Original Paper

Hydrogen Sulfide Inhalation Improves Neurological Outcome via NF- κ B-Mediated Inflammatory Pathway in a Rat Model of Cardiac Arrest and Resuscitation

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Key Words

Cardiac arrest • Inflammation • Brain recovery • Hydrogen sulfide • NF- κ B

Abstract

Background/Aims: The effects of H₂S on cerebral inflammatory reaction after cardiac arrest (CA) and cardiopulmonary resuscitation (CPR) remain poorly understood. In this study, we investigated the effects of exogenous 40 ppm and 80 ppm H₂S gas on inflammatory reaction and neurological outcome after CA/CPR. **Methods:** CA was induced by ventricular fibrillation and followed by CPR. Forty or 80 ppm H₂S was inhaled for 1 h immediately following CPR. The levels of IL-1 β , IL-6 and TNF- α , the myeloperoxidase (MPO) activity, the expression of iNOS and ICAM-1, and the phosphorylation and translocation of NF- κ B were evaluated at 24 h after CA/CPR. The tape removal test, survival rate and hippocampal neuronal counts were investigated at 14 d after CA/CPR. **Results:** CA/CPR induced significant increases in IL-1 β , IL-6, TNF- α and MPO activity. The phosphorylation and translocation of NF- κ B, and the expression of iNOS and ICAM-1 were increased significantly. Inhalation of 40 or 80 ppm H₂S gas decreased these inflammatory cytokines. Furthermore, 40 or 80 ppm H₂S inhibited the activation of NF- κ B and the downstream proinflammatory mediators iNOS and ICAM-1. H₂S inhalation also improved neurological function, 14-d survival rate, and reduced hippocampal neuronal loss. **Conclusion:** These results indicated that inhalation of H₂S protected against brain injury after CA/CPR. The mechanisms underlying protective effects of H₂S were associated with the inhibition of CA/CPR-induced inflammation reactions by reducing IL-1 β , IL-6 and TNF- α , and concomitantly inhibiting the activation and infiltration of neutrophils. The beneficial effects of H₂S might be mediated by downregulation of NF- κ B and the downstream proinflammatory signaling pathway.

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Introduction

Sudden cardiac arrest (CA) is a serious public health problem affecting more than 450,000 individuals annually [1]. Among these patients, only 10 to 40% survive. Worse, more than half of the survivors have various permanent neurological consequences, with only 5 to 17% of CA and cardiopulmonary resuscitation (CA/CPR) patients discharged without a serious neurological problem [1-3]. Apart from induced hypothermia, no other specific treatment is available to reduce neuronal death and improve functional outcome after CA/CPR till now.

Previous studies had shown that the inflammatory response were a major contributor to exacerbate brain injury following cerebral ischemia [4]. It has been reported that cerebral ischemia leads to significantly elevated levels of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which further induce the recruitment and activation of various types of leukocytes including the neutrophils [5, 6]. Those mediators accumulate in the capillaries and impair cerebral blood flow, extravasate into the brain parenchyma, produce excessive reactive oxygen species, or induce matrix metalloproteinase-9 and exert deleterious effects on the progression of tissue damage. Therefore, inhibition of the inflammatory response may provide a potential treatment target for neuroprotection in brain ischemia.

Additional data suggest that hydrogen sulfide (H₂S) plays important roles in cardiovascular, neuronal, renal, respiratory, gastrointestinal, hepatic and endocrine systems [7, 8]. Recent studies have demonstrated that H₂S donor (Na₂S or NaHS) have protective effects after brain ischemia and reperfusion including CA/CPR [9, 10]. However, there are few data about the effects of exogenous H₂S gas on CA/CPR. In our previous study, we revealed that inhalation of 80 ppm H₂S gas improved brain metabolism and reduced neurohistopathological damage after CA/CPR in rabbits [11]. But more direct evidence for the neuroprotective effect of H₂S gas is still lacking. In recent studies, the anti-inflammatory actions of H₂S in different models were detected [12-16]. Therefore, the present study was aimed to investigate the effect of exogenous H₂S gas on neuroinflammation in the early reperfusion period after CA/CPR. Furthermore, the effects of H₂S on neurological outcome and neuronal survival were also investigated.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of Harbin Medical University in Harbin, Heilongjiang, China, and followed national guidelines (Guidelines on Administration of Laboratory Animals in China & Guidelines on the Humane Treatment of Laboratory Animals in China) for the treatment of animals.

Subjects and surgical procedures

We tested male Sprague-Dawley rats weighing 280–300 g in this study. Animals had access to food and water *ad libitum* until the morning of the experiment. Animals were anesthetized with 1–2% halothane and 30% oxygen. Ventilation was controlled with a small animal ventilator (Inspira ASV, Harvard Apparatus Inc., MA, USA) to maintain arterial pH at 7.35–7.45, PaCO₂ at 35–45 mmHg, and PaO₂ over 90 mmHg. Pericranial temperature was monitored using a thermistor probes (BIOPAC Systems Inc., CA, USA) percutaneously placed adjacent to the skull. The pericranial temperature was maintained at 37 ± 0.5°C throughout the experiments. A standard lead II ECG was recorded continuously using subdermal needle-type electrodes placed in the limbs. The right femoral artery and vein were cannulated with PE-50 catheters for continuous monitoring of blood pressure, measurement of blood gases, infusion of fluid, and drug administration. All of the physiology data were monitored and recorded using the BIOPAC MP150 physiometer (BIOPAC Systems Inc.). The number of animals and the flow diagram of the experimental groups were shown in Figure 1.

CA protocol

We induced ventricular fibrillation using an esophageal electrode (5F, Bard Peripheral Vascular Inc., Tempe, AZ) by a 1-min impulse of 12 V at 50 Hz alternating current. If spontaneous defibrillation occurred,

Fig. 1. Flow diagram of the experimental groups. ROSC = return of spontaneous circulation; MPO = Myeloperoxidase; Sham = sham group, CA = cardiac arrest group; H₂S 40 = H₂S 40 group; H₂S 80 = H₂S 80 group.

we delivered additional 20 s impulses [17]. CA was confirmed by an abrupt decrease in MAP to less than 15 mmHg; when that occurred, ventilation was stopped.

After 6 min of CA, CPR was implemented by reconnecting the ventilator with FiO₂ = 1.0, administering epinephrine (0.02 mg/kg) and sodium bicarbonate (1 mEq/kg), and applying sternal compressions at a rate of 200 per min. After 2 min of CPR, we applied external defibrillation (biphasic, 2 J; Zoll M-series Defibrillator, ZOLL Medical U.K. Ltd, Cheshire, England). The cycle of 1 min of chest compression followed by defibrillation was repeated, if necessary, until the return of spontaneous circulation (ROSC) or the elapse of more than 10 min. Successful ROSC was defined as an initial return of a sinus ECG rhythm with an obvious arterial wave form and MAP of 50 mmHg or greater lasting at least 5 min. Resuscitated rats were randomly assigned into the CA group (FiO₂ = 0.5, without H₂S gas), the H₂S 40 group, and the H₂S 80 group (FiO₂ = 0.5, with 40 ppm or 80 ppm H₂S gas for 1 h mechanically ventilation). We monitored blood gas analyses at 15 and 30 min after ROSC, and appropriate ventilator adjustments were made, if necessary. After ROSC had been established for 1 h, the vascular catheters were removed. Surgical wounds were sutured and infiltrated with 0.25% bupivacaine (total 0.5 mg). Rats were then weaned from the ventilator and extubated. To avoid hypoxic episodes, rats were placed in a chamber enriched with 50% oxygen for another 1 h. The oxygen administration contained 40 ppm or 80 ppm H₂S for the H₂S treatment groups and no H₂S for the CA group. After that rats were returned to their cages with easily accessible food and water. Shams underwent all same procedures as were administered to the CA and the H₂S groups, except that the shams received no ventricular fibrillation or CPR.

Assessment of neurologic outcome and survival rate

We familiarized all animals to the neurologic test for 3 consecutive days and evaluated them before the experiment to ensure normal neurologic function. We determined neurologic outcomes at 1, 3, and 14 d after ROSC and 14-d survival rates. We evaluated sensorimotor integration using a modified tape removal test (TRT) [18]. We recorded the time to remove both adhesive tapes, with all times greater than 180 s recorded as 180 s. The same investigator, who was blinded to treatment, carried out all evaluations.

Neuronal counts

For hippocampal neuronal counts, the rats that survived for 14 d and those in the sham group were investigated. Immediately after euthanization, rats were perfused through the heart with phosphate-buffered saline and 4% paraformaldehyde. We examined 5 µm coronal serial sections of the hippocampus of fixed brains embedded in paraffin. Sections corresponded to bregma -3.3 cm, according to the atlas of

Paxinos and Watson [19]. Neurons were judged to be viable, if they showed a distinct nucleus and nucleolus; non-viable ischemic neurons exhibited nuclear pyknosis, karyorrhexis, and vacuolization. One observer, who was blinded to the experimental protocol, counted the numbers of viable pyramidal neurons per high-power field ($\times 400$).

Cerebral cytokine levels and MPO activity in brain tissue

At 24 h after ROSC, animals were deeply anesthetized by halothane. Brain tissues were rapidly dissected out and frozen immediately at -80°C . Each brain tissue sample was homogenized in phosphate-buffered saline and centrifuged at 10,000 *g* at 4°C for 10 min. The supernatants from brain tissues were collected and, using the commercially available ELISA kits (Abcam, Cambridge, MA), we measured the concentrations of IL-1 β , IL-6, TNF- α , and MPO activity. We prepared reagents, samples, and working standards according to the manufacturer's instructions at room temperature. Each sample was run in duplicate and evaluated using a microplate reader (BioTek Instruments Inc, Winooski, VT), as recommended by the manufacturer. One unit of MPO activity was defined as the amount that degraded 1 μmol hydrogen peroxide per min at 25°C . The activity was expressed as units/g tissue.

Western blot analysis for NF-κB, iNOS, and ICAM-1

Protein homogenates of brain samples were prepared by rapid homogenization in Tissue Extraction Reagent II (Invitrogen Corporation, Carlsbad, CA), according to the manufacturer's instructions. The supernatant was used for western blot analyses of inducible nitric oxide (iNOS) and intercellular adhesion molecule-1 (ICAM-1) expressions. In the case of the NF-κB activation, including phosphorylation and translocation to the nucleus, analyses were carried out on nuclear extracts. Nuclear and cytoplasmic proteins were separated according to the protocol described in Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL). Protein concentrations were measured using a BCA Protein Assay Kit (Beyotime Biotech, China). Proteins were separated by 10% SDS polyacrylamide gels, separated by gel electrophoresis (Mini Protean III, Biorad, NZ), and transferred onto a 0.45 μm polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). The membranes were blocked 5% skim milk powder in TBST and then incubated overnight at 4°C with anti-ICAM-1 (1:1000; BIOS, China), anti-iNOS (1:500; BIOS, China), anti-NF-κB p65 (1:500; Abcam, Cambridge, MA), anti-NF-κB p65 (phospho S536) (1:500; Abcam, Cambridge, MA), anti-actin (1:2000; Zhongshan Golden Bridge Biotechnology, China), and anti-Histone H2A (1:500, Santa Cruz, CA). After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:3000, Zhongshan Golden Bridge Biotechnology, China). Bands were detected using the enhanced chemiluminescence detection system (Beyotime Biotech, China), and protein band densities were digitally quantified and normalized to β -actin or Histone H2A using Image J software (NIH, USA).

Statistical Analysis

TRT scores were expressed as median and interquartile range (25th to 75th percentiles) and analyzed by the Kruskal-Wallis test, followed by post-hoc Mann-Whitney U tests, when the overall Kruskal-Wallis result was significant. Other data are presented as the mean $\pm$ SD and analyzed by one-way ANOVA, with Bonferroni correction for post hoc comparisons between multiple experimental groups. Kaplan-Meier survival curves were compared using the log-rank test. We considered probability values of $P < 0.05$ to be significant. Statistical analysis was performed using SPSS® software (SPSS Inc., Chicago, IL).

Results

Overall Survival Rate and TRT after ROSC

All 5 of the rats in the sham group survived through the 14 d observation period. Of the 20 rats in the CA group, 10 died and the remaining 10 survived through 14 d. Four rats died in the H₂S 40 group and 5 rats died in the H₂S 80 group. Finally, 10 of 20 rats (50%) in the CA group, 16 of 20 rats (80%) in the H₂S 40 group, and 15 of 20 rats (75%) in the H₂S 80 group survived until day 14. As depicted in Figure 2, there was a significant difference in the 14-d survival rate between the CA group and H₂S 40 or H₂S 80 group (both $P < 0.05$).

Fig. 2. Kaplan-Meier plot of cumulative survival 14 days after cardiac arrest and resuscitation in Sham (n = 5), CA (n = 20), H₂S 40 (n = 20) and H₂S 80 (n = 20) groups. Sham = Sham group; CA = cardiac arrest group; H₂S 40 = H₂S 40 group; H₂S 80 = H₂S 80 group. # *P* < 0.05 vs. CA group.

Fig. 3. Tape removal test (TRT) at day 1, day 3, and day 14 after cardiac arrest and resuscitation. Open circles indicate values for individual animals. Horizontal bars indicate median values. Sham = Sham group; CA = cardiac arrest group; H₂S 40 = H₂S 40 group; H₂S 80 = H₂S 80 group. * *P* < 0.05 vs. Sham group, # *P* < 0.05 vs. CA group.

Fig. 4. The histological images of neuron and viable neuronal counts in the hippocampus CA1 region in Sham, CA, H₂S 40 and H₂S 80 groups at 14 d after cardiac arrest and resuscitation. (A) Representative histological images of neuron in the hippocampus CA1 region. (B) The hippocampus CA1 region. (C) The number of neurons in different groups. Sham = Sham group; CA = cardiac arrest group; H₂S 40 = H₂S 40 group; H₂S 80 = H₂S 80 group. * *P* < 0.05 vs. Sham group, # *P* < 0.05 vs. CA group.

Before the experiment, all animals removed the TRT adhesive tapes rapidly and there were no differences between groups. After ROSC, dramatic increases in TRT latencies showed a severe neurologic deficit in all resuscitated animals. The times needed in TRT trials increased from 4 s (3-6 s) before CA to 142 s (103-180 s) on day 1 and then fell to 130 s (47-180 s) on day 3 and 54 s (46-75 s) on day 14 in the CA group. As can be seen in Figure

Fig. 5. Cytokines levels (A) and MPO activities (B) in brain tissue at 24 h after resuscitation in Sham, CA, H₂S 40 and H₂S 80 groups. Sham = sham group; CA = cardiac arrest group; H₂S 40 = H₂S 40 group; H₂S 80 = H₂S 80 group. * $P < 0.05$ vs. Sham group, # $P < 0.05$ vs. CA group.

3, compared with the CA group, the H₂S 40 group differed significantly from day 1 (86 s (47-180 s), $P < 0.05$) to day 3 (40 s (25-79 s), $P < 0.05$) and day 14 (17 s (9-32 s), $P < 0.05$). The similar trend was also observed in H₂S 80 group from day 1 (78 s (44-140 s), $P < 0.05$) to day 3 (54 s (44-73 s), $P < 0.05$) and day 14 (16 s (9-25 s), $P < 0.05$) when compared with CA group. No significant differences were observed between the H₂S 40 group and the H₂S 80 group from day 1 to day 14 (Fig. 3).

Neuronal Counts

As shown in Figure 4, hematoxylin-eosin staining showed a massive reduction of vital neurons in all resuscitated animals. Nuclear pyknosis, karyorrhexis, and vacuolization were also observed in the hippocampus CA1 region in the CA group. However, the neuronal density and cell morphology were relatively well preserved in the H₂S 40 and H₂S 80 group. For comparison, the number of viable neurons in the CA group (20 ± 6) was significantly less than in the sham group (53 ± 10 , $P < 0.05$). A larger number of viable neurons was observed in the H₂S 40 group (33 ± 8 , $P < 0.05$) and H₂S 80 group (35 ± 8 , $P < 0.05$) than in the CA group, but both still less than that in the sham group (both $P < 0.05$).

Effects of H₂S on IL-1β, IL-6, TNF-α, and MPO activity in brain tissue

At 24 h after ROSC, the brains of CA rats showed significantly higher levels of IL-1β than that from sham group rats (38.62 ± 5.43 pg/mg vs. 15.99 ± 3.86 pg/mg, $P < 0.05$). Compared

Fig. 6. Effects of H₂S on NF-κB phosphorylation (A) and translocation (B) in Sham, CA, H₂S 40 and H₂S 80 groups after CA/CPR. Representative immunoblots for P65, phospho-P65 (p-P65), β-actin and Histone H2A are shown above the bars. Sham = sham group; CA = cardiac arrest group; H₂S 40 = H₂S 40 group; H₂S 80 = H₂S 80 group. * $P < 0.05$ vs. Sham group, # $P < 0.05$ vs. CA group.

with the CA group, the levels of IL-1β were significant decreased in the H₂S 40 group (29.17 ± 4.40 pg/mg, $P < 0.05$) and H₂S 80 group (22.30 ± 7.74 pg/mg, $P < 0.05$). There was no significant difference between H₂S 40 group and H₂S 80 group ($P > 0.05$) (Fig. 5A).

CA/CPR led to significant increases of IL-6 levels in the CA group (54.65 ± 10.68 pg/mg), H₂S 40 group (35.60 ± 8.27 pg/mg) and H₂S 80 group (32.03 ± 2.12 pg/mg) when compared with the sham group (16.99 ± 4.19 pg/mg, both $P < 0.05$). But inhalation of H₂S led to a significant decrease in IL-6 levels in the H₂S 40 group and H₂S 80 group when compared with the CA group (both $P < 0.05$) (Fig. 5A).

Fig. 7. Effects of H₂S on the expression of iNOS and ICAM-1 protein in brain tissue following CA/CPR in Sham, CA, H₂S 40 and H₂S 80 groups after CA/CPR. Representative western blots for iNOS, ICAM-1 and β-actin are shown above the bars. Sham = sham group; CA = cardiac arrest group; H₂S 40 = H₂S 40 group; H₂S 80 = H₂S 80 group. * $P < 0.05$ vs. Sham group, # $P < 0.05$ vs. CA group.

TNF-α levels were significantly elevated in CA rats compared with sham rats (25.06 ± 2.47 pg/mg vs. 10.11 ± 1.80 pg/mg, $P < 0.05$) at 24 h after ROSC, but these increases were significantly inhibited by 40 ppm H₂S (19.73 ± 1.60 pg/mg, $P < 0.05$) and 80 ppm H₂S (17.64 ± 2.21 pg/mg, $P < 0.05$) treatment. However, the levels of TNF-α in the H₂S 40 group and H₂S 80 group were not significantly different ($P > 0.05$) (Fig. 5A).

Significantly increased MPO activity in brain tissue was observed in the CA group when compared with the sham group at 24 h (0.33 ± 0.08 U/g vs. 0.09 ± 0.05 U/g, $P < 0.05$) after ROSC (Fig. 5B). Compared with the CA group, these increases were significantly inhibited in the H₂S 40 group (0.17 ± 0.07 , $P < 0.05$) and H₂S 80 group (0.19 ± 0.11 , $P < 0.05$).

Inhalation of H₂S inhibits NF-κB activation in ischemic brain

CA/CPR resulted in a statistically significant increase in the expression of NF-κB p65 phosphorylation (3.63 folds to sham, $P < 0.05$, Fig. 6A) as shown in CA group. In the H₂S-treated group, the phosphorylation of p65 was significantly reversed in H₂S 40 group (2.01 folds to sham, $P < 0.05$) and H₂S 80 group (1.93 folds to sham, $P < 0.05$).

To further examine whether H₂S regulates NF-κB p65 translocation to the nucleus after CA/CPR, the nucleus and cytoplasm fractions were extracted. In sham group, immunoblotting for p65 was predominately located in the cytoplasm. After CA/CPR, we observed a 4.4-fold increase in nucleus/cytoplasm ratio in CA group ($P < 0.05$ vs. sham, Fig. 6B), indicating the remarkable translocation of p65 from cytoplasm to nucleus. Inhalation of 40 ppm H₂S (2.56 folds to sham, $P < 0.05$) or 80 ppm H₂S (2.62 folds to sham, $P < 0.05$) significantly prevented the nuclear translocation of NF-κB p65 when compared with CA group. Taken together, these data suggested that inhalation of H₂S could inhibit NF-κB activation/translocation induced by CA/CPR.

Inhalation of H₂S inhibits NF-κB-dependent protein expression

NF-κB is a well-known regulator of various pro-inflammatory factors. Therefore, the pro-inflammatory mediators including iNOS and ICAM-1 in the ischemic brain tissue were tested. Immunoblotting results showed that after 24 h of CA/CPR, the values of iNOS elevated significantly in the CA group (3.73 folds to sham, $P < 0.05$) when compared with sham group (Fig. 7). However, the levels of increased iNOS dropped significantly in H₂S 40 group (2.05 folds to sham, $P < 0.05$) and H₂S 80 group (2.16 folds to sham, $P < 0.05$) when compared with CA group. Meanwhile, H₂S also significantly decreased the expression of ICAM-1 in the 40 ppm group (2.08 folds to sham, $P < 0.05$) and 80 ppm group (1.98 folds to sham, $P < 0.05$) when compared with CA group (3.45 folds to sham, $P < 0.05$). However, we observed no significant difference between the H₂S 40 group and H₂S 80 group in iNOS and ICAM-1 (both $P > 0.05$).

Discussion

Using a model of CA/CPR in rats, we found that inhalation of 40 or 80 ppm H₂S gas at an early ROSC stage was accompanied by better neurologic outcomes and survival rate for 14 d. These improvements were associated with an inhibition of inflammation reaction as shown by IL-1β, IL-6, TNF-α, and MPO measurements at 24 h after CPR. The inhibition of NF-κB and pro-inflammatory cytokines iNOS and ICAM-1 is a possible mechanism for H₂S treatment.

In previous mice study, the neuroprotective effects of H₂S had been partly attributed to hypothermia [20]. However, these results had been questioned deeply in larger animals even in rats [21-23]. In our previous study with rabbit, no significant differences in rectal, cortex, and hippocampus temperature were observed after 80 ppm H₂S inhalation [11]. Stein and colleagues further pointed out that rats were largely insensitive to H₂S-induced low body temperature unless O₂ tension was decreased to 10.5 % [23]. To exclude the interference of hypothermia, the pericranial temperature in this study were maintained at 37.5 ± 0.5°C throughout the experiments. However, the neuroprotective effects of H₂S were also observed with better neurologic outcomes and survival rate after CA/CPR. Therefore, the neuroprotective effect of H₂S in this study was not dependent on hypothermia.

There is increasing evidence demonstrating that inflammatory processes play pivotal roles in the pathophysiology of brain damage after CA/CPR [24-27]. The inflammation is characterized by activation of the complement system, cytokines, and leukocytes. Those effects may be observed as early as 3 h after CA/CPR and may last for several days, as demonstrated by Adrie et al., with a description of "sepsis-like syndrome" [26]. Using 6 min of CA in rats, Teschendorf et al. demonstrated that inflammatory markers (such as TNF-α and ICAM-1) were significantly upregulated on the mRNA level in brain tissue as early as 6 h after CA [24]. The findings of the present study further confirm that IL-6, TNF-α, and MPO activities were increased in the brain tissue of rats after CA/CPR. There was some evidence that high IL-6 and TNF-α serum levels were associated with early neurological worsening and a higher mortality rate [5, 26]. In agreement with these findings, our data suggested that the inflammation after CA/CPR might have a similar impact, because IL-1β, IL-6 and TNF-α levels were significantly elevated in the CA group with bad neurological outcome.

H₂S has recently been shown to exert several anti-inflammatory effects in different models. For instance, it had been shown that administration of H₂S donor (NaHS) would attenuate myocardial injury through inhibition of ICAM-1 expression and polymorphonuclear leukocyte accumulation [12]. In the gastrointestinal tract, H₂S had been shown to promote the resolution of mucosal inflammation to heal ulcers, while suppression of endogenous H₂S synthesis resulted in an increased granulocyte infiltration [13]. H₂S was also reported to induce neutrophil apoptosis and to suppress NSAID-induced granulocyte infiltration, as well as the expression of some leukocyte, endothelial adhesion molecules, and TNF-α [14, 15]. Moreover, Yin and coauthors showed that, after global cerebral ischemia and reperfusion, exogenous H₂S attenuated pro-inflammatory markers TNF-α and monocyte chemoattractant protein-1 in a dose-dependent manner [28]. Additionally, recent research by Xuan and colleague revealed that exogenous NaHS reduced the over-expression in IL-1β and TNF-α, as well as the extensive astrogliosis and microgliosis in the hippocampus of rat model of Alzheimer's disease [29]. All these studies suggested that H₂S played an important role in anti-inflammatory effects. Similar to these previous studies, in the present work, the pro-inflammatory cytokine IL-1β, IL-6 and TNF-α were inhibited in the H₂S group. Further, a relatively better neurological outcome was observed at the same time. The present findings, in conjunction with previously published data [12-15, 28, 29], suggested that H₂S might improve neurological outcomes through attenuating CA/CPR-induced inflammation.

Recent studies had shown that the pathogenic role of neutrophils including accumulation in cerebral capillaries and infiltration into the brain parenchyma was induced by neuroinflammatory mediators that released from ischemic brain tissue, and MPO activity was always used as an index of neutrophil activation and infiltration [12-14]. Inhibition of neutrophil activation and infiltration into the ischemic brain was associated with an

improving neurological outcome [15, 28]. In this study, we found here that inhalation of 40 or 80 ppm H₂S markedly inhibited the increased MPO activities induced by CA/CPR, and improved neurological results were observed. It is therefore reasonable to postulate that H₂S protects against brain injury after CA/CPR through reducing neutrophils activation or infiltration.

The NF-κB pathway is considered to play important roles in a variety of central nervous system injury such as traumatic brain injury, transient focal ischemia and global ischemia [30]. Under normal conditions, NF-κB exists in the cytoplasm in an inactive form. Once activated, subunits of NF-κB translocate into the nucleus and bind to their cognate DNA binding sites to mediate transcription of various inflammatory genes or their products such as iNOS, ICAM-1, TNF-α, IL-1β and IL-6 [30]. Thus, inhibitors of NF-κB activation may have therapeutic potential for brain protection. Transgenic mice with inactivation of astroglial NF-κB shown enhanced neuroprotection in autoimmune encephalomyelitis by suppressing central nervous system inflammation [31]. Additionally, inhibition of NF-κB *in vivo* reduced astrogliosis and suppressed neuroinflammation in a transgenic mouse model of Alzheimer disease [32]. Similarly, Williams et al. demonstrated that modulation of NF-κB activity with MLN519 attenuated the expression of ICAM-1, TNF-α, IL-1β and IL-6, and reduced neutrophil infiltration into the brain [33].

iNOS and ICAM-1 are downstream target genes of NF-κB [34-36]. The up-regulation of iNOS resulted in the further production of large amounts of nitric oxide, which played critical roles in controlling most inflammatory processes [34, 36]. Previously study had shown that ICAM-1 was transcriptionally regulated by NF-κB, and NF-κB p65 transactivated the ICAM-1 promoter in a κB site-dependent manner [37]. The up-regulation of ICAM-1 played a key role in the recruitment and extravasation of circulating leukocytes at sites of tissue injury [34-36].

In our experiment, the phosphorylation and translocation of NF-κB p65 increased significantly after CA/CPR, while inhalation of 40 ppm or 80 ppm H₂S attenuated the phosphorylation and translocation of NF-κB, reflecting the effective blockage of CA/CPR-induced NF-κB activation. Further, our data suggested that inhalation of H₂S efficiently attenuated the levels of iNOS, ICAM-1, TNF-α, IL-1β and IL-6. Taken all these together, we demonstrated that the neuroprotection offered by H₂S was at least partially attributed to its anti-inflammatory properties and this neuroprotection was more likely associated with downregulation of NF-κB and the downstream proinflammatory signaling pathway.

In this study, 40 ppm and 80ppm H₂S were choose to explore the dose-dependent effects of H₂S on CA/CPR because these doses were safe and low concentrations for rat [38], and were widely used in previous animal studies and no obvious toxic effects including the histological and behavioral outcome were observed [11, 21, 39].

Last, but not least, we must point out that the dose-dependent protective effect of H₂S was not demonstrated in this study. Inhalation of 40 ppm or 80 ppm H₂S produced the same effects in nearly all the parameters measured. Hence we infer that there might be a maximal effects by inhalation H₂S. Future studies on different concentration and duration of H₂S are warranted to determine the optimum concentration and therapeutic schedule of H₂S. In addition, the translational implication of H₂S in the human clinical setting is uncertain and needs to be investigated.

Conclusion

In summary, the findings presented here provide evidence suggesting that inhalation of H₂S protects against brain injury after CA/CPR. The mechanisms underlying protective effects of H₂S are associated with the inhibition of CA/CPR-induced inflammation reactions by reducing the levels of IL-1β, IL-6 and TNF-α, and concomitantly inhibiting the activation and infiltration of neutrophils. The beneficial effects of H₂S might be mediated by downregulation of NF-κB and the downstream proinflammatory signaling pathway.

Disclosure Statement

The authors declare no conflict of interest.

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