Protective effect of Shenfu on gut epithelium in a porcine model of hemorrhagic shock

Yong Liang,[1] Chunsheng Li ,[1] Wei Yuan,[1] Junyuan Wu,[1] Qiang Zhang,[2] Mingqing Zhang,[3] Hongjie Qin,[4] Zexuan Li[1]

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[1]Emergency Medicine Clinical Research Center, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China
[2]ICU, Peking University Third Hospital, Beijing, China
[3]Emergency, Beijing Jishuitan Hospital, Beijing, China
[4]Emergency, Beijing Luhe Hospital, Capital Medical University, Beijing, China

Correspondence to
Dr Chunsheng Li, Emergency Medicine Clinical Research Center, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China; lcscyyy@163.com

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ABSTRACT

This study aimed to explore the protective effect of Shenfu on the hemodynamics and gut integrity in a porcine model of hemorrhagic shock. Hemorrhagic shock was induced in 32 domestic pigs with a rapid bleeding via the arterial sheath to a mean arterial pressure of 40 mm Hg within 10 min. Animals with hemorrhagic shock were then randomly assigned into the negative control group (n=8), receiving neither blood transfusion nor drug treatment; the blood transfusion group, in which animals were given blood transfusion alone; the saline group, in which animals were blood transfused and resuscitated with saline (3 mL/kg); and the Shenfu group, in which animals received blood transfusion and resuscitation with Shenfu (3 mL/kg). Blood tumor necrosis factor-alpha (TNF-α) and interleukin-6 were measured using ELISAs. Tissue levels of superoxide dismutase (SOD), malondialdehyde (MDA), Na^+/K^+-ATPase, Ca++ATPase, myeloperoxidase (MPO), and fatty acid binding protein 2 (FABP2) were determined using respective quantitation kits. Fluid resuscitation with Shenfu significantly improved HR, CI, and MAP of pig with hemorrhagic shock, which was accompanied with mitigation of tissue damages in intestinal epithelium. Blood TNF-α was reduced in the Shenfu group. Bcl-2 and cleaved caspase-3 expression in intestinal tissues were elevated and decreased, respectively, in pigs treated with Shenfu. Notably, treatment with Shenfu suppressed oxidative stress markers MDA, MPO, and FABP2 in the intestine. Oppositely, SOD, Na^+/K^+-ATPase and Ca++ATPase levels in intestinal tissues were promoted by Shenfu treatment. Shenfu demonstrates significant protective effect on the hemodynamics and gut epithelium of pigs with hemorrhagic shock.

Significance of this study

What is already known about this subject?

- Hemorrhagic shock represents a common clinical manifestation of many emergency diseases.
- The underlying pathogenesis of hemorrhagic shock is attributed to the hypoperfusion and sympathetic compensation by peripheral vasoconstriction.
- Currently, blood transfusion combined with fluid resuscitation is the key treatment modality to restore the volume and oxygenation in the scenario of acute blood loss.

What are the new findings?

- The use of Shenfu could protect animals with hemorrhagic shock against death by improving the hemodynamics.
- The integrity of the gut epithelium is of paramount importance to the prognosis of patients with hemorrhagic shock.
- The effect of Shenfu on ischemia/reperfusion injury has remained to be fully characterized.

How might these results change the focus of research or clinical practice?

- Shenfu could demonstrate significant protective effect on the hemodynamics and gut epithelium of pigs with hemorrhagic shock, of which the underlying mechanism would be attributed to its suppression on apoptosis and oxidative damages on organs.

BACKGROUND

Hemorrhagic shock represents a common clinical manifestation of many emergency diseases. Hemorrhagic shock is characterized by an acute reduction in blood volume which, if not promptly managed, can result in mortality. Indeed, the mortality rate of hemorrhagic shock is unacceptably high.[1] The underlying pathogenesis of hemorrhagic shock is attributed to the hypoperfusion and sympathetic compensation by peripheral vasoconstriction.[2] Currently, blood transfusion combined with fluid resuscitation is the key treatment modality to restore the volume and oxygenation in the scenario of acute blood loss; however, this treatment approach can potentially result in massive vascular endothelial damage and therefore multiple organ failure, a clinical scenario commonly known as ischemia/reperfusion (I/R) injury.[3] Due to its high-energy demand and rapid turnover, gut epithelium is sensitive to hypoperfusion and hypoxia, and indeed, gut damage on I/R injury is an important determinant of clinical outcome in hemorrhagic shock.[4] On reperfusion, the excessively generated, highly reactive free radicals

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initiate lipid peroxidation, causing structural damages of plasma membrane and triggering influx of inflammatory cells, which further exacerbate tissue damages.[5 6] In this context, therapeutic agents that can mitigate the initiation of I/R cascade on reperfusion treatment for hemorrhagic shock are urgently needed.

Shenfu injection, formulated with red Radix Ginseng (Hongshen) and Radix Aconitum Carmichaeli (Fupian), is a traditional Chinese medicine that has been clinically used for years for treatment of diseases associated with cardiovascular failures like congestive heart failure, shock, acute myocardial infarction and pulmonary heart disease.[7] The modulating effect of Shenfu injection on the cardiovascular system has been well studied, including dilating coronary artery, improving microcirculation, increasing blood pressure and strengthening myocardial contractility.[8–10] A randomized clinical trial has shown that Shenfu is able to improve clinical outcomes in patients with return of spontaneous circulation after in-hospital cardiac arrest (CA).[11] Hemorrhagic shock and CA both involve I/R injury as the common pathophysiological feature. The clinical benefit of Shenfu on hemorrhagic shock management has therefore been examined; however, most studies have emphasized on the mechanisms by which Shenfu increases vascular perfusion and improves hemodynamics. The protective effect of Shenfu on major organs other than the cardiovascular system like the intestine has remained to be fully elucidated. As such, the present study was aimed to investigate whether fluid resuscitation with Shenfu would protect the intestine against tissue damages in a porcine model of hemorrhagic shock. The findings would provide novel insights into the refinement of management strategy for patients with hemorrhagic shock.

METHODS

Hemorrhagic shock model and treatment

Thirty-two domestic male pigs (Sus scrofa domestics), weighted from 25 to 32 kg, were employed in the present study. The procedure of inducing hemorrhagic shock followed the published protocol.[12] In brief, after an overnight fasting, pigs were intramuscularly premedicated with midazolam (0.3 mg/kg; Dormicum, Germany) and ketamine (10 mg/kg; Ketanest, Germany), and were then anesthetized by intravenous administration of fentanyl (3 μg/kg, fentanyl; Janssen, Germany), ketamine (2 mg/kg), and flunitrazepam (0.25 mg/kg, Roche). After that, the trachea was intubated after an injection of pancuronium (0.3 mg/kg; DeltaSelect, Germany), allowing a pressure-controlled mechanical ventilation by a ventilator. After a stabilization for 30 min, to induce shock, pigs were rapidly bled via the arterial sheath (femoral artery) to a mean arterial pressure (MAP) of 40 mm Hg within 10 min. The blood pressure was then maintained at 40 mm Hg for 60 min. Hemodynamic and circulatory data of pigs were closely monitored using transpulmonary thermodilution (ie, pulse contour cardiac output).

Drug treatment and study parameters

Pigs with hemorrhagic shock were randomly assigned into different groups (n=8 per group): negative control, blood transfusion, saline group and Shenfu group. The negative control (ie, no treatment) was pig with hemorrhagic shock receiving neither resuscitation nor drug treatment, and the blood transfusion group (ie, mock) received blood transfusion alone. The saline and Shenfu group received blood transfusion together with conventional volume resuscitation with saline (3 mL/kg) and Shenfu (3 mL/kg), respectively.

Measurements of heart rate (HR), Cardiac Index (CI) and MAP and collection of blood samples were performed at baseline, and 0 1, 2, 3, 4 and 6 hours after the onset of resuscitation. On the completion of the in-live experiment, that is, 6 hours postresuscitation, animals were anesthetized and euthanized by lethal injection with potassium chloride (40 mL, 1 mol/L). Tissue samples of intestine and heart were collected for analysis.

Transmission electron microscopy

Tissue architecture of the intestine was examined using scanning electron microscopy following our established protocol. In brief, the collected tissues were fixed, dehydrated and embedded for preparation of tissue sections with a thickness of 50–60 nm. Sections were stained with uranium (IV) acetate and lead citrate, and were scanned using transmission electron microscopy (model JEM-1400; JEOL, Massachusetts, USA).

ELISA and oxidative stress marker determination

Blood tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) was measured using Quantikine porcine TNF-α Immunoassay (catalog no. PTA00; R&D Systems, Minnesota, USA) and Quantikine porcine IL-6 Immunoassay (catalog no. P6000B, R&D Systems), respectively. Respective quantitation kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) was used to determine the tissue levels of superoxide dismutase (SOD) (catalog no. A001-3-2), malondialdehyde (MDA) (catalog no. A003-1-2), Na^+/K^+-ATPase (catalog no. A070-2-2), Ca++ATPase (catalog no. A070-4-1), myeloperoxidase (MPO) (catalog no. A044-1-1), and fatty acid binding protein 2 (FABP2) (catalog no. H265-1-2). Assays were performed following manufacturers' instructions. In brief, blood or tissue samples were added to antibody-coated plates. After washing, the bound target-of-interest was detected using horseradish peroxidase-conjugated detection antibody. Signal was then developed using 3,3′,5′5′-tetramethylbenzidine substrate.

Immunohistochemistry (IHC) and immunoblotting

Intestinal expression of Bcl-2 was examined using IHC. In brief, intestinal tissues were formalin-fixed, paraffin-embedded, and sectioned for staining with anti-porcine Bcl-2 antibody (5 μg/mL; Cell Signaling Technology, Massachusetts, USA). Immunoblotting was also employed to determine the level of Bcl-2, BAX, and caspase-3 in the intestine. All primary antibodies were obtained from Cell Signaling Technology.

Statistical analysis

All data were analyzed using statistical software SPSS V.20.0. One-way analysis of variance and t-test were used to compare data between groups. Significant difference was indicated by p values of <0.05.

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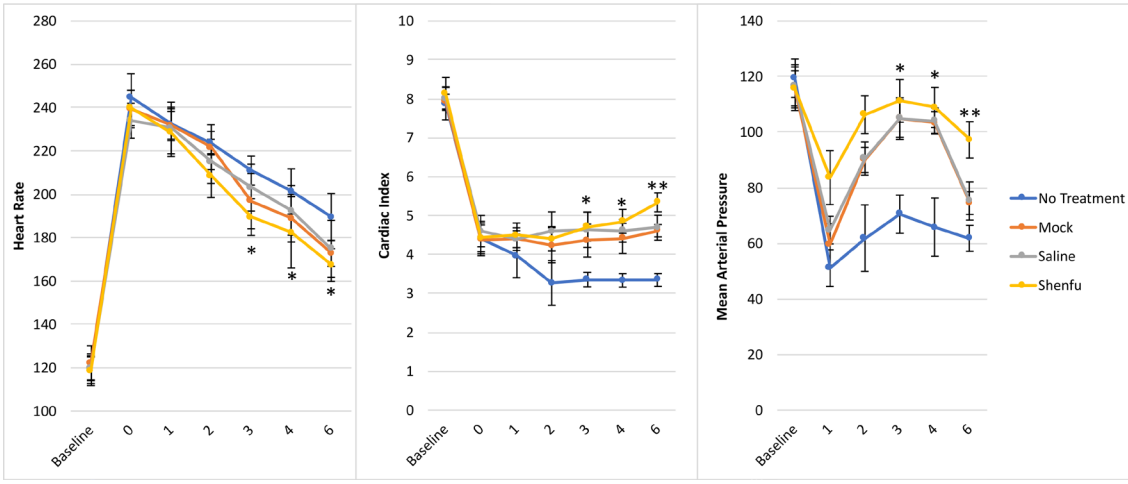

Figure 1 Hemodynamics of pigs with hemorrhagic shock after different treatment. Heart rate, Cardiac Index and mean arterial pressure were measured at baseline and 0, 1, 2, 3, 4, and 6 hours after the onset of fluid resuscitation. pigs with hemorrhagic shock were randomly assigned into different groups (n=8). In the no treatment group, three hemorrhagic pigs died and in the mock-control; one pig died during the in-live study period. Shown are the mean value of each group at different time points with error bars representing SD *, significant difference (p<0.05) between the Shenfu group and the no treatment group; **, significant difference (p<0.05) between the Shenfu group and the other three treatment groups.

RESULTS

Protection of pigs against mortality resulted from hemorrhagic shock

The survival of pigs after the onset of hemorrhagic shock was followed. Among the pigs that received no treatment/resuscitation, two and one pig died at 2 and 4 hours after the onset of hemorrhagic shock, respectively. One pig receiving resuscitation alone died 4 hours postresuscitation. All saline and Shenfu-treated reperfused animals survived.

The HR, CI and MAP of animals were monitored (figure 1 and table 1). The induction of hemorrhagic shock sharply increased the HR of all animals. The HR gradually decreased, but the changes in animals resuscitated with Shenfu were found more significant. Opposite to HR, both CI and MAP decreased after the onset of hemorrhagic shock. The further drop in CI was prevented by resuscitation, and the use of Shenfu could further enhance the improvement of CI. Similar to CI, resuscitation could bring MAP towards the range comparable to the normal. The improvement in MAP was most significant in animals treated with Shenfu injection. Taken together, the use of Shenfu could protect animals with hemorrhagic shock against death by improving the hemodynamics.

Table 1 HR, CI and MAP of pigs with hemorrhagic shock after different treatments

			Time points after reperfusion											
	Baseline		0 hour		1 hour		2 hours		3 hours		4 hours		6 hours	
	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
HR														
No treatment	119.50	5.50	244.75	10.93	232.50	7.73	223.63	5.40	211.00	6.69	201.33	10.42	189.60	10.78
Mock	122.25	7.83	239.38	8.62	231.88	6.38	221.75	10.36	196.88	12.74	189.00	11.01	172.71	5.94
Saline	119.50	6.82	234.00	8.07	230.63	11.94	215.25	10.21	203.13	10.82	192.63	11.49	174.88	13.12
Shenfu	118.63	6.84	239.88	8.17	228.50	10.99	208.63	10.11	189.50*	8.47	182.50*	16.41	167.38*	7.56
CI														
No treatment	7.88	0.42	4.41	0.34	3.96	0.56	3.27	0.57	3.36	0.18	3.34	0.17	3.35	0.16
Mock	7.93	0.19	4.39	0.42	4.39	0.22	4.24	0.45	4.36	0.42	4.41	0.39	4.61	0.16
Saline	8.02	0.29	4.60	0.40	4.39	0.29	4.59	0.50	4.64	0.46	4.61	0.29	4.69	0.32
Shenfu	8.13	0.42	4.44	0.42	4.49	0.32	4.41	0.31	4.72*†	0.37	4.85*†	0.31	5.34*†‡	0.24
MAP														
No treatment	119.38	6.91			51.13	6.58	61.88	11.97	70.50	6.86	65.83	10.46	61.80	4.66
Mock	115.75	6.32			59.63	7.48	90.00	4.54	104.88	6.71	103.38	4.03	74.50	4.07
Saline	116.50	7.76			64.75	4.98	90.50	5.95	104.88	7.45	104.13	4.58	75.25	6.86
Shenfu	115.63	7.84			83.63	9.68	106.25	6.80	111.25*	7.67	108.88*	7.20	97.25*†‡	6.52

*P<0.05 comparing to no treatment.
†P<0.05 comparing to mock.
‡P<0.05 comparing to saline.
CI, Cardiac Index; HR, heart rate; MAP, mean arterial pressure.

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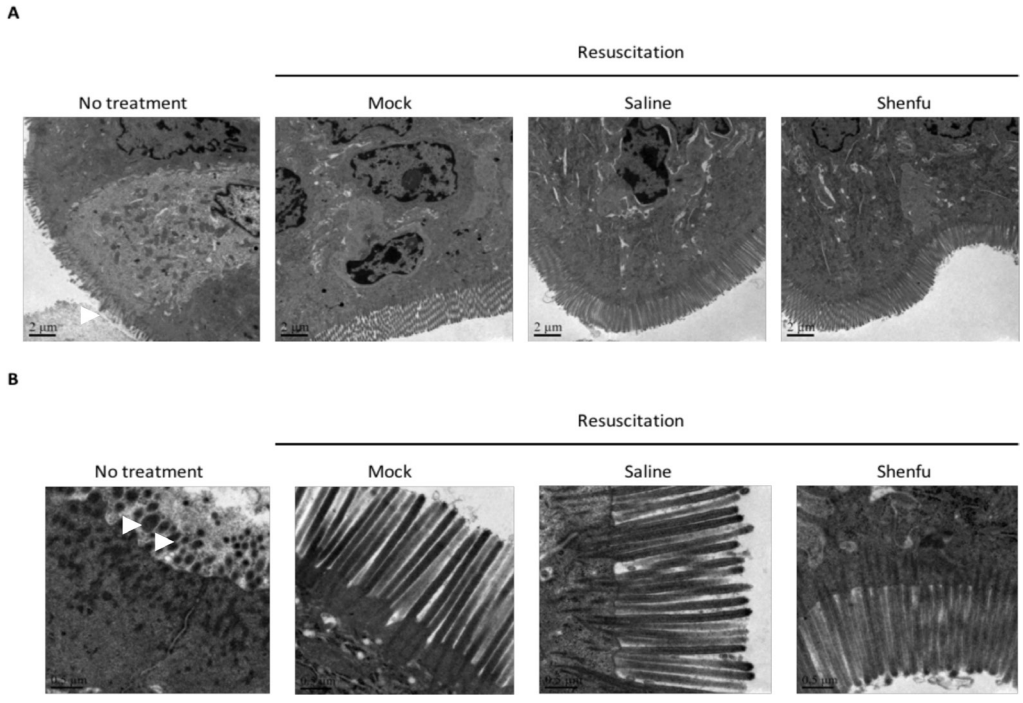

Figure 2 Transmission electron microscopy was employed to examine the tissue architecture of pig intestine: (A) Overall tissue architecture of intestinal epithelium and (B) structure of microvilli of the intestine. The excessive damaged intestinal microvilli in pigs with hemorrhagic shock receiving no treatment are shown by white arrowheads.

Effect of Shenfu on tissue architecture of the intestine

The architecture of intestine tissues of pigs receiving different treatments was examined using transmission electron microscopy (figure 2). Intestine was damaged by the onset of hemorrhagic shock as evidenced by the poor intestine architecture of pigs receiving no treatment (figure 2A). Resuscitation alone slightly improved tissue integrity; however, treatment with Shenfu resulted in the most significant improvement in architecture. In addition to the overall integrity of intestine, the detailed structures of intestinal villa were also investigated (figure 2B). Hemorrhagic shock led to complete destruction of the villa resuscitation alone, and resuscitation with saline slightly improved the villus structure. Substantial protection of villi was seen in pigs reperfused with Shenfu. These data clearly indicated the protective effect of Shenfu against the tissue damage on intestine resulting from hemorrhagic shock.

Effect of Shenfu on tissue expression of apoptosis mediators

The intestinal expression of mediators involved in apoptosis was examined using immunohistochemistry and immunoblotting (figure 3). Immunohistochemical staining of intestine sections showed that resuscitation with Shenfu elevated Bcl-2 expression in pig intestine, which was indicated by the accumulated intense brownish red signal in tissue sections (figure 3A). The increased Bcl-2 expression resulting from resuscitation with Shenfu was confirmed using immunoblotting (figure 3B). Compared with pigs receiving neither drug nor resuscitation, pigs treated with resuscitation of Shenfu overexpressed Bcl-2 in the intestine. Resuscitated pigs presented slightly higher levels of Bax comparing to pigs receiving no treatment; however, the elevations were not significant. For the level of cleaved caspase-3 (ie, 15 kDa fragment), the expression levels were elevated in pigs receiving no treatment and those treated with resuscitation alone. These data demonstrated the modulating effect of Shenfu on apoptotic mediator expression.

Effect of Shenfu on blood inflammatory cytokines

Whether resuscitation with Shenfu would affect the blood levels of certain inflammatory cytokines like TNF-α and IL-6 was examined using ELISAs (online supplemental figure 1). Pigs receiving no treatment and those having resuscitation alone and resuscitation with saline presented comparable levels of TNF-α in blood; however, notably, resuscitation with Shenfu resulted in a significant drop in blood TNF-α. For IL-6, the circulating levels were elevated in pigs receiving resuscitation, of which the levels among these animals were similar to each other. The modulating effect of Shenfu on inflammatory cytokines in hemorrhagic shock was therefore clearly demonstrated.

Effect of Shenfu on oxidative stress in intestine and heart

The potential suppressive effect of Shenfu on oxidative damage was assessed by looking at the differential expression of oxidative stress markers in the intestine and heart (online supplemental figure 2). Hemorrhagic shock led to elevation in multiple oxidative stress markers including MDA, MPO, and FABP2 (online supplemental figure 2A, upper panel). Resuscitation alone and with saline could suppress MDA and MPO. Further reduction in MDA and MPO resulted from resuscitation with Shenfu. For FABP2, only resuscitation with Shenfu could suppress FABP2 in the intestine.

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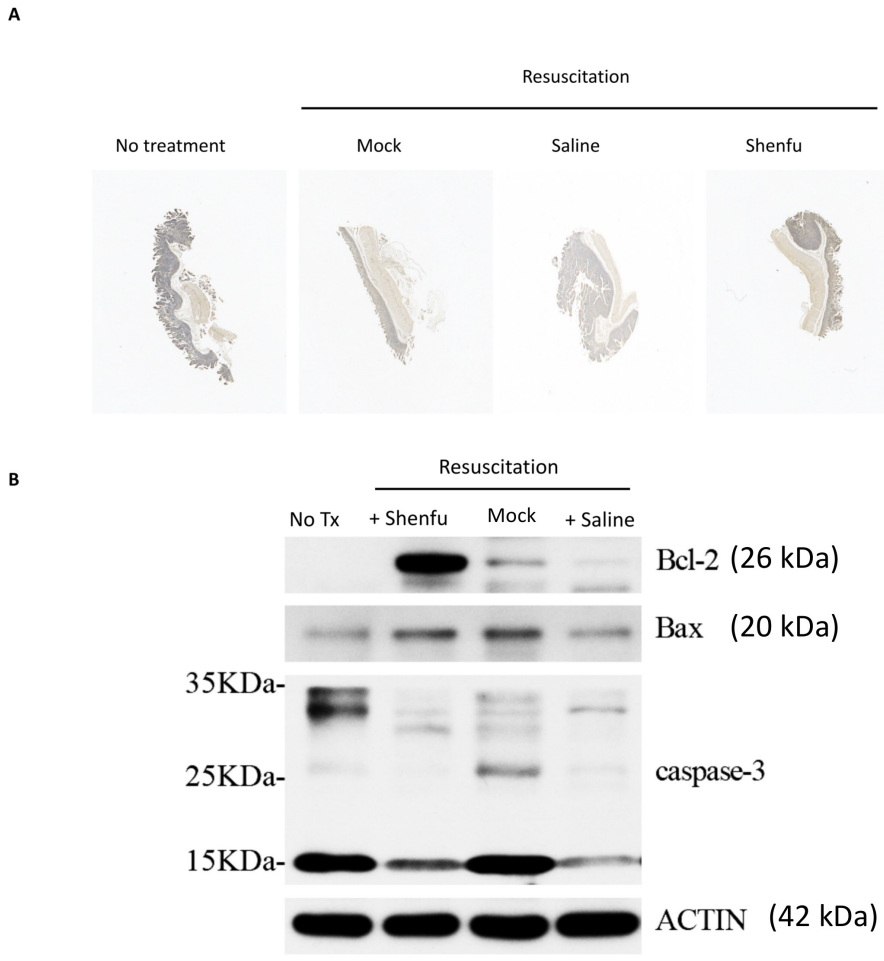

Figure 3 IHC and immunoblotting were used to determine the intestinal tissue expression of apoptotic mediators. (A) IHC indicated the elevation of Bcl-2 protein in the intestine of pigs treated with Shenfu. Expression of Bcl-2 was illustrated as the brownish red signal in tissue sections. (B) The promotion of Bcl-2 level by Shenfu was confirmed using immunoblotting. IHC, immunohistochemistry.

In addition to these markers, intestinal levels of SOD, Na^+/K^+-ATPase and Ca++ATPase were also examined (online supplemental figure 2A, lower panel). Resuscitation, either alone or with saline or Shenfu, could increase SOD, Na^+/K^+-ATPase and Ca++ATPase levels in the intestine.

The oxidative stress markers expressed in the heart were also examined (online supplemental figure 2B). Like in the intestine, MDA level in the heart was reduced by resuscitation, regardless of whether it was given alone or together with either saline or Shenfu. Again, resuscitation could increase Na^+/K^+-ATPase level in pigs with hemorrhagic shock. Surprisingly, the increase of ATPase by Shenfu was found less significant compared with resuscitation alone and resuscitation with saline.

DISCUSSION

The present study examined the protective effect of Shenfu in a porcine model of hemorrhagic shock. Results showed that blood transfusion combined with fluid resuscitation of Shenfu could protect pigs with hemorrhagic shock against mortality and could mitigate tissue damages of the intestine, which was evidenced by the findings that the Shenfu group presented a well-organized microvilli of gut epithelium. Resuscitation with Shenfu also showed modulating effects on oxidative stress, inflammatory cytokines, as well as apoptotic mediators. These findings would be essential to the management of patients with hemorrhagic shock, given gut injury in I/R is the most important determinant of clinical outcome in hemorrhagic shock.[4] The integrity of the gut epithelium is of paramount importance to the prognosis of patients with hemorrhagic shock. Damaged intestinal epithelium shows an increased permeability, allowing dissemination of luminal bacteria and toxins into the circulation, exacerbating the systemic inflammation and its sequel of multiple organ failure in hemorrhagic shock.[13 14]

Shenfu is a traditional Chinese medicine formula. Modern pharmacologial studies have identified ginsenoside and aconite total alkaloids as the major bioactive components. Ginsenoside has been shown to have a bidirectional regulation of blood pressure, while alkaloids have been demonstrated to have excitatory effects on both alpha and beta-adrenergic receptors.[15] The role of Shenfu in maintaining blood pressure within a normal range has also been examined in clinical trials.[8] Along with these published works, the present study indicated Shenfu could improve the hemodynamics of pigs with hemorrhagic shock, protecting them against mortality.

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The findings of the present work have suggested that the protective effect of Shenfu on gut epithelium would be at least partially attributed to the modulating effect of Shenfu on apoptotic mediators. Treatment with Shenfu significantly elevated Bcl-2 expression in intestinal tissues. Apoptosis is a programmed cell death process governed by the balance between proapoptotic and antiapoptotic proteins. Bcl-2 was reported to initiate an antiapoptosis response via an ERK1/2-mediated pathway.[16] Resuscitation with Shenfu could also preserve the structural integrity of mitochondria of intestinal epithelium (data not shown) and prevent the cleavage of caspase-3. The normal functionality of mitochondria and tight regulation on caspase-3 activation are essential to the survival of cells under stress like shock.[17] These findings collectively strengthened the antiapoptotic role of Shenfu in a porcine model of hemorrhagic shock.

Quantitative assays of this study have shown that Shenfu could suppress intestinal levels of MDA, MPO, and FABP2, and at the same time increased the levels of SOD, Na^+/K^+-ATPase and Ca++ATPase. MDA, MPO, and FABP2 are widely accepted markers for oxidative stress,[18–20] while SOD, Na^+/K^+-ATPase and Ca++ATPase are actually molecular targets of which the degradation would be accelerated by oxidative stress.[21] These data have collectively suggested Shenfu would efficiently ameliorate lipid peroxidation of plasma membrane, preserving the tissue integrity of the gut. The intact intestinal epithelium is essential to the prognosis of patients with hemorrhagic shock, given damaged intestines, of which the permeability is significantly increased, and would permit uncontrolled passage of luminal bacteria and toxins to the circulation, triggering systemic inflammation and hence organ failures. Indeed, in this study, the suppression on oxidative stress markers was accompanied with a significant reduction in proinflammatory cytokine TNF-α, suggesting the mitigation in peroxidation would result in the reduction in the magnitude of systemic inflammatory response. On the other hand, oxidative stress was reported to affect the normal function of gut by promoting degradation of certain protein targets, suggesting targeting proteasome activity with specific inhibitors could be a novel therapeutic approach for hemorrhagic shock.[22] It is of interest to explore whether the combined use of such proteasome inhibitors with Shenfu would further enhance the therapeutic efficacy. The molecular targets of Shenfu in the context of hemorrhagic shock have remained to be fully understood. The hypoxic adenosinergic mechanism is central to the pathophysiology of I/R injury of the gut. Whether treatment with Shenfu would modulate the hypoxic adenosinergic mechanism has to be studied. In addition, the effect of Shenfu on I/R injury has remained to be fully characterized. This study provided little insights into the safety as well as the potential adverse effects associated with the use of Shenfu. Further investigations are warranted.

CONCLUSION

In conclusion, Shenfu could demonstrate a significant protective effect on the hemodynamics and gut epithelium of pigs with hemorrhagic shock, of which the underlying mechanism would be attributed to its suppression on apoptosis and oxidative damages on organs.

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Competing interests None declared.

Patient consent for publication Not required.

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Data availability statement Data are available upon reasonable request.

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ORCID iD

Chunsheng Li http://orcid.org/0000-0002-9509-6249

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