Study on the Neuroprotective Mechanism of Hydrogen Sulfide in Regulating the Nrf2/HO-1 Signaling Pathway in Mice with Parkinson's Disease

Jia Xu[1,a,*], Li Yuqian[2,b]

[1]*Beijing Fengtai Youanmen Hospital*
[2]*Beijing No.5 High School*
[a] *anny7451@sina.com,* [b] *yedda0228@outlook.com*
**corresponding author*

Abstract: *This study investigates the effects of hydrogen sulfide (H2S) molecules on the Nrf2 (nuclear factor E2-related factor 2)/HO-1 (heme oxygenase-1) pathway in a mouse model of Parkinson's disease (PD), aiming to elucidate the neuroprotective mechanism of hydrogen sulfide therapy for PD. Real-time fluorescence quantitative PCR was employed to measure the expression levels of glutathione peroxidase (GSH-Px) mRNA, superoxide dismutase (SOD), and Nrf2 in the striatum of the mice. Additionally, Western blot analysis was conducted to assess the protein expression levels of TH and the Nrf2/HO-1 pathway in the striatal tissue. The results indicated that the expression levels of CAT, GSH-Px mRNA, SOD, and Nrf2 showed significant differences between the experimental group and the control group, with notable increases in TH and Nrf2/HO-1 expression ($P<0.05$). In conclusion, hydrogen sulfide can upregulate the expression levels of Nrf2/HO-1 in the cerebral striatum of PD mice, contributing to its therapeutic effects.*

Key words: *hydrogen sulfide; Parkinson's disease; Oxidative stress; Nrf2/HO-1*

1.Introduction

Currently, Alzheimer's disease is the most prevalent age-related neurodegenerative disorder, followed by Parkinson's disease (PD) as the second most common condition. PD is characterized by its insidious and complex onset, significantly impacting both the physical and mental health of patients as well as their overall quality of life. Clinical manifestations primarily include postural instability, muscle rigidity, bradykinesia (slow movement), resting tremor, cognitive dysfunction—such as progressive memory loss—and various neuropsychiatric symptoms. This undoubtedly imposes a substantial economic burden on both society and individuals [1].A hallmark pathological feature of PD is the deposition of eosinophilic Lewy bodies (LB) and the degeneration and necrosis of dopaminergic neurons within the nigrostriatal pathway [2]. With the increasing prevalence of PD, the global number

of cases has now reached 6 million [3], representing a 2.5-fold increase in the past 30 years, thereby exerting immense pressure on societal resources.The specific mechanisms underlying the pathogenesis of PD remain unclear, with numerous hypotheses put forth. Therefore, it is crucial to explore potential therapeutic targets for this disease. As illustrated in Figure 1, oxidative stress is identified as one of the most critical factors in PD and plays a significant role in its treatment [4-5]. Among the therapeutic targets for PD, the Nrf2 (nuclear factor E2-related factor 2)/HO-1 (heme oxygenase-1) signaling pathway stands out for its notable therapeutic effects, demonstrating the capacity to significantly improve behavioral disorders and enhance the quality of life for patients [6-8].Experimental findings suggest that the Nrf2/HO-1 pathway plays an essential role in treating PD, with certain molecules targeting this pathway able to alleviate the pathological features associated with the disease [9]. However, there are limited clinical reports on the use of hydrogen sulfide molecules to activate the Nrf2/HO-1 pathway for PD treatment [10]. This experiment was designed to investigate the mechanism by which hydrogen sulfide may aid in the treatment of PD in mice through the Nrf2/HO-1 pathway.

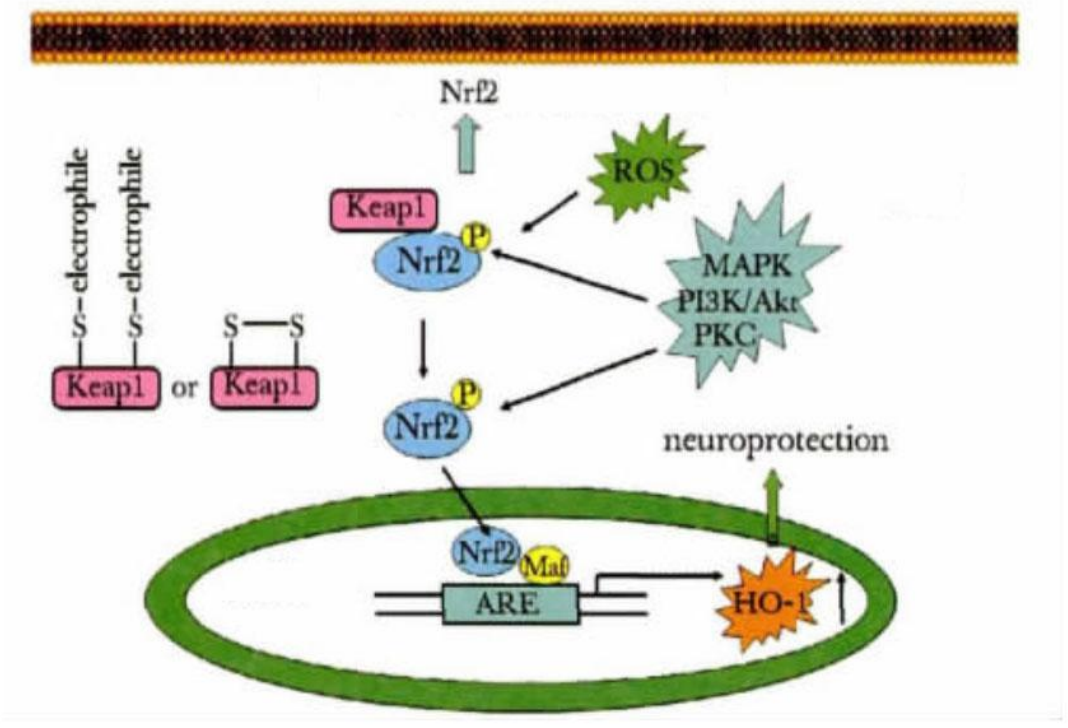


Figure 1. The Nrf$_2$/HO-1 pathway and a proposedmechanism responsible for its activation

2.Materials and Methods

2.1 Experimental Animals

A total of 30 C57BL/6 mice, each weighing (18±2) g and aged 8 weeks, were obtained from

Hunan Slack Jingda Experimental Animal Co., Ltd. All mice were SPF grade and confirmed to be healthy prior to experimentation.

2.2 Reagents

The following reagents were utilized in this study: Sodium Hydrosulfide (NaHS; American Sigma Corporation).Primary antibodies (Wuhan Sanying Biotechnology Co., Ltd.).Anti-mouse secondary antibodies (Wuhan Xavier Biotechnology Co., Ltd.).Nrf2 (Batch No. 16396-1-AP).HO-1 (Batch No. 10701-1-AP).BCA Protein Concentration Assay Kit (Biosharp).Glutathione Peroxidase (GSH-Px; Wuhan Bolf Biotechnology Co., Ltd.). Superoxide Dismutase (SOD; Batch No. NM-013671).Tyrosine Hydroxylase (TH).Catalase (CAT).1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine Hydrochloride (MPTP; Shanghai Aladdin Biochemical Technology Co., Ltd.).

2.3 Instruments

The following instruments were employed during the study:Real-time fluorescence quantitative PCR instrument (Bio-Rad). Enzyme labeling apparatus.X-30R desktop refrigerated centrifuge (Beckman Coulter, USA).JY92IIN ultrasonic cell disruptor (Ningbo Xinzhi Biotechnology Co., Ltd.).

2.4 Drug Administration Protocol

To establish the Parkinson's disease (PD) mouse model, after a 7-day acclimatization period, the 30 C57BL/6 mice were randomly assigned into three groups: model group, experimental group, and control group. Ten mice per group received an intraperitoneal injection of MPTP at a dose of 30 mg/kg, diluted with 0.9% sodium chloride, once daily for seven consecutive days.

Indicators of successful PD model replication included notable weight loss, reduced mobility (manifested by difficulty in raising limbs), and behavioral changes such as lack of grooming, rigidity, and tremors. Concurrently, mice in the experimental group received daily intraperitoneal injections of NaHS at a dose of 30 μmol/kg (equivalent to 1.68 mg/kg) for three weeks. Mice in the control group were administered an equal volume of 0.9% sodium chloride solution intraperitoneally once per day. All treatments and observations were conducted at fixed times each day, ensuring consistency across groups.

2.5 Experimental Methods

For the immunohistochemical analysis, four mice from each group (model, experimental, and control) were anesthetized with pentobarbital sodium at a concentration of 40 mg/kg. Following anesthesia, brain samples were collected and fixed in 4% paraformaldehyde.

To prepare the samples for quantitative PCR, 25 mg of striatal tissue was stored at -80°C, from which 2 μL was used to synthesize cDNA via reverse transcription. The cDNA was then diluted tenfold to create a fluorescence quantitative PCR reaction mix. GAPDH served as the internal control, and relative gene expression was quantified using the 2-AT method.

For protein analysis, the supernatant was extracted from the fresh striatal tissue (25 mg) after centrifugation for 15 minutes post-homogenization. 5× loading buffer was added to the supernatant, followed by denaturation by boiling at 95°C before electrophoresis. The proteins were transferred to a PVDF membrane, which was sealed with 5% skim milk powder for 1 hour after rinsing with TBST solution.

The brains of the remaining PD mice from all three groups were processed on ice and stored at -80°C in Eppendorf tubes for later analysis. Primary antibodies against β-actin (1:1000), TH (1:1000), Nrf2 (1:5000), and HO-1 (1:1000) were applied and incubated overnight at 4°C. Following this, HRP-conjugated secondary antibodies (1:2000) were applied for 1 hour at 4°C. The membranes were washed with TBST (3 times for 5 minutes each) and visualized using an ECL substrate. Densitometric analysis of the bands was performed using ImageJ software.

2.6 Experimental Data Processing

Experimental data are presented as mean ± standard deviation (± SD) using GraphPad Prism 8.0 software. Statistical comparisons between groups were conducted using one-way ANOVA, and results were considered statistically significant when $P< 0.05$ (LSD test).

3.Results

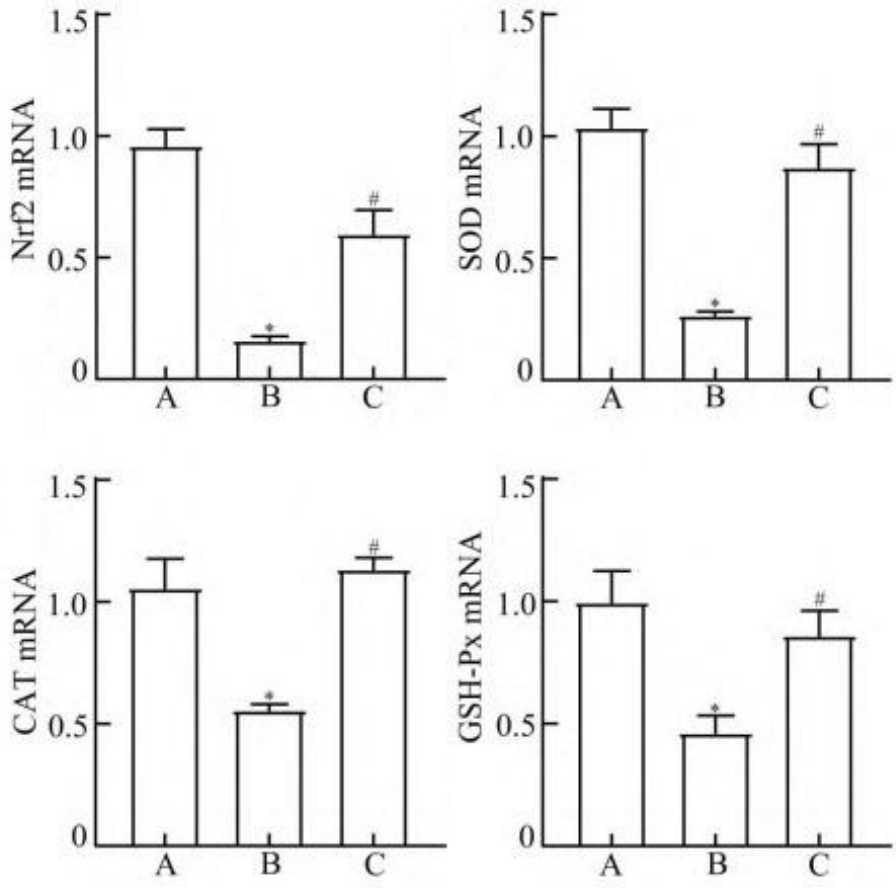
3.1 Comparison of the Expression Levels of GSH-Px mRNA, SOD, Nrf2, and CAT in the Striatum of Three Groups of Mice

As illustrated in Figure 2, the expression levels of GSH-Px mRNA, SOD, Nrf2, and CAT in the striatum of the model group were significantly lower compared to the control group ($P<0.05$). In contrast, the experimental group exhibited a notable increase in the expression levels of GSH-Px mRNA, SOD, Nrf2, and CAT in the striatum ($P<0.05$).

3.2 Comparison of the Expression Levels of Nrf2, HO-1, and TH Proteins in the Striatum of Three Groups of Mice

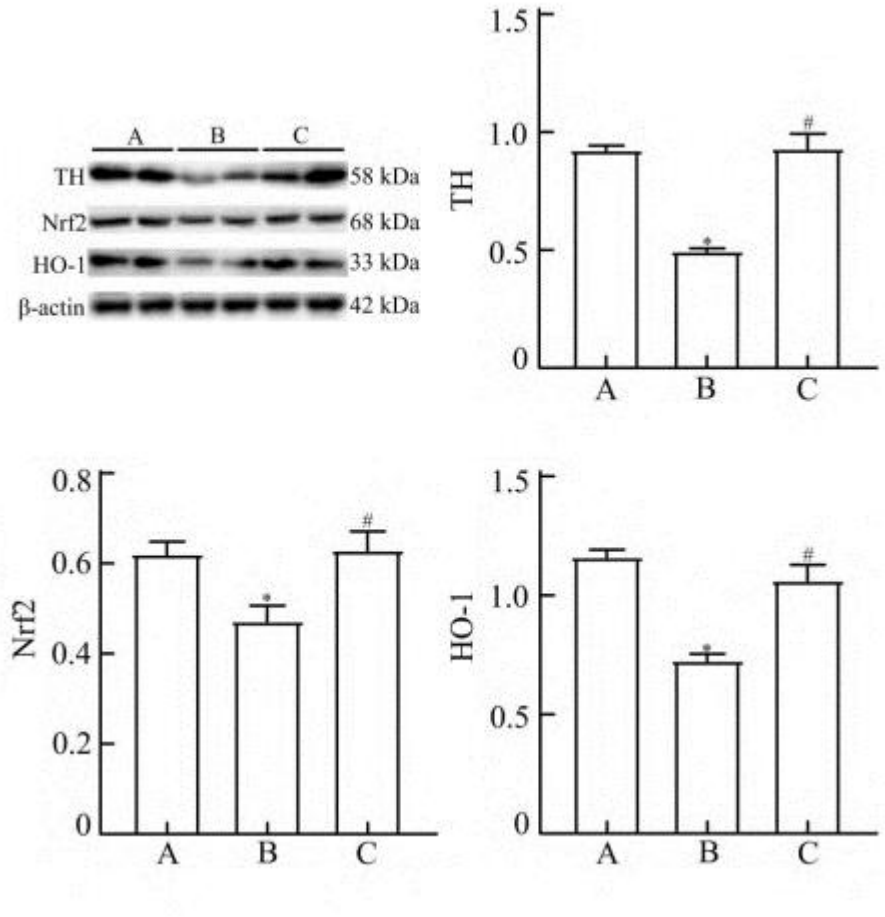
As shown in Figures 3 and 4, the protein expression levels of Nrf2, HO-1, and TH in the striatum of the model group were significantly reduced when compared to the control group ($P<0.05$). Conversely, the

experimental group demonstrated a significant increase in the expression levels of Nrf2, HO-1, and TH in the striatum (P<0.05).



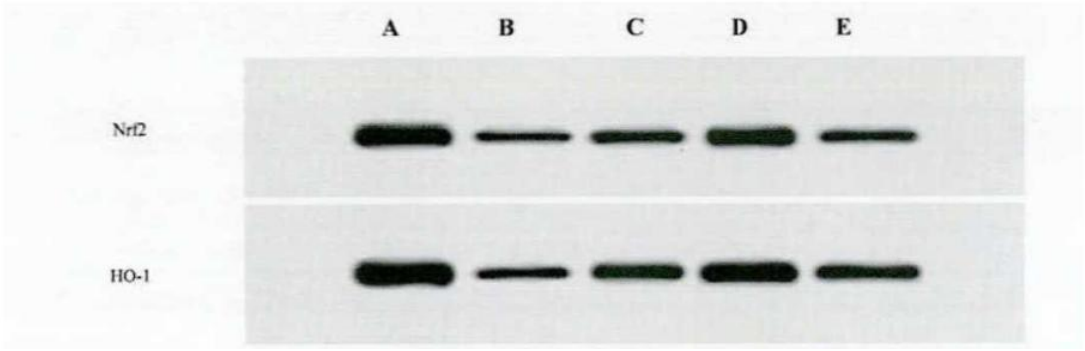
（Note: A. Control group; B. Model group; C. Electroacupuncture group; Compared with the control group, *P<0.05; Compared with the model group, *P<0.05.）

Figure 2. Comparison of GSH-Px mRNA, SOD, Nrf_2, CAT expression levels in the striatum of 3 groups of mice (x±s, n=6)



（Note: A. Control group; B. Model group; C. Electroacupuncture group; Compared with the control group, **P*<0.05; Compared with the model group, **P*<0.05.）

Figure 3. Comparison of Nrf_2, HO-1, TH proteins expression levels in the striatum of 3 groups of mice (x±s, *n*=6)



（Note: A. Experimental double dose group; B. Model group; C. Control group; D: Electroacupuncture group; E: Pathway inhibition group.）

Figure 4. Expression of Nrf_2 /HO-1 proteins in brain tissue of rats in each group

4.Discussion

The decrease in dopamine levels in the striatum is attributed to the degeneration and necrosis of dopaminergic neurons in the dense part of the substantia nigra, which is a hallmark of PD [11]. Tyrosine hydroxylase (TH), as a marker protein for dopaminergic neurons and the rate-limiting enzyme for DA synthesis, serves as an indirect indicator of dopaminergic neuronal activity based on its expression level in the striatum [12-14]. Abnormalities in the enzyme antioxidant system may lead to oxidative stress, which plays a critical role in the pathogenesis of PD [15-16]. The oxidative stress status in the body is reflected by the levels of GSH-Px, catalase (CAT), and superoxide dismutase (SOD), as these are key components of the enzymatic antioxidant defense system [17]. It has been reported that the reduction of neuronal damage in PD models primarily relies on lowering oxidative stress levels [18].

Our experimental results indicated that the expression levels of GSH-Px mRNA, SOD, and CAT in the brains of PD mice were significantly decreased, suggesting extensive oxidative damage to the brain tissue in the model group compared to the control groups. Hydrogen sulfide (H_2S) gas molecules were observed to elevate the expression levels of GSH-Px mRNA, SOD, and CAT, indicating that H_2S treatment may alleviate oxidative stress in the brain.

As depicted in Figure 5, Nrf2 plays a pivotal role in regulating the body's antioxidant responses as a transcription factor. Under physiological conditions, Nrf2 binds to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. In response to oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to antioxidant response elements (ARE), and activates the expression of downstream HO-1 antioxidant genes [19-20]. Therefore, the Nrf2/HO-1 pathway is crucial for protecting cells against oxidative damage.

Liu et al. demonstrated that moxibustion protects dopaminergic neurons by enhancing the expression of HO-1, a downstream factor that activates the Nrf2/ARE pathway, thereby mitigating oxidative stress induced by 6-hydroxydopamine hydrochloride in the striatum of model rats [21]. Our results showed effective activation of the Nrf2/HO-1 pathway in the experimental group compared to the model group. Notably, the protein expression levels of Nrf2 and HO-1 in the striatum were significantly increased following H_2S administration, indicating that H_2S may reduce oxidative stress and protect dopaminergic neurons.

However, given the complex interactions among various pathogenic factors in PD and the

synergistic pharmacological effects of H_2S on other signaling pathways observed in this study, further investigation is needed to elucidate the specific mechanisms by which H_2S contributes to the treatment of PD.

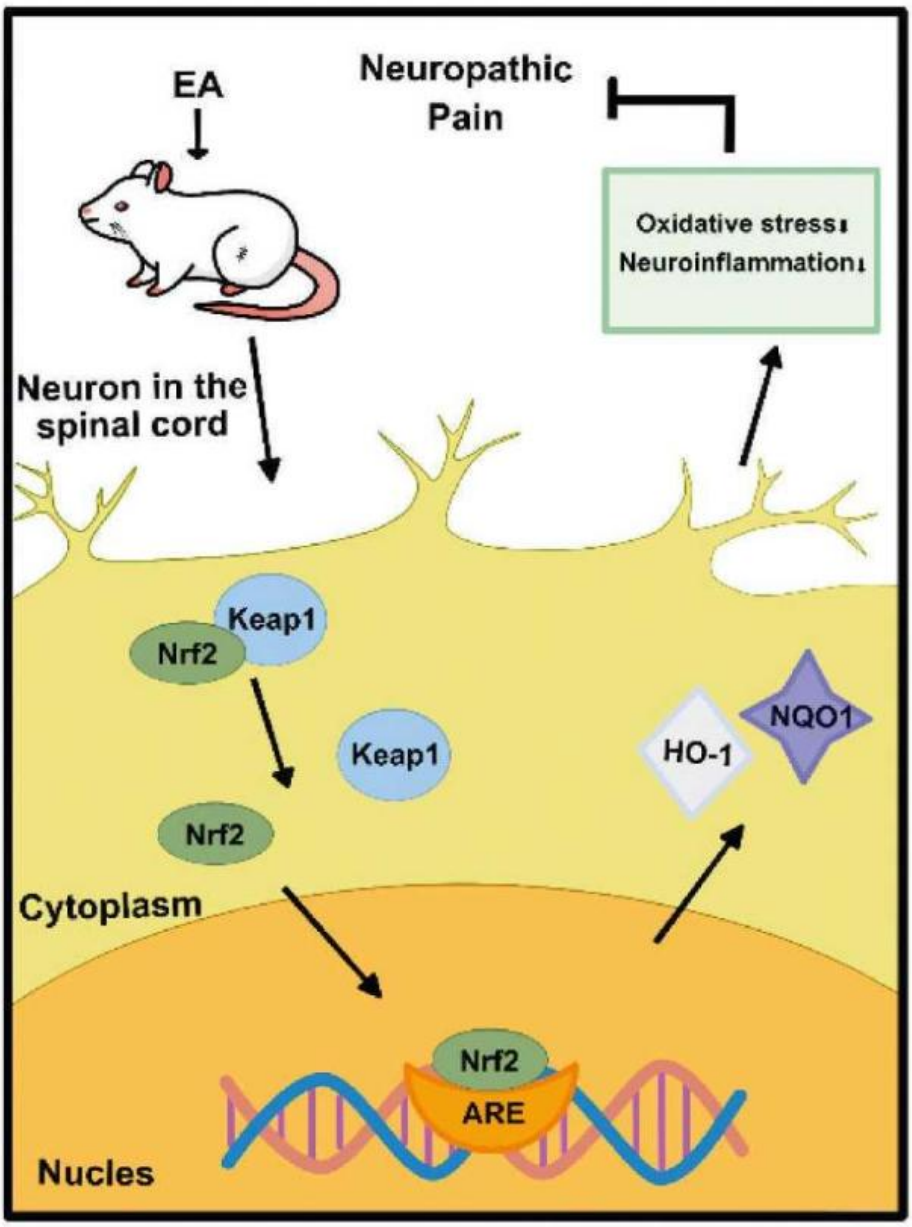


Figure 5. Schematic mechanism showing the change in Nrf_2/HO-1 in spinal cord neurons of CCI rats.

4.Conclusion

In summary, the experimental design involved randomly dividing thirty mice into three groups: the experimental group, the control group, and the model group, with ten mice in each group. Mice in

both the experimental and model groups were injected intraperitoneally with 30 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to establish a Parkinson's disease (PD) mouse model. The experimental group received sodium hydrosulfide (NaHS) as a hydrogen sulfide (H2S) donor, which was dissolved in water to generate H2S, with the NaHS solution concentration being three times that of H2S after ionization (30 μmol/kg or 1.68 mg/kg). Following continuous administration for three weeks, daily observations and recordings of the mice's behavior were conducted, while the other two groups remained unaffected by treatment.

The results indicated significant differences in the expression levels of catalase (CAT), GSH-Px mRNA, superoxide dismutase (SOD), and nuclear factor erythroid 2-related factor 2 (Nrf2) between the experimental and model groups. Notably, the expression levels of tyrosine hydroxylase (TH) and the Nrf2/HO-1 pathway were significantly elevated ($P<0.05$). Therefore, we conclude that hydrogen sulfide upregulates the expression of Nrf2/HO-1 in the cerebral striatum of PD mice, contributing to its therapeutic effects.

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