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Original Article

Effects of heme oxygenase-1 in the intestine on the intestinal barrier function of rats with hemorrhagic shock

Xinyue Gao, Jianping Zhang, Dawei Yang, Qiang Tao, Li Liu, Jianrong Guo

Department of Anesthesiology, Gongli Hospital Affiliated to The Second Military Medical University, Shanghai, China

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Abstract: Objective: This study aimed to investigate effects of heme oxygenase -1 (HO-1) at different concentrations on intestinal barrier function. Methods: Male SD rats (n=50) were assigned into lactococcus lactis expressing HO-1 (LL-HO-1) group (HO group; n=10), Gln group (n=10), lactococcus lactis group (LL group; n=10), zinc protoporphyrin (ZnPP group; n=10) and PBS group (n=10) randomly. Results: When compared with ZnPP group, mortality, Chiu's score, and MPO activity reduced significantly, while HO-1 content and IL-10 concentration as well as PaO_2 and MAP increased significantly in HO group, Gln group, LL group and PBS group ($P<0.05$) after fluid infusion. When compared with LL group and PBS group, mortality, Chiu's score and MPO activity reduced significantly, but HO-1 content and IL-10 concentration, PaO_2 and MAP increased significantly in HO group and Gln group ($P<0.05$) after fluid infusion. When compared with Gln group, mortality, Chiu's score and MPO activity reduced significantly, while HO-1 content and IL-10 concentration, PaO_2 and MAP increased significantly in HO group ($P<0.05$) after fluid infusion. Conclusion: Oral LL-HO-1 and Gln may stimulate HO-1 expression in intestine of rats with hemorrhagic shock, exerting protective effects on intestinal barrier function, which is associated with inhibition of intestinal inflammation and improvement of PaO_2 and MAP after fluid infusion.

Keywords: Heme oxygenase-1, hemorrhagic shock, glutamine, intestinal barrier, intestinal inflammation

Introduction

Hemorrhagic shock (HS) is a major cause of high mortality in both peacetime and war, and has high incidence of complications and high mortality [1]. Therapy of HS requires comprehensive measures including timely surgical hemostasis and fluid infusion. Although a lot of measures have been developed for the therapy of shock, and great progresses have been achieved in some of these measures such as increasing blood volume, blood pressure regulation, usage of hemostatic and tourniquet, and resuscitation with limited volume, clinicians usually focus on the protection of important organs (such as heart, lung and kidney) when neglect changes occur in intestinal function in clinical practice [2]. Actually, intestinal function is damaged as early as immediately after shock [2]. Intestine is the largest immune organ, and macrophages and activated neutrophils are involved in release of different pro-inflammatory mediators and oxygen free radicals, as well as the ischemia/reperfusion induced intestinal barrier dysfunction, which are crucial for the occurrence and progression of systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF) [3, 4].

Heme oxygenase (HO) family has three isozymes: HO-1, HO-2 and HO-3. HO-1, also known as heat shock protein 32, has been found to be a unique inducible one. HO-1 may degrade free heme released by aging or damaged red blood cells to generate carbon monoxide (CO), biliverdin (subsequently metabolized into bilirubin in intestine) and divalent iron ions. HO-1 and metabolites of heme have anti-oxidative capability and are important anti-oxidants [5]. Under a physiological condition, HO-2 is the major type of HO in gastrointestinal tract, while HO-1

is not expressed in the intestine. Under a stress condition (such as ischemia/hypoxia) or in presence of an inducer, HO-1 expression is induced to exert important physiological effects [6, 7].

Early parenteral nutrition with glutamine (Gln) has been found to reduce the incidence of MODS and mortality of severe trauma patients, which is ascribed to the protection on intestinal barrier function, compromised bacterial translocation and attenuation of intestinal inflammation [8]. In animal experiments, findings also confirmed that Gln was able to stimulate HO-1 expression in intestine of HS rats and endotoxemia rats, suggesting protective effects of Gln on intestinal barrier function [9-11] which was consistent with our previous findings [12].

In our previous study, genetic engineering technique was employed to construct recombinant lactococcus lactis expressing HO-1 (LL-HO-1) which was orally administered in healthy rats. Then, HS and/or endotoxemia was introduced to these rats, and results showed that biologically active HO-1 increased significantly in intestine, exerting protective effects on intestinal barrier function and attenuating intestinal inflammation [13-17].

This is one of our serial studies. On the basis of protective effects of LL-HO-1 and Gln, this study was undertaken to investigate HO-1 expression in the intestine of HS rats which received pretreatment with different inducers. According to blood gas analysis and detections of mean arterial pressure (MAP) and expressions of proinflammatory and anti-inflammatory cytokines, potential mechanism underlying protective effects of HO-1 on the intestinal barrier function was further explored.

Methods

Drugs and reagents

LL-HO-1 was kindly provided by the Key Laboratory of Anesthesiology in Jiangsu Province (Xuzhou, China). Gln (Amersco, USA), zinc protoporphyrin (ZnPP; Sigma), PBS, TNF-α and IL-10 immunohistochemistry kits (Boster Biotech Co., Ltd), HO-1 antibody (Sigma, USA), HO-1 immunohistochemistry kit (Stressgen, Canada), and MPO activity detection kit (Nanjing Jiancheng Biotech Co., Ltd) were used in this study.

Grouping and treatments

This study has been approved by the Ethics Committee of Gongli Hospital Affiliated to the Second Military Medical University (GJ-2013-10) and the international guidelines for animal welfare were followed. A total of 50 healthy, specific pathogen free male SD rats weighing 280-320 g were purchased from the Experimental Animal Center of Xuzhou Medical Collage in Jiangsu Province, China. Animals were randomly assigned into LL-HO-1 group (HO group; n=10), Gln group (n=10), lactococcus lactis group (LL group; n=10), ZnPP group (n=10) and PBS group (n=10). In Gln group, Gln in PBS (1 mL) was administered orally at 0.75 g/kg 6 h before experiment; in ZnPP group, ZnPP was intraperitoneally administered at 10 mmol/kg 1 h before experiment. In the remaining groups, animals were orally treated with 1 mL of LL-HO-1 or LL (2.5×10^9 CFU/mL) or PBS 24 h before experiment. Then, HS was introduced to these animals, and sample collection was done 1 h later. All the animals were housed for 7 days to accommodate to the environment and received food deprivation for more than 12 h. After experiments, animals were given *ad libitum* access to water and food and housed in cages separately. The room temperature was maintained at 22°C-25°C.

Establishment of HS animal model

In brief, rats were intraperitoneally anesthetized with 10% chloral hydrate at 300-350 mg/kg and then placed in a supine position. The hair at the groin and thigh were removed, followed by sterilization. The right femoral artery and left femoral vein were separated under an aseptic condition. A 22 G trocar was used to puncture the femoral vein and artery and then connected to the three-way valves, blood pressure transducer and patient 401 (Biomedical Systems Inc.) multifunctional monitor. The blood pressure of the femoral artery and the pulse rate were measured continuously (catheter was pre-treated with 7.5 U/ml heparin). When the blood pressure became stable, bloodletting was done through the femoral artery intermittently within 5 min, and timekeeping began when the MAP reduced to 40 mmHg. Intermittent bloodletting and blood transfusion via the femoral vein were done to maintain MAP at 35-40 mmHg for 60 min (rate of bloodletting

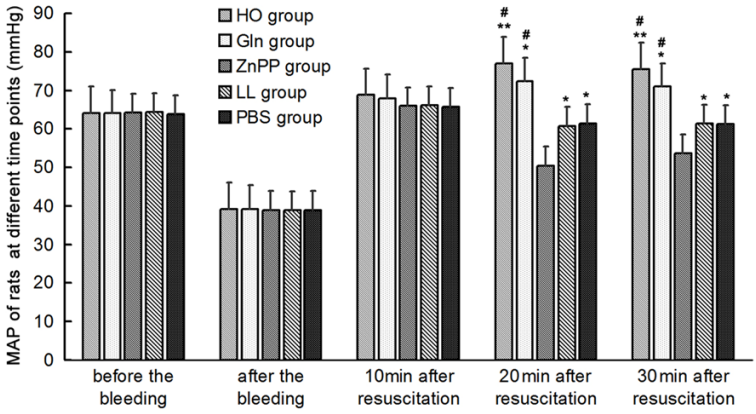

Figure 1. MAP of rats in different groups before and after bloodletting, and at 10, 20 and 30 min after resuscitation. *$P<0.05$: when compared with ZnPP group, MAP in HO group, Gln group, LL group and PBS group increased significantly at 20 min and 30 min after resuscitation; #$P<0.05$: when compared with LL group and PBS group, MAP in HO group and Gln group increased significantly at 20 min and 30 min after resuscitation; **$P<0.05$: when compared with Gln group, MAP in HO group increased significantly at 20 min and 30 min after resuscitation.

was 1-1.5 mL/min, and blood was preserved in 1-m sterile syringe containing 7.5 U of heparin). Then, the blood collected and Ringer's lactate solution (2 times of the volume of blood collected) were used for resuscitation. After the blood had been transfused via the femoral vein, fluid was transfused, which was performed within 30 min. Successful resuscitation was defined as the blood pressure equal to or higher than 90% of normal blood pressure. Finally, the trocar was removed, the blood vessels were ligated, and wound was closed under an aseptic condition. The rats were assured to breathe air freely and the anal temperature was monitored continuously. The rat temperature was maintained at 37°C-37.5°C by heating with a lamp.

Sample collection and detections

Blood (0.1 mL) was collected via the femoral artery for blood gas analysis before bloodletting, before resuscitation and after resuscitation with a COMPACT 3 analyzer (COMPACT, Swiss). At the same time, the MAP was measured at 10, 20 and 30 min of fluid infusion. At 1 h after establishment of HS animal model, laparotomy was performed under an aseptic condition, and a fraction of small intestine (2-3 cm in length) was excised at the site near the terminal ileum. A part of small intestine (1 cm) was fixed in 10% formaldehyde solution and the remaining intestine was stored at -80°C for use. The mortality was calculated. Colorimetry was employed to measure the MPO activity of the intestine, and immunohistochemistry to detect the expressions of TNF-OMPACT, Swiss). At the same time, the MAP was measured at 10, 20 and 30 min of fluid infusion. At 1 h after establishment of HS animal model, laparotomy was performed under an aseptic condition, and a fraction of small intestine (2-3 cm in length) was excised at the site near the terminal ileum. A part of small intestine (1 cm) was fixed in 10% formaldehyde solution and the remaining intestine was stored at -80°C for use. The mortality was calculated. Colorimetry was employed to measure the MPO activity of the intestine, and immunohistochemistry to detect the expressions of TNF-α, IL-10 and HO-1. Ten spots were randomly selected from each field, and the difference in the optical density (OD) between the spot and the background was used as the spot OD. ELISA was performed to measure the HO-1 expression in the intestine. In addition, the intestine was processed for histological examination. Chiu's 6-grade scoring system was employed to evaluate the severity of intestinal mucosal injury: grade 0, normal villi; grade 1, subepithelial Gruenhagenna space (edema), usually at the apex of the villus; grade 2, extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria; grade 3, massive epithelial lifting down the sides of villi; a few tips may be denuded; grade 4, denuded villi with lamina propria and dilated capillaries exposed; grade 5, Digestion and disintegration of lamina propria; haemorrhage and ulceration [18].

Statistical analysis

Quantitative data are expressed as mean ± standard deviation (x±s). Intragroup comparisons were done with *t* test, and intergroup comparisons with one way analysis of variance. Qualitative data were compared with chi square

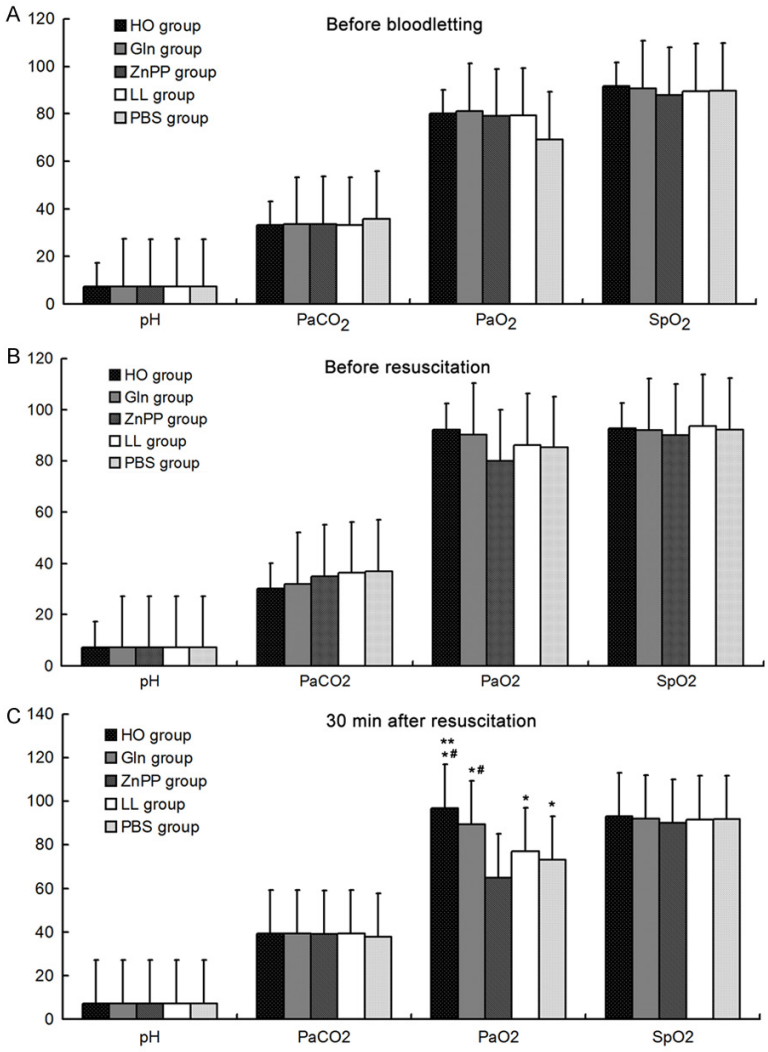

Figure 2. pH, $PaCO_2$, PaO_2 and SpO_2 of HS rats before bloodletting (A), before resuscitation (B), and at 30 min after resuscitation (C). *$P<0.05$: when compared with ZnPP group, PaO_2 in HO group, Gln group, LL group and PBS group increased significantly at 30 min after resuscitation; #$P<0.05$: when compared with LL group and PBS group, PaO_2 in HO group and Gln group increased significantly at 30 min after resuscitation; **$P<0.05$: when compared with Gln group, PaO_2 in HO group increased significantly at 30 min after resuscitation.

test. Categorical data were tested with rank sum test. Statistical analysis was performed with SPSS version 19.0. A value of $P<0.05$ was considered statistically significant.

Results

Blood loss and mortality

The blood loss was 4.1±1.7 mL in HO group, 4.0±1.9 mL in Gln group, 4.0±1.8 mL in LL group, 4.0±2.0 mL in ZnPP group, and 4.1±1.9 mL in phosphate buffer solution (PBS) group, showing no significant difference among them. At 1 h after establishment of animal model, the mortality was 10% (1/10) in HO group, 20% (2/10) in Gln group, 30% (3/10) in LL group and PBS group, and 40% (4/10) in ZnPP group. When compared with ZnPP group, the mortality reduced significantly in HO group, Gln group, LL group and PBS group; when compared with LL group and PBS group, the mortality reduced significantly; when compared with Gln group, the mortality reduced significantly in HO group ($P<0.05$). There was no significant difference in the blood loss among 5 groups, suggesting that the animal model was successfully established. The low mortality in HO group and Gln group indicated that HO-1 was protective and LL-HO-1 was effective to induce HO-1.

MAP and blood gas analysis

As shown in **Figure 1**, the MAPs in HO group, Gln group, LL group and PBS group were significantly higher than in ZnPP group at 20 min and 30 min after resuscitation. When compared with LL group and PBS group, the MAP increased significantly in HO group and Gln group at 20 min and 30 min after resuscitation. When compared with Gln group, MAP in HO group increased significantly at 20 min and 30 min after resuscitation ($P<0.05$).

As shown in **Figure 2**, the PaO_2 in ZnPP group was comparable to that in HO group, Gln group, LL group and PBS group before bloodletting and before resuscitation. At 30 min after resuscitation, the PaO_2 in HO group, Gln group, LL group and PBS group was significantly higher than in ZnPP group; when compared with LL group and PBS group, the PaO_2 increased significantly in HO group and Gln group at 30 min after resuscitation; when compared with Gln

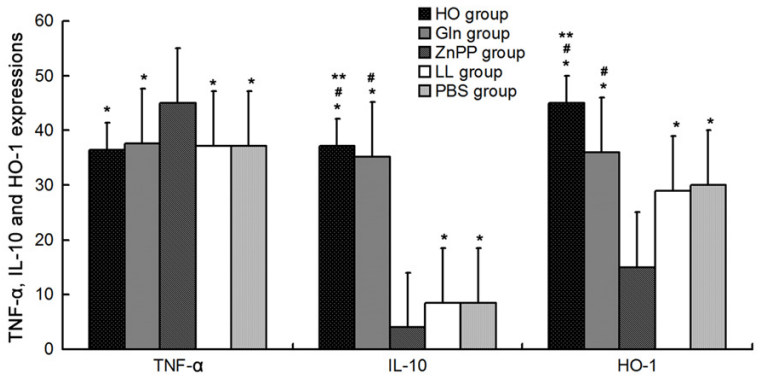

Figure 3. Contents of TNF-α, IL-10 and HO-1 in the intestine of five groups. *$^{*}P<0.05$: when compared with ZnPP group, TNF-α content reduced significantly and IL-10 and HO-1 contents increased significantly in HO group, Gln group, LL group and PBS group; $^{\#}P<0.05$: when compared with LL group and PBS group, IL-10 and HO-1 contents increased significantly in HO group and Gln group; $^{**}P<0.05$: when compared with Gln group, IL-10 and HO-1 contents increased significantly in HO group.

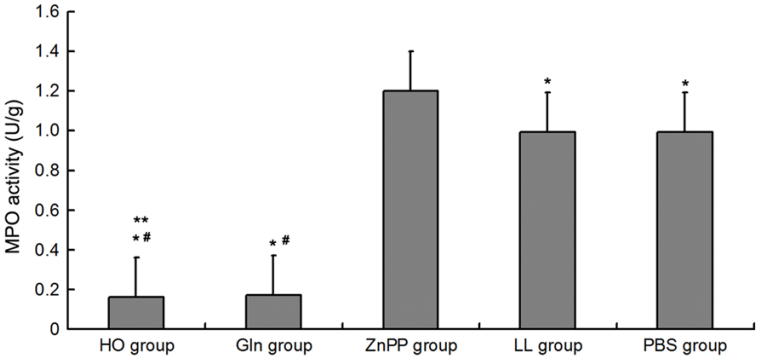

Figure 4. MPO activity of the intestine of 5 groups (U/g). *$^{*}P<0.05$: when compared with ZnPP group, MPO activity reduced significantly in HO group, Gln group, LL group and PBS group; $^{\#}P<0.05$: when compared with LL group and PBS group, MPO activity reduced significantly in HO group and Gln group; $^{**}P<0.05$: when compared with Gln group, MPO activity reduced significantly in HO group.

group, the PaO_2 increased significantly in HO group at 30 min after resuscitation ($P<0.05$). This suggests that HO and Gln had no significant influence on the blood gases during HS, but were effectively to improve MAP and PaO_2 at middle to late stage of resuscitation (20 and 30 min), which is helpful for the focal blood supply and oxygen supplement. This may be ascribed to the initiation of HO-1 expression in the rat intestine of HO group and Gln group.

Expressions of TNF-stage of resuscitatindmyeloperoxidase (MPO) activity

As shown in **Figures 3** and **4**, when compared with ZnPP group, the TNF-α expression reduced significantly, IL-10 and HO-1 expressions increased markedly, and MPO activity reduced dramatically in HO group, Gln group, LL group and PBS group; when compared with LL group and PBS group, the IL-10 and HO-1 expressions increased significantly, and MPO activity reduced significantly in HO group and Gln group; when compared with Gln group, the IL-10 and HO-1 expressions increased significantly and MPO activity reduced significantly in HO group ($P<0.05$). However, the TNF-α expression was comparable among HO group, Gln group, LL group and PBS group. This suggests that the HO-1 expression in the intestine of HO group and Gln group was effective to inhibit MPO activity and increase anti-inflammatory cytokines, protecting the intestine against the occurrence and development of inflammation.

HO-1 content (ELISA) and ChiuChiu's score

As shown in **Figures 5** and **6**, when compared with ZnPP group, the HO-1 content increased significantly and Chiu's score reduced significantly; when compared with LL group and PBS group, HO-1 content of the intestine increased significantly and Chiu's score reduced significantly in HO group and Gln group; when compared with Gln group, the intestinal HO-1 content increased significantly and Chiu's score reduced significantly in HO group ($P<0.05$). This suggests that HO and Gln are able to increase the expression of bioactive HO-1 in the intestine, exerting protective effects on the intestinal barrier function, as shown by the reduction in Chiu's score.

Discussion

The pathological basis of HS is microcirculation dysfunction (mismatch of oxygen supplement and oxygen consumption), resulting in hypoxia.

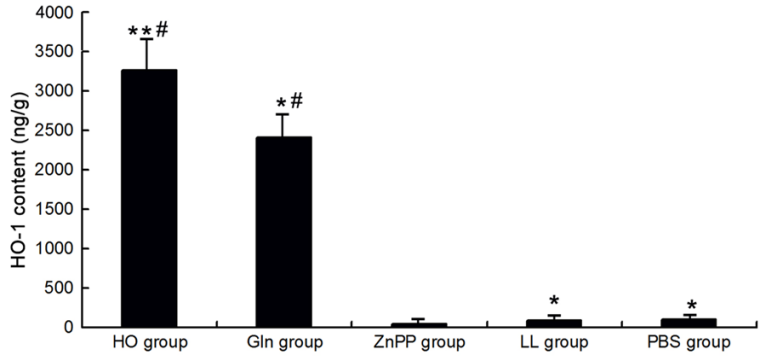

Figure 5. HO-1 expression in the intestine of 5 groups (ELISA; pg/g). *$^{*}P<0.05$: when compared with ZnPP group, HO-1 expression increased significantly in HO group, Gln group, LL group and PBS group; $^{\#}P<0.05$: when compared with LL group and PBS group, HO-1 expression increased significantly in HO group and Gln group; $^{**}P<0.05$: when compared with Gln group, HO-1 expression increased significantly in HO group.

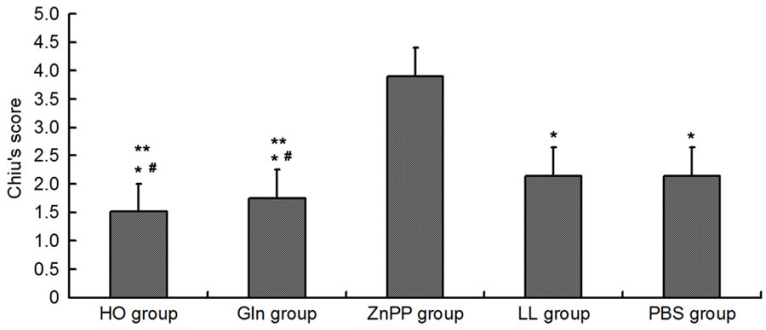

Figure 6. Chiure 6d significantly in HO groupgroups. *$^{*}P<0.05$: when compared with ZnPP group, Chiu compared with ZnPP group, roup a significantly in HO group, Gln group, LL group and PBS group; $^{\#}P<0.05$: when compared with LL group and PBS group, Chiu compared with LL group and PBS g significantly in HO group and Gln group; $^{**}P<0.05$: when compared with Gln group, Chiu compared with Gln group, Gln gr significantly in HO group.

reflecting the intestinal function [18].

In the presence of gastrointestinal dysfunction, a lot of oxygen reactive intermediates are produced, and inflammation related cells such as polynuclear neutrophils accumulate [3] and secret a variety of pro-inflammatory mediators (TNF-oxygen reactive intermediates are produced) as well as oxygen free radicals to participate in the occurrence and progression of MODS and MOF, deteriorating the primary diseases [4]. The activated polynuclear neutrophils exist in the terminal capillaries of some organs and may release some inflammatory mediators and oxidative substances, which may damage the endothelial cells, increase the vascular permeability, cause tissue edema and organ damage and dysfunction, leading to SIRS and MODS [21]. MPO is mainly found in the neutrophils, and monocytes and macrophages have a low MPO activity. MPO activity is closely related to the number of neutrophils and may reflect the change in neutrophils in tissues [22].

Persistent reduction in cardiac output may lead to reduction in oxygen supply or oxygen content, resulting in irreversible shock, and tissue hypoxia may further progress and cause MOF [19]. During HS, the major pathological response is to increase the cardiac output and perfusion pressure to meet the oxygen supply to important organs (such as heart and brain), which may reduce the blood supply to the abdominal cavity and intestine, compromising the intestinal barrier function and causing SIRS [2], usually having a high risk for MODS and/or MOF with a high mortality [20]. Intestinal mucosal damage is characterized by mucosal edema, villous rupture and damaged intercellular conjunction. Chiu's grading system is often used to evaluate the intestinal mucosal damage and has been used as a morphological indicator

In the presence of stress-induced damage to tissues and cells, to regulate the HO expression is one of promising strategies to counteract this damage. During HS, the intestine which has no HO-1 expression under the normal condition may begin to express HO-1 following ischemia/hypoxia, and HO-1 expression peaks at 6-12 h after resuscitation [7]. The protective effects of HO-1 are related to its anti-inflammatory, anti-apoptotic and anti-fibrotic activities [23] and may be ascribed to the metabolites of heme (CO, biliverdin, bilirubin and ferritin) after catalysis by HO-1 because HO-1 itself and these metabolites have the anti-oxidative activity and have been found to be important anti-oxidants [24]. There was evidence showing that HO-1 was protective toHS via improving the organ function. On the contrary, to block HO-1 expres-

sion may deteriorate organ injury [25]. Clinical trials also revealed that to induce HO-1 overexpression in the intestinal epithelial cells in early stage of HS might improve the survival rate, and reduce post-shock complications and mortality [6]. In addition, study also finds that the protective effect of HO-1 is dependent on the level of HO-1 expression: appropriate expression of HO-1 is helpful to reduce protein oxidation, lipid peroxidation and cell death; overexpression of HO-1 may facilitate the lactate dehydrogenase release, reduce glutathione S-transferase content and damage the integrity of cell membrane [26].

As for inflammatory mediators, TNF-α has been found to play a central role in the pathogenesis of SIRS and MODS and serve as an initiator of SIRS and MODS [27]. In HS, the intestine is a major organ and resource of TNF-α [7]. IL-10 is an important anti-inflammatory cytokine and expressed following inflammatory stimulation. The IL-10 in the blood and tissues is closely related to the severity of inflammation, and may attenuate the inflammation of focal and distal organs. In addition, IL-10 is able to inhibit the activity of TNF-α and has been used as an anti-inflammatory marker of the intestine [28]. The anti-inflammatory effect of IL-10 is mediated by HO-1, and IL-10 has a crosslink with HO-1 in a positive feedback manner [29]. Thus, the TNF-α content, MPO activity, IL-10 content and HO-1 expression of the intestine may accurately reflect the extent of released inflammatory mediators in the intestine and the severity of inflammation.

Gln is a non-essential amino acid and widely distributed in the blood, tissue fluid and milk of mammalians. In the presence of tissue injury, cells release a large amount of Gln, and endogenous Gln becomes insufficient. Thus, to supplement exogenous Gln is necessary under pathological conditions [30]. Studies have confirmed that Gln may reduce the release of inflammatory cytokines and stimulate the expression of HSP family members to exert protective effects on pathological conditions (ischemia/reperfusion injury, lung injury, endotoxin induced vasoactive increase and endotoxin mediated cardiac dysfunction [31]. HO-1 is also known as heat shock protein 32 and HO-1 expression is induced by Gln following HS induced oxidative damage and ischemia/reperfusion injury, which may confer anti-oxidative and anti-apoptotic effects [10]. Experiments have confirmed that additional Gln is able to promote the growth of intestinal mucosal villi, inhibit the bacterial growth and maintain the integrity of intestinal mucosa, which are protective for the morphology and function of intestinal mucosal barrier [32]. After treatment with Gln at 0.75 g/kg, HSP70 expression was induced, which could significantly attenuate the cardiac injury of rats with septic shock or endotoxemia and maintain the circulatory stability [31, 33]. This pretreatment was done at 6 h before experiment, because HSP70 expression often occurs at 6-12 h after Gln treatment [31]. In another study, rats were intravenously injected with Gln at 0.75 g/kg before HS, and the increase in HO-1 expression was also observed [33]. In the present study, Gln at 0.75 g/kg was orally administered before experiment considering the delayed HO-1 expression and the gastric emptying time.

The goal of resuscitating severe HS is to maintain the hemodynamic stability and the sufficient oxygen supply to important organs [34]. Animal experiments and clinical trials have confirmed that early fluid infusion is able to improve the cardiac function and assure the normal hemodynamic in the major organs [35]. Moreover, the change in microcirculation has no relationship with the change in blood pressure of cardiovascular system: the intestinal microcirculation remains unchanged and the intestine has no sufficient blood perfusion even MAP and cardiac output may significantly increase after application of adrenalines [36]. Sufficient oxygen supply is important for the normal physiological function of the gastrointestinal tract, and low blood perfusion may further compromise the intestinal barrier function [37]. Thus, to maintain the sufficient oxygen supply to the intestine plays an important role. HO-1 is able to maintain the stability of microcirculation, but whether it is also able to maintain the microcirculation of the intestine is required to be confirmed.

In this study, oral LL-HO-1 and Gln increased the expression of bioactive HO-1 in the intestinal epithelial cells of rats with HS, exerting protective effects: (1) The mortality, Chiu's score and MPO activity in HO group and Gln group reduced significantly, suggesting the attenua-

tion of intestinal inflammation; (2) HO-1 expression and IL-10 content increased significantly in HO group and Gln group, which may exert anti-inflammatory effect and reduce the release of inflammatory mediators via the intestine; (3) ZnPP (a specific inhibitor of HO-1) significantly deteriorated the intestinal inflammation; (4) In HS rats, the PaO_2 and MAP increased significantly after fluid infusion, which assures the blood perfusion and oxygen supply to focal tissues and is helpful to stabilize the intestinal microcirculation and reduce the incidence of intestinal barrier dysfunction. As compared with Gln, LL-HO-1 was more effective to stimulate the HO-1 expression in the intestine of HS rats, but more findings are required to support the superiority of LL-HO-1 in the therapy of HS.

Our results showed that oral LL-HO-1 and Gln not only increased the HO-1 expression in the intestine, but elevated the PaO_2 and MAP, which were beneficial to increase the oxygen supply and blood perfusion of the intestine, reduce cell swelling and necrosis, and decrease the release and synthesis of inflammatory cytokines. However, we could not confirm that HO-1 was able to improve the oxygen supply on the basis of PaO_2 and MAP because there were no indicators able to directly reflect the microcirculation in this study. Thus, we only evaluated the microcirculation of the intestine indirectly according to PaO_2 and MAP, and more studies are required to confirm these findings. In addition, we only investigated the intestinal inflammation at 1 h after resuscitation, and the long-term effects of fluid infusion were not evaluated. Furthermore, the dose of drugs, the ways in which drugs were administered, and the timing of drug administration are needed to be evaluated in future studies. These were limitations of this study, which will be resolved in future studies.

In HS rats, oral LL-HO-1 and Gln are able to stimulate the HO-1 expression in the intestine, exerting protective effects on the intestinal barrier function, which is related to the attenuation of intestinal inflammation and improvement of PaO_2 and MAP after fluid infusion.

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Disclosure of conflict of interest

None.

Address correspondence to: Jianrong Guo, Department of Anesthesiology, Gongli Hospital Affiliated to the Second Military Medical University, No. 219 Miaopu Road, Shanghai 200135, China. E-mail: guojianrong_sh@163.com

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