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Protective effects of hydrogen-rich saline on necrotizing enterocolitis in neonatal rats☆,☆☆

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Abstract

Purpose: The aim of this study was to test the hypothesis that hydrogen-rich saline (HRS) might have protective effects on the development of necrotizing enterocolitis (NEC) in a neonatal rat model.

Methods: NEC was induced in male newborn Sprague–Dawley rats by formula feeding, exposure to asphyxia and cold stress. Sixty-four rat pups were divided randomly into four groups: C+NS (n=11), C+H_2 (n=11), NEC+NS (n=20), and NEC+H_2 (n=22). Rats in the former two groups were mother-fed. Pups received intra-peritoneal injection of HRS (10 ml/kg, 10 min before asphyxia stress twice a day) or the same dose of normal saline. Rats were monitored until 96 h after birth. Body weight, histological NEC score, survival time, malondialdehyde, antioxidant capacity, inflammatory mediators, and mucosal integrity were assessed.

Results: HRS treatment maintained the body weight, reduced the incidence of NEC from 85% (17/20) to 54.5% (12/22), increased the survival rate from 25% (5/20) to 68.2% (15/22), and attenuated the severity of NEC. In addition, HRS inhibited the mRNA expression of pro-inflammatory mediators (inducible nitric oxide synthase, tumor necrosis factor-α, and interleukin-6), down-regulated lipid peroxidation, enhanced total antioxidant capacity, and prevented the increase of diamine oxidase in serum. However, no significant influence of HRS on the interleukin-10 mRNA expression was observed.

Conclusions: HRS showed beneficial effects on neonatal rats with NEC via decreasing oxidative stress, increasing antioxidant capacity, suppressing inflammation, and preserving mucosal integrity.

Necrotizing enterocolitis remains an enigma despite recent advances in etiology and pathogenesis [1–3]. A proposed mechanism of intestinal injury in NEC involves over-production of reactive oxygen species (ROS) and reduction of antioxidant defense system [4]. Therefore, many researchers focused on using enzymatic or non-enzymatic antioxidants to treat NEC. For example, melatonin and N-acetylcysteine therapy significantly reduced the severity of intestinal damage in NEC [5,6]. Recently, it has been reported that hydrogen exerted anti-oxidative, anti-inflammatory and anti-apoptotic effects by selectively reducing hydroxyl radicals and peroxynitrites, and showed

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protective effects on graft injury after small intestinal transplantation [7–10].

Here, we hypothesized that hydrogen-rich saline could ameliorate intestinal damage in NEC rats. We used the well-established experimental neonatal rat model to verify the hypothesis, and found that hydrogen treatment could decrease the incidence and severity of NEC, reduce the oxidative stress injury and inflammatory mediators, and preserve the gut barrier integrity.

1. Materials and methods

1.1. Experimental design and animal model

All experimental protocols included in this study were approved by the Animal Care Committee of the Children's Hospital of Shanghai and performed according to the Guide for the Care and Use of Laboratory Animals (8th edition, National Institutes of Health, USA).

Newborn male Sprague–Dawley (SD) rats were obtained from the Experimental Animal Center of Xinhua Hospital, and reared in a neonatal incubator to control body temperature (pups were kept at 30 °C and 60% relative humidity). We used the neonatal rat NEC model described by Caplan et al. [11]. Rat pups were hand-fed trans-orally with artificial formula using a 24 G catheter (Togo Medikit, Miyazaki, Japan). The formula consisted of 15 g Similac (Abbott Laboratories, USA) in 75 ml canine milk replacement, and provided calories 840 kJ/kg per day. Feeding were started at 0.1 ml every 3 h and advanced slowly to 0.3–0.4 ml every 4 h as tolerated. Neonatal rats were subjected to asphyxia stress (60 s exposure to 100% nitrogen) followed by cold exposure (4 °C for 10 min) twice a day. Body weights were recorded daily. Routine care included daily cleaning and stimulation of bowel and bladder function using a cotton swab.

Sixty-four neonatal rats, originating from 6 different litters, were divided randomly into 4 experimental groups. Group 1 (C+NS, n=11) rats were mother-fed and treated with intraperitoneal injection of normal saline 10 min before asphyxia stress twice a day (10 ml/kg). Rats in group 2 (C+H_2, n=11) were also mother-fed, but hydrogen-rich saline instead of normal saline was administrated (10 ml/kg). Group 3 (NEC+NS, n=20) consisted of neonates that were hand-fed, stressed with asphyxia and hypothermia, and treated with normal saline via peritoneal injection (10 ml/kg). Newborn rats in group 4 (NEC+H_2, n=22) were treated in a similar fashion to those in Group 3, however, they were injected hydrogen-rich saline instead (10 ml/kg). Animals were sacrificed via decapitation at the time of illness (abdominal distention, discolored abdominal walls or bloody stools) or 96 h after birth.

1.2. Preparation of hydrogen-rich saline

HRS was provided by Second Military Medical University (Shanghai, China) and the detailed information for the preparation was described previously [12]. Hydrogen was dissolved in normal saline for 6 h under high pressure (0.4 MPa) to a supersaturated level. The saturated hydrogen saline was sterilized by gamma radiation and stored under atmospheric pressure at 4 °C. It was freshly produced every week to maintain a constant concentration of more than 0.6 mM.

1.3. NEC evaluation

After the killing, the gastrointestinal tract was removed and a macroscopic assessment of the gut for typical signs of NEC was performed. A 2 cm section of terminal ileum was cut, fixed in 10% formalin for 24 h, paraffin-embedded, microtome sectioned at 3 μm (Leica RM 2235, Germany), and stained with hematoxylin and eosin (H&E) for histological evaluation of NEC (light microscope, OLYMPUS BX51). The rest of the ileum was excised, washed with cold normal saline (4 °C), and stored in liquid nitrogen for protein and RNA analysis. Histopathological changes in the ileum were evaluated blindly by an experienced pathologist (X. Wang) and graded as follows: grade 0, normal; grade 1, focal mild injury confined to villous tips; grade 2, partial loss of villi; grade 3, necrosis extending to submucosa; grade 4, transmural necrosis. Tissues with scores 2 or more were assessed as NEC positive [11] (Fig. 1).

1.4. Immunohistochemical (IHC) evaluation for inducible nitric oxide synthase (iNOS)

IHC staining was performed on the fully automated IHC system (Bond-Max, Leica Microsystems, Germany) with Bond Polymer Refine Detection Kit (Catalog No: DS 9800, Leica Biosystems Newcastle Ltd, UK). In brief, the kit works as follows: First, microtome sections were deparaffinized and rehydrated. Then, sections underwent heat-induced epitope retrieval (Bond Epitope Retrieval Solution 1, Catalog No: AR 9961, Leica Biosystems Newcastle Ltd, UK), and were incubated with 3% (v/v) hydrogen peroxide to quench endogenous peroxidase activity. Tissues were stained with diluted primary antibody of iNOS (1:200, Catalog No: sc-8310, Santa Cruz Biotechnology, USA). Post Primary IgG linker reagent localized mouse antibodies. Polymeric horseradish peroxidase (HRP) IgG reagent localized rabbit antibodies. The substrate chromogen, 3,3′-diaminobenzidine tetrahydrochloride (DAB), visualized the complex via a brown precipitate. Hematoxylin (blue) counterstaining allowed the visualization of cell nuclei. For control staining, normal rabbit IgG was used as the primary antibody instead of anti-iNOS antibody. No staining was detected in control slides. Sections were examined by a blinded evaluator (X. Wang).

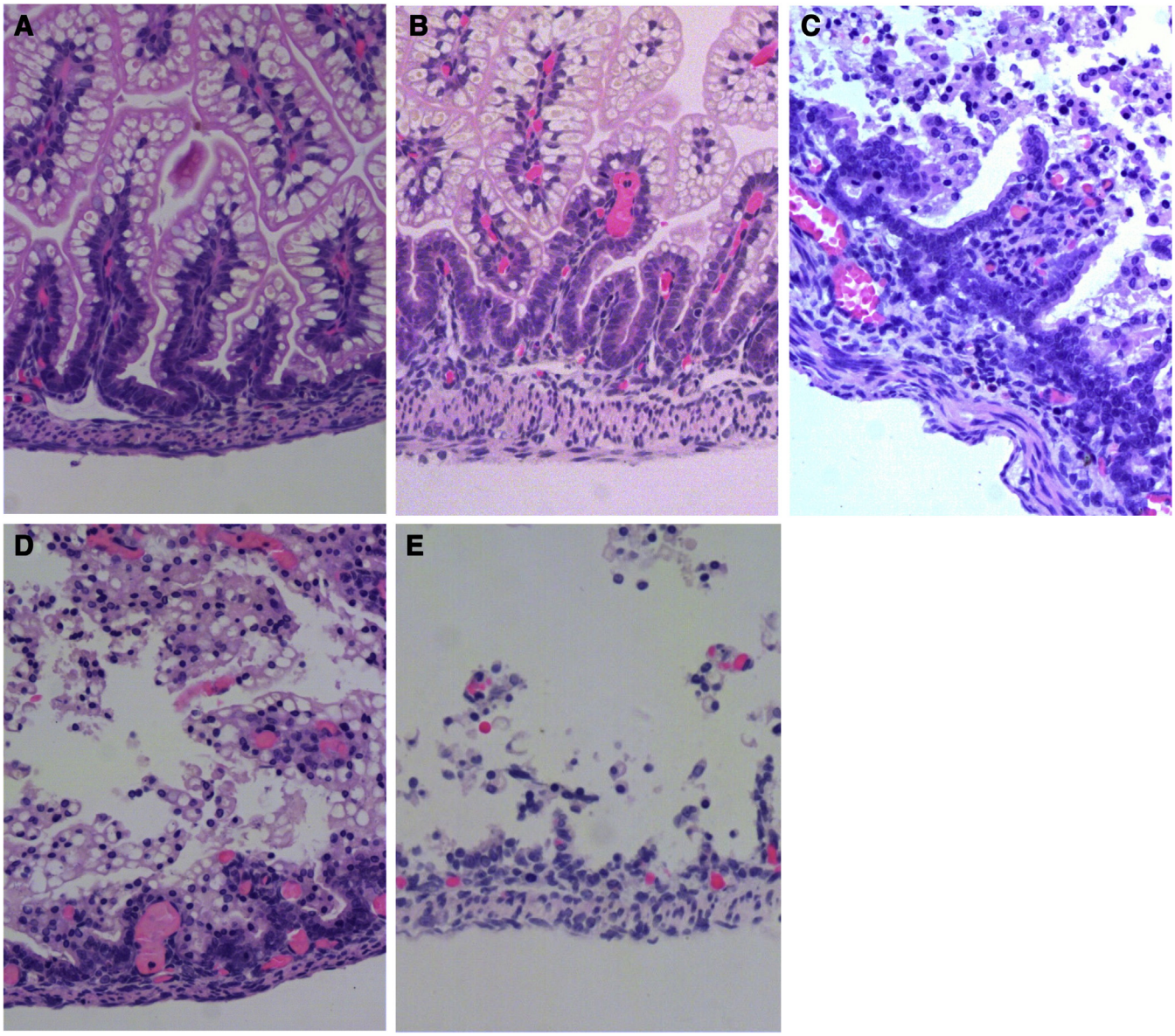

Fig. 1 Histological changes of distant ileum in neonatal rats were graded as described in the methods section. In brief, grade 0 (A), normal ileum; grade 1 (B), mild injury in villous tips; grade 2 (C), partial loss of villi; grade 3 (D), severe damage to submucosa; grade 4 (E), full necrosis. Rats were considered as NEC positive with score ≥ 2. Original magnification: 200×.

1.5. Real-time RT-PCR assay

Total RNA was extracted from terminal ileum according to the instructions described in the manufacturer's protocol (TRIzol Reagent, Catalog No: 15596-026, Life Technologies, USA). The purity and concentration of RNA were determined by Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). mRNA levels of beta-actin, interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), iNOS, and IL-10 were detected in triplicate using SYBR Green, two-step RT-PCR (PrimeScript RT Master Mix, Catalog No: DRR036A. SYBR Premix Ex Taq [Tli RNaseH Plus], Catalog No: DRR420A. Takara Biotechnology, Dalian, China). cDNA was synthesized from 500 ng of total RNA. Primer sequences were listed in Table 1. The fold changes in related cytokines expression were calculated by the comparative C_T method also referred to as the $2^{-\Delta C}{}_T$ method [13].

1.6. Measurement of ileum oxidative injury and total antioxidant capacity

Ileum tissues were prepared by homogenization on ice in RIPA buffer (25 mM Tris•HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS. Catalog No: 89900, Thermo Scientific, USA). Then, samples were centrifuged at 13,000 rpm for 10 min at 4 °C to remove tissue debris.

Table 1 Sequences of oligonucleotide primers.

Target gene	Primer sequences (5′ to 3′)	Product length
beta-actin (β-actin)	(F) GCGTCCACCCGCGAGTACAA (R) CGACGACGAGCGCAGCGATA	100 bp
Inducible nitric oxide synthase (iNOS)	(F) GCCCGCTTTGCCACGGAAGA (R) AGGCAGCAGGCACACGCAAT	145 bp
Tumor necrosis factor-alpha (TNF-α)	(F) TGGGTCCAACTCCGGGCTCA (R) TGGAATCCTTGCCGGTGGCG	117 bp
Interleukin-6 (IL-6)	(F) GTCTCGAGCCCACCAGGAACG (R) AGGGAAGGCAGTGGCTGTCAAC	132 bp
Interleukin-10 (IL-10)	(F) GCAAGGCAGTGGAGCAGGTGA (R) TGCAGTCCAGTAGATGCCGGGT	156 bp

The protein concentration was determined by the bicinchoninic acid (BCA) method using a commercially available BCA protein assay kit (Catalog No: 23225, Pierce Biotechnology, USA). Intestinal malondialdehyde (MDA) content, a marker of lipid peroxidation, was measured to evaluate the severity of gut oxidative injury by the thiobarbituric acid (TBA) colorimetric method [14]. Procedures were performed as described in the MDA assay kit (Catalog No: s0131, Beyotime Institute of Biotechnology, Jiangsu, China). The concentration of MDA was calculated from the standard curve and expressed as μmol/g protein. Tissue samples were also analyzed for reductive capacity by the ferric reducing ability of plasma (FRAP) method [15] using a total antioxidant capacity assay kit (Catalog No: s0116, Beyotime Institute of Biotechnology, Jiangsu, China). Results were expressed as mmol/g protein.

1.7. Serum diamine oxidase (DAO) assay

Blood samples from the pups were obtained via cardiac puncture, clotted for 30 min before centrifugation at 3000 rpm for 10 min. Rat DAO level, as a marker of the integrity of the intestinal mucosa [16], was determined by a rat DAO enzyme-linked immunosorbent assay (ELISA) kit (Catalog No: CK-E 30013R, R&D systems, USA). The Stop Solution changes the color from blue to yellow and the intensity of color is measured at 450 nm using a microplate reader (Synergy H1 Hybrid Multi-Mode, Biotek Instruments, USA). The DAO concentration in the samples was then measured by comparing the Optical Density of the samples to the standard curve. Results were expressed as U/l.

1.8. Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software package. The incidence of NEC was compared using χ^2 test. Survival time was analyzed by Kaplan–Meier analysis (with log-rank test). The severity of NEC was analyzed using Mann–Whitney U test. Other results were expressed as mean ± standard deviation (SD). The differences among four groups were detected by one-way analysis of variance (ANOVA). Between groups, variance was determined using Students–Newman–Keuls (SNK) post hoc test. $P < 0.05$ was considered statistically significant.

2. Results

2.1. Effects of hydrogen-rich saline on body weight gain and survival rate

The body weight changes in four experimental groups were depicted in Table 2. Mother-fed pups in group 1 and group 2 (C+NS and C+H_2) showed steady increases in body weight during the study period. Body weights in group 3 (NEC+NS) gradually decreased throughout the experiment. But neonatal rats in group 4 (NEC+H_2) could maintain the body weight during the same period. Differences in body weight between NEC+NS and NEC+H_2 groups became statistically significant at Day 4 ($P = 0.011$). There were no statistically significant differences between C+NS and C+H_2 groups.

Table 2 Body weight change during the experiment.

Group	D1	D2	D3	D4
C+NS	6.24±0.50	7.15±0.68	8.25±0.73	9.57±0.69
C+H_2	6.15±0.45	6.92±0.54	7.93±0.74	9.20±0.38
NEC+NS	6.49±0.36	6.56±0.37 *	6.47±0.40 *	6.17±0.45 *
NEC+H_2	6.45±0.31	6.55±0.33 *	6.40±0.36 *	6.60±0.34 *, **

Data are presented as mean±SD.
* Statistical significance with $P < 0.05$ (NEC+NS and NEC+H_2 vs. C+NS or C+H_2).
** Statistical significance with $P < 0.05$ (NEC+NS vs. NEC+H_2).

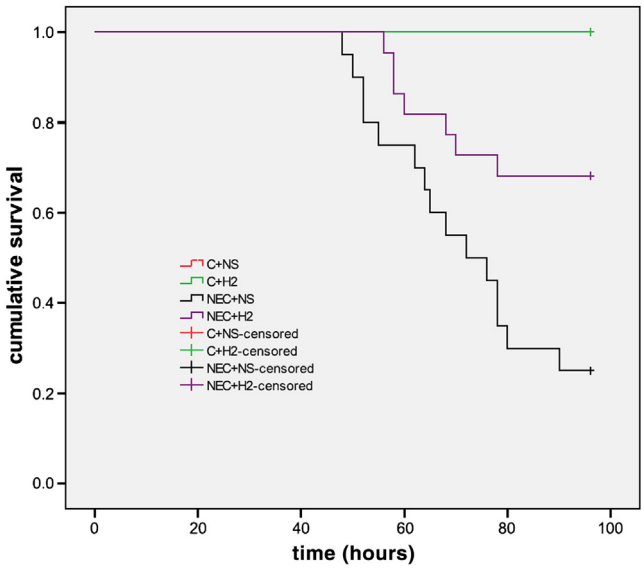

Fig. 2 Kaplan–Meier survival analysis.

Newborn rats developed signs of NEC between 48 h and 96 h after the beginning of the experiment. Administration of hydrogen-rich saline increased the survival rate (96 h) from 25% (5/20) in the NEC+NS group to 68.2% (15/22) in the NEC+H_2 group (P=0.007, log-rank test, Fig. 2 and Table 3). The mean survival time in the NEC+NS group was 73.5 h (73.5±17.3), while 85.8 h in the NEC+H_2 group (85.8±15.9). Because the censored data in the NEC+H_2 group was over 50%, we only got the median survival time in the NEC+NS group (72 h).

2.2. Effects of hydrogen-rich saline on the incidence and severity of NEC in neonatal rats

Rat pups in the mother-fed groups (C+NS and C+H_2) did not develop NEC and exhibited no abnormal intestinal architecture. Mean histological scores in these two groups were 0.18 and 0.27. 85% (17/20) of rats in the NEC+NS group showed NEC changes in the ileum characterized as moderate (grade 2, n=6), severe (grade 3, n=8), and full necrosis (grade 4, n=3), whereas 54.5% (12/22) of rats in the NEC+H_2 group developed NEC (Fig. 3 and Table 3).

Table 3 Effects of hydrogen-rich saline on neonatal rats.

Group	Incidence rate of NEC	Survival rate	Histological score (mean±SD)
C+NS	0% (0/11)	100% (11/11)	0.18±0.40
C+H_2	0% (0/11)	100% (11/11)	0.27±0.47
NEC+NS	85% (17/20)	25% (5/20)	2.55±0.94
NEC+H_2	54.5% (12/22)*	68.2% (15/22)*	1.77±0.81*

* Statistical significance with P<0.05 between NEC+NS and NEC+H_2 groups.

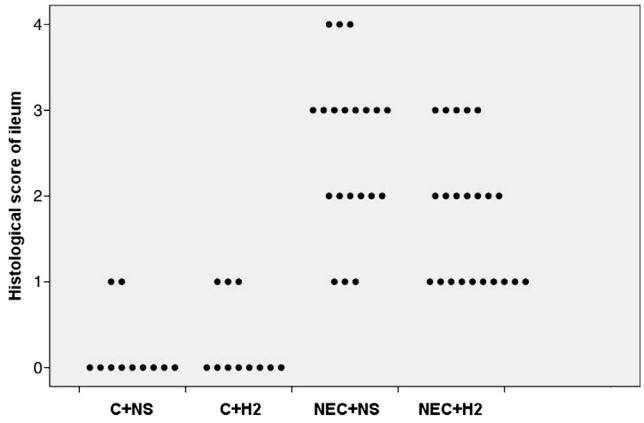

Fig. 3 Histological NEC scores of ileum in each group. Rats were considered to have NEC if the histological score was ≥2.

Hydrogen-rich saline significantly reduced the degree of intestinal injury in the NEC+H_2 group compared with that in the NEC+NS group, with a mean NEC score of 2.55 vs. 1.77 (P=0.01).

2.3. iNOS, TNF-α, IL-6 and IL-10 mRNA levels in ileum

The ileum mRNA expression of pro-inflammatory mediators such as iNOS, TNF-α, and IL-6 was significantly induced in the NEC+NS group (P<0.05, Fig. 4 and Table 4, 16-, 3-, and 8-fold increase compared with those in the C+NS group, respectively, using the $2^{-\Delta C_T}$ method). Intraperitoneal injection of hydrogen-rich saline markedly decreased the induction of those inflammatory mediators (Fig. 4 and Table 4, 4.6-, 1.7-, and 3.5-fold increase compared with those in the C+NS group, respectively, using the $2^{-\Delta C_T}$ method). Differences in mRNA expression (iNOS, TNF-α, and IL-6) between NEC+NS and NEC+H_2 groups were statistically significant (P<0.05). The mRNA of IL-10, as an anti-inflammatory cytokine, was up-regulated in the NEC+NS and NEC+H_2 group. There was no statistical difference of IL-10 mRNA expression observed between NEC+NS and NEC+H_2 rats (P=0.105). No differences of all the four gene expression were detected between two mother-fed groups.

2.4. Oxidative stress injury reduced in ileum

The ileal concentration of MDA in the rats in the NEC+NS group was found to be markedly increased compared with the two mother-fed groups (P<0.05, Fig. 4 and Table 4). Hydrogen-rich saline produced a dramatic decrease of the MDA in the ileum (P<0.05). The reductive capacity of ileum was determined by evaluation of Fe^{2+}-tripyridyltriazine (Fe^{2+}-TPTZ) reduced from Fe^{3+}-TPTZ. There was no difference between the C+NS and C+H_2 groups. Rats in the NEC+NS group showed a decrease of

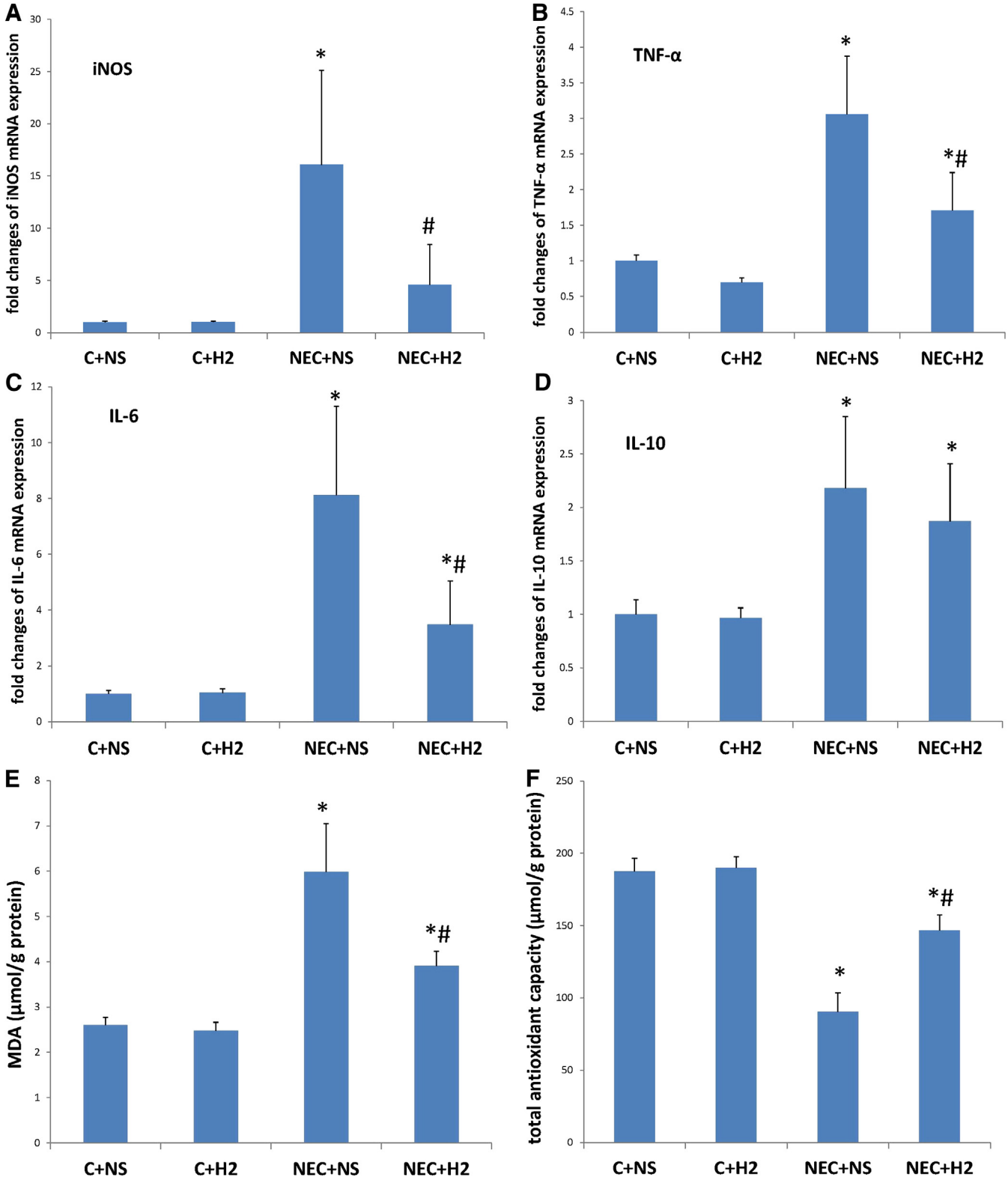

Fig. 4 Fold changes of relative expression of iNOS (A), TNF-α (B), IL-6 (C) and IL-10 (D) mRNA, MDA concentrations (E), and total antioxidant capacity (F) in the ileum of neonatal rats among four groups. * depicts statistical significance with $P<0.05$ (NEC+NS and NEC+H_2 vs. C+NS or C+H_2). # depicts statistical significance with $P<0.05$ (NEC+NS vs. NEC+H_2).

Table 4 Relative expression of cytokines and biochemical evaluations for each group.

Group	C+NS	C+H_2	NEC+NS	NEC+H_2
iNOS	1.69E-05±1.59E-06	1.73E-05±1.38E-06	2.71E-04±1.52E-04 *	7.70E-05±6.53E-05 **
TNF-α	3.96E-04±3.30E-05	2.76E-04±2.44E-05	1.21E-03±3.24E-04 *	6.78E-04±2.10E-04 *,**
IL-6	9.49E-04±1.16E-04	9.87E-04±1.35E-04	7.70E-03±3.02E-03 *	3.31E-03±1.48E-03 *,**
IL-10	8.31E-05±1.13E-05	8.04E-05±7.90E-06	1.81E-04±5.56E-05 *	1.56E-04±4.46E-05 *
MDA(μmol/g protein)	2.61±0.17	2.48±0.18	5.99±1.06 *	3.91±0.32 *,**
TAC(mmol/g protein)	187.6±8.8	189.9±7.8	90.4±13.3 *	146.7±10.8 *,**
DAO(U/L)	241.4±12.3	248.7±15.0	474.2±80.6 *	340.8±21.8 *,**

Data are presented as mean±SD.
* Statistical significance with P<0.05 (NEC+NS and NEC+H_2 vs. C+NS or C+H_2).
** Statistical significance with P<0.05 (NEC+NS vs. NEC+H_2).

the total antioxidant power in the ileum (P<0.05, Fig. 4 and Table 4). Administration of hydrogen-rich saline was able to restore the reductive capacity significantly (P<0.05).

2.5. DAO in rat serum

Fig. 5 showed high levels of serum DAO activity in the NEC+NS group compared with the C+NS or C+H_2 groups (P<0.05). However, hydrogen-rich saline treatment significantly decreased the levels of DAO in the serum (P<0.05, Fig. 5 and Table 4). There were no statistically differences between the C+NS and C+H_2 groups.

2.6. iNOS expression decreased in the epithelium of ileum

Samples from mother-fed rat pups (C+NS and C+H_2 groups) showed extremely low levels of iNOS expression

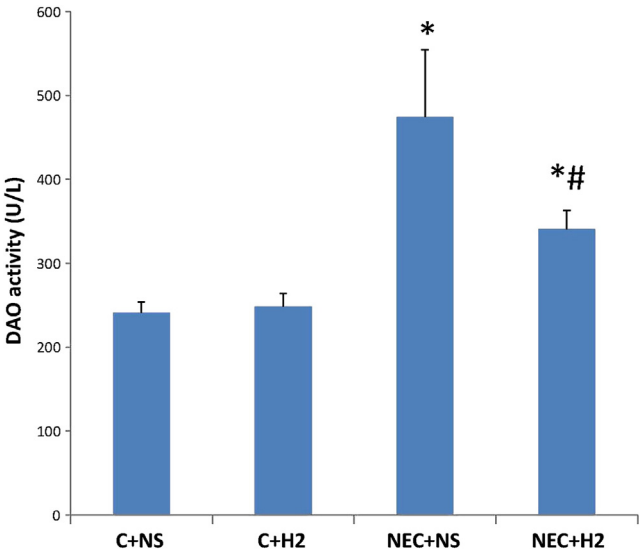

Fig. 5 DAO activity in rat serum. * depicts statistical significance with P<0.05 (NEC+NS and NEC+H_2 vs. C+NS or C+H_2). # depicts statistical significance with P<0.05 (NEC+NS vs. NEC+H_2).

(Fig. 6A), whereas rats in the NEC+NS group displayed very strong iNOS staining of enterocytes (Fig. 6D). In neonatal rats with NEC scores of 4, positive cells were abundant in the intestinal lumen (Fig. 6E). IHC revealed that iNOS protein was localized primarily to the epithelial cells in the apical and lateral membrane of villi. Hydrogen-rich saline treatment reduced the expression of iNOS compared with the NEC+NS group (Fig. 6B and C).

3. Discussion

In the current study, we found that HRS administration reduced the incidence and severity of NEC, maintained the body weight, and increased the 96 h survival rate from 25% to 68.2%. In addition, the protective effects of hydrogen on NEC were associated with decreasing oxidative stress, increasing antioxidant capacity, inhibiting pro-inflammatory mediators, and preserving gut barrier integrity.

NEC is a leading cause of morbidity and mortality among infants in the NICU. The combination of intestinal immaturity, an imbalance in circulatory regulation, abnormal bacterial colonization, hypoxic-ischemia, and immunologic overreaction of intestinal mucosa, leads to a confluence of predisposing factors [1–3,17,18]. Several lines of evidence have suggested that the excessive production of free radicals and reduction of antioxidant defense system play important roles in the development of NEC [4–6,19–21]. ROS and reactive nitrogen species (RNS) such as hydroxyl radicals and peroxynitrites are able to react with nucleic acids, lipids, and proteins, causing damage to tissues and organs.

Recently, a growing number of studies have shown that hydrogen exerted beneficial effects on small intestine, kidney and liver after transplantation or ischemia–reperfusion via antioxidant and anti-inflammatory activity [7–10,22,23]. But, to our knowledge, this is the first report on the application of HRS in a neonatal rat NEC model.

We speculate that the possible mechanisms contributing to the beneficial effects of HRS on NEC may be related to many factors. First, HRS administration was capable of reducing the lipid peroxidation and up-regulating antioxidant

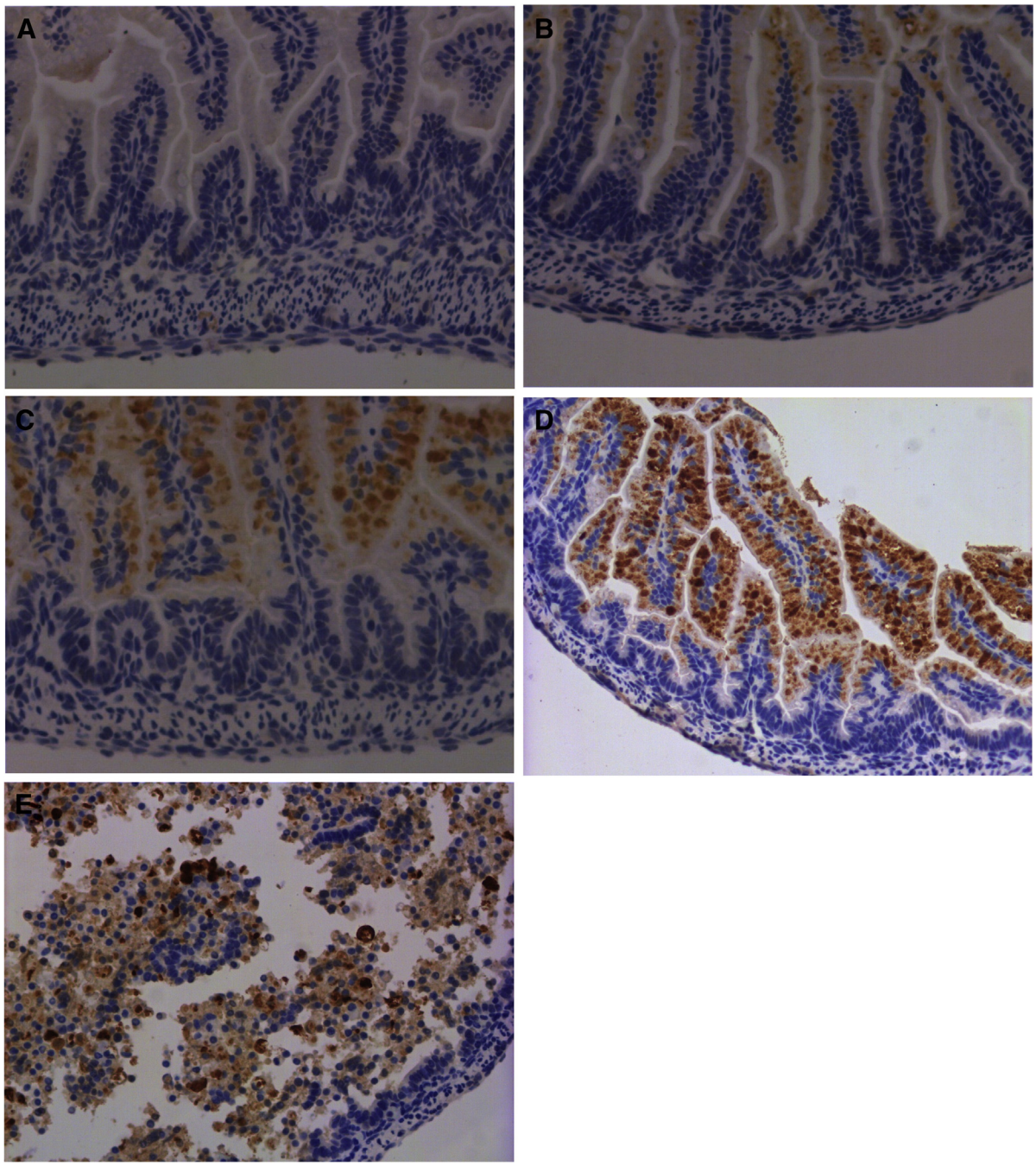

Fig. 6 Immunohistochemical staining for iNOS in representative ileum sections. Panel (A) shows extremely low levels of iNOS expression and is considered negative. In panels (B), (C) and (D), enterocytes stained positive for iNOS increased significantly. In samples (NEC score 4) where villus structure was severely destroyed, numerous iNOS positive cells could be seen in the lumen of ileum (E). iNOS cytoplasmic staining is restricted to the epithelial cells (B, C and D). Original magnification: 200×.

system. Unlike most antioxidants, which are unable to successfully target organelles, hydrogen has advantages in distribution characteristics for its capability to penetrate biomembranes and diffuse into cytosol, mitochondria, and nucleus. Hydrogen can detoxify strong ROS/RNS, including hydroxyl radicals and peroxynitrite. MDA, the product of lipid peroxidation, is widely used as an index to estimate the overall oxidative stress status [5–7,14]. Here, we observed the increase of MDA, and the decrease of total antioxidant capacity in ileum of rats in the NEC+NS group. Furthermore, we showed that HRS (10 ml/kg, intra-peritoneal injection) significantly improved the antioxidant power and reduced the MDA level. These results suggested that up-regulation of antioxidant capacity, which led to elimination of excessive free radicals, might attribute to the protection of HRS administration.

Second, HRS treatment reduced the gene expression of pro-inflammatory mediators. Overproduction of pro-inflammatory mediators (platelet activating factor, TNF-α, IL-1β, IL-6, IL-8, IL-11, etc.) has been implicated in the development of NEC [1,24–27]. Xie et al. [28] provided evidence that the increased levels of oxidative stress and high-mobility group box 1 (HMGB1) were attenuated by hydrogen treatment in murine polymicrobial sepsis model. Our findings showed that HRS alleviated inflammation by down-regulating mRNA expression of iNOS, TNF-α, and IL-6. Additionally, iNOS protein expression also decreased in the epithelium of ileum. Inflammation and oxidative stress seem to be two sides of the same coin in newborn babies both contributing to intestinal injury. The mRNA of IL-10, known as an anti-inflammatory cytokine, was also induced after NEC in the present study. However, HRS treatment did not significantly influence the IL-10 expression. In a recent study, Emani et al. [29] reported that IL-10 played a protective role in the pathogenesis of NEC by attenuating the degree of intestinal inflammation in IL-10-deficient mice. One possible explanation to this difference is that hydrogen exerted protective effects via non-IL-10-dependent manner.

Third, HRS preserved the mucosal integrity of ileum demonstrated by decreasing the serum DAO concentration. DAO is an enzyme synthesized primarily in the gastrointestinal mucosal cells. Luk et al. [16] demonstrated that levels of DAO activity could closely reflect the degree of mucosal damage during its injury, while other digestive enzymes, such as lactase, maltase, and sucrase could not provide as a sensitive marker of progressive mucosal injury. Serum levels of DAO have been used as an indicator of the integrity and/or functional mass of the intestinal mucosa. The intestinal mucosa is known to be vulnerable to insults, such as ischemia–reperfusion, induced ROS injury. Maintenance of mucosal integrity could have important systemic benefits, such as diminished bacterial translocation [30]. In current study, histological examination of the ileum confirmed that hydrogen significantly prevented mucosal structural damage. Our findings agreed with what Buchholz et al. [9,10] reported in their studies, which showed hydrogen inhalation significantly prevented an increase in the gut permeability.

One weakness of our study involved the lack of quantitative hydrogen level in blood samples before and after HRS intra-peritoneal injection. The rat pups only weighed between 5.0 g and 7.0 g following birth. But a volume of 5 ml blood is required to determine the hydrogen gas concentration by gas chromatography. It is almost unrealistic to perform the procedure on neonatal rats. However, Ohsawa et al. [7] reported in their article that the amount of hydrogen dissolved in venous blood (10 ng/ml) was less than that in arterial blood (20 ng/ml) after 2% hydrogen inhalation, suggesting that hydrogen had been utilized in the tissues of adult SD rats. And Xie et al. [28,31] showed in their studies that hydrogen inhalation at a 2% or 4% concentration had no notable effects on arterial pH, PO_2, and PCO_2 in adult mice.

Medical gas therapy is a new and relatively unexplored field. Guven et al. [32,33] showed that hyperbaric oxygen and ozone therapy could protect the intestine in NEC pups by modulating antioxidant defense and anti-inflammatory protection. Hydrogen therapy might partly share the same mechanism with hyperbaric oxygen and ozone. However, some researchers already started pre-clinical trials of hydrogen-rich water (consumption of 1.5–2 l per day for 8 weeks, hydrogen concentration: 0.55–0.65 mM), and found hydrogen administration was generally safe, well-tolerated, and had beneficial effects on the participants [34]. The primary molecular target of hydrogen remains unknown. Obviously, there are many questions yet to be answered about the mechanism, the optimal dose, long-term effects etc. before the practical clinical application of HRS.

In summary, the data from our study indicated that administration of HRS had protective effects on neonatal rats with NEC via decreasing oxidative stress, suppressing pro-inflammatory mediators, and preserving gut mucosal integrity. However, further studies are required to better understand the molecular mechanisms underlying the beneficial effects of hydrogen.

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