

Original Research Article

Hydrogen sulfide ameliorates isoflurane-induced cognitive impairment in mice: Implication of caspase-3 activation

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Abstract

Purpose: Isoflurane could induce cognitive impairment and activate caspase-3. However, the mechanism of action is unclear and target interventions are unavailable. The present study examined the potential protective function of hydrogen sulfide (H₂S) against isoflurane-induced cognitive impairment.

Methods: Effects of NaHS (5 mg/kg) on cognitive impairment induced by isoflurane (1.4% for 2 h) were assessed using a fear-conditioning test in a group of 8-month old mice. H4 human neuroglioma cells, which were transfected with upregulated human amyloid precursor protein were treated for 3 or 6 h with 2% isoflurane, in the presence of 100- μ M NaHS in the mice. A group of mice treated with normal saline in place of the NaHS in each case served as control. Western blotting, fluorescence assay, and a mitochondrial swelling assay were employed to observe the results of caspase-3 activation, mitochondrial dysfunction, and ROS and ATP levels.

Results: NaHS significantly mitigated isoflurane-induced cognitive impairment in mice. In cultured cells, NaHS reduced caspase-3 activation, ROS, mitochondria membrane reduction, mitochondrial permeability transition pore opening, and cellular ATP level. NaHS could ameliorate cognitive impairment induced by isoflurane through inhibiting caspase-3 activation, oxidative stress, and mitochondrial dysfunction.

Conclusion: These results indicate that hydrogen sulfide (H₂S) has potential protective function against isoflurane-induced cognitive impairment. Further investigation of NaHS as an intervention to attenuate anesthesia-associated neurotoxicity is vital.

Keywords: Hydrogen sulfide, isoflurane-cognition, fear conditioning, neurotoxicity

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INTRODUCTION

Alzheimer's disease (AD) is a prevailing reason for dementia in adults [1]. Symptoms typically start at age of 60 years and might involve memory loss, failure in performing daily activities,

and personality change. The evidence so far shows that exposure to general anesthetics could interact with AD-associated pathological mechanisms, and promote the progression of AD [2].

A randomized controlled trial showed that sevoflurane could accelerate the progression of cognitive impairment, which often precedes AD [3]. A case-control study in China established that anesthesia during surgery is associated with the risk of dementia [4]. Another study indicated that people in the middle age without cognitive impairment are more likely to have rapid cognitive decline after surgery and anesthesia [5]. Several other studies, however, failed to establish this association in anesthesia and dementia [6-8]. It was previously reported that isoflurane might promote caspase-3 activation [9], increase amyloid beta-protein (A β) level in the brain [10-12], and impair learning and memory [13]. The mechanism on how isoflurane enhances caspase activation is not completely understood, but may result from increased levels of ROS, mitochondrial dysfunction and decreased ATP level [14,15].

In addition to being a gaseous neuromodulator, H₂S is also an anti-oxidant and could produce anti-apoptotic effects in neurons and glial cells [16-23]. Accumulating evidence indicates the protective functions of H₂S in the CNS in the process of AD, vascular dementia, and Parkinson's disease [24-27].

In this study, we hypothesized that sodium hydrosulfide (NaHS) might inhibit caspase-3 activation via ROS-, mitochondria-, and ATP-associated mechanisms in glioma cells transfected with amyloid precursor. Therefore, the purpose of this study was to explore the underlying mechanisms of caspase-3 activation *in vitro* and examine the potential effect of NaHS on cognitive impairment in isoflurane-induced mice model.

EXPERIMENTAL

In vivo Experiments

Following ethical approval by the Animal Care and Use Committee of Shanghai Tenth People's Hospital, Tongji University School of Medicine, this study was carried out following the Regulations of Experimental Animal Administration. Wild-type C57BL/6J mice (8 months old) of both sexes (20 females, and 20 male, with 5 males and 5 females in each group) were placed under a reversed 12h/12h light/dark cycle at 20–22°C for a period of 1 week before the experiments in Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China. At random, each mouse in the treatment group (10 mice; with 5 males and 5 females) received an intraperitoneal injection of 5 mg/kg NaHS (Cat #: 161527, Sigma-Aldrich, St

Louis, MO, US) while each mouse in the control group (10 mice; with 5 males and 5 females) received injection of 0.1 ml saline. The dosage of NaHS used was based on previous studies, which showed that 5 mg/kg for rats would have desirable results [28-32]. Thirty minutes after NaHS or saline dose, the mice were exposed to 1.4% isoflurane in 100% O₂ for 2 hr, using a home-made chamber. Isoflurane concentration was verified, and adjusted when needed using a gas analyzer (GE Healthcare, MA, USA). All animals were consistently kept at 37 ± 0.5°C during the experiments.

Fear conditioning test was carried out as described previously [15], with slight modifications using a standard chamber from Stoelting (Wood Dale, IL, USA). On the first day after isoflurane, mice were habituated for 3 min before the presentation of the auditory tone (60 s, 3600 Hz, 80 dB). In the final 2 seconds of the tone, mice received gentle foot shock (0.8 mA, 0.5 sec) through the floor grid. After the foot shock, mice were kept in the testing chamber for 30 seconds before sending to the home cage. On the second day, mice first received a context test (without any stimulus). Two hours later, a tone test was carried out. Briefly, mice were placed in a novel context (distinct wall color and odor) for 3 min before the presentation of the tone for 3 min. During the context and tone experiments, the time during which the mice exhibited freezing behavior (failure to make any movement except respiration) was noted and evaluated with an Any-Maze video tracking system with the following settings: first, freezing on threshold for 10 sec and then freezing off threshold for 20 sec and last minimum freezing duration for mere 1 sec). The chamber was cleaned with 75% ethanol after each session.

In vitro Experiments

The *in vitro* experiments were carried out with human neuroglioma cells H4 with transfection of human amyloid precursor protein (H4-APP cells, a gift from Dr. Zhongcong Xie at the Massachusetts General Hospital, Boston, MA, USA) [15]. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Lonza, MD, USA) with 9 percent heat-inactivated fetal calf serum (Atlanta Biologicals, GA, USA), 100 µg/mL streptomycin, 2 mM L-glutamine (G1146, Sigma-Aldrich, MO, USA), 100 U/ml penicillin, and 220 µg/mL G418 (Corning Mediatech, Manassas, VA, USA) at 37 °C in humid atmosphere having 21% O₂ and 5% CO₂. Cells were treated with NaHS (final concentration: 100 µM) or vehicle in 6-well plates at 1 × 10⁶ cells in 1.5 mL as described previously, and 30 min prior

to isoflurane exposure (2%). MPTP opening, MMP, and ATP levels were examined 3 hours later; caspase-3 activation and ROS were examined 6 hours later, as detailed in previous study [15]. A Datex infrared gas analyzer was adopted to closely observe isoflurane concentration during the exposure (Puritan-Bennett, Tewksbury, MA, USA).

Western blot assay

Cells were lysed using a buffer containing 150 mM NaCl, 10 mM Tris-HCl, 0.5% Nonidet P-40, 2 mM EDTA, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin A [15]. Lysate obtained was then centrifuged at 2500×g for 15 min and the supernatant was probed using a polyclonal rabbit-anti-mouse anti-caspase-3 antibody (Cat #: 9662, 1:1100; Cell Signaling Technology, MA, USA) that recognizes the full-length (35–40 kDa) and 17–20 kDa caspase-3. Anti-rabbit IgG (#7074, 1:5000; Cell Signal Technology) was used as secondary antibody. An enhanced chemiluminescence method (Thermo Fisher, USA) was employed and then analyzed on NIH ImageJ (US National Institutes of Health, USA). The results obtained were normalized with the β-actin control. Protein concentration was measured using a bicinchoninic method. All experiments were done six times and the mean was reported.

ROS measurement

ROS concentration was measured using OxiSelect Intracellular ROS Kit and an OxiSelect In Vitro ROS/RNS Kit (Cell Biolabs, CA, USA) as described previously [15]. The fluorescence value was maintained at 480/530 nm.

Isolation of mitochondria

Active Motif kit (Carlsbad, CA, USA) was used to isolate the mitochondria. Cell homogenate and the supernatant were centrifuged at 800 × g and 10,000 × g, at 4°C for 20 min to obtain the mitochondrial (pellet) and a cytosolic fraction (supernatant), respectively [33].

Mitochondrial swelling

Mitochondrial swelling was examined as previously described [33,34]. The mitochondria preparation (protein concentration: 0.5 mg/ml) was placed in a buffer containing 10 mM Tris-Mops (pH 7.4), 125 mM KCl, 1 mM KH₂PO₄, 2.5 mM malate, 5 mM glutamate, and 10 µM EGTA-Tris (pH 7.4) for 5 min with or without adding 1 µM cyclosporine A. Time-dependent absorbance change at 540 nm in mitochondrial suspensions

from isoflurane or control groups was examined using a Beckman DU 640 spectrophotometer (Beckman, CA, USA).

Evaluation of MMP

Mitochondrial membrane potential (MMP) was estimated through tetraethylbenzimidazolylcarbocyanine iodides (JC-1) fluorescence ratio detection and tetramethylrhodamine ethyl ester and perchlorate (TMRE). JC-1 MMP detection kit (30001, Biotium, CA, USA) were used as described previously [15]. MMP was calculated as the ratio of red fluorescence to green fluorescence by fluorometric plate reader (Thermo Fisher Scientific, MA, USA). For measuring MMP by TMRE, cells were treated and evaluated under a 100 X objective lens fluorescence microscope (BZ 9000, Keyence, Itasca, IL, USA).

ATP measurement

ATP (A22066 Invitrogen, CA, USA) concentration was examined as described previously [15]. Briefly, to calculate the ATP, fluorescence was measured and compared with a curve obtained from samples of known ATP.

Data analysis

Data are presented as mean values ±SD and analysed using ANOVA and post-hoc Bonferroni as appropriate. At 95% confidence interval, p<0.05 was regarded to be statistically significant.

RESULTS

NaHS ameliorates cognitive impairment induced by isoflurane

FC tests showed that mice in isoflurane group had decreased freezing time to context (Figure 1A) ($F = 12.027$, $P < 0.0001$) and to tone (Figure 1B) ($F = 6.482$, $P = 0.004$) compared to control group. Decreased freezing time suggests that the isoflurane induced the cognitive deficit. Furthermore, in the isoflurane NaHS group, the decreased freezing time was alleviated compared to isoflurane group (Figure 1A-B).

NaHS inhibits Caspase-3 activity caused by isoflurane

WB showed increased caspase-3 fragments, but not full-length caspase-3, upon isoflurane exposure ($p=0.034$) (Figure 2). NaHS attenuated the increase of caspase-3 fragments induced by isoflurane ($p = 0.046$) but did not affect caspase-

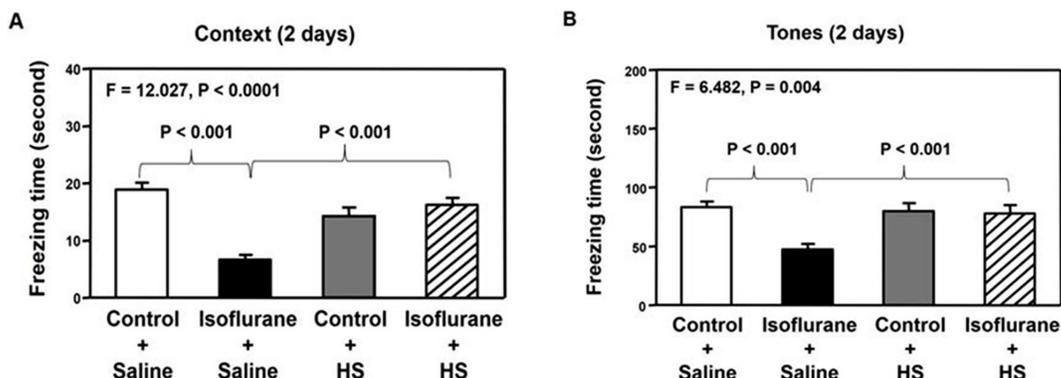


Figure 1: NaHS ameliorates isoflurane-induced cognitive impairment. *A:* Results of the FCT context test in mice treated with control conditions plus saline, isoflurane (1.4%) plus saline, control conditions plus NaHS (5 mg/kg) and isoflurane (1.4%) plus NaHS (5 mg/kg) for 2 h. *B:* Results of the FCT tone test in mice treated with control conditions plus saline, isoflurane (1.4%) plus saline, control conditions plus NaHS (5 mg/kg) and isoflurane (1.4%) plus NaHS (5 mg/kg). The two-way ANOVA and a post hoc Bonferroni test were used to analyze the results

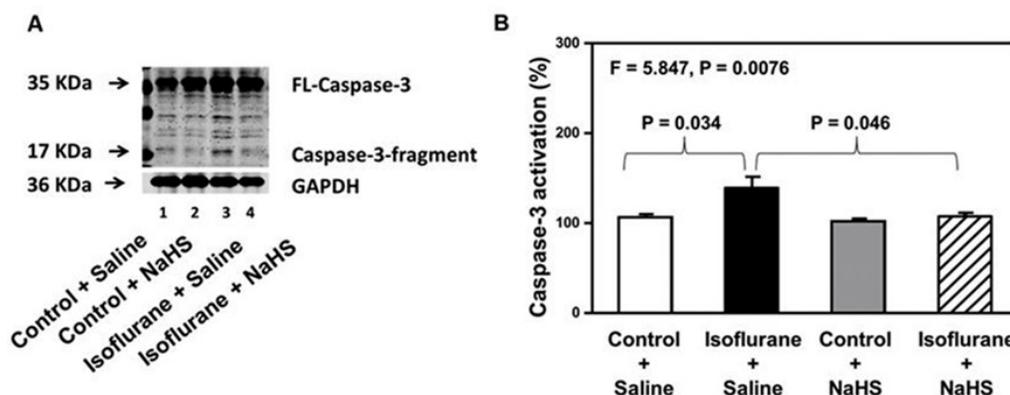


Figure 2: NaHS attenuates isoflurane-induced caspase-3 activation in H4-APP cells. *A:* Western blot showing caspase-3 activation after 6-h exposure to 2% isoflurane (lane 3) versus the control (lane 1), and the effect of 100- μ M NaHS (lane 4) in H4-APP cells. GAPDH was used as a loading control. *B:* Statistical summary of the Western blot data. $N = 6$ per group. FL: full length.

3 fragment in cells not exposed to isoflurane. Isoflurane and NaHS had significant interaction ($F = 5.847, p = 0.0076$).

isoflurane) but did not affect mitochondria swelling in cells not exposed to isoflurane (Figure 4).

NaHS attenuates ROS increase induced by isoflurane

Isoflurane and NaHS had a significant effect on ROS level ($F = 34.12, p < 0.001$) (Figure 3). NaHS attenuated the increase in isoflurane-induced ROS level ($p < 0.001$) and decreased ROS in cells not exposed to isoflurane ($p < 0.001$).

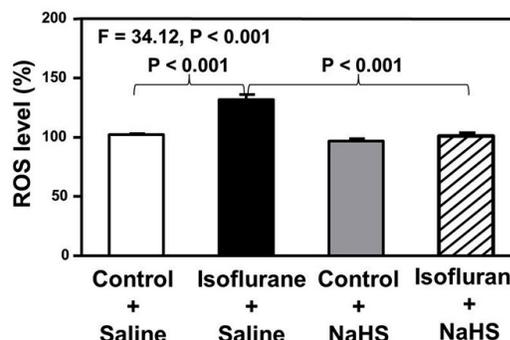


Figure 3: NaHS attenuates isoflurane-induced ROS accumulation in H4-APP cells. Fluorescence staining for ROS showing the effect 6-h exposure to 2% versus the control, and the effect of 100- μ M NaHS. ROS: reactive oxygen species

NaHS attenuates isoflurane-induced mPTPs opening

Ca^{2+} induced significant mitochondria swelling in isoflurane-exposed cells ($p < 0.01$) NaHS attenuated Ca^{2+} -induced mitochondria swelling and permeability transition ($P < 0.01$ for

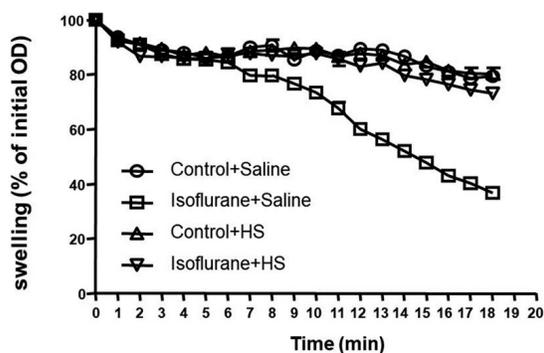


Figure 4: NaHS attenuates isoflurane-induced opening of mPTPs. Opening of mPTPs was assessed by measuring mitochondrial swelling. Data are represented as the relative percentage to the control OD at 540 nm absorbance plotted against time. Bars indicate the standard deviation of six experiments

NaHS attenuates isoflurane-induced MMP reduction

TMRE showed decrease in MMP in isoflurane group compared to control (Figure 5A). NaHS attenuated isoflurane-induced reduction in the MMP which was confirmed by the JC-1 fluorescence ratio (Figure 5B). Isoflurane and NaHS interacted significantly ($F = 18.16$, $p < 0.01$) (Figure 5B)

NaHS attenuates isoflurane-induced ATP reduction

A significant interaction was observed between isoflurane and NaHS ($F = 7.128$, $p = 0.0046$) (Figure 6). The post-hoc Bonferroni analysis suggested isoflurane decreased ATP ($p = 0.009$) inhibited by NaHS ($p = 0.024$).

DISCUSSION

The FCT results demonstrate that NaHS could mitigate isoflurane-induced cognitive impairment. An experiment in cultured cells showed that NaHS mitigated caspase-3 activation induced by isoflurane, ROS accumulation, ATP reduction, and mitochondrial dysfunction. This may be the first study showing NaHS could inhibit isoflurane-mediated cytotoxicity whose underlying mechanism of reduced cytotoxicity could be attributed to suppression of isoflurane-induced ROS accumulation, ATP reduction, and mitochondrial malfunction.

Oxidative stress often results in different neurodegenerative diseases. It was reported that H_2S provides protection to primary neurons and immortalized mouse hippocampal cells from

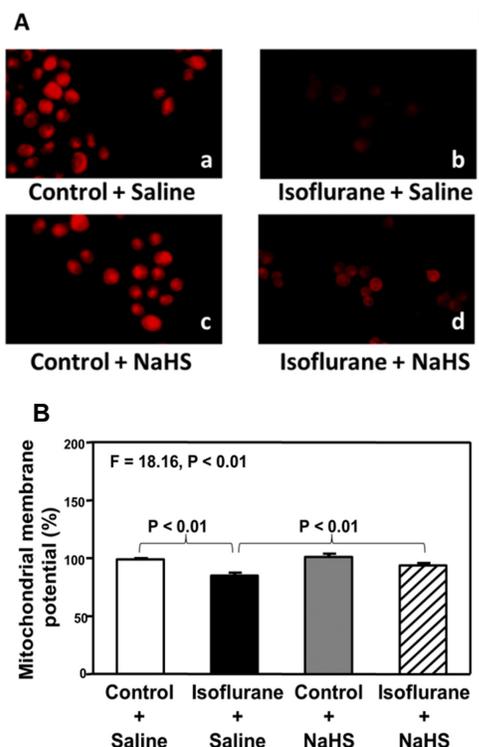


Figure 5: NaHS attenuates the isoflurane-induced reduction in mitochondrial membrane potential (MMP) in H4-APP cells. A. Tetramethylrhodamine ethyl ester and perchlorate staining showing the effect of 3-h exposure to 2% isoflurane versus the control, and the effect of 100- μ M NaHS in H4-APP cells. $N = 6$ per group. B. Tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorescence analysis showing the effect of 3-h exposure to 2% isoflurane versus the control, and the effect of 100- μ M NaHS in H4-APP cells. $N = 6$ per group

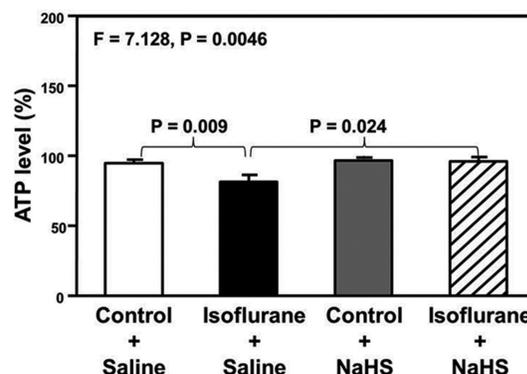


Figure 6: NaHS attenuates the isoflurane-induced reduction in ATP levels in H4-APP cells. Bioluminescence assay showing the effect of 3-h exposure to 2% isoflurane versus the control, and the effect of 100- μ M NaHS in H4-APP cells. $N = 6$ per group.

oxidative glutamate toxicity [19,22]. Additionally, Lu et al. discovered that H_2S enhances glutamate uptake and increases glutathione

production to prevent excessive glutamate accumulation in synaptic clefts, thus protecting astrocytes from oxidative toxicity [23]. Similarly, our findings indicate that NaHS could attenuate ROS accumulation in cells induced by isoflurane, suggesting that NaHS could mitigate the oxidative toxicity of isoflurane.

The mPTP opening decreases the potential of the membrane and releases apoptogenic proteins to amplify the apoptotic mechanism [35-38]. Oxidative and other cellular stresses cause mPTP opening [39]. In contrast, inhibition of mPTP opening protects neurons from apoptosis [34]. Hu et al. have shown that H₂S inhibits apoptosis by rotenone in SH-SY5Y cells by protecting mitochondria [21]. Xie et al. proposed that mPTP opening and MMP reduction contribute to caspase-3 activation by isoflurane, which can be attenuated by a hydrogen-rich saline [15,39]. Our findings are consistent with these findings as they show that NaHS inhibited isoflurane-induced mPTP opening, and thus attenuated caspase-3 promotion by isoflurane. These results supported the notion that caspase-3 promoted by isoflurane involved mitochondrial dysfunction and ROS mechanisms.

Our FCT experiments in mice demonstrated that NaHS could attenuate cognitive impairment caused by isoflurane exposure. This finding encourages future investigation of NaHS as a treatment for neurobehavioral decline induced by anesthesia. Various behavior studies have shown that H₂S could ameliorate cognitive and memory impairments by attenuating ROS production and the activation of apoptotic pathways. Previous studies using Morris maze test showed that H₂S (i.e., NaHS) or S-propargylcysteine (SPRC), could ameliorate lipopolysaccharide-induced cognitive impairment [28,40]. Chu et al. showed that NaHS could alleviate memory impairment in mice due to surgery [30]. A study using A β rat models of AD showed that NaHS could attenuate spatial memory impairment [29]. He et al. demonstrated that NaHS improved memory acquisition and spatial learning in APP/PS1 transgenic mice [25]. Altogether, these results indicated that exogenous H₂S could attenuate cognitive dysfunction due to a variety of etiological factors.

First important limitation of this study was that we did not examine the dose-response relationship as Yin et al. showed that NaHS protected from I/R injury by regulating oxidative stress in a dose-dependent manner (e.g., 0.2 and 0.4 μ mol/kg) [41]. It is possible that, at different doses, NaHS may have distinct or even opposing effect on caspase-3 activity induced by isoflurane, ROS,

mitochondria, and ATP. Second, the behavioral effects of NaHS were examined using only FCT. Third, we did not check the potential functions of different H₂S donors (e.g. SPRC). Effects of H₂S-releasing agents with sustained and controlled release that more faithfully mimic physiological conditions are essential. Despite these limitations, the present study showed that NaHS could attenuate cognitive impairments induced by isoflurane exposure.

CONCLUSION

This study indicated that NaHS could ameliorate isoflurane-induced cognitive impairments, likely through attenuating caspase-3 activation induced by isoflurane, mPTP opening, ROS increase, and MMP reduction. Future studies using other H₂S donors (e.g., SPRC) and other anesthetics (e.g., sevoflurane) are warranted.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. *XZ and CL designed and contributed equally this work. DC, YF, LZ, XYN, XS and XZN collected data while DC wrote the manuscript which was read and approved for submission by all authors.*

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