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Hydrogen-rich saline protects against intestinal ischemia/reperfusion injury in rats

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Abstract

Hydrogen gas was reported to reduce reactive oxygen species and alleviate cerebral, myocardial and hepatic ischemia/reperfusion (I/R) injuries. This paper studied the effect of hydrogen-rich saline, which was easier for clinical application, on the intestinal I/R injury. Model of intestinal I/R injury was induced in male Sprague-Dawley rats. Physiological saline, hydrogen-rich saline or nitrogen-rich saline (5 ml/kg) was administered via intravenous infusion at 10 min before reperfusion, respectively. The intestine damage was detected microscopically and was assessed by Chiu score system after I/R injury. In addition, serum DAO activity, TNF-α, IL-1β and IL-6 levels, tissue MDA, protein carbonyl and MPO activity were all increased significantly by I/R injury. Hydrogen-rich saline reduced these markers and relieved morphological intestinal injury, while no significant reduction was observed in the nitrogen-rich saline-treated animals. In conclusion, hydrogen-rich saline protected the small intestine against I/R injury, possibly by reduction of inflammation and oxidative stress.

Keywords: *Hydrogen-rich saline, intestinal, ischemia/reperfusion, inflammation, oxidative stress*

Introduction

Intestinal ischemia/reperfusion (I/R) injury is a common and often devastating clinical disorder, which generally occurs in arterial occlusion by thrombi or embolisms, small bowel transplantation or frequently in non-occlusive processes forming low mesenteric flow such as hypovolemia, cardiac insufficiency, sepsis and severe burns [1]. Intestinal I/R is accompanied by decreased contractile activity, increased microvascular permeability and dysfunction of mucosal barrier, which could eventually cause systemic inflammatory response syndrome [2]. I/R leads to the release of reactive oxygen species (ROS) or reactive nitrogen species (RNS), such as superoxide anion, hydroxyl radical, hydrogen peroxide and peroxynitrite [3]. In addition, I/R recruits and activates neutrophils in the intestine. Activated neutrophils augments ischemic injury by releasing cytotoxic free radicals and proteolytic enzymes [4]. Free radicals can damage cellular membrane and subcellular structures, which contain large amounts of phospholipids and protein, resulting in lipid peroxidation and sequentially structural and metabolic alterations, leading to cell apoptosis and necrosis [5].

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Hydrogen (H_2) gas has been used in medical applications to prevent decompression sickness (DCS) in deep divers for safety profiles [6]. Recently, Ohsawa et al. [7] found that molecular hydrogen could selectively reduce cytotoxic reactive oxygen species, such as $^{\bullet}OH$ and ONOO- *in vitro* and exert therapeutic antioxidant activity in a rat middle cerebral artery occlusion model. In addition, the inhalation of H_2 gas has been demonstrated to reduce the infarct size in the rat model of myocardial ischemia–reperfusion injury [8] and suppress hepatic ischemia/reperfusion injury in mice [9].

H_2 gas would be much cheaper then other anti-oxidative drugs if it could be applied in clinical practice. However, clinical application of H_2 gas inhalation is not convenient and may be dangerous because it is inflammable and explosive. On the other hand, H_2 gas saturated physiological saline, which is called hydrogen-rich saline, is easy to apply and safe. Therefore, we investigated whether administration of hydrogen-rich saline exerted a protective effect in the model of intestinal ischemia/reperfusion injury. We examined oxidative stress and inflammation response after hydrogen-rich saline treatment in intestinal I/R injury in rats.

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing 220–250 g were used and the protocols were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University in Shanghai, China. All animals were given free access to normal rat diet and tap water and were maintained on a 12:12-h light/dark cycle (lights on at 06:00 h). The animals were fasted for 12 h with water *ad libitum* before operation.

Hydrogen-rich saline production

Hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4 MPa) to a supersaturated level using hydrogen-rich water-producing apparatus produced by our department. The saturated hydrogen saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. Hydrogen-rich saline was sterilized by gamma radiation. Hydrogen-rich saline was freshly prepared every week, which ensured that a concentration of more than 0.6 mmol/L was maintained. Gas chromatography was used to confirm the content of hydrogen in saline by the method described by Ohsawa et al. [7].

Experimental protocols

Animals were divided into four groups consisting of eight rats each: (1) sham-operated plus vehicle physiological saline treatment; (2) intestinal ischemia/reperfusion plus vehicle physiological saline treatment; (3) intestinal ischemia/reperfusion plus hydrogen-rich saline treatment; and (4) intestinal ischemia/reperfusion plus nitrogen-rich saline treatment. A rat small intestinal I/R model was adapted as described previously [10]. Briefly, rats were anaesthetized with pentobarbital (50 mg/kg) intraperitoneally. Through a midline laparotomy, the superior mesenteric artery (SMA) was clamped and ischemia was maintained for 45 min. Reperfusion was initiated by removing the clamp and was confirmed by the return of pulsation of the vascular arcade. A jugular venous cannula was inserted for fluid and drug administration. Sham-operated animals underwent the same surgical procedures without clamping. Physiological saline, hydrogen-rich saline or nitrogen-rich saline (5 ml/kg) was given via jugular venous cannula infusion within 10 s at 10 min before reperfusion initiation, respectively. Two hours after reperfusion initiation, blood was drawn from the inferior vena cava and then animals were sacrificed. The entire small intestine was removed and washed three times with ice-cold physiological saline. Blood samples were spun at 1000 rpm for 10 min and serum samples were collected.

Morphologic observation

A 5 cm segment of small intestine removed from 5 cm proximal to the terminal ileum was fixed in 10% buffered formaldehyde-saline. Three 1 cm segments of intestinal specimen were embedded in paraffin and stained with hematoxylin and eosin. Morphological damages were assessed by Chiu histological injury scoring system of intestinal villi (0 = normal mucosa, 1 = slight-, 2 = moderate-, 3 = massive subepithelial detachments, 4 = denudes villi, 5 = ulceration) [11]. Two independent and blinded researchers performed the histological scoring.

Biochemical analysis

For determination of malondialdehyde (MDA), the intestinal mucosa was scraped off, homogenized with 10 volume of 20 mmol/L potassium phosphate buffer (pH 7.4) containing 30 mmol/L KCl and then centrifuged at 1500 g for 15 min. MDA was assessed spectrophotometrically with the method defined by Ohkawa et al. [12] as MDA reacted with thiobarbituric acid and formed a pink, maximum absorbent complex at 532 nm wavelength.

The extent of protein oxidation was determined by measuring the protein carbonyl content. Briefly, the intestinal mucosa homogenate was mixed with streptomycin followed by centrifugation and then it was incubated with DNPH for 1 h followed by

precipitation with TCA. The pellet was washed with ethanol–ethyl acetate to remove excess DNPH and resuspended in guanidine hydrochloride. The solution was centrifuged and incubated at 37°C in a water bath for 10 min. Finally, the absorbance was measured at 366 nm wavelength and the results were expressed as nmol/mg protein [13].

For myeloperoxidase (MPO) activity measurement, the mucosa was homogenized in 10 volume of ice-cold 20 mmol/L potassium phosphate buffer (pH 7.4) containing 30 mmol/L KCl. The homogenate was centrifuged at 12 000 rpm for 10 min at 4°C. The pellet was then rehomogenized with an equivalent volume of 50 mmol/L acetic acid containing 5% hexadecyltrimethylammonium bromide (HETAB). MPO activity was assessed by measuring the H_2O_2 dependent oxidation of 0-dianizidine 2HCl. One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance of 1.0/min at 460 nm and 37°C [14].

Serum diamine oxidase (DAO) activity was measured using the method described by Takagi et al. [15]. It was based on a coupled reaction with peroxidase and a new chromogen, 10-(carboxymethyl-aminocarbonyl)- 3,7- bis (dimethylamino) phenothiazine sodium salt. The absorbance was measured at 668 nm. One unit of enzyme activity was defined as μmol substrates converted in 1 min at 37°C.

Protein concentration determination was made on the homogenates by BCA assay [16]. All of the analyses were performed in duplicate.

Cytokine determination

Cytokine levels in serum were determined by highly sensitive enzyme-linked immunosorbent assay (ELISA) kits from R&D systems according to the manufacturer's recommendations.

Statistical analysis

Data are expressed as means $\pm$ SEM for each group. The differences among experimental groups were detected by one-way analysis of variance. Between groups, variance was determined using the Student-Newman–Keuls post hoc test. A p-value of less than 0.05 was considered to be statistically significant.

Results

Hydrogen-rich saline treatment attenuates intestinal injury

We observed histological I/R injuries featured by shortening of the villi, loss of villous epithelium and prominent mucosal neutrophil infiltration. All of these changes were ameliorated by administration of hydrogen-rich saline. Chiu scoring and microphotographs are shown in Figure 1. No mucosal injury was
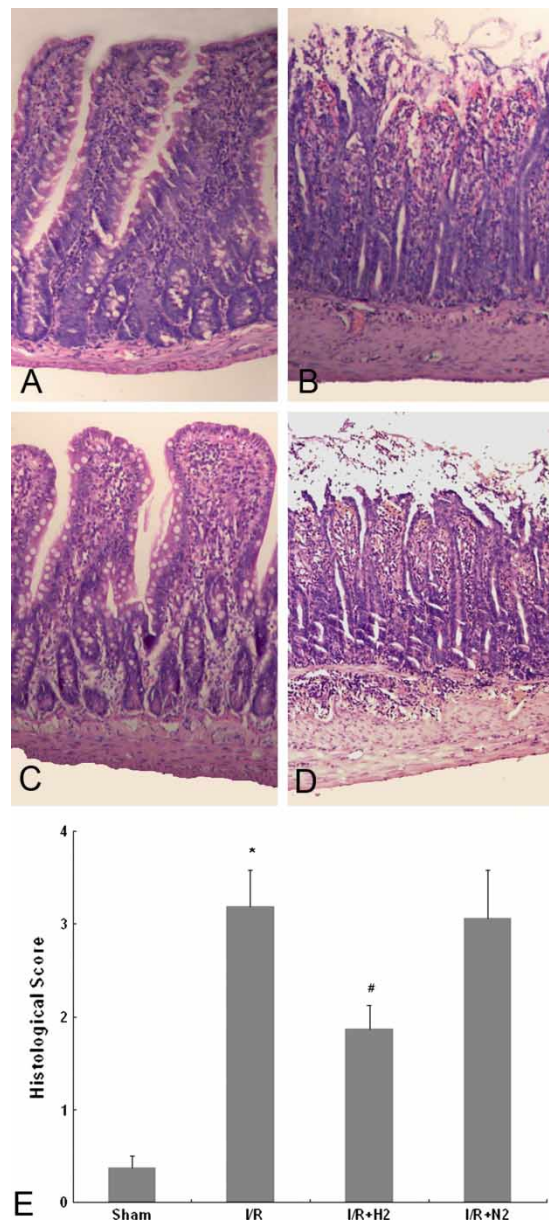

Figure 1 (Continued)

Figure 1. Morphologic observation of the intestinal tissue in sham-operated animals, I/R animals and I/R animals treated with hydrogen-rich saline or nitrogen-rich saline. Photomicrographs of the intestinal tissue stained by the hematoxylin and eosin (100×). (A) Sham: Histological features of normal intestinal tissue were observed, (B) I/R: The villi are denuded to the level of the lamina propria and dilated capillaries, (C) I/R + H_2: Erosion of the surface epithelium while the architecture of the villi are preserved, (D) I/R + N_2: Erosion of the surface epithelium while the architecture of the villi are not preserved. (E) Intestinal mucosal injury evaluated by Chiu scoring system in sham-operated animals, I/R animals and I/R animals treated with hydrogen-rich saline or nitrogen-rich saline. Grading as (0 =normal mucosa, 1 =slight-, 2 =moderate-, 3 =massive subepithelial detachments, 4 =denudes villi, 5 =ulceration). Data are expressed as means $\pm$ SEM for at least triplicate independent experiments (n =8 per group). $^\star p < 0.01$ vs Sham; # $p < 0.05$ vs I/R group.

observed in the sham-operated group. I/R injuries were pronounced when compared with the sham-operated group (3.2 ± 1.1 vs 0.38 ± 0.35, $p < 0.01$).
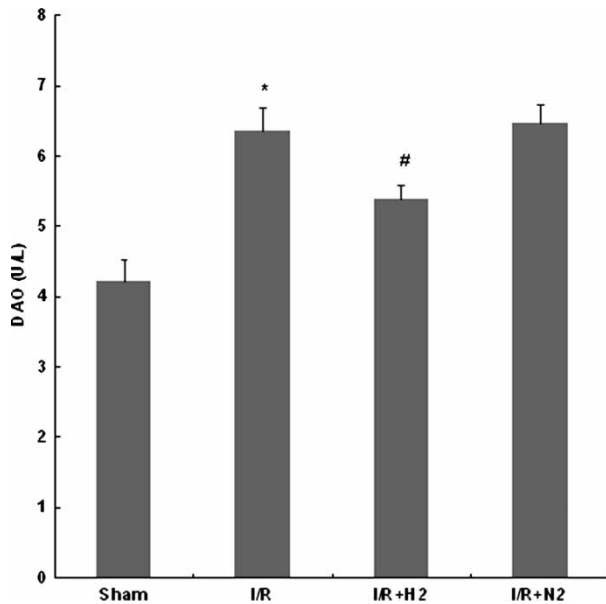
Figure 2. Serum diamine oxidase (DAO) activity levels in sham-operated animals, I/R animals and I/R animals treated with hydrogen-rich saline or nitrogen-rich saline. Data are expressed as means $\pm$ SEM for at least triplicate independent experiments ($n = 8$ per group). $\star p < 0.01$ vs Sham; # $p < 0.05$ vs I/R group.

Hydrogen-rich saline administration significantly reduced the mucosal injury caused by I/R (1.9 ± 0.69 vs 3.2 ± 1.1, $p < 0.05$). Nitrogen-rich saline showed no significant reduction of the intestinal injury (3.1 ± 0.51 vs 3.2 ± 1.1, $p > 0.05$).

Figure 2 shows high levels of serum DAO activity in I/R rats when compared with sham-operated rats (6.34 ± 0.35 vs 4.21 ± 0.32, $p < 0.01$). Hydrogen-rich saline treatment significantly reduced the levels of serum DAO activity in rats with intestinal I/R (5.37 ± 0.20 vs 6.34 ± 0.35, $p < 0.05$). Nitrogen-rich saline had no significant effect on the serum DAO activity (6.45 ± 0.28 vs. 6.34 ± 0.35, $p > 0.05$).

Hydrogen-rich saline treatment reduces oxidative stress

The rats subjected to intestinal I/R displayed an increase of the MDA levels in intestinal mucosa compared with the sham-operated rats (2.30 ± 0.19 vs 1.01 ± 0.11, $p < 0.01$). Administration of hydrogen-rich saline to the rats with intestinal I/R resulted in a marked reduction of the MDA levels (1.70 ± 0.14 vs 2.30 ± 0.19, $p < 0.05$). Nitrogen-rich saline did not reduce the MDA levels significantly (2.18 ± 0.25 vs 2.30 ± 0.19, $p > 0.05$). (Figure 3) Figure 4 illustrates that protein carbonyl contents significantly elevated in the intestinal of I/R treated rats compared to the control rats (2.20 ± 0.07 vs 1.20 ± 0.08, $p < 0.01$) and hydrogen-rich saline treatment was able to reduce protein carbonyl contents effectively in contrast to the I/R treated rats (1.83 ± 0.10 vs 2.20 ± 0.07, $p < 0.05$). Nitrogen-rich saline was unable to reduce protein carbonyl contents significantly (2.29 ± 0.20 vs 2.20 ± 0.07, $p > 0.05$).
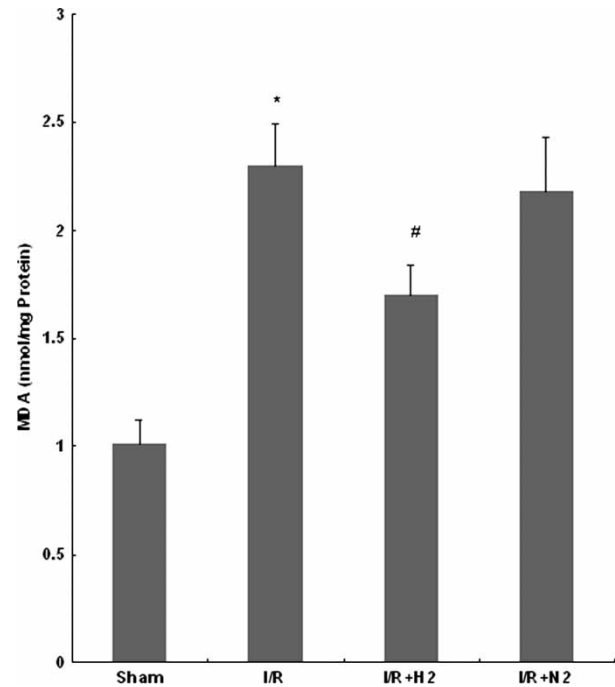

Figure 3. Intestinal mucosa malondialdehyde (MDA) levels in sham-operated animals, I/R animals and I/R animals treated with hydrogen-rich saline or nitrogen-rich saline. Data are expressed as means $\pm$ SEM for at least triplicate independent experiments ($n = 8$ per group). $\star$ $p < 0.01$ vs Sham; # $p < 0.05$ vs I/R group.

Hydrogen-rich saline treatment alleviates neutrophil infiltration

MPO activity examined as an indicator of neutrophil infiltration was shown in Figure 5. Compared with the sham-operated group, MPO activity in the intestinal mucosa tissues in the I/R group was found to have significantly increased (215.7 ± 13.81 vs 12.83 ± 0.88, $p < 0.01$). Hydrogen-rich saline produced a significant decrease of the MPO activity in I/R rats (118.24 ± 11.67 vs 215.7 ± 13.81, $p < 0.05$). Nitrogen-rich saline showed no significant decrease of the MPO activity (210.56 ± 18.7 vs 215.7 ± 13.81, $p > 0.05$).

Hydrogen-rich saline treatment decreases pro-inflammatory cytokines

As shown in Table I, serum TNF-α, IL-1β and IL-6 concentrations were significantly elevated in intestinal I/R animals when compared with those in sham-operated animals. Hydrogen-rich saline administration lowered the I/R-induced elevation of serum TNF-α, IL-1β and IL-6 concentrations. No significant reduction of these cytokines was produced by nitrogen-rich saline administration.

Discussion

Even though free radical scavengers have been demonstrated to reduce intestinal I/R damage
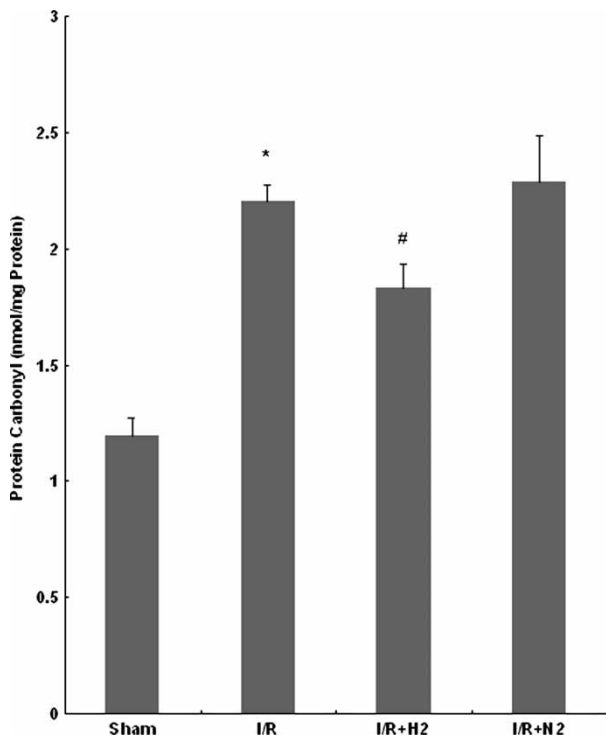
Figure 4. Intestinal mucosa protein carbonyl levels in sham-operated animals, I/R animals and I/R animals treated with hydrogen-rich saline or nitrogen-rich saline. Data are expressed as means $\pm$ SEM for at least triplicate independent experiments ($n = 8$ per group). $\star p < 0.01$ vs Sham; # $p < 0.05$ vs I/R group.

[17–19], this is the first observation that hydrogen-rich saline decreases intestinal I/R injury in an animal model. We observed that hydrogen-rich saline reduced not only morphological injury, but also serum DAO, which served as a marker of the integrity of the intestinal mucosa [20]. We especially investigated the effect of hydrogen-rich saline on oxidative stress and inflammation in this small intestine I/R injury model. Oxidative stress plays an important role in the intestinal I/R injury. In the intestinal tissue subjected to I/R, activated neutrophils induce tissue injury through the production and release of reactive oxygen species and cytotoxic proteins (for example, pro-teases, MPO, lactoferrin). MPO activity is commonly used to measure the extent of neutrophils infiltration in intestinal tissues subjected to I/R injury. In the present study, intestinal I/R caused an elevation in tissue MPO activity, indicating the presence of enhanced neutrophils recruitment in the mucosa tissue. We have found that hydrogen-rich saline decreased neutrophils infiltration.

Lipid peroxidation caused by reactive oxygen species is one of the most critical mechanisms operated in cellular damage and death. Many oxygenated compounds, particularly aldehydes such as malondialdehyde (MDA), are produced during the attack of free radicals to membrane lipoproteins and polyunsaturated fatty acids. The hydroxyl radical is the most reactive product of reactive oxygen species
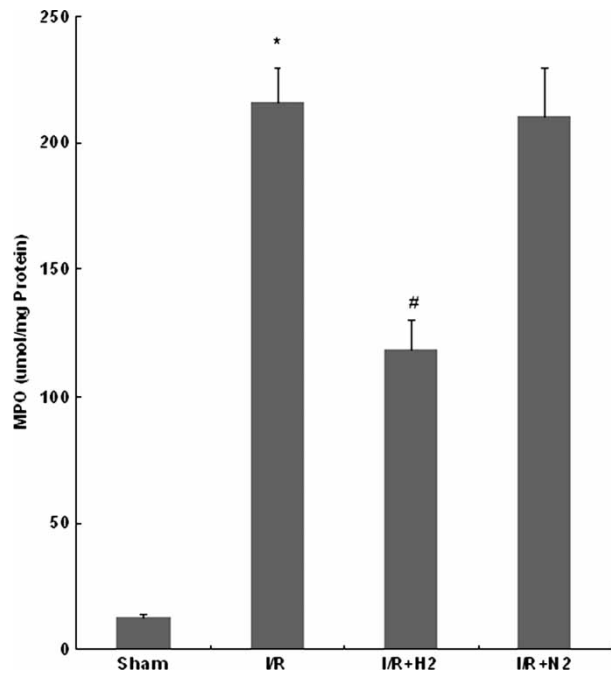

Figure 5. Intestinal mucosa myeloperoxidase (MPO) activity levels in sham-operated animals, I/R animals and I/R animals treated with hydrogen-rich saline or nitrogen-rich saline. Data are expressed as means $\pm$ SEM for at least triplicate independent experiments ($n = 8$ per group). $\star p < 0.01$ vs Sham; # $p < 0.05$ vs I/R group.

generated in cells. Hydroxyl radicals easily react with cellular macromolecules, including DNA, proteins and lipids, to exert a strong cytotoxic effect. It is well known that the reaction of hydroxyl radicals and lipids results in lipid peroxidation. The observed increase of intestinal MDA levels was an indicator of lipid peroxidation, which also verified the oxidative damage in the intestinal tissue in this animal model. Hydrogen-rich saline reduced oxidative stress represented again by lipid peroxidation. Similarly, protein carbonyl as an indicator of protein oxidation was also demonstrated to increase in the model of intestinal I/R injury [21]. Our result shows that hydrogen-rich saline could reduce protein oxidation, which might be another protective mechanism of its anti-oxidative effects.

The rise of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, is another important

Table I. Serum TNF-α, IL-1β and IL-6 concentrations in sham-operated animals, I/R animals and I/R animals treated with hydrogen-rich saline or nitrogen-rich saline.

	TNF-α	IL-1β	IL-6
Sham	21.6 ± 5.0	9.1 ± 2.4	30.7 ± 4.5
I/R	$137.2 \pm 26.0\star$	$37.6 \pm 5.8\star$	$134.3 \pm 18.0\star$
I/R+H$_2$	$75.7 \pm 10.8^{\#}$	$22.8 \pm 2.9^{\#}$	$82.5 \pm 13.3^{\#}$
I/R+N$_2$	127.9 ± 16.3	41.3 ± 5.5	141.2 ± 22.1

Data are expressed as means $\pm$ SEM for at least triplicate independent experiments ($n = 8$ per group). $\star p < 0.01$ vs Sham; #$p < 0.05$ vs I/R group.

pathophysiological process in I/R injury [22–24]. Elevation of TNF-α, IL-1β and IL-6 causes leukocyte infiltration to damaged tissue [25]. Large amounts of resident macrophages exist in the lamina propria of the intestinal mucosa. In addition, some other cells, such as enterocytes and Paneth cells, also have the ability of producing pro-inflammatory cytokines [26,27]. So the small intestine has been recognized as an important source of these cytokines. Intestinal I/R caused a significant elevation of TNF-α, IL-1β and IL-6 in serum [28] while hydrogen-rich saline reduced these pro-inflammatory cytokines. It cues us hydrogen-rich saline treatment might inhibit the excessive intestinal inflammatory response and its cascade.

In several recent studies, H$_2$ inhalation was reported to protect cerebral [7], myocardial [8] and hepatic [9] I/R injury in animal models. In addition, Buchholz et al. [29] reported that hydrogen inhalation ameliorates oxidative stress in transplantation-induced intestinal graft injury. In this study, we demonstrated that molecular hydrogen might be involved in the reduction of inflammation and oxidative stress and ameliorated morphological injury to the intestine after I/R. Furthermore, a recent study indicated that drinking hydrogen water reduced oxidative stress in brain and prevented stress-induced decline in learning and memory caused by chronic physical restraint in mice [30]. Drinking hydrogen-rich water decreased lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance [31]. We used hydrogen-rich saline injection in the intestinal I/R injury model for the first time, which is safe and easy to apply. It may have great potential for clinical use.

Furthermore, we tested the nitrogen-rich saline in an intestinal I/R model as control. Nitrogen-rich saline was also a de-oxygenated solution in the same manner as the prepared hydrogen-rich saline. Neither intestinal injury nor oxidative stress in intestinal I/R rats was reduced by nitrogen-rich saline treatment. It helps us to conclude that the observed protection by hydrogen-rich saline is being mediated via an anti-oxidant action.

In conclusion, hydrogen-rich saline protected the small intestine against I/R injury, possibly by reduction of inflammation and oxidative stress. However, the exact mechanism and signalling pathway involved in the protection role of hydrogen in the intestinal I/R injury need to be studied in the future.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Yasuhara H. Acute mesenteric ischemia: the challenge of gastroenterology. Surg Today 2005;35:185–195.

[2] Pierro A, Eaton S. Intestinal ischemia reperfusion injury and multisystem organ failure. Semin Pediatr Surg 2004;13:11–17.

[3] Schoenberg MH, Beger HG. Reperfusion injury after intestinal ischemia. Crit Care Med 1993;21:1376–1386.

[4] Simpson R, Alon R, Kobzik L, Valeri CR, Shepro D, Hechtman HB. Neutrophil and nonneutrophil-mediated injury in intestinal ischemia-reperfusion. Ann Surg 1993;218:444–453; discussion 453–444.

[5] Otamiri T. Oxygen radicals, lipid peroxidation, and neutrophil infiltration after small-intestinal ischemia and reperfusion. Surgery 1989;105:593–597.

[6] Fontanari P, Badier M, Guillot C, Tomei C, Burnet H, Gardette B, Jammes Y. Changes in maximal performance of inspiratory and skeletal muscles during and after the 7.1-MPa Hydra 10 record human dive. Eur J Appl Physiol 2000;81:325–328.

[7] Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 2007;13:688–694.

[8] Hayashida K, Sano M, Ohsawa I, Shinmura K, Tamaki K, Kimura K, Endo J, Katayama T, Kawamura A, Kohsaka S, Makino S, Ohta S, Ogawa S, Fukuda K. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. Biochem Biophys Res Commun 2008;373:30–35.

[9] Fukuda K, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. Biochem Biophys Res Commun 2007;361:670–674.

[10] Megison SM, Horton JW, Chao H, Walker PB. A new model for intestinal ischemia in the rat. J Surg Res 1990;49:168–173.

[11] Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. Arch Surg 1970;101:478–483.

[12] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–358.

[13] Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464–478.

[14] Hillegass LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. J Pharmacol Methods 1990;24:285–295.

[15] Takagi K, Nakao M, Ogura Y, Nabeshima T, Kunii A. Sensitive colorimetric assay of serum diamine oxidase. Clin Chim Acta 1994;226:67–75.

[16] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.

[17] Tomatsuri N, Yoshida N, Takagi T, Katada K, Isozaki Y, Imamoto E, Uchiyama K, Kokura S, Ichikawa H, Naito Y, Okanoue T, Yoshikawa T. Edaravone, a newly developed radical scavenger, protects against ischemia-reperfusion injury of the small intestine in rats. Int J Mol Med 2004;13:105–109.

[18] Poussios D, Andreadou I, Papalois A, Rekka E, Gavalakis N, Aroni K, Kourounakis PN, Fotiadis C, Sechas MN. Protective effect of a novel antioxidant non-steroidal anti-inflammatory agent (compound IA) on intestinal viability after acute mesenteric ischemia and reperfusion. Eur J Pharmacol 2003;465:275–280.

[19] Ozacmak VH, Sayan H, Arslan SO, Altaner S, Aktas RG. Protective effect of melatonin on contractile activity and

oxidative injury induced by ischemia and reperfusion of rat ileum. Life Sci 2005;76:1575–1588.

[20] Luk GD, Bayless TM, Baylin SB. Plasma postheparin diamine oxidase. Sensitive provocative test for quantitating length of acute intestinal mucosal injury in the rat. J Clin Invest 1983;71:1308–1315.

[21] Abu-Zidan FM, Winterbourn CC, Bonham MJ, Simovic MO, Buss H, Windsor JA. Small bowel ischaemia-reperfusion increases plasma concentrations of oxidised proteins in rats. Eur J Surg 1999;165:383–389.

[22] Yao YM, Sheng ZY, Yu Y, Tian HM, Wang YP, Lu LR, Xu SH. The potential etiologic role of tumor necrosis factor in mediating multiple organ dysfunction in rats following intestinal ischemia-reperfusion injury. Resuscitation 1995;29:157–168.

[23] Yamada T, Murase N, Maeda T, Ye Q, Sakamoto T, Terakura M, Starzl TE, Todo S. Protective effect of TNF-alpha and IL-1 beta inhibitor FR167653 on ischemia-reperfusion injury in rat small intestinal transplantation. Transplant Proc 1998;30:2638.

[24] Sun Z, Wang X, Lasson A, Borjesson A, Leveau P, Haraldsen P, Andersson R. Roles of platelet-activating factor, interleukin-1beta and interleukin-6 in intestinal barrier dysfunction induced by mesenteric arterial ischemia and reperfusion. J Surg Res 1999;87:90–100.

[25] Nezu Y, Nezu Y, Shigihara K, Harada Y, Yogo T, Hara Y, Tagawa M. Effects of small intestinal ischemia and reperfusion on expression of tumor necrosis factor-alpha and interleukin-6 messenger RNAs in the jejunum, liver, and lungs of dogs. Am J Vet Res 2008;69:512–518.

[26] Keshav S, Lawson L, Chung LP, Stein M, Perry VH, Gordon S. Tumor necrosis factor mRNA localized to Paneth cells of normal murine intestinal epithelium by in situ hybridization. J Exp Med 1990;171:327–332.

[27] Ogle CK, Mao JX, Hasselgren PO, Ogle JD, Alexander JW. Production of cytokines and prostaglandin E2 by subpopulations of guinea pig enterocytes: effect of endotoxin and thermal injury. J Trauma 1996;41:298–305.

[28] Nezu Y, Tagawa M, Sakaue Y, Hara Y, Tsuchida S, Ogawa R. Kinetics of endotoxin concentration and tumor necrosis factor-alpha, interleukin-1beta, and interleukin-6 activities in the systemic and portal circulation during small intestinal ischemia and reperfusion in dogs. Am J Vet Res 2002;63:1680–1686.

[29] Buchholz BM, Kaczorowski DJ, Sugimoto R, Yang R, Wang Y, Billiar TR, McCurry KR, Bauer AJ, Nakao A. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. Am J Transplant 2008;8: 2015–2024.

[30] Nagata K, Nakashima-Kamimura N, Mikami T, Ohsawa I, Ohta S. Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. Neuropsychopharmacology 2009;34:501–508.

[31] Kajiyama S, Hasegawa G, Asano M, Hosoda H, Fukui M, Nakamura N, Kitawaki J, Imai S, Nakano J, Ohta M, Adachi T, Obayashi H, Yoshikawa T. Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. Nutr Res 2008;28:137–143.