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Immune recovery after fluid resuscitation in rats with severe hemorrhagic shock*

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Abstract: Objective: To investigate the effects of resuscitation with normal saline (NS), hypertonic saline (HTS), and hydroxyethyl starch (HES) on regulatory T cells (Tregs), helper T 1 (Th1)/Th2 and cytotoxic T 1 (Tc1)/Tc2 profiles in the treatment of hemorrhagic shock. Methods: Rats subjected to severe hemorrhagic shock were resuscitated for 30 min with NS (n=8), HTS (n=8), or HES (n=8); sham (n=8) and naive control (n=8) groups were used for comparison. Following fluid resuscitation, the whole shed blood was reinfused for 30 min, and the rats were observed with continuous hemodynamic monitoring for 120 min. $CD4^+CD25^+Foxp3^+$ Treg proportions, Th1/Th2 and Tc1/Tc2 profiles in spleen were analyzed by three-color flow cytometry. Results: The proportion of $CD4^+CD25^+Foxp3^+$ Tregs and ratios of Th1/Th2 and Tc1/Tc2 did not differ among control, sham, and HTS groups, but were significantly lower in NS and HES groups (both P<0.05 vs. sham); NS and HES levels were similar. The level of Tc1 was significantly increased in HTS (P<0.05 vs. sham), and levels of Tc2 were increased in NS, HES, and HTS groups compared to sham (all P<0.05), but did not differ from each other. Conclusions: HTS resuscitation has a greater impact on immune system recovery than NS or HES by preserving the proportion of Tregs and maintaining the balance between Th1/Th2 and Tc1/Tc2 cells in the spleen. Thus, HTS resuscitation provides potential immunomodulatory activity in the early stage after hemorrhagic shock.

Key words: Regulatory T cells; Helper T cells; Cytotoxic T cells; Hemorrhagic shock
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1 Introduction

Hemorrhagic shock is an emergency event primarily caused by low organ perfusion, which results in marked and widespread oxidative tissue damage. Crucial treatment of this condition involves timely control of hemorrhage and adequate intravascular volume replacement. Even with successful fluid resuscitation, acute respiratory distress syndrome, systemic inflammatory response syndrome, or sepsis and multiple organ dysfunction syndrome can develop. These are the leading causes of death in middle and late phases of hemorrhagic shock. These conditions are thought to result from hyperinflammation or immunodepression (Menger and Vollmar, 2004; Moore *et al.*, 2004; 2006; Brøchner and Toft, 2009).

Fluids for resuscitation include normal saline (NS), hypertonic saline (HTS), and hydroxyethyl starch (HES), although controversy remains on which strategy is superior (Angele *et al.*, 2008). Recently, the immunologic effects of resuscitation with HTS solutions have been examined (Yip *et al.*, 2007; Murao *et al.*, 2009; Vincenzi *et al.*, 2009; Isayama *et al.*, 2012). HTS stimulates the rapid release of adenosine

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triphosphate (ATP) in a time- and concentration-dependent manner, which enhances T-cell activation by increasing Ca^{2+} influx (Yip *et al.*, 2007). In addition, HTS resuscitation suppresses the release of proinflammatory cytokines (Isayama *et al.*, 2012), and thus might improve immunosuppressive reactions after hemorrhagic shock. Indeed, our previous studies indicate that HTS alleviates immune disorders and limits tissue injury after hemorrhagic shock (Lu *et al.*, 2007; 2008; 2010).

Although serious immune dysfunction often appears in the middle or late stage, immune and inflammation systems are involved from the onset of hemorrhagic shock. Hence, it is essential to reveal the initial impacts of resuscitation with different fluids on the immune system to provide advice for early recovery and immune intervention strategies. The purpose of this study was to compare the effects of three resuscitation fluids on immune cell regulation in the early stage of hemorrhagic shock. Specifically, we evaluated the proportion of regulatory T cells (Tregs), which help maintain immune balance, and can limit inflammatory tissue damage caused by infection, autoimmunity, allogeneic immune responses, and antitumor immunity (Belkaid and Rouse, 2005; Zhao *et al.*, 2013); we also evaluated the ratios of helper T (Th) and cytotoxic T (Tc), which can execute many crucial immune functions (Marcu *et al.*, 2007; Zhang *et al.*, 2008; Chen *et al*., 2014).

2 Materials and methods

2.1 Ethics statement

This study was approved by the Ethics Committee of the School of Medicine, Zhejiang University, Hangzhou, China. Animals were handled according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

2.2 Animals

Forty male Sprague-Dawley rats, weighing 280–330 g, were purchased from the Laboratory Animal Center of the Medical Institute of Zhejiang Province, China and were maintained on standard rat diet and tap water ad libitum before the experiment. Animals were housed under controlled light/dark conditions for at least 3 d; the light period was from 8:00 a.m. to 5:00 p.m. in specific pathogen-free conditions.

2.3 Experimental protocol

Rats were anesthetized with 40 mg/kg pentobarbital intraperitoneally (IP) and placed in a supine position on a warm pad (25 °C). After sterilization with 10% (0.1 g/ml) povidone-iodine solution, the right carotid artery was isolated and cannulated with a polyethylene catheter, which was used for blood withdrawal and connected to a pressure transducer and computerized physiograph system (model 1290C; Hewlett-Packard Co., Palo Alto, CA, USA) for continuous hemodynamic monitoring. In the same way, the left femoral vein was cannulated for fluid infusion, and rats were heparinized (500 U/kg). Rats were observed for 10 min post-cannulation to ensure stable blood hemodynamics and spontaneous breathing. Blood loss during cannulation was collected by gauze for measurement by weight; only rats with <0.22 g blood loss were used for fluid resuscitation experiments.

Severe and controlled hemorrhagic shock was induced according to our previous studies (Lu *et al.*, 2007; 2008; 2010). Briefly, while under the mild anesthesia (40 mg/kg pentobarbital IP), acute hemorrhage was initiated (time=0 min) with four controlled blood withdrawals via the right carotid arterial cannula: 10 ml/kg per 5 min for the first two withdrawals, then 5 ml/kg per 5 min for the second two, to yield a total hemorrhage volume of 30 ml/kg. The shed blood was collected in glass syringes with heparin for later reinfusion. Fluid resuscitation was administered after 30 min. In the third stage, at 60 min, rats were reinfused with the whole shed blood to represent in-hospital emergency treatment. An observation stage then ensued (time=90–210 min) via continuous hemodynamic monitoring. Rats were then sacrificed by heart puncture and the spleens were removed for preparation of cell suspensions used in subsequent analyses.

2.4 Grouping of animals

Forty rats were randomly assigned to one of five groups (n=8 each group). In the control group, rats received no operative intervention. In the sham group, rats only received anesthesia, cannulation, heparinization, and observation. In the fluid resuscitation

phase, rats in the NS resuscitation group received 0.9% (9 g/L) NaCl infusion for a 3:1 ratio against the blood loss volume (Gurfinkel *et al.*, 2003; Alam *et al.*, 2004; Barros *et al.*, 2011; Maier and Alam, 2011). The HTS group received 6.0 ml/kg body weight of 7.5% (0.075 g/ml) NaCl solution for a 1:5 ratio against blood loss volume (Murao *et al.*, 2003a; 2003b). The HES group received 30 ml/kg body weight of 6% HES (200/0.5; Fresenius Kabi, Baden Humboldt, Germany) solution for a 1:1 ratio against blood loss volume (Barros *et al.*, 2011).

2.5 Flow cytometry

RBC Lysis Buffer was used to remove the red blood cells from the sample. The monoplast suspension of spleen was prepared according to our previous protocol (Lu *et al.*, 2013). Spleen cell suspensions were cultured for 4 h at 37 °C at 5% CO_2 in RPMI-1640 media (Sigma-Aldrich, St. Louis, MO, USA) containing phorbol myristate acetate, ionomycin, monensin (Beyotime, China), and fetal bovine serum.

Three-color flow cytometer (FC500, FACSCalibur; Beckman-Coulter, Brea, CA, USA) was then used to detect the various cell types. The software FACSCalibur (Beckman-Coulter, USA) was used for flow cytometric analysis. Anti-rat CD4 FITC (fluorescein isothiocyanate), CD25 PE (phycoerythrin), and Foxp3 PerCP-Cy5 (peridinin chlorophyll protein-cyanine 5) antibodies, and their isotype controls (eBioscience, San Diego, CA, USA) were used for detecting Tregs ($CD4^+CD25^+Foxp3^+$). Anti-rat CD3 FITC, CD8a PerCP, interleukin (IL)-4 PE, and interferon (IFN)-γ PE antibodies, and their isotype controls (BD Biosciences, San Jose, CA, USA) were used for detecting Th1 ($CD3^+IFN\text{-}\gamma^+CD8^-$), Th2 ($CD3^+IL\text{-}4^+CD8^-$), Tc1 ($CD3^+IFN\text{-}\gamma^+CD8^+$), and Tc2 ($CD3^+IL\text{-}4^+CD8^+$) cells.

2.6 Statistical analysis

Data analyses were performed using SPSS Version 13.0 software (SPSS Inc., Chicago, IL, USA). Cell type percentages and ratios were compared by the homogeneity test, and then one-way analysis of variance (ANOVA) and the least significant difference *t*-test were applied for comparisons among the five groups. A two-way repeated-measures ANOVA was performed to compare arterial pressures among the groups. Data are presented as mean±standard deviation (SD) with a $P<0.05$ considered statistically significant.

3 Results

3.1 Mean arterial pressure

All rats undergoing acute hemorrhagic shock survived the experiments, with about 50% loss of blood volume based on weight. Mean arterial pressures (MAPs) measured throughout the experiment are presented in Fig. 1. Blood pressure levels for rats in the control group were not obtained, as they did not receive polyethylene catheters for monitoring. Acute hemorrhage initially induced a dramatic decrease in MAP, to about 20 mmHg at 20 min, which increased to about 30 mmHg at 30 min by self-compensation. Fluid resuscitation restored pressures within 15 min, with MAPs approaching 90 mmHg at the 45 min time-point. The MAPs then remained stable throughout the emergency treatment (60–90 min) and observation (90–210 min) stages. MAP levels remained unchanged throughout the entire experiment for rats in the sham group.

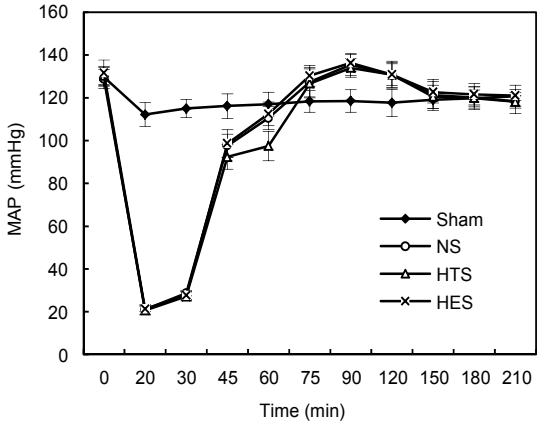

Fig. 1 Mean arterial pressure (MAP) monitoring
Sham: sham group (n=8); NS: normal saline group (n=8); HTS: hypertonic saline group (n=8); HES: hydroxyethyl starch group (n=8). Data are represented as mean±SD

A two-way repeated-measures ANOVA was performed with fluids as the between-subjects factors and MAPs obtained at different time-points as within-subject variables. As a Mauchly's test of sphericity indicated that variances in differences among the

groups were not equal (df=54, P<0.05), degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε=0.34). As a result, no significant difference in MAP was found among NS, HTS, and HES groups during the experiment (F[6.78, 50.82]=1.943, P>0.05).

3.2 Changes of $CD4^+CD25^+Foxp3^+$ Tregs in spleen

The representative illustration of flow cytometry for Tregs is shown in Fig. 2. The percentage of $CD4^+CD25^+Foxp3^+$ Tregs in rats receiving HTS fluid resuscitation did not differ from those in sham and control rats (Table 1). However, the percentages of Tregs in NS and HES groups were similar and significantly lower than those in the control and sham groups (P<0.05).

3.3 Changes of Th1/Th2 and Tc1/Tc2 ratios in spleen

The representative illustration of flow cytometry for Th and Tc is shown in Fig. 3. Ratios of Th1/Th2 and Tc1/Tc2 in rats receiving HTS resuscitation did

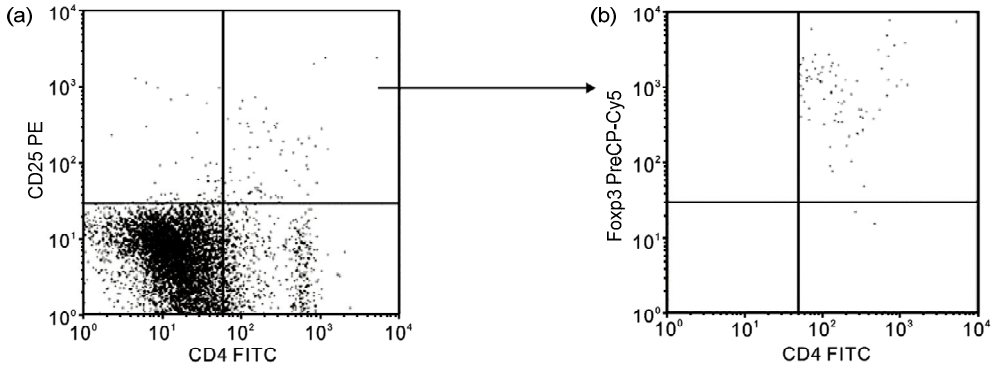

Fig. 2 Representative illustration of flow cytometry for Tregs

(a) Upper right quadrant represents $CD4^+CD25^+$ cells. (b) Upper right quadrant represents $CD4^+CD25^+Foxp3^+$ Tregs

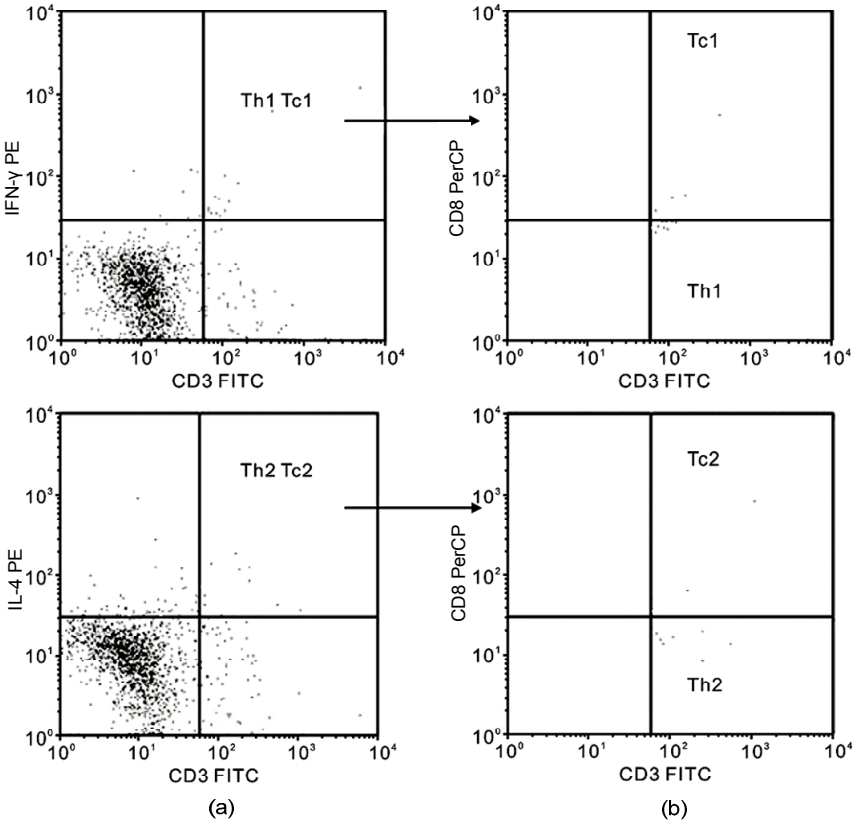

Fig. 3 Representative illustration of flow cytometry for Th and Tc cells

(a) Upper right quadrants represent $CD3^+$interferon (IFN)-γ^+ (top) and $CD3^+$interleukin (IL)-4^+ (bottom) cells, respectively. (b) Upper right quadrant represents $CD3^+IFN\text{-}\gamma^+CD8^+$ Tc1 cells and lower right quadrant represents $CD3^+IFN\text{-}\gamma^+CD8^-$ Th1 cells (top); upper right quadrant represents $CD3^+IL\text{-}4^+CD8^+$ Tc2 cells and lower right quadrant represents $CD3^+IL\text{-}4^+CD8^-$ Th2 cells (bottom)

not differ from those in the control and sham rats (Table 2). However, both ratios were significantly lower in rats receiving NS and HES than in the control and sham groups (P<0.05). Interestingly, the percentage of Tc1 cells was significantly higher only in the HTS group, whereas the levels of Tc2 in all three groups receiving fluid resuscitation were equally and significantly improved (P<0.05 vs. control and sham; Fig. 4).

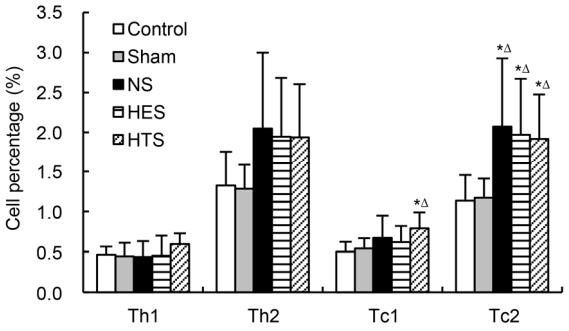

Fig. 4 Percentages of Th and Tc in spleen

Control: control group (n=8); Sham: sham group (n=8); NS: normal saline group (n=8); HTS: hypertonic saline group (n=8); HES: hydroxyethyl starch group (n=8). Data are represented as mean±SD. $^{*}P$<0.05, vs. control; $^{\Delta}P$<0.05, vs. sham by one-way ANOVA and the least significant difference t-test

Table 1 Percentages of $CD4^+CD25^+Foxp3^+$ Tregs

Group	n	Tregs (%)
Control	8	2.21±0.35
Sham	8	1.96±0.30
NS	8	$1.36±0.19^{*\Delta}$
HTS	8	$2.10±0.31^{\#\S}$
HES	8	$1.24±0.34^{*\Delta}$
F		17.072
P		0.000

HES: hydroxyethyl starch; HTS: hypertonic saline; NS: normal saline. Data are represented as mean±SD. $^{*}P$<0.05, vs. control; $^{\Delta}P$<0.05, vs. sham; $^{\#}P$<0.05, vs. NS; $^{\S}P$<0.05, vs. HES

Table 2 Th1/Th2 and Tc1/Tc2 ratios

Group	n	Th1/Th2	Tc1/Tc2
Control	8	0.37±0.08	0.46±0.10
Sham	8	0.35±0.08	0.47±0.11
NS	8	$0.22±0.06^{*\Delta}$	$0.33±0.04^{*\Delta}$
HTS	8	$0.32±0.07^{\#\S}$	$0.42±0.08^{\#\S}$
HES	8	$0.23±0.07^{*\Delta}$	$0.32±0.08^{*\Delta}$
F		7.051	5.395
P		0.000	0.002

HES: hydroxyethyl starch; HTS: hypertonic saline; NS: normal saline. Data are represented as mean±SD. $^{*}P$<0.05, vs. control; $^{\Delta}P$<0.05, vs. sham; $^{\#}P$<0.05, vs. NS; $^{\S}P$<0.05, vs. HES

4 Discussion

Tregs mediate immune and inflammatory responses in many pathophysiologic processes. A sufficient quantity of Tregs is critical in keeping inflammation balance and reducing tissue injury (Li *et al*., 2009; Fogle *et al*., 2010b; Tang *et al*., 2014; Zhao *et al*., 2015; Zhang *et al*., 2016). Excessive activity or quantity of Tregs can promote infectious deterioration, sepsis, or tumor immune escape, whereas an insufficient amount can result in overactive inflammatory responses (Burgents *et al*., 2010; Fogle *et al*., 2010a; Carambia *et al*., 2014). Tregs have been shown to be differentially influenced by various resuscitation fluids after hemorrhagic shock (Murao *et al*., 2009; Isayama *et al*., 2012). In the present study, resuscitation with HTS did not alter the percentage of Tregs in the spleen, whereas NS and HES significantly lowered it. Thus, resuscitation with a small volume (1:5 ratio against blood loss volume) of HTS can stabilize spleen Tregs in the early phase, and may play a beneficial role in reducing immunologic stress after hemorrhagic shock. Indeed previous studies have shown that HTS resuscitation can reduce the injury to lungs and intestinal mucosa after hemorrhagic shock (Fernandes *et al*., 2009; Gao *et al*., 2009; Lu *et al*., 2010). The proposed mechanism by which HTS can mediate these effects is by shrinking cells and mechanically altering membranes, resulting in the release of ATP and promotion of T-cell function (Loomis *et al*., 2003; Woehrle *et al*., 2010; Ledderose *et al*., 2016). Whether a similar mechanism is involved in the effect on Tregs in our rat hemorrhagic shock model requires further research.

The ratios of Th1/Th2 and Tc1/Tc2 can reflect the balance of immune stability to some degree. Several studies have shown that hemorrhagic shock disturbs this balance by enhancing the differentiations of Th2 and Tc2 cells and suppressing the differentiations of Th1 and Tc1 cells, which increases the risks

of sepsis and multiple organ failure (Marcu *et al*., 2007; Miller *et al*., 2007; Chen *et al*., 2014). In the present study, resuscitation with HES and NS significantly lowered the ratios of Th1/Th2 and Tc1/Tc2. However, the quantities of Tc1 and Tc2 cells were increased with HTS, whereas only Tc2 cells increased with NS and HES. It is possible that HTS resuscitation preserves Th1 and Tc1 cell differentiation, and may have more effective immunomodulation activity than NS and HES in the early stage after hemorrhagic shock. Consistent with this, Zhang *et al*. (2008) showed that the differentiation of Th1 cells was enhanced after resuscitation with HES.

Peripheral blood was not analyzed in the present study in consideration of the effect of blood reinfusion on immune function of peripheral blood mononuclear cells. However, the results of the present study indicated that resuscitation with all three fluids equally restored MAP levels in the early stage of hemorrhagic shock. Although the MAP in rats receiving HTS was slightly lower than those in the other groups at the end of the fluid resuscitation stage, it was still high enough ((97.50±6.83) mmHg) to maintain blood supply to vital organs, based on previous reports (Lu *et al*., 2010; Barros *et al*., 2011; Vallet, 2011; Cui *et al*., 2014). Fluid resuscitation with NS or HES may result in hypoosmolar dehydration, thus stimulating an immune system response and inflammatory injury, whereas HTS may promote a more beneficial state of immunologic stress. The effect of resuscitation with these fluids on coagulation and liver/kidney function still needs to be explored to provide more useful information for future clinical application.

In summary, this study shows that resuscitation with a small volume of HTS has a stronger impact on the spleen immune system than HES or NS. Specifically, HTS preserves Treg proportions in the spleen, while increasing the numbers of Th1 and Tc1 cells and maintaining the balance between Th1/Th2 and Tc1/Tc2. Additional studies are needed to further investigate the related mechanisms.

Compliance with ethics guidelines

Feng YAO, Yuan-qiang LU, Jiu-kun JIANG, Lin-hui GU, and Han-zhou MOU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：严重失血性休克大鼠液体复苏后的免疫修复

目　的：严重失血性休克大鼠模型早期阶段使用不同的液体复苏，比较脾脏组织中调节性 T 细胞（Tregs）、辅助性 T 细胞 1（Th1）/Th2 以及细胞毒性 T 细胞 1（Tc1）/Tc2 的不同变化，初步探讨其免疫修复机制。

创新点：（1）脾脏为机体重要免疫器官，检测其中的免疫细胞变化，比外周血更具敏感性和特异性；（2）将免疫反应中多环节的免疫细胞变化进行协同分析，结果更具创新性和科学性，为临床上形成规范的救治方案提供了科学的实践资料。

方　法：将 SD 雄性大鼠随机分成 5 组，其中对照组和 Sham 组（假手术）仅作为比较，其余三组在建立严重失血性休克大鼠模型后，采用不同的液体复苏：等渗盐水（NS 组）、高渗盐水（HTS 组）和羟乙基淀粉（HES 组）。然后再灌注 30 分钟，并持续监测血液动力学 120 分钟，最后心脏穿刺，取脾脏组织，通过三色荧光标记流式细胞术进一步分析 $CD4^+CD25^+Foxp3^+$ Treg 细胞含量，以及 Th1/Th2 和 Tc1/Tc2 的比值。

结　论：液体复苏后大鼠脾脏中 $CD4^+CD25^+Foxp3^+$ Tregs 细胞含量、Th1/Th2 和 Tc1/Tc2 的比值在对照组、Sham 组和 HTS 组中无差异，并都显著高于 NS 组和 HES 组。与 Sham 组比较，HTS 组中 Tc1 水平明显升高，而 NS 组、HES 组和 HTS 组中 Tc2 水平均有升高，且三组之间 Tc2 水平无差别。因此，对于维持脾脏中 Treg 细胞含量、Th1/Th2 和 Tc1/Tc2 平衡的作用上，HTS 液体复苏对免疫系统的影响大于 NS 和 HES。综上所述，在失血性休克后的早期阶段 HTS 复苏可提供潜在的免疫修复作用。

关键词：调节性 T 细胞；辅助性 T 细胞；细胞毒性 T 细胞；失血性休克