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Intestinal epithelial injury induced by maternal separation is protected by hydrogen sulfide☆

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ABSTRACT

Purpose: Oxidative stress has been implicated in the pathogenesis of various neonatal diseases involving the intestine. Hydrogen sulfide (H_2S) has been shown to protect against oxidative stress. We hypothesized that administration of sodium hydrosulfide (NaHS), an H_2S donor, to neonatal mice can decrease the intestinal epithelial injury associated with maternal separation (MS).

Methods: C57BL/6 mice received either intraperitoneal phosphate buffered saline (PBS; n = 10) or NaHS (1 mg/kg/day; n = 10), followed by MS for 3 h daily between postnatal day P5 and P9. Control neonatal mice were untreated and were not exposed to MS (n = 10). Proximal colon was harvested and analyzed for crypt length, goblet cell number per crypt, oxidative stress and inflammation. Groups were compared using one-way ANOVA with Bonferroni post-test.

Results: Compared to controls, MS + PBS mice had shorter crypt lengths, fewer goblet cells per crypt, reduced glutathione peroxidase activity, increased expression of thiobarbituric acid reactive substances and inducible nitric oxide synthase mRNA, as well as increased *IL-6*, *TNFα* and myeloperoxidase. Administration of NaHS significantly counteracted these negative effects of MS.

Conclusions: H_2S protects the colon from the epithelial damage, oxidative stress and inflammation caused by maternal separation. This study provides insights on the pathogenesis of neonatal bowel diseases and indicates the potential for a pharmacological intervention to rescue the colonic epithelium.

Level of evidence: n/a – animal and laboratory study.

Maternal separation (MS) is a phenomenon that occurs when neonatal mammals are separated from their mothers, resulting in acute and chronic effects [1]. Animals subjected to MS not only develop anxiety [2], but also changes in colonic morphology and physiology [3–5]. In a model of early MS, we have shown that neonatal mice develop colonic epithelial disruption and increased trans-cellular permeability [6,7]. These intestinal derangements were associated with endoplasmic reticulum stress and with ensuing goblet cell impairment [8].

Premature human infants are often subjected to MS as they are nursed in incubators away from their mothers, but so far the impact of separation in the neonatal period has not been investigated. In fact, this could contribute to the development of neonatal conditions such as retinopathy of prematurity, chronic lung disease, and necrotizing enterocolitis (NEC). Oxidative stress and production of reactive oxygen species (ROS) are involved in the pathogenesis of these diseases [9–12]. There is experimental evidence that ROS production is involved in NEC, resulting in disruption of the intestinal epithelial barrier, enterocyte apoptosis, and tight and gap junction disruption, thus altering gut permeability [13].

Recently, we have shown that hydrogen sulfide (H_2S) decreases intestinal epithelial cell injury by reducing oxidative stress and inflammatory cytokines *in vitro* [14]. H_2S is an endogenously produced gaseous mediator that has anti-inflammatory and cytoprotective functions, and contributes to mucosal defense against infection in the gastrointestinal tract [15–19].

Herein, we hypothesize that oxidative stress is involved in the intestinal injury induced by early MS, and that administration of H_2S decreases this injury in our MS mouse model.

1. Methods

1.1. Animals

Following ethical approval (IACUC# 32,238), neonatal C57BL/6 mice were randomly assigned to receive an intraperitoneal injection of

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- analysis and interpretation of data: BL; ZM
- drafting of manuscript: BL; CL; AH
- critical revision of manuscript: AZ; AP

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phosphate buffered saline (PBS, n = 10) or NaHS, an H_2S donor (10 mg/kg/day, n = 10). MS was performed for 3 h daily between postnatal day five (P5) and nine (P9) as described [6]. Since mouse pups subjected to intraperitoneal injections of PBS did not develop signs of bowel injury, we used neonatal mice not receiving intraperitoneal injection or subjected to MS as control (n = 10). On P9, the mice were sacrificed.

1.2. Intestinal morphology evaluation

At sacrifice, proximal colon specimens were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for 5 μm thickness. The slides were stained with hematoxylin and eosin (H&E) for crypt length analysis and with alcian blue (ScyTek, Logan, UT, USA) for goblet cell as previously reported [8]. Image J analysis software (v1.45 s; NIH, Bethesda, MD, USA) was used to measure crypt length and the number of goblet cells per crypt was scored by three blinded independent investigators (BL, CL, AZ).

1.3. Oxidative stress

To investigate whether MS induced oxidative stress, we measured glutathione peroxidase (GPx) activity, thiobarbituric acid reactive substances (TBARS) as previously reported [8], and inducible nitric oxide synthase (iNOS).

iNOS RNA expression was measured by real-time PCR. RNA was isolated from tissues by TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Total RNA (1 μg) was reverse transcribed by qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD, USA) and SYBR green-based qRT–PCR was performed with advanced qPCR supermix (Wisent Inc., Quebec, Canada), with primer sequences for iNOS, forward CTGCTGGT GGTGACAAGCACATTT and reverse ATGTCATGAGCAAAGGCGCAGAA C.

1.4. Gut inflammation

To investigate gut inflammation in the colon, we measured interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), and myeloperoxidase (MPO), a marker of neutrophil infiltration.

1. Inflammatory cytokines were measured by real-time PCR as described above, with primer sequences reported previously [8].
2. Protein was isolated from colonic tissue by homogenization in tissue extraction buffer (Invitrogen, *CA*, USA), containing Protease Inhibitor Single-Use Cocktail (Sigma Aldrich, St. Louis, MO, USA). Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific, IL, USA).
   MPO: MPO concentration was determined using a Colorimetric Activity Assay Kit (Sigma Aldrich, St. Louis, MO, USA), measured with a micro-plate reader at an optical density of 412 nm (Molecular Devices SpectraMax Gemini EM, Sunnyvale, CA, USA). Concentrations were determined using a standard curve and expressed as nmol/mg protein.
   Western Blots: Proteins were separated by NuPAGE 4–12% Bis Tris gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane using iBlot Gel Transfer Device (Life Technologies, MD, USA). The membrane was probed with antibodies for TNFα (Cell Signaling Technology, Danvers, MA) and HRP-conjugated secondary antibodies. Immuno-positive bands were detected using an ECL Plus kit (Invitrogen, CA, USA) according to the manufacturer's instructions. Band intensities were determined using an Odyssey scanner (LI-COR Biosciences, Lincoln, USA).

1.5. Statistics

Data were compared using one-way ANOVA with Bonferroni post-test. As results were normally distributed (Kolmogorov–Smirnov test), they are presented as mean ± SD. $p < 0.05$ was considered significant.

2. Results

2.1. Morphology

Colonic crypt length was decreased in MS + PBS group compared to control (control = 122.20 ± 14.29 μm, MS + PBS = 73.49 ± 8.89 μm, $p < 0.001$), but was improved by the administration of NaHS (MS + NaHS = 96.77 ± 14.15 μm, $p < 0.05$ to MS + PBS, Fig. 1A).

The number of goblet cells per crypt was significantly reduced in MS + PBS group (6.17 ± 1.17) compared to control (13.17 ± 2.13, $p < 0.001$, Fig. 1B). However, administration of NaHS increased the number of goblet cells per crypt (8.53 ± 1.88) compared to MS + PBS ($p < 0.05$, Fig. 1B).

2.2. Oxidative stress

GPx activity was significantly decreased in MS + PBS group in comparison to control (control = 175.6 ± 89.20 units/mg protein, MS + PBS = 62.22 ± 18.33 units/mg protein, $p < 0.05$, Fig. 2A), but administration of NaHS restored GPx activity back to control levels (MS + NaHS = 184.7 ± 107.4 units/mg protein, $p < 0.05$ to MS + PBS, p = n.s. to control, Fig. 2A).

TBARS levels were upregulated in MS + PBS group (3.44 ± 1.35 units/mg protein) compared to control (1.14 ± 0.42 units/mg protein, $p < 0.001$, Fig. 2B), but decreased after NaHS treatment (MS + NaHS = 1.78 ± 0.51 units/mg protein, $p < 0.01$ 05 to MS + PBS, p = n.s. to control, Fig. 2B).

Similarly, compared to control, MS + PBS mice had higher relative *iNOS* mRNA expression (control = 1.0 ± 0.44, MS + PBS = 8.96 ± 8.19, $p < 0.01$, Fig. 2C); however, NaHS was associated with reduced *iNOS* mRNA expression (MS + NaHS = 0.28 ± 0.06, $p < 0.001$, Fig. 2C).

2.3. Gut inflammation

IL-6 mRNA expression levels were increased by MS (MS + PBS = 4.40 ± 1.88) in comparison to control (1.0 ± 0.43, $p < 0.001$), but were reduced to normal levels by the administration of NaHS (0.65 ± 0.15, $p < 0.001$ to MS + PBS, p = n.s. to control, Fig. 3A).

Similarly, relative mRNA levels of *TNFα* were significantly increased because of MS (control = 1.0 ± 0.42, MS + PBS = 3.49 ± 2.23, $p < 0.001$), but were significantly lowered when NaHS was given in the MS group (MS + NaHS = 0.63 ± 0.20, $p < 0.001$ to MS + PBS, p = n.s. to control, Fig. 3B). We confirmed these mRNA changes by TNFα protein expression (Fig. 3D).

Finally, to further validate our finding, we have studied the MPO expression, which has previously been shown to be a reliable indicator of inflammation [20]. Protein expression of MPO was significantly increased after MS (control = 186.2 ± 19.73 nmol/mg protein, MS + PBS = 537.4 ± 83.36 nmol/mg protein, $p < 0.001$, Fig. 3C), and was decreased following treatment with NaHS (MS + NaHS = 368.4 ± 41.02 nmol/mg protein, $p < 0.001$ to MS + PBS, p = n.s. to control).

3. Discussion

This study has shown for the first time that oxidative stress is involved in the colonic injury induced by maternal separation in neonatal mice. Moreover, we have demonstrated that administration of H_2S improves intestinal morphology, reduces oxidative stress and decreases mucosal inflammation in mice exposed to maternal separation during the first few days after birth.