Original Paper

Protective Effects of Hydrogen Sulfide Against Cigarette Smoke Exposure-Induced Placental Oxidative Damage by Alleviating Redox Imbalance via Nrf2 Pathway in Rats

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Key Words

Hydrogen sulfide • Nuclear factor erythroid 2-related factor 2 • Oxidative stress • Placental damage • Redox imbalance

Abstract

Background/Aims: Cigarette smoke exposure (CSE) during pregnancy is a well-recognized health hazard that causes placental damage. Hydrogen sulfide (H₂S) has been reported to protect multiple organs from injury. However, the protective effects of H₂S have not been tested in the placenta. This study aimed to explore the potential of H₂S in protecting placenta against oxidative injury induced by CSE during pregnancy and the possible underlying mechanisms. **Methods:** Pregnant SD rats were randomly divided into 4 groups: NaCl, NaHS (a donor of H₂S), CSE and CSE+NaHS. Placental oxidative damage was detected by 8-hydroxy-2-deoxyguanosine (8-OHdG) stain and malondialdehyde (MDA) assay. Placental redox status was assessed by measuring reactive oxygen species (ROS), total antioxidant capacity (T-AOC) and glutathione (GSH) levels, as well as copper/zinc SOD (SOD1), manganese SOD (SOD2), catalase (CAT) and glutathione peroxidase (GPx) activities and expressions. Meanwhile, nuclear factor erythroid 2-related factor 2 (Nrf2) was analyzed by immunohistochemistry, real-time PCR and Western blot. **Results:** We found that NaHS markedly reduced the elevated levels of 8-OHdG and MDA induced by CSE. Further, NaHS treatment effectively mitigated CSE-induced placental redox imbalance by inhibiting ROS production, restoring T-AOC level, increasing GSH/GSSG ratio, and augmenting SOD1 SOD2, CAT and GPx activities and expressions. More notably, NaHS administration also reversed the aberrant decrease of Nrf2 due to CSE in rat placentas. **Conclusion:** Our data demonstrate that H₂S can protect against CSE-induced placental oxidative damage probably by alleviating redox imbalance via Nrf2 pathway.

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Introduction

Cigarette smoke is one of the major environmental health risk factors affecting almost all the organs or systems of human body [1-4]. A growing body of evidence indicates that maternal smoking or cigarette smoke exposure (CSE) during pregnancy is associated with a variety of placental complications, including alterations to the development and function of the placenta [5, 6]. Histologically, the placentas from smokers display changes including the thickened trophoblastic basement membrane, increased collagen in the villous stroma [7, 8], and degenerated organelles in syncytiotrophoblasts [9]. Functionally, these modifications appear to alter placental blood flow [10], and disturb progesterone production [11], estrogen metabolism [12], amino acid transport [13], as well as the activity of drug-metabolizing enzymes [14]. It is worth noting that CSE during pregnancy is also associated with increased risks of placenta-associated syndromes [15], such as preterm birth, placenta previa and placental abruption [16, 17].

Cigarette smoke is a highly complex mixture containing nearly 4500 chemical constituents, including nicotine, carbon monoxide, toxic metals, and a substantial amount of reactive oxygen species (ROS) [6, 18]. Moreover, cigarette smoke also triggers ROS formation in a variety of cells [19]. A vast array of evidence indicates that excessive ROS production, exceeding the anti-oxidative capacity of cells, may damage cell components such as DNA, protein, and lipids, and then cause cellular dysfunction [20]. Meanwhile, a number of adverse events associated with oxidative stress are observed in tissues following CSE, which include cardiac hypertrophy [21], pulmonary fibrosis [22] as well as endothelial cells injury [23]. In mammalian cells, ROS is scavenged by a host of enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and nonenzymatic antioxidants, such as glutathione (GSH) [20, 24]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that regulates the transcriptional activation of anti-oxidative genes [25]. Under physiological conditions, Nrf2 is bound to its natural inhibitor Kelch-like-ECH-associated protein 1 (Keap1) in the cytoplasm [25]. In the presence of oxidative or xenobiotic stimuli, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it binds to the antioxidant response element, and initiates the transcription of anti-oxidative genes encoding the respective proteins such as SOD, CAT, GPx and heme oxygenase-1 [26]. Deficiency of Nrf2 was shown to exacerbate cerebral infarction and neurologic deficits in animal models [27, 28]. Further, studies displayed that Nrf2 disruption made mice highly susceptible to CSE-induced emphysema [29, 30]. By contrast, activation of Nrf2 could alleviate lung oxidative stress response, alveolar destruction, alveolar cells apoptosis, and pulmonary hypertension imposed by chronic CSE in mice [31]. In addition, growing evidence suggests that Nrf2 has protective effects in various organs and tissues, including the brain [32], heart [33] and liver [34]. Nevertheless, to date, no previous studies have investigated whether Nrf2 can protect the placenta against CSE-induced oxidative damage.

Hydrogen sulfide (H_2S), which can be synthesized by cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CGL) and 3-mercaptopyruvate sulfurtransferase (3-MST) in mammal cells, has been considered as the third endogenous gaseous signaling molecule, along with nitric oxide and carbon monoxide, to regulate a variety of physiological and pathological processes in organs [35, 36]. Animal models and human studies have identified that H_2S possess potent anti-oxidative activity and other physiological functions. For instance, H_2S plays an important role in regulating the response to ischemia/reperfusion injuries in heart [37], liver [38] and brain [39]. A previous study showed that inhalation of H_2S could alleviate cotton smoke-induced lung injury by reducing the production of inducible nitric oxide synthase and nitric oxide in rats [40]. In addition, an *in vivo* study revealed that H_2S could improve cigarette smoking-induced left ventricular systolic dysfunction via inhibition of apoptosis and autophagy in rats [41]. In our previous works, we found that H_2S could alleviate medullary respiratory centers injury induced by *in utero* CSE in neonatal rats [42],

and that H₂S could also protect against acute hypoxia-induced medullary respiratory centers impairment via anti-oxidant and anti-apoptotic effects in adult rats [43]. However, whether H₂S can protect the placenta against CSE-induced oxidative injury is still unclear.

Given the anti-oxidative activity of H₂S and the critical role of Nrf2 in cellular redox pathways, we aimed therefore in the present study to explore whether H₂S can protect against CSE-induced placental oxidative damage in rats, and to clarify whether the Nrf2 pathway is involved in the protection.

Materials and Methods

Animal grouping

Adult Sprague-Dawley rats (body weight: female, 240-260 g; male, 360-380 g) were obtained from Sichuan University Experimental Animal Center. The rats were housed in groups in a room with a light/dark cycle of 12 h/12 h at 22 ± 1°C and were provided with access to standard pellet diet and water ad libitum. Animals were acclimatized to the environment for 7 days prior to the experiments. Pregnancies were established by mating nulliparous female rats with fertile male rats at a ratio of 2:1 overnight. Pregnancy was confirmed by the presence of spermatozoa on the vaginal smear and the day was considered as gestational day (gd) 0. Pregnant rats were randomly divided into 4 groups: NaCl, NaHS (donor of H₂S), CSE and CSE+NaHS. All animal experiments were carried out in strict accordance with the recommendation in the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 8023) revised 1978, and approved by the Animal Care and Use Committee of Sichuan University.

Maternal cigarette smoke exposure

Maternal CSE was performed as previously published [42, 44]. Briefly, pregnant rats were placed in a restraining exposure box (80 cm × 60 cm × 50 cm) with cigarette smoke delivered cyclically (2 cigarette/12 min, 10 min with the box closed and the remaining 2 min with the box open, repeated five times) by lighting cigarettes (Tianxiang, 11 mg of tar and 1 mg of nicotine per cigarette, China Tobacco Chuanyu Industrial Co., China). CSE was conducted twice a day (starting at 9:00 a.m. and 16:00 p.m., respectively) during gd 7-20.

In addition to the CSE treatment as described above, the pregnant rats in CSE and CSE+NaHS groups received an equivalent volume of physiological saline and NaHS (56 μmol/kg), respectively, intraperitoneally administered at 2.5 ml/kg body weight 30 min before the first smoke exposure each day. Previous studies in our laboratory have determined the serum cotinine level by means of ELISA. Using this regimen, the serum cotinine concentration (92.3±15.7 ng/ml) [42] achieves a level of smoke exposure that simulates active smoking during pregnancy [45, 46]. The pregnant rats in NaCl and NaHS groups were exposed to air under a similar condition and were intraperitoneally injected with an equivalent volume of physiological saline and NaHS (56 μmol/kg), respectively.

On gd 21, the pregnant rats from all groups were anesthetized with 10% chloral hydrate (3 ml/kg) via intraperitoneal injection and cesarean sections were performed to harvest the placentas, and the fetuses were euthanized by decapitation after ether inhalation. Placentas from the litters with 10~12 fetuses were collected (8 litters each group). The placentas were carefully dissected from the maternal mesometrial triangle and umbilical cords, cleaned amniotic fluid as well as blood. Then, the placentas were immediately fixed in 4% paraformaldehyde for immunohistological staining, or placed in RNALater for real-time PCR, or frozen in liquid nitrogen for Western blot and biochemical analysis.

Immunohistological staining

One placenta was randomly chosen from each litter and fixed in 4% paraformaldehyde overnight at 4°C, then the placentas were bisected and oriented during the paraffin embedding procedure so that the cut face exhibited a transverse view of the placenta. Serial sections were cut at an interval of 50 μm, and total three intervals were made, then the sections (5 μm) were deparaffinized, rehydrated and rinsed in distilled water. Endogenous peroxidase activity was blocked by a 30 min incubation in 3% H₂O₂ at room temperature, then antigens were retrieved by heating in 0.01 M sodium citrate (pH 6.0) for 20 min. After three washes in phosphate buffered saline (PBS), the sections were blocked with 3% normal goat serum (Chemicon International Inc., Temecula, CA, USA) for 60 min in room temperature. The sections were

incubated at 4°C overnight with rabbit anti-8-OHdG (1:200; Proteintech, Chicago, IL, USA) and Nrf2 (1:250; Proteintech, Chicago, IL, USA), respectively, in PBS containing 1% goat serum. After washed in PBS, sections were incubated for 60 min at room temperature with goat anti-rabbit IgG peroxidase-polymer secondary antibody (Proteintech, Chicago, IL, USA). The signals were developed with acetate-imidazol buffer containing 2.5% nickel sulfate and 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) for 5 min, then the sections were counterstained with hematoxylin. Finally, the sections were dehydrated through graded ethanol series, cleared in xylene, and covered with Cytoseal (Stephens Scientific, Kalamazoo, MI, USA). For the negative reagent control, the primary antibody was omitted. The sections were processed at the same time using the same chemical reagents to avoid batch-to-batch variation during immunostaining.

To analyze the expressions of 8-OHdG and Nrf2 in placentas, the labyrinthine zones were chosen to photograph using a microscope (Olympus BX51T, Olympus Corp., Tokyo, Japan). As previous study described [47], the immunoreactivity intensity was quantified with Image Pro-Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) by an operator blind to the placental groups. Values of optical density (OD) were calculated by the equation: $\Sigma \text{integral optical density} / \Sigma \text{area}$, where integral optical density is the integral optical density in a region of interest, and area is the area of a region of interest. In this study, $\Sigma \text{integral optical density}$ is the sum of integral optical density of all cells in the photograph and Σarea is the total area of all cells in the photograph.

Lipid peroxidation evaluation

The level of MDA was evaluated using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) as previous study described [48]. Briefly, the placental tissues were homogenized independently in ice-cold saline (1:10, wt/vol), and incubated with thiobarbituric acid at 95°C for 40 min, then the reaction mixtures were cooled to room temperature and centrifuged at 3500 rpm for 10 min. The supernatants were collected and measured spectrophotometrically at 532 nm according to the manufacturer's instructions. Each measurement was performed in duplicate. Samples were normalized for differences in the amount of protein as determined by a DC protein concentration assay (Bio-Rad, Hercules, CA, USA).

Fluorescent staining

The level of ROS in placenta was detected using CellROX® Deep Red Reagent (Life Technologies, Carlsbad, CA, USA) as previous study described [49]. In brief, the frozen placentas were cut at an interval of 50 µm, and total three intervals were made, then the sections (12 µm) were washed in PBS, and incubated with CellROX® Deep Red (5 µM) in PBS for 30 min at 37°C in a humidified chamber protected from light. The placental labyrinthine zone was chosen for imaging at an excitation wavelength of 633 nm (emission range of 640-680 nm) with a fluorescence microscope (Olympus BX51TR, Olympus Corp., Tokyo, Japan). Three visual fields were randomly photographed with a 40× objective, and the camera parameters were kept constant during imaging. For quantitative analysis of the ROS, integral fluorescence density of the region of interest was measured with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) by an operator blind to the placental groups, as previous study described [47].

Total antioxidant activity assessment

The level of T-AOC was measured using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Briefly, placental tissues were homogenized independently in ice-cold saline (1:10, wt/vol), and the homogenate was fully blend and kept still for 10 min, then the supernatant was centrifuged at 1000 g at 4°C, and the pellet was discarded and the supernatant was collected and measured spectrophotometrically at 520 nm according to the manufacture's instructions.

Glutathione measurement

Total GSH and GSSG in placental homogenates were measured using a GSH/GSSG assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Reduced GSH was calculated as a difference between total GSH and GSSG, and the GSH/GSSG ratio was determined. The protein concentration for each sample was determined by a DC protein concentration assay (Bio-Rad, Hercules, CA, USA).

Antioxidant enzyme activities assay

The enzymatic activities of SOD1, SOD2, CAT and total GPx in placental homogenates were assessed, respectively, using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Briefly, the placentas were homogenized independently and centrifuged at 3500 rpm for 10 min at 4°C, the supernatants were incubated, respectively, with total SOD, SOD1, CAT and GPx reaction solutions, and then measured spectrophotometrically at 550 nm (total SOD and SOD1), 240 nm (CAT) and 340 nm (total GPx), respectively, according to the manufacture's instructions. The activity of SOD2 was calculated as a difference between total SOD and SOD1.

Quantitative real-time PCR analysis

The quantitative real-time PCR analysis was performed as previous study described [50]. Briefly, total RNA was extracted from placental tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and the RNA concentration was quantified by a spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA). Reverse transcription of an equal amount of total RNA was performed using a Thermo Scientific Revert Aid First Strand cDNA kit (Thermo Scientific, Waltham, MA, USA). The primer pairs for *Sod1*, *Sod2*, *Cat*, *GPx1* and *Nrf2* were shown in Table 1. Quantitative real-time PCR was performed with SsoFast EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 0.5 µg of cDNA. *β-actin* was used as the reference gene. Samples were run in triplicate to ensure amplification integrity. The final volume of the PCR reaction mixture was 20 µl, which consisted of 10 µl 2 × SYBR supermix, 0.5 µl 10 µM forward primer and reverse primer, 1 µl cDNA, and 8 µl Rnase/DNase-free water. The PCR cycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 5 sec. Data were collected during each cycle at the 60°C extension step. The amplification efficiency was tested in standard curves using serial cDNA dilutions. Amplification specificity was checked using melting curves. The quantitation values of target genes were normalized to the endogenous *β-actin* control gene. The relative fold changes in gene expression levels were obtained by comparing the $2^{-\Delta\Delta C_t}$ data of the different groups.

Western blot analysis

The Western blot analysis was performed as previous study described [51]. Briefly, the placental tissue was homogenized independently in RIPA buffer (Beyotime, Nanjing, Jiangsu, China) with 1% phenylmethylsulfonyl fluoride (Beyotime, Nanjing, Jiangsu, China) and protease inhibitor (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer's instructions at 4°C. After centrifugation at 12,000 rpm for 10 min, the supernatant was collected and quantified by BCA (bicinchoninic acid) Protein Assay Kit (Beyotime, Nanjing, Jiangsu, China) and stored at -80°C. An equal amount of total protein (30 mg) was loaded and separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBS-T (10 mmol/L Tris, 150 mmol/L NaCl and 0.1% Tween 20, pH7.5) for 2 h at room temperature, and then incubated with diluted primary antibodies: rabbit SOD1 (1:800), SOD2 (1:800), CAT (1:600), GPx1 (1:600), Nrf2 (1:400) and *β-actin* (1:2000) (Proteintech,

Table 1. Primers sequences for the real time quantitative PCR

Gene	NCBI accession No.	Primer sequences (5'-3')	Product size (bp)
Sod1	NM_017050.1	For: AATGTGTCCATTGAAGATCGTGTGA Rev: ATGTTTCTGACCTTTACGACCTTCG	127
Sod2	NM_017051.2	For: GACCCAAAGTCACGCTTGATA Rev: ATCCCGAGTCCAAACAGGTC	102
Cat	NM_012520.2	For: AGCCAGAAGAGAAACCCACA Rev: TGAAAGAACAAGTCGCTGGC	104
GPx1	NM_030826.4	For: ATGCCTTAGGGGTTGCTAGG Rev: AGATATAGCCCAAGCTACAGC	129
Nrf2	NM_031789.2	For: GAGACGGCCATGACTGAT Rev: AATGAGTAGCTAGGGGAGTG	120
<i>β-actin</i>	NM_031144.3	For: GGTGAAGGTCGGAGTCAACG Rev: CCAGTAGGTACTGTTGAAAC	112

Chicago, IL, USA) at 4°C overnight. On the following day, the membranes were washed in TBS-T and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature. The target protein bands were visualized with chemiluminescence luminol reagents (ECL; millipore, Billerica, MA, USA). Blots were imaged by Molecular Image®ChemiDoc™ XRS⁺ with Image Lab™ Software (Bio-Rad, Hercules, CA, USA). The tests were performed three times and quantification was analyzed by Image J 1.50 b Gel Analyzer (National Institutes of Health, Washington, DC, USA). Western blot data were normalized relative to the density of the β -actin bands.

Statistical analysis

All data were presented as mean $\pm$ standard error. Statistical analysis of all the data was done using two-way ANOVA with SPSS software (version 17.0). Statistical significance was set at $P < 0.05$.

Results

NaHS alleviates placental oxidative damage induced by CSE

To investigate the effects of CSE and H₂S on placental oxidative damage, we detected the level of 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker for DNA oxidative damage, in placentas. Results showed that the immunoreactivity of 8-OHdG was scarce and sparsely distributed in placentas of the NaCl and NaHS groups (Fig. 1A and B). By contrast, abundant 8-OHdG immunoreactivity was observed in the placentas of the CSE group (Fig. 1C), whereas the increased 8-OHdG immunoreactivity was reduced with NaHS treatment in the CSE+NaHS group (Fig. 1D). Quantitative analysis (Fig. 1E) revealed that the mean level of 8-OHdG in the CSE group was significantly higher than that in the NaCl group ($P < 0.05$), whereas the increased 8-OHdG due to CSE was decreased with NaHS treatment in the CSE+NaHS group ($P < 0.05$). There was no significant difference between NaHS and NaCl groups in terms of 8-OHdG levels ($P > 0.05$).

To test the effects of CSE and H₂S on placental oxidative damage, we also measured the content of malondialdehyde (MDA), a marker for lipid peroxidation, in placentas. As displayed in Fig. 2, The

Fig. 1. NaHS alleviates CSE-induced DNA oxidative damage in placentas. (A-D) Representative images for 8-OHdG immunostaining of placentas in the NaCl, NaHS, CSE and CSE+NaHS groups, respectively. (E) Comparisons of optical density (OD) values of 8-OHdG in the NaCl, NaHS, CSE and CSE+NaHS groups. * $P < 0.05$ vs. NaCl group; # $P < 0.05$ vs. CSE group. $n = 6$ per group.

Fig. 2. NaHS alleviates CSE-induced lipid peroxidation in placentas. Comparisons of the contents of MDA in the NaCl, NaHS, CSE and CSE+NaHS groups. * $P < 0.05$ vs. NaCl group; # $P < 0.05$ vs. CSE group. $n = 6$ per group.

MDA content was markedly increased in the CSE group when compared with the NaCl group ($P < 0.05$). With NaHS treatment, however, the MDA content was significantly decreased in the CSE+NaHS group compared with the CSE group ($P < 0.05$). In addition, the MDA contents did not vary significantly in the NaHS group compared with the NaCl group ($P > 0.05$). Taken together, these results indicate that NaHS application could alleviate placental oxidative damage induced by CSE in rats.

NaHS ameliorates placental redox imbalance caused by CSE

To investigate the effects of CSE and H_2S on redox status in placentas of our model, three sets of experiments were performed. First, the ROS was detected by using ROS Fluorescent Probe-CellROX in placentas (Fig. 3A). As shown in Fig. 3B, the mean fluorescence intensity of CellROX in the CSE group was strikingly increased compared with that in the NaCl group ($P < 0.05$), whereas the increased fluorescence intensity due to CSE was lowered with NaHS treatment in the CSE+NaHS group ($P < 0.05$); and there was no significant difference in levels of fluorescence intensity between the NaHS and NaCl groups ($P > 0.05$).

Second, we probed the levels of total antioxidant activity (T-AOC), GSH, oxidized glutathione (GSSG) and GSH/GSSG ratio in placentas. As illustrated in Fig. 4A, the level of T-AOC was significantly lower in the CSE group compared with that in the NaCl group ($P < 0.05$). With administration of NaHS, however, the T-AOC level was strikingly increased in the CSE+NaHS group compared with that in the CSE group ($P < 0.05$). There was no significant difference between the NaHS and NaCl groups in terms of T-AOC levels ($P > 0.05$). As shown in Fig. 4B-D, in the CSE group, the GSH content was decreased, the GSSG content was increased, and then the ratio of GSH/GSSG was significantly decreased, compared with the NaCl group ($P < 0.05$); in contrast to the CSE group, these effects were obviously reversed with NaHS treatment in the CSE+NaHS group ($P < 0.05$). No significant difference was found in GSH and GSSG contents as well as the GSH/GSSG ratio between the NaHS and NaCl groups ($P > 0.05$).

Third, we analyzed the anti-oxidative enzymes activities and expressions in placental homogenates. As shown in Fig. 4E-F, the activities of copper/zinc SOD (SOD1) and manganese SOD (SOD2) in the CSE group were significantly lower than those in the NaCl group ($P < 0.05$). However, SOD1 and SOD2 activities were restored by NaHS administration in the CSE+NaHS group compared to the CSE group ($P < 0.05$). There were not significant differences in activities of SOD1 and SOD2 between the NaCl and NaHS groups ($P > 0.05$). In contrast to the NaCl group, the CAT activity in the CSE group was significantly decreased ($P < 0.05$), whereas the reduced CAT activity was normalized with NaHS treatment in the CSE+NaHS group ($P < 0.05$).

Fig. 3. NaHS inhibits ROS production induced by CSE in placentas. (A) Representative fluorescent images of placentas stained with CellROX in the NaCl, NaHS, CSE and CSE+NaHS groups, respectively. (B) Comparisons of the mean fluorescence intensity of CellROX in the NaCl, NaHS, CSE and CSE+NaHS groups. * $P < 0.05$ vs. NaCl group; # $P < 0.05$ vs. CSE group. $n = 6$ per group.

Fig. 4. NaHS alleviates placental redox imbalance caused by CSE. (A) Comparisons of the levels of T-AOC in the NaCl, NaHS, CSE and CSE+NaHS groups. (B-D) Comparisons of the contents of GSH, GSSG and GSH/GSSG ratio in placental homogenates from the NaCl, NaHS, CSE and CSE+NaHS groups, respectively. (E-H) Comparisons of the activities of SOD1, SOD2, CAT and total GPx in placental homogenates from the NaCl, NaHS, CSE and CSE+NaHS groups. (I-L) Comparisons of the mRNA expression levels of *Sod1*, *Sod2*, *Cat* and *GPx1* in placentas from the NaCl, NaHS, CSE and CSE+NaHS groups. (M-P) Representative immunoblots (upper) and densitometric analysis (lower), respectively, of SOD1, SOD2, CAT and GPx1 protein expressions in the NaCl, NaHS, CSE and CSE+NaHS groups, respectively. * $P < 0.05$ vs. NaCl group; # $P < 0.05$ vs. CSE group. $n = 6$ per group.

Meanwhile, CAT activities did not differ significantly between the NaCl and NaHS groups ($P > 0.05$) (Fig. 4G). Similarly, the activity of total GPx was also significantly decreased in the CSE group compared to the NaCl group ($P < 0.05$). However, the GPx activity was elevated with NaHS treatment in the CSE+NaHS group ($P < 0.05$); and there was no significant difference in GPx activity between the NaHS and NaCl groups ($P > 0.05$) (Fig. 4H). As displayed in Fig. 4I-L, the mRNA expression levels of *Sod1*, *Sod2*, *Cat* and *GPx1* were significantly decreased in the CSE group when compared with the NaCl group ($P < 0.05$). However, the decreases in mRNA expressions of the anti-oxidative genes due to CSE were significantly enhanced with NaHS administration in the CSE+NaHS group ($P < 0.05$); and none of the mRNA in the NaHS group was significantly different from that in the NaCl group ($P > 0.05$). In line with mRNA expression, the protein levels of SOD1, SOD2, CAT and GPx1 (Fig. 4M-P) were also significantly decreased in the CSE group when compared to the NaCl group ($P < 0.05$), whereas the lowered protein levels due to CSE were normalized with NaHS application in the CSE+NaHS group

($P < 0.05$). These results clearly indicate that H_2S could ameliorate placental redox status imbalance caused by CSE in rats.

NaHS augments placental Nrf2 expression suppressed by CSE

To confirm whether the anti-oxidative effects of H_2S were associated with Nrf2, a key regulator of endogenous antioxidants, we measured Nrf2 expression in placentas. As shown in Fig. 5A-D, abundant Nrf2 immunoreactivity was observed in placentas of the NaCl and NaHS groups. However, sparse immunoreactivity for Nrf2 was observed in the CSE group (Fig. 5C), which was normalized with NaHS treatment in the CSE+NaHS group (Fig. 5D). Quantitative analysis (Fig. 5E) revealed that the level of Nrf2 in the CSE group was significantly lower compared with that in the NaCl group ($P < 0.05$), whereas the decreased Nrf2 due to CSE was reversed with NaHS treatment ($P < 0.05$). There was no significant difference between the NaHS and NaCl groups in terms of Nrf2 levels ($P > 0.05$).

As shown in Fig. 5 F-G, the mRNA and protein expression levels of Nrf2 in the CSE group were also significantly decreased compared with those in the NaCl group ($P < 0.05$). However, in contrast to the CSE group, the decreases both in mRNA and protein expressions of Nrf2 were dramatically elevated with NaHS treatment in the CSE+NaHS group ($P < 0.05$). Additionally, there was no significant difference in Nrf2 expressions between the NaHS and NaCl groups ($P > 0.05$). Taken together, these results suggest that H_2S was a potent inducer for Nrf2 as well as Nrf2-regulated anti-oxidative genes expressions in response to CSE in rat placentas.

Discussion

The results presented in this study showed that H_2S donor NaHS could protect against CSE-induced placental oxidative damage in rats. Histological staining revealed that NaHS could alleviate placental DNA damage due to CSE. Meanwhile, NaHS markedly reduced CSE-induced lipid peroxidation in placentas. Further, NaHS effectively mitigated CSE-induced placental redox imbalance by suppressing ROS production, restoring T-AOC level, increasing GSH/GSSG ratio, and augmenting SOD1, SOD2, CAT and GPx activities and expressions. More notably, NaHS could also induce Nrf2 mRNA and protein expressions in CSE placentas. Thus, our findings suggest that H_2S could ameliorate CSE-induced placental damage, which might be associated with attenuation of redox imbalance via up-regulation of Nrf2 and the

Fig. 5. NaHS augments Nrf2 expression suppressed by CSE in placentas. (A-D) Representative images for Nrf2 immunostaining in placentas of the NaCl, NaHS, CSE and CSE+NaHS groups, respectively. (E) Comparisons of optical density (OD) of Nrf2 in the NaCl, NaHS, CSE and CSE+NaHS groups. (F) Comparisons of the mRNA expression levels of Nrf2 in placentas from the NaCl, NaHS, CSE and CSE+NaHS groups. (G) Representative immunoblots (upper) and densitometric analysis (lower), respectively, of Nrf2 protein expressions in the NaCl, NaHS, CSE and CSE+NaHS groups. * $P < 0.05$ vs. NaCl group; # $P < 0.05$ vs. CSE group. n=6 per group.

expression of Nrf2-mediated anti-oxidative gene targets in placentas.

Cigarette smoke contains scores of toxins which have detrimental effects on placentation, affecting both the vascular and trophoblast compartments [6]. Previously, a clinical study revealed that the levels of 8-OHdG, a reliable biomarker of oxidative DNA damage, was significantly increased in the bronchial epithelial cells of smoking patients [52]. In our investigation, with the CSE rat model, we observed that the 8-OHdG was markedly increased in the CSE rat placentas, which was in line with previous research wherein a high level of 8-OHdG was noticed in the placentas of women smokers [53]. Interestingly, in the present study, the increased 8-OHdG in the placentas of rats exposed to cigarette smoke was distinctly reduced with NaHS treatment. Consistent with our findings, studies revealed that the levels of 8-OHdG and MDA were decreased upon NaHS application in *in vivo* model of cigarette smoke-induced emphysema [54] and *in vitro* model of hypoxia/reoxygenation-induced cardiomyocytes injury [55]. Moreover, our previous studies also proved that treatment with NaHS could antagonize hypoxia-induced increase in MDA content in medulla oblongata of adult rats [43] and in medullary slices of neonatal rats [56]. The amounts of MDA often reflect the degree of lipid peroxidation in tissues and indirectly reflect the degree of cell damage. We herein measured the MDA content and found a sharp rise of MDA content in the placentas of rats exposed to cigarette smoke. However, as expected, our results showed that the accumulation of MDA due to CSE in placentas was reduced with NaHS application, which implicates a protective role of H₂S against CSE-induced placental oxidative damage.

Excess production of ROS or inadequate anti-oxidative capacity may alter the cellular redox balance resulting in a phenomenon known as oxidative stress [57]. ROS emitted by cigarette smoke and produced from the inflammatory cells and the structural cells lead to direct or indirect damage of nucleic acids, proteins, and lipids, and are involved in the pathogenesis of various diseases [54, 58, 59]. Consistent with this notion, by using the CSE rat model, we found that the ROS level in the CSE placentas was markedly increased, however, the excess ROS were noticeably cleared with NaHS application. In line with our findings, a recent study reported that chronic CSE enhanced myocardial ROS levels and resulted in left ventricular remodeling and dysfunction, whereas H₂S remarkably ameliorated these adverse events via attenuation of the oxidative stress in rats [60]. Besides the dramatic increase in ROS, we also observed a distinct decrease of GSH/GSSG ratio in the placentas of CSE rats. In mammalian cells, GSH is considered as a major antioxidant that scavenges free radicals and other reactive nitrogen species directly or indirectly through enzymatic reaction [24]. When oxidative stress occurs, GSH will become GSSG during the process of binding free radicals, and the GSH/GSSG ratio rapidly decreases [61]. Also, we found in this study that the T-AOC, an indicator of total antioxidant status reflecting the degree of oxidative damage in cells [62], was markedly decreased in CSE rat placentas, whereas the decreased T-AOC due to CSE was antagonized with NaHS administration.

In mammals, there are three distinctive SODs, SOD1, SOD2 and the extracellular SOD (SOD3) [63]. SOD1 is the major intracellular form of SOD, accounting for ~80% total SOD proteins. SOD2 is exclusively localized in the mitochondrial matrix, whereas SOD3 is the secreted form that is mainly associated with the extracellular matrix of different tissues [63]. Together, these SOD enzymes scavenge superoxide anion into molecular oxygen and hydrogen peroxide, then CAT and GPx reduce hydrogen peroxide, thus preventing production of highly toxic hydroxyl radical [64]. Growing evidence indicates that cigarette smoking or CSE significantly decrease the activities of anti-oxidative enzymes both *in vitro* and *in vivo* researches [65, 66]. In the present study, our results displayed a significant reduction in activities of SOD1, SOD2, CAT and total GPx in CSE rat placentas, however, the decreased activities of the anti-oxidative enzymes were improved with NaHS application. In line with elevation of anti-oxidative enzymes activities, we also observed that NaHS application dramatically increased anti-oxidative genes mRNA and protein expression levels in CSE rat placentas. Accumulating evidence indicates that H₂S can selectively scavenge hydroxyl radicals and peroxynitrite, and is involved in modulating anti-oxidative genes expression under oxidative stress conditions [67]. For instance, a previous study revealed

that administration of NaHS could effectively ameliorate CSE-induced pulmonary fibrosis via attenuation of oxidative stress in rats [22]. Consistent with these findings, we found in this study that the mRNA and protein expressions of anti-oxidants in the CSE rat placentas were up-regulated with NaHS treatment. Taken together, these results unequivocally demonstrate that CSE during pregnancy can give rise to placental redox imbalance, however H_2S is capable of ameliorating the redox imbalance, which might be due to its potential to reduce ROS production, increase GSH/GSSG ratio and augment anti-oxidative enzymes activities and expressions in rats.

To explore the underlying mechanism of anti-oxidative effects for H_2S , we further investigated the effects of NaHS on Nrf2 expression in rat placentas. Although emerging evidence has shown that Nrf2 is one of the major cellular defense lines against oxidative stress, and its influences on anti-oxidative genes have been widely established [68], the effects of H_2S on Nrf2 pathway have never been described in the placenta. Several studies have suggested that Nrf2 plays a protective role in pulmonary diseases incurred by cigarette smoking including acute lung injury [26], chronic obstructive pulmonary disease [69] and asthma [70]. Clinically, Nrf2 protein expression was found to be significantly decreased in both lung tissues and alveolar macrophages from smokers [71]. In animals, activation of Nrf2 has been found to attenuate airway inflammation and emphysema incurred by CSE in mice [54]. In the current study, we found that the levels of Nrf2 mRNA and protein were distinctly lowered in CSE rat placentas, however, the decrease in Nrf2 was normalized with NaHS treatment. It suggests that H_2S may, at least in part, augment Nrf2 expression that is suppressed by CSE in rats, and consequently initiate Nrf2-mediated anti-oxidative genes including *Sod1*, *Sod2*, *CAT* and *GPx1* expressions in placentas. Nevertheless, whether CSE and H_2S alter the levels of nuclear Nrf2 and some other downstream antioxidants such as heme oxygenase-1 needs to be further investigated.

In conclusion, the present study demonstrates that H_2S can protect against CSE-induced placental oxidative damage in rats, perhaps through H_2S free radical scavenging activities, and by alleviating placental redox imbalance via regulation of Nrf2 pathway. Although the molecular mechanisms of H_2S actions remain unclear, our findings provide new insight into the biological effects of H_2S and suggest a potential therapeutic approach for placental injury caused by CSE.

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Disclosure Statement

No conflict of interests exists.

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