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ORIGINAL ARTICLE

# Hydrogen-rich saline solution attenuates renal ischemia–reperfusion injury

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**Abstract**
*Purpose* Renal ischemia–reperfusion (I/R), an important cause of acute kidney injury, is unavoidable during various types of operations, including renal transplantation, surgical revascularization of the renal artery, partial nephrectomy, and treatment of suprarenal aortic aneurysms. Exacerbation of I/R injury is mediated by reactive oxygen species (ROS). A recent study has shown that hydrogen has antioxidant properties. In this study, we tested the hypothesis that a hydrogen-rich saline solution (HRSS) attenuates renal I/R injury in a rodent model.
*Methods* Rats were treated with an intravenous injection of HRSS or control saline solution followed by renal I/R. After 24 h of treatment, we performed a histological examination and transmission electron microscopy, and measured serum levels of 8-OHdG.
*Results* Histological analysis revealed a marked reduction of interstitial congestion, edema, inflammation, and hemorrhage in renal tissue harvested 24 h after HRSS treatment compared to saline administration. Renal I/R injury, which led to altered mitochondrial morphology, was also inhibited by HRSS. Furthermore, serum 8-OHdG levels were significantly lower in rats treated with HRSS and subjected to renal I/R.
*Conclusions* These protective effects were likely due to the antioxidant properties of HRSS. These results suggest that HRSS is a potential therapeutic candidate for treating various I/R diseases.

**Keywords** Hydrogen · 8-OHdG · Saline solution · Ischemia–reperfusion injury · Renal

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## Introduction

Ischemic/reperfusion (I/R) injury, including arterial occlusion, shock, and organ transplantation, is a common finding in clinical settings. 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 [1–3]. Despite a growing number of clinical care methods and extensive pathophysiological research, mortality due to AKI remains high and unchanged in the last several decades [4].

A major event in I/R-induced kidney injury is the generation of cytotoxic oxygen radicals [5, 6]. An increase in cytotoxic oxygen radicals leads to increased cellular injury, including DNA damage, protein oxidation and nitrosylation, lipid peroxidation, and apoptosis [7]. Endothelial dysfunction and inflammatory responses arise as I/R injury evolves in the kidney. Consequently, renal function deteriorates, resulting in renal dysfunction [8]. However, cells are equipped with enzymatic and nonenzymatic free radical scavenging systems which serve as defense mechanisms against free radicals [9]. Moreover, several studies using I/R injury models have clearly demonstrated the protective effects of antioxidant drugs against free radicals and reactive oxygen species (ROS) [10, 11].

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Cytotoxic oxygen radicals produced either endogenously or exogenously can attack lipids, proteins, and nucleic acids within cells. In both nuclear and mitochondrial DNA, 8-hydroxydeoxyguanosine (8-OHdG) is an oxidized nucleoside that is frequently used to detect and study DNA lesions, making it a suitable biomarker for oxidative stress [12].

Hydrogen exists virtually everywhere in nature, and is found in almost every chemical compound. However, hydrogen does not exist as a natural gas. Recently, Ohsawa et al. [13] demonstrated that hydrogen gas had a protective effect against cerebral I/R injury by reducing the amount of cytotoxic oxygen radical species. In addition, various studies have reported that hydrogen gas has protective effects against organ dysfunction induced by various I/R injuries [14–16].

In vivo studies have revealed that treatment with hydrogen can protect against various organ injuries through its antioxidant effect, which reportedly reduces I/R-induced tissue damage and is cytoprotective [13–16]. Although colorless and odorless, hydrogen reacts with various chemicals and is combustible, making its use in the clinical setting very challenging. We hypothesized that a hydrogen-rich saline solution (HRSS) would be easy to use and could attenuate AKI-induced renal I/R injury. In order to test this hypothesis, we administered HRSS in rats and measured its impact on serum 8-OHdG levels. Kidney histopathology was also assessed following I/R-induced renal injury.

## Materials and methods

### Animal and treatment protocol

Male Wistar rats weighing 250–300 g were used in the experiments (Kyudou, Saga, Japan). All rats had access to unlimited food and water prior to and after the treatment protocol. All protocols conformed to the National Institute of Health (NIH) guidelines, with all rats receiving humane care in compliance with the Principles of Laboratory Animal Care. This study was approved by the Ethics Committee of Animal Research at Oita University Faculty of Medicine.

HRSS was prepared by gassing with hydrogen for 60 min. Hydrogen concentration was measured by gas chromatography (GC-8A; Shimadzu Corporation, Kyoto, Japan). Dissolved oxygen levels were measured by the electrode method (DO142; Shimadzu Corporation, Kyoto, Japan).

Rats were randomly assigned to one of three groups. In the noninjured control group ($n = 6$), rats received sham treatment, followed by continuous intravenous administration of a 0.9% NaCl solution (1 ml/kg/h). In the I/R group ($n = 6$), the right kidney was surgically removed and the left renal artery was occluded, followed by continuous intravenous administration of a 0.9% NaCl solution (1 ml/kg/h). Occlusion of the left renal artery was performed for 60 min under sevoflurane anesthesia. In the HRSS-I/R group ($n = 6$), the right kidney was surgically removed and the left renal artery was occluded, followed by continuous intravenous administration of HRSS (1 ml/kg/h). Occlusion of the left renal artery was performed as described above. All rats were anesthetized with 4% sevoflurane (Maruishi Pharmaceutical Co., Osaka, Japan) during surgical treatments. Rats received continuous saline or HRSS administration for 24 h.

### Kidney function

Serum was obtained from blood samples collected 24 h after renal I/R injury. Serum blood urea nitrogen (BUN) and creatinine were measured by standard methods on an i-STAT 300F autoanalyzer (Fuso Pharmaceutical Industries, Osaka, Japan).

### Histological analysis

Rats under sevoflurane anesthesia were killed 24 h after renal I/R injury. Histological specimens were fixed in 10% formalin, embedded in paraffin, and sectioned on a microtome. Each section was stained with hematoxylin and eosin. Samples were blindly analyzed by a pathologist who determined the extent of kidney injury based on a technique outlined by Erdogan et al. [17]. Briefly, 24 areas corresponding to the kidney proximal tubules were graded for the degree of renal damage based on each of the following parameters: tubular cell necrosis, cytoplasmic vacuole formation, hemorrhage, and tubular dilatation. The semi-quantitative scale ranged from 0 to 4 [0, normal kidney; 1, minimal damage (0–5% injury); 2, mild damage (5–25% injury); 3, moderate damage (25–75% injury); and 4, severe damage (75–100% injury)]. The mean score for each parameter was determined and subjected to statistical analysis.

### Transmission electron microscopy

Rats under sevoflurane anesthesia were killed 24 h after renal I/R injury. Histological specimens were immersed in a fixative solution containing 4% paraformaldehyde, 1% $CaCl_2$, and 7% sucrose for 30 min at 4°C. Fixed specimens were then cut into small pieces using a razor blade, post-fixed in 2% osmium for 2 h at 4°C, dehydrated through a graded ethanol series, and embedded in Epok 812 (Ohken, Tokyo, Japan). Ultrathin sections (~90–95 nm) were cut using a diamond knife on an ultramicrotome

(Reichert-Nissei Ultracut S; Lecia, Vienna, Austria) and examined on a JEM-1200 EX II electron microscope (JEOL, Tokyo, Japan).

### Determination of serum 8-OHdG levels

8-OHdG levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd., Shizuoka, Japan). Absorbance at 450 nm was determined using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

### Statistical analysis

All data are presented as mean ± standard deviation (SD) and were evaluated using post hoc tests of one-way analysis of variance (ANOVA). A $p$ value of <0.05 was considered significant.

## Results

### Concentrations of hydrogen and oxygen in HRSS

The initial concentration of hydrogen in HRSS upon infusion was 640 μM (a similar concentration to a saturated solution of hydrogen). However, this rapidly decreased to approximately 10 μM by the end of the infusion period. Prior to gassing with hydrogen, the concentration of oxygen in the solution was 8.58 mg/l. This dramatically deceased to 0.6 mg/l after gassing with hydrogen. However, the concentration of oxygen gradually increased upon infusion, and reached 4.7 mg/l by the end of the infusion period.

### Effect of HRSS on kidney tissue after renal ischemia–reperfusion injury

Histological specimens corresponding to kidney tissue were obtained 24 h after renal I/R injury in the presence or absence of HRSS. Light microscopy revealed normal kidney morphology in the noninjured control group (Fig. 1a, b). However, tubular cell necrosis, cytoplasmic vacuoles, hemorrhage, and tubular dilatation were observed in histological specimens from the I/R group (Fig. 1c, d). These four types of histological alterations were markedly reduced in specimens from the HRSS-I/R group (Fig. 1e, f). All histological scores for renal injury were significantly higher in specimens from the I/R group compared to the control group, whereas intermediate scores were obtained in specimens from the HRSS-I/R group (Fig. 1g; $p < 0.05$).

Electron microscopy of kidney specimens from the control group revealed intact tubular cells with mitochondrial orientations perpendicular to the basement membrane (Fig. 2a). Mitochondria in the I/R group appeared swollen with abnormal cristae and tiny flocculent particles. In addition, microvilli were observed shedding into the lumen of the tubules, and tubular cells had lost their normal morphology (Fig. 2b). However, the HRSS-I/R group showed less damage to tubular cells and had intact microvilli and mitochondrial morphology (Fig. 2c).

### Kidney function

Effects of HRSS on kidney function were analyzed 24 h after renal I/R injury. In the control group, mean creatinine and BUN serum levels were 0.4 ± 0.1 and 15.0 ± 2.0 mg/dl, respectively (Table 1). These markers were significantly elevated in the I/R group (creatinine: 4.1 ± 1.0 mg/dl; BUN: 164.0 ± 20.4 mg/dl; Table 1). However, the HRSS-I/R group showed significant improvement, with mean creatinine and BUN levels of 2.3 ± 0.7 and 119.3 ± 29.5 mg/dl, respectively (Table 1; $p < 0.05$).

### Effect of HRSS on serum 8-OHdG levels

Blood samples were collected from rats 24 h after renal I/R injury in the presence or absence of HRSS. While 8-OHdG levels were elevated in the I/R group, these levels were significantly reduced in the HRSS-I/R group (Fig. 3; $p < 0.05$).

## Discussion

In this study, we demonstrated that HRSS significantly attenuates renal I/R injury. HRSS treatment reduced serum 8-OHdG levels compared to the I/R group, potentially through an antioxidant effect.

The most common isotope of hydrogen is protium, which has a single proton and no neutrons. However, hydrogen does not exist as a natural gas. In 2007, Ohsawa et al. [13] were the first to report that hydrogen gas has a protective effect against cerebral I/R injury. In addition, other studies have reported that hydrogen gas can protect against organ dysfunction induced by various I/R injuries [14, 16]. In this study, we demonstrated that HRSS significantly improves renal function, maintaining tissue structure after I/R injury. Taken together, our findings are in line with published results which show that hydrogen has a protective effect against I/R-induced organ dysfunction.

We found that 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels, which were increased in the I/R group, were significantly reduced with HRSS treatment. Several lines of

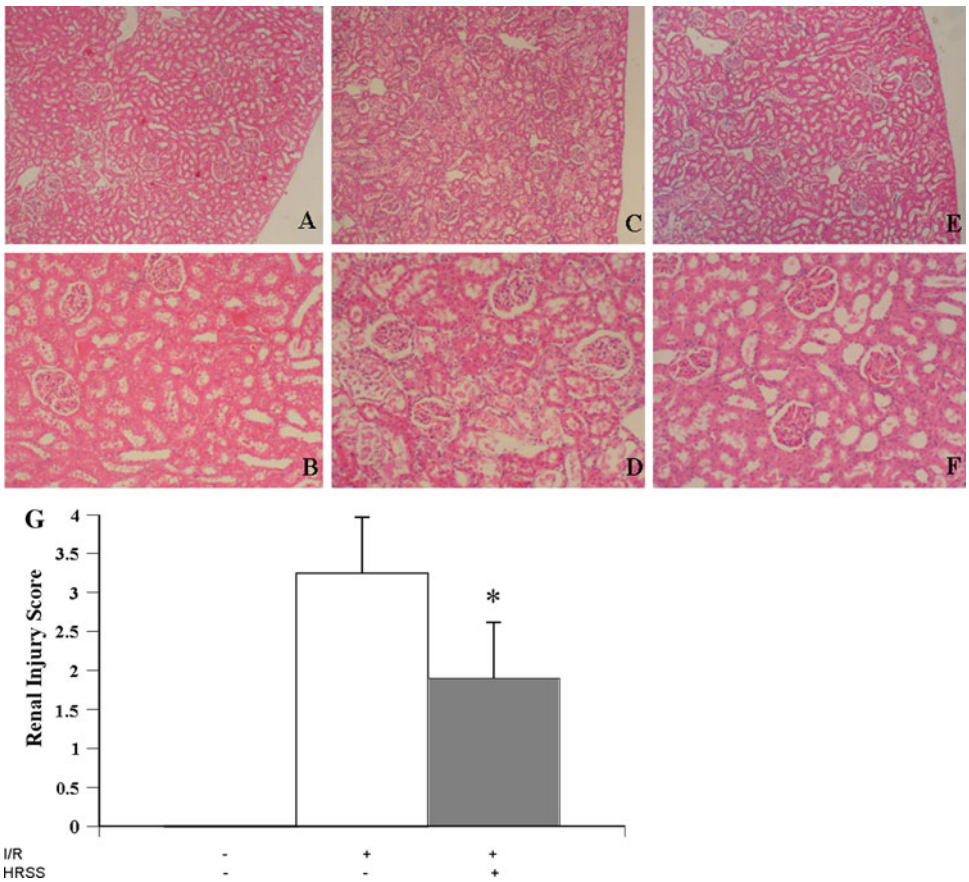


**Fig. 1** Changes in kidney histology. Kidney tissue from rats of the various treatment groups were fixed, embedded, sectioned, and stained with hematoxylin and eosin. Shown are representative histological specimens from the saline-treated control group (**a** ×40 magnification; **b** ×100 magnification), saline-treated renal I/R injury group (I/R group; **c** ×40 magnification; **d** ×100 magnification), and I/R injury group treated with hydrogen-rich saline solution (HRSS-I/R group; **e** ×40 magnification; **f** ×100 magnification). **g** Histological changes observed 24 h after renal I/R injury included tubular cell necrosis, cytoplasmic vacuole formation, hemorrhage, and tubular dilatation. Injury scores are expressed as mean ± SD. Semi-quantitative injury scores range from 0 to 4 [0, normal kidney; 1, minimal damage (0–5% injury); 2, mild damage (5–25% injury); 3, moderate damage (25–75% injury); and 4, severe damage (75–100% injury)]. The *white bar* represents the injury score obtained from the I/R group, while the *gray bar* represents the injury score from the HRSS-I/R group. The control group (no bar depicted) had a mean score of 0. *Denotes a significant difference ($p < 0.05$) relative to the I/R group

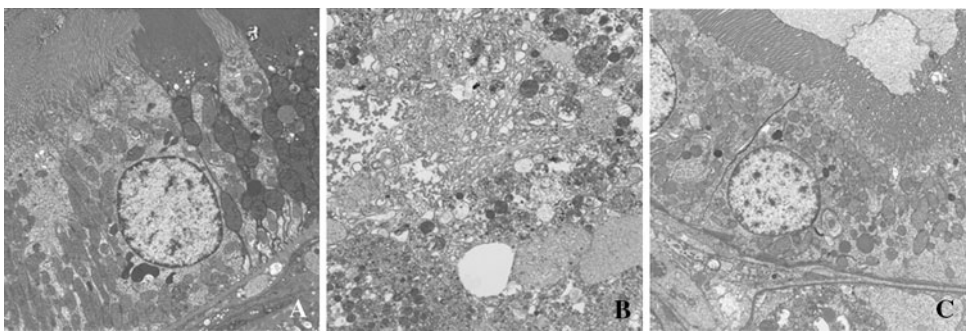


**Fig. 2** Electron microscopy analysis of the effect of hydrogen-rich saline solution on renal ischemia–reperfusion injury. Tissue specimens were obtained from rats 24 h after treatment. These specimens were fixed, embedded, sectioned, stained, and subjected to transmission electron microscopy as described in the "Materials and methods" section. Micrographs shown were taken at ×10,000 magnification. **a** Control group; **b** ischemia–reperfusion (I/R) group; **c** I/R group treated with hydrogen-rich saline solution (HRSS-I/R group)

evidence support the assumption that oxidative stress plays a crucial role in the pathogenesis of I/R injury [18]. Episodes of acute oxidative stress occurring after I/R can be especially dangerous because these types of injury cause ROS concentrations to increase at a rate that overwhelms the body's defense mechanisms and can be severely

**Table 1** Effect of hydrogen-rich saline solution treatment on I/R-induced renal dysfunction

| | Control group | I/R group | HRSS-I/R group |
|---|---|---|---|
| Creatinine (mg/dl) | 0.4 ± 0.1* | 4.1 ± 1.0 | 2.3 ± 0.7* |
| BUN (mg/dl) | 15.0 ± 2 .0* | 164.0 ± 20.4 | 119.3 ± 29.5* |

Kidney function was measured 24 h after renal I/R injury in rats ($n = 6$ per group). Evaluated markers for renal function included blood urea nitrogen (BUN) and creatinine. Data are expressed as mean ± SD

* Significant difference ($p < 0.05$) relative to the I/R group

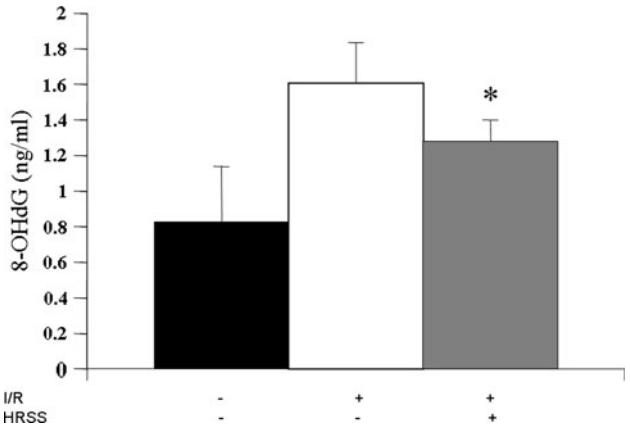


**Fig. 3** Effect of renal ischemia–reperfusion on serum 8-OHdG levels. Serum 8-OHdG levels were measured 24 h following renal I/R-induced injury. Control group (*black bar*); ischemia–reperfusion (I/R) group (*white bar*); and I/R group treated with hydrogen-rich saline solution (HRSS-I/R group; *gray bar*). Data are expressed as mean ± SD ($n = 6$ per group). *Denotes a significant difference ($p < 0.05$) relative to the I/R group

damaging to affected tissue [19]. On the other hand, 8-OHdG is a modified base that exists in DNA due to attack by hydroxyl radicals that are formed as products of the repair of oxidized guanine lesions during oxidative stress. 8-OHdG has been identified as a biomarker for oxidative stress [20]. Renal I/R injury is characterized by oxidative stress in mitochondria within the proximal tubule [21]. Increased oxidative stress results in mitochondrial dysfunction and altered morphology, eventually leading to organ failure [22]. We found that HRSS minimizes changes to mitochondrial morphology associated with I/R injury. We therefore speculate that the reduction in 8-OHdG levels and minimal changes to mitochondrial morphology can be attributed to attenuated oxidative stress.

Several studies have shown that hydrogen exerts cytoprotective effects after various types of injuries [13–16]. Hydrogen is thought to exert beneficial effects through various mechanisms, one of which relates to hydrogen's antioxidative properties [13]. In this study, we found that continuous HRSS administration significantly reduced 8-OHdG levels after I/R injury, suggesting that hydrogen gas reduced the extent of oxidative stress. In this study, we found that the concentration of oxygen was significantly reduced in HRSS. Furthermore, the low oxygen concentration in HRSS may have contributed to low ROS production. As a result, HRSS reduced oxidative stress following renal I/R injury.

Numerous antioxidative drugs, such as edaravone, have been shown to improve various organ injuries [23–25]. While these drugs have failed in clinical settings, several studies indicate that hydrogen is effective against various diseases [13–16]. Despite this, there are safety issues with using hydrogen, such as the possibility of detonations, fires resulting from mixing with air, and asphyxiation. Furthermore, hydrogen is known to dissolve metals. This can result in hydrogen gas leaks, which in turn can result in spontaneous ignition when mixed with air. Hydrogen flames are extremely hot and essentially invisible, leading to the possibility of burns.

Given its high combustibility and danger, we decided to use hydrogen as a saline solution in order to test its effect in a rat model of renal I/R injury. HRSS had not previously been tested due to the aforementioned reasons, but we hypothesized that HRSS could be used safely. Our results demonstrated that HRSS reduces renal I/R injury, providing support for its therapeutic potential. We also considered intravenous HRSS administration to be more advantageous than conventional antioxidant therapies.

Several limitations of our study should be noted. First, we have not assessed delayed treatment with HRSS after renal I/R injury. Second, our rat model of renal I/R injury is not entirely analogous to clinical I/R conditions seen in patients. Third, we have established neither serum hydrogen levels nor its safety for long-term administration.

In conclusion, we found that HRSS treatment attenuates renal I/R injury in a rat model. Taken together, our results suggest that HRSS has antioxidant effects and may have therapeutic potential against various clinical conditions involving I/R injury.

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