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Title: Hydrogen-rich saline protects against renal ischemia/reperfusion injury in rats

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Dear editor:

Enclosed please find a revised manuscript “Hydrogen-rich saline protects against renal ischemia/reperfusion injury in rats” (JSURGRES-D-10-00797). We thank the reviewers for their careful review and insight comments. We have made some changes in the manuscript (shown in red colour in the revised manuscript). We believe that we have carefully addressed all the concerns. In doing so, the paper has been improved and strengthened. What follows is a response (shown in red colour) to the reviewers:

Reviewer #1: Subject: Hydrogen-rich saline protects against renal ischemia/reperfusion injury in rats

1. Summary

1.1. This is the report of a basic project in a rat model of renal ischemia and reperfusion.

1.2. The authors created four groups of rats with N=8 in each group. The groups included (1) sham-operated plus physiological with saline treatment; (2) renal I/R plus physiological saline treatment; (3) renal I/R plus hydrogen-rich saline treatment; and (4) renal I/R plus nitrogen-rich saline treatment.

1.3. All had the same surgical procedure. Groups 2, 3 and 4 had a renal ischemia and reperfusion injury.

1.4. Twenty four hours later, the animals were sacrificed and studied.

1.5. The outcome variables included serum measures of renal function, light microscopy of the kidney, TUNEL assays for apoptosis, renal MDA levels, Renal 8-hydroxydeoxyguanosine (8-OHdG) Levels Assay, activities of superoxide dismutase (SOD) and catalase (CATRenal Myeloperoxidase (MPO) ActivityDetermination of TNF-a, IL-1<beta> and IL-6 Levels

1.6. The inter-group differences of the data were tested by one-way ANOVA followed by LSD-t Test for multiple comparisons.

1.7. The manuscript includes one table, five figures and 20 references.

2. Comments

2.1. The manuscript is precise and well written.

Response: Thanks for your comment.

2.2. The data appears to support the authors' conclusions.

Response: Thanks for your comment.

3. Recommendation: Accept for publication.

Reviewer #2: Wang et al evaluated the effects of intraperitoneal hydrogen-rich saline on a host of factors after renal ischemia-reperfusion injury and demonstrated a reduction in oxidative stress and inflammation. The findings from this study are compatible with studies of the effects of hydrogen-rich saline on ischemia-reperfusion injury to other organs.

1. The authors might want to reference a paper using a slightly different model of renal ischemia-reperfusion that demonstrated a decrease in 8-OhdG after intravenous injection (J Anesth 2010 Aug 24(4):569-74).

Response: We have added this paper as a reference in discussion section of the revised manuscription (Line 4, Page 10).

2. Why did the authors choose 5 minutes prior to reperfusion for the timing of the hydrogen rich saline? Also why intraperitoneal instead of intravenous?

Response: We referenced a paper to determin 5 minutes prior to reperfusion for the timing of the hydrogen-rich saline (Nature Medicine 2007 May 13:688 – 694). This referenced paper revealed that hydroen exerted its protective effect only when it was inhaled during reperfusion; Howerver, when hydrogen was inhaled during ischemia, brain infarct volume was not significantly decreased. In another referenced paper that investigated the effect of inhaled hydrogen on mycaridal ischemia-reperfusion injury, the arterial hydrogen levels started to increase 2 min after inhalation of hydrogen and reached a maximum level after 5 min. The incremental rate of hydrogen saturation for the non-ischemic myocardium was similar to that observed in arterial blood (Biochem Biophys Res Commun 2008 Aug 373(1):30-5). Because hydrogen is permeable to cell membranes

and can target organelles, according to the above two paper, we speculate that hydrogen concentration may be very high in the time of reperfusion initiation by adopting 5 minutes prior to reperfusion for the timing of hydrogen-rich saline in order to achieve the significantly protective effective on ischemia-reperfusion injury.

Also why intraperitoneal instead of intravenous?

Response: Our group has already done several experiments of the protective effects of intraperitoneal injection of hydrogen-rich saline on ishcemia-reperfusion-induced injury to other vulnerable organs (Brain Res 2009 Feb 1256:129-37, Exp Biol Med 2009 Oct 234(10):1212–1219) and discovered the more significantly protective effects of hydrogen-rich saline on injuried organs by intraperitoneal injection than by intravenous injection. A possible reason, we suggest, is that hydogen dissolved in the saline is more likely to diffuse in the lung by intravenous injection than by intraperitoneal injeciton due to the low solubility of hydrogen, which might be solved by intravenous infusion and we will conduct comparative research between intraperitoneal injection and intravenous infusion in the near future.

3. There are a number of papers from the Shanghai group looking at the effects of hydrogen-rich saline on ischemic injury to other organs, and all demonstrate reduction in inflammatory and oxidative markers. The authors have demonstrated an effect on renal ischemia-reperfusion on a wide range of markers. Could the authors expound further on how they plan to elucidate the effects of hydrogen-rich ischemia, now that they have demonstrated effects in multiple organs?

Response: Kidney is rich in blood supply thus vulnerable to ischemia-reperfusion (I/R) injury. I/R is the primary cause of acute kidney injury (AKI), particularly in patients hospitalized in intensive care units. AKI often leads to renal cell death, delayed graft function, renal graft rejection, and permanent impairment of renal function. Mortality due to AKI remains high and unchanged in the last several decades (Nat Clin Pract Nephrol 2006 July 2:364–77), which makes it urgent to develop a new strategy to protect against renal ischemia-reperfusion injury in several clinical settings such as shock, kidney

transplantation and suprarenal AAA repair. Although studies have demonstrated a protective effect of hydrogen-rich saline on I/R injury in multiple organs (brain, intestine, lung, liver and heart), the effect of hydrogen-rich saline on renal I/R injury need to be elucidated further.

4. There is certainly clinical applicability of a fluid that can improve outcome after renal ischemia -- i.e. during suprarenal AAA repair. Are there any potential harms from intraperitoneal use of hydrogen rich saline?

Response: In a randomized, double-blind, placebo-controlled, crossover clinical trial which investigated the effect of supplementation of hydrogen-rich water on type 2 diabetes, intake of hydrogen-rich saline orally improved lipid and glucose metabolism without any adverse effects (Nutr Res 2008 Mar 28(3):137-43). As far as we known, no clinical trials have been conduted investigating the effect of hydrogen-rich saline by intraperitoneal use or intravenous use. The potential harms from intraperitoneal use of hydrogen-rich saline, we speculate, include possible intestinal perforation, possible peritoneal infection and possible intraperitoneal hemorrhage. It is concerned that intraperitoneal use of hydrogen-rich saline might exacerbate renal function in the case of ischemia-reperfusion injury. If so, hydrogen can be dissolved in peritoneal dialysis solution instead of saline and hydrogen-rich peritoneal dialysis solution might supply hydrogen as effectively as hydrogen-rich saline. All of the concerns on intraperitoneal use of hydrogen-rich saline need to be explored in the future studies.

Thanks for your help in evaluating our manuscript and for your care in shepherding this paper through review. We appreciate the careful and thoughtful comments of you and the reviewers in evaluating our work.

Yours sincerely,

Dr. Wang Fei

Revised (2010-10-25)

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

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Abstract

Backgrounds/Aims: Recently it has been demonstrated that hydrogen, as a novel antioxidant, can selectively reduce hydroxyl radicals (·OH) and peroxynitrite anion (ONOO-) in vitro and exert therapeutic antioxidant activity in many diseases. This study was designed to investigate the effect of hydrogen-rich saline on the renal ischemia/reperfusion (I/R) injury in rats. ***Methods:*** A rat model of renal I/R injury was induced by 45-min occlusion of the bilateral renal pedicles and 24-h reperfusion. Physiological saline, hydrogen-rich saline or nitrogen-rich saline (8 ml/kg) was administered intraperitoneally at 5 min before reperfusion, respectively. ***Results:*** After I/R injury, serum BUN, Cr, tissue MDA, 8-OhdG, TNF-a, IL-1β, IL-6 levels and MPO activity were all increased significantly, while tissue SOD and CAT activities were all decreased significantly. Hydrogen-rich saline reversed these changes and relieved morphological renal injury and I/R-induced apoptosis, while no significant changes was observed in the nitrogen-rich saline-treated group compared with physiological saline-treated group. ***Conclusions:*** Hydrogen-rich saline is able to attenuate the renal I/R injury, which is possibly by reduction of oxidative stress and inflammation.

Key words: ischemia/reperfusion; hydrogen; antioxidant; oxidative stress

Introduction

Renal I/R injury is common in several clinical situations, including renal transplantation and shock. I/R-induced acute renal failure is associated with decreased allograft survival in patients with transplanted kidneys and high mortality and morbidity in patients with native kidneys [1]. It was demonstrated that reactive oxygen species (ROS) and reactive nitrogen species (RNS) increase in the areas of ischemia and reperfusion, which are responsible for renal damage. Meanwhile, inflammation also plays an important role in the pathogenesis of renal I/R injury, through leukocyte activation and expression of adhesion molecules and cytokines [2-7]. Free radicals and pro-inflammatory cytokines 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.

Recently, it has been demonstrated that molecular hydrogen could selectively reduce cytotoxic ROS and RNS, such as ·OH and ONOO- in vitro and exert therapeutic antioxidant activity in a rat middle cerebral artery occlusion model [8]. However, the potential effect of hydrogen on renal I/R injury has not been examined. Therefore, the present study investigated the possible therapeutic effects of hydrogen-rich saline on renal I/R injury in rats.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 200–220g were given free access to normal rat diet and tap water and maintained in a temperature-controlled room with a 12:12-h light/dark cycle (lights on at 06:00 h). All procedures performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the use of experimental animals.

Hydrogen-rich Saline Production

Hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4MPa) to a supersaturated level using hydrogen-rich water-producing apparatus produced by our department. The saturated hydrogen saline was stored under atmospheric pressure at 48C 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 0.6mmol/L was maintained. Gas chromatography was used to confirm the content of hydrogen in saline by the method described by Ohsawa et al [8].

Renal Ischemia/Reperfusion

The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and placed in a supine position. Body temperature was maintained between 36°C and 37°C. After laparotomy and dissection of both renal pedicles, bilateral ischemia was induced by occluding the renal pedicles with atraumatic microvascular clamp for 45 min. The immediate color change of the kidneys signifying the stoppage of blood flow, indicates successful occlusion. During reperfusion, clamps were removed and the blood flow to the kidneys was re-established with visual verification of blood return.

Experimental Groups

Animals were divided into four groups consisting of 8 rats each: (1) sham-operated plus physiological saline treatment; (2) renal I/R plus physiological saline treatment; (3) renal I/R plus hydrogen-rich saline treatment; (4) renal I/R plus nitrogen-rich saline treatment. Sham-operated animals underwent the same surgical procedures except that the bilateral renal pedicles were not clamped. Physiological saline, hydrogen-rich saline or nitrogen-rich saline (8 ml/kg) was intraperitoneally injected at 5 min before the

reperfusion, respectively. Twenty-four hours after reperfusion initiation, blood was drawn from the abdominal inferior cava vein immediately before induced death. The left kidney was obtained and perfused with phosphate buffered saline (PBS) to remove all blood, then weighed and stored at -80°C until assayed. Blood samples were spun at 1000 rpm for 15 min and serum samples were collected.

Analysis of Renal Function

Serum blood urea nitrogen (BUN) and creatinine (Cr) was used for the evaluation of renal function. The samples were analyzed on a COBAS Mira chemical analyzer (Roche, Basel, Switzerland), using commercial kits from Sigma (St Louis, MO, USA).

Histological Analyses

Renal samples were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin eosin. Evaluation was performed with light microscopy without knowledge of the study groups. Assessment was carried out by expertise observers.

In Situ Apoptosis Assay

Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) assay was performed to detect apoptosis in situ. 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 (Oncogene Research products, Boston, USA) according to the protocol. Apoptotic cells were identified as those with a brown-stained nucleus. TUNEL-positive renal tubular 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 cells in 20 non-overlapping serial scopes taken from each

slide, beginning from a random start, at ×400 magnification.

Renal Malondialdehyde (MDA) Measurement

Renal MDA levels were determined using a BIOXYTHCH MDA-586 Assay kit (OxisRe-search, Oregon, USA). Briefly, frozen renal tissues were homogenized in the presence of butylated hydroxytoluene. After centrifugation, free MDA in the supernatant was converted to a stable carbocyanin dye (maximum absorption at 586 nm) by the chemical reaction with N-methyl-2-phenylindole. Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA) using BSA as a standard. MDA levels were normalized against protein (umol/mg).

Renal 8-hydroxydeoxyguanosine (8-OHdG) Levels Assay

The supernatant from kidney homogenate was prepared for detecting the levels of 8-OHdG levels with a commercial ELISA kit following the instructions of the manufacturer. Briefly, the samples were added to plate wells precoated with rat monoclonal anti-8-OHdG antibody (Japan Institute for the Control of Aging, Fukuroi, Japan). They were incubating for 120 min at 37°C. After washed for 3 times, the wells were treated with Biotinylated rat monoclonal anti-8-OHdG antibody for 60 min at 37°C. After washed for 3 times, the wells were treated with Streptavidin-Horseradish Peroxidase(HRP) for 30 min at 37°C. After the wells were washed for 3 times, a substrate containing 3,3’,5,5’-tetramethylbenzidine(TMB) was added and the wells were incubated for 15 min at 37°C. The reaction was terminated by the addition of a sulphuric acid. The absorbance was read at a wavelength of 450nm.

Antioxidant Enzymatic Activity Assay

The renal tissue homogenates were prepared in chilled PBS (0.1 M, pH 7.4), and were centrifuged at 10, 000 g at 4°C for 10 minutes. The supernatants were collected, aliquoted, stored at -80°C until the

following analysis.

The activities of superoxide dismutase (SOD) and catalase (CAT) were measured using commercial kits purchased from CaymanChemical Company (Ann Arbor, MI, USA). According to the manufacturer's instructions, total SOD activity was assayed by detecting superoxide radicals generated by xanthine oxidase and hypoxanthine. The reaction was monitored at 450 nm and one unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The CAT activity was assayed by measuring the reduction of hydrogen peroxide at 540 nm and one unit was defined as the amount of enzyme that would cause the formation of 1.0 nmol of formaldehyde per minute at 25°C. All spectrophotometric readings were performed by using a spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA). All assays were conducted in triplicates. The tissue protein concentration was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

Renal Myeloperoxidase (MPO) Activity Assay

The supernatant from kidney homogenate was prepared for detecting the activity of MPO, an indicator of neutrophil infiltration in the renal tissue. MPO activity was defined as the quantity of enzyme degrading 1μmol of peroxide per minute at 37℃ and was expressed in unit per gram weight of wet tissue. The change in absorbance was measured spectrophotometrically at 590 nm by spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA).

Determination of TNF-a, IL-1β and IL-6 Levels in the Renal Tissues

The supernatant from kidney homogenate was prepared for detecting the levels of TNF-a, IL-1β and IL-6 with a commercial enzyme-linked immunosorbent assay (ELISA) kit following the instructions of the manufacturer. The absorbance was read on a microplate reader and the concentrations were calculated

according to the standard curve. Protein content in the sample was determined by Coomassie blue assay and the results were corrected per microgram of protein.

Statistical Analysis

Results were expressed as mean ± SD. Statistical analysis was performed with the SPSS11.0 software package. The inter-group differences of the data were tested by one-way ANOVA followed by LSD-t Test for multiple comparisons. A P value of less than 0.05 was considered to be statistically significant.

Results

Hydrogen-rich Saline Treatment Ameliorated Renal Function

After 24 h reperfusion, serum BUN and Cr levels were used to assess the effect of hydrogen-rich saline on renal I/R injury. As shown in Fig.1, compared with I/R group, hydrogen-rich saline treatment had a significantly reduction in serum BUN and Cr levels, while nitrogen-rich saline treatment showed no significant reduction.

Hydrogen-rich Saline Treatment Attenuated Renal Tissue Injury

The rats of renal I/R group demonstrated an increased infiltration of neutrophils, and had more significant tubular necrosis and apoptosis than those of hydrogen-rich saline treatment group. This injury also included widespread loss of tubular dilation, and cast formation. Kidneys of hydrogen-rich saline treatment group rats had only patchy necrosis with less cast formation and loss or shrinkage of nuclei of the tubule and dramatically decreased neutrophil plugging (Fig. 2a).

Cell apoptosis was evaluated by TUNEL immunostaining to localize DNA fragmentation. As shown in Fig. 2b, kidneys from rats in the I/R group showed extensive TUNEL-positive staining, predominantly in

the outer medulla. In contrast, kidneys from rats in the hydrogen-rich saline treatment group had very little TUNEL-positive staining.

Nitrogen-rich saline treatment showed no significant reduction of the renal injury and cell apoptosis.

Hydrogen-rich Saline Treatment Alleviated Oxidative Stress

Oxidant stress in renal tissue was evaluated by measuring renal MDA and 8-OHdG levels after 24 h reperfusion. Hydrogen-rich saline treatment not nitrogen-rich saline treatment significantly decreased the MDA and 8-OHdG levels compared with I/R group (Fig. 3).

Hydrogen-rich Saline Treatment Improved the Antioxidant Enzymatic Activities

As shown in Fig. 4, administration of hydrogen-rich saline treatment significantly elevated the SOD and CAT activities in comparison with the I/R group, while nitrogen-rich saline had no significant effect on the antioxidant enzymatic activities.

Hydrogen-rich Saline Treatment Attenuated Neutrophil Infiltration

MPO activity examined as an indicator of neutrophil infiltration and accumulation in tissues were measured (Fig. 5). Compared with I/R group, hydrogen-rich saline produced a significant decrease of the MPO activity while nitrogen-rich saline showed no significant decrease of the MPO activity.

Hydrogen-rich Saline Treatment Decreased Pro-inflammatory Cytokines Levels

ELISA detection showed that administration of hydrogen-rich saline treatment significantly decreased the levels of TNF-a, IL-1β and IL-6 (Table 1). No significant reduction of these cytokines was produced by nitrogen-rich saline administration.

Discussion

Our present study found that intraperitoneal injection of hydrogen-rich saline significantly attenuated

I/R-induced apoptosis, MPO activity, MDA, 8-OhdG, TNF-a, IL-1β and IL-6 levels and increased SOD and CAT activities in the renal tissues compared with those in I/R plus physiological saline treatment rats, which was in consistent with a recent study that demonstrated a decrease in the serum level of 8-OhdG after continuous intravenous administration of hydrogen-rich saline [9].

Lipid peroxidation caused by reactive oxygen species is one of the most critical mechanisms involved in cellular damage and death. Membrane-associated polyunsaturated fatty acids are readily attached to hydroxyl radical in a process that leads to peroxidation of lipids, which can disrupt membrane fluidity and cell compartmentation, resulting in cell lysis [10]. As a biomarker of oxidative DNA damage, 8-OHdG in tissue or body fluid is known as a sensitive indicator. 8-Hydroxydeoxyguanosine, a major product of oxidative DNA damage, is produced by enzymatic cleavage after 8-hydroxylation of the guanine base of DNA [11]. Our study demonstrated that hydrogen-rich saline treatment significantly alleviated oxidative stress by reducing renal MDA and 8-OHdG levels following I/R injury.

A growing number of studies have demonstrated that excessive production of ROS and reduction of antioxidant defense systems play an important role in the development of renal I/R injury [12, 13]. As the case in many other organs, the kidney is also vulnerable to oxidative stress resulting from I/R. It is well known that ROS include many types such as superoxide anion, ·OH, hydrogen peroxide (H_2O_2), and so on. One type of ROS can be converted into another type via antioxidant enzymes in vivo. The primary ROS produced in aerobic organisms are superoxide, which are highly reactive agents. SOD converts superoxide anion radical into H_2O_2, which is detoxified into H_2O by either glutathione peroxidase (GSH-Px) or CAT. Accordingly, their deficiencies can cause excessive oxidative stress. In the present study, we found that hydrogen-rich saline treatment significantly improved the activities of CAT and SOD subjected to renal I/R injury.

Polymorphonuclear neutrophil infiltration in a tissue is characteristic of acute inflammation, indicating the collective action of chemotactic mediators. Once neutrophils migrate into the ischemic area they release ROS, proteases, elastase, MPO, cytokines, all of which are involved in tissue injury. In our study, MPO activity, an index of tissue neutrophil infiltration, was increased by renal I/R injury, whereas hydrogen-rich saline treatment significantly inhibited neutrophil infiltration and protected the tissue against further injury.

Renal I/R injury is an important clinical problem that results in the release of the pro-inflammatory cytokines, such as TNF-a, IL-1β and IL-6 [14]. These cytokines play important roles in the induction of neutrophils activation and infiltration and induce not only localized tissue injury, but also remote organ injury [15]. In the present study, the renal tissue levels of the pro-inflammatory cytokines TNF-a, IL-1β and IL-6 were significantly elevated in I/R-induced renal injury. Furthermore, hydrogen-rich saline treatment reversed the levels of the inflammatory mediators while protecting the renal tissue against I/R-induced oxidative injury.

Furthermore, our study investigated the nitrogen-rich saline in an renal 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 oxidative stress nor inflammation in renal I/R rats were reduced by nitrogen-rich saline treatment, which helped us to conclude that the observed protection by hydrogen-rich saline was mediated via an antioxidant action.

The potential for hydrogen to scavenge free radicals has been proposed by several studies, especially ·OH and ONOO-, which are the strongest oxidants and react indiscriminately with nucleic acids, lipids and proteins resulting in DNA fragment, lipid peroxidation, and inactivation of protein [8]. As a novel antioxidant, hydrogen gas has been used in protecting cerebral [16], myocardial [17] and hepatic

[19] I/R injury in animal models and neonatal cerebral hypoxia-ischemia [13], and improving lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance by selectively reducing cytotoxic oxygen radicals [18]. More recently, it had also been demonstrated that hydrogen-rich saline treatment has a protective effect on intestinal ischemia/reperfusion (I/R) injuries [20] and lung injury induced by intestinal I/R [21]. Therefore, the ability to selectively and directly reduce or eliminate ·OH and ONOO- may be responsible for the protective effect of hydrogen-rich saline in renal I/R observed in this study, which attenuated oxidative stress and inflammation. However, the exact mechanism and signalling pathway involved in the protection role of hydrogen in the renal I/R injury need to be elucidated in the future.

Acknowledgements

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Table legends

Table1. Levels of TNF-a, IL-1β and IL-6 in renal tissues

Paramters	Sham	I/R	I/R+H_2	I/R+N_2
TNFα(pg/mg protein)	287.56±28.98	566.68±22.58*	391.00±15.14$^{\#}$	552.68±21.38
IL-1β(pg/mg protein)	247.30±34.22	503.90±19.68*	352.83±20.88$^{\#}$	512.91±21.35
IL-6(pg/mg protein)	364.94±35.45	744.70±72.50*	502.35±26.02$^{\#}$	732.71±67.84

Data are expressed as mean ± SD, n = 8.*$p < 0.01$ vs Sham; $^{\#}p < 0.05$ vs I/R group.

Figure legends

Fig. 1. Hydrogen-rich saline treatment attenuated renal I/R injury after 45 minutes of ischemia and 24 hours of reperfusion (mean ± SD; n = 8). (a) Effect of hydrogen-rich saline treatment on the serum BUN; (b) Effect of hydrogen-rich saline treatment on the serum Cr. *p < 0.01 vs Sham; $^{\#}$p < 0.01 vs I/R group.

Fig. 2. (a) Histological evaluations and TUNEL staining of renal tissue. Representative photographs of HE and TUNEL used to determine morphological changes (upper column, ×400) and apoptosis (lower column, ×400) in renal tissues were taken in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. (b) Significant inhibition of I/R-induced apoptosis were found in I/R plus hydrogen-rich saline treatment group in comparison with I/R. Data were represented by mean ± SD, n = 8.*p < 0.01 vs Sham; $^{\#}$p < 0.01 vs I/R group.

Fig. 3. (a) Renal tissue MDA levels in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups; (b) Renal tissue 8-OHdG levels in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. Data are expressed as means ± SD for at least triplicate independent experiments (n = 8 per group).*p < 0.01 vs Sham; $^{\#}$p < 0.05 vs I/R group.

Fig. 4. (a) Renal tissue SOD activity in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups; (b) Renal tissue CAT activity in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. Data are expressed as means ± SD for at least triplicate independent experiments (n = 8 per group).*p < 0.01 vs Sham; $^{\#}$p < 0.05 vs I/R group.

Fig. 5. Renal tissue MPO activity in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. Data are expressed as means ± SD for at least triplicate independent experiments (n = 8 per group).*p < 0.01 vs Sham; $^{\#}$p < 0.05 vs I/R group.

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

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Abstract

Backgrounds/Aims: Recently it has been demonstrated that hydrogen, as a novel antioxidant, can selectively reduce hydroxyl radicals (·OH) and peroxynitrite anion (ONOO-) in vitro and exert therapeutic antioxidant activity in many diseases. This study was designed to investigate the effect of hydrogen-rich saline on the renal ischemia/reperfusion (I/R) injury in rats. ***Methods:*** A rat model of renal I/R injury was induced by 45-min occlusion of the bilateral renal pedicles and 24-h reperfusion. Physiological saline, hydrogen-rich saline or nitrogen-rich saline (8 ml/kg) was administered intraperitoneally at 5 min before reperfusion, respectively. ***Results:*** After I/R injury, serum BUN, Cr, tissue MDA, 8-OhdG, TNF-a, IL-1β, IL-6 levels and MPO activity were all increased significantly, while tissue SOD and CAT activities were all decreased significantly. Hydrogen-rich saline reversed these changes and relieved morphological renal injury and I/R-induced apoptosis, while no significant changes was observed in the nitrogen-rich saline-treated group compared with physiological saline-treated group. ***Conclusions:*** Hydrogen-rich saline is able to attenuate the renal I/R injury, which is possibly by reduction of oxidative stress and inflammation.

Key words: ischemia/reperfusion; hydrogen; antioxidant; oxidative stress

Introduction

Renal I/R injury is common in several clinical situations, including renal transplantation and shock. I/R-induced acute renal failure is associated with decreased allograft survival in patients with transplanted kidneys and high mortality and morbidity in patients with native kidneys [1]. It was demonstrated that reactive oxygen species (ROS) and reactive nitrogen species (RNS) increase in the areas of ischemia and reperfusion, which are responsible for renal damage. Meanwhile, inflammation also plays an important role in the pathogenesis of renal I/R injury, through leukocyte activation and expression of adhesion molecules and cytokines [2-7]. Free radicals and pro-inflammatory cytokines 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.

Recently, it has been demonstrated that molecular hydrogen could selectively reduce cytotoxic ROS and RNS, such as ·OH and ONOO- in vitro and exert therapeutic antioxidant activity in a rat middle cerebral artery occlusion model [8]. However, the potential effect of hydrogen on renal I/R injury has not been examined. Therefore, the present study investigated the possible therapeutic effects of hydrogen-rich saline on renal I/R injury in rats.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 200–220g were given free access to normal rat diet and tap water and maintained in a temperature-controlled room with a 12:12-h light/dark cycle (lights on at 06:00 h). All procedures performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the use of experimental animals.

Hydrogen-rich Saline Production

Hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4MPa) to a supersaturated level using hydrogen-rich water-producing apparatus produced by our department. The saturated hydrogen saline was stored under atmospheric pressure at 48C 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 0.6mmol/L was maintained. Gas chromatography was used to confirm the content of hydrogen in saline by the method described by Ohsawa et al [8].

Renal Ischemia/Reperfusion

The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and placed in a supine position. Body temperature was maintained between 36°C and 37°C. After laparotomy and dissection of both renal pedicles, bilateral ischemia was induced by occluding the renal pedicles with atraumatic microvascular clamp for 45 min. The immediate color change of the kidneys signifying the stoppage of blood flow, indicates successful occlusion. During reperfusion, clamps were removed and the blood flow to the kidneys was re-established with visual verification of blood return.

Experimental Groups

Animals were divided into four groups consisting of 8 rats each: (1) sham-operated plus physiological saline treatment; (2) renal I/R plus physiological saline treatment; (3) renal I/R plus hydrogen-rich saline treatment; (4) renal I/R plus nitrogen-rich saline treatment. Sham-operated animals underwent the same surgical procedures except that the bilateral renal pedicles were not clamped. Physiological saline, hydrogen-rich saline or nitrogen-rich saline (8 ml/kg) was intraperitoneally injected at 5 min before the

reperfusion, respectively. Twenty-four hours after reperfusion initiation, blood was drawn from the abdominal inferior cava vein immediately before induced death. The left kidney was obtained and perfused with phosphate buffered saline (PBS) to remove all blood, then weighed and stored at -80°C until assayed. Blood samples were spun at 1000 rpm for 15 min and serum samples were collected.

Analysis of Renal Function

Serum blood urea nitrogen (BUN) and creatinine (Cr) was used for the evaluation of renal function. The samples were analyzed on a COBAS Mira chemical analyzer (Roche, Basel, Switzerland), using commercial kits from Sigma (St Louis, MO, USA).

Histological Analyses

Renal samples were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin eosin. Evaluation was performed with light microscopy without knowledge of the study groups. Assessment was carried out by expertise observers.

In Situ Apoptosis Assay

Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) assay was performed to detect apoptosis in situ. 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 (Oncogene Research products, Boston, USA) according to the protocol. Apoptotic cells were identified as those with a brown-stained nucleus. TUNEL-positive renal tubular 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 cells in 20 non-overlapping serial scopes taken from each

slide, beginning from a random start, at ×400 magnification.

Renal Malondialdehyde (MDA) Measurement

Renal MDA levels were determined using a BIOXYTHCH MDA-586 Assay kit (OxisRe-search, Oregon, USA). Briefly, frozen renal tissues were homogenized in the presence of butylated hydroxytoluene. After centrifugation, free MDA in the supernatant was converted to a stable carbocyanin dye (maximum absorption at 586 nm) by the chemical reaction with N-methyl-2-phenylindole. Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA) using BSA as a standard. MDA levels were normalized against protein (umol/mg).

Renal 8-hydroxydeoxyguanosine (8-OHdG) Levels Assay

The supernatant from kidney homogenate was prepared for detecting the levels of 8-OHdG levels with a commercial ELISA kit following the instructions of the manufacturer. Briefly, the samples were added to plate wells precoated with rat monoclonal anti-8-OHdG antibody (Japan Institute for the Control of Aging, Fukuroi, Japan). They were incubating for 120 min at 37°C. After washed for 3 times, the wells were treated with Biotinylated rat monoclonal anti-8-OHdG antibody for 60 min at 37°C. After washed for 3 times, the wells were treated with Streptavidin-Horseradish Peroxidase(HRP) for 30 min at 37°C. After the wells were washed for 3 times, a substrate containing 3,3’,5,5’-tetramethylbenzidine(TMB) was added and the wells were incubated for 15 min at 37°C. The reaction was terminated by the addition of a sulphuric acid. The absorbance was read at a wavelength of 450nm.

Antioxidant Enzymatic Activity Assay

The renal tissue homogenates were prepared in chilled PBS (0.1 M, pH 7.4), and were centrifuged at 10, 000 g at 4°C for 10 minutes. The supernatants were collected, aliquoted, stored at -80°C until the

following analysis.

The activities of superoxide dismutase (SOD) and catalase (CAT) were measured using commercial kits purchased from CaymanChemical Company (Ann Arbor, MI, USA). According to the manufacturer's instructions, total SOD activity was assayed by detecting superoxide radicals generated by xanthine oxidase and hypoxanthine. The reaction was monitored at 450 nm and one unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The CAT activity was assayed by measuring the reduction of hydrogen peroxide at 540 nm and one unit was defined as the amount of enzyme that would cause the formation of 1.0 nmol of formaldehyde per minute at 25°C. All spectrophotometric readings were performed by using a spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA). All assays were conducted in triplicates. The tissue protein concentration was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

Renal Myeloperoxidase (MPO) Activity Assay

The supernatant from kidney homogenate was prepared for detecting the activity of MPO, an indicator of neutrophil infiltration in the renal tissue. MPO activity was defined as the quantity of enzyme degrading 1μmol of peroxide per minute at 37℃ and was expressed in unit per gram weight of wet tissue. The change in absorbance was measured spectrophotometrically at 590 nm by spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA).

Determination of TNF-a, IL-1β and IL-6 Levels in the Renal Tissues

The supernatant from kidney homogenate was prepared for detecting the levels of TNF-a, IL-1β and IL-6 with a commercial enzyme-linked immunosorbent assay (ELISA) kit following the instructions of the manufacturer. The absorbance was read on a microplate reader and the concentrations were calculated

according to the standard curve. Protein content in the sample was determined by Coomassie blue assay and the results were corrected per microgram of protein.

Statistical Analysis

Results were expressed as mean ± SD. Statistical analysis was performed with the SPSS11.0 software package. The inter-group differences of the data were tested by one-way ANOVA followed by LSD-t Test for multiple comparisons. A P value of less than 0.05 was considered to be statistically significant.

Results

Hydrogen-rich Saline Treatment Ameliorated Renal Function

After 24 h reperfusion, serum BUN and Cr levels were used to assess the effect of hydrogen-rich saline on renal I/R injury. As shown in Fig.1, compared with I/R group, hydrogen-rich saline treatment had a significantly reduction in serum BUN and Cr levels, while nitrogen-rich saline treatment showed no significant reduction.

Hydrogen-rich Saline Treatment Attenuated Renal Tissue Injury

The rats of renal I/R group demonstrated an increased infiltration of neutrophils, and had more significant tubular necrosis and apoptosis than those of hydrogen-rich saline treatment group. This injury also included widespread loss of tubular dilation, and cast formation. Kidneys of hydrogen-rich saline treatment group rats had only patchy necrosis with less cast formation and loss or shrinkage of nuclei of the tubule and dramatically decreased neutrophil plugging (Fig. 2a).

Cell apoptosis was evaluated by TUNEL immunostaining to localize DNA fragmentation. As shown in Fig. 2b, kidneys from rats in the I/R group showed extensive TUNEL-positive staining, predominantly in

the outer medulla. In contrast, kidneys from rats in the hydrogen-rich saline treatment group had very little TUNEL-positive staining.

Nitrogen-rich saline treatment showed no significant reduction of the renal injury and cell apoptosis.

Hydrogen-rich Saline Treatment Alleviated Oxidative Stress

Oxidant stress in renal tissue was evaluated by measuring renal MDA and 8-OHdG levels after 24 h reperfusion. Hydrogen-rich saline treatment not nitrogen-rich saline treatment significantly decreased the MDA and 8-OHdG levels compared with I/R group (Fig. 3).

Hydrogen-rich Saline Treatment Improved the Antioxidant Enzymatic Activities

As shown in Fig. 4, administration of hydrogen-rich saline treatment significantly elevated the SOD and CAT activities in comparison with the I/R group, while nitrogen-rich saline had no significant effect on the antioxidant enzymatic activities.

Hydrogen-rich Saline Treatment Attenuated Neutrophil Infiltration

MPO activity examined as an indicator of neutrophil infiltration and accumulation in tissues were measured (Fig. 5). Compared with I/R group, hydrogen-rich saline produced a significant decrease of the MPO activity while nitrogen-rich saline showed no significant decrease of the MPO activity.

Hydrogen-rich Saline Treatment Decreased Pro-inflammatory Cytokines Levels

ELISA detection showed that administration of hydrogen-rich saline treatment significantly decreased the levels of TNF-a, IL-1β and IL-6 (Table 1). No significant reduction of these cytokines was produced by nitrogen-rich saline administration.

Discussion

Our present study found that intraperitoneal injection of hydrogen-rich saline significantly attenuated

I/R-induced apoptosis, MPO activity, MDA, 8-OhdG, TNF-a, IL-1β and IL-6 levels and increased SOD and CAT activities in the renal tissues compared with those in I/R plus physiological saline treatment rats, which was in consistent with a recent study that demonstrated a decrease in the serum level of 8-OhdG after continuous intravenous administration of hydrogen-rich saline [9].

Lipid peroxidation caused by reactive oxygen species is one of the most critical mechanisms involved in cellular damage and death. Membrane-associated polyunsaturated fatty acids are readily attached to hydroxyl radical in a process that leads to peroxidation of lipids, which can disrupt membrane fluidity and cell compartmentation, resulting in cell lysis [10]. As a biomarker of oxidative DNA damage, 8-OHdG in tissue or body fluid is known as a sensitive indicator. 8-Hydroxydeoxyguanosine, a major product of oxidative DNA damage, is produced by enzymatic cleavage after 8-hydroxylation of the guanine base of DNA [11]. Our study demonstrated that hydrogen-rich saline treatment significantly alleviated oxidative stress by reducing renal MDA and 8-OHdG levels following I/R injury.

A growing number of studies have demonstrated that excessive production of ROS and reduction of antioxidant defense systems play an important role in the development of renal I/R injury [12, 13]. As the case in many other organs, the kidney is also vulnerable to oxidative stress resulting from I/R. It is well known that ROS include many types such as superoxide anion, ·OH, hydrogen peroxide (H_2O_2), and so on. One type of ROS can be converted into another type via antioxidant enzymes in vivo. The primary ROS produced in aerobic organisms are superoxide, which are highly reactive agents t. SOD converts superoxide anion radical into H_2O_2, which is detoxified into H_2O by either glutathione peroxidase (GSH-Px) or CAT. Accordingly, their deficiencies can cause excessive oxidative stress. In the present study, we found that hydrogen-rich saline treatment significantly improved the activities of CAT and SOD subjected to renal I/R injury.

Polymorphonuclear neutrophil infiltration in a tissue is characteristic of acute inflammation, indicating the collective action of chemotactic mediators. Once neutrophils migrate into the ischemic area they release ROS, proteases, elastase, MPO, cytokines, all of which are involved in tissue injury. In our study, MPO activity, an index of tissue neutrophil infiltration, was increased by renal I/R injury, whereas hydrogen-rich saline treatment significantly inhibited neutrophil infiltration and protected the tissue against further injury.

Renal I/R injury is an important clinical problem that results in the release of the pro-inflammatory cytokines, such as TNF-a, IL-1β and IL-6 [14]. These cytokines play important roles in the induction of neutrophils activation and infiltration and induce not only localized tissue injury, but also remote organ injury [15]. In the present study, the renal tissue levels of the pro-inflammatory cytokines TNF-a, IL-1β and IL-6 were significantly elevated in I/R-induced renal injury. Furthermore, hydrogen-rich saline treatment reversed the levels of the inflammatory mediators while protecting the renal tissue against I/R-induced oxidative injury.

Furthermore, our study investigated the nitrogen-rich saline in an renal 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 oxidative stress nor inflammation in renal I/R rats were reduced by nitrogen-rich saline treatment, which helped us to conclude that the observed protection by hydrogen-rich saline was mediated via an antioxidant action.

The potential for hydrogen to scavenge free radicals has been proposed by several studies, especially ·OH and ONOO-, which are the strongest oxidants and react indiscriminately with nucleic acids, lipids and proteins resulting in DNA fragment, lipid peroxidation, and inactivation of protein [8]. As a novel antioxidant, hydrogen gas has been used in protecting cerebral [16], myocardial [17] and hepatic

[19] I/R injury in animal models and neonatal cerebral hypoxia-ischemia [13], and improving lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance by selectively reducing cytotoxic oxygen radicals [18]. More recently, it had also been demonstrated that hydrogen-rich saline treatment has a protective effect on intestinal ischemia/reperfusion (I/R) injuries [20] and lung injury induced by intestinal I/R [21]. Therefore, the ability to selectively and directly reduce or eliminate ·OH and ONOO- may be responsible for the protective effect of hydrogen-rich saline in renal I/R observed in this study, which attenuated oxidative stress and inflammation. However, the exact mechanism and signalling pathway involved in the protection role of hydrogen in the renal I/R injury need to be elucidated in the future.

Acknowledgements

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Table legends

Table1. Levels of TNF-a, IL-1β and IL-6 in renal tissues

Paramters	Sham	I/R	I/R+H_2	I/R+N_2
TNFα(pg/mg protein)	287.56±28.98	566.68±22.58*	391.00±15.14$^{\#}$	552.68±21.38
IL-1β(pg/mg protein)	247.30±34.22	503.90±19.68*	352.83±20.88$^{\#}$	512.91±21.35
IL-6(pg/mg protein)	364.94±35.45	744.70±72.50*	502.35±26.02$^{\#}$	732.71±67.84

Data are expressed as mean ± SD, n = 8.*$p < 0.01$ vs Sham; $^{\#}p < 0.05$ vs I/R group.

Figure legends

Fig. 1. Hydrogen-rich saline treatment attenuated renal I/R injury after 45 minutes of ischemia and 24 hours of reperfusion (mean ± SD; n = 8). (a) Effect of hydrogen-rich saline treatment on the serum BUN; (b) Effect of hydrogen-rich saline treatment on the serum Cr. *p < 0.01 vs Sham; $^{\#}$p < 0.01 vs I/R group.

Fig. 2. (a) Histological evaluations and TUNEL staining of renal tissue. Representative photographs of HE and TUNEL used to determine morphological changes (upper column, ×400) and apoptosis (lower column, ×400) in renal tissues were taken in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. (b) Significant inhibition of I/R-induced apoptosis were found in I/R plus hydrogen-rich saline treatment group in comparison with I/R. Data were represented by mean ± SD, n = 8.*p < 0.01 vs Sham; $^{\#}$p < 0.01 vs I/R group.

Fig. 3. (a) Renal tissue MDA levels in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups; (b) Renal tissue 8-OHdG levels in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. Data are expressed as means ± SD for at least triplicate independent experiments (n = 8 per group).*p < 0.01 vs Sham; $^{\#}$p < 0.05 vs I/R group.

Fig. 4. (a) Renal tissue SOD activity in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups; (b) Renal tissue CAT activity in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. Data are expressed as means ± SD for at least triplicate independent experiments (n = 8 per group).*p < 0.01 vs Sham; $^{\#}$p < 0.05 vs I/R group.

Fig. 5. Renal tissue MPO activity in sham-operated, I/R and I/R treated with hydrogen-rich saline or nitrogen-rich saline groups. Data are expressed as means ± SD for at least triplicate independent experiments (n = 8 per group).*p < 0.01 vs Sham; $^{\#}$p < 0.05 vs I/R group.

Figure1

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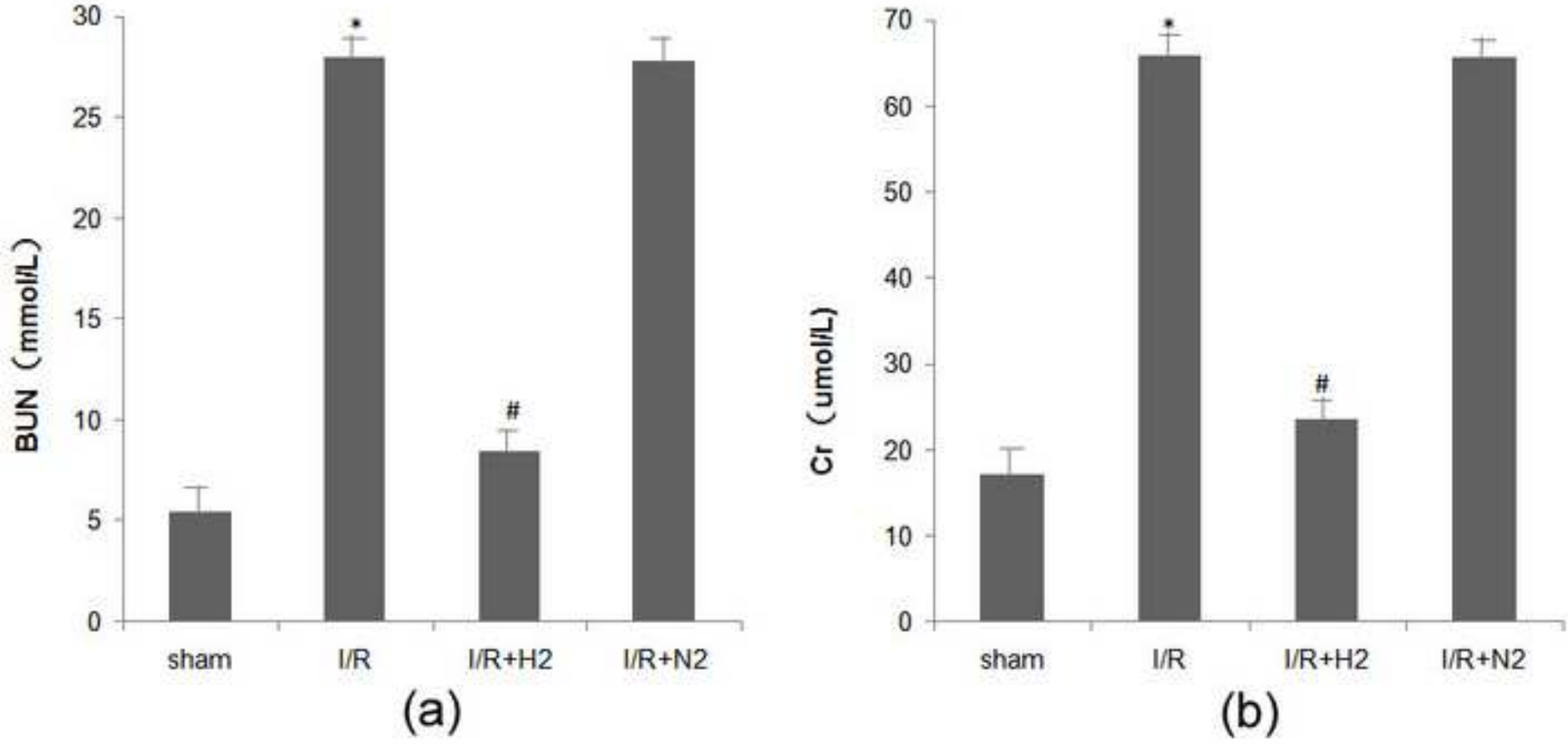

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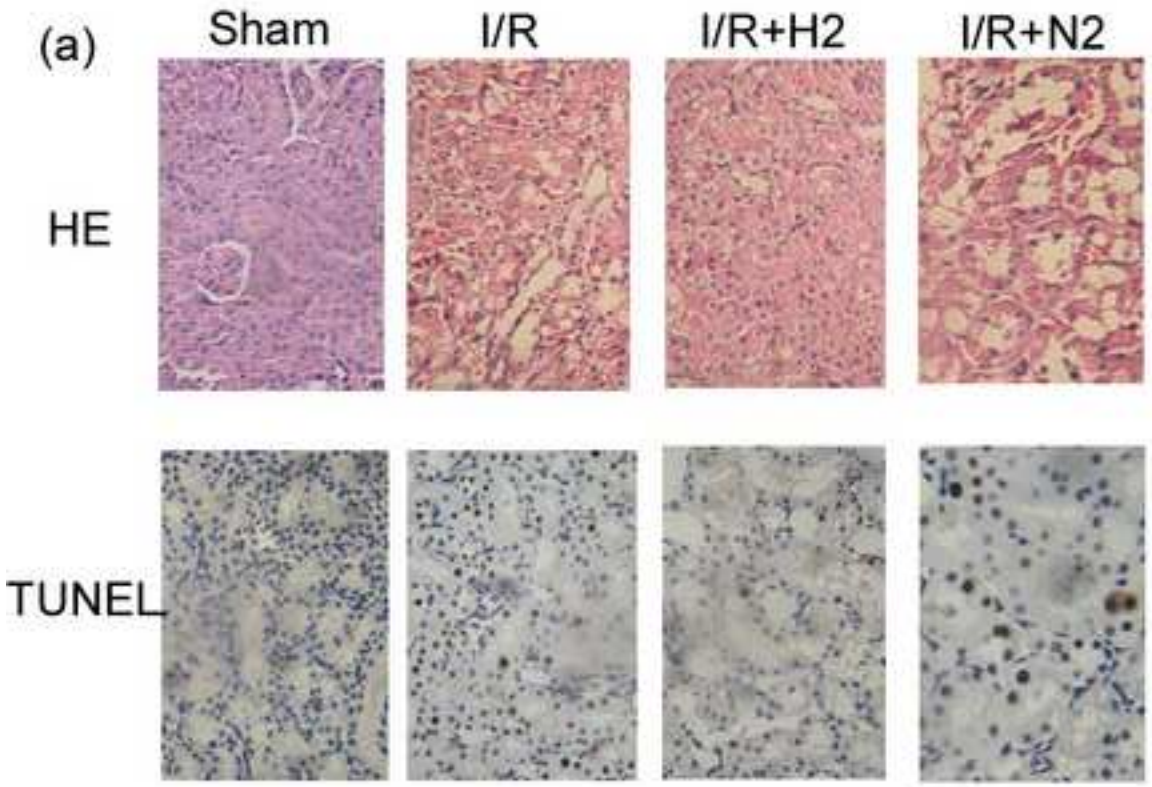

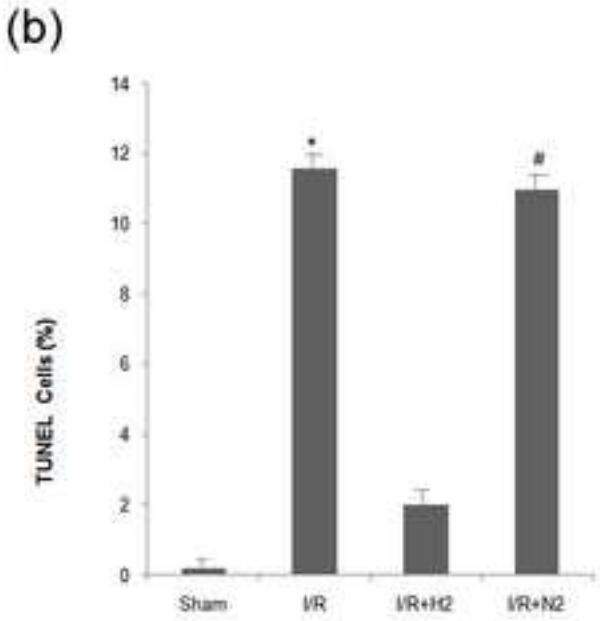

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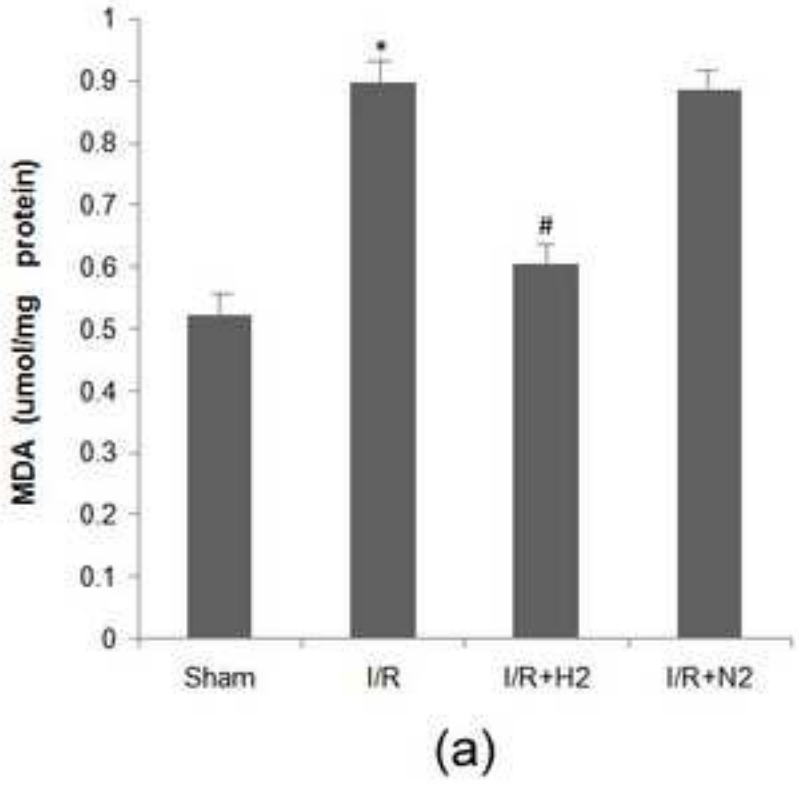

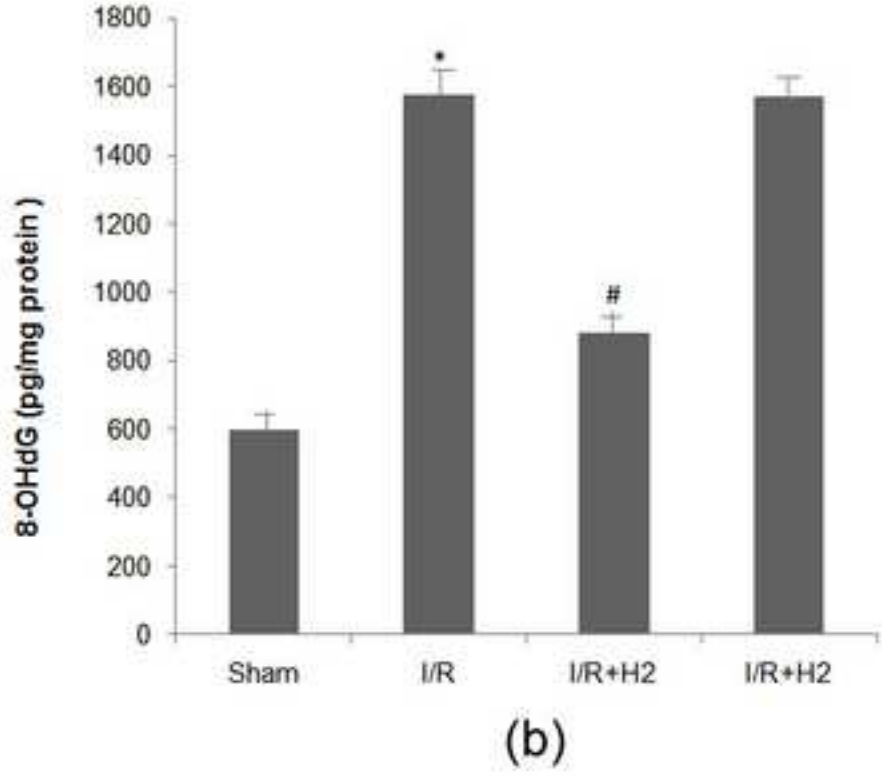

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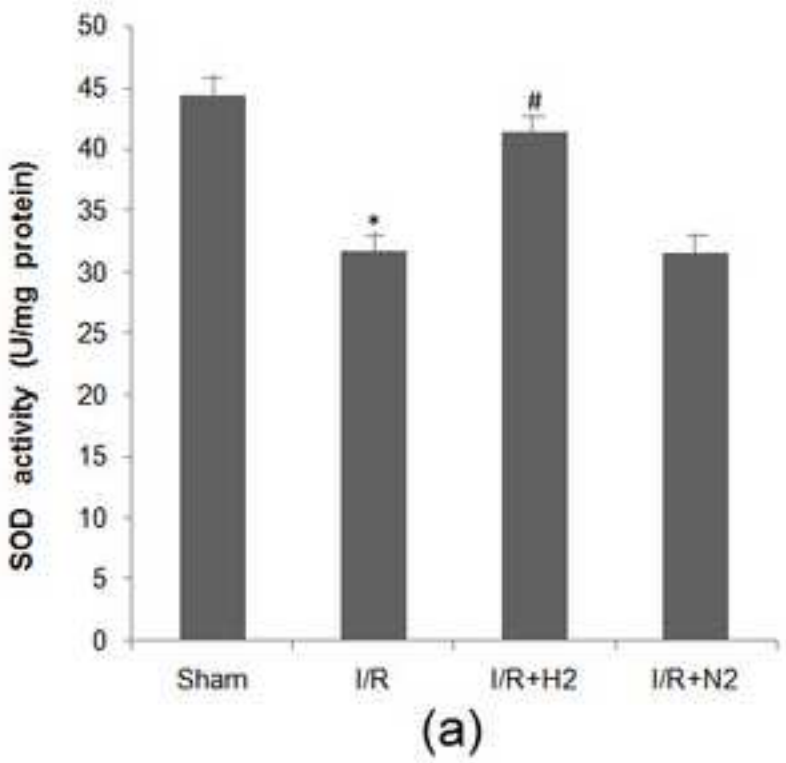

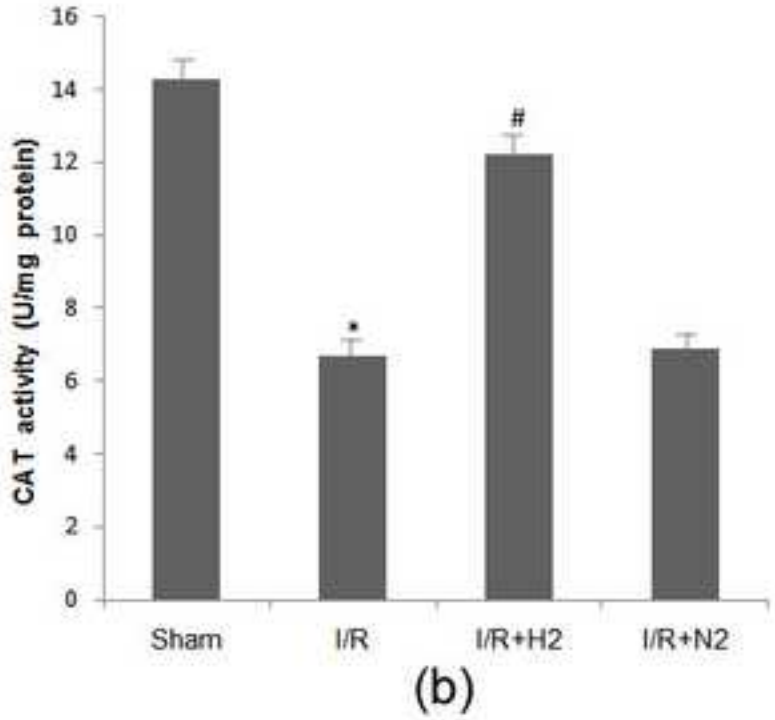

Figure5

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