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The effects of hydrogen-rich saline on the contractile and structural changes of intestine induced by ischemia–reperfusion in rats

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Abstract

Background. Hydrogen has been considered as a novel antioxidant that prevents injuries resulted from ischemia-reperfusion (I/R) injury in various tissues. The study was designed to determine the effect of hydrogen-rich saline on the smooth muscle contractile response to KCl, and on epithelial proliferation and apoptosis of intestine subjected to I/R.

Methods. Intestinal I/R injury was induced in Sprague-Dawley rats using bulldog clamps in superior mesenteric artery by 45 min ischemia followed by 1 h reperfusion. Rats were divided randomly into four groups: sham-operated, I/R, I/R plus saline treatment, and I/R plus hydrogen-rich saline treatment groups. Hydrogen-rich saline (> 0.6 mM, 6 ml/kg) or saline (6 ml/kg) was administered respectively via tail vein in 30 min prior to reperfusion. Following reperfusion, segments of terminal jejunum were rapidly taken and transferred into isolated organ bath and responses to KCl were recorded. Samples of terminal jejunum were also taken for measuring malondialdehyde and myeloperoxidase. Apoptosis in intestinal epithelium was determined with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling technique (TUNEL). Expression and distribution of proliferating cell nuclear antigen (PCNA) were detected with immunohistochemistry.

Results. Hydrogen-rich saline treatment significantly attenuated the severity of intestinal I/R injury with inhibiting I/R-induced apoptosis and promoting enterocytes proliferation. Moreover, Hydrogen-rich saline treatment significantly limited the neutrophil infiltration, lipid oxidation and ameliorated the decreased contractility response to KCl in the intestine subjected to I/R.

Conclusions. These results suggest that hydrogen treatment has a protective effect against intestinal contractile dysfunction and damage induced by intestinal I/R. This protective effect is possibly due to its ability to inhibit I/R-induced oxidative stress, apoptosis and to promote epithelial cell proliferation.

Keywords: Hydrogen, Intestinal ischemia/reperfusion, Antioxidant, Oxidative stress, Contractility, Apoptosis

Introduction

The intestinal ischemia-reperfusion (I/R) injury is a devastating syndrome which occurs in a variety of clinical settings including abdominal aortic aneurysm, small intestinal transplantation, strangulated hernia, and neonatal necrotizing enterocolitis. The consequence of intestinal I/R injury not only alternates absorptive function of intestine, but also may cause intestinal bacteria colony shifting, and even lead to the multiple organ failure (MOF) [1-4]. The small intestine is highly sensitive to I/R. The alterations in intestinal motor function and mucosal integrity observed in intestinal I/R have been widely documented. Reduced gut motility, decreased contractile response [5, 6] and delayed intestinal transit [7] have been shown to result from intestinal I/R generated in various experimental models. It has been demonstrated that occlusion of the superior mesenteric artery (SMA) followed by reperfusion can cause apoptosis in the intestinal epithelium [8].

Overproduction of toxic hydroxyl radicals has been shown to play an important role in the pathogenesis of intestinal I/R injury, which thereby promoting the lipid peroxidation, DNA oxidation, thiyl radical formation and mitochondrial depolarization and eventually leading to cellular apoptosis and necrosis [9]. Toxic hydroxyl 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, and leading to cell death and necrosis [10]. Hydrogen (H_2), which could react with hydroxyl radical to produce water, was reported to be a novel antioxidant and has protective effect on transplantation induced intestinal graft injury [11], chronic liver inflammation [12], arteriosclerosis [13], and myocardial I/R injury [14]. Recently, Ohsawa et al. revealed that inhalation of H_2 gas markedly suppressed brain injury after cerebral I/R injury by buffering the effect of oxidative stress [15]. They found that H_2 could selectively reduce the hydroxyl radical, the most cytotoxic of reactive oxygen species (ROS), and effectively protect cells.

In addition, in our recent work it has been found that hydrogen-rich saline has a protective effect on intestinal I/R [16], lung injury induced by intestinal I/R injury [17]

and neonatal cerebral hypoxia-ischemia [18] in rats, which could be more convenient and appropriate in clinical practice as H_2 gas inhalation is potentially flammable and explosive. The protective effect of hydrogen-rich saline on the small intestine I/R injury had been revealed possibly by reduction of inflammation and oxidative stress [16]. However, none of the previous studies evaluated the effects on intestinal contractile response, and proliferation, apoptosis in villus and crypt of epithelial cells. The study was to further investigate the protective effect of hydrogen-rich saline on intestinal I/R injury.

Materials and methods

Animals. Six-week-old male Sprague-Dawley rats (180 - 200 g) were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences and fed with standard laboratory chow *ad libitum*. All rats received humane care according to the Guide for the Care and Use of Laboratory Animals by the Chinese Academy of Sciences.

Hydrogen-rich saline preparation. The hydrogen-rich saline was prepared as we previously described [18]. Briefly, hydrogen was dissolved in normal saline for 2 h under high pressure (0.4 MPa) to the supersaturated level using a self-designed hydrogen-rich water-producing apparatus. The saturated hydrogen saline was stored under atmospheric pressure at 4°C in an aluminum bag without dead volume. Hydrogen-rich saline was freshly prepared every week to ensure a constant concentration more than 0.6 mM.

Experimental design. The intestinal I/R injury was performed as previously described [19]. Briefly, the rats were anesthetized with 10% chloralhydrate (4 ml/kg, i.p.) and a laparotomy was performed. The SMA was identified and carefully isolated by blunt dissection. A micro-bulldog clamp was placed at the root of SMA to cause complete cessation of blood flow for 45 min, and thereafter the clamp was loosened to form reperfusion injury. During procedure, animals were positioned under a heating lamp in order to prevent heat loss. The animals were randomly divided into four groups as described: (1) Sham-operated group (n = 8): All the surgical steps were performed, except that intestinal I/R injury was not induced. Animals were kept under

anesthesia for the duration of the intestinal I/R procedure. The sham group served as control of I/R group; (2) I/R group (n = 8): the SMA was occluded for 45 min followed by 1 h reperfusion, which served as controls of saline- or hydrogen-rich saline-administered groups; (3) I/R + saline (n = 8): the SMA was occluded for 45 min followed by 1 h reperfusion, but saline solution (6 ml/kg) was administered via tail vein 30 minutes before reperfusion; (4) I/R + hydrogen-rich saline (n = 8): hydrogen-rich saline (6 ml/kg) instead of saline was administered via tail vein 30 minutes before reperfusion. Rats were sacrificed at the end of the reperfusion. Intestinal tissue was taken for detection as follow.

***In Vitro* jejunal smooth muscle contractility.** The segment of terminal jejunum contractility was measured as previously described [20]. Briefly, a 0.5 cm segment of jejunum from each rat was obtained and intestinal content was cleared by flushing with saline solution. Each jejunal segment was mounted on two wire hooks that traversed the length of the segment under 2.45 mN (0.25 g weight) passive load in a 5 ml organ bath. The organ bath was filled with modified Kreb's solution (mM: NaCl 119, KCl 4.7, $CaCl_2$ 1.6, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 22, HEPES 8, Glucose 10), gassed with a mixture of CO_2 5% and O_2 95% and kept at 37°C. The modified Kreb's solution was changed every 15 min. After a 60-min period of equilibration at 2.45 mN for of basal tension, the spontaneous contractility was recorded for 45 min. Tension was recorded with RM6240 Multichannel Acquisition System for Physiological Signals (Chengdu instrument factory, Chengdu, China) at a sampling rate of 0.4 s. After finishing the spontaneous contractility detection, the induced contractile activity was further measured in organ bath under depolarizing stimulation by elevating potassium chloride concentration to 30 mM in modified Kreb's solution. Active force development was recorded for 15 min, which was normalized by converting the tension divided by jejunal segment weight after blotted dry (g/ g tissue), and reported as g/g tissue/s. In the preparation of high K^+ solutions, NaCl was exchanged for equimolar amounts of KCl in order to maintain the physiological osmolarity of the Kreb's solution.

Histological and immunohistochemical assessment. Jejunal specimens were

embedded in paraffin after 48 h fixation with 10% neutral buffered formalin, then sectioned and stained with hematoxylin-eosin (HE) for histopathological examination. To determine the number of proliferating cells in intestine epithelium, the expression and distribution of proliferating cell nuclear antigen (PCNA) were detected with immunohistochemical method. Briefly, these specimens were dewaxed and incubated with 3% H_2O_2 in methanol at 37°C for 10 min to quench endogenous peroxidase activity. After blocked at room temperature for 30 min, the sections were incubated with mouse monoclonal antibody against PCNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. The labeled antigen was visualized with an Envision^{TM} detection kit (Gene Tech, Shanghai, China) followed by the diaminobenzidine reaction. Finally, the specimens were counterstained by immersion in hematoxylin. The positive staining cells and their distribution were observed under 400 times microscope. Sixty intestinal villi and crypts per time point were required for counting, and then the ratio of positive cells were calculated and analyzed, respectively.

Enterocyte apoptosis. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling method (TUNEL) was used for histological identification of apoptotic cells. Briefly, the slides were digested with proteinase K, incubated with terminal deoxyribonucleotidyl transferase enzyme, and subsequently incubated with antidigoxigenin peroxidase, using the in situ apoptosis detection kit (R&D Systems, Minneapolis, MN, USA) according to the protocol. Peroxidase was applied for color development under microscopic control. Apoptotic cells were identified as those with a brown-stained nucleus. Only the intestinal epithelial cells were counted. TUNEL-positive intestinal epithelial cells (%) were quantified on image analysis software (Image J, NIH, USA) by investigators blinded to the samples, in which the apoptotic index was defined as the proportion of TUNEL-positive cells to the total number of intestinal epithelial cells at villi or crypts in 20 nonoverlapping serial scopes taken from each slide, beginning from a random start, at a magnification of ×400.

Intestinal Malondialdehyde (MDA) content. Intestinal MDA content was measured

to evaluate the severity of intestinal peroxidation injury by thiobarbituric acid colorimetric method using an MDA assay kit (Nanjing Jiancheng Corp., Nanjing, China). The intestine was 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. The absorbance of the supernatant was measured by spectrophotometry at 532 nm for MDA content, as MDA reacted with thiobarbituric acid and formed a pink, maximum absorbent complex at 532 nm wavelength [21]. The MDA concentration was calculated from the standard curve and expressed as nmol/mg protein.

Intestinal myeloperoxidase (MPO) activity assay. For MPO activity assay, the intestine was harvested and homogenized immediately on ice in five volumes of normal saline. The MPO activity was measured using an MPO assay kit (Nanjing Jiancheng Corp., Nanjing, China), following the manufacturer's recommendations. One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 37 °C and MPO activity of tissue was expressed as unit per gram (U/g).

Statistical analysis. Values were presented as mean ± SD. Statistical analysis was done using the SPSS 13.0 by one-way analysis of variance (ANOVA) to establish whether the difference among the four groups was statistically significant. Between groups, variance was determined using the Student-Newman-Keuls post hoc test. *P* value less than 0.05 was considered statistically significant.

Results

Hydrogen-rich saline treatment ameliorated intestinal I/R injury

The effects of hydrogen-rich saline treatment on histological changes of jejunal tissue in rats with intestinal I/R injury were showed in Fig. 1. The typical histological features which occurred in I/R and I/R + saline-treated groups were characterized 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.

Quantitative immunohistochemical results for PCNA expression were evaluated in Fig. 2. PCNA was weakly expressed in intestinal tissues of the sham-operated group, with positive particles mostly distributing in the nuclei of the lower third of crypt cells.

The positive cellular rate for PCNA in the I/R, I/R + saline-treated and I/R + hydrogen-rich saline groups markedly increased at intestinal villi compared with the sham-operated group ($P < 0.01$). However, at intestinal crypts the hydrogen-rich saline administration significantly elevated the positive rate of PCNA ($P < 0.01$). The intestinal epithelial cells located in the intestinal wall with nucleus-positive staining and non-staining plasma were identified as intestinal apoptotic cells on TUNEL assay. There were few apoptotic cells in sham-operated group. The apoptotic index in I/R and saline-treated groups markedly increased compared with sham-operated group ($P < 0.01$). When rats treated with hydrogen-rich saline, fewer TUNEL-positive cells were seen and the apoptotic index significantly decreased as compared to the I/R or I/R + saline-treated group ($P < 0.01$; Fig. 2).

Hydrogen-rich saline recovered intestinal smooth muscle contractility after I/R

As shown in Fig. 3A, the jejunal segments from sham-operated animals generated regular spontaneous contractions and the intestinal contractile activity totally disappeared in the intestinal I/R injury and saline, hydrogen-rich saline treated groups. After stimulation with potassium chloride at 30 mM, all groups demonstrated a sustained increase in tension. Although it appeared more regular and rhythmic, the KCl induced intestinal contractile response in I/R + saline group was intermittent and weak. However, in hydrogen-rich saline treated group it was continuous and much stronger as compared with I/R + saline group. Compared with I/R or I/R + saline group, the intestinal smooth muscle contractility in the hydrogen-rich saline treatment animals recovered more significantly ($P < 0.01$), but the enhanced contractility in hydrogen-rich saline treated group was still significant lower than that in sham-operated animals ($P < 0.05$; Fig. 3B).

Hydrogen-rich saline treatment reduced oxidative stress after I/R

As an indicator of lipid peroxidation, MDA content reflected the level of oxygen free radical and inflammatory response of the tissue. The intestinal MDA content increased significantly in intestinal I/R and I/R + saline groups when compared with sham-operated group ($P < 0.05$). More importantly, treatment of rats with hydrogen-rich saline at a dose of 6 ml/kg significantly attenuated the increasing in

intestinal MDA level ($P < 0.05$; Fig. 4A).

Hydrogen-rich saline attenuated neutrophil infiltration after I/R

To estimate the leukocyte recruitment to intestine tissue, MPO activity examined as an indicator of neutrophil infiltration and accumulation in tissues was measured. The results showed a significant increase in MPO activity in tissues of intestinal I/R and I/R + saline-treated groups as compared with that in the sham-operated group ($P < 0.05$). Treatment with hydrogen-rich saline 30 min before reperfusion at 6 ml/kg, however, clearly attenuated the increase in MPO activity in intestine ($P < 0.05$; Fig. 4B), suggesting that hydrogen prevents leukocyte recruitment to the intestine.

Discussion

This study demonstrated that hydrogen-rich saline treatment reduced the severity of intestinal I/R injury in a rat model. The protective effect of hydrogen against I/R in intestinal epithelium is supported by the results from ameliorated histological findings, as well as its abilities to inhibit I/R-induced apoptosis and to promote epithelial cell proliferation. In addition, hydrogen-rich saline treatment has been shown significantly recovers intestinal smooth muscle contractility after intestinal I/R, accompanied by reduction of the MDA level and MPO activity in the intestinal tissues.

It is well recognized that the small intestine is very sensitive to the deleterious effects of I/R. Normally, the intestinal epithelium undergoes rapid and continuous self-renewal along the crypt-villus axis and it keeps a delicate balance between proliferation and apoptosis. Several studies have suggested that I/R injury can induce an apoptotic response in the adult rat intestinal epithelium, although they did not quantitate this response along the crypt-villus axis [8, 22]. Meanwhile, as PCNA is a significant cell-cycle regulated nuclear protein for DNA-polymerase, the PCNA-labeled nuclei had been shown to identify cells in the late G1 and early S phases of the cell cycle, as well as cells undergoing DNA repair [23, 24]. In present study, we found that intestinal I/R injury, following occlusion of the SMA, led to statistically significant increment of expression of PCNA and apoptosis in undifferentiated epithelial cells located in the proliferative compartment of the adult rat's small intestine, as well as increment of apoptosis in differentiated epithelial cells

distributed in the villus. However, the administration of hydrogen-rich saline could significantly increase protein expression of PCNA and reduce apoptosis in mucosal cells, especially in crypt epithelial cells.

Oxidative stress plays an important role in the intestinal I/R injury. The intestinal mucosa is known to be particularly sensitive to I/R generated ROS injury. The I/R injury leads to the release of ROS or reactive nitrogen species (RNS), such as superoxide anion, hydroxyl radical, hydrogen peroxide and peroxynitrite [25]. During the reperfusion, when the oxygen was re-supplied, tissue injury could be further exacerbated depending on the time and intensity of the ischemia. Meanwhile, it produced excess of xanthine oxidase and oxygen free radicals, which would aggressively and indiscriminately damage cellular macromolecules, including DNA, proteins and lipids [26]. As has been demonstrated previously, the development of I/R injury is associated with two representative pathological processes: neutrophil accumulation and lipid peroxidation [17]. Hence, two corresponding biochemical markers, MPO activity and MDA content, were also evaluated in present study. We found that the MDA content was upregulated after intestinal I/R injury and remarkably downregulated after hydrogen-rich saline administration. In addition, another hallmark of intestinal reperfusion injury as determined by MPO activity was downregulated after hydrogen-rich saline treatment as well.

The potential for hydrogen to scavenge free radicals has been proposed by several studies, especially the hydroxyl radical and peroxynitrite anion [15]. The hydroxyl radical is the neutral form of the hydroxide ion and the normal product in the cell metabolism. The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of approximately 10^{-9} s [27]. In addition, cells produce both the superoxide anion and nitric oxide (NO) during the oxidative burst triggered during inflammatory processes [28]. Under these conditions, superoxide anion and NO may react together to produce significant amounts of oxidative active molecule, peroxynitrite anion, which is a potent oxidizing agent that can cause DNA fragmentation and lipid oxidation [27]. Peroxynitrite anion also reacts with carbon dioxide (CO_2) present in biological fluids to form reactive intermediates

that can oxidize thiols and nitrate phenolic compounds, such as tyrosine [27]. After intestinal I/R injury, ROS and RNS, such as the hydroxyl radical, superoxide anion, hydrogen dioxide (H_2O_2), NO, peroxynitrite anion, appear to play a critical role in cell death. Moreover, supplementation of antioxidant has been reported to successfully attenuate I/R injury-induced tissue damage [29-31]. As a novel antioxidant, hydrogen has been used in suppressing brain injury after cerebral I/R injury and neonatal cerebral hypoxia-ischemia, and improving lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance by selectively reducing cytotoxic oxygen radicals, and revealing marked therapeutic potential [13, 15, 18, 32-34]. More recently, it had also been demonstrated that hydrogen-rich saline treatment has a protection effect on lung injury induced by intestinal I/R injury [17]. Therefore, the ability of hydrogen to reduce or eliminate hydroxyl radical and peroxynitrite anion may be responsible for the protective effect in intestinal I/R observed in this study.

The majority of the intestinal epithelial cells are absorptive enterocytes that are responsible for the absorption of digested nutrients and fluids. Based on previous studies, 30 min of intestinal ischemia followed by immediate reperfusion significantly decreased mucosal substrate absorption when compared with uninjured, control rat intestine [35]. Utilizing a rat model of intestinal I/R, previous work had shown that epithelial damage/apoptosis caused both acute and chronic alterations of the intestinal absorptive function, which featured as significantly decreased D-xylose absorption lasted for 4 week and became abnormal again at 8 and 12 weeks, and significantly increased fecal fat excretion at 4 and 12 week after intestinal I/R compared to the sham control [36]. The decreased intestinal absorptive capacity after I/R may, in fact, reflect the other important side of the pathophysiological changes of the intestinal I/R, and the issue whether the hydrogen-rich saline treatment improve intestinal absorption still need to be further investigated in our model of intestinal I/R.

In summary, our present study, for the first time, show that hydrogen-rich saline restores the reduction of intestinal smooth muscle contractile response to KCl in intestinal I/R model. Our results suggest that hydrogen-rich saline treatment may ameliorate structural and functional damages observed in an experimental I/R, due

mainly to reducing inflammation and inhibiting lipid peroxidation. Application of hydrogen-rich saline treatment shows promising results in animal model and may become powerful tools in the clinical treatment of acute intestinal I/R injury that occurs in intestinal transplantation and small intestinal surgery.

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Figure legend

Fig. 1. Representative photomicrographs of the rat intestine sections from the sham-operated, I/R, I/R + saline and I/R + hydrogen groups after 45 min of intestinal ischemia followed by 1 h of reperfusion. Normal histopathology presented in sham-operated group. The features which occurred in I/R and I/R+saline groups were characterized 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. Routine hematoxylin and eosin stained (×100 upper panel, ×200 lower panel).

Fig. 2. The effect of hydrogen-rich saline treatment on enterocytes proliferation and apoptosis. Rats were subjected to a 45 min of intestinal ischemia followed by 1 h reperfusion and the hydrogen-rich saline was administered 30 min prior to reperfusion. Representative photographs of immunohistochemistry and TUNEL used to determine expression of PCNA (upper column, ×200) and apoptosis (lower column, ×100) in intestinal epithelium were taken in sham-operated, I/R, I/R+saline and I/R+hydrogen groups (A). Significant increase in PCNA expression (B) and inhibition of I/R-induced apoptosis (C) were found in I/R+hydrogen group in comparison with I/R

or I/R+saline group (* $P < 0.01$). Data were represented by mean ± SD, n = 8.

Fig. 3. The effect of hydrogen-rich saline treatment on intestinal contractile activity. Rats were subjected to a 45 min of intestinal ischemia followed by 1 h reperfusion and the hydrogen-rich saline was administered 30 min prior to reperfusion. The contractility of intestinal smooth muscle in rats was measured in sham-operated, I/R, I/R+saline and I/R+hydrogen groups. (A) Representative traces of jejunal smooth muscle contractile activity (Left column, basal spontaneous activity; right column; 30 mM KCl induced contractile response). (B) Calculated jejunal smooth muscle contractility in response to 30 mM KCl. Significant improvement was measured in animals treated with hydrogen-rich saline (* $P < 0.01$ vs. I/R or I/R+saline group), but the enhanced contractility still significant lower than that in sham-operated animals (** $P < 0.05$). Data were represented by mean ± SD, n = 8.

Figure 4. Jejunal tissue levels of malondialdehyde (MDA) and myeloperoxidase (MPO) in sham-operated, I/R, I/R+saline and I/R+hydrogen groups. Compared with the I/R or I/R+saline group, the MDA and MPO levels in the I/R+hydrogen group were both significantly decreased (* $P < 0.05$ vs. I/R or I/R+saline group). Data were expressed as mean ± SD, n = 8.

Figure

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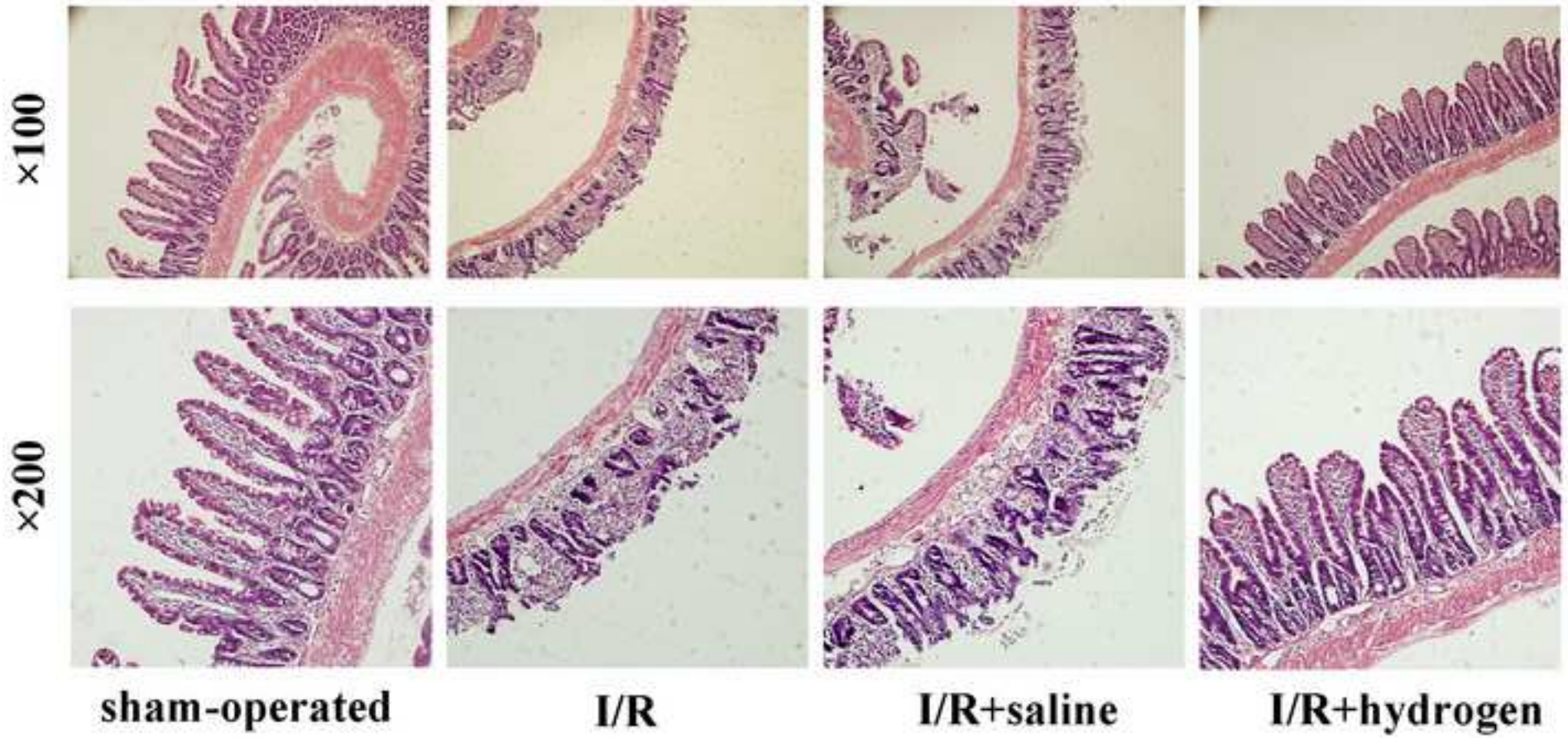

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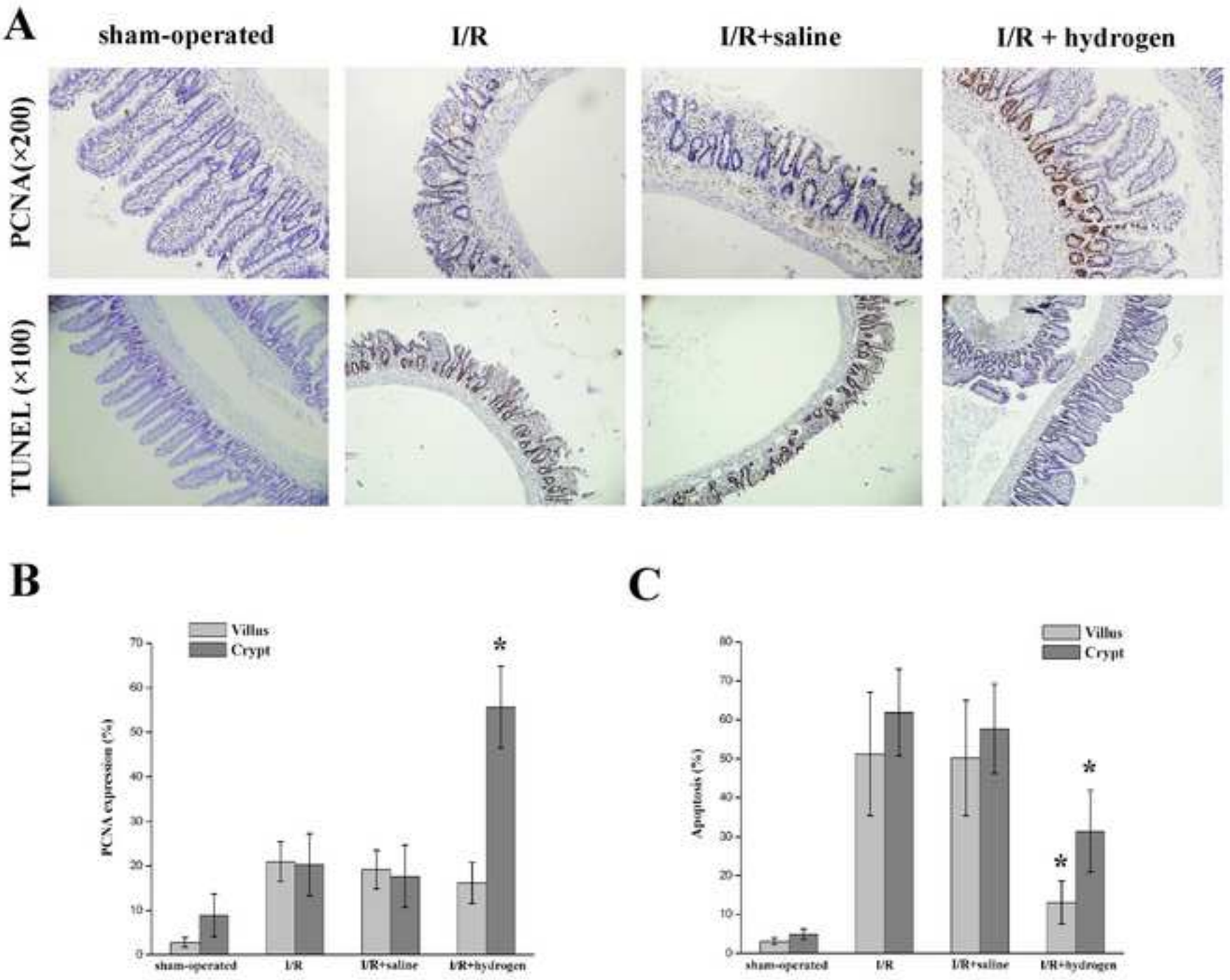

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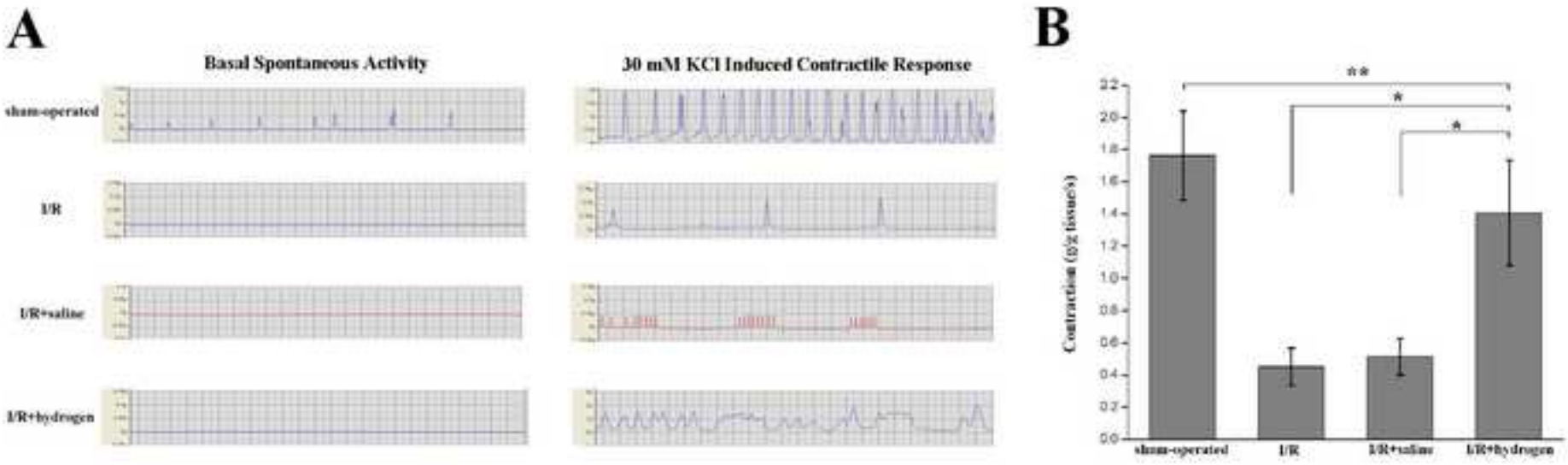

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