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Original Article

Three hydrogen-rich solutions protect against intestinal injury in uncontrolled hemorrhagic shock

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Abstract: Intestinal tissue got largely decreased blood supply in uncontrolled hemorrhagic shock, because of limited blood mainly supporting brain, heart, kidney *etc*. This makes intestine as the primary injury target after uncontrolled hemorrhagic shock. However, limited studies focus on how to protect intestine against hemorrhagic shock. Ringer's solution, pentoxifylline and hypertonic saline are widely used to resuscitate in haemorrhagic shock and sepsis tissue injury. Evidence showed that hydrogen inhibited inflammation and reduced oxidative damage. Here we tested the hypothesis whether hydrogen rich Ringer's, pentoxifyline and hypertonic saline solutions increase the benefit in protecting small intestine from injury in uncontrolled hemorrhagic shock rat model. We tested the anti-inflammation effect of H-Ringer's, HHES and HHSH administration. We found hydrogen-rich solutions treatment groups showed the decreased MDA, MPO, IL-6 and TNF-α levels, and increased SOD, IL-10 comparing with those of non-hydrogen solutions administration groups. Our histological results showed that these three solutions with saturation hydrogen alleviatived the intestinal injury including the intact intestinal villi and less neutrephil infiltration. Our results indicate that these three hydrogen-rich solutions can protect intestinal injure after uncontrolled hemorrhagic shock. The protective effect might be through inhibiting proinflammatory factors, promoting anti-inflammatory cytokines and reducing inflammatory cells infiltration. Our study has potential clinical importance of uncontrolled hemorrhagic shock patient's resuscitation.

Keywords: Hydrogen, hemorrhagic shock, intestinal injure, neutrephil infiltration, pro-inflammatory factors

Introduction

Approximately 30% of the deaths caused by trauma are due to hemorrhagic shock. Uncontrolled hemorrhagic shock causes body's low blood capacity and low perfusion of visceral organs. Limited blood supply mainly maintains brain, heart and kidney' function, leaving intestine blood supply largely decreased. Thus intestine mucosa is the first-affected site in uncontrolled hemorrhagic shock [1]. Ischemia/reperfusion could induce decreased intestinal contractile activity and increased microvascular permeability. Moreover, bacteria and endotoxin transformation caused by intestine mucosa injury may induce systemic inflammatory response syndrome [2]. Thus, intestine mucosa injury is pathological basis of multiple organ failure. Dantzker took intestinal tract as "the canary of the body" [3].

Complicated reasons cause ischemia/reperfusion injury following hemorrhagic shock. Evidence showed that oxygen free radical is the culprit during the early ischemia/reperfusion. It has been reported that Ringer's solution, hypertonic saline (HTS), hydroxyethyl starch (HES), and hypertonic sodium chloride hydroxyethyl starch (HSH) had expansion ability and could increase the osmotic pressure, thus these saline solutions are widely used in shock treatment [4]. Previous study showed that hydrogen-rich saline could act as antioxidants and selectively reduce hydroxyl radicals (OH) and peroxynitrite anion (ONOO-), which protect the brain after mild traumatic brain injury [5, 6]. Our previous study showed that hydrogen-rich saline could inhibit serum IL-6 and TNF-α activity, decrease the MDA production and SOD consumption, these results indicate that hydrogen-rich saline can reduce the inflammatory reac-

tion and the oxidative damage [7]. This raises the possibility that the hydrogen-rich solutions including hydrogen saturated Ringer's solution (H-Ringer), hydrogen saturated HES (H-HES) and hydrogen saturated HSH (H-HSH) might have better abilities of anti-shock, anti-inflammation and antioxidant.

We tested that whether administration of hydrogen-rich solutions including H-Ringer, H-HES and H-HSH could protect against intestinal injury in rat model. We observed the levels of malonaldehyde (MDA), superoxide dismutase (SOD), myeloperoxidase (MPO), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10), as while as the intestinal pathological changes. We found UHS could induce intestinal injury and inflammatory responses and hydrogen-rich solutions treatment could protect intestinal injury. Our study provides fundamental basis for clinical use of hydrogen-rich solutions.

Materials and methods

Preparation of hydrogen-rich solutions

The protocol of preparing hydrogen-rich solutions was according to previous study [8]. Hydrogen was dissolved in Ringer, HES and HSH respectively under high pressure (0.4 MPa). The dissolving procedure last 6 hr to achieve the supersaturated level. All the solutions were stored in an aluminum bag without dead volume at 4°C under atmospheric pressure.

Preparation of rat UHS model

Male Wistar rats (290-320 g) were obtained from Experimental Animal Center of Shandong University. Animals were raised under controlled conditions: the temperature maintained at 25 ± 2°C, the humidity was about 55%, and 12-h light/12-h dark cycle. Animals were deprived of water and food for 12 hr before experiment.

The UHS preparation procedure was similar with previous study [9]. Rats were anesthetized with pentobarbital (0.4 ml/100 g intraperitoneally). And rats were placed on the warming pad with spontaneously breathing, and then the left side of the femoral vein was isolated about 2 cm, Heparinization PE-50 hexene catheter was insert in the femoral vein about 2 cm, Heparin saline (50 U/100 g) was injected for further intravenous infusion. Same procedure was performed in the right side of the femoral vein. And the right side of the femoral vein was used for gathering blood sample. Physiological signal recorder (MP150, BIOPAC) was used to detect the mean arterial pressure and heart rate.

Grouping of animals

Seventy rats were randomly divided into seven groups, including:

Sham group: only received anaesthesia, cannulation, heparinization, and observation.

Ringer's solution treatment group: intravenous infusing Ringer's solution after UHS, 10 animals.

HES treatment group: intravenous infusing HES after UHS, 10 animals.

HSH treatment group: intravenous infusing HSH after UHS, 10 animals.

H-Ringer's solution treatment group: intravenous infusing H-Ringer's solution after UHS, 10 animals.

H-HES treatment group: intravenous infusing H-HES after UHS, 10 animals.

H-HSH treatment group: intravenous infusing H-HSH after UHS, 10 animals.

Biochemical analysis

For comparing the results between groups, we chose 0.2-1 g intestinal mucosa 5 cm below Ligament of Treitz. The intestinal mucosa was scraped off, and the cold normal saline washed mucosa to remove the blood. Mix the sample with 0.86% saline solution in the 1:9 ratios. Homogenized the tissue 10 s × 3~5 with 30 s interval. Centrifuged the sample at 3000 g for 15 min. follow the kit protocol to detect MDA, SOD, MPO, TNF-α, IL-6 and IL-10 levels. Testing kits of MDA, SOD and MPO were purchased from Nanjing JianCheng biological engineering institute. ELISA kits of TNF-α, IL-6 and IL-10 were from Shanghai Lengton Biological Technology Co. Ltd.

MDA reacted with thiobarbituric acid and formed a pink, thus UV-VIS spectrophotometer (T6-190-1-100NM PGeneral Co. Ltd, Beijing) could assessed intestinal MDA levels. SOD could eliminate superoxide anion free radical which was from Xanthine/xanthine oxidase

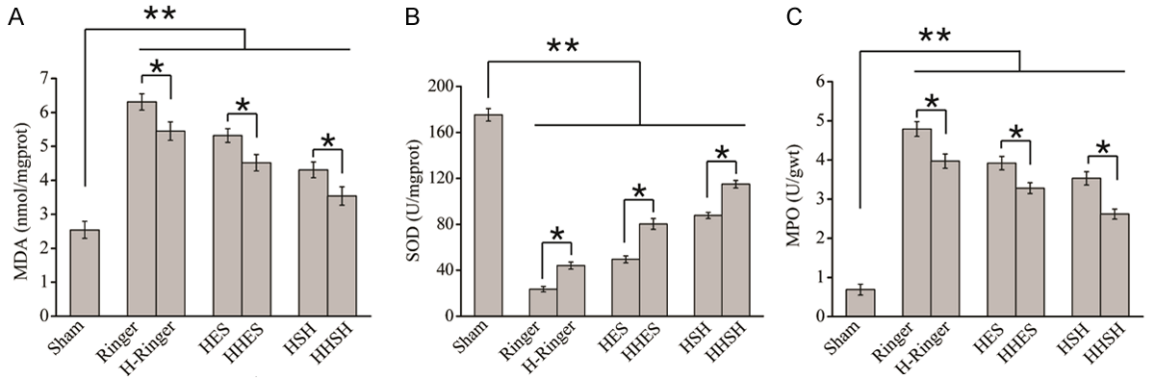

Figure 1. Hydrogen-rich saline solutions treatment reduced oxidative stress. A: MDA levels in sham operated animals, non hydrogen saline solutions and hydrogen-rich saline solutions treatment animals. B: SOD activities in sham operated, non hydrogen saline solutions and hydrogen-rich saline solutions treatment groups. C: MPO in sham operated animals, non hydrogen saline solutions treatment animals and hydrogen-rich saline solutions treatment animals. Data are expressed as means ± SEM, n = 10 for each group.

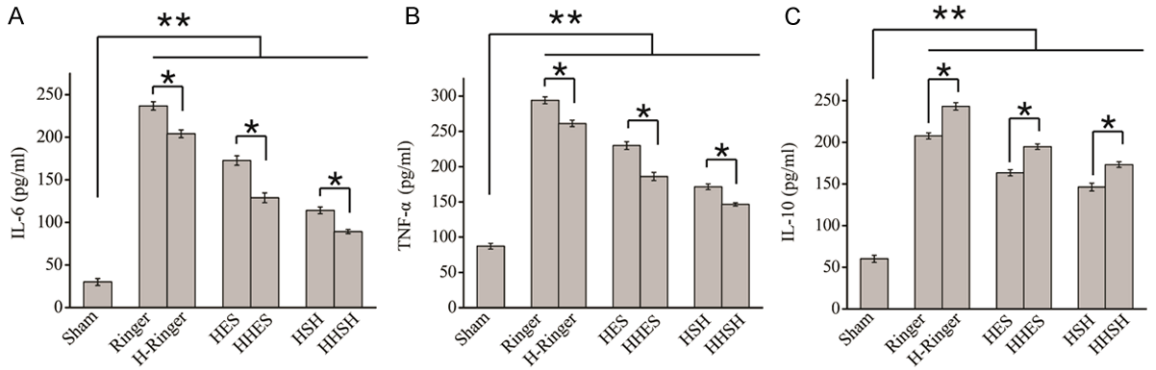

Figure 2. IL-1b, TNF-α and IL-6 concentrations in sham operated animals, non hydrogen saline solutions treatment animals and hydrogen-rich saline solutions treatment animals. Data are expressed as means ± SEM, n = 10 for each group.

reaction and protect cells against oxidative stress. Superoxide anion free radical could oxide hydroxylamine to nitrite, thus nitrite could evaluate SOD activity.

Statistical analysis

SPSS 13.0 software was used for statistical analysis, and all the data were presented as mean ± SEM. The differences between groups were determined by one-way analysis of variance. Significant differences were determined by $P < 0.01$.

Results

Hydrogen-rich solutions treatment reduced oxidative stress

We tested whether hydrogen-rich solutions could reduce the intestinal oxidative stress after UHS. MDA and SOD are the indicators of oxidative stress. We observed that all the UHS groups (including both hydrogen-rich solutions and non-hydrogen saline solutions) had increased MDA and decreased SOD comparing with Sham-operation group ($P < 0.01$, **Figure 1A** and **1B**). This indicated that UHS could induce intestinal oxidative stress. However, administration of hydrogen-rich solutions reduced the MDA levels and improved SOD levels comparing with the non-hydrogen saline groups ($P < 0.01$, **Figure 1A** and **1B**). MPO was the indicator of neutrophil, MPO deficient neutrophil infiltration could induce oxidative stress which caused cellular injury. We found that hydrogen-rich solutions treatment could reduce MPO levels comparing with non-hydrogen saline solutions ($P < 0.01$, **Figure 1C**). These results suggest that hydrogen-rich solutions treatment could reduce intestinal oxidative stress.

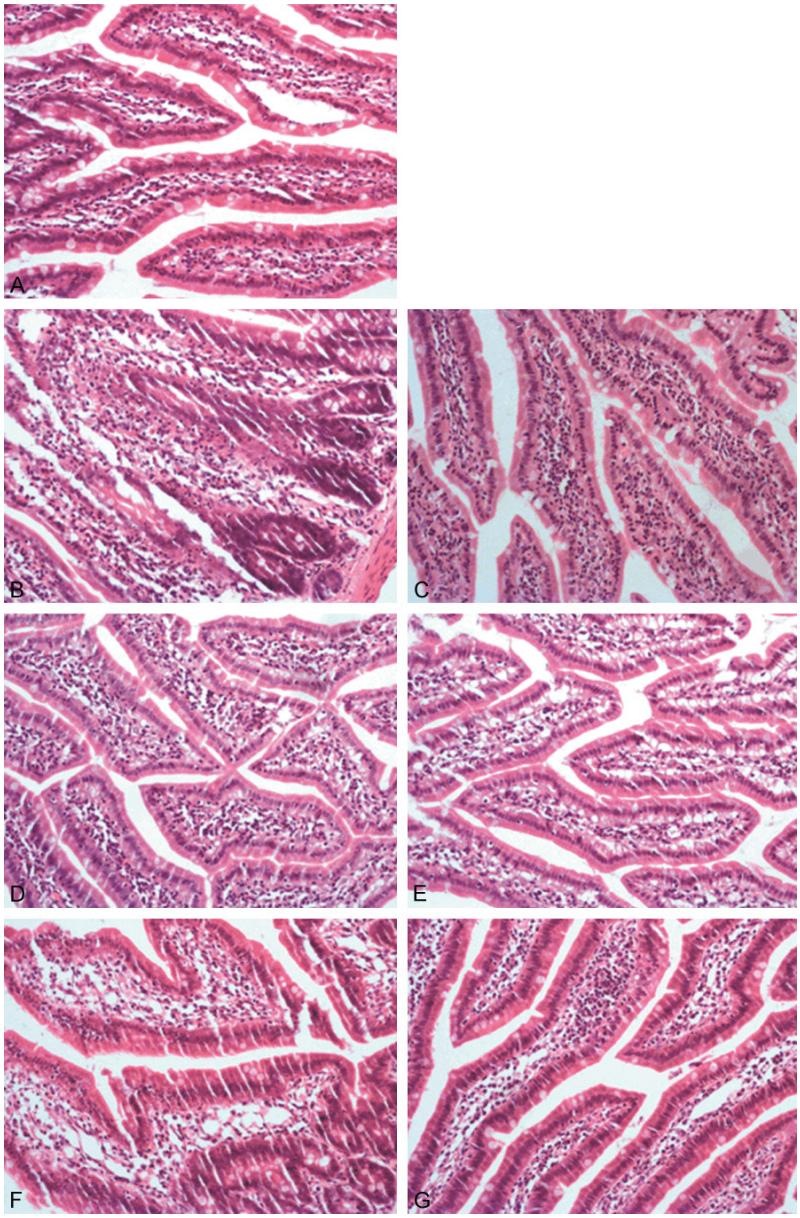

Figure 3. Administration of hydrogen-rich saline solutions alleviated intestinal histological injury. A: No histological features of intestinal tissue were observed in Sham-operation group. B, D and F present the results that massive intestinal injuries were observed in Ring's solution, HES and HHS treatment groups. C, E and G show the slightly intestinal injuries in H-Ring's solution, H-HES and H-HHS treatment groups.

Hydrogen-rich solutions treatment decreased pro-inflammatory factors and increased anti-inflammatory cytokine level

We tested the activities of cytokines to investigate the anti-inflammation effect of hydrogen-rich solutions. As shown in **Figure 2A-C**, UHS induced significantly elevated IL-6, TNF-α, IL-10 in intestine ($P < 0.01$). However, the concentrations of pro-inflammatory factors including IL-6 and TNF-α were significant lowered in hydrogen-rich solutions treatment groups comparing with those in non-hydrogen saline solutions ($P < 0.01$, **Figure 2A** and **2B**). The anti- inflammatory cytokine IL-10 was significant increased when rats were administrated by hydrogen-rich solutions ($P < 0.01$, **Figure 2C**).

Hydrogen-rich solutions ameliorated UHS induced intestinal injury

Previous evidence showed that UHS could induce intestinal damage. Thus we observed intestinal histological changes under different solutions treatment conditions. As shown in **Figure 3A**, No intestinal injury was observed in the sham-operated group. In non hydrogen- rich saline solutions group including Ringer's solution, HES and HSH treatment, all the rat intestinal mucosa showed obvious histological injury. Plenty of intestinal villi were stripped. Massive neutrophil infiltrated the intestinal tissue. And the capillaries were hyperemia (**Figure 3B**, **3D** and **3F**). However, administration of hydrogen-rich solutions rescued the histological injury. We observed that in the hydrogen-rich solutions group, the injury of intestinal mucosa was alleviative, including the intact intestinal villi, less infiltrated inflammatory cells and slightly hyperemia capillaries (**Figure 3C**, **3E** and **3G**).

Discussion

In this study we demonstrated that hydrogen-rich solutions including H-Ringer's solution, H-HES, H-HSH could have increased benefit comparing with Ringer's solution, HES and HSH

in protecting intestine against injury in uncontrolled hemorrhagic shock. Hydrogen-rich solutions could protect intestinal histological injury, decrease intestinal oxidative stress and inflammatory responses.

Hydrogen (H_2) is a natural gas, which could react with hydroxyl radical to produce water. Ohsawa et al showed hydrogen could act as an antioxidant and have potential preventive and therapeutic applications [10]. Abundant evidence showed that hydrogen-rich saline had anti-inflammatory effect and could protect brain, lung, heart and pancreas following hemorrhagic shock [11-13]. Recent study showed that Hydrogen-rich saline can decrease histological renal injury and I/R-induced apoptosis, and the protect mechanisms might be reducing oxidative stress and inflammation [14]. Chen et al showed intraperitoneal injecting Hydrogen-rich saline significantly limited the neutrophil infiltration and lipid oxidation [2].

Small intestine has abundant of bacteria and lymphocytes. Uncontrolled hemorrhagic shock induced intestinal ischemia reperfusion injury [15], this injury caused bacteria and toxins transposition through the circulatory system [2]. Massive cytokines and inflammatory factors were activated by bacteria and toxins, consequently even multiple organ dysfunction syndrome was induced [16]. Inflammation played an important role in shock progressing. Shock could activate inflammation, meanwhile enhanced inflammation factor and accumulated leukocytes exacerbate shock, thus vicious circle occurred [11, 17]. Tumor necrosis factor-alpha (TNF-alpha) plays a crucial role in inflammation. TNFα is one of the cytokines that mediate the acute phase reaction. TNFα was produced primarily by macrophages as while as other cell types including lymphoid cells, endothelial cells et al. TNF can induce fever, apoptosis and inflammation et al [18]. Multiple tissues can produce Interleukin-6 (IL-6). IL-6 has two opposing effects on the inflammatory response. Previous study showed that IL-6 stimulated acute phase protein synthesis, amplified inflammatory response and induced tissue injuries [19, 20]. Interleukin-10 (IL-10) alleviated inflammatory response through stimulating IL-1ra production in the monocyte, it seemed that IL-10 could counteract the effects of the proinflammatory cytokines including TNFα and IL-6 et al [21]. Thus the amounts of TNF-α, IL-6 and IL-10 might perform the inflammatory injuries. It is reported that increased cytokines including TNF-α, IL-6 and IL-10 were produced, and these cytokines could activate and aggregate neutrophil which injured the tissues [22]. Xu et al reported that hydrogen saline has the anti-inflammation effect not only through limiting the neutrophil infiltration, but also inhibiting TNFα production from macrophagocyte by suppressing TNFα mRNA activity [23]. In this study, we treated uncontrolled hemorrhagic shock rats with hydrogen-rich solutions including H-Ringer, HHES and HHSH. Comparing with the non hydrogen-rich solutions treatment group, we found H-Ringer, HHES and HHSH treatment could decrease IL-6 and TNFα expression and promote IL-10 production. Histological evidence showed that H-Ringer, HHES and HHSH treatment induced significant decreased inflammatory cells infiltration and limited injured villus in the intestinal tissue. These results suggest that hydrogen-rich solutions could decrease proinflammatory cytokines and increase anti-proinflammatory cytokines, which could protect against intestinal ischemia/reperfusion injury after UHS.

Xanthine Dehydrogenase transforms to Xanthine Oxidase, because of lack of ATP supply in the ischemia condition. And Xanthine Oxidase catalyzes Hypoxanthine to produce Xanthine and Uric Acid, meanwhile produce large oxygen free radical [24]. Oxygen free radical plays an important role in the pathogenesis of ischemia/reperfusion injury [25]. Intestinal mucosa is susceptible to oxygen free radical, thus oxidative stress plays an important role in intestinal injury [26]. Hemorrhagic shock could increase peroxide product and cause oxidative stress [27]. MDA reflects the degree of lipid peroxidation and is widely used as a biomarker to measure the oxidative stress in an organism. MPO is a peroxidase an enzyme that is contained in neutrophilic granulocyte lysosomes, it can be used to assess neutrophil infiltration in intestinal tissue [28]. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, thus SOD serves a key antioxidant role to protect cells against oxidative stress. SOD as antioxidant indicator can be used to elevate intestinal recovery after UHS. In this study, we used MDA, MPO and SOD as indicators to test hydrogen-rich solutions protective function. We

found that hydrogen-rich solutions treatment rats had significantly decreased MDA and MPO comparing with non-hydrogen-rich solutions treatment rats while SOD level was lower in hydrogen-rich solutions treatment group than that in non-hydrogen-rich solutions. These results indicate that the protective effect of hydrogen might be through inhibition of lipid oxidation reaction, neutrephil infiltration and increasing ability of scavenging free radical.

We compared the protective effects of different hydrogen-rich solutions. We found that HHSH solution was the best and H-Ringer's solution the last protective. We propose the possible reasons might be: (1) the expansion ability of Ringer's solution is not good enough [29], Plasma colloid osmotic pressure is decreased after Ringer's solution injection, this causes tissue edema because of water permeating to interstitial fluid. Tissue edema leads to the increased distance between cells and blood capillary, which results in reduced oxygen uptake and abundant free oxygen. (2) HES solution has good expansion ability and also can protect blood capillary. HES can decrease capillary leak syndrome probability and improve the oxygen carrying capacity [4]. (3) HSH can quickly expand plasma volume, and transiently recover the macro-hemodynamics [30]. Thus HSH transfusion can increase blood supply for intestine and decrease the oxygen radical by sufficient ATP [31, 32].

In this study, we investigated hydrogen-rich solutions could protect against intestinal ischemia-reperfusion injury after uncontrolled hemorrhagic shock in rats. Hydrogen increases the benefit of Ringer's, HES and HSH solutions. This study has potential importance in uncontrolled hemorrhagic shock field.

Disclosure of conflict of interest

None.

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