshirsagar *et al.* 2021). Our findings indicate that H_2S could reverse MMC-induced learning and memory impairment in mice (Figure 2).

Studies have shown that H_2S could upregulate BDNF expression and BDNF/TrkB signaling, significantly reducing hippocampal endoplasmic reticulum stress and cell apoptosis in rats exposed to homocysteine, thereby preventing cognitive impairment caused by homocysteine (Juan *et al.* 2021). Similarly, upregulation of BDNF and TrkB expression has been associated with improved neuronal survival, enhanced neural plasticity, and neurogenesis, significantly improving learning and memory disorders induced by lipopolysaccharides (Abdelaziz *et al.* 2024). In PC12 cells, the BDNF/TrkB signaling pathway mediated the neuroprotective effect of H_2S against formaldehyde-induced

cytotoxicity, oxidative stress, and apoptosis (Jiang *et al.* 2015). Furthermore, Sun *et al.*'s experiments confirmed that BDNF/TrkB dysfunction led to hippocampal-dependent learning and memory deficits in adult mice, while TrkB receptor agonists, such as 7,8-DHF, successfully restored stress-induced spatial memory loss in mice (Sun *et al.* 2023). However, how MMC exposure affects the activity of the BDNF/TrkB pathway and whether H_2S reverses the effect of MMC remained unresolved. To understand this, we used immunoblotting to detect BDNF/TrkB activity and found that MMC exposure inhibited the BDNF/TrkB signaling pathway activity, while NaHS intervention alleviated MMC's inhibitory effect on this signaling pathway (Figure 3). Further elucidating the impact of MMC on learning and memory in mice, we employed the TrkB receptor agonist 7,8-DHF to counteract MMC. Our findings confirmed that MMC induced learning and memory disorders in mice by inhibiting the activity of the BDNF/TrkB pathway (Figure 4).

CONCLUSION

Our findings demonstrated that H_2S ameliorated learning memory deficits in mice by reversing the inhibitory effects of MMC on BDNF/TrkB signaling pathway (Figure 5).

DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

This work was supported by the NSFC (National Natural Science Foundation of China) [No.31660271], Jiangsu Province Industry-university-research cooperation project (BY20221030), the Jiangsu Innovation and Entrepreneurship Talent Program (JSSCBS20211166), the Science and Technology Project of Jiangxi Provincial Education Department (GJJ180580, GJJ211014), the Yancheng Innovation and Entrepreneurship Leading Talent Program [Yanwei(2020)40], and Jiangsu Vocational College of Medicine research fund project [20204102, 20200010].

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