

Original Article

Protective effect of hydrogen sulfide against neuronal apoptosis via inhibiting neuroinflammation in Parkinson rats

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Abstract: Objective: Parkinson's disease (PD) is an age-related progressive degenerative disorder. Hydrogen sulfide (H₂S) is an endogenous gasotransmitter and exerts crucial physiological functions in central nervous system. Whether H₂S protects PD through inhibiting neuro-inflammation remains unknown. Methods: In this study, a 6-OHDA induced PD rat model was established and the protective role of H₂S was detected via H₂S donor NaHS. Results: Our results demonstrated that H₂S inhibited the activation of NF-κB signaling pathway, cytokines release in PD rats and significantly reduced the expression of caspase 3, caspase 9 and cytochrome C, and decreased apoptotic cell numbers. Conclusion: H₂S may preserve the function of dopaminergic neurons in PD rats possibly through inhibiting neuro-inflammation in Parkinson rats.

Keywords: Parkinson's disease, hydrogen sulfide, neuronal apoptosis

Introduction

Parkinson's disease (PD) is an age-related progressive degenerative disorder, which is associated with the loss of dopaminergic neurons in the substantia nigra (SN) and leads to motor disorder like bradykinesia, resting tremor, rigidity, as well as postural instability.

Hydrogen sulfide (H₂S) is an endogenous gasotransmitter and has been recognized to have crucial physiological functions in central nervous system. Lots of effects H₂S have been investigated in previous studies. Reports have suggested that H₂S is involved in introducing long-term potentiation (LTP) [1, 2], regulating calcium homeostasis [3], suppressing oxidative stress [4] and so on. Furthermore, in addition to the above mentioned physiology functions, H₂S also plays important roles in pathological processes of neurodegenerative diseases.

NF-κB is a classical pathway involved in various kinds of inflammation. As indicated, low grade chronic inflammation and NF-κB pathway acti-

vation were detected in clinical and experimental Parkinson disease [5]. Also, high expression of the HMGB1-TLR4 axis was found in patients with Parkinson [6]. Altogether, inhibiting the NF-κB pathway is an effective step to alleviate PD [7].

Previous studies have indicated that H₂S is able to attenuate neuro-inflammation induced by lipopolysaccharide [8] and amyloid-β [9], suppress oxidative stress induced by hydrogen peroxide. However, whether H₂S could inhibit neuronal apoptosis remained unknown. Based on these reports, it was speculated that H₂S could inhibit neuronal apoptosis via inhibiting neuro-inflammation in Parkinson's rats. Thus, H₂S may have a potential therapeutic value [10, 11] to treat Parkinson's disease.

Materials and methods

Chemicals and reagents

All chemicals, antibodies for detecting NF-κB pathway activity and LDH assay kit were pur-

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chased from Sigma (Sigma, St. Louis, MO). The lactate dehydrogenase (LDH) AssayKit was purchased from Cayman Chemical (Ann Arbor, Michigan). Antibodies for detecting cell apoptosis were purchased from SantaCruz Biotechnology (Santa Cruz, CA). Other reagents not mentioned were purchased from sigma (St. Louis, MO). This research was approved by Zhebei Mingzhou Hospital Ethics Committee.

Animals

Male Sprague-Dawley (SD) rats (180-220 grams) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of China. All animal works were carried out strictly in accordance with IACUC regulations.

LDH release assay

Serum was prepared as previous described. Equal amounts of lactate dehydrogenase assay substrate, enzyme and dye solution were mixed. A half volume of the above mixture was added to one volume of medium supernatant. After incubation at room temperature for 30 minutes, the reaction was terminated by the addition of 1/10 volume of 1 N HCl to each sample. Spectrophotometrical absorbance was measured at a wavelength of 490 nm and reference wavelength of 690 nm.

Superoxide dismutase (SOD) activity determination

SOD activity was measured in cells using the Cayman Chemical Superoxide Dismutase Assay Kit (Cayman Chemicals, Inc., Ann Arbor, MI). Briefly, cells were sonicated in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose, on ice. After centrifugation, the supernatant was collected. The reaction was initiated by adding diluted xanthine oxidase to all wells, and then the plate was incubated on a shaker at room temperature for 20 minutes. The absorbance was read at 450 nm.

PD rat model establishment

Male Sprague-Dawley (SD) rats (180-220 g) were anesthetized with ketamine (75 mg/kg, i.p.)

and xylazine (10 mg/kg, i.p.). After that, the rats were placed in a stereotaxic apparatus (Stoelting Instruments, Wood Dale, IL, USA). 6-OH-DA (8 mg 6-OHDAhydrobromide dissolved in 4 ml sterile saline containing 0.02% ascorbic acid) was unilaterally injected into the left striatum (coordinates from bregma: AP, +1.0 mm; ML, +3.0 mm; DV, 24.5 mm) with a Hamilton syringe (0.46 mm in diameter, bluntip) at a rate of 0.5 ml per minute. The needle was left in place for 3 minutes and then slowly withdrawn in the subsequent two to three minutes. Sham-operated rats were injected with 4 ml saline containing 0.02% ascorbic acid into the left striatum and served as controls in this study. After surgery, the rats were kept in cages and exposed to a 12:12 h light-dark cycle with unrestricted access to tap water and food.

Behavioral test

Three weeks after surgery, the animals' tendency to rotate in response to apomorphine (0.5 mg/kg, s.c.) was tested. This test was re-performed one week later, i.e. four weeks after surgery. Only those rats consistently showing at least 7 turns per minute in both tests were considered as the successfully induced PD-like model. These PD-like rats were then divided into different groups receiving different treatments, vehicle- or Sodium hydrosulfide (NaHS) - administered group. In addition, the sham-operated rats also received vehicle treatment. These treatments continued for another 3 weeks. The rotational behavior was monitored at one week interval till the end of treatment, and the behavior tests were conducted before drug treatments in order to avoid disturbance. After 3 weeks of treatment, the rats were euthanized by CO₂ and brain tissues were collected for assays.

Measurement of serum TNF- α , IL-1 β levels

Blood samples were collected to measure TNF- α and IL-1 β level in serum using enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc. (Minneapolis, MN, USA).

Immunofluorescence

Immunofluorescence staining was performed according to the procedures as previously described with some modification [23]. The brain was then collected and immersed into 4% PFA

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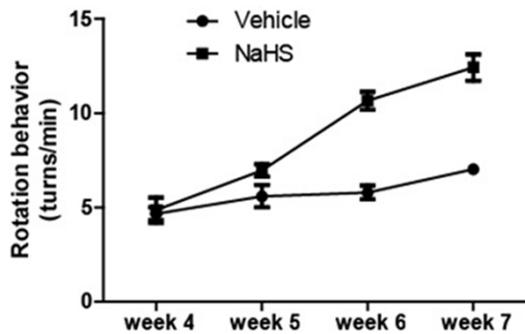


Figure 1. Treatment with NaHS ameliorated the rotational behavior in the unilateral 6-OHDA-lesioned rats. Data are presented as mean \pm SD, $n = 9$. $^{***}P < 0.01$ compared with the values in Vehicle group at the corresponding time point.

for post fix at 4°C overnight. These brain samples were transferred into 15% sucrose in phosphate buffered saline (PBS) overnight at 4°C and subsequently to 30% sucrose solution till the brain sunk to the tube bottom. Thereafter, the brain were sectioned on a cryostat at a thickness of 30 μ m and mounted onto the poly-L-lysine coated slides. The sections were permeabilized with 0.3% Triton X-100/PBS for 10 minutes and blocked with 10% BSA in PBS for another 30 minutes. After that, the sections were incubated with mouse monoclonal anti-TH antibody (1:500, Sigma, St. Louis, MO, USA) for 2 hours at room temperature and followed with appropriate goat anti-mouse secondary antibody incubation for one hour.

Tunnel staining

Apoptosis was quantified in paraffin-embedded aortic using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) Assay kit (TUNEL Apo-Green Detection Kit, Biotool, Houston, USA) according to manufacturer instructions. Green fluorescence staining indicated positive TUNEL staining.

Western blot analysis

The translocation of NF- κ B and its activity were determined using Western Blot as previous described [12]. Protein was extracted using a total protein extraction kit or nuclear protein extraction kit (keyGEN BioTECH, China), and qualified by BCA protein assay kit (Beyotime, China). Equal amounts of protein from tissue

samples were separated on 7.5-20% SDS-PAGE and then transferred to a PVDF membrane (Immobilon-P, USA). The PVDF membrane was probed by antibodies respectively. A primary antibody against β -actin (Bioss, China) for total protein was used as loading control. Peroxidase-conjugated anti-rabbit/mouse IgG (Thermo, USA) was used as secondary antibodies at 1:1000 for 60 minutes at room temperature. Then, ECL (Beyotime, China) detection was performed using the MultiSpectral imaging system (UVP, Cambridge, UK).

Statistical analysis

Data were analyzed using IBM SPSS statistics program (Microsoft, USA). Comparisons between two groups were performed using the student's t-test. To compare three or more groups, analysis of variance (ANOVA) followed by a post hoc (Bonferroni) test was employed. More than six measurements were performed for each experiment. Data are expressed as the mean \pm standard deviation (SD). $P < 0.05$ indicated a significant difference.

Results

NaHS ameliorated behavioral symptoms in PD rats

To evaluate the therapeutic effect of NaHS on Parkinson's disease, the unilateral 6-OHDA lesion rat model was established. Four weeks after 6-OHDA lesion, the PD rat was injected with vehicle or NaHS daily for another 3 weeks. As shown in **Figure 1**, NaHS significantly ameliorated the rotation behavior after 2 weeks treatment, which indicated that the administration of NaHS may alleviate the behavior disorder in PD.

NaSH attenuated the degeneration of dopaminergic neuronal in SN

The loss of dopaminergic neurons in the SN is a main reason to cause the movement dysfunction in PD model. From the immunostaining results, unilateral 6-OHDA lesion destroyed most of the tyrosine hydroxylase positive (TH+) neurons in SN pars compacta in the injured hemisphere, while the administration of NaHS remarkably attenuated the effects. These data suggest that NaHS may preserve the function of dopaminergic neurons in PD rats (**Figure 2**).

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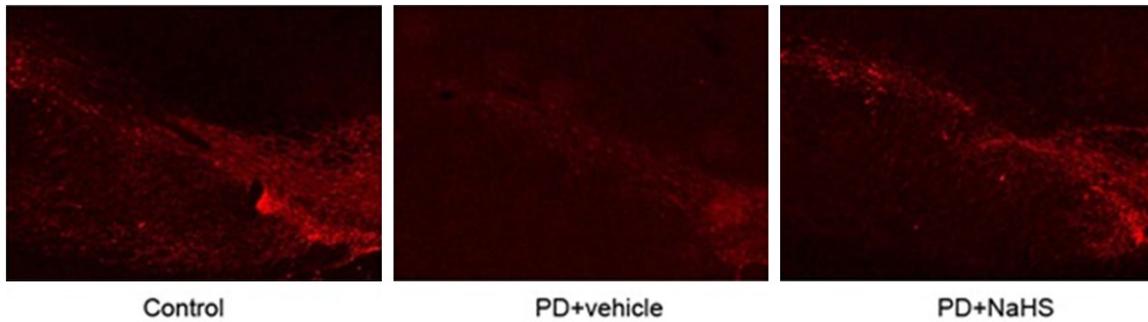


Figure 2. Effect of NaSH on 6-OHDA-induced TH⁺ neuronal degeneration. Photos were taken at $\times 100$ magnification, and the white bar indicated 0.1 mm. Samples were collected from two independent experiments.

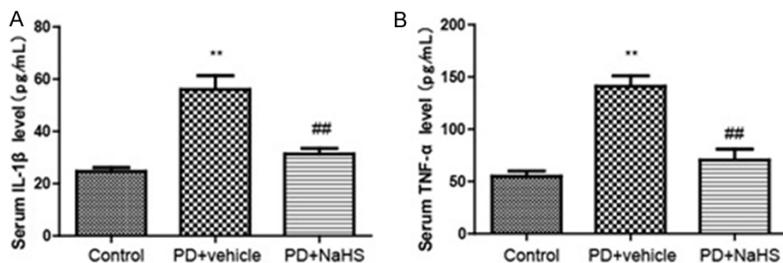


Figure 3. NaHS inhibited cytokines expression in PD rats. Data are presented as mean \pm SD, n = 9. **P < 0.01 compared with control group, ##P < 0.05 compared with vehicle group.

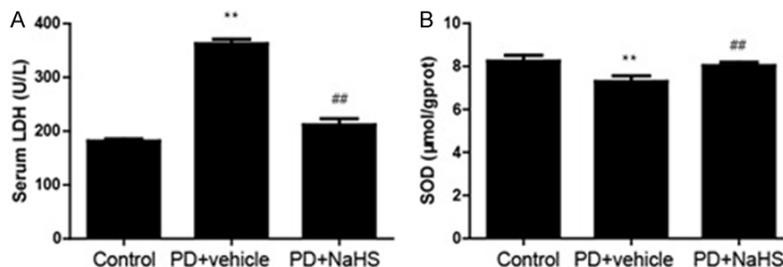


Figure 4. Effect of NaHS on SOD activity and LDH release in PD rats. Data are presented as mean \pm SD, n = 9. **P < 0.05 compared with control group, ##P < 0.01 compared with vehicle group.

NaHS inhibited cytokines expression in PD rats

As shown in **Figure 3**, serum IL-1 β and TNF- α level was significantly increased in PD rats, and markedly decreased after NaHS treatment. The results indicated a potential anti-inflammatory effect of NaHS in PD rats.

Effect of NaHS on SOD activity and LDH release in PD rats

SOD level and LDH release were detected to assess cell injury. SOD is an important enzyme

in cellular defense against oxidative stress and the production of LDH is regarded as an early indicator of cell death. As shown in **Figure 4**, in PD rats, serum LDH level was elevated and SOD level was decreased, however, after administration of NaSH, the level of SOD and LDH were significantly increased and decreased, respectively. These indicated a potential protective effect of NaSH on PD rats.

NaSH treatment inhibits I κ B α degradation and NF- κ B translocation in PD rats

The pro-inflammatory mediator, NF- κ B is a nuclear transcription factor that plays a central role in PD, and inhibiting NF- κ B activity is beneficial to reduce cell apoptosis.

To examine the influence of NaSH on NF- κ B signal pathway, I κ B α degradation, NF- κ B/p65 nuclear translocation and phosphorylation were measured by Western blotting. As shown in **Figure 5**, in PD rat, I κ B degradation was inhibited, and the inhibition was reversed by NaSH treatment. The translocation of NF- κ B/p65 into the nucleus as well as phosphorylation of p65 was then investigated. 6-OHDA injection significantly induced the translocation of NF- κ B/p65 and phosphorylation of p65, and treatment with NaSH partially blocked TNF- α -induced NF- κ B/p65 translocation and p65 phosphoryla-

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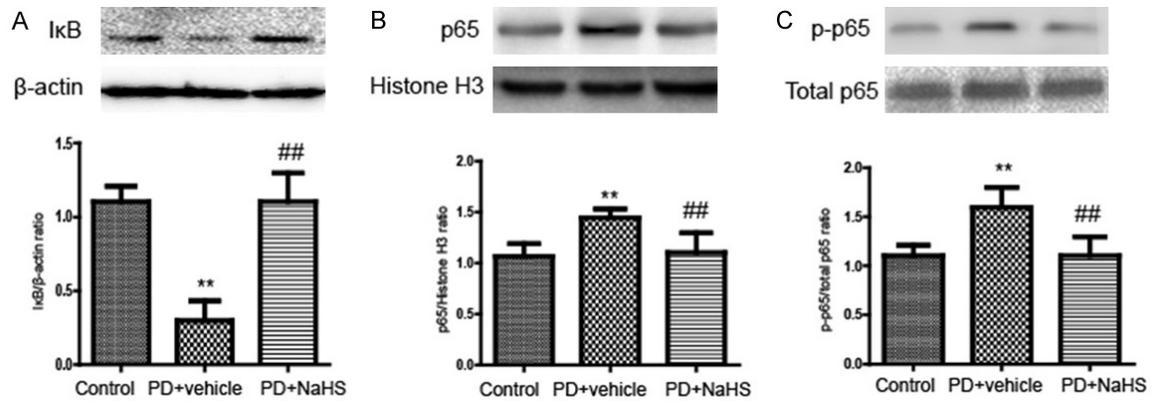


Figure 5. Effect of NaHS on NF-κB activity in PD rats. Data are presented as mean ± SD, n = 9. **P < 0.01 compared with control group, ##P < 0.01 compared with vehicle group.

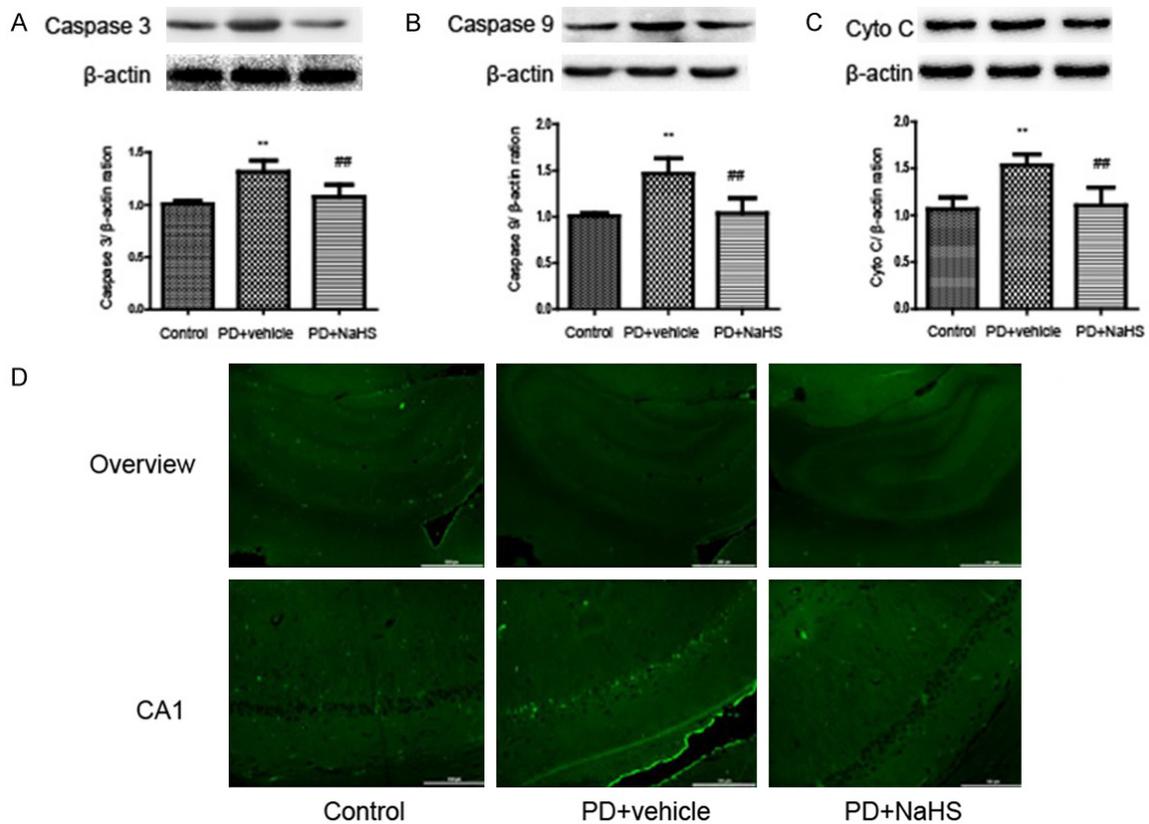


Figure 6. Effect of NaHS on cell apoptosis in PD rats. A. Caspase 3 expression. B. Caspase 9 expression. C. Cytochrome C (cyto C) expression. D. TUNEL staining. Data are presented as mean ± SD, n = 9. **P < 0.01 compared with control group, ##P < 0.01 compared with vehicle group.

tion. These results suggest that NaSH inhibited the nuclear translocation of the NF-κB/p65 subunit to the nucleus, indicating that NaHS may inhibit neuron inflammation via NF-κB signaling pathway.

NaHS treatment inhibited cell apoptosis in PD rats

Cell apoptosis plays a crucial role in the pathogenesis of PD disease. Thus, to evaluate the

effect of NaHS on apoptosis, caspase-3, caspase-9 and cytochrome C expression was determined by Western blotting. As shown in **Figure 6**, a significantly increased expression of caspase-3 and caspase-9 in PD rats was also downregulated after NaHS administration. Additionally, as shown in **Figure 6D**, TUNEL staining analysis showed that the number of apoptotic cells was significantly increased in PD rats, whereas NaHS obviously reduced the number of apoptotic cells, indicating the intense anti-apoptotic effect of NaHS. The above results demonstrated the effects of NaHS on inhibiting cell apoptosis.

Discussion

The symptoms of Parkinson's disease are associated with the loss of dopaminergic neurons and the deficiency of dopamine in the SN and striatum, and oxidative stress plays a crucial role in the pathology of neurodegeneration [13, 14]. Its prevalence rate has become increasingly with the ageing of the population and has become one of the serious diseases that affect human's health. Its prevention is not only a major medical problem but also a serious social problem. However, the current clinical treatment methods are not ideal for the treatment of PD. Therefore, investigation of the pathogenesis of PD and its new prevention strategies has important practical significance.

In this study, protective effects of hydrogen sulfide in PD rats were investigated in 6-OHDA induced experimental PD models. By detecting NF- κ B pathway activity and inflammatory related cytokines (IL-1 β and TNF- α) level, the effect of hydrogen sulfide on neuro-inflammation was found. Additionally, by detecting apoptosis related protein expression and TUNEL staining, hydrogen sulfide has shown to reduce neuronal apoptosis. Moreover, TH⁺ staining showed hydrogen sulfide attenuated the degeneration of dopaminergic neuronal.

Hydrogen sulfide is a newly discovered third type of gas signal-modulating molecule that has been involved in various functions in vivo. Studies have shown that hydrogen sulfide can regulate the synaptic activity, affect long-term potentiation of the hippocampus, regulate neuroendocrine and vasodilator. Early studies found that hydrogen sulfide can antagonize the damage effects of hydrogen peroxide and MPP⁺ on PC12 cells [15]. There is also evidence that, H₂S has been recognized as an

anti-oxidant [16-18] and previous studies have demonstrated the protective effect of H₂S on 6-OHDA and rotenone-induced PD models [19]. A recently published report indicated that H₂S S-sulfhydrated Keap 1 at cysteine-151, which enhanced the release of Nrf-2 from Keap-1 and upregulated Nrf-2 activity [20]. Although NaHS was able to protect the cells against apoptosis and oxidative stress, whether NaHS ameliorates PD via anti-inflammation remains unknown. In this study, anti-inflammatory effect of NaHS in PD rats was verified.

Detection of rotational behavior in rats is the usual measure of the consequences of unilateral 6-OHDA injury [21, 22]. It is generally believed that the rotation behavior is caused by the selective destruction of dopaminergic neurons in the injection zone by 6-OHDA, which causes the destruction of dopaminergic neurons in the ipsilateral side of the injection, decreased dopamine content, and dopamine receptor hypersensitivity.

At this time, the use of the dopamine receptor direct synergist APO combined with the neurotransmitter dopamine released by the nigrostriatal dopaminergic neurons can cause the animal to generate a rotational behavior toward the healthy side. In this study, by detecting rotation behavior, the protective role of NaHS in PD rats was found.

Neuronal cell death is a pathophysiologic consequence of many brain insults that trigger PD, and has been implicated as a causal factor in PD. Increase apoptosis cell level has been demonstrated in PD in previous study [23]. In the current study, NaSH treatment significantly reduced caspase 3, caspase 9 and cytochrome C expression, and decreased apoptotic cell numbers, indicating a protective effect of NaHS.

Conclusion

In summary, H₂S alleviates cell apoptosis and neuroinflammation of PD, suggesting it might be a novel approach for treating PD.

Disclosure of conflict of interest

None.

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