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Effects of three fluid resuscitation methods on apoptosis of visceral organs in rats with hemorrhagic shock

LU Yuan-qiang (陆远强)†[1], CAI Xiu-jun (蔡秀军)[1], GU Lin-hui (顾琳慧)[2],
FAN Yu-jing (樊宇靖)[1], WANG Qi (王 琦)[1], BAO De-guo (鲍德国)[1]

([1]*Department of Emergency, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China*)
([2]*Cancer Institute, Zhejiang Tumor Hospital, Hangzhou 310022, China*)
†E-mail: yuanqiang609@sina.com

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Abstract: Objective: To observe the effects of three fluid resuscitation methods on apoptosis of visceral organs in rats with hemorrhagic shock. Methods: A model of rat with severe hemorrhagic shock and active bleeding was established in 32 SD (Sprague-Dawley) rats. The rats were randomly divided into control group, no fluid resuscitation group (NF group), controlled fluid resuscitation group (NS40 group) and rapid large scale fluid resuscitation group (NS80 group). Each group contained 8 rats. The curative effects were compared. At the same time, the apoptosis in liver, kidney, lung and small intestinal mucosa of survivors after hemorrhage and resuscitation was detected by light microscopy in HE (hematoxylin and eosin) stained tissue sections, flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Results: The survival rate of early fluid resuscitation (14/16) was markedly higher than that of NF group (3/8). There was some apoptosis in liver, kidney, lung and small intestinal mucosa of all survivors. Compared with NF and NS40 groups, the apoptosis of liver, kidney and small intestinal mucosa of NS80 group was obviously increased. Conclusions: Among three fluid resuscitation methods, controlled fluid resuscitation can obviously improve the early survival rate and the apoptosis of liver, kidney and small intestinal mucosa in rats with severe and uncontrolled hemorrhagic shock, and may benefit improvement of prognosis.

Key words: Shock, Hemorrhagic, Resuscitation, Apoptosis
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INTRODUCTION

Hemorrhagic shock is a common clinic emergency case. Successful treatment includes surgical control of hemorrhage and restoration of tissue perfusion. Current guidelines for presurgical treatment of patients with hemorrhagic shock recommend rapid volume resuscitation to normal blood pressure as quickly as possible. The practice is controversial because aggressive restoration of intravascular volume and rapid increasing of blood pressure before securing hemostasis may exacerbate hemorrhage and worsen outcome.

Controlled resuscitation allows prehospital treatment to work with compensatory mechanisms. The concept is to restore some intravascular fluid while taking into consideration hemostatic mechanisms. Such a scheme would balance the seemingly mutually exclusive processes of tissue perfusion and hemostasis.

Our study used a model of rat with severe hemorrhagic shock and active bleeding to test the effects of different resuscitation methods on apoptosis of visceral organs.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Sir Run Run Shaw Hospital, School of

Medicine, Zhejiang University. Forty-two male Sprague-Dawley (SD) rats, weighing 270~460 g, were obtained from the Medical Institute of Zhejiang Province, China. They had unlimited access to food and water before the experiments. After being weighed, the rats were anesthetized with pentobarbital (40 mg/kg intraperitoneally) and 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. 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, thirty-two fulfilled the inclusion criteria.

The model of rat with severe hemorrhagic shock and active bleeding was established (Capone *et al*., 1995). Under light anesthesia, the injury began (time=0) 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 late two times). The shed blood was collected in glass syringes with heparin and reinfused during resuscitation. At 30 min, uncontrolled hemorrhagic shock was added to the volume-controlled shock by amputation of the tail at 75 percent of its length, measured from the tip. The bleeding tail was immediately directed into a container (with heparin) and the amount of shed blood was measured. This phase was called "prehospital phase" and continued for 60 more minutes. During this period, the rats were early resuscitated by infusing normal saline (NS). Fluids were administered via the femoral vein with an infusion pump at the rate of 2 ml/(kg·min). The pump was turned on and off to maintain the MAP (mean arterial pressure) goal.

At 90 min, a phase simulating hospital treatment (hospital phase) began. Hemostasis was achieved by tail wound closure. Simultaneously, resuscitation began with infusion of blood and normal saline solution. The "hospital phase" lasted 60 min and the end points were Hct (hematocrit) of 30 percent and MAP of 80 mmHg.

Thirty-two rats were randomly divided into four groups of eight rats each with the sequence of the experiments randomized in blocks of four (one from each group): group 1 (control group), neither fluid resuscitation nor hemostasis in the "prehospital" or "hospital phases" (no treatment); group 2 (no fluid resuscitation group, NF group), no fluid resuscitation in the "prehospital phase" (no field fluid resuscitation); group 3 (controlled fluid resuscitation group, NS40 group), NS infusion during the "prehospital phase" to reach and maintain MAP at 40 mmHg, beginning immediately after the tail cut (field fluid resuscitation to MAP 40 mmHg); and group 4 (rapid large scale fluid resuscitation group, NS80 group), NS infusion during the "prehospital phase" beginning immediately after the tail cut, to reach and sustain MAP of 80 mmHg (field fluid resuscitation to MAP 80 mmHg). Groups 2, 3, and 4 had a "hospital phase" with the same end points: control of bleeding, fluid resuscitation with blood and NS to Hct of 30 percent and MAP of 80 mmHg.

Blood samples (0.3 ml/sample) were taken separately from rats for complete blood count at 0, 120 and 150 min, and blood samples (0.2 ml/sample) were collected separately for determining serum lactate levels at 0, 30, 60 and 90 min.

Rats who lived for 150 min were regarded as survivors. The surviving rats were immediately sacrificed after resuscitation and hemostasis. The liver, kidneys, lungs and small intestine were taken out quickly and flushed with 0.01 mol/L cold phosphate buffer solution (pH 7.4).

The left part of liver, kidneys, lungs and part of the small intestine were fixed with 10% buffered formaldehyde for routine pathological examination and 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 (in situ Cell Detection Kit, AP) was purchased from American Promega Corporation. The detection procedure was mainly done according to the instructions provided by the corporation. Under high power

field (400× magnification) of fluorescent microscope, the number of apoptotic cells characterized by the positive staining nucleus in TUNEL staining sections was also counted in 10 randomly selected fields per section. The mean number of apoptotic cells per one field for each rat was calculated for further statistical analysis.

The right part of organs and part of the small intestine were immediately sent to the central laboratory of Sir Run Run Shaw Hospital for apoptosis assaying by flow cytometer (FCM, Beckman Coulter EPICS XL), and the method of Annexin-V/PI staining was used. The hypodiploid peak appearing before the G_1 phase on the histogram indicating reduced DNA content in apoptotic cells was shown by FCM analysis. For each sample, 5000 cells were measured.

Data were presented as mean±*SEM*. With SPSS 10.0 software package, statistical analysis for comparing mean values from the four groups was made by one way analysis of variance (ANOVA) and least significant difference-*t* test (LSD-*t*). Survival rate was compared using Fisher's exact test. Differences were considered significant at $P<0.05$.

RESULTS

Characteristics of the animal model

The rats of the uncontrolled severe hemorrhagic shock model suffered approximately 50% blood volume loss based on animal body weight. Without treatment in control group, eight rats all survived at 30 min, four rats survived at 90 min, no one survived at 150 min. ANOVA showed that no statistically significant differences occurred in weight, Hct, PLT (platelet) and HB (hemoglobin) among the rats of the four groups.

Variation of mean arterial pressure (MAP)

Pre-hemorrhage MAP was (114±12) mmHg for control group, (124±9) mmHg for NF group, (128±10) mmHg for NS40 group, and (129±7) mmHg for NS80 group. At this point, MAP in NS40 or NS80 groups were higher than that in control group ($P<0.05$), but there was no significant difference among NF, NS40, and NS80 group.

At the conclusion of blood examination (20 min), MAP had decreased to (24±3) mmHg in control group, (21±3) mmHg in NF group, (22±2) mmHg in NS40 group, and (21±2) mmHg in NS80 group. Thirty minutes before the tail cut, MAP had slightly increased to (33±6) mmHg in control group, (30±4) mmHg in NF group, (31±4) mmHg in NS40 group, and (30±2) mmHg in NS80 group. There was no statistically significant difference in MAP among the four groups at 20 and 30 min.

Fluid volumes administered

The volume of normal saline given during the "prehospital phase" was (3.86±1.12) ml per 100 g in NS40 group and (13.95±3.46) ml per 100 g in NS80 group ($P<0.01$ for NS80 group compared with NS40 group). Control and NF groups received no fluid in the "prehospital phase".

Survival rate

All rats survived in the first 30 min of the experiment. When the "hospital phase" began (90 min), the number of survivors was four in control group, three in NF group, eight in NS40 group, and six in NS80 group. At the conclusion of the "hospital phase" (150 min), the number of survivors was zero in control group, three in NF group, eight in NS40 group, and six in NS80 group.

Compared with NF group (3/8), the higher survival rate of NS40 and NS80 groups (14/16) showed significant difference ($P<0.05$). The survival rate of NS40 group (8/8) was significantly higher than that of NF group (3/8), with the comparison showing significant difference ($P<0.05$). The survival rate of NS40 group (8/8) was slightly higher than that of NS80 group (6/8), the survival rate of NS80 group (6/8) was slightly higher than NF group (3/8), the comparison showed no statistically significant difference.

Serum lactate level

There was no statistically significant difference in serum lactate level at 0 and 30 min of the "prehospital phase". After fluid resuscitation, lactate levels in the NS40 and NS80 groups obviously decreased. Compared with control and NF groups, there was statistically significant difference in serum lactate level at 60 and 90 min of the "prehospital phase". The dynamic changes in serum lactate level are detailedly listed in Table 1.

Histological results

The liver, kidneys, lungs and small intestine mucosa of survivors were stained with hematoxylin and eosin (HE) for routine microscopic examinations. In HE stained sections, none of the visceral organs had necrosis or other visible abnormalities in histological structure.

TUNEL staining results

In TUNEL stained sections of visceral organs of NF, NS40 and NS80 groups, some positive staining cells (apoptotic cell) were observed. Fluorescent microscopy revealed that TUNEL positive cells presented green fluorescence and contracted or fragmented nuclei. Occasionally, apoptotic bodies could be found (Figs.1~3).

The TUNEL staining results in visceral organs of NF, NS40 and NS80 groups are shown in Table 2. The number of TUNEL positive cells of kidney and small intestinal mucosa was obviously more in NS80 group than that in NF or NS40 group, with the differences showing statistical significance. However, between NS40 group and NF group, there was no significant difference in the number of TUNEL positive cells of visceral organs.

Flow cytometric analysis

Table 3 particularly shows the FCM results of apoptosis in visceral organs of NF, NS40 and NS80 groups. The apoptotic rates of liver, kidney and small intestinal mucosa were obviously higher in NS80 group than that in NF or NS40 group, and the comparison showed statistically significant difference. In addition, the apoptotic rate of small intestinal mucosa in NS40 group was higher than that of NF group (P<0.05). However, between NF group and NS40 group, there were no significant differences in apoptotic rates of liver, kidney and lung.

Table 1 Serum lactate level changes of four groups rats in the "prehospital phase" (mmol/L, $\bar{\chi} \pm s$)

Group	0 min		30 min		60 min		90 min	
	n	Lactate level	*n*	Lactate level	*n*	Lactate level	*n*	Lactate level
Control group	8	1.51±0.08	8	8.35±1.88	5	8.95±1.42	4	9.91±1.69
NF group	8	1.66±0.15	8	9.31±1.38	5	9.41±2.40	3	8.79±1.47
NS40 group	8	1.62±0.22	8	8.71±1.15	8	5.56±0.81$^{▲Δ}$	8	4.62±0.79$^{▲Δ}$
NS80 group	8	1.58±0.18	8	9.08±0.95	8	5.16±0.95$^{▲Δ}$	6	3.64±0.44$^{▲Δ}$
F value		1.19		0.74		15.81		40.40
P value		>0.05		>0.05		<0.01		<0.01

$^{▲}P$<0.01 compared with control group; ^{Δ}P<0.01 compared with NF group

Table 2 TUNEL staining results in visceral organs of three groups rats (number/high power field, $\bar{\chi} \pm s$)

Group	*n*	Small intestinal mucosa	Liver	Kidney	Lung
NF group	3	32.4±4.4$^{▲}$	1.4±0.5	1.3±0.7^{Δ}	6.3±1.3
NS40 group	8	37.8±5.6$^{▲}$	1.3±0.4	1.3±0.6$^{▲}$	5.6±2.0
NS80 group	6	48.0±4.0	1.9±0.3	2.6±0.8	5.0±1.6
F value		12.23	3.30	8.76	0.61
P value		<0.01	>0.05	<0.01	>0.05

$^{▲}P$<0.01 compared with NS80 group; ^{Δ}P<0.05 compared with NS80 group

Table 3 FCM results of apoptosis in visceral organs of three groups rats (%, $\bar{\chi} \pm s$)

Group	*n*	Small intestinal mucosa	Liver	Kidney	Lung
NF group	3	2.55±0.28$^{▲*}$	0.44±0.14^{Δ}	1.29±0.18$^{▲}$	2.68±0.43
NS40 group	8	5.59±2.43$^{▲}$	0.34±0.12$^{▲}$	1.67±1.18$^{▲}$	2.69±1.00
NS80 group	6	9.98±1.01	0.74±0.24	4.62±1.19	2.11±0.42
F value		18.88	9.57	15.09	1.12
P value		<0.01	<0.01	<0.01	>0.05

$^{▲}P$<0.01 compared with NS80 group; ^{Δ}P<0.05 compared with NS80 group; $^{*}P$<0.05 compared with NS40 group

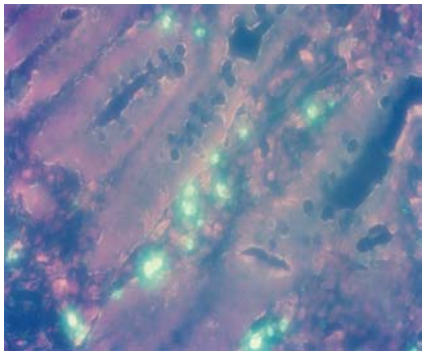

Fig.1 Small intestinal mucosa tissue, apoptosis detection by TUNEL method (original magnification ×400)

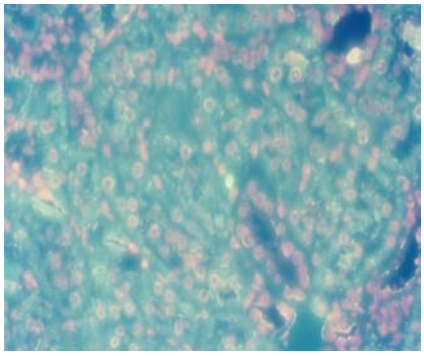

Fig.2 Kidney tissue, apoptosis detection by TUNEL method (original magnification ×400)

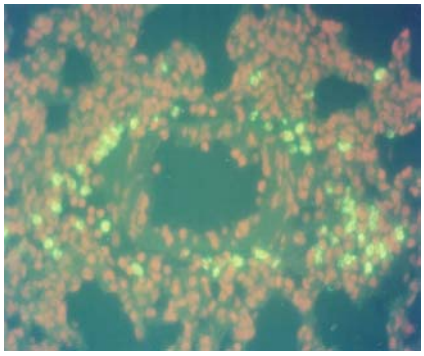

Fig.3 Lung tissue, apoptosis detection by TUNEL method (original magnification ×400)

DISCUSSION

Conventional guidelines for prehospital treatment of hypotension secondary to hemorrhage after trauma recommend rapid infusion of crystalloid solution to restore normal blood pressure as quickly as possible. This premise is based, in part, on clinical studies and on substantial laboratory data that showed hemorrhagic shock in animals produced with a controlled hemorrhage model was reversible when shed blood was replaced with two to three times that volume of crystalloid solution. Although controlled hemorrhage is a well-defined laboratory model, resuscitation of the patients with multiple injuries and active bleeding may present very different pathophysiology. Sometimes, increased blood pressure from rapid fluid resuscitation can lead to a worsened outcome by disruption of early soft thrombus, coagulopathy, hemodilution and rebleeding.

The body is good at compensating for hemorrhagic shock. Left undisturbed, it will decrease blood loss and maintain organ perfusion to some extent through a series of stress reactions. However, compensatory mechanisms are limited in magnitude and duration. Profound initial blood losses or conditions of prolonged transport to the operating room can overwhelm compensation and result in death. On the basis of the above-mentioned theories, some scholars advocate the concept of "controlled fluid resuscitation", which only administers moderate fluid infusion to prolong the compensatory time of patients with traumatic hemorrhagic shock before surgical hemostasis (Capone *et al*., 1995; Burris *et al*., 1999; Kim *et al*., 1997). Controlled fluid resuscitation means restoring some intravascular volume while taking into consideration hemostatic mechanisms, which allows prehospital treatment to work with compensatory mechanisms, balances the seemingly mutually exclusive processes of tissue perfusion and hemostasis.

The model of blood withdrawal combined with active bleeding by tail cut produced severe hemorrhagic shock, in which initial blood loss was about 50 percent of the circulation blood volume. Early fluid infusion could obviously improve short-term survival rate of rats. In addition to increased survival rate, fluid resuscitation partly improved tissue perfusion and metabolic parameter as indicated by serum lactate level in our study.

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 researches 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

(Guan *et al*., 1998; Xu *et al*., 1997; Yu *et al*., 2002; Meldrum *et al*., 1997; Angele *et al*., 1999). The apoptosis in pathological conditions also can reflect the severity of disordered internal environment of the body. In particular, a great deal apoptosis of small intestinal mucosa can result in impairment of mucosa barrier and immune function, which is bound up with endogenous infection and MODS.

Our study showed that apoptosis of different extent happened in visceral organs of the rat survivors, especially in the small intestinal mucosa. It showed that intestinal mucosa was the first-affected and rapidly-changed site when shock occurred. Furthermore, the apoptosis of liver, kidney and small intestinal mucosa was statistically higher in NS80 group than that in NF or NS40 group. This implies that, in prehospital treatment of severe and controlled hemorrhagic shock, brief and moderate hypotension may be helpful for maintaining organ function and decreasing the later complications of traumatic patients. In recent years, a few clinical observations also supported the same opinions (Bickell *et al*., 1994; Revell *et al*., 2003; Chen *et al*., 2004). The inherent mechanism is not completely clear, but may be related to the following factors: (1) rapid large scale fluid infusion seriously disturbs the internal environment and exacerbates cellular metabolism, which can induce abnormal apoptosis of visceral organs; (2) high perfusion pressure of tissue and organ followed with rapid large scale fluid resuscitation can cause severe ischemia-reperfusion injury by inducing a large amount of oxygen-derived free radicals (Zhang *et al*., 2004). In addition, the apoptosis of small intestinal mucosa in NS40 group was higher than that of NF group, probably because controlled fluid resuscitation can still induce ischemia-reperfusion injury to some extent.

In sum, our results showed that in severe hemorrhagic shock, some fluid must be given in proper time to improve tissue perfusion and avoid early death. The data also confirmed that rapid large scale fluid resuscitation to restore MAP of 80 mmHg during uncontrolled hemorrhage obviously increased the apoptosis of liver, kidneys and small intestinal mucosa in rats, which may increase the risk and extent of organ dysfunction. Controlled fluid resuscitation to MAP of 40 mmHg in the treatment of severe and uncontrolled hemorrhagic shock can obviously improve the early survival rate and the apoptosis of visceral organs, which may benefit improvement of prognosis.

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