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

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ABSTRACT

Background: Rhabdomyolysis is a leading cause of acute kidney injury. 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 acute kidney injury.

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

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

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1. 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 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 an 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 oxidized 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 et al. [19] for the first time showed that a low dose of hydrogen (H2) can significantly improve rats after stroke, and proved that the inhalation of H2 gas markedly suppressed brain injury by buffering the effects of oxidative stress. Later, they also proved that inhalation of 2% H2 can treat reperfusion injury in the liver and myocardium [20,21]. Because of 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 ischemic brain injury and 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 cardiorenal injury [29], cisplatin-induced nephrotoxicity [30–32], and chronic allograft nephropathy [33]. Dissolved H2 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, H2 has several advantages. H2 can easily penetrate biomembranes and diffuse into the cytosol, mitochondria, and the nucleus because of 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. [35] administrated 500 mL HRS to four patients and improved acute erythematous skin diseases.

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.

2. Materials and methods

2.1. Animals

Male Wistar rats, specific pathogen free, weighing 180–200 g, 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 12 h 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.

2.2. HRS production

HRS was prepared as previously described [25]. H2 gas was dissolved in physiological saline for 2 h under high pressure (0.4 MPa) to a supersaturated level using HRS producing apparatus made by the Institute of Atherosclerosis, Taishan Medical School, China. 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 0.6 nmol/L was maintained.

2.3. Study design

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

Group 1 serves as the control group. The animals were treated with saline (10 mL/kg/d, intraperitoneal [i.p.]) for 7 d, deprived of drinking water for 24 h on the sixth day, then were
given saline (10 mL/kg, intramuscular [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 (10 mL/kg/d, i.p.) for 7 d, deprived of drinking water for 24 h on the sixth day, then were given 50% glycerol (10 mL/kg, i.m.). Half the dose was administered to each hind limb muscle.

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

Group 4 is the low dosage HRS treatment group (Gly + HRS, 5 mL/kg/d). The animals were treated with HRS (5 mL/kg/d HRS plus 5 mL/kg/d saline, i.p.) for 7 d, deprived of drinking water for 24 h on the sixth day, then were given 50% glycerol (10 mL/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 24 h urine collections while allowed free access to food and water. At the end of the 24 h, rats were anesthetized with 2% pentobarbital sodium (40 mg/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 30 min (4000 g for 10 min 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 histologic sectioning.

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**Fig. 1** — Effect of HRS on serum urea (A) and Cr (B). Con, control group; Gly, glycerol treated group; Gly + HRS 10 mL/kg/d, HRS (10 mL/kg/d i.p.) treated group; Gly + HRS 5 mL/kg/d, HRS (5 mL/kg/d i.p.) treated group. **P < 0.01 versus Con group; ##P < 0.01 versus Gly group; ^^P < 0.01 versus Gly + HRS 10 mL/kg/d group.

**Fig. 2** — Periodic acid-Schiff staining of kidney sections (original magnification ×400). (A) Control group. (B) Glycerol treated group. (C) HRS (10 mL/kg/d i.p.) treated group. (D) HRS (5 mL/kg/d i.p.) treated group. (Color version of figure is available online.)
2.4. Muscle enzymes

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

2.5. Renal function

Serum creatinine (Cr) and urea were detected using AU5400 automatic biochemistry analyzer (Beckman coulter Inc).

2.6. Oxidative stress index and inflammation index

Kidney tissue 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 enzyme-linked immunosorbent assay kits (Nanjing Jiancheng Bioengineering Co, Ltd, China) following the instructions from the manufacturer, and malondialdehyde (MDA) and superoxide dismutase (SOD) levels were tested with colorimetry according to the manufacturer’s instructions.

2.7. Kidney morphologic studies

Kidney tissues were embedded in paraffin wax, and 3 μm sections were stained with periodic acid-Schiff. The pathologic changes of kidney tissue were examined by light microscopy. Slides were reviewed blindly and scored with a semiquantitative 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).

2.8. Statistical analysis

Statistical analyses were performed with Prism5.0 (Graph Pad, San Diego, CA). Data were expressed as mean ± standard deviation. We used the t-test and one-way analysis of variance followed by least significant difference test for the comparisons between two groups and among many groups, respectively. A value of \( P < 0.05 \) was accepted as statistically significant.
3. Results

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

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

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

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

3.3. HRS-pretreatment protects rats from renal tubular injury by glycerol

By light microscopy, the control group had no obvious abnormalities (Fig. 2A). In the glycerol group, the basic histologic abnormalities were tubular necrosis, cast formation, brush border loss, and interstitial edema (Fig. 2B). Compared with the glycerol group, tubular cell necrosis and cast formation partially decreased with high dosage HRS and low dosage HRS treatment (Fig. 2C and D). The mean histologic damage score for the glycerol group was significantly higher than control group (P < 0.01), the mean histologic damage scores for the high dose HRS treatment group and the low dose HRS treatment group were lower than control group (P < 0.01). The mean histologic damage score in high dose HRS treatment group was lower than in low dosage HRS treatment group (P < 0.01; Fig. 3).

3.4. 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; Fig. 4A–C).

3.5. HRS-pretreatment changes the expression of the antioxidative stress index

The activity of SOD decreased and GSH-PX increased after glycerol injection. HRS (10 mL/kg/d and 5 mL/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; Fig. 5A and B).

3.6. 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 (10 mL/kg/d and 5 mL/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; Fig. 6A and B).

4. 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 on the alterations observed with glycerol injections. Intramuscular injection of glycerol in rats can result in rhabdomyolysis AKI. CK is the most sensitive damage index for muscle cells [33]. When the level of CK is >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.
accompanied by intense renal vasoconstriction [7]. Glycerol-induced renal damage is commonly used as 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, whereas the 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 antioxidant 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 H2 gas treatment and H2 in solution, not different doses of H2 in solution.

5. Conclusions

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 h after injury, further samples and a survival analysis of the rats may reveal more information about the longer term effectiveness of this treatment. Also, because 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 afterward has not yet been confirmed. Further experimental study is required.

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