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Hypertonic saline resuscitation maintains a more balanced profile of T-lymphocyte subpopulations in a rat model of hemorrhagic shock

LU Yuan-qiang[†1], CAI Xiu-jun[1], GU Lin-hui[2], MU Han-zhou[2], HUANG Wei-dong[1]

([1]*Department of Emergency and General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China*)

([2]*Cancer Institute, Zhejiang Tumor Hospital, Hangzhou 310022, China*)

[†]E-mail: lls201@163.com; luyuanqiang609@yahoo.com.cn

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Abstract: Objective: To investigate the potential and early effect of hypertonic saline resuscitation on T-lymphocyte subpopulations in rats with hemorrhagic shock. Methods: A model of rat with severe hemorrhagic shock was established in 18 Sprague-Dawley (SD) rats. The rats were randomly divided into Sham group, HTS group (hypertonic saline resuscitation group) and NS group (normal saline resuscitation group). Each group contained 6 rats. The $CD4^+$ and $CD8^+$ subpopulations of T-lymphocytes in peripheral blood were detected respectively before shock and after resuscitation by double antibody labelling and flow cytometry. Results: In the early stage after hemorrhagic shock, fluid resuscitation and emergency treatment, the $CD4^+$ lymphocytes of peripheral blood in HTS and NS groups markedly increased. Small volume resuscitation with HTS also induced peripheral $CD8^+$ lymphocytes to a certain extent, whereas NS resuscitation showed no effect in this respect. Consequently, compared with Sham and HTS groups, $CD4^+/CD8^+$ ratio of peripheral blood in NS group was obviously increased, and showed statistically differences. Conclusion: In this model of rat with severe hemorrhagic shock, small volume resuscitation with HTS is more effective than NS in reducing immunologic disorders and promoting a more balanced profile of T-lymphocyte subpopulations regulating network.

Key words: Hemorrhagic shock, Resuscitation, Sodium chloride solution, Hypertonic saline, T-lymphocyte subpopulations, Flow cytometry

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INTRODUCTION

Hemorrhagic shock accounts for a large portion of civilian and military trauma deaths (Bellamy, 1984; Moore *et al*., 2004). The body's initial response to traumatic injury and hemorrhage is generally characterized by an excessive innate immune activation and by an overwhelming inflammatory reaction (Giannoudis, 2003). In severely injured patients, even when adequately treated early after injury, sepsis and multiorgan dysfunction (MOD) are frequent outcomes. The immune system is clearly involved in this phenomenon (Marshall and Cohen, 2000; Maier, 2000; Rotstein, 2003). Regulated inflammatory responses are generally considered as a beneficial host response to injury (Oberholzer *et al*., 2000), while post-traumatic hyperinflammation and ensuing immune incompetence are considered to be maladaptive and often auto-destructive (Giannoudis, 2003).

One of the current guidelines applied to the early management of hemorrhagic shock is the aggressive administration of fluid to correct the deranged hemodynamic status. Crystalloid solutions have generally been considered first-line therapy. However, recent evidences suggest that isotonic crystalloid solutions may actually aggravate the immune dysfunction (Rhee *et al*., 2000).

A small volume resuscitation with hypertonic saline (HTS) is a relatively new conceptual approach to shock therapy. HTS solutions usually consist of 7.5% 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 (Ro-

cha-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, a number of researches disclosed previously unsuspected properties of HTS resuscitation, amongst them the correction of endothelial and red cell edema accompanying significant consequences in capillary blood flow (Victorino *et al*., 2003; Homma *et al*., 2005; Hoppen *et al*., 2005; Cruz *et al*., 2006).

In addition to these logistical advantages, some experimental studies and clinical observations revealed that HTS resuscitation had favorable immunomodulatory effects by affecting cellular immune function and preventing the inflammatory response in trauma victims (Homma *et al*., 2005; Junger *et al*., 1994; 1997a; 1997b; 1998; Rizoli *et al*., 1998; 2006; Deitch *et al*., 2003; Rotstein, 2000; Kolsen-Petersen, 2004; Powers *et al*., 2003; Shields *et al*., 2003; Coimbra *et al*., 2005).

Although the immune response to trauma or hemorrhage is complex and involves all inflammatory cells, T-lymphocytes perform an important regulatory function and may play a major role in the dysfunctional regulation of immune function after trauma. Thus, the current study was undertaken to assess the early effect of HTS resuscitation on T-lymphocyte response by determining T-lymphocyte subpopulations of peripheral blood in the treatment of severe hemorrhagic shock.

MATERIALS AND METHODS

Animals

Thirty male Sprague-Dawley (SD) rats, weighing 250~350 g, were obtained from the Medical Institute of Zhejiang Province, China.

Experimental protocol

This study was approved by the Ethics Committee of Sir Run Run Shaw Hospital affiliated to School of Medicine, Zhejiang University, China. The test animals had unlimited access to water before the experiments. After being weighed, they were anesthetized with pentobarbital (40 mg/kg intraperitoneally) and then 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. The animals were heparinized (500 U/kg). Blood losses of 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. Only animals whose blood losses were lower than 0.2 ml during the above-mentioned procedure and were spontaneously breathing 10 min after the procedure were included in the study. Among the test rats, eighteen fulfilled the inclusion criteria.

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

At 60 min, a phrase simulating hospital emergency treatment (hospital phrase) began. Resuscitation began with infusion of shed blood and normal saline solution. The "hospital phrase" lasted 30 min. After "hospital phrase", survivors were strictly monitored and observed for 120 min (observation phrase).

Grouping of animals

Eighteen rats were divided into three groups of six rats each with the sequence of the experiments randomized in blocks of three (one from each group): Group 1 (Sham group), only receiving anaesthesia, cannulation, heparinization and observation; Group 2 (hypertonic saline resuscitation group, HTS group), HTS [5.71 ml of 7.5% NaCl solution per kilogram of

body weight, according to the dosage recommended by Cai *et al*.(2002)] infusion during the late 30 min of "pre-hospital phase"; and Group 3 (normal saline resuscitation group, NS group), NS (0.9% NaCl, three times of blood loss volume) infusion during the late 30 min of "pre-hospital phase".

Collection of samples

Peripheral blood samples (0.5 ml/sample) were taken separately from arterial catheter of rats for determining the $CD4^+$ and $CD8^+$ subpopulations of T-lymphocytes at 0 and 210 min.

Flow cytometry analysis

T-lymphocyte surface receptors were determined by double antibody labelling and flow cytometry. T-lymphocyte subpopulations analyzed included CD4/helper and CD8/suppressor.

Fresh anti-coagulating blood (100 μl) was taken and incubated at room temperature in the dark with an equal volume of fluorescence-labelled monoclonal antibodies (FITC-IgGl/PE-IgGl, FITC-CD4/PE-CD8, Caltag, USA). Five hundred microlitres optilyse C was added, and the mixture was re-incubated at room temperature for 8 min. Two millilitres PBS was added after complete lysis of the red blood cells. After washing and centrifugation (1100 r/min), the samples were thoroughly mixed with 1 ml PBS and then analyzed by flow cytometry (Beckman-Coulter, FACSCalibur, USA).

Statistics

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 paired-samples *t* test, 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 rats of severe hemorrhagic shock model suffered approximately 50% blood volume loss based on animal body weight. Our previous studies indicated that rats all could survive acute blood loss (Lu *et al*., 2005; 2006). Simultaneously, we also found from this study that eighteen test rats all survived the above-mentioned three phases. The weight was (311.0±35.5) g in Sham group, (308.0±35.8) g in HTS group, and (313.5±33.3) g in NS group. ANOVA showed that no statistically significant difference occurred in weight among the rats of the three groups ($F=0.037$, $P=0.963$).

Variation of MAP (mean arterial pressure)

Table 1 shows MAP changes of the three rats groups in the "pre-hospital phase". At 0 min, there was no significant difference in MAP among Sham, HTS and NS groups. Furthermore, between HTS and NS groups, there were no significant differences in MAP at 20 min and 30 min.

Table 1 MAP changes of three groups rats in the "pre-hospital phase" (mmHg, $\bar{\chi}\pm s$)

Group	*n*	0 min	20 min	30 min
Sham group	6	128±8	124±8	125±6
HTS group	6	133±7	21±3▲	25±3▲
NS group	6	132±6	20±2▲	24±4▲

▲$P<0.01$ compared with Sham group

Variation of T-lymphocyte subpopulations in peripheral blood

The rats of Sham group showed no obvious change in T-lymphocyte subpopulations of peripheral blood after undergoing anaesthesia, cannulation and heparinization. In the early stage after hemorrhagic shock, fluid resuscitation and emergency treatment, the peripheral $CD4^+$ T-lymphocytes of HTS and NS groups markedly increased, with the difference showing statistical significance compared with Sham group (Table 2). Simultaneously, the peripheral $CD8^+$ T-lymphocytes of HTS group also increased to some extent, while those of NS group showed no apparent change. Between HTS group and NS group, there was significant difference in $CD8^+$ T-lymphocytes of peripheral blood (Table 3).

The $CD4^+/CD8^+$ ratio in peripheral blood of Sham, HTS and NS groups is shown in Table 4. The peripheral $CD4^+/CD8^+$ ratio of NS group increased obviously after hemorrhagic shock and resuscitation, and showed statistically significant differences compared with Sham and HTS groups. The peripheral $CD4^+/CD8^+$ ratio of HTS group slightly increased.

However, between Sham group and HTS group, there was no significant difference in the $CD4^+/CD8^+$ ratio of peripheral blood.

Table 2 Peripheral $CD4^+$ T-lymphocytes changes of three groups rats (%, $\bar{\chi} \pm s$)

Group	*n*	0 min	210 min	*t*	*P*
Sham group	6	44.44±5.86	50.33±4.48	1.572	0.177
HTS group	6	41.44±5.99	58.77±6.96▲	4.613	0.006
NS group	6	45.11±6.25	64.47±5.36Δ	7.761	0.001
F	−	0.630	9.376	−	−
P	−	0.546	0.002	−	−

▲$P<0.05$ compared with Sham group; Δ$P<0.01$ compared with Sham group

Table 3 Peripheral $CD8^+$ T-lymphocytes changes of three groups rats (%, $\bar{\chi} \pm s$)

Group	*n*	0 min	210 min	*t*	*P*
Sham group	6	26.62±3.94	28.32±2.07	1.006	0.361
HTS group	6	25.29±5.19	30.78±3.73▲	3.572	0.016
NS group	6	28.63±5.44	25.24±4.06	1.179	0.292
F	−	0.703	3.999	−	−
P	−	0.510	0.041	−	−

▲$P<0.05$ compared with NS group

Table 4 Peripheral $CD4^+/CD8^+$ ratio changes of three groups rats ($\bar{\chi} \pm s$)

Group	*n*	0 min	210 min	*t*	*P*
Sham group	6	1.69±0.27	1.75±0.20Δ	0.659	0.539
HTS group	6	1.65±0.20	1.94±0.38▲	3.461	0.018
NS group	6	1.59±0.12	2.62±0.55	4.525	0.006
F	−	0.349	7.878	−	−
P	−	0.711	0.005	−	−

▲$P<0.05$ compared with NS group; Δ$P<0.01$ compared with NS group

DISCUSSION

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 dies from complications brought on by the host's immune response to trauma and hemorrhage. The excessive inflammatory response leads to the dysfunction of immune cells, resulting in serious tissue damage and, ultimately, end organ failure. Consequently, balanced inflammatory responses are generally considered as a beneficial host response to injury.

The inflammatory response is dependent on intercellular communication through modification of cytokine response and fluctuation of peripheral immune cells such as natural killer (NK) cells, B cells, and T-lymphocyte subpopulations ($CD4^+$ and $CD8^+$ cells). It is well-known that T-lymphocytes express either the CD4 or the CD8 receptor on their surface. (1) The $CD4^+$ T-lymphocyte subpopulation is also called Help T cell (TH cell), and can be generally divided into three subpopulations of TH_0, TH_1 and TH_2. TH_0 cells are the original $CD4^+$ T-lymphocytes, and can differentiate into the mature TH_1 or TH_2 cells by signal-activating in the microenvironment. TH_1 cells mainly participate in promoting cell-mediated immune response, augmenting IgM and IgG2 synthesis by B cells, and activating macrophages. TH_2 cells are concerned with promoting antibody-mediated immune response, leading to IgG1 and IgE responses, and increasing numbers of local and/or circulating eosinophils. (2) The $CD8^+$ T-lymphocyte subpopulation can be divided into two subpopulations of Suppressor T cell (TS cell) and Cytotoxic T cell (TC cell). TS cells can quickly terminate or suppress immuno-inflammatory responses and TC cells can effectively kill the target through one of at least two distinct mechanisms. (3) The $CD4^+$ and $CD8^+$ subpopulations collaborate and restrict each other, playing important modulation roles in the immuno-inflammatory reactions of the body to infection and injury. Consequently, the ratio of $CD4^+/CD8^+$ cells is fairly constant among individuals, which can effectively reflect balanced condition between pro-inflammatory and anti-inflammatory processes (Cheadle *et al*., 1993).

At present, it is acknowledged that unbalanced immuno-inflammatory reactions triggered by shock or trauma is linked to MOD and death. Recently, several interesting studies found that small volume resuscitation with HTS could alter the immuno-inflammatory responses to shock or trauma, alleviate tissue damage and attenuate post-shock MOD (Homma *et al*., 2005; Junger *et al*., 1994; 1997a; 1997b; 1998; Rizoli *et al*., 1998; 2006; Deitch *et al*., 2003; Rotstein, 2000; Kolsen-Petersen, 2004; Powers *et al*., 2003; Shields *et al*., 2003; Coimbra *et al*., 2005). The inherent mechanisms of HTS resuscitation are not completely clear yet.

Data derived from both in vitro human leukocyte studies and animal models of hemorrhagic shock revealed that HTS resuscitation decreases neutrophil activation/adherence (Homma *et al*., 2005; Junger *et*

al., 1998; Rizoli *et al*., 1998; Deitch *et al*., 2003), stimulates lymphocyte proliferation (Loomis *et al*., 2001), inhibits pro-inflammatory but stimulates anti-inflammatory cytokine production by macrophages (Junger *et al*., 1997a; Powers *et al*., 2003; Shields *et al*., 2003), and reduces hormone secretion (Cross *et al*., 1989; Wade *et al*., 1991). In the in vivo setting of human, HTS resuscitation has been shown to mitigate the development of inflammation (Rizoli *et al*., 2006). For example, Deitch *et al*.(2003) established a rat model of trauma-hemorrhagic shock and revealed that resuscitation from hemorrhagic shock with HTS was associated with less polymorphonuclear neutrophil (PMN) activation than resuscitation with Ringer's lactate (RL) through comparing the ability to modulate PMN CD11b, CD18, or L-selectin expression. Powers *et al*.(2003) indicated HTS inhibited TNF-alpha production while enhancing IL-10 release by modulating alveolar macrophage function, when used for resuscitation of hemorrhagic shock. It proved convincingly that systemic administration of HTS exerted an immunomodulatory effect on alveolar macrophages and presented salutary effect on lung injury after resuscitated hemorrhagic shock, by shifting the balance of pro- and counter-inflammatory cytokine production in favor of an anti-inflammatory response. Ozguc *et al*.(2003) established a rat model of liver ischemia-reperfusion by total hepatic inflow occlusion under hypovolemic conditions. Subsequently, they found that HTS/6% dextran resuscitation seemed to be more effective than RL in decreasing the liver tissue damage.

However, there are some arguments against the immunomodulatory effects of HTS resuscitation. Bahrami *et al*.(2006) found that HTS resuscitation reduced the inflammatory response but not lung tissue damage or mortality after severe hemorrhagic shock.

Our data from the present experiment showed that the peripheral $CD4^+$ subpopulation of T-lymphocytes in HTS and NS groups markedly increased in the early stage after hemorrhagic shock, fluid resuscitation and emergency treatment. Furthermore, small volume resuscitation with HTS also induced the peripheral $CD8^+$ subpopulation of T-lymphocytes to a certain extent, whereas NS resuscitation showed no effect in this respect. Consequently, compared with Sham and HTS groups, the $CD4^+/CD8^+$ ratio of peripheral blood in NS group was obviously increased, and showed statistically significant differences. It indicates that, in the early stage after hemorrhagic shock and resuscitation, there is more severe disorder or imbalance of T-lymphocyte subpopulations regulating network in NS group than that in Sham group or HTS group.

In summary, we found that in the rat model of severe hemorrhagic shock, small volume resuscitation with HTS is more effective than NS resuscitation in ameliorating the initial immuno-inflammatory disorders and promoting a balanced profile of T-lymphocyte subpopulations regulating network, which may be one of the immunomodulatory mechanisms of HTS resuscitation. Because of their logistical advantages and immunomodulatory properties, HTS may be ideal replacements for conventional isotonic resuscitation fluids.

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