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Hypertonic saline resuscitation reduces apoptosis of intestinal mucosa in a rat model of hemorrhagic shock*

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Abstract: Objective: To investigate the early effects of hypertonic and isotonic saline solutions on apoptosis of intestinal mucosa in rats with hemorrhagic shock. Methods: A model of rat with severe hemorrhagic shock was established in 21 Sprague-Dawley (SD) rats. The rats were randomly divided into the sham group, normal saline resuscitation (NS) group, and hypertonic saline resuscitation (HTS) group, with 7 in each group. We detected and compared the apoptosis in small intestinal mucosa of rats after hemorrhagic shock and resuscitation by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), FITC (fluorescein-iso-thiocyanate)-Annexin V/PI (propidium iodide) double staining method, and flow cytometry. Results: In the early stage of hemorrhagic shock and resuscitation, marked apoptosis of small intestinal mucosa in the rats of both NS and HTS groups was observed. The numbers of apoptotic cells in these two groups were significantly greater than that in the sham group (P<0.01). In the HTS group, the apoptic cells significantly decreased, compared with the NS group (P<0.01). Conclusion: In this rat model of severe hemorrhagic shock, the HTS resuscitation of small volume is more effective than the NS resuscitation in reducing apoptosis of intestinal mucosa in rats, which may improve the prognosis of trauma.

Key words: Hemorrhagic shock, Resuscitation, Sodium chloride solution, Hypertonic saline, Apoptosis, Intestinal mucosa, Flow cytometry, In situ nick-end labelling

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INTRODUCTION

Hemorrhagic shock accounts for a large portion of civilian and military trauma deaths (Bellamy, 1984; Moore *et al*., 2004). Successful treatment includes surgical control of hemorrhage and restoration of tissue perfusion. The fluid category of resuscitation of the hypotensive trauma patients is open to debate.

At present, hypertonic saline (HTS) has been applied as an alternative resuscitation strategy in patients of hemorrhagic shock. HTS solutions usually consist of 7.5% (w/v) NaCl. It was originally based on the idea that a relatively large circulating blood volume expansion could be obtained by administering a relatively small volume of fluid, taking advantage of osmosis (Rocha-e-Silva *et al*., 1986; Cai *et al*., 2002; Cruz *et al*., 2006). It was soon realized that the physiological vasodilator property of hypertonicity was a useful byproduct of small volume resuscitation in that it induced reperfusion of previously ischemic territories. Subsequently, recent studies disclosed that HTS resuscitation might have favorable immunomodulatory effects and alleviate ischemia-reperfusion injury of organs (Attuwaybi *et al*., 2004; Bulger *et al*., 2007; Lu *et al*., 2007b).

In recent years, a number of studies have indicated that intestinal mucosa is the first-affected and rapidly-changed site when shock or other low-flow conditions occur (Murao *et al*., 2003; Powers *et al*., 2005; Lu *et al*., 2006). Consequently, the intestinal

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tract was defined as "the canary of the body" by Dantzker (1993). Thus, in the current study we used the rat model of severe hemorrhagic shock to compare the early effects of hypertonic and isotonic saline solutions on apoptosis of intestinal mucosa in rats.

MATERIALS AND METHODS

Animals

Thirty male Sprague-Dawley (SD) rats weighing 250~350 g were obtained from the Medical Institute of Zhejiang Province, China, and were used in all experiments. Animals were allowed standard rat diet and tap water ad libitum, and were maintained under controlled conditions: 12-h light/12-h dark schedule at (24±2) °C. This study was approved by the Ethics Committee of Medical College, Zhejiang University, China.

Experimental procedure

SD rats were weighed and anesthetized with pentobarbital (40 mg/kg intraperitoneally) and then were placed in a supine position on a warming pad (25 °C). After applying povidone-iodine solution, the right carotid artery was isolated and cannulated with polyethylene catheter through a neck incision. The arterial catheter was used for blood withdrawal and was connected to a pressure transducer and computerized physiograph system for continuous hemodynamic monitoring. In the same way, the left femoral vein was cannulated for fluid infusion and reinfusion of the shed blood. The animals were heparinized (500 U/kg). Blood losses of the rats during the procedure were measured by mopping all blood from the incision with preweighed gauze sponges, which were then reweighed. A transformation formula of 1 g=0.9 ml of blood was used. Twenty-one rats that had lower than 0.2 ml blood losses during the procedure and had spontaneously breathed for 10 min after the procedure were included in the study.

Using the method created by Capone *et al*.(1995), the rat model of severe hemorrhagic shock was established. Under light anesthesia, the injury began (time=zero) with blood withdrawal through the carotid arterial cannula for 4 times (1 ml per 100 g per 5 min in the first 2 times, 0.5 ml per 100 g per 5 min in the next 2 times). The shed blood was collected in glass syringes with heparin and reinfused during emergency treatment. This phase was called "pre-hospital phase" and lasted for 60 min. During the late 30 min of this period, the rats were early resuscitated by administering different crystalloid fluids.

At 60 min, a phase simulating hospital emergency treatment (hospital phase) began. Resuscitation began with reinfusion of the shed blood. The "hospital phase" lasted for 30 min, after which survivors were strictly monitored and observed for 120 min (observation phase).

Grouping of animals

Twenty-one rats were randomly divided into 3 groups, with 7 in each. The sham group only received anaesthesia, cannulation, heparinization, and observation. The normal saline resuscitation group (NS group) received 0.9% (w/v) NaCl (3 times of blood loss volume) infusion during the late 30 min of "pre-hospital phase." The hypertonic saline resuscitation group (HTS group) received 5.71 ml of 7.5% (w/v) NaCl solution (per kg of body weight) infusion during the late 30 min of "pre-hospital phase" according to the dosage recommended by Cai *et al*.(2002).

Collection and detection of samples

Rats that had lived for 210 min were regarded as survivors and were immediately sacrificed after the "observation phase." The distal portion of small intestine (the ileum) was taken out quickly and flushed with 0.01 mol/L cold phosphate buffer solution (PBS, pH 7.4). A part of the ileum was fixed with 10% (w/v) buffered formaldehyde for terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). All histological specimens were examined by a pathologist who was blind to the animals' resuscitation protocol. The TUNEL detection kit was purchased from American Promega Corporation and the detection procedure followed the manufacturer's instructions. Under the high power field (400× magnification) of fluorescent microscope, the number of apoptotic cells characterized by the positive staining nucleus in TUNEL staining sections was counted in 10 randomly selected fields per section. The mean number of apoptotic cells per field for each rat was calculated for further statistical analysis.

The mucosa was scraped from the other part of the ileum, and was immediately sent to the laboratory

in Cancer Institute of Zhejiang Tumor Hospital for apoptosis study. An FC500 flow cytometer (FACSCalibur, Beckman-Coulter, USA) was used, and the samples were stained with a researcher who was blind to the animals' resuscitation protocol using Annexin-V/PI (propidium iodide) staining kit (Bender MedSystems Corporation, Austria). For each sample, 10000 cells were measured.

Statistical analysis

Data were presented as mean±standard deviation (*SD*). SPSS 13.0 software package was used for statistical analysis for comparing mean values from the three groups by homogeneity test, one-way analysis of variance (ANOVA) and least significant difference-*t* test (*LSD*-*t*). Differences were considered significant at $P<0.05$.

RESULTS

Characteristics of the animal model

The 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. ANOVA showed that no statistically significant difference occurred in weight among the rats of the three groups ($F=0.134$, $P=0.875$). All the rats suffered approximately 50% blood loss based on their body weight and survived in all the above-mentioned three phases. These results are consistent with our previous studies which reported that rats could survive acute blood loss to 50% (Lu *et al*., 2005; 2006; 2007a; 2007b).

Variation of mean arterial pressure (MAP)

Fig.1 shows MAP changes of the rats in the three groups. Pre-hemorrhage 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 the MAP in the sham group was stable throughout the experiment, a slow arterial blood withdrawal occurred in both HTS and NS groups over time. At the conclusion of blood withdrawal (20 min), MAP had decreased to (18.9±4.4) mmHg in the NS group and to (20.3±4.5) mmHg in the HTS group. After ten more minutes, MAP had slightly increased to (21.7±8.8) mmHg in the NS group and to (24.8±6.3) mmHg in the HTS group.

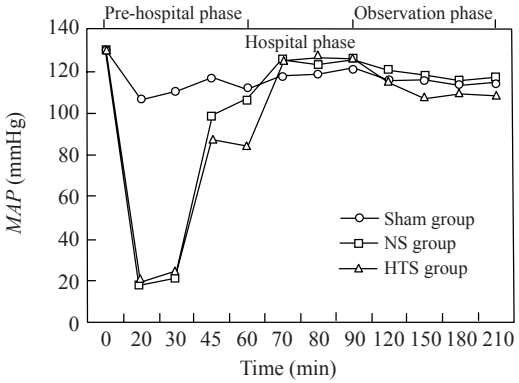

Fig.1 Changes of MAP in the sham, NS, and HTS groups. The blood withdrawal began at 0 min, NS and HTS infusion began at 30 min, and the reinfusion of the shed blood began at 60 min

After receiving normal saline and hypertonic saline in NS and HTS groups, respectively, MAP began to restore. At the conclusion of bolus infusion (45 min), MAP increased to (99.0±9.4) mmHg in the NS group and to (87.1±18.4) mmHg in the HTS group. At the end of "pre-hospital phase" (60 min), MAP in the NS group was found to be (106.9±7.8) mmHg, significantly higher than that [(85.4±11.7) mmHg] in the HTS group ($P<0.05$).

Furthermore, no significant changes in MAP were observed in the three groups during the next "hospital phase" and "observation phase" of the experiment.

TUNEL staining

In TUNEL stained sections of the small intestinal mucosa of the sham, NS, and HTS groups, apoptotic cells were observed. They presented green fluorescence and contracted or fragmented nuclei under fluorescent microscopy (Table 1). The numbers of TUNEL positive cells in the NS and HTS groups were markedly greater than that in the sham group ($P<0.01$).

Table 1 Results of apoptosis in the small intestinal mucosa of the three groups rats

Group	*n*	Number of TUNEL positive cells in high power field	*FCM* (%)
Sham group	7	5.77±2.31	0.35±0.41
NS group	7	33.54±7.71*	6.96±1.68*
HTS group	7	22.19±4.78$^{*\#}$	4.57±1.27$^{*\#}$
F value		46.676	51.006
P value		0	0

$^{*}P<0.01$, compared with the sham group; $^{\#}P<0.01$, compared with the NS group; FCM: flow cytometry

The apoptotic cells decreased significantly in the HTS group compared to the NS group ($P<0.01$).

Flow cytometry analysis

Table 1 shows apoptosis in the small intestinal mucosa of the sham, NS, and HTS groups by flow cytometry analysis. The apoptotic rates of the small intestinal mucosa in the NS and HTS groups were significantly higher than that in the sham group ($P<0.01$). The apoptotic rate of the small intestinal mucosa in the NS group was significantly higher than that in the HTS group ($P<0.01$).

DISCUSSION AND CONCLUSION

Hemorrhage remains one of the leading causes of death among trauma victims (Hoyt, 2004). A large number of victims die of exsanguination, while another considerable portion die from complications resulting from the host response to trauma and hemorrhage. The excessive response leads to the dysfunction of immune cells, resulting in serious tissue damage and, ultimately, organ failure.

Conventional guidelines for pre-hospital treatment of hypotension secondary to hemorrhage after trauma recommend a rapid infusion of large volume isotonic crystalloid solution to restore normal blood pressure as quickly as possible. However, recent evidence suggests that isotonic crystalloid solutions may actually aggravate immune dysfunction and organ failure (Rhee *et al*., 2000). Recognition of the limitations of isotonic crystalloid resuscitation has led to the search for alternative resuscitation strategies that might better limit the development of trauma-hemorrhage-induced organ dysfunction and systemic inflammation. In recent years, a few clinical observations have indicated that HTS might have significant applications as a novel fluid resuscitation strategy in hemorrhagic shock (Rizoli *et al*., 2006; Bulger *et al*., 2007; 2008).

The main causes of death in the late stage of patients with severe trauma or hemorrhagic shock are infection, multiple organ dysfunction syndrome (MODS), or multiple organ failure (MOF). Recently, several research groups have found that apoptosis was obviously induced in visceral organs in the early stage of polytrauma combined with shock, which may play a role in early organ injury and later multiple organ failure (Xu *et al*., 1997; Schmieg *et al*., 2000; Yu *et al*., 2002). The apoptosis in pathological conditions can also reflect the severity of disordered internal environment and tissue damage (van Way *et al*., 2003). In particular, significant apoptosis of the intestinal mucosa can result in abnormal gut permeability and impairment of mucosa barrier and immune function, which is bound up with endogenous infection and MODS.

Our study shows clearly that a great deal of the apoptosis of the intestinal mucosa of rats occurred in the early period after hemorrhagic shock and resuscitation. Hotchkiss *et al*.(2000) also demonstrated that the apoptosis of intestinal epithelial and lymphoid tissues occurred extremely rapidly after injury and shock. Apoptotic loss of intestinal epithelial cells may compromise bowel wall integrity and be a mechanism for bacterial or endotoxic translocation into the systemic circulation. Apoptosis of lymphocytes may impair immunologic defenses and predispose to infection. More recently, Diebel *et al*.(2005) revealed the pivotal role of tumor necrosis factor-α in signaling apoptosis in intestinal epithelial cells under shock conditions by Caco2 intestinal cell monolayers.

In the present study, HTS treatment significantly reduced the apoptosis of the small intestinal mucosa, compared to NS treatment. This indicates that in pre-hospital treatment of severe hemorrhagic shock, the resuscitation of a small volume of HTS may be helpful for maintaining organ function and decreasing the later complications of trauma and hypotension. The mechanisms of HTS resuscitation may lie on the following factors. Firstly, HTS resuscitation effectively ameliorates the cellular metabolisms by inducing a considerable microcirculatory improvement and restoring intestinal perfusion, which can reduce abnormal apoptosis of small intestinal mucosa (Murao *et al*., 2003; Shires *et al*., 2005). This positive effect of HTS is related to the correction of endothelial or red cell edema and selective vasodilation of the precapillary arteriole, accompanying significant consequences in capillary blood flow (Victorino *et al*., 2003; Vajda *et al*., 2004; Homma *et al*., 2005; Hoppen *et al*., 2005; Zakaria *et al*., 2006). Secondly, hemorrhagic shock causes a whole body ischemia-reperfusion injury, leading to multiple organ dysfunctions. Several laboratory studies have shown that

ischemia-reperfusion injury can induce apoptosis (Zhao *et al*., 2003; Zhang *et al*., 2004; Wu *et al*., 2004). HTS resuscitation might prevent gut ischemia-reperfusion injury by inducing overexpression of some cytoprotective proteins such as heme oxygenase-1 (HO-1), reducing the production of oxygen-derived free radicals, regulating the inflammatory response, and decreasing oxidative stress (Ozgüç *et al*., 2003; Attuwaybi *et al*., 2004; Powers *et al*., 2005; Gonzalez *et al*., 2006; Lu *et al*., 2007b).

In conclusion, we created the rat model of hemorrhagic shock and examined the effects of HTS and NS on the apoptosis of rat intestinal mucosa. We found that small volume resuscitation with HTS is more effective than NS resuscitation in reducing the initial apoptosis of intestinal mucosa, which may improve the prognosis of trauma. To further elucidate the mechanisms, we plan to evaluate the relationship of HO-1 mRNA expression and apoptosis of the intestinal mucosa, and the effects of specific blockers for caspase-3 and Bcl-xL. Based on the logistical advantages and favorable properties, it appears that HTS may be an ideal replacement for conventional isotonic resuscitation fluids.

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