



Hydrogen sulfide from a NaHS source attenuates dextran sulfate sodium (DSS)-induced inflammation via inhibiting nuclear factor- κ B

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Abstract: This study investigated the alleviating effects of hydrogen sulfide (H₂S), derived from sodium hydrosulfide (NaHS), on inflammation induced by dextran sulfate sodium (DSS) in both in vivo and in vitro models. We found that NaHS injection markedly decreased rectal bleeding, diarrhea, and histological injury in DSS-challenged mice. NaHS (20 μ mol/L) reversed DSS-induced inhibition in cell viability in Caco-2 cells and alleviated pro-inflammation cytokine expression in vivo and in vitro, indicating an anti-inflammatory function for H₂S. It was also found that H₂S may regulate cytokine expression by inhibiting the nuclear factor- κ B (NF- κ B) signaling pathway. In conclusion, our results demonstrated that H₂S alleviated DSS-induced inflammation in vivo and in vitro and that the signal mechanism might be associated with the NF- κ B signaling pathway.

Key words: Hydrogen sulfide (H₂S), Inflammation, Nuclear factor- κ B (NF- κ B), Dextran sulfate sodium (DSS)
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1 Introduction

Inflammatory bowel disease (IBD) patients suffer from chronic inflammation with the most common symptoms being weight loss, abdominal pain, (bloody) diarrhea, fatigue, and frequently extra-intestinal symptoms such as joint pain or skin and eye inflammation (Dubeau *et al.*, 2013; Liu *et al.*, 2014; Mileva *et al.*, 2014; Malago *et al.*, 2015; Xu *et al.*, 2016). Kaplan (2015) reported that the incidence of IBD in the world is continuing to rise, with increasing prevalence in both industrialized and developing countries. While the exact etiology of IBD remains obscure, inflammation has been identified as a factor contributing to disease progression (Hirai and Matsui,

2015; Shimshoni *et al.*, 2015).

The nuclear factor- κ B (NF- κ B) signaling pathway has been found to be involved in differentiation, immune response, proliferation, cell adhesion, angiogenesis, oxidative stress, and apoptosis (Watanabe *et al.*, 2015). Compelling evidence indicates that NF- κ B is associated with various inflammatory diseases, including ulcerative colitis and Crohn's disease (Sun and Zhang, 2007). TLR4/Myd88, an upstream signal of NF- κ B, can be activated in response to various inflammatory and infectious diseases. After activation, TLR4/Myd88 mediates the inflammatory response by activating NF- κ B (Cao *et al.*, 2014; Wang *et al.*, 2015). Inhibitors of the NF- κ B signaling pathway have been widely used to alleviate IBD (Sunil *et al.*, 2010; McCann *et al.*, 2015).

Hydrogen sulfide (H₂S) is a gaseous molecule with various physiological functions, including neuromodulation, oxidative stress, regulation of blood

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pressure and cardiac function, inflammatory response, cellular energetics and apoptosis (Kabil *et al.*, 2014). The beneficial role of H₂S in various inflammatory responses has been validated (Gemici *et al.*, 2015; Howell *et al.*, 2015; Zhang *et al.*, 2015), but there is little reference to the effects of H₂S, or its mechanisms of action, in IBD. In this study we therefore evaluated the pharmacological effects of H₂S from a sodium hydrosulfide (NaHS) source on inflammation and the NF- κ B signal in dextran sulfate sodium (DSS)-induced inflammation in both in vivo and in vitro models of IBD.

2 Materials and methods

2.1 Animal model and groups

Thirty-two male ICR mice weighing 22–24 g were used in the experiment. Mice were divided into three groups each containing 10 animals: a control group (Cont), a DSS group (DSS), and a NaHS+DSS group (NaHS). In the control group, mice were allowed free access to tap water for drinking. Mice in the other two groups were allowed free access to a 5% (0.05 g/ml) DSS solution supplied as drinking water for 7 d to induce colonic inflammation. Mice from the NaHS group received freshly prepared NaHS solution (14 μ mol/kg; Sigma-Aldrich) via intraperitoneal injection twice a day. Mice in the control and DSS groups received the same volume of sterile saline alone. The NaHS dosage was according to a previous report (Benetti *et al.*, 2013). All mice were housed in polycarbonate cages at room temperature (25 \pm 3) °C, humidity (50 \pm 5)%, and a 12-h cycle of light and dark. During the experimental period, all mice were allowed free access to laboratory strip chows.

Afterwards, each mouse was weighed to calculate the average weight gain and then sacrificed. Colonic length and weight were measured. In addition, colonic samples from each mouse were collected and immediately frozen in liquid nitrogen and stored at –70 °C for further analyses.

2.2 Clinical evaluation of DSS colitis

Rectal bleeding and diarrhea from each mouse were recorded daily. The rectal bleeding was determined using Haemocult kits (Beckman Coulter, Inc., CA, USA). The score of rectal bleeding was classified as follows: 0 for no blood (normal); 2 for positive

haemocult; and 4 for gross bleeding. The diarrhea score was classified as follows: 0 for well-formed pellets; 2 for pasty and semiformal stools; and 4 for liquid stools (Vlantis *et al.*, 2015).

2.3 Histomorphometry determination

Haematoxylin and eosin (HE) staining (Yin *et al.*, 2015b) was used for morphological evaluation after DSS treatment. Briefly, colon samples (0.5 cm) were kept in 4% neutral buffered 10% formalin, processed using routine histological methods and mounted in paraffin blocks. Then 6- μ m-thick sections were cut and stained with HE. All specimens were examined under a light microscope (Nikon, Japan).

The histological examination was performed in a blinded fashion using a scoring system previously validated and described: severity of inflammation (0–3: none, slight, moderate, severe), depth of injury (0–3: none, mucosal, mucosal and submucosal, transmural), crypt damage (0–4: none, basal 1/3 damaged, basal 2/3 damaged, only surface epithelium intact, entire crypt and epithelium lost), and percentage of the involved area (0–4: 0%, 1%–10%, 10%–25%, 25%–50%, 50%–100%). Total scores, including the individual parameters added together, could range from 0 to 14.

2.4 Serum immunoglobulins

Orbital blood was collected and centrifuged at 3000 r/min for 10 min after 4 h clotting at 4 °C. Serum was separated and stored for further analyses. Assay kits for the analysis of serum immunoglobulins were obtained from Nanjing Jiancheng (China). Serum immunoglobulin A (IgA), IgG, and IgM were determined using an Automatic Biochemistry Radiometer system (Au640, Olympus).

2.5 Cell culture and treatment

Human colorectal adenocarcinoma-derived intestinal epithelial cells (Caco-2) (ATCC, Manassas, VA, USA) were grown in DMEM/F12 supplemented with 1 mmol/L sodium pyruvate, 20% fetal bovine serum, and 50 U/ml penicillin-streptomycin. Cells were treated with 2% (0.02 g/ml) DSS for 4 d to induce inflammation (Nighot *et al.*, 2013). Cell viability was determined by the CKK-8 assay (Sigma-Aldrich, MO, USA) according to the manufacturer's instructions. Briefly, 8 \times 10³ cells were seeded in 96-well plates. In the following day, cells were incubated

with 1, 5, 10, 20, 50, and 100 $\mu\text{mol/L}$ NaHS for 2 d and then assayed.

2.6 NF- κ B activity

Cellular NF- κ B activity after DSS and NaHS treatment was measured via an ELISA kit (Cell Biolabs, USA).

2.7 Complementary DNA (cDNA) synthesis and quantification of mRNA by real-time PCR analysis

RNA was isolated from colon and cell tissues with TRIZOL reagent according to the manufacturer's instructions. Synthesis of the first strand (cDNA) was conducted using oligo (dT) 20 and Superscript II reverse transcriptase (Invitrogen, USA).

Primers were designed with Primer 5.0 according to the gene sequence of mouse; the sequences are shown in Table 1. Real-time PCR analysis was conducted according to previous studies (Yin *et al.*, 2013a; 2014). The relative expression of different genes was normalized and presented as a ratio to their expression in the control group.

2.8 Nuclear protein extraction and Western blot analysis

Nuclear proteins were extracted using nuclear and cytoplasmic extraction reagents in accordance

with the manufacturer's instructions (Thermo Fisher Scientific Inc., USA). Western blot was performed (Yin *et al.*, 2015a) and NF- κ Bp65 (Abcam, Inc., USA) was used as the primary antibody. Rabbit proliferating cell nuclear antigen (PCNA) antibody (Sigma) was used as the nuclear protein loading control. The expression ratio of NF- κ B was normalized against PCNA.

2.9 Statistical analysis

All statistical analyses were performed using SPSS 17.0 software. Group comparisons were performed using analysis of variance (ANOVA) and followed with Tukey's multiple comparison test.

3 Results

3.1 Effects of NaHS on clinical indices in DSS-induced colitis

DSS treatment significantly reduced final body weight, daily weight gain, and colonic length, and increased colonic weight, rectal bleeding score, and diarrhea score ($P < 0.05$; Fig. 1). Although NaHS administration failed to alleviate DSS-dysregulated body weight, colonic length, and colonic weight ($P > 0.05$), it markedly decreased scores for rectal bleeding and diarrhea ($P < 0.05$). HE staining results revealed that DSS caused colonic histological injury which was mitigated by NaHS ($P < 0.05$).

3.2 Effects of NaHS on inflammatory cytokines in DSS-induced colitis

Colonic interleukin-1 β (IL-1 β), IL-6, IL-10, IL-17, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α) mRNA were measured by reverse transcription (RT)-PCR to evaluate the inflammatory response after DSS treatment in mice (Fig. 2). The results showed that adding 5% DSS to drinking water induced colonic inflammation in mice evidenced by the upregulation of IL-1 β , IL-6, IL-10, IL-17, and TNF- α expression ($P < 0.05$). Compared with the DSS group, NaHS administration significantly down-regulated colonic IL-1 β , IL-17, and TNF- α expression ($P < 0.05$), which indicated an anti-inflammatory function for NaHS.

Table 1 PCR primer sequences: the forward (F) primers and the reverse (R) primers

Gene	Nucleotide sequences of primers (5'-3')
<i>β-Actin</i>	F: GTCCACCTTCCAGCAGATGT R: GAAAGGGTGTAACGCAGC
<i>IL-1β</i>	F: CTGTGACTCGTGGGATGATG R: GGGATTTTGTCTGTTGCTTGT
<i>IL-6</i>	F: TGCAAGAGACTTCCATCCAGT R: GTGAAGTAGGGAAGGCCG
<i>IL-10</i>	F: ACAGCCGGGAAGACAATAAC R: CAGCTGGTCCCTTTGTTTGAAG
<i>IL-17</i>	F: TACCTCAACCGTTCCACGTC R: TTTCCCTCCGCATTGACAC
<i>IFN-γ</i>	F: ATGAACGCTACACACTGCATCTTGGCTT R: CCTCAAACCTGGCAATACTCATGAATGC
<i>TNF-α</i>	F: AGGCACTCCCCAAAAGAT R: TGAGGGTCTGGGCCATAGAA
<i>TLR4</i>	F: TTCAGAACTTCAGTGGCTGGATT R: CCATGCCTTGTCTTCAATTGTTT
<i>Myd88</i>	F: GCATGGTGGTGGTTGTTTCTG R: GAATCAGTCGCTTCTGTTGG

3.3 Effects of NaHS on serum immunoglobulins in DSS-induced colitis

As shown in the Table 2, DSS treatment significantly reduced serum IgG and IgA ($P<0.05$). Although NaHS injection tended to alleviate DSS-induced inhibition of IgG and IgA levels, the difference was insignificant ($P>0.05$).

Table 2 Serum immunoglobulins after DSS exposure

Group	IgG (g/L)	IgM (g/L)	IgA (g/L)
Cont	12.93±1.71 ^a	12.15±2.07	8.16±1.30 ^a
DSS	8.70±1.56 ^b	10.67±1.29	5.12±0.36 ^b
NaHS	9.15±2.04 ^{ab}	11.17±1.13	6.29±0.50 ^{ab}

Data are expressed as mean±standard deviation (SD) ($n=10$). Values in the same column with different superscripts are significant ($P<0.05$).

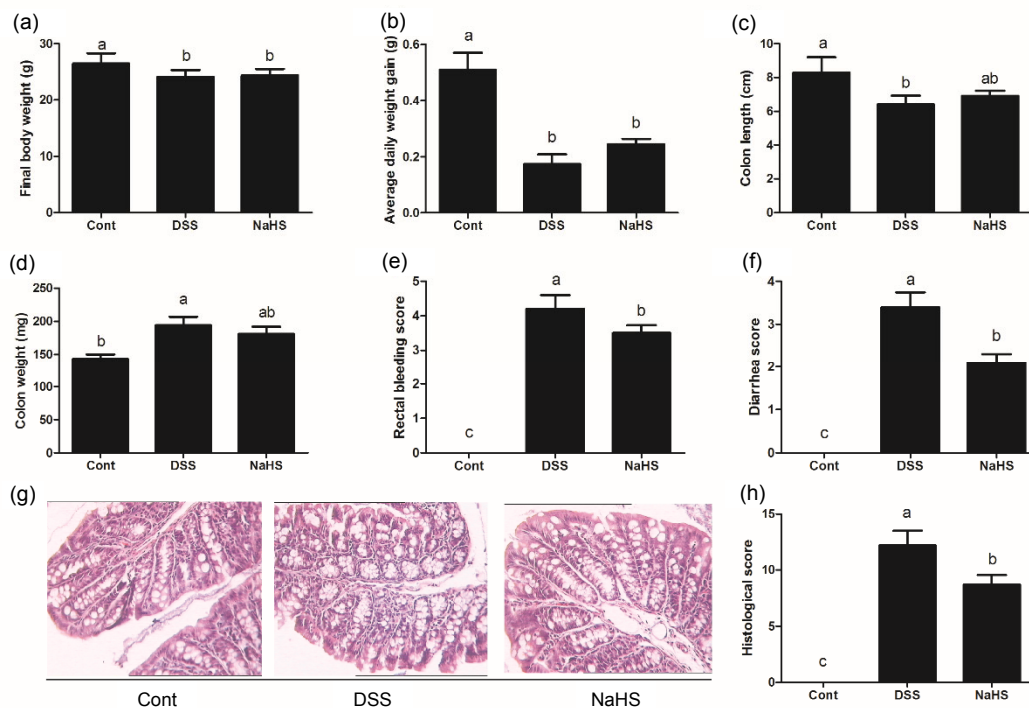


Fig. 1 Effects of NaHS on clinical parameters in DSS-induced colitis in mice

(a) Final body weight; (b) Average daily weight gain; (c) Colon length; (d) Colon weight; (e) Rectal bleeding score; (f) Diarrhea score; (g) HE staining; (h) Histological score. Data are expressed as mean±SD ($n=10$). Different letters above the columns are significant ($P<0.05$)

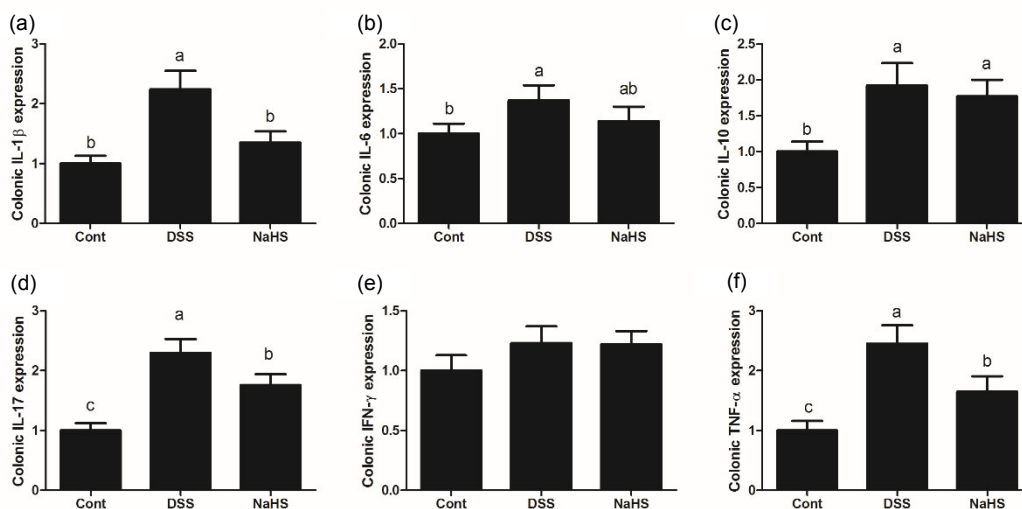


Fig. 2 Effects of NaHS on pro-inflammation cytokine expression in DSS-challenged mice

Expression of colonic IL-1 β (a), IL-6 (b), IL-10 (c), IL-17 (d), IFN- γ (e), and TNF- α (f). Data are expressed as mean±SD ($n=10$). Different letters above the columns are significant ($P<0.05$)

3.4 Effects of NaHS on inflammatory cytokines in DSS-challenged Caco-2 cells

We examined the role of NaHS in DSS-induced inflammatory response in a cell culture model. Cell viability was measured after treatment with different concentrations of NaHS (1, 5, 10, 20, 50, and 100 $\mu\text{mol/L}$). The 2% DSS inhibited cell viability ($P<0.05$; Fig. 3), whereas 20 $\mu\text{mol/L}$ NaHS markedly reversed this inhibition in Caco-2 cells ($P<0.05$). Therefore, 20 $\mu\text{mol/L}$ was used as the experimental dose for other tests.

DSS significantly enhanced IL-1 β , IL-6, IL-10, IL-17, and TNF- α mRNA abundances in Caco-2 cells ($P<0.05$), whereas NaHS alleviated DSS-induced inflammation by downregulating IL-1 β , IL-17, and TNF- α expression ($P<0.05$). The in vitro results further validated the anti-inflammatory effect of NaHS.

3.5 Effects of NaHS on the NF- κB signal in DSS-challenged in vivo and in vitro models

In the mouse model, DSS significantly activated the TLR4/Myd88 signal compared with the control

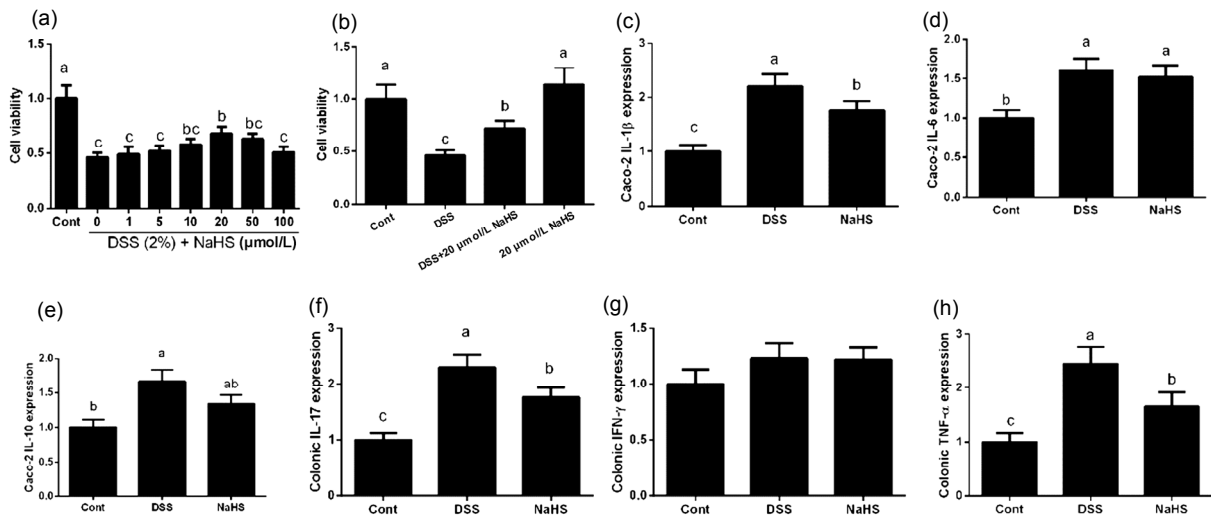


Fig. 3 Effects of NaHS on pro-inflammation cytokine expression in DSS-challenged Caco-2 cells (a, b) Cell viability; (c) IL-1 β expression; (d) IL-6 expression; (e) IL-10 expression; (f) IL-17 expression; (g) IFN- γ expression; (h) TNF- α expression. Data are expressed as mean \pm SD ($n=10$). Different letters above the columns are significant ($P<0.05$)

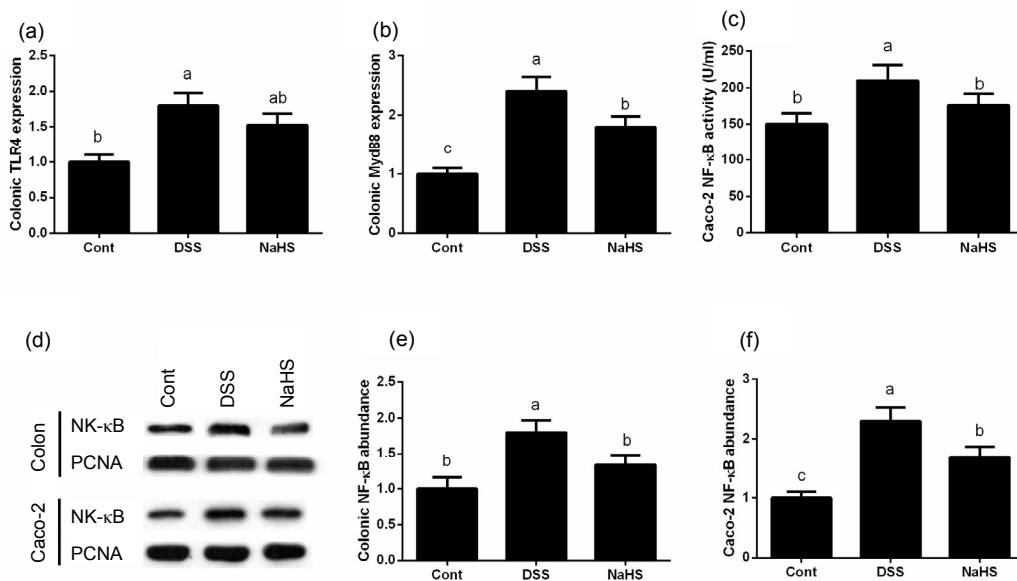


Fig. 4 Effects of NaHS on the NF- κB signal in DSS-induced inflammation in in vivo and in vitro models (a) TLR4 expression; (b) Myd88 expression; (c) NF- κB activity; (d) Western blot result; (e) NF- κB abundance in mice; (f) NF- κB abundance in Caco-2 cells. Data are expressed as mean \pm SD ($n=10$). Different letters above the columns are significant ($P<0.05$)

group ($P < 0.05$; Fig. 4). Although NaHS failed to down-regulate TLR4 expression, the Myd88 mRNA result demonstrated that DSS markedly enhanced the nuclear translocation of NF- κ B and NaHS inhibited level in the NaHS group was significantly lower than that in the DSS group ($P < 0.05$). The Western blotting result demonstrated that DSS markedly enhanced nuclear translocation of NF- κ B and NaHS inhibited NF- κ B activation ($P < 0.05$). The ELISA and Western blotting results in Caco-2 cells further revealed that NaHS alleviated DSS-induced inflammation by inhibiting the NF- κ B signal in vivo and in vitro.

4 Discussion

Accumulating evidence suggests that H₂S may serve as an important biological gasotransmitter. H₂S at physiological concentrations has been shown to protect cells in retinal neurons and act as a potential therapeutic target for retinal degeneration (Mikami *et al.*, 2011; Eastep and Chen, 2015). H₂S also regulates cellular Ca²⁺ homeostasis in microglial cells (Lee *et al.*, 2006) and protects neurons from oxidative stress injury (Kimura and Kimura, 2004). In immune and inflammatory responses, H₂S has been demonstrated to exhibit anti-inflammatory effects in various pathological situations (Bhatia, 2015; Pozsgai *et al.*, 2015). However, some reports suggest that sulfur-containing compounds, including H₂S released from the bacterial metabolism of non-absorbed sulfate, may be the injurious agents in the development of colitis, while Furne *et al.* (2000) confirmed that blocking fecal release of H₂S via bismuth subsalicylate failed to alleviate intestinal inflammation. Although other sources of H₂S have been reported in various types of inflammation, this study focused on the effect of NaHS-H₂S on the DSS-induced colonic inflammatory response in in vivo and in vitro models to estimate the beneficial function of H₂S.

We found that H₂S, using NaHS as its source, had a clinically protective effect against DSS-induced colonic injury in mice. H₂S markedly decreased rectal bleeding and diarrhea, and alleviated colonic histological injury. A previous study had shown that a marked increase in H₂S generation contributes to ulcer healing and inflammation resolution in experimental colitis (Flannigan *et al.*, 2014). Inhibition of

endogenous H₂S generation after cystathionine γ -lyase inhibitor treatment, a primary synthetase of H₂S in the gastrointestinal tract, significantly exacerbated DSS-induced colitis (Hirata *et al.*, 2011; Flannigan *et al.*, 2014). These results indicate that H₂S serves a beneficial role in DSS-induced colitis.

Compelling evidence in human and animal models has demonstrated that the generation of inflammatory cytokines and the inflammatory response in the gastrointestinal tract are involved in the progression of IBD (Beloqui *et al.*, 2013; Sánchez-Fidalgo *et al.*, 2013; Scharl *et al.*, 2013; McCann *et al.*, 2015). In this study, we found that DSS exposure significantly up-regulated IL-1 β , IL-6, IL-10, IL-17, and TNF- α expression both in vivo and in vitro, whereas NaHS treatment alleviated this dysregulation; DSS treatment also decreased serum IgG and IgA, which was not alleviated by NaHS administration in our experiments. This may be because circulating immunoglobulins play an important role in the immune response: changes in immunoglobulins have been observed during the inflammatory response, suggesting their use as a potential therapy (Novokmet *et al.*, 2014). Intransplantation-induced lung injury, NaHS injection has been demonstrated to inhibit the production of IL-1 β and improve pulmonary function (Wu *et al.*, 2013). Xu *et al.* (2015) reported that pre-treatment with NaHS ameliorates high glucose-induced inflammation in H9c2 cardiac cells, evidenced by the inhibition of IL-1 β , IL-6, and TNF- α expression. Furthermore, treatment with an H₂S donor in a rat model of non-erosive esophagitis markedly alleviated the inflammatory response and regulated serum IL-17 concentration (Zayachkivska *et al.*, 2014). Thus, we speculate that H₂S may serve as an anti-inflammatory agent in DSS-induced inflammation in vivo and in vitro.

NF- κ B has been considered to be a key pro-inflammatory transcription factor involved in the expression of various genes, including cytokines (Shori and Baba, 2015; Yin *et al.*, 2015b). Under normal conditions, NF- κ B is sequestered in the cytoplasm via its inhibitory proteins, I κ Bs (Yin *et al.*, 2013b). Various reports have revealed the relationship between I κ Bs and inflammation (Shin *et al.*, 2012). Phosphorylation of I κ Bs is associated with its degradation and NF- κ B activation (Yan and Polk, 1999). Compelling evidence suggests that NF- κ B is

activated in IBD and other inflammatory diseases (Cheon *et al.*, 2006; Vinod and Guruvayoorappan, 2014; Rashti and Koohsari, 2015). Thus, inhibition of the NF- κ B signaling pathway has been considered to be a potential target for IBD therapy. In this study, both in vivo and in vitro data showed that DSS exposure activated the NF- κ B signal and NaHS treatment significantly inhibited this activation. Similarly, Zhou *et al.* (2014) reported that H₂S exerts anti-inflammatory effects by inhibiting NF- κ B signaling in high glucose-induced inflammation (McCann *et al.*, 2015). As an upstream signal of NF- κ B, TLR4/Myd88 is also activated by DSS exposure and down-regulated by NaHS treatment, further demonstrating the anti-inflammatory effect of H₂S.

In conclusion, the present study provides in vivo and in vitro evidence that H₂S derived from NaHS ameliorates the negative effects of DSS exposure in mice and Caco-2 cells and that this beneficial role may be associated with inhibition of the NF- κ B signaling pathway.

Compliance with ethics guidelines

Xi CHEN and Xi-shuang LIU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题目: 硫化氢通过抑制 NF- κ B 信号通路对葡聚糖硫酸钠 (DSS) 诱导的炎症反应起到缓解效果

目的: 硫化氢 (H₂S) 具有抗氧化和抗炎反应的作用, 但是在 DSS 诱导的结肠炎模型中的研究鲜有报道。因此, 本文采用小鼠和人结肠上皮细胞系 Caco-2 为实验模型, 研究了 H₂S 在 DSS 诱导的炎症模型中的缓解效果。

创新点: (1) 本研究采用体内和体外模型分别对 H₂S 对 DSS 诱导的炎症缓解效果进行了验证, 结果发现 H₂S 具有抗炎作用; (2) 本研究发现 H₂S 能够抑制核转录因子 κ B (NF- κ B) 信号通路, 从而对炎症起到缓解作用。

方法: 采用 DSS 建立小鼠结肠炎模型, 腹腔注射 H₂S 供体硫化氢钠 (NaHS); 采用 DSS 诱导 Caco-2 炎症, 然后处理 H₂S 供体 NaHS。收集小鼠结肠组织和细胞, 进行反转录聚合酶链反应 (RT-PCR) 和蛋白质免疫印迹法 (Western blot) 分析炎症基因和 NF- κ B 表达情况。

结论: H₂S 对 DSS 诱导的体内和体外炎症反应具有一定的缓解作用, 其机制可能是通过影响了 NF- κ B 信号通路。

关键词: 硫化氢(H₂S); 炎症反应; 核转录因子 κ B(NF- κ B); 葡聚糖硫酸钠 (DSS)