Background: Trauma-hemorrhagic shock (T/HS) has been associated with multiorgan dysfunction, including bone marrow failure. This study examined apoptosis and morphologic alterations in bone marrow mononuclear cells (BMMNCs) with different volume therapies after T/HS.

Methods: T/HS was induced in groups of male Sprague-Dawley rats through a fracture of the left femur and continual bleeding for 30 minutes, followed by resuscitation with Ringer’s lactate solution (RL), 6% hydroxyethyl starch solution (HES), or 5% albumin (ALB). Mean arterial blood pressure was monitored during the T/HS and resuscitation, and the impacts of various resuscitative fluids on apoptosis and morphology of BMMNCs at 24 hours and 48 hours after resuscitation were examined using flow cytometry, transferase-mediated dUTP nick-end labeling assay, and hematoxylin and eosin staining.

Results: Fluctuations in mean arterial blood pressure were homogenous among the three treatment groups. The percentage of early BMMNC apoptosis increased significantly at 24 hours and 48 hours (24.65% ± 5.41% and 29.09% ± 2.07%, respectively; p < 0.05), and the percentage of late BMMNC apoptosis increased to 13.43% ± 2.82% (p < 0.05) at 48 hours in the T/HS + RL group. In contrast, resuscitation with HES alone dramatically attenuated the apoptosis. Resuscitation with ALB alleviated BMMNC apoptosis, except for late apoptosis at 48 hours. A greater number of apoptotic BMMNCs as well as morphologic alterations were shown using the transferase-mediated dUTP nick-end labeling assay and hematoxylin and eosin stain in the T/HS + RL group than in the HES or ALB groups.

Conclusion: Intravascular volume replacement with HES showed prevention of BMMNC apoptosis at first 48 hours after T/HS compared with RL and ALB. These findings provide new insights into the intervention mechanism of HES on T/HS-related multiorgan dysfunction.

Key Words: Trauma-hemorrhagic shock, Bone marrow mononuclear cells, Hydroxyethyl starch solution, Apoptosis.

Hemorrhagic shock after critical trauma that involves hypovolemia and subsequent tissue malperfusion and organ dysfunction has shown high morbidity and mortality in a clinical setting.1–4 Shortly after multiple injuries combined with shock, apoptosis occurs in many organs, such as the thymus, spleen, liver, lung, and intestine, resulting in multiple organ failure.4–6 Moreover, traumatic injury-induced immunosuppression has been shown associated with marked alteration of many immune functions, including T-cell activation, proliferation, and cytokine release, which resulting an increased susceptibility to sepsis.7–11 We have previously demonstrated that an imbalance in Th1 and Th2 responses may be a response to trauma-hemorrhagic shock (T/HS)-related immunodisorders.12

Bone marrow (BM) has been indicated as one of the prominent organs for self-homeostasis after injury. In this regard, recent studies have shown that BM dysfunction occurred in experimental animal models of hemorrhagic shock,7,13,14 hypoxia,15 and soft tissue injury,16 etc., and that the apoptosis play a partial role in the development of BM failure after severe shock. In addition, human BM has also been shown to be depressed by trauma and hemorrhagic shock.8,17,18

An early sufficient intravascular fluid therapy is a fundamental principle applied to the management of T/HS. Recent data indicate that resuscitation with different solutions not only affects organ perfusion but also modulates T/HS-related inflammatory and immune responses. The question of which fluid is most suitable is still a controversial subject of debate. A growing body of evidence suggests that the isotonic crystalloid solution may actually aggravate the T/HS-related inflammatory injury and organ apoptosis.5,19 Although many studies have addressed the relative benefits of certain natural colloids such as albumin (ALB),13,20,21 the negative perception of ALB for resuscitation should not be ignored.22–25 Hydroxyethyl starch solution (HES)26 has been reported to have pharmacokinetic and pharmacodynamic advantages, which reduced splanchnic ischemia, inflammatory response, capillary leakage, and CD4+ T-cell apoptosis, and correcting imbalances in Th1 and Th2 responses12,27–30 without coagu-
lopathy. However, there is only limited information available about the effects of various volume replacement regimens on BM response after T/HS.\textsuperscript{14}

The goals of this study were to examine the effects of various resuscitation fluids (Ringer’s lactate solution [RL], HES, and 5% ALB) on T/HS-induced BM mononuclear cell (BMMNC) apoptosis and morphologic alteration in a rat model of T/HS for a reasonable resuscitation scenario.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats aged 8 weeks to 10 weeks and weighing 200 g to 250 g were purchased from the Animal Resource Center at the Zhejiang University Medical College (Hangzhou, China). All animal research protocols used in this study were approved by the institutional laboratory review board and based on the principle stated in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1985).

Animal Grouping

A total of 32 rats were randomly divided into three self-controlled groups: (a) the T/HS + RL group sustained T/HS and was resuscitated with RL (n = 16); (b) the T/HS + HES group was resuscitated with 6% HES (n = 16); and (c) the T/HS + ALB group was resuscitated with 5% ALB (n = 16).

Establishment of the T/HS Model

The experimental T/HS model for rats was established, as described previously, with minor modifications. Briefly, rats were anesthetized intraperitoneally with 200 mg/kg chloral hydrate, restrained in a supine position. After a small incision, the right jugular vein, right carotid artery, and left femoral artery were catheterized for fluid resuscitation, monitoring of mean arterial blood pressure (MAP), and bleeding, respectively. The left femur was dissected from the connective tissue to expose the distal epiphysis and subjected an incision sites. Then, MAP were reduced to 30 mm Hg quickly by blood withdrawal using left femoral artery device, and the shed blood was stored in heparinized syringes, at the temperature of 37°C. After rinsing with PBS, BMMNCs were isolated by Ficoll-diatrizoate density gradient separation (2,500 rpm/min for 10 minutes) and were washed twice in phosphate buffered saline.

Analysis of BMMNC Apoptosis by Flow Cytometry

The level of BMMNC apoptosis was analyzed using flow cytometry with an apoptosis detection kit (Immunotech, Marseille, France) according to the manufacturer’s instructions. After binding with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI), BMMNCs were analyzed using flow cytometry.

Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

BMMNCs were detected using a terminal deoxynucleotide TUNEL assay (Catalog No. 11684817910, In Situ Cell Death Detection Kit, POD; Roche, Mannheim, Germany), according to the specifications and instructions of the manufacturer. Briefly, BMMNCs were loaded onto polylysine-coated slides at a concentration of 2 × 10^4 cells per slide and fixed in 4% ice-cold paraformaldehyde dissolved in PBS for 30 minutes at room temperature. The cells were then rinsed twice with PBS buffer and incubated with blocking solution (0.3% H2O2 in methanol) for 30 minutes at room temperature. After rinsing with PBS, BMMNCs were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 10 minutes on ice. Slides were then rinsed twice and incubated for 60 minutes at 37°C with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-conjugated dUTP. Then, slides were incubated with sheep antifluorescein antibody conjugated to hors eradish peroxidase for 30 minutes at 37°C, giving the TUNEL signal, observed using the 3,3’-diaminobenzidine substrate kit (Vector Labs, Burlingame, CA), a brown color. Finally, the slides were counterstained by immersion in hematoxylin. Negative controls were prepared with distilled water in place of the terminal deoxynucleotidyl transferase enzyme in the working solution. Positive controls were prepared with DNase I (1 mg/mL; Sigma, CA) to induce DNA fragments before incubating with the TUNEL reaction mixture. Positive cells were examined under a light microscope.

Morphologic Examination

BMMNCs were loaded onto slides and stained with hematoxylin and eosin. Morphologic changes were observed, using light microscopy, by an experienced hematologist who was unaware of the treatment used.

Statistical Analysis

The data were presented as means ± standard deviation and were analyzed by the one-way analysis of variance using the Student-Newman-Keuls test using SPSS 13.0 software (SPSS, Chicago, IL). Statistical significance was defined as p < 0.05.
Flow cytometry has recently become a technique of choice for the quantitative analysis of apoptosis because of its ability to discriminate between apoptosis and necrosis. In our study, there was no difference in baseline apoptosis among three groups. However, the percentage of early apoptotic BMMNCs (both annexin V-positive and PI-negative) increased to 24.65% ± 5.41% at 24 hours after resuscitation in the T/HS + RL group. Furthermore, BMMNC apoptosis was aggravated at 48 hours because the percentage of early apoptotic cells increased to 29.09% ± 2.07% and late apoptotic cell (both annexin V-positive and PI-positive) increased to 13.43% ± 2.82% (p < 0.05 compared with the other two groups). In contrast, the frequency of BMMNC apoptosis in the T/HS + HES group had no significant increase at 24 hours or 48 hours, indicating that resuscitation with HES may alleviate T/HS-related spontaneous BMMNC apoptosis in vivo. Similarly, resuscitation with 5% ALB resulted in no significant increase in BMNNC apoptosis at 24 hours, but a remarkable increase in the early and late apoptotic percentage at 48 hours after resuscitation (13.55% ± 2.58% and 22.46% ± 1.46%, respectively; p < 0.05 vs. baseline and 24 hours value), indicating that resuscitation with 5% ALB was not able to exert the same antiapoptosis activity as HES. Therefore, resuscitation with different fluids had different effects on spontaneous BMMNC apoptosis in vivo. Although no significant alteration was detected using the TUNEL method in the T/HS + ALB group, an increased percentage of apoptosis in the T/HS + ALB group was identified using flow cytometry. Hence, flow cytometry should be regarded as a decisive technique for the assessment of apoptosis in this study.

**RESULTS**

**MAP Fluctuation With Different Fluids in the T/HS Model**

MAPs for the four groups of rats are plotted with time in Figure 1. In the sham group, MAP maintained a stable level, which differed from the other groups that underwent T/HS and resuscitation. During the induction of T/HS, MAP slightly decreased immediately after bone fracture and then rapidly decreased to a level of 30 mm Hg after bleeding. During the shock phase, MAP increased commensurately followed by a gradual elevation toward baseline after resuscitation, regardless of the fluid transfused. This fluctuation of MAP demonstrated the establishment of a successful T/HS model. Furthermore, there was no significant difference in the MAP at the end of resuscitation among three treatment groups, indicating that all three fluids corrected T/HS in the model. No distinction in the MAP was noticed among three groups after 1 hour postresuscitation.

**Resuscitation With HES Reduces T/HS-Related BMMNC Apoptosis**

T/HS usually causes oxidative stress and overactivation of nuclear factor-kB and may lead to apoptosis. To test this hypothesis, BMMNC apoptosis was characterized ex vivo using flow cytometry (Fig. 2) and TUNEL method (Fig. 3).

Recently, the TUNEL method has been used worldwide despite its limitations in specificity (failure to distinguish apoptosis versus necrosis) and a high false-positive rate in certain cases. However, the TUNEL method is useful in detecting apoptosis if used in conjunction with other techniques. In our study, the TUNEL assay (Fig. 3) showed TUNEL-positive cells increased dramatically in the T/HS + RL group at 24 hours and 48 hours after resuscitation (5.83% ± 1.14% and 6.50% ± 0.97%, respectively, p < 0.05 compared with the other two groups), which were rarely observed in the T/HS + HES or the T/HS + ALB groups.

**DISCUSSION**

The effects of different resuscitation solutions were examined on apoptosis and morphology of BMMNCs in a rat model of T/HS followed by resuscitation with RL, HES, or ALB. The results indicated that rats subjected to T/HS sustain significant increases in apoptosis in BMMNCs after resuscitation with RL, which were not observed in resuscitation with HES and partially seen with ALB.

Apoptosis or programmed cell death is a tightly regulated form of active cell suicide, which occurs in many organs, such as the thymus, spleen, lung, and gut, in response to traumatic injury and hemorrhage. With respect to BM response, many recent studies have shown that an increase in BM apoptosis either in patients or in an animal model is thought to be a crucial mechanism responsible for hematopoietic abnormalities and immunoregulatory disorders.
The mechanism of organ apoptosis after trauma is also unclear because of involving many factors and pathways. The loss of the proper balance between proapoptotic and compensatory antiapoptotic factor9 plays the key role on organ apoptosis while inflammation,10 endotoxin33 and ischemia-reperfusion injury28 are also involved in severe trauma. Many reports have indicated that the majority of cellular injury might occur during resuscitation and not during the ischemia period.34,35 As the end-organ, BM is susceptible to ischemia-reperfusion injury resulting from generation of reactive oxygen species and inflammatory caspase. Microcirculatory instability also plays a critical role on organ impairment after reperfusion. There were reports that demonstrated that patients might have inadequate regional organ oxygen delivery and decreased oxygen consumption on the cellular level after hemorrhagic shock despite apparently normal systemic perfusion and that tissue oxygen saturation predicted the development of organ dysfunction rather than the traditional parameters.3,27 On the other hand, Badami et al.36 have demonstrated that a small number of hematopoietic progenitor cells left the BM and recently entered the peripheral circulation especially to the injury sites. Although, these stem cell could play some role in self-repair, the increased mobilization of cells from BM which were trapped in injured tissue and failed to come back to the BM might be one mechanism for BM dysfunction after shock. Hence, it seems very complicated of BM response, and more extensive studies should be done.

The choice of resuscitation strategy seems so important, because different fluids can have a widely divergent impact on immune response, inflammatory activation, and tissue injury. Recently, the ancillary and immunomodulatory effects of various resuscitation solutions have begun to be elucidated, and several studies have shown the advantages of colloid solutions over crystalloid volume therapy.14,27,30 Resuscitation with RL has been associated with neutrophil...
activation, tissue swelling, and immunodisorders. Furthermore, the influence on microcirculatory stability with different volume therapy has been studied. The tissue perfusion and tissue oxygen tension decreased significantly although systemic hemodynamics maintained stable using RL, indicating that the RL did not restore microvascular perfusion for its distribution in the interstitium. Lang et al. revealed that HES showed an improved tissue oxygen tension during human perioperative period compared with a crystalloid-based volume replacement strategy, which was probably due to its significant reduction effect of tissue edema and capillary permeability. Boldt et al. has demonstrated that HES may increase flow rate in organs and tissues because of the decreased viscous flow resistance, improved blood fluidity, and decreased erythrocyte aggregation. HES also exerts a protective role through its inherent specific effects on platelet function, plasma viscosity, and blood corpuscle-endothelial cell interactions. In this study, although the systemic hemodynamics parameters such as MAP fluctuation was similar among the three treatments, a remarkable increase of apoptosis and morphologic changes at both 24 hours and 48 hours after resuscitation has been shown in RL group. In contrast, neither the ratio of apoptosis nor morphologic changes showed significant differences using HES for resuscitation. The diversity in BMMNC apoptosis with different resuscitative fluids should be in part attributed to microhemodynamic heterogeneity in BM. Other research has shown that HES is an appropriate resuscitation fluid based on inhibiting NF-κB activation, maintaining the Bcl-2/Bax ratio, and preventing oxidative stress after acute hemorrhagic shock.

The protective role of ALB in BMMNC apoptosis was significant compared with RL in this study, similar to the results of Osband et al., who explained that ALB has been found to bind a toxic inhibitor of erythropoiesis, which when unbound, inhibits BM progenitor cell growth. Other reasons for its protective effect might be the antioxidant and scavenger properties by repleting thiol store. ALB also has been shown to improve microcirculation blood flow, modulate apoptosis, and reduce the inflammatory response. However, resuscitation with ALB has also been independently associated with some severe complications including altering coagulation, decreasing myocardial contractility, and aggravating edema. Therefore, resuscitation with ALB showed a significant increase of apoptosis at 48 hours in our present

Figure 3. TUNEL assay of BMMNCs. A, 24 hours after resuscitation with RL; B, 48 hours after resuscitation with RL. ↑: apoptosis-positive cells stained in brown (original magnification ×400). C, Percentage of TUNEL-positive cell in three groups of rats at 24 hours and 48 hours. *p < 0.05 THS + RL group versus THS + HES group and THS + ALB groups.
study, indicating that ALB was not able to completely inhibit BMMNC apoptosis after T/HS.

This study was limited to measurement of the BMMNCs apoptosis during the first 48 hours after T/HS. Whether there are alterations in apoptosis using different fluid replacement regime at time points later than 48 hours remains to be determined in further study. However, the advantage of HES which prevented BMMNC apoptosis during the first 48 hours might benefit the patients by earlier intervention of BM injury after shock. Moreover, only MAP was chosen as the hemodynamic treatment benchmark to ensure that our resuscitation scheme with different type of fluid in the T/HS model was similar and comparable. Despite of the absence of the other parameters such as lactate level and base excess in our study, some experiments also have shown that there was no significant different in lactate concentration between RL and other colloid fluid used in resuscitation. A recent study showed no significant differences in hemodynamic outcome related to the type of fluid and indicated that timely resuscitation is effective irrespective of the type of nonsanguineous fluid used. However, more microhemodynamic parameter including tissue oxygen tension and saturation should be considered more valuable in evaluating the efficiency of different fluid and be examined in our future study.

In summary, this study using a rat model of T/HS indicated that resuscitation with HES reduced morphologic abnormalities of BMMNCs by inhibiting early and late apoptosis, which provide new insights into understanding the positive effects of HES resuscitation. In our study, we have not examined criteria of inflammation or microcirculatory blood flow which may help explain the possible benefit of HES or ALB observed after correction of BMMNC apoptosis.

CONCLUSION

Intravascular volume replacement with HES showed prevention of BMMNC apoptosis at first 48 hours after T/HS compared with RL and ALB. These findings provide new insights into the intervention mechanism of HES on T/HS-related multiorgan dysfunction. Whether HES is of benefit to decrease post-shock complication such as hematopoietic abnormalities and immune dysfunction should be elucidated in clinic studies.

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