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Effect of hydrogen-rich water on acute peritonitis of rat models

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ABSTRACT

Objective: To study the effect of hydrogen-rich water (HRW) on acute peritonitis with three different rat models.
Methods: Acute peritonitis was induced by three methods including intraperitoneal injection of lipopolysaccharide (LPS), rats' feces or cecal ligation and puncture (CLP) operation. For each model, male Sprague Dawley rats were used and distributed into saline control group, HRW control group, saline plus model group, and HRW plus model group. Saline or HRW (3 ml per rat) was orally administered by gavage for 7 days beforehand and 3 days after modeling. The efficacy was tested by detecting concentrations of white blood cells (WBCs), plasma endotoxin, interleukin (IL)-6 and tumor necrosis factor (TNF)-α. The activities of malondialdehyde (MDA), myeloperoxidase (MPO) and glutathione (GSH) in visceral peritoneum tissues were also evaluated. Meanwhile, histopathology examination of visceral peritoneum was performed using hematoxylin and eosin staining. The expression and location of nuclear factor kappaB (NF-κB) in the visceral peritoneum were detected by immunohistochemistry.
Results: Three models showed the same result that hydrogen-rich water had an efficient protective effect on acute peritonitis. HRW could significantly lower the levels of WBCs, plasma endotoxin and cytokines, enhance GSH activity and reduce MPO and MDA activities in the peritoneum tissue when compared with that of groups with only saline treated. Simultaneously, we found that HRW could also decrease the NF-κB expression in the peritoneum tissues.
Conclusion: Hydrogen-rich water could alleviate the severity of acute peritonitis, and it might perform this function by its anti-inflammation, anti-oxidation and anti-bacterial effects and reducing NF-κB expression in the peritoneum tissues.

1. Introduction

Peritonitis is a common postoperative complication that can develop into lethal sepsis in case of delayed diagnosis or inappropriate treatment, whose mortality doubled in the past decades [1,2]. In the United States, about 750,000 people developed into sepsis each year, with a mortality rate of 28.6% and an approximate cost of $16.7 billion [3,4]. Although high-class antibiotics and advanced intensive care have proven to be effective on the treatment, the morbidity and mortality remain kept at a high level. The pathophysiology of peritonitis is complicated and is involved in various processes, of which, the most important one is the inflammatory reaction [5]. Local intra-abdominal focus of inflammation caused by the microorganisms can promote the synthesis and secretion of massive inflammatory cytokines, which would destroy the endothelial junctions and provide access for bacteria into the systemic circulation leading to lethal bacteremia [6,7]. A more severe inflammatory response process usually indicates a much higher mortality. Meanwhile, the oxidative stress induced by the direct effect of bacteria and indirect effect of inflammation also contributes to the severity of peritonitis [8]. Overproduction of reactive oxygen can not only result in the direct organ injury but also exacerbate the inflammatory reaction simultaneously [9]. In terms of the deeper molecule mechanisms, it is supposed that the microorganisms and their components can immediately activate the transcription factors—nuclear factor-κB (NF-κB). NF-κB can initiate gene expression of cytokines, adhesion molecules, chemokines, and cytotoxic enzymes, which are considered to be directly responsible for the organ injury and death [10–13]. During the pathological process of the peritonitis, NF-κB plays an activating role in the inflammatory reaction, which might be a potential therapeutic target in the future clinical work [14].

Hydrogen therapy is a new medical approach which has gotten a rapid development in the past several years [15]. In 2007, Ohsawa et al. found that inhalation of hydrogen gas significantly suppressed brain injury by buffering the effects of oxidative stress in an acute focal ischemia and reperfusion rat model [16]. And then in 2008,

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Buchholz et al. found that the hydrogen therapy could inhibit the inflammatory reaction in the rat model of small intestinal transplantation [17]. Contemporarily, Chen et al. proved that the hydrogen therapy had a protective effect against acute pancreatitis for its ability to inhibit oxidative stress, apoptosis, NF-κB activation, etc [18]. In 2010, Xie et al. found that hydrogen had the protective effects on sepsis and sepsis-associated organ damage mainly relied on its anti-oxidative property [19]. Up to now, three main methods including inhalation, oral intake of hydrogen-rich water (HRW) and injection of hydrogen-saturated saline have been developed and proved to be valid and reliable to deliver the hydrogen.

The most common experimental models used in acute peritonitis research generally pursue three strategies, including injection of lipopolysaccharide (LPS) into the abdominal cavity [20], injection of feces into the peritoneal cavity [21], and cecal ligation and puncture (CLP) operation [22]. Our study was designed to investigate the potential therapeutic effects of hydrogen on the peritonitis in three above-mentioned rodent models and try to find out the possible mechanism.

2. Materials and methods

2.1. Experimental animals and HRW

The study was conducted using male Sprague Dawley rats (210–260 g) (Animal Feeding Center of Xi'an Jiaotong University Medical School). All rats were housed (5 per cage) in conventional animal facilities with 12:12 light/dark cycle. The study was approved by the Animal Research Committee in Xi'an Jiaotong University Medical School. Animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. The hydrogen-rich water adopted Izumio drinking water (Naturally Plus Japan International Co.). The main technology of this product is dissolving the hydrogen in water under high pressure to the supersaturated level using a gas-rich water-producing apparatus, and storing it under atmospheric pressure at 4 °C in an aluminum bag with no dead volume. The gas chromatography was used to confirm the content of hydrogen (hydrogen concentration of the HRW we used in this research: 0.62–0.82 mM).

2.2. Experimental design

Acute peritonitis was induced by three different experimental methods: 1) by intraperitoneal administration of LPS, 2) by intraperitoneal administration of rats' fecal suspension and 3) by cecal ligation and puncture (CLP) operation.

Model 1. Acute peritonitis was induced by intraperitoneal injection with LPS (10 mg/kg, LPSL2880, from *Escherichia coli* 055:B5, Sigma Chemical Co, St. Louis, MO, USA) [20,23].

Model 2. Acute peritonitis was induced by intraperitoneal injection of fecal slurry (6.25 ml/kg), which was prepared from the bowel contents of a rat from the same batch, suspended in saline and filtered to remove fibrous material [24,25].

Model 3. Acute peritonitis was induced by cecal ligation and puncture (CLP) operation. All animals were anesthetized, shaved and prepared with iodine. Through a midline laparotomy, the cecum was filled with feces by milking the stools back from the descending colon and then ligated just below the ileocecal valve with a 3-0 silk ligature. The anterior mesenteric cecal surface was punctured twice with a 23-gauge needle below the ligature, the bowel was placed back into the peritoneal cavity, and the abdomen was closed in two layers. The operative procedure was done under aseptic conditions [22,26].

Overall, for each model, male Sprague Dawley rats were divided into four groups randomly, consisting of 6–17 animals each: saline control group, HRW control group, model group and HRW plus model group. Saline or HRW (6 ml/kg per rat) was orally administered by gavage for 7 days beforehand and 3 days after modeling (daily at 10:00 AM). On the 8th day, acute peritonitis was induced by the aforementioned three methods. Normal control and HRW control groups were given intraperitoneal injection of saline (models 1 and 2) or suture following laparotomy only (model 3). One milliliter of preheat sterile saline was administered s.c. for fluid resuscitation in models. On the 10th day, rats were sacrificed by euthanasia to collect the blood and tissue samples. The detailed experimental protocol was shown in the Supplementary Fig. 1.

2.3. Analytical measurements

Blood samples were collected from the cut tail (6 h, 24 h, and 48 h after modeling) and cardiac puncture (72 h after modeling) and divided into whole blood and plasma for further assays. The concentration of circulating white blood cells (WBCs) was determined using a hematology analyzer. Plasma concentrations of TNF-α and IL-6 were measured using enzyme-linked immunosorbent assays. Endotoxin concentrations were measured by the Limulus Amebocyte Lysate test (Dakewe Biotech Co.)

2.4. Visceral peritoneum enzymatic activity assay

Activities of malondialdehyde (MDA), myeloperoxidase (MPO) and glutathione (GSH) from the visceral peritoneum (greater omentum) tissue were measured with the activity assay kits from NanJing JianCheng Bioengineering. The harvested greater omentum was homogenized with 10 volumes of potassium phosphate buffer (20 mmol/L, 0.1 M, pH 7.4) containing potassium chloride (30 mmol/L) and then centrifuged at 1500 *g* for 15 min. The supernatants were collected, liquoted, and stored at −80 °C until the following analysis. The detection was conducted following the reference manual.

2.5. RNA isolation and quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis

Visceral peritoneum tissue samples from each group were snap-frozen in liquid nitrogen and stored at −70 °C until the experiments. Total RNA was isolated from cells using the RNAfast200 Kit (Fastagen Biotech, Shanghai, China). Reverse transcription was performed using the PrimeScript RT reagent Kit (TaKaRa Biotechnology, Dalian, China). The mRNA expression was assayed in triplicate and normalized to the β-actin mRNA expression. The relative levels were calculated using the Comparative-Ct Method (ΔΔCt method). The following primers were used for qRT–PCR, NF-κB:

5′-CAGCCTTCCCCACTAAAATAACC-3′ (sense) and
5′-ACCCACAAAAACCCTGCTCTG-3′ (antisense); β-actin:
5′-ATCGTGCGTGTGACATTAAGGAG-3′ (sense) and 5′-AGGAAGGA AGGCTGGAAGAGTG-3′ (anti-sense); All primer pairs were synthesized by TaKaRa.

2.6. Histological and immunohistochemistry study

Samples from the visceral peritoneum were fixed in 10% formalin solution and embedded in paraffin after completion of the routine follow-up. Serial sections of 5-μm thickness were obtained and stained with hematoxylin/eosin (HE) to evaluate gastric morphology. To establish the immunolocalization of NF-κB, a mouse polyclonal antibody (Beijing Biosynthesis Biotechnology Co., LTD) was used at a working dilution of 1:50. The antibody was applied directly to sections, and slides were incubated overnight at 4 °C in a humidified chamber. Immune complexes were subsequently treated with the secondary antibody (containing anti-rabbit and anti-mouse immunoglobulins) and detected via application of streptavidin peroxidase treatment for 20 min at room temperature. After rinsing sections with three changes

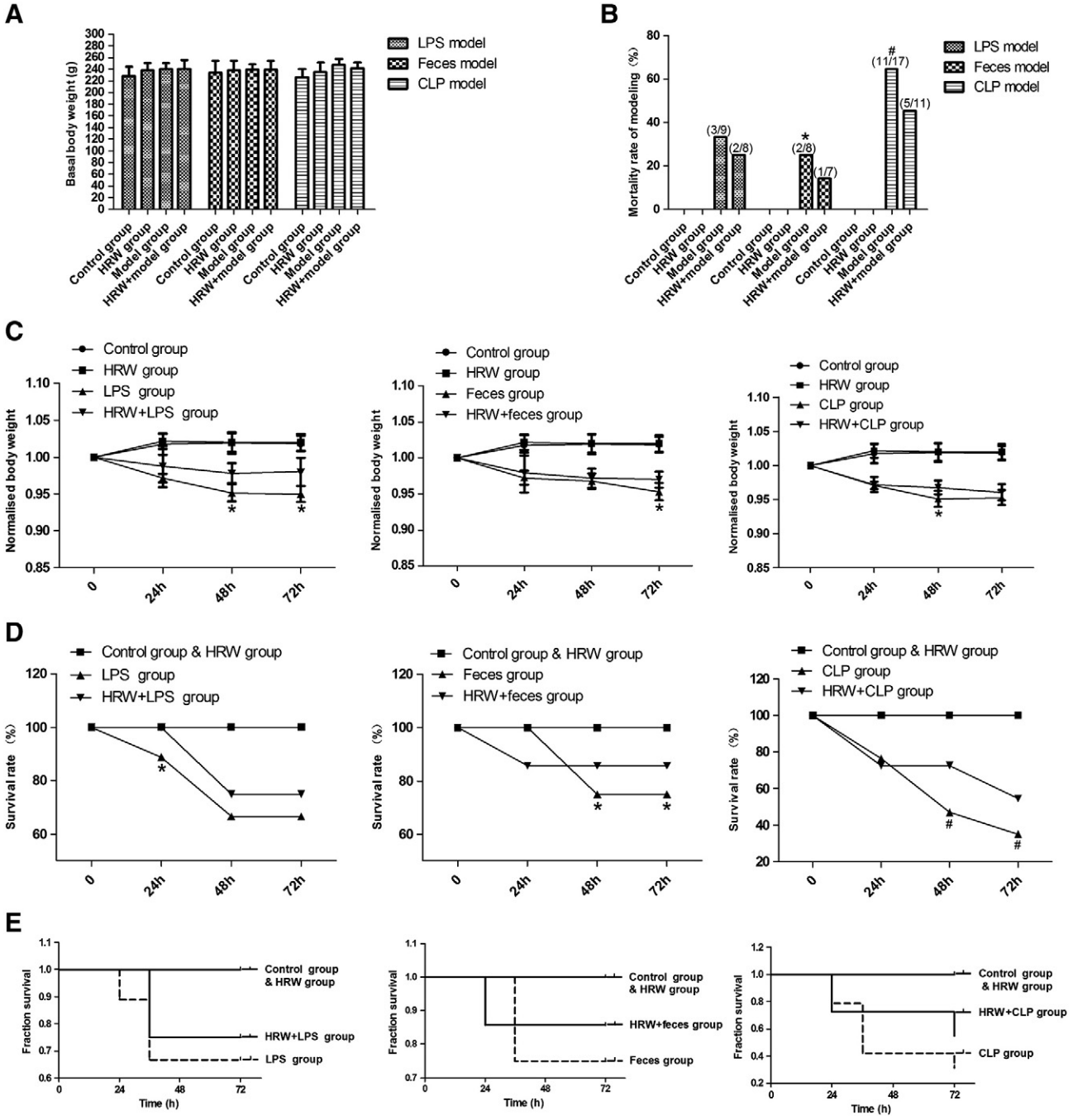

Fig. 1. Hydrogen-rich water reduced the mortality rate of acute peritonitis modeling and improved the survival rate of peritonitis rats. The values are expressed as mortality or survival percentage. A. The basal body weight of the rats in the study. B. Effects of HRW on the final mortality rate of peritonitis rats. $^{*}P < 0.05$ vs hydrogen + model group; $^{\#}P < 0.01$ vs hydrogen + model group. C. The body weight normalized by that on day 0 as a function of days after modeling in the four research groups. D and E. Effects of HRW on the survival rate of acute peritonitis in three models at 0 h, 24 h, 48 h and 72 h after modeling. $^{*}P < 0.05$ vs hydrogen + model group; $^{\#}P < 0.01$ vs hydrogen + model group. E.

of PBS, immunoreactivity was visualized with diamine benzidine (DAB)-hydrogen peroxide (20 min). Sections were gently rinsed in distilled water, counterstained with H&E, and taken photomicrographs under a microscope (Olympus Optical Co.,Tokyo, Japan). The IHC was carried out by two researchers under blind conditions. The intensity of immunohistochemical staining was scored as 0 (negative), 1 (weak), 2 (moderate strong) or 3 (strong). The extent of staining was assessed based on the percentage of positive staining cells: 0 (negative), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The final staining score for each sample was the mean of the intensity and extent scores from five fields. The expression was considered as low if the final score was 1–5 and as high if the final score was 6–12.

2.7. Statistical analysis

The survival and mortality rates are expressed as percentages. The measurement data are expressed as mean ± standard deviation (SD). Differences between experimental and control groups were assessed by either the analysis of variance (ANOVA) or nonparametric tests, as

applicable, using SPSS 18.0 (SPSS, 165 Inc.). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Hydrogen-rich water improves the survival rate and decreases the mortality rate in the acute peritonitis models

In this research, we investigated the efficacy of hydrogen therapy on acute peritonitis with three different models. In the CLP model, the rats of the operation group showed apathy, astasia, anorexia, and hypothermia in the first 24 h. And in the LPS and feces models, rats displayed little abnormality in the first 6 h but got rapidly worse in the next 6–24 h. They were all in a bad situation of spirit during the first 72 h. The basal body weights of the rats in all models were shown in Fig. 1A, and the BW normalized by that of day 0 as an indicator of function after modeling improved at different levels when treated with HRW (Fig. 1C). The 24/48/72 h survival rates after acute peritonitis modeling were 88.89%/66.67%/66.67% (LPS model), 100%/75%/75% (Feces model) and 76.47%/47.06%/35.23 (CLP model), while HRW could improve them to 100%/75%/75%, 85.71%/85.71%/85.71%, and 72.73%/72.73%/54.54% respectively (Fig. 1D and E). The 3-day mortality rate after acute peritonitis modeling was 33.33% (LPS model), 25% (Feces model) and 64.7% (CLP model), and the hydrogen therapy could reduce them to 25%, 14.29% ($P < 0.05$) and 45.45% ($P < 0.01$) respectively (Fig. 1B).

3.2. Hydrogen-rich water alleviates the inflammatory reaction in the acute peritonitis models

We investigated the effect of hydrogen therapy on the inflammatory reaction of acute peritonitis. We detected the WBCs, plasma endotoxin, TNF-α and IL-6 levels at 6/24/48/72 h to show the severity and time effect of the inflammation. Our results suggested that the inflammation

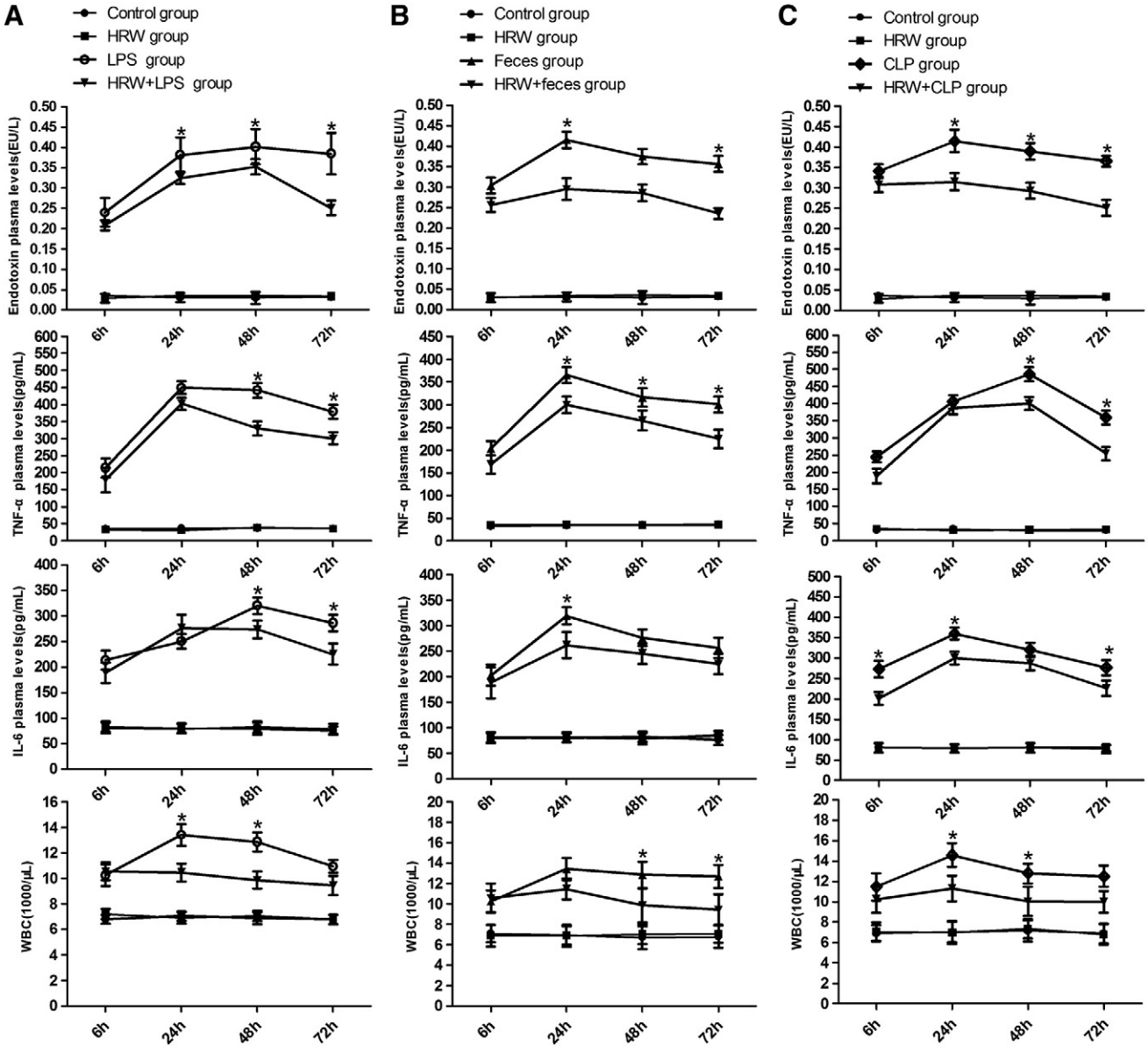

Fig. 2. Hydrogen-rich water decreased the WBCs, plasma endotoxin, TNF-α, and IL-6 concentrations in the acute pancreatitis. A, LPS-induced acute pancreatitis models. B, feces-induced acute pancreatitis models. C, CLP-induced acute pancreatitis models. These indicators were measured at 6 h, 24 h, 48 h and 72 h after modeling or sham dispose. Hydrogen-rich water exhibited the inhibition of inflammation reaction from 6 h after modeling and kept the protective role during the experiment. The values are expressed as means ± SD (n = 6–16 per group). $^{*}P < 0.05$ vs hydrogen + model group.

reached peak at about 24–48 h after modeling and mitigated gradually in the following hours. Treatment with HRW could decrease the WBCs, endotoxin, TNF-α, and IL-6 levels in plasma to different extents from 6 h after modeling. In the LPS-induced acute peritonitis, the peak level of the endotoxin and IL-6 was 0.402 ± 0.106 EU/L and 320.14 ± 36.35 pg/mL at 48 h, while HRW water treatment could reduce the damage to 0.353 ± 0.089 EU/L and 273.46 ± 27.12 pg/mL, respectively ($P < 0.05$). The TNF-α and WBCs reached the top level at 24 h after modeling (450.35 ± 58.24 pg/mL and 13.426 ± 2.389 × 10^3/μL), and the hydrogen could decrease them to much lower levels (402.22 ± 47.24 pg/mL and 10.476 ± 1.957 × 10^3/μL, $P < 0.05$) (Fig. 2A). The details of the inflammation indicators at different time in three models were shown in Fig. 2.

3.3. Hydrogen-rich water assuages the oxidative stress in the acute peritonitis models

At the end of the research (72 h), we tested the oxidative stress and tissue enzymatic activity in the visceral peritoneum. The rats in the model group showed a significant increase of MDA and MPO activities in visceral peritoneum, which could otherwise be attenuated by HRW treatment. We also found that HRW could increase the GSH activity (Fig. 3). In the feces-induced acute peritonitis, MDA and MPO activities in the visceral peritoneum increased significantly in the model group compared with the HRW treatment group (23.76 ± 2.918 vs 20.88 ± 1.684 nmol/mg.prot, $P < 0.05$ and 4.260 ± 0.405 vs 3.024 ± 0.266 U/g tissue, $P < 0.01$), and the protective indicator GSH increased significantly from 4.517 ± 0.329 mg/g protein to 8.660 ± 0.679 mg/g protein after the administration use of HRW ($P < 0.05$) (Fig. 3B). The detailed MDA, MPO and GSH activities in three models were shown in Fig. 3.

3.4. Hydrogen-rich water alleviates visceral peritoneum injury and inhibits NF-κB expression and immunostaining

We studied the morphological change of the visceral peritoneum in the three acute peritonitis models using H&E staining and Immunostaining. The H&E staining showed a discrete infiltrate of inflammatory cells and clusters of leukocytes in the peritoneum in the model group and HRW + model group, while the latter group displayed much more mild infiltration (Fig. 4A—H&E staining from the rats in CLP-induced peritonitis model). Immunostaining of the CLP-induced peritonitis model showed an increased immunoreactivity of NF-κB in endothelial and perivascular inflammatory cells in the model group and a reduction effect of HRW on its expression (Fig. 4A). The NF-κB score of the IHC staining in the HRW + model group was obviously

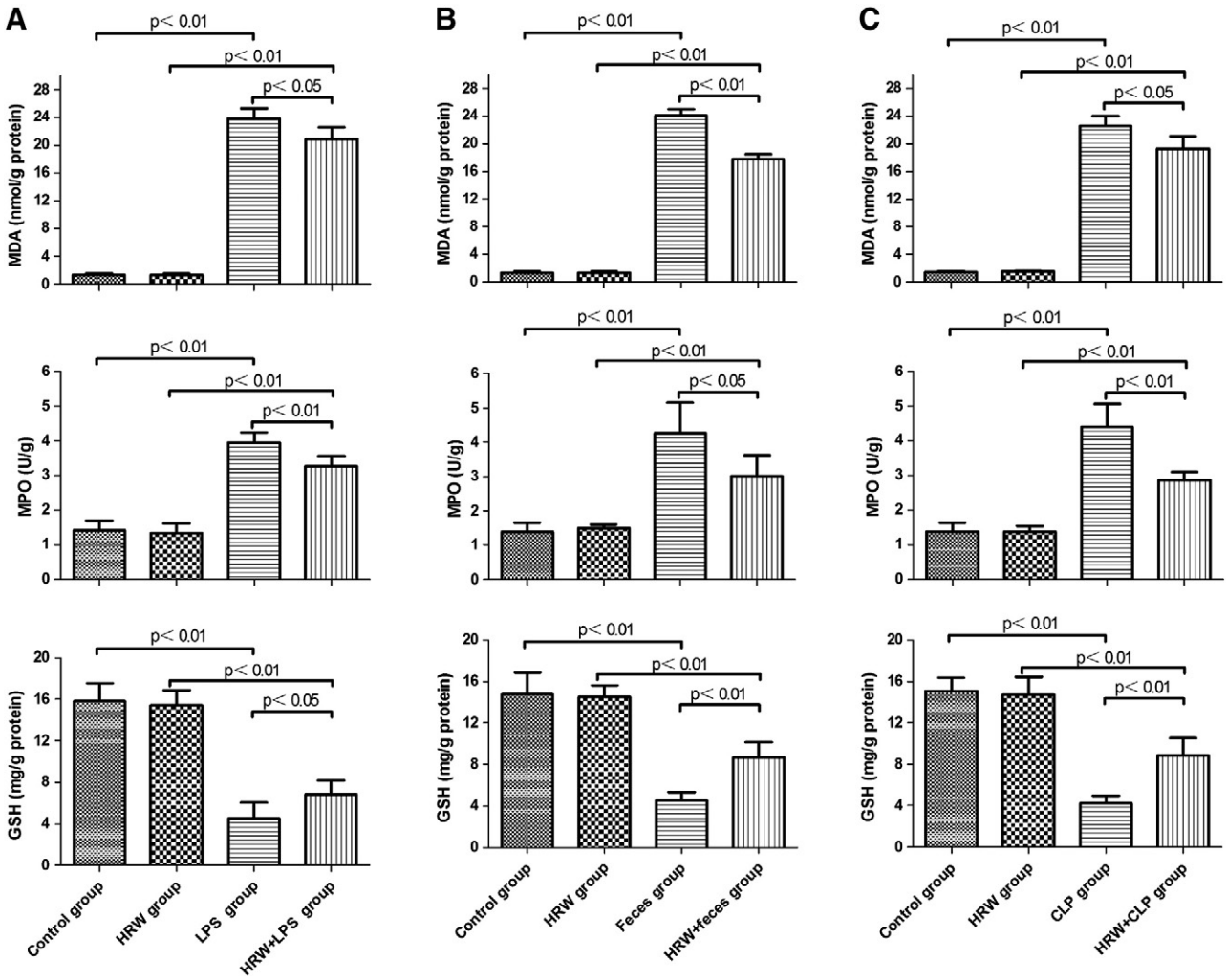

Fig. 3. Hydrogen therapy attenuated oxidative stress injury of visceral peritoneum in the acute pancreatitis. A, LPS-induced acute pancreatitis models. B, feces-induced acute pancreatitis models. C, CLP-induced acute pancreatitis models. The administration of HRW significantly increased the GSH and reduced the increase of MDA and MPO in acute pancreatitis in all three models. The values are expressed as means ± SD (n = 6 per group).

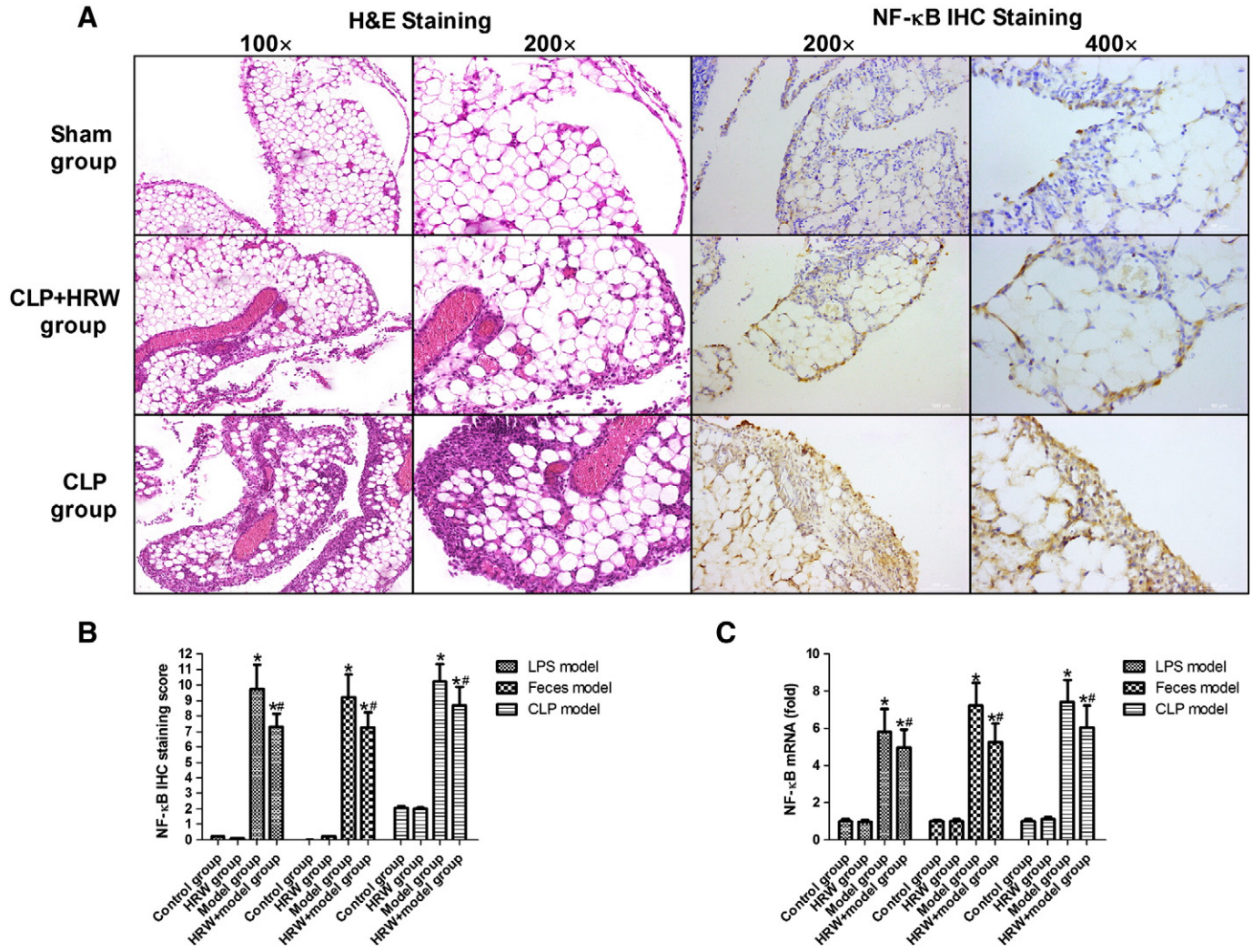

Fig. 4. Representative photomicrographs of morphological change in the rats with acute pancreatitis. A. Hemalun–eosine staining in the visceral peritoneum of CLP modeling or HRW plus CLP-modeling mice. Representative photographs of NF-κB expression in the visceral peritoneum tissues. The LPS-induced rats had more NF-κB immunopositive staining as indicated by brown color, while treatment with HRW can significantly reduce NF-κB immunostaining. B. The NF-κB IHC staining score in the three models indicated that hydrogen therapy could significantly lower the staining score and reduce its activation and expression. $^{*}P < 0.01$ vs shame group. $^{\#}P < 0.05$ vs model group. C. The relative NF-κB mRNA levels in the three acute pancreatitis models. $^{*}P < 0.01$ vs shame group. $^{\#}P < 0.05$ vs model group.

lower than that in the model group (8.67 ± 1.230 vs 10.25 ± 1.120, $P < 0.05$, Fig. 4B). Moreover, HRW could significantly reduce the mRNA level of NF-κB in visceral peritoneum from 7.410 ± 1.192 in the model group to 6.028 ± 1.211 in the HRW + model group ($P < 0.05$, Fig. 4C).

4. Discussion

Acute peritonitis differs from other infections because of the broad variety of causes, severity of the infection, polymicrobial pathogenesis and complex pathological process [27]. In the present study, we used three animal models including LPS or feces intraperitoneal administration and CLP operation to study the effect of the hydrogen-rich water on acute peritonitis. The results showed that the hydrogen therapy could effectively reduce the severity of acute peritonitis in all three models. Hydrogen could significantly ameliorate the increased WBCs, plasma endotoxin, TNF-α and IL-6 levels, and could also reduce the oxidative stress mainly by decreasing the MAO and MPO activities and increasing the GSH activity. The protective effect was also supported by the results from ameliorated histological findings and its ability to inhibit NF-κB activation.

Acute peritonitis is one of the most headachy postoperative complications, which is an important cause of death in surgical practice and intensive care units [28]. The most serious consequence of acute peritonitis is sepsis, often leading to an unacceptably high morbidity and mortality [29]. So the research of acute peritonitis and sepsis is always the hotspot of surgery and critical care medicine. The animal model is one of the most important methods in the scientific research. It can not only provide convenience in deriving a better understanding of the pathophysiology of disease, but also provide important and indispensable tools to explore the therapy of disease. It is the bridge between the fundamental research and clinical application. To date, there are mainly three different models to study peritonitis, including intraperitoneal administration of LPS, fecal suspension and CLP operation. The LPS administration model is mostly used to study the spontaneous peritonitis or dialysis related peritonitis, because the pathogenic bacterium of these diseases is always the *Escherichia coli* [30,31]. The CLP operation model is most commonly used to study acute peritonitis, which has an advantage on promoting similar pathophysiology of the secondary peritonitis in human beings and can better resemble the clinical condition of a bowel perforation and mixed bacterial infection [32]. The fecal administration model is the simplified CLP model which is also induced by the mixed bacterial infection, but lacks the operation stimulation [21]. In fact, three models represent a cascade progressive-like stage in the pathophysiological process of peritonitis. CLP model mimics the clinical

process of peritonitis that is mostly caused by the surgical operation. Fecal model imitates the detailed contaminant of peritonitis and LPS model simulates the preliminary mechanism of the peritonitis process (Supplementary Fig. 2). The most straling characteristic in our study is that we focus on the effect of hydrogen on peritonitis from both horizontal and vertical sights with three models, and the results show that hydrogen could obviously alleviate the lesions in all models. Another finding is that hydrogen can decrease the endotoxin levels, which is released from bacterial wall and an indicator of bacterial quantity. So we speculate that hydrogen may have a potential anti-bacterial effect except for its anti-inflammation and anti-oxidant effects. In fact, this similar effect has been observed in several studies but not proposed [19,33,34]. Because of its low molecular weight and high penetration ability, hydrogen can enter into the bacteria easily and may interfere with the process of bacterial proliferation and metabolism to inhibit bacteria. On the other hand, hydrogen may promote the innate antibacterial responses in the body just like the vitamins do [35]. This is the first evidence that hydrogen may have anti-bacterial effect, and further studies need to be launched to verify the effect, explore the mechanisms and promote the clinical use.

Ultimately, in the process of peritonitis, bacteria are the main source of local and systemic infections. Bacterial pathogens and their products trigger the inflammatory response by transcriptional activation of inflammatory genes, leading to the release of large number of inflammatory mediators, including cytokines, chemokines, adhesion molecules, reactive oxygen and so on, which can easily cause sepsis when they are uncontrolled and excessive [36]. This process is mediated by the activation of inducible transcription factors, such as NF-κB, which play a pivotal role in the immune and inflammatory responses. Previous investigators have found that acute peritonitis and sepsis were associated with the activation of the transcription factor NF-κB in various organs and tissues [37–39], which can regulate the synthesis of TNF-α, IL-6, inducible nitric oxide synthase, cyclooxigenase-2 and many other molecules involved in the inflammatory reaction [40]. Hydrogen is a colorless, odorless, tasteless and also the lightest and most abundant chemical element in nature. Masses of experiments have confirmed its effective function in the antioxidant, anti-apoptotic, anti-inflammatory, anti-allergy and anti-cancer procedures [41]. Hydrogen can selectively reduce hydroxyl radicals which are the strongest ones of the oxidant species in human body [16]. Contemporarily, as a means of compensation, hydrogen can increase the activities of antioxidant enzymes such as catalase, superoxide dismutase or heme oxygenase-1 to keep redox equilibrium [42]. Inflammation and oxidation processes are reciprocally related. ROS can activate TNF-α expression by up-regulating the NF-κB signaling pathway while TNF-α can activate NADPH-oxygenase (NOX) expression that generates ROS from NADPH [43,44]. Hydrogen can also suppress the tissue-destructive production including pro-inflammatory cytokines, TNF-α and IFN derived from the activated lymphocytes, mainly through inhibiting NF-κB signaling pathway. Take Chen et al. for instance, they found that the hydrogen therapy could inhibit NF-κB activation in the acute pancreatitis [18], showing similar results with ours. Based on these recognitions, we postulated that the ameliorated effect of hydrogen therapy on acute peritonitis may be explained by the reduced NF-κB activation and the consecutively reduced inflammation and oxidative stress (Supplementary Fig. 5). But, unfortunately, we have to admit that the detailed mechanism of hydrogen inhibiting NF-κB activation is still an enigma.

As a result of the present study, we confirmed that hydrogen might have a potential protective effect in acute peritonitis. Although hydrogen could not provide a thorough treatment, the results of our study showed its enormous potential in mitigating the lesions. Based on the results of our research, the consumption of HRW is beneficial to prevent peritonitis and reduce its severity, which may be helpful for the patients who are going to take selective operations if they drink the hydrogen-rich water days before the operations. On the other hand, oral intake of hydrogen-rich water is a humanized drug-delivery way, which can be more easily accepted when it is adopted in the future. The future research could focus on the combination of hydrogen therapy and traditional antibiotics, which might be more efficient than using antibiotics alone.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2014.04.011.

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