Letter to the Editor

Effect of Hypertonic Versus Isotonic Saline Resuscitation on Heme Oxygenase-1 Expression in Visceral Organs Following Hemorrhagic Shock in Rats*

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To compare the early effects of hypertonic and isotonic saline resuscitation on heme oxygenase-1 (HO-1) expression in organs of rats with hemorrhagic shock. Rats were randomly divided into hypertonic saline resuscitation (HTS), normal saline resuscitation (NS) and sham groups. HO-1 mRNA, protein expression and apoptosis were evaluated in organs. In the HTS group, significant difference was noted in HO-1 protein in small intestinal mucosa and liver compared with the NS and sham groups, and in HO-1 mRNA in liver and kidney compared with the sham group. The apoptosis of small intestinal mucosa, liver, heart, and lung was significantly lower in the HTS group than that in the NS group. In this study, small volume resuscitation with HTS can efficiently up-regulate the expression level of HO-1 in small intestinal mucosa and liver, which may be one of the mechanisms alleviating organ damage.

Hemorrhagic shock (HS) triggers a systemic inflammatory response that culminates in multiple organ failure with significantly high mortality. Hypoxia/reoxygenation and neutrophil activation enhance this systemic inflammatory response by generating profound oxygen-derived free radicals, which ultimately leads to organ or tissue injury[1-2]. Hypertonic saline (HTS), consisting of 7.5% (w/v) NaCl, is an alternative resuscitation strategy for HS patients. By taking advantage of osmosis, a relatively large circulating blood volume expansion can be obtained by administering a relatively small volume of fluid. HTS resuscitation regulates immunological reactions, protects organs from damage, and alleviates ischemia-reperfusion injury[3-6].

Modulation of the heme oxygenase (HO) system is one of the most promising approaches to attenuate organ damage[7]. HO-1 induction plays a fundamental role against oxidative processes mediated by free-heme, and may protect organs from damage[8-9]. HO-1 and HO-2 are two identified isozymes of HO. HO-1 is induced in response to various stress stimuli, whereas HO-2 is constitutively expressed and not inducible. HO-1 is up-regulated by a variety of inducers, such as endotoxin, hydrogen peroxide, prostaglandins, and cytokines. Increased expression of HO-1 has also been shown in numerous pathophysiological states (e.g., trauma, sepsis, pancreatitis and ischemia-reperfusion injuries). Over-expression of HO-1 is cytoprotective and immunoregulatory in a variety of tissues[8-10].

In our previous study, small-volume resuscitation with HTS was more effective than isotonic saline resuscitation in reducing initial apoptosis in the small intestinal mucosa in a rat HS model[4]. Therefore, we hypothesized that resuscitation with hypertonic saline during hemorrhagic shock confers increased expression of HO-1 and cytoprotection against apoptosis in visceral organs.

This study was approved by the Ethics Committee of the School of Medicine, Zhejiang University, China. Twenty-one male Sprague-Dawley rats, weighing 250-350 g, were obtained from the Medical Institute of Zhejiang Province, China. According to a previously reported method[3-4], a rat model with severe and controlled hemorrhagic shock was established. Under mild anesthesia, severe injury was initiated (time=zero) with controlled blood withdrawal via the right carotid arterial cannula four times (at a rate of 1 mL/100 g per 5 min for the first two times; 0.5 mL/100 g per 5 min for the final two times). The shed blood was collected in glass syringes with heparin and reinfused during emergency therapy. This phase was called the “pre-hospital phase” and lasted for 60 min. During the last 30 min of this period, the rats were resuscitated early by administering hypertonic or isotonic saline solution. At 60 min, phase simulating hospital emergency treatment began (the “hospital phase”). Resuscitation began with reinfusion of the shed blood. The hospital phase lasted for 30 min, after which survivors were strictly monitored for 120 min (the “observation phase”).

Twenty-one rats were randomly divided into three groups (seven in each group). The sham group only received anaesthesia, cannulation, and heparinization. The normal saline resuscitation group (NS) received 0.9% (w/v) NaCl infusion (three times the blood loss volume according to previous studies and clinical practice[11-12]) during the last

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30 min of the pre-hospital phase. The hypertonic saline resuscitation group (HTS) received 6.0 mL/kg body weight of 7.5% (w/v) NaCl solution infusion during the last 30 min of the pre-hospital phase, according to the dosage recommended by our previous studies[3-5].

HO-1 mRNA and protein expression in small intestinal mucosa, liver, heart, lung, and kidney were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot analysis. Total RNA extraction was performed according to the manufacturer's instructions. The primer sequences were: 5'-TGC TCG CAT GAA CAC TCT GGA GAT-3' (HO-1 forward), 5'-ATG GCA TAA ATT CCC ACT GCC ACG-3' (HO-1 reverse), 5'-TCA TCA CTA TCG GCA ATG AGC GGT-3' (β-actin forward), and 5'-ACT CCT GCT TGC TGA TCC ACA TCT-3' (β-actin reverse). The qRT-PCR was performed by an ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA). Real-time PCR results were recorded as threshold cycle (Ct) values. To adjust for different concentrations of total cDNA in the samples, a ΔCt was calculated based on the difference in Ct values between the target gene and the housekeeping gene β-actin. In data analysis, ΔCt was converted into an expression index based on the formula: $1000\times2^{(-\Delta Ct)}$.

The frozen specimens of heart, liver, kidney, lung, and small intestinal mucosa were homogenized on ice in cell lysis buffer (Beyotime Biotech Co., Jiangsu, China), incubated for 1 h at 4 °C, and centrifuged at 14 000 rpm for 5 min. Western blot analysis was conducted according to standard protocols using primary HO-1 antibody (Abcam Ltd., Hong Kong, China) and peroxidase-conjugated secondary anti-mouse antibody (Jingmei Genevale Technology Co., Beijing, China). The protein bands in the blot were detected by the ECL SuperSignal detection kit (Beyotime Biotech Co., Jiangsu, China) according to the manufacturer's instructions. β-actin was used as a control and detected by a specific primary antibody (Jingmei Genevale Technology Co.) and the same secondary antibody. Densitometry data were analyzed by Quantity One (ChemiDoc XPS, Bio-Rad Laboratories, Inc., Hercules, CA, USA) for semi-quantification of HO-1 and β-actin. The relative amounts of HO-1 protein were normalized to β-actin.

Apoptosis in the heart, liver, kidney, lung, and small intestinal mucosa was analyzed by flow cytometry (FC500, FACSCalibur, Beckman-Coulter, Miami, Fla., USA). The samples were stained with a fluorescein-isothiocyanate-annexin V/propidium iodide double staining kit (Bender MedSystems Co., Vienna, Austria). A total of 10 000 cells were measured for each sample.

Statistical analysis was performed with the SPSS version 13.0 software package (SPSS Institute, Chicago, IL, USA). Normally distributed data are presented as mean±standard deviation and were compared by the homogeneity test, one-way analysis of variance, and the least significant difference-*t* test. Abnormal data are presented as the median and interquartile range (25th and 75th percentiles), and were analyzed by the Kruskal-Wallis H test for three-group comparisons and the Nemenyi test for two-group comparisons. A *P* value of <0.05 was considered statistically significant.

Characteristics of the Animal Model Body weight was 312.4±34.7 g in the sham group, 315.4±29.6 g in the NS group, and 306.7±31.4 g in the HTS group (F=0.134, P=0.875). All the rats suffered approximately 50% blood loss based on their body weight and survived all three phases.

Variation of Mean Arterial Pressure (MAP) Pre-hem-orrhage MAP was 129.3±4.4 mmHg in the sham group, 128.7±6.0 mmHg in the NS group, and 130.0±7.5 mmHg in the HTS group. While MAP in the sham group was stable throughout the experiment, slow arterial blood withdrawal occurred in the HTS and NS groups over time. At the conclusion of withdrawal (20 min), MAP had decreased to 18.9± 4.4 mmHg in the NS group and 20.3±4.5 mmHg in the HTS group. Ten minutes later, MAP had slightly increased to 21.7±8.8 mmHg in the NS group and 24.8±6.3 mmHg in the HTS group (Figure 1).

After receiving normal saline and hypertonic saline, MAP began to increase in the NS and HTS groups. At the end of the pre-hospital phase (60 min), MAP in the NS group was significantly higher than that in the HTS group (106.9±7.8 *vs* 85.4±11.7 mmHg). No significant changes in MAP were observed in the three groups during the subsequent hospital and observation phases.

Expression of HO-1 mRNA in Visceral Organs The qRT-PCR results of HO-1 mRNA in the organs of the sham, NS, and HTS groups are shown in Table 1. In the small intestinal mucosa, among the three groups, HO-1 mRNA from the HTS group had the highest level of expression. However, by the Kruskal-Wallis H test, the differences were not statistically significant (P=0.612). In liver tissue, HO-1 mRNA in the HTS group had the highest level of expression, and this was significantly higher than that in the sham group (P=0.013).

Only weak expression of HO-1 mRNA was observed in the heart, lung, and kidney tissues in all three groups. HO-1 mRNA expression in kidney tissue was significantly higher in the HTS group than that in the sham group (P=0.013). However, HO-1 mRNA expression in heart and lung tissues was not significantly different among the three groups (P=0.692, 0.117).

Expression Levels of HO-1 Protein in Visceral Organs There was only weak or negative expression of HO-1

protein in the heart, lung, and kidney tissues of the three groups, and detection of expression was unreliable. HO-1 protein expression in the small intestinal mucosa in the NS (0.10±0.03 protein expression index) and HTS (0.19±0.06 protein expression index) groups was significantly higher than that in the sham group (0.03±0.01 protein expression index), and HO-1 protein expression in the HTS group was significantly higher than that in the NS group (both $P<0.01$, Table 1). Furthermore, HO-1 protein expression in liver tissue was significantly higher in the HTS (2.58±1.72 protein expression index) group than that in the sham (0.48±0.21 protein expression index) and NS (1.25±0.37 protein expression index) groups (both $P<0.05$).

Apoptosis in Visceral Organs The apoptotic rates of the small intestinal mucosa, liver, heart, lung, and kidney were significantly higher in the NS group than those in the sham group ($P<0.01$ or $P<0.05$, Figure 2). The apoptotic rates of the small intestinal mucosa and lung in the HTS group were higher than those in the sham group (both $P<0.01$). The apoptotic rates of the small intestinal mucosa, heart, and lung were significantly higher in the NS group than those in the HTS group (all $P<0.01$).

Conventional guidelines for pre-hospital treatment of hypotension secondary to hemorrhage may actually aggravate immune dysfunction and organ failure. In the clinic, HTS might have significant applications as a novel fluid resuscitation strategy in HS. HO-1 (molecular weight, 32 kD), also known as heat shock protein 32, is a stress-inducible protein, and its over-expression provides considerable protection against ischemia-reperfusion injury[13-14].

In our study, the highest level of expression of HO-1 protein was observed in the small intestinal mucosa and liver in the HTS group. Furthermore, the highest level of HO-1 mRNA expression was also observed in the small intestinal mucosa, liver, heart, lung, and kidney in the HTS group. However, HO-1 mRNA expression was higher in the HTS group than the sham group in the liver and kidney. The reason for our findings may be due to the fact that mRNA is an intermediate product in the process of transcription and translation, and may be unstable. However, protein expression assayed by western blotting is more stable than qRT-PCR. Therefore, HTS resuscitation may significantly induce the expression level of HO-1 in visceral organs, especially in small intestinal mucosa and liver, compared with NS resuscitation.

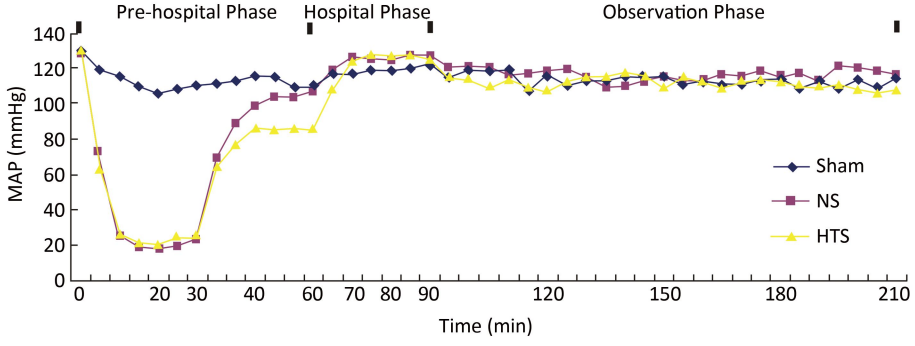

Figure 1. Changes of mean arterial pressure (MAP) in sham, normal saline (NS), and hypertonic saline (HTS) groups. Blood withdrawal began at 0 min, NS and HTS infusion began at 30 min, and reinfusion of shed blood began at 60 min.

Table 1. HO-1 mRNA Expression in Rat Visceral Organs (Expression Index, $\bar{x} \pm s$)

Group	*n*	HO-1 mRNA expression									
		Small intestinal mucosa		Liver		Heart		Lung		Kidney	
		median	25th and 75th percentiles	median	25th and 75th percentiles	median	25th and 75th percentiles	median	25th and 75th percentiles	median	25th and 75th percentiles
Sham	7	1.7003	0.2278-3.6447	0.6443	0.1611-1.2886	0.3700	0.0654-0.5609	0.1308	0.0076-0.5609	0.0701	0.0327-0.5609
NS	7	1.8223	0.3453-2.7621	1.1218	0.1983-54.4070	0.1503	0.0164-2.0933	0.1611	0.0654-2.7621	0.0925	0.0496-1.2886
HTS	7	3.6447	1.0466-16.7476	2.9604	1.5864-233.1002*	0.3966	0.3453-1.4802	0.1726	0.1402-15.6250	1.2023	0.7662-1.9531*
P		0.612		0.041		0.692		0.117		0.024	

Note. *$P=0.013<0.017$ *vs* sham.

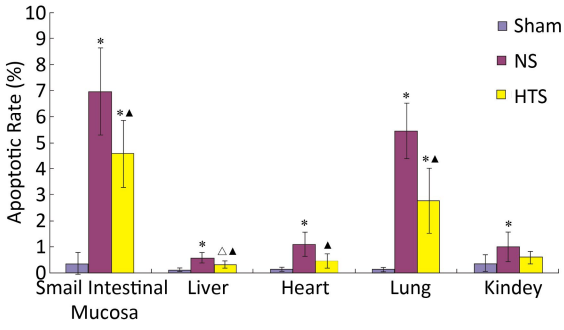

Figure 2. Flow cytometric results of apoptosis in the visceral organs of the three groups of rats. Data are presented as mean± SD for n=7 animals per group. $^{*}P$<0.01 compared with sham; $^{\triangle}P$<0.05 compared with sham; $^{\blacktriangle}P$<0.0 compared with NS.

HS causes whole body ischemia-reperfusion injury, leading to multiple organ dysfunction. HS elicits the formation of a significant amount of oxygen-derived free radicals in visceral organs, not only by organic hypoxia/reoxygenation itself, but also by immune activation due to endotoxin/bacterial translocation, which leads to oxidative tissue injury. Therefore, HS induces HO-1 expression in visceral organs, and this process plays a significant protective role against HS-induced injury. HTS resuscitation prevents organ ischemia-reperfusion injury by inducing over-expression of cytoprotective proteins, such as HO-1, reducing the production of oxygen-derived free radicals, regulating inflammatory responses and cellular respiration, attenuating apoptosis of organs, and decreasing oxidative stress[15-16]. The results from the current study are in agreement with these previous results.

In our previous studies[17], a large amount of apoptosis occurred in visceral organs in the early period after HS and resuscitation, and this might play a role in early organ injury and later multiple organ failure. Apoptosis in pathological conditions sensitively reflects the severity of organ dysfunction and disorder of the internal environment. Therefore, apoptosis is considered as a sensitive indicator of tissue or organ damage[18]. In our study, varying amounts of apoptosis occurred in the visceral organs of rats in the early period of HS and resuscitation, especially in the small intestinal mucosa and lung. Furthermore, apoptosis in the small intestinal mucosa, liver, heart, and lung was significantly lower in the HTS group than that in the NS group. This finding suggests that, in the pre-hospital treatment of severe HS, resuscitation with a small volume of HTS may maintain the function of visceral organs and decrease late complications of traumatic hypotension.

Considering the consistency between HO-1 expression and apoptosis in visceral organs after HS and resuscitation, especially in small intestinal mucosa and liver, we speculate that up-regulation of HO-1 expression leads to a strong cytoprotective effect and attenuates organ damage. The mechanism of how HTS resuscitation induces HO-1 is unclear, but is likely to be multi-factorial. HTS resuscitation has profound effects on early signal transduction, which regulates stress gene expression of intestinal epithelial cells[19]. Hypoxia-ischemia in gastrointestinal mucosa occurs earliest and severe after shock[20], and HTS resuscitation can effectively recover blood supply and reduce apoptosis in the gastrointestinal mucosa. HO-1 is naturally expressed in hepatic cells, and this may also be one of the reasons.

In summary, in our severe HS rat model, a small volume of resuscitation with HTS up-regulates the expression level of HO-1 more effectively than NS resuscitation, at least in the small intestinal mucosa and liver. Over-expression of HO-1 in the small intestinal mucosa and liver may be one of the molecular mechanisms of alleviating organ damage by HTS resuscitation.

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