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Pretreatment with hydrogen-rich saline reduces the damage caused by glycerol-induced rhabdomyolysis and acute kidney injury in rats

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Running title: Pretreatment with hydrogen-rich saline protects
Abstract

**Background:** Rhabdomyolysis is a leading cause of acute kidney injury (AKI). The pathophysiological process involves oxidative stress and inflammation. Hydrogen-rich saline (HRS) is an antioxidant and anti-inflammatory. This study explored the protective effect of pretreatment with HRS on the development of glycerol-induced rhabdomyolysis AKI.

**Materials and methods:** 48 rats were randomly divided into four equal groups. Group 1 served as the control, group 2 was given 50% Glycerol (10 ml/kg, i.m.), group 3 was given glycerol after 7 days pretreatment with high dose HRS (10ml/kg/d, i.p.), and group 4 was given glycerol after 7 days pretreatment with low dose HRS (5ml/kg/d, i.p.). Renal health was monitored by serum creatinine (Cr), urea and histological analysis, rhabdomyolysis by creatine kinase (CK) levels and. Oxidative stress was monitored by kidney tissue reactive oxygen species (ROS), malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OH-dG), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) levels. Inflammation was monitored by interleukin -6 (IL-6) and tumor necrosis factor alpha (TNF -α) evaluation.

**Results:** Glycerol administration resulted in an increase in the mean histological damage score, serum Cr, Urea and CK, and kidney tissue ROS, MDA, 8-OH-dG,
GSH-PX, IL-6 and TNF-α, and a decrease in kidney tissue SOD activity. All these factors were significantly improved by both doses of HRS but the mean histological damage score, urea, Cr, CK, ROS, 8-OH-dG, GSH-PX, IL-6 and TNF-α for the high dose HRS treatment group were even lower.

Conclusions: Pretreatment by HRS ameliorated renal dysfunction in glycerol-induced rhabdomyolysis by inhibiting oxidative stress and the inflammatory response.

Key words: Acute kidney injury; Hydrogen-rich saline; Inflammation; Oxidative stress; Rhabdomyolysis
Introduction

Rhabdomyolysis refers to the breakdown of striated muscle, which results in the release of potentially toxic compounds into the circulation that may affect kidney function. Rhabdomyolysis is one of the most common reasons for acute kidney injury (AKI) and accounts for 15% of cases with a mortality of 5% [1]. Rhabdomyolysis can have many causes such as crush injury, medications, infections, myopathies and muscular dystrophies, and various other diseases. A common cause is excessive training, this is called exertional rhabdomyolysis and is often seen in military training [2] [3]. Rhabdomyolysis caused AKI in 51% of cases in a hospital study, with 32% fatalities [4]. The most widely used model of myoglobinuric acute renal failure (ARF) is by intramuscular injection of hypertonic glycerol [5]. This when utilized in rats produces myoglobinuria and the resulting responses typical of the human rhabdomyolysis syndrome.

The oxidative stress damage caused from the free-iron-catalyzed Fenton reaction, myoglobin redox cycling and generation of oxidized lipids can lead to rhabdomyolysis renal failure [6, 7]. The inflammatory reaction also participates in rhabdomyolysis AKI [8, 9]. The standard treatment for rhabdomyolysis AKI is aggressive rehydration and this might be sufficiently effective for patients with a mild form of the disease. However, the evidence suggests that pharmacologic agents that inhibit myoglobin redox cycling might represent the best therapeutic intervention for patients with a more severe form of this disease [6]. Studies have shown that vitamin
C [10-12]. Alpha-melanocyte-stimulating hormone [13], montelukast [14], resveratrol [15] and L-carnitine [16, 17] can protect against rhabdomyolysis AKI by rectifying detrimental changes in the antioxidant profile and systemic cytokines. At present the studies are largely on vitamin C. Oxidative metmyoglobin, the oxidised form of myoglobin, is a toxic molecule that triggers oxidative stress reactions, such as lipid peroxidation that lead to muscle ischemia-reperfusion injury [7]. Vitamin C has been shown to be able to effectively inhibit the formation of metmyoglobin [11] and appears to be a promising candidate for the prevention of rhabdomyolysis AKI [18]. However, vitamin C can make urine weakly acidic and reducing urine pH even slightly is adverse for patients. In addition, vitamin C reacts with free radicals, which can produce toxic metabolites that subsequently need to be removed.

In 2007, Ohsawa and his team for the first time showed that a low dose of hydrogen can significantly improve rats after stroke, and proved that the inhalation of H$_2$ gas markedly suppressed brain injury by buffering the effects of oxidative stress [19]. Later they also proved that inhalation of 2% hydrogen can treat reperfusion injury in the liver and myocardium [20, 21]. Due to the great limitations of breathing the drug, some scholars applied special equipment to prepare hydrogen-rich saline (HRS) [22, 23]. It has been shown that HRS can protect rats from ischemia and irrigation damage of the brain and in diabetic retinopathy [24, 25]. It has since been demonstrated using animal models that HRS may be beneficial to a wide range of diseases and ailments [26], including renal ischemia/reperfusion injury [27, 28], ischemia-induced cardio-renal injury [29], cisplatin-induced nephrotoxicity [30-32]
and chronic allograph nephropathy [33]. Dissolved hydrogen has also been studied in a clinical trial for its effectiveness in preventing chronic inflammation during hemodialysis [34]. So it is possible that HRS could also be a valuable tool against rhabdomyolysis AKI. HRS has been found to be a safe and effective antioxidant [19] and anti-inflammatory [20]. Compared with the traditional antioxidants, hydrogen has several advantages. Hydrogen can easily penetrate bio-membranes and diffuse into the cytosol, mitochondria and the nucleus due to its low molecular weight; it is mild enough not to disturb metabolic oxidation-reduction reactions or disrupt reactive oxygen species (ROS) mediated cell signaling. Many animal experiments have confirmed the antioxidant effect of the HRS [26], for example Ono et al administered 500 ml HRS to 4 patients and improved acute erythemtous skin diseases [35].

We hypothesized that pretreatment with HRS could protect against rhabdomyolysis AKI by antioxidant and anti-inflammatory methods. We applied the glycerol-induced rhabdomyolysis AKI rat model to validate HRS by the way of its antioxidant and anti-inflammatory protection against rhabdomyolysis AKI.

**Materials and methods**

**Animals**

Male Wistar rats, specific pathogen free (SPF), weighing 180~200g, were bought from Shandong University of Traditional Chinese Medicine, and bred in Mount Taishan Medical University animal center. The rats were fed with conventional rat
feed, free feeding and drinking. They were housed in an air-conditioned room with 12h light-dark cycles, where the temperature (21±2°C) and relative humidity (60-65%) were kept constant. The study protocol was approved by the Ethics Committee of No.88 Hospital of PLA.

**HRS production**

HRS was prepared as previously described [16, 25]. Hydrogen gas was dissolved in physiological saline for 2h under high pressure (0.4MPa) to a supersaturated level using HRS producing apparatus made by the atherosclerosis Institute of Taishan Medical School. The saturated HRS was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. HRS was sterilized by gamma radiation. HRS was freshly prepared every week, which ensured that a concentration of 0.6nmol/L was maintained.

**Study design**

Rats were randomly divided into 4 groups, each comprising of 12 animals. The animals were allowed free access to food but deprived of drinking water for 24h before glycerol injection.

Group 1 serves as the control group (Con). The animals were treated with saline (10ml/kg/d, i.p.) for 7d, deprived of drinking water for 24h on the sixth day, then were given saline (10ml/kg, i.m.). Half the dose was administered to each hind limb muscle.

Group 2 is the glycerol group (Gly). The animals were treated with saline (10ml/kg/d, i.p.) for 7d, deprived of drinking water for 24h on the sixth day, then were given 50%
glycerol (10ml/kg, i.m.). Half the dose was administered to each hind limb muscle.

Group 3 is high dosage HRS treatment group (Gly+HRS, 10ml/kg/d). The animals were treated with HRS (10ml/kg/d, i.p.) for 7d, deprived of drinking water for 24h on the sixth day, then were given 50% glycerol (10ml/kg, i.m.). Half the dose was administered to each hind limb muscle.

Group 4 is low dosage HRS treatment group (Gly+HRS, 5ml/kg/d). The animals were treated with HRS (5ml/kg/d HRS plus 5ml/kg/d saline, i.p.) for 7d, deprived of drinking water for 24h on the sixth day, then were given 50% glycerol (10ml/kg, i.m.). Half the dose was administered to each hind limb muscle.

The animals were placed in individual metabolic cages after the glycerol injection for 24h urine collections while allowed free access to food and water. At the end of the 24h, rats were anesthetized with 2% pentobarbital sodium (40mg/kg, i.p). After complete anesthesia, a midline abdominal incision was performed, and then their blood was collected via intracardiac puncture. Blood samples were centrifuged after 30min (4000g for 10min at 4°C), and samples were stored at -80°C until assay. After blood collection, the kidneys were harvested. The left kidney was frozen at -80°C for further enzymatic analysis; the right kidney was fixed in 4% paraformaldehyde solution for histological sectioning.

*Muscle enzymes*

Serum creatine kinase (CK) was detected using Beckman coulter AU5400 automatic biochemistry.

*Renal function*
Serum creatinine (Cr) and urea were detected using Beckman coulter AU5400 automatic biochemistry.

**Oxidative stress index and inflammation index**

Kidney tissue reactive oxygen species (ROS), 8-hydroxydeoxyguanosine (8-OH-dG), glutathione peroxidase (GSH-PX), Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels from the harvested and frozen kidney tissue were measured with commercial ELISA kits (Nanjing Jiancheng Bioengineering Co., Ltd., Chin) following the instructions from the manufacturer, and malondialdehyde (MDA) and superoxide dismutase (SOD) levels were tested with colorimetry according to the manufacturer's instructions.

**Kidney morphological studies**

Kidney tissues were embedded in paraffin wax, and 3µm sections were stained with periodic acid-Schiff (PAS). The pathological changes of kidney tissue were examined by light microscopy. Slides were reviewed blindly and scored with a semi quantitative scale evaluating changes found in AKI [17]. Specifically, for each kidney 100 cortical tubules (×200) from at least 10 different areas were scored. Higher scores represented more severe damage (maximum score per tubule was 10), with points given for the presence and extent of tubular epithelial cell flattening (1 point), brush border loss (1 point), cell membrane bleb formation (1 or 2 points), interstitial edema (1 point), cytoplasmic vacuolization (1 point), cell necrosis (1 or 2 points), and tubular lumen obstruction (1 or 2 points).

**Statistical analysis**
Statistical analyses were performed with Prism 5.0 (Graph Pad, San Diego, CA). Data were expressed as mean ± SD. We used the t test and One-Way analysis of variance followed by LSD test for the comparisons between two groups and among many groups respectively. A value of $P<0.05$ was accepted as statistically significant.

**Results**

*HRS-pretreated rats inhibit the increase of serum CK by glycerol-induced renal dysfunction*

Glycerol injection significantly increased serum CK [5971±3236 U/L vs. 652±165 U/L ($P<0.01$)]. HRS (10ml/kg/d and 5ml/kg/d) treatment significantly decreased CK compared with the glycerol group [2638±1960 U/L and 2873±1785 U/L vs. 5971±3236 U/L] ($P<0.05$).

*HRS-pretreated rats have marked protection from glycerol-induced renal dysfunction*

Glycerol injection significantly increased serum Urea and Cr. HRS (10ml/kg/d and 5ml/kg/d) treatment significantly improved this by decreasing back towards the control values in Urea and Cr. The high dose HRS treatment group was obviously superior to the low dose HRS treatment group (Figure 1A, B).

*HRS-pretreatment protects rats from renal tubular injury by glycerol*

By light microscopy, the control group had no obvious abnormalities (Figure 2a). In the glycerol group, the basic histological abnormalities were tubular necrosis, cast formation, brush border loss and interstitial edema (Figure 2b). Compared with the glycerol group, tubular cell necrosis and cast formation partially decreased with high
dosage HRS and low dosage HRS treatment (Figure 2c, d). The mean histological damage score for the glycerol group was significantly higher than control group ($P<0.01$), the mean histological damage score for the high dose HRS treatment group and the low dose HRS treatment group were lower than control group ($P<0.01$). The mean histological damage score in high dose HRS treatment group was lower than in low dosage HRS treatment group ($P<0.01$). (Figure 3)

**HRS-pretreatment inhibits the increase in the renal oxidative stress index induced by glycerol**

The levels of kidney tissue ROS, MDA and 8-OH-dG significantly increased with glycerol treatment ($P<0.01$). Both high dose HRS and low dose HRS treatments showed significant reduction in ROS, MDA and 8-OH-dG levels ($P<0.01$). The levels of ROS and 8-OH-dG decreased more in the high dose HRS treatment than in low dose HRS treatment ($P<0.05$). (Figure 4A, B, C).

**HRS-pretreatment changes the expression of the antioxidative stress index**

The activity of SOD decreased and GSH-PX increased after glycerol injection. HRS (10ml/kg/d and 5ml/kg/d) treatment significantly increased SOD and decreased GSH-PX ($P<0.01$). The effect of GSH-PX in the high dose HRS treatment was superior to the low dose HRS treatment ($P<0.05$). (Figure 5A, B.)

**HRS-pretreatment inhibits the increase in the renal inflammation index induced by glycerol**

Glycerol injection significantly increased IL-6 and TNF-α ($P<0.01$). The groups treated with HRS (10ml/kg/d and 5ml/kg/d) showed significantly decreased IL-6 and
TNF-α \((P<0.01)\). The levels of IL-6 and TNF-α in the high dose HRS treatment group were lower than in the low dose HRS treatment group \((P < 0.05)\). (Figure 6A, B.)

**Discussion**

In this study, we investigated the consequences of HRS on renal dysfunction caused by glycerol, and found that pretreatment of animals with HRS significantly reduced renal dysfunction and improved upon the alterations observed with glycerol injections.

Intramuscular injection of glycerol in rats can result in rhabdomyolyis AKI. CK is the most sensitive damage index for muscle cells [33]. When the level of CK is more than 5000 U/L, AKI easily occurs. In this study, serum CK increased after intramuscular injection of glycerol to a mean value of 7000 U/L.

The etiology of rhabdomyolyis AKI results from the lysis of myocytes and the release of their content into the circulation, leading to circulating myoglobin that is deposited in the kidney, causing renal tubular obstruction and necrosis, accompanied by intense renal vasoconstriction [7]. Glycerol-induced renal damage is commonly used as a rhabdomyolysis AKI model. This study found that renal tubular protein casts appeared and serum urea and Cr increased after the animal’s intramuscular injection of glycerol.

Glycerol-induced renal damage was accompanied by oxidative stress and an inflammatory reaction. Our experimental results showed that levels of ROS, MDA,
8-OH-dG, GSH-PX, ROS, IL-6 and TNF-α in kidney tissue significantly increased, while activity of SOD in kidney tissue significantly reduced. Research has shown that exercise from long distance running may cause lipid peroxidation damage in the skeletal muscle and kidney [34]. Increasing studies have shown that myoglobin mediated oxidative damage plays a key role in the development of rhabdomyolysis AKI. Oxidative stress and inflammatory reactions are involved in rhabdomyolysis AKI occurrence and development [6-9].

HRS abrogates rhabdomyolysis AKI by rectifying detrimental changes in antioxidant profiles and systemic cytokine production. Our study showed that pretreatment of animals with HRS significantly reduced the levels of serum Cr and urea which indicated that HRS had a protective effect on glycerol-induced rhabdomyolysis AKI in rats. In the treatment group, the levels of kidney tissue ROS, MDA, 8-OH-dG and GSH-PX significantly dropped, activity of kidney tissue SOD increased, and concentrations of kidney tissue IL-6, TNF-α decreased. These results clearly demonstrated that HRS, probably via its antioxidation stress and anti-inflammatory properties, ameliorates glycerol-induced rhabdomyolysis AKI. This study also found that a high dose was better than a low dose contradicting the conclusions of the review by Ohno et al [26] where they say that there is no dose response; however, they came to their conclusion based on comparisons between hydrogen gas treatment and hydrogen in solution, not different doses of hydrogen in solution.

In summary, our results support the hypothesis that HRS can reduce the damage
from renal dysfunction caused by glycerol. HRS ameliorated renal dysfunction in glycerol-induced rhabdomyolysis by inhibiting oxidative stress and the inflammatory response. HRS is a meaningful pretreatment for rhabdomyolysis in sports medicine and military medicine. However, these results will require further work to fully evaluate the potential of HRS; in this study we took a sample at one time point, 24 hours after injury, further samples and a survival analysis of the rats may reveal more information about the longer term effectiveness of this treatment. Also since some cases, such as those resulting from crush injuries, are unpredictable, it is not always possible to be pretreated with HRS before rhabdomyolysis. Whether HRS can treat rhabdomyolysis AKI afterwards has not yet been confirmed. Further experimental study is required.
Disclosures: None of the authors have any disclosures or conflicts of interest to report

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References


**Figure legends**

**Figure. 1.** Effect of Hydrogen-rich saline (HRS) on serum urea and creatinine (Cr).

Con, control group; Gly, glycerol treated group; Gly+HRS 10ml/kg/d, HRS (10ml/kg/d i.p.) treated group; Gly+HRS 5ml/kg/d, HRS (5ml/kg/d i.p.) treated group.  
**P < 0.01 versus Con group; **P < 0.01 versus Gly group; **P < 0.01 versus Gly+HRS 10ml/kg/d group.

**Figure. 2.** Periodic acid-Schiff (PAS) staining of kidney sections (original magnification × 400).

(a) control group (b) glycerol treated group (c) hydrogen-rich saline (HRS) (10ml/kg/d i.p.) treated group (d) HRS (5ml/kg/d i.p.) treated group

**Figure. 3.** Kidney tissue histological damage score.

Con, control group; Gly, glycerol treated group; Gly+HRS 10ml/kg/d, hydrogen-rich saline (HRS) (10ml/kg/d i.p.) treated group; Gly+HRS 5ml/kg/d, HRS (5ml/kg/d i.p.) treated group. **P < 0.01 versus Con group; **P < 0.01 versus Gly group; **P < 0.01 versus Gly+HRS 10ml/kg/d group.

**Figure. 4.** Effect of hydrogen-rich saline (HRS) on kidney tissue reactive oxygen species (ROS), malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OH-dG).

Con, control group; Gly, glycerol treated group; Gly+HRS 10ml/kg/d, HRS (10ml/kg/d i.p.) treated group; Gly+HRS 5ml/kg/d, HRS (5ml/kg/d i.p.) treated group. **P < 0.01 versus Con group; **P < 0.01 versus Gly group; **P < 0.01 versus Gly+HRS 10ml/kg/d group.

**Figure. 5.** Effect of hydrogen-rich saline (HRS) on kidney tissue superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX).
Con, control group; Gly, glycerol treated group; Gly+HRS 10ml/kg/d, HRS (10ml/kg/d i.p.) treated group; Gly+HRS 5ml/kg/d, HRS (5ml/kg/d i.p.) treated group. **P<0.01 versus Con group; ## P<0.01 versus Gly group; ▲ P<0.05 versus Gly+HRS 10ml/kg/d group.

**Figure. 6.** Effect of hydrogen-rich saline (HRS) on kidney tissue interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α).

Con, control group; Gly, glycerol treated group; Gly+HRS 10ml/kg/d, HRS (10ml/kg/d i.p.) treated group; Gly+HRS 5ml/kg/d, HRS (5ml/kg/d i.p.) treated group. **P<0.01 versus Con group; ## P<0.01 versus Gly group; ▲ P<0.05 versus Gly+HRS 10ml/kg/d group.