

## Original Article

# Dai-Huang-Fu-Zi-Tang ameliorate the extent of acute lung injury by regulating AQP5 and CC16 after hemorrhagic shock resuscitation in rats

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**Abstract:** Hemorrhagic shock (HS) resuscitation often leads to systemic inflammatory response syndrome and acute lung injury (ALI) in clinic, which endanger the life of patients. Dai-Huang-Fu-Zi-Tang (DHFZT) is a famous traditional Chinese prescription for many diseases; we carried out this study to investigate whether DHFZT could relieve ALI after HS resuscitation and try to find its underlying mechanism. Forty-eight Sprague-Dawley (SD) rats were randomly divided into three groups: the sham group, the control group and the DHFZT group. Each group was further divided into two subgroups by the time points of 12 h and 24 h after hemorrhagic shock resuscitation. The control/DHFZT group was given 2 ml normal saline/DHFZT enema at 0 h and 12 h after resuscitation. The histological observation showed that in the control group large amounts of inflammatory cells were seen in the alveoli and pulmonary interstitium, with severe pulmonary capillary congestion and interstitial edema. But in the DHFZT group, there was a small amount of inflammatory cell infiltration, mild pulmonary capillary congestion and interstitial edema. In addition, compared to the sham group, the pulmonary W/D ratio and serum endotoxin level in the control group were significantly elevated; the expression level of AQP5 and CC16 in lung tissue was significantly decreased both at 12 h and 24 h after resuscitation, but these results were obviously reversed in the DHFZT group. These results suggest that DHFZT can ameliorate the extent of ALI after HS resuscitation by up-regulating AQP5 and CC16 in lung tissue.

**Keywords:** Hemorrhagic shock, Dai-Huang-Fu-Zi-Tang, acute lung injury, AQP5, CC16

## Introduction

Hemorrhagic shock is commonly seen in acute bleeding caused by trauma and gastrointestinal ulcer, which can cause decrease of effective circulating blood volume and inadequate perfusion of tissues, and has a high mortality rate without active treatment [1]. When HS occurs, the body redistributes the systemic circulation to ensure the blood supply of vital organs such as heart and brain, this together with the "retaliatory contraction" of the intestinal vessels after resuscitation can exacerbate the intestinal ischemia, leading to bacterial translocation and endotoxemia [2-5]. The "waterfall pattern" cascade reaction of cytokine after HS resuscitation is mostly triggered by monocyte-macrophage system stimulated

by gut bacteria/endotoxin translocation [6, 7]. The uncontrolled inflammation gradually involves the whole body presenting as systemic inflammatory response syndrome (SIRS). Lungs bear the brunt during a series of injuries caused by SIRS, and ALI can occur at an early stage and rapidly develop into acute respiratory distress syndrome (ARDS) without effective intervention [8, 9]. ALI is characterized by pulmonary capillary endothelial cell damage presented as increased extra-pulmonary capillary fluid volume and migration of neutrophils to lungs at an early stage, which is closely related to the development of pulmonary edema [10, 11].

Aquaporins (AQPs) are a class of membrane water channels containing six longer transmembrane and two shorter non-transmembrane

**Table 1.** Herbal compositions of DHFZT

Scientific name	Herbal name	Quantity (dry, g)
Rheum palmatum Linn	Radix et Rhizoma Rhei (DH)	9.0
Aconitum carmichaeli	Debeaux Radix Aconiti Lateralis Praeparata (FZ)	9.0
Asarum heterotropoides F. Schmidt var. mandshuricum	Radix et Rhizoma Asari (XX)	3.0
Total		21.0

DHFZT = Dai-Huang-Fu-Zi-Tang. DH = Dai Huang. FZ = Fu Zi. XX = Xi Xin.

helical segments, their primary function is to facilitate the passive transport of water in various eukaryotes and prokaryotes, and they are widely distributed in organs of mammals [12-14]. Presently, there are thirteen kinds of AQPs, and at least eight of them have been shown to transport water in humans and rodents. AQP5 is a protein with a molecular weight of 27 kD, which is highly expressed in type I alveolar epithelial cells [15]. AQP5 can maintain the water balance between the blood vessels and the pulmonary interstitium by specifically transporting water molecules and clearing the water in alveolar space, which can produce a “barrier effect” on the alveolar epithelium [16, 17]. Clara cells are some short columnar and non ciliated cells, which are the major cells in the epithelium of the bronchioles. Clara cell secretory protein 16 (CC16) is the predominant product from Clara cells [18], it has the biological characteristics such as anti-inflammation, immunomodulating and antioxidant [19, 20].

DHFZT is a famous traditional Chinese prescription which has been widely used to treat various inflammatory diseases, and our preliminary study demonstrated that DHFZT could alleviate rat lung injury with severe acute pancreatitis [21]. In this study, we wanted to further investigate whether DHFZT could alleviate ALI after HS resuscitation in rats, and try to explain its underlying mechanism by detecting the expression of AQP5 and CC16.

## Materials and methods

### Animals

A total of 48 SD male rats, weighing 250-300 g, were purchased from the Experimental Animal Center of Dalian Medical University. Cages were individually ventilated at  $20 \pm 2^\circ\text{C}$  and 45-65% relative humidity with a photoperiod of 12 h of light and 12 h of dark. All rats were adaptively fed for 1 week before the experi-

ment. All procedures were conducted in conformity with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Ethics Committee of affiliated Zhongshan Hospital of Dalian University. Anesthetic drugs and all other necessary measures were used to reduce animal suffering during experimental procedures.

### Preparation and quality control of DHFZT

DHFZT is composed of 3 species of herbal plants, each dried crude drug of which were purchased from Tong Ren Tang Group Co., Ltd. (Beijing, China). The formula of DHFZT is described in **Table 1**, and voucher specimen of *Rheum palmatum* Linn (No.00000022), *Aconitum carmichaeli* Debeaux (No. 018-14237), and *Asarum heterotropoides* F.S Schmidt var. *mandshuricum* (No. 00916696) are kept in Institute of Botany, the Chinese Academy of Sciences. To keep the consistency of the herbal chemical ingredients, all of the herbal components were originally obtained from the standard native sources as stated above with GAP grade and the drugs were extracted with standard methods according to Chinese Pharmacopoeia III (edition 2010). According to the original prescription from the “Jin Kui Yao Lue”, DH, FZ and XX were mixed in the ration of 3:3:1 (w/w). First, FZ were soaked in water (1:25) for 30 min, followed by extraction in boiling water (100°C) for 1 h. Then DH was added and boiled for 10 min. Finally, XX was added and boiled for 5 min. The DHFZT was concentrated by rotary evaporator (Heidolph Instruments, Germany) and lyophilized to obtain dry extract through freeze-drying system (Labconco, United States) at  $-80^\circ\text{C}$ , yielding final 3.72 g (extraction ratio 17.71%), and stored at  $4^\circ\text{C}$  for use. The lyophilized DHFZT extract was dissolved in an appropriate volume of distilled water prior to administering to rats.

## DHFZT ameliorate ALI by regulating AQP5 and CC16

**Table 2.** Pulmonary W/D ratio of each group

Groups	12 h (n = 8)	24 h (n = 8)
Sham	4.44 ± 0.16	4.50 ± 0.24
Control	5.31 ± 0.33**	6.09 ± 0.26**
DHFZT	4.61 ± 0.26*	5.24 ± 0.22*

Values are mean ± SD. \*\* $P < 0.01$ , control group versus sham group at 12 h and 24 h. \* $P < 0.05$ , DHFZT group versus control group at 12 h and 24 h. DHFZT = Dai-Huang-Fu-Zi-Tang. W/D = wet/dry.

### Experimental process

After 1 week of acclimation, a total of 48 rats were randomly divided into three groups ( $n = 16$ ): the sham group (catheterization only), the control group (HS + resuscitation + normal saline enema) and the DHFZT group (HS + resuscitation + DHFZT enema). Each group was further divided into two subgroups according to 12 h and 24 h after hemorrhagic shock resuscitation ( $n = 8$ ). The rats were fasted for 8 h, with water deprivation for 4 h before operation. Animals were anesthetized with pentobarbital sodium (40 mg/kg). With aseptic technique, a poly-ethylene (PE50) catheter was inserted into the right carotid artery for continuous mean arterial pressure (MAP) monitoring with a multi-functional physiological recorder (BIOPAC, USA) and two other catheters were respectively inserted into the left femoral artery for blood withdrawal and in the right femoral vein for fluid infusion. Heparin saline (800 IU/kg) was injected slowly into the right femoral vein to complete systemic heparin. The hemorrhagic shock model was made according to the Wiggers' modified method [22]. The MAP was gradually reduced to 40 mmHg by withdrawing blood (1-1.2 ml/min) and then was maintained around 40 mmHg for 1 h before resuscitation was initiated. Resuscitation was done by transfusing the autologous blood and equal volume of Ringer's solution. MAP stable over 100 mmHg was regarded as a successful recovery. The control/DHFZT group was given 2 ml normal saline/DHFZT enema 0 and 12 hours after resuscitation.

Twelve and 24 hours after resuscitation, blood samples were drawn from the abdominal aorta for measuring serum endotoxin and CC16, then the left lung was harvested to determine W/D weight ratio, the right lung was harvested to detect the expression of AQP5 and CC16 with

immunohistochemistry and ELISA, and also for the histological observation by HE staining.

### Measurement of serum endotoxin, CC16 and AQP5

Blood samples were centrifuged at 15,000 rpm under 4°C and then stored at -80°C. The serum concentration of endotoxin was measured by EKT-5 M set dynamic Gram-negative bacteria test kit (Jin Shanchuan technology development Co., Ltd., Beijing, China) through kinetic turbidimetric assay. The serum CC16 level was detected using a commercial ELISA kit (Wes-tang Bio-tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The lung homogenates were prepared to detect AQP5 level by an ELISA kit (Fusheng Industrial Co., Ltd., Shanghai, China).

### Pulmonary W/D ratio

The inferior lobe of left lung was excised and measured immediately to obtain the "wet weight value", and then dried in an oven maintaining the temperature at 50°C for 72 hours and measured again to obtain the "dry weight value". Subsequently, the W/D ratio was calculated.

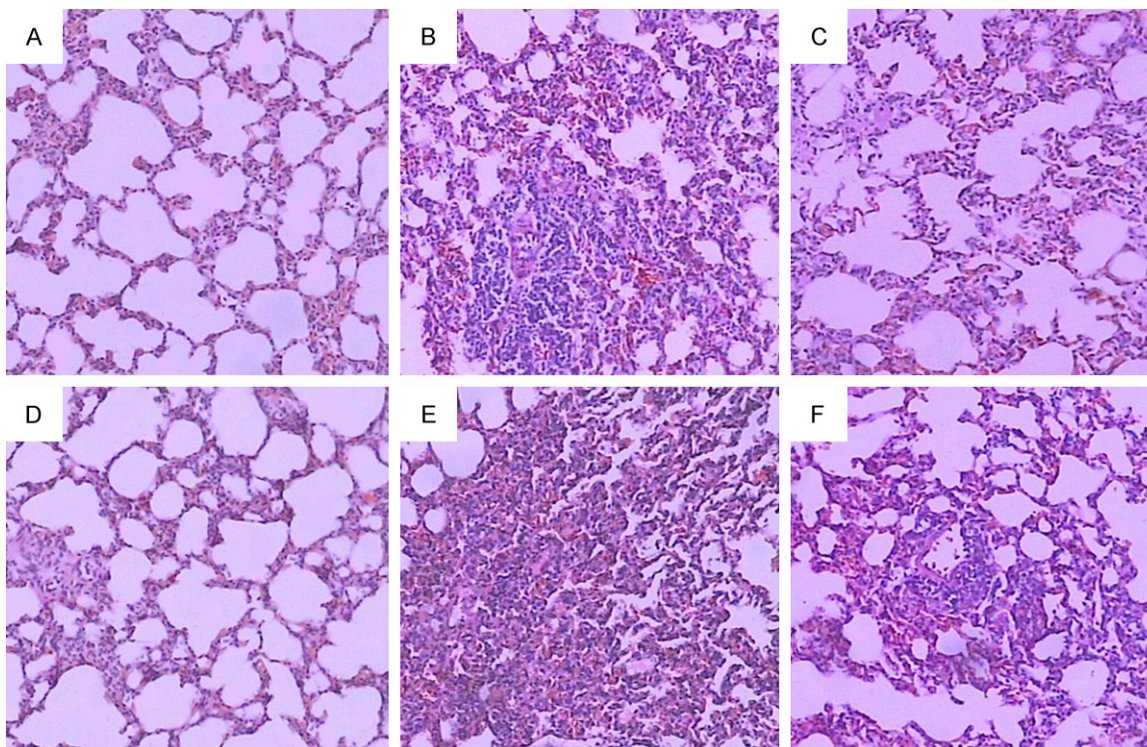
### Immunohistochemical and histological detection

Immunohistochemical (IHC) staining was performed using the DAB chromogenic detection kit (Bio-high TechnologyDeve Co., Ltd., Hebei, China) according to the manufacturer's instructions. The following antibodies were used: rat anti-AQP5 polyclonal antibody (Fusheng Industrial Co., Ltd., Shanghai, China), rat anti-CC16 polyclonal antibody (Fusheng Industrial Co., Ltd., Shanghai, China). Immunohistochemical score was measured according to the immunoreactive score developed by Remmele and Stagner [23]. Paraffin sections of the lung tissue were routinely stained with HE for histological observation. After IHC and HE staining, images were captured by a microscope (Olympus Corp, Japan) and recorded under the identical optical condition using ISCapture imaging software.

### Statistical analysis

Data are presented as the mean ± SD. All statistical analyses were performed using the





**Figure 1.** Histological observation of lung tissue with HE staining. HE staining was performed in lung tissue in sham group (A, D), control group (B, E), and DHFZT group (C, F) at 12 h (A-C) and 24 h (D-F) after hemorrhagic shock resuscitation, respectively. DHFZT = Dai-Huang-Fu-Zi-Tang; HE = hematoxylin-eosin.

**Table 3.** Serum endotoxin level in each group

Groups	12 h (pg/ml, n = 8)	24 h (pg/ml, n = 8)
Sham	12.37 ± 1.44	13.01 ± 1.32
Control	17.62 ± 1.15**	18.04 ± 1.77**
DHFZT	15.23 ± 1.14*	15.83 ± 1.50*

Values are mean ± SD. \*\* $P < 0.01$ , control group versus sham group at 12 h and 24 h. \* $P < 0.05$ , DHFZT group versus control group at 12 h and 24 h. DHFZT = Dai-Huang-Fu-Zi-Tang.

SPSS (Version 17.0). All data were evaluated for statistical significance with one-way analysis of variance (ANOVA) by least significant difference (LSD) test.  $P < 0.05$  was considered statistically significant.

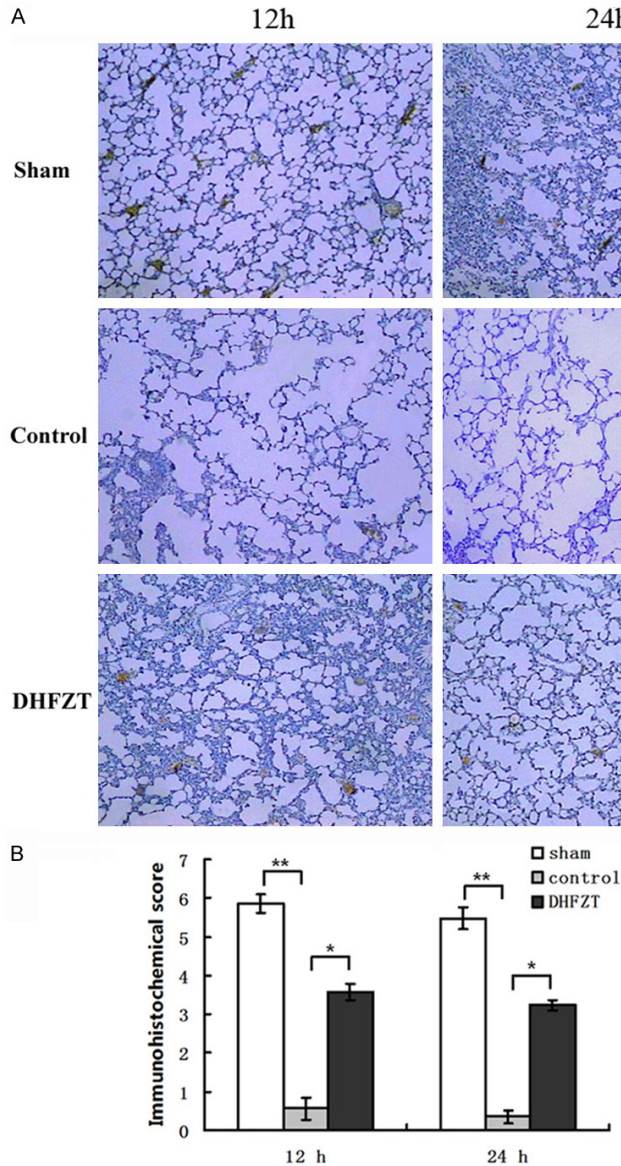
## Results

### *DHFZT reduced the degree of ALI after HS resuscitation*

We carried out pulmonary W/D ratio measurement and histological observation to test the occurrence of acute lung injury after HS resus-

citation. The pulmonary W/D ratio of the control group was significantly higher compared with that of the sham group at both 12 h and 24 h after resuscitation. But compared to the control group, the pulmonary W/D ratio of the DHFZT group was markedly lowered at the corresponding two time points. The results were showed in **Table 2**. And the histological observation with HE staining showed that the alveolar structure was clear and complete, the alveolar septum was normal, and there was no inflammatory infiltration in the sham group (**Figure 1A, 1D**). But in the control group large amounts of inflammatory cells were seen in the alveoli and pulmonary interstitium, and also with severe pulmonary interstitial edema, widening of alveolar septum and pulmonary capillary congestion (**Figure 1B, 1E**). In the DHFZT group, there was a small amount of inflammatory cell infiltration and a slight increase in alveolar septa, as well as mild pulmonary capillary congestion and interstitial edema (**Figure 1C, 1F**). These results showed ALI occurred after hemorrhagic shock resuscitation, and DHFZT could alleviate the extent of ALI.

## DHFZT ameliorate ALI by regulating AQP5 and CC16



**Figure 2.** The expression of CC16 in lung tissue after hemorrhagic shock resuscitation. A. CC16 IHC staining was performed in lung tissue in sham group, control group and DHFZT group at 12 h and 24 h after hemorrhagic shock resuscitation, respectively. B. Immunohistochemical score for CC16 in each group at 12 h and 24 h after hemorrhagic shock resuscitation, respectively. \*\* $P < 0.01$ , \* $P < 0.05$ ,  $n = 8$  per group. DHFZT = Dai-Huang-Fu-Zi-Tang; CC16 = Clara cell secretory protein 16; IHC = immunohistochemical.

**Table 4.** Serum CC16 content in each group

Groups	12 h (ng/l, $n = 8$ )	24 h (ng/l, $n = 8$ )
Sham	21.29 ± 1.48	22.20 ± 1.68
Control	38.95 ± 1.33**	45.68 ± 1.09**
DHFZT	26.21 ± 1.71*	30.22 ± 1.69*

Values are mean ± SD. \*\* $P < 0.01$ , control group versus sham group at 12 h and 24 h. \* $P < 0.05$ , DHFZT group versus control group at 12 h and 24 h. DHFZT = Dai-Huang-Fu-Zi-Tang. CC16 = Clara cell secretory protein 16.

*DHFZT reduced the serum endotoxin level after HS resuscitation*

The serum endotoxin level in the control group was significantly higher than that in the sham group at both 12 h and 24 h after resuscitation. Nevertheless, the serum endotoxin level in DHFZT group was significantly lowered compared with that in the control group. The results were showed in **Table 3**.

*CC16 content increased in lung tissue but decreased in serum under the intervention of DHFZT after HS resuscitation*

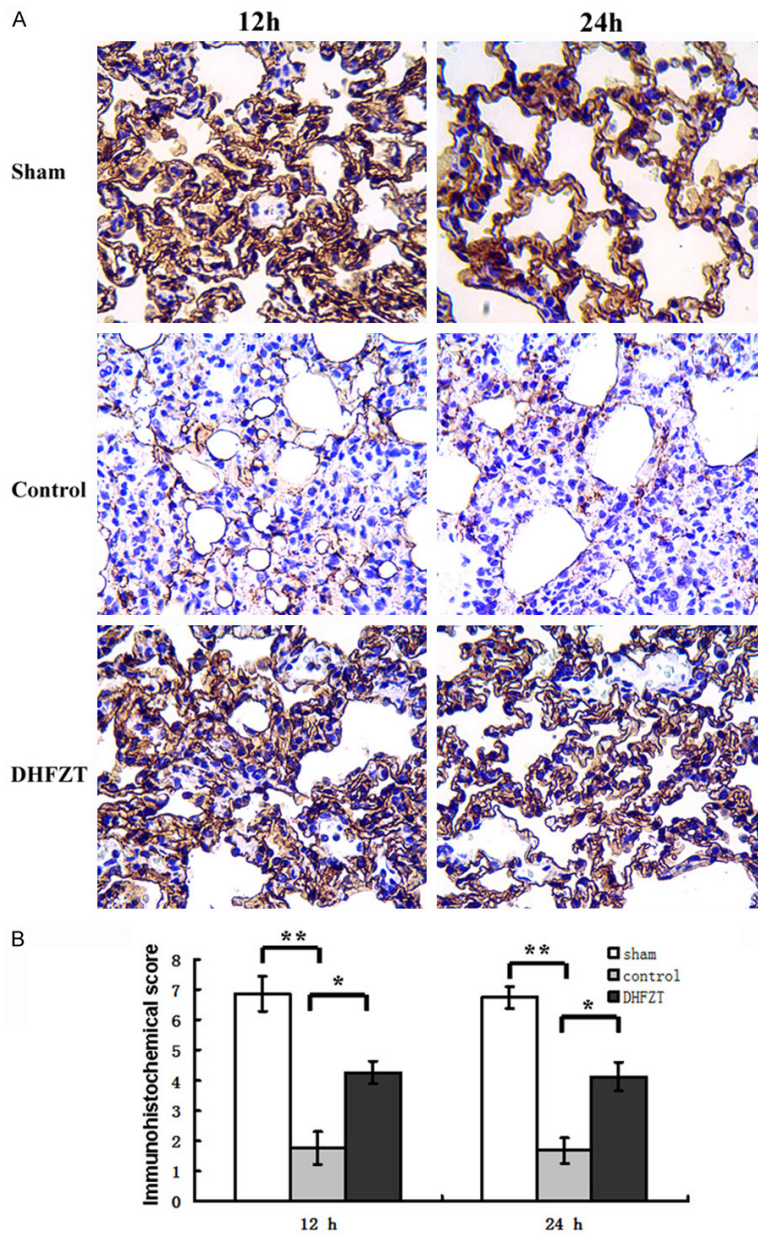
Immunohistochemical score of CC16 in the control group was significantly lower than that in the sham group both at 12 h and 24 h (**Figure 2A, 2B**). Besides, compared to the sham group, the result of ELISA test showed CC16 content in serum was significantly increased in the control group (**Table 4**). Instead, compared to the control group, the immunohistochemical score of the DHFZT group was obviously increased (**Figure 2A, 2B**), and the CC16 content in serum was significantly decreased both at 12 h and 24 h (**Table 4**).

*AQP5 content increased in lung tissue under the intervention of DHFZT after HS*

*resuscitation*

Immunohistochemical score of AQP5 in the control group was significantly lower than that in the sham group both at 12 h and 24 h (**Figure 3A, 3B**). Further, the result of ELISA test showed lung tissue AQP5 content in the control group was also significantly decreased compared to the sham group (**Table 5**). But quite the contrary, compared to the control group, the immu-





**Figure 3.** The expression of AQP5 in lung tissue after hemorrhagic shock resuscitation. A. AQP5 IHC staining was performed in lung tissue in sham group, control group and DHFZT group at 12 h and 24 h after hemorrhagic shock resuscitation, respectively. B. Immunohistochemical score for AQP5 in each group at 12 h and 24 h after hemorrhagic shock resuscitation, respectively. \*\* $P < 0.01$ , \* $P < 0.05$ ,  $n = 8$  per group. DHFZT = Dai-Huang-Fu-Zi-Tang; AQP5 = Aquaporin 5; IHC = immunohistochemical.

nohistochemical score and lung tissue AQP5 content were significantly increased in the DHFZT group both at 12 h and 24 h (Table 5; Figure 3A, 3B).

**Discussion**

It is generally believed that the polymorphonuclear leukocytes, inflammatory cytokines and

chemokines, as well as lung tissue ischemia/reperfusion damage, intestinal barrier dysfunction and bacterial endotoxin translocation have played very important roles in the pathological process of acute lung injury after hemorrhagic shock resuscitation. Some researchers have tried to explain the underlying mechanism at molecular level, such as factor-kappa B and toll-like receptor 4 [24-26]. But so far, the mechanism of the pathogenesis of ALI after HSR has not been completely elucidated yet. The pathological characteristics of ALI include fluid accumulation in the alveolar and interstitial lung, due to imbalance of lung tissue fluid formation and reflux. AQP5 can maintain the water balance between the blood vessels and the pulmonary interstitium, and CC16 has the biological characteristics such as anti-inflammation, immunomodulating and antioxidant, so we choose these two proteins to try to find the potential mechanism of ALI after HSR.

Our research showed that the serum endotoxin content in the control group was increased obviously after hemorrhagic shock resuscitation in rats. However, the serum endotoxin content in the DHFZT group was decreased significantly compared to the control group, which indicated that reducing the serum endotoxin level was an important way for DHFZT to alleviate ALI after hemorrhagic shock resuscitation. Our preliminary studies have found DHFZT could obviously increase the intestinal blood flow and improve the intestinal immune barrier function in rats after HS resuscitation [27], which is accordance with this study. This study also showed that the decrease of lung tissue AQP5 and increase of pulmonary W/D ratio were accompanied by the aggravated pulmonary interstitial edema after HS resuscitation.

**Table 5.** Lung tissue AQP5 content in each group

Groups	12 h (ng/g, n = 8)	24 h (ng/g, n = 8)
Sham	15.81 ± 0.62	15.65 ± 0.68
Control	9.06 ± 0.46**	8.68 ± 0.59**
DHFZT	13.05 ± 0.21*	12.63 ± 0.15*

Values are mean ± SD. \*\* $P < 0.01$ , control group versus sham group at 12 h and 24 h. \* $P < 0.05$ , DHFZT group versus control group at 12 h and 24 h. DHFZT = Dai-Huang-Fu-Zi-Tang. AQP5 = Aquaporin 5.

tation. But the pulmonary W/D ratio was decreased and the AQP5 content was increased significantly under the intervention of DHFZT at the both time points, and the pulmonary edema was alleviated at the same time. So we thought the pulmonary edema after HS resuscitation was related to the down-regulation of AQP5 expression in lung tissue, and DHFZT could alleviate pulmonary edema by up-regulating AQP5 indirectly. Meanwhile, we also found the CC16 content was decreased in the lung tissue, but instead it was increased in the serum after HS resuscitation. We thought the reason for this was that HS resuscitation could cause damage to alveolar membrane and capillary barrier, so CC16 entered the peripheral blood from the epithelial lining fluid due to large concentration difference [28], resulting in elevated serum CC16 level over time. Furthermore, the role of CC16 in inhibiting the inflammatory response was weakened, and more proinflammatory cytokines were accumulated in the lung tissue [29, 30]. Then the alveolar capillary membrane was further damaged and the capillary permeability was more increased, leading to serious alveolar and interstitial pulmonary edema, and so acute lung injury occurred eventually [31, 32]. DHFZT could inhibit this pathological process and reduce pulmonary edema, with a protective effect on lung tissue after HS resuscitation.

In summary, ALI occurred after HS resuscitation following increased serum endotoxin level and decreased expression of AQP5 and CC16 in lung tissue. DHFZT could reduce the level of serum endotoxin, relieve the degree of acute pulmonary edema by increasing the expression of AQP5 and CC16 in lung tissue. However at present, the mechanism of DHFZT has not been well illustrated, and there is little research

on its effect on hemorrhagic shock. The present study is limited to animal experiment, further clinical and experimental studies are still needed to understand the mechanism of DHFZT and provide a new approach for the treatment of ALI after hemorrhagic shock resuscitation.

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### Disclosure of conflict of interest

None.

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