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Ziqi Dihuang decoction ameliorates thrombosis in septic rats by inhitbiting plasminogen activator inhibitor-1

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ABSTRACT

Introduction: Sepsis is now a global medical burden with high morbility and mortality. The focus of this study was to evaluate the effects of Ziqi Dihuang (ZQDH) decoction on inflammatory and thrombosis-related parameters in septic rats.
Mothods: A rat model of sepsis was established by cecal ligation and puncture (CLP). Male Sprague-Dawley rats were randomly divided into Sham group, CLP group, ZQDH-1ow group (0.735 g/kg) and ZQDH-high group (1.47 g/kg). Rats in ZQDH groups were given ZQDH decoction by gavage for 7 days before CLP. White blood cells (WBC), inflammatory cell infiltration of liver, kidney and lung, as well as serum levels of tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and reactive oxygen species (ROS) were used to assess systemic inflammatory response. Coagulation and fibrinolytic indexes included platelet count, coagulation function, fibrin deposition, and levels of tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) in serum, liver, kidney and lung.
Results: LPS rats showed significant changes in inflammatory and thrombosis-related parameters such as increased WBC and inflammatory factors, decreased platelet counts, and increased tPA and PAI-1 concentrations in serum and organs. ZQDH decoction pretreatment can significantly inhibit the infiltration of inflammatory cells in the lung, and inhibit the production of TNF-α, IL-6 and ROS in a dose-dependent manner. ZQDH decoction also ameliorated thrombocytopenia, renal fibrin deposition, and tPA and PAI-1 levels in serum and organs.
Conclusion: These results suggest that ZQDH decoction can dose-dependently relieve systemic inflammatory injury and regulate fibrinolysis system in septic rats, which may be mediated by PAI-1.

1. Introduction

Sepsis is an acute syndrome triggered by infection that can lead to life-threatening organ dysfunction.[1] With an annual incidence above 30 million cases and an acute mortality rate about 26.0%, sepsis has become a global burden, and researchers have been struggling to develop new treatments for it.[2] Sepsis is often accompanied by coagulation and fibrinolytic dysfunction, which put patients at risk for both blood clots and bleeding, manifested as thrombocytopenia, increased fibrinogen degradation products and microvascular thrombosis, which can lead to disseminated intravascular coagulation (DIC). Plasminogen activator inhibitor −1 (PAI-1) is an inhibitor of fibrinolysis, and an important element of the imbalance between clot formation and fibrinolysis.[3] As a risk factor for thrombosis, it is also known to be a considerable predictor of severity and mortality in sepsis.[4,5]

Sepsis-induced DIC is characterized by inhibition of fibrinolysis and is prone to develop into multiple organ dysfunctions. At present, there is no definite treatment for it.[6] In traditional Chinese medicine, sepsis belongs to the category of febrile diseases. The theory of blood stasis and heat stasis proposed by a famous Chinese medicine scholar Zhou Zhongying provides a new idea for the treatment of sepsis-related coagulopathy with the method of cooling blood and dispersing blood stasis.[7] The Ziqi Dihuang (ZQDH) formula is just based on this theory. It contains five herbs,

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List of abbreviations

APTT	Activated partial thromboplastin time
CLP	Cecal ligation and puncture
DIC	Disseminated intravascular coagulation
ELISA	Enzyme-linked immunosorbent assays
FIB	Fibrinogen
HE	Hematoxylin-eosin
IL-6	Interleukin-6
MSB	Martius-Scarlet-Blue
MPV	Mean platelet volume
N %	Percentage of neutrophils
PAI-1	Plasminogen activator inhibitor-1
PLT	Platelet count
PT	Prothrombin time
ROS	Reactive oxygen species
tPA	Tissue plasminogen activator
TNF-α	Tumor necrosis factor
uPA	Urokinase type plasminogen activator
WBC	White blood cell count
ZQDH	Ziqi Dihuang

among which Rehmanniae and Rhubarb are sovereign drugs to clear the heat of blood division and restore the Yin of injury, Lithospermum and Radix Paeoniae Rubra are minister drugs for promoting blood circulation and dissipating blood stasis, and Panax notoginseng is warm in nature as an assistant drug. These five herbs play the role of cooling blood, dispersing blood stasis, clearing heat and restoring Yin together. We intend to investigate the effect of ZQDH decoction on sepsis-induced coagulopathy and thrombosis in a rat model of sepsis induced by cecal ligation and puncture (CLP).

2. Materials and methods

2.1. Drugs and chemicals

ZQDH decoction consists of five herb granules, including Rehmanniae (Di Huang, 30g), Rhubarb (Da Huang, 10g), Lithospermum (Zi Cao, 10g), Radix Paeoniae Rubra (Chi Shao, 10g), and Panax notoginseng (San Qi, 10g). All the herb granules were purchased from Tianjiang Pharmaceutical Co Ltd, Taizhou, China. Enzyme-linked immunosorbent assays (ELISA) kits for tPA, PAI-1, tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and reactive oxygen species (ROS) were obtained from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China).

2.2. Animals and experimental protocol

Sprague-Dawley rats, weighing 200 ± 20 g and 8–10 weeks old, were provided by the Animal Laboratory of Jiangsu Province Hospital of Chinese Medicine, with animal study approval number of SCXK 2019-0010. Rats were housed in cages with a temperature of 20 ±1 °C and relative humidity of 45%, and had free access to tap water and standard laboratory feed. Rats were randomly divided into four groups: Sham group (n = 6), CLP group (n = 6), ZQDH-low (7.35 g/kg, n = 6) and ZQDH-high group (14.7 g/kg, n = 6). The normal dose for rat (7.35 g/kg.d) was calculated based on the normal dose for adult human (70 g/d), and the body surface area ratio between adult human and rat. In this study, the normal dose was referred to as “low dose” and twice the normal dose was referred to as “high dose”. ZQDH herb granules were dissolved in distilled water to a final concentration of 0.735 g/ml or 1.47 g/ml, and were administered to rats by oral gavage for 7 days before CLP operation. Rats in Sham and CLP groups were gavaged with the same volume of normal saline. All procedures were implemented in accordance with the Animal Management Regulations of the Ministry of Health of China.

Septic rat model was established by cecal ligation and puncture (CLP) surgery.[8] Rats were fasted and given free access to water for 12 h prior to CLP surgery. After anesthetized with 1% pentobarbital sodium (40 mg/kg, ip), rats were subjected to a midline incision of ~2 cm on the anterior abdomen. The cecum was carefully separated, exteriorized, and ligated with 3-0 suture at half of the cecum. The ligated cecum was punctured using a 12G needle to create two holes and was gently squeezed to discharge a small amount of fecal content. Thereafter, put the cecum back into the abdominal cavity, suture and close the abdomen. Rats in the Sham group were subjected to identical abdominal incision and intestinal manipulation, but the cecum was neither ligated nor punctured. All surgical procedures were completed within 15 min. All rats were subcutaneously injected with normal saline (0.04 ml/g) for fluid resuscitation and were allowed free access to food and water after surgery. All rats were sacrificed 12 h after operation. Blood samples were collected by heart puncture, and tissue specimens (liver, kidney and lung) were preserved for subsequent experiments.

Another 32 rats (8 in each group) were selected to observe the 7d survival rate.

2.3. Histopathological testing

Tissue specimens (liver, kidney and lung) were fixed with 4% paraformaldehyde at 4 °C for >24 h, embedded in paraffin and serially sectioned (5 μm). Hematoxylin-eosin (HE) and Martius-Scarlet-Blue (MSB) were used to quantify the inflammatory cell infiltration and fibrin deposition, respectively. For each assessment, 40 × high power field images of affected hepatic lobules, glomeruli, or alveoli were captured and 10 images from each group were randomly selected. Image J software was used for image process and analysis. The number of red pixels corresponding to fibrin in MSB image was automatically calculated using the threshold color plug-in and represented as a histogram.

2.4. Blood cell test and coagulation function

White blood cell count (WBC), percentage of neutrophils (N %), platelet count (PLT) and mean platelet volume (MPV) were tested using an automatic haematology analyzer (SysmexXS-800i, Japan). Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (FIB) were detected with an automatic coagulation analyzer (STAGOSTA-R MAX, France).

2.5. ELISA tests of tPA, PAI-1, TNF-α, IL-6 and ROS

ELISA kits were used to detect the levels of tPA and PAI-1 in serum and organs (liver, kidney and lung), and the levels of TNF-α, IL-6 and ROS in serum. Serum samples were obtained by centrifugation of blood samples at 3000 g for 15 min at 4 °C. Tissue supernatant samples were obtained by centrifuging the tissue homogenate at 5000 g for 15 min at 4 °C. All procedures were carried out according to the manufacturer's protocol.

2.6. Statistical analysis

SPSS software (version 26.0, IBM Corp) was used for data analysis. All continuous data were presented as the mean ± standard deviation and analyzed using one-way ANOVA. Pairwise

comparisons were performed with LSD post-hoc test when equal variances assumed, or Tamhane T2 post-hoc test when equal variances not assumed. Survival analysis was tested by Kaplan-Meierand Log-Rank method. Pearson correlation coefficient was used to calculate the correlation between PAI-1 and each inflammatory factor. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Survival rate

The 7d survival rate was 100% in the sham group, 25.0% in the CLP group, 37.5% in the ZQDH-low group, and 50.0% in the ZQDH-high group (Fig. 1). There was no significant difference among CLP group, ZQDH-low group, and ZQDH-high group.

3.2. HE staining of liver, kidney and lung

As shown in Fig. 2, there was no significant difference in the infiltration of inflammatory cells in liver and kidney among the four groups of rats. In the liver, CLP only caused swelling and necrosis of some hepatocytes and infiltration of a few inflammatory cells. In the kidney, CLP induced increase of glomerular interstitial, capillary congestion, interstitial edema and some inflammatory cell infiltration. In the lung, however, the alveolar wall was notably thickened and a large number of inflammatory cells infiltrated into the interstitium after CLP. The infiltration of inflammatory cells in the lung of the two ZQDH groups was significantly alleviated, especially in the high dose group.

3.3. MSB staining of liver, kidney and lung

As shown in Fig. 3, there was no significant difference in liver MSB staining among four groups of rats. The fibrin deposition volume in kidney and lung of CLP group were considerably larger than that of Sham group. With ZQDH pretreatment, the fibrin deposition in glomeruli was significantly reduced, and it was further attenuated in ZQDH-high group than ZQDH-low group. But this finding was not seen in the lung.

3.4. Blood cell test

No significant difference was seen in N% and MPV among four groups of rats (Fig. 4B and D). WBC increased significantly and PLT decreased significantly after CLP procedure. ZQDH decoction pretreatment could effectively inhibit the PLT reduction induced by LPS (Fig. 4C).

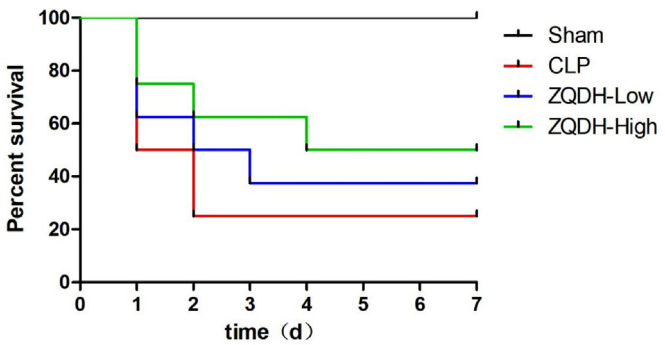

Fig. 1. Survival curve of rats.

3.5. Coagulation function

As shown in Fig. 5, no significant difference was observed in PT and APTT among four groups. Compared with Sham group, the serum FIB level in CLP group was significantly increased. After pretreatment with ZQDH prescription, there was a trend of improvement of FIB, but the difference was not statistically significant.

3.6. tPA and PAI-1 in serum, liver, kidney and lung

Fig. 6 shows that tPA and PAI-1 levels were significantly increased in serum and liver, kidney, and lung tissues after CLP, while pretreatment with high-dose ZQDH decoction reduced most tPA and PAI-1 levels.

3.7. Inflammatory factors of TNF-α, IL-6 and ROS

As shown in Fig. 7, the serum levels of TNF-α, IL-6, and ROS increased significantly after CLP, while pretreatment with ZQDH decoction notably reduced them in a dose-dependent manner (Fig. 7A, B, 7C). Moreover, the concentrations of these factors were positively correlated with serum PAI-1 levels (Fig. 7D, E, 7F).

4. Discussion

CLP model is the closest approximation to the mechanism of human sepsis and is now considered to be the gold standard for sepsis modeling.[8] This model focuses on local necrosis caused by distal cecal ligation and systemic inflammatory response induced by leakage of intestinal content into the peritoneum. It should be noted that the severity of CLP is affected by the length of the ligated cecum, the size of the perforation needle and fluid resuscitation treatment. To minimize differences in the experiment, we chose to perform ligation at half the distance between the base and the distal pole of the cecum, to keep the severity of sepsis consistent, and ZQDH decoction was administered before CLP to avoid variations in treatment. It can be seen from Fig. 1 that the survival rate of CLP rats is only 25%, which is basically consistent with previous literatures, indicating a successful modeling.

The continuous updating of sepsis management guidelines, from the initial 6 h-bundle to 3 h-bundle, emphasizes the importance of early intervention.[9,10] Therefore, compared with the observation point of 24 h after CLP in most studies,[11] we chose an earlier time window of 12 h after CLP to simulate clinical sepsis, so as to provide a theoretical basis for early identification and rescue of septic patients.

Platelet activation during sepsis is the main reason for platelet count decrease, the key to the formation of fibrin, and an important factor in the progression of sepsis.[12] On the one hand, activated platelets participate in inflammatory reaction through aggregation, adhesion and deformation. Thus, peripheral thrombocytopenia and the increase of mean platelet volume indicate endothelial cell damage and platelet activation.[13] On the other hand, activated platelets is a key regulator of blood clotting and a major source of plasma PAI-1, which plays an important role in thrombotic diseases. PAI-1, a 50 kDa glycoprotein, is the main physiological inhibitor of tissue type and urokinase type plasminogen activator (t-PA and u-PA). tPA and uPA can convert inactive plasminogen into fibrin-degrading plasminogen. Under normal circumstances, the concentration of PAI-1 in plasma and tissue remains at a low level, and only increase under pathological conditions, such as inflammation and thrombosis.[14] Sepsis just consistent with this pathological process. With the platelet activation in sepsis, the levels of tPA and PAI-1 in plasma and tissue increase. When PAI-1 level far exceeds

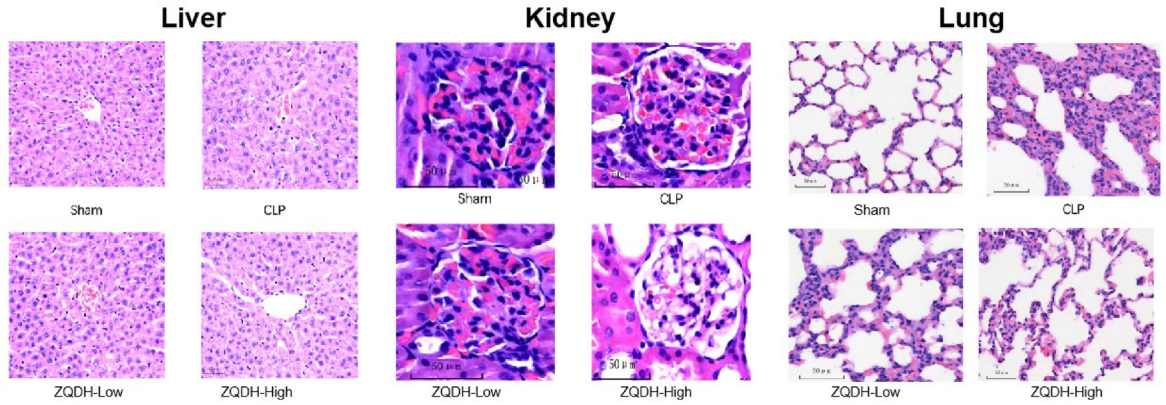

Fig. 2. HE staining of liver, kidney and lung of rats.

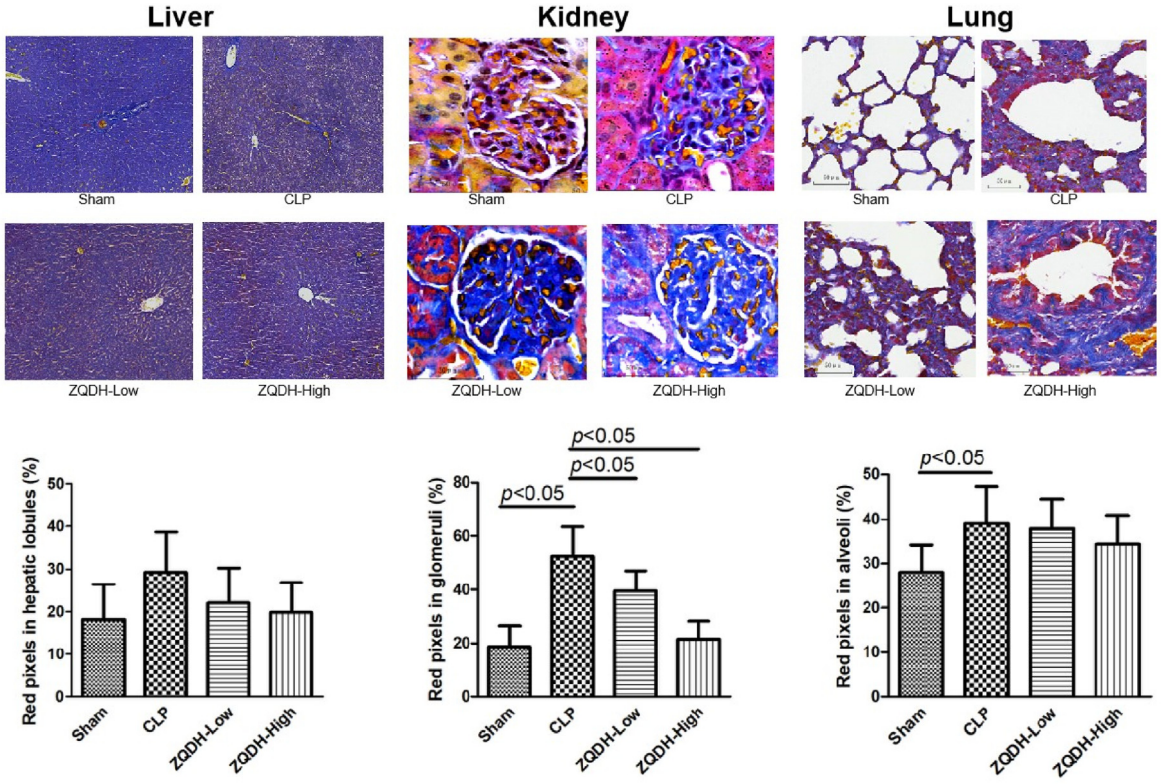

Fig. 3. MSB staining and relative volume fraction of fibrin in liver, kidney and lung of rats.

tPA, fibrin cannot be degraded, leading to microthrombosis; whereas when tPA level far exceeds PAI-1, the risk of bleeding is greatly increased.

In traditional Chinese medicine, it is widely believed that sepsis belongs to febrile disease, as described in ancient Chinese medicine works like *Treatise on Febrile Diseases (ShangHan Lun)*. The theory of clearing heat and toxin, promoting blood circulation and removing blood stasis has similar works with the theory of sepsis in modern medicine, and is currently commonly used in animal and clinical research of sepsis.[15] In the composition of ZQDH decoction in this study, Rhubarb has pharmacological effects such as anti-inflammation, regulating immunity, reducing endotoxin, improving microcirculation, and anti-thrombosis.[16,17] Among the components of Rhubarb, rhein and emodin have the strongest anti-inflammatory effect, while chrysophanol can inhibit platelet aggregation induced by collagen and thrombin.[18] Catalpol in Rehmanniae has the effects of anti-inflammation, anti-oxidation and protection of vascular endothelium.[19] Shikonin is abundant in Lithospermum, which can inhibit NF-κB signaling pathway and reduce PAI-1 level.[20] Radix Paeoniae Rubra is widely used in thrombotic diseases. Paeoniflorin is the main active ingredient of Radix Paeoniae Rubra, which not only has effects of anti-inflammation, sedation and anti-coagulation, but also protects vascular endothelial and inhibits platelet activation.[21] Panax notoginseng is a classic Chinese medicine with the dual effects of hemostasis and promoting circulation. In its active components, panax notoginseng saponins have anti-inflammatory effect, dencichine can improve platelet count, and ginsenosides have effects of antithrombosis and vascular endothelial protection.[22]

Experimental data in this research showed that ZQDH

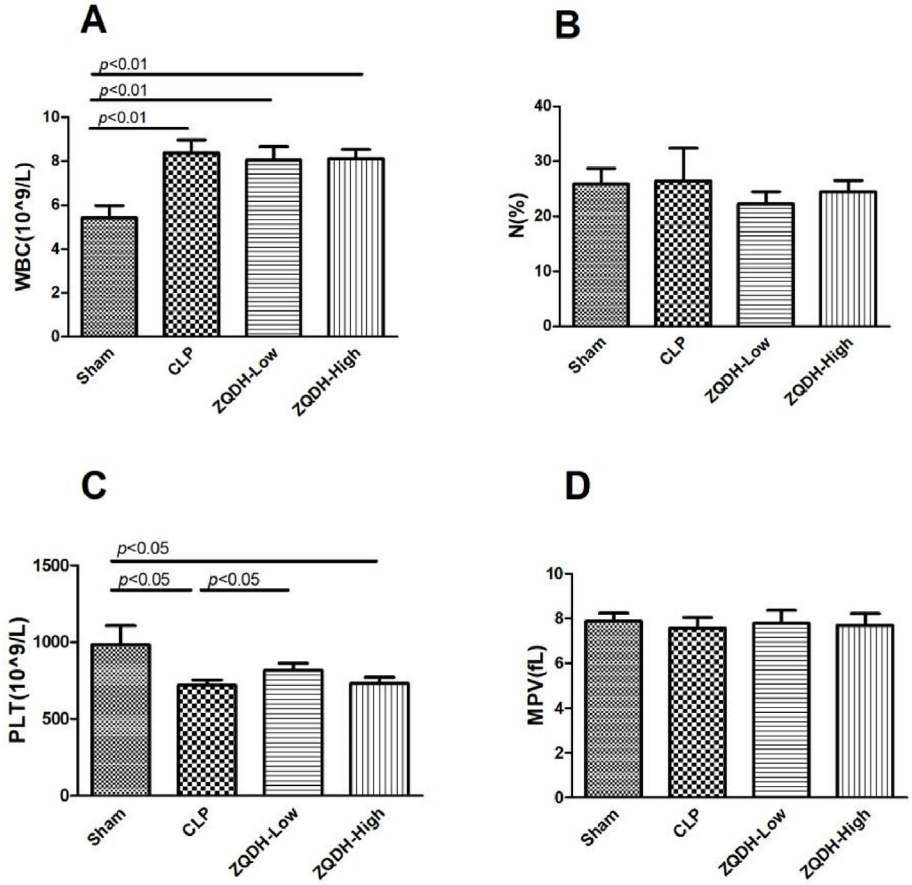

Fig. 4. White blood cell count (WBC, Fig. 4A), percentage of neutrophils (N%, Fig. 4B), platelet count (PLT, Fig. 4C) and mean platelet volume (MPV, Fig. 4D) in the blood.

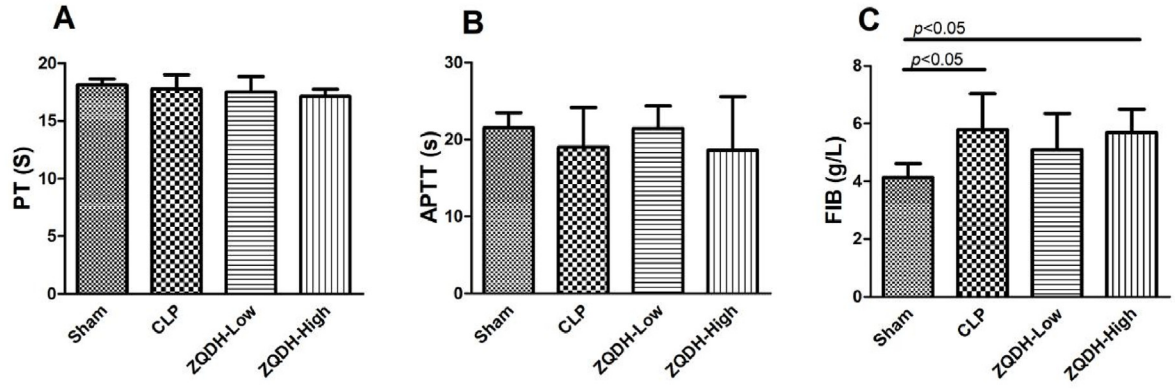

Fig. 5. Serum levels of prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (FIB).

decoction, especially at high dosage, can significantly increase platelet counts, improve the infiltration of inflammatory cells in the lung, and reduce pro-inflammatory cytokines in the blood plasma of septic rats, which proves that ZQDH decoction can effectively inhibit inflammation response of sepsis. Simultaneously, with the platelet activation during sepsis, tPA and PAI-1 accumulate in tissues, causing an imbalance of coagulation and fibrinolytic systems, resulting in the suppression of fibrinolytic processes. Under this condition, kidney may bear the brunt of fibrin deposition and microthrombus formation. ZQDH decoction can significantly reduce renal fibrin deposition and levels of tPA and PAI-1 in the kidney, suggesting that ZQDH may alleviate the inhibition of fibrinolysis by regulating the activities of tPA and PAI-1 in sepsis. The protective effect of ZQDH decoction on lung inflammation and renal fibrin deposition is also consistent with the pathophysiological mechanism of acute lung injury and acute kidney injury in the early stage of sepsis. Moreover, findings from this trial are consistent with a trial showing that PAI-1 level is positively correlated with other acute-phase proteins during acute inflammation such as pro-inflammatory factors and reactive oxygen species (ROS).[14]

The major limitations of our study are as follows: First, the dose difference between two treatment groups was not very large,

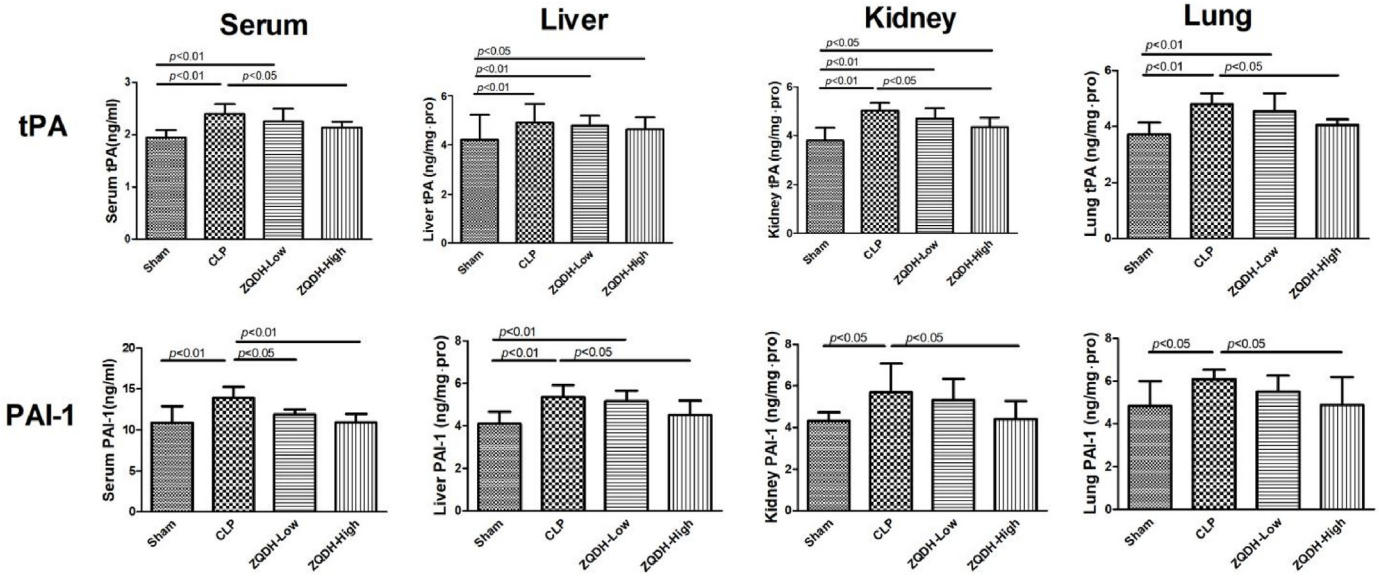

Fig. 6. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) in serum and organs of rats.

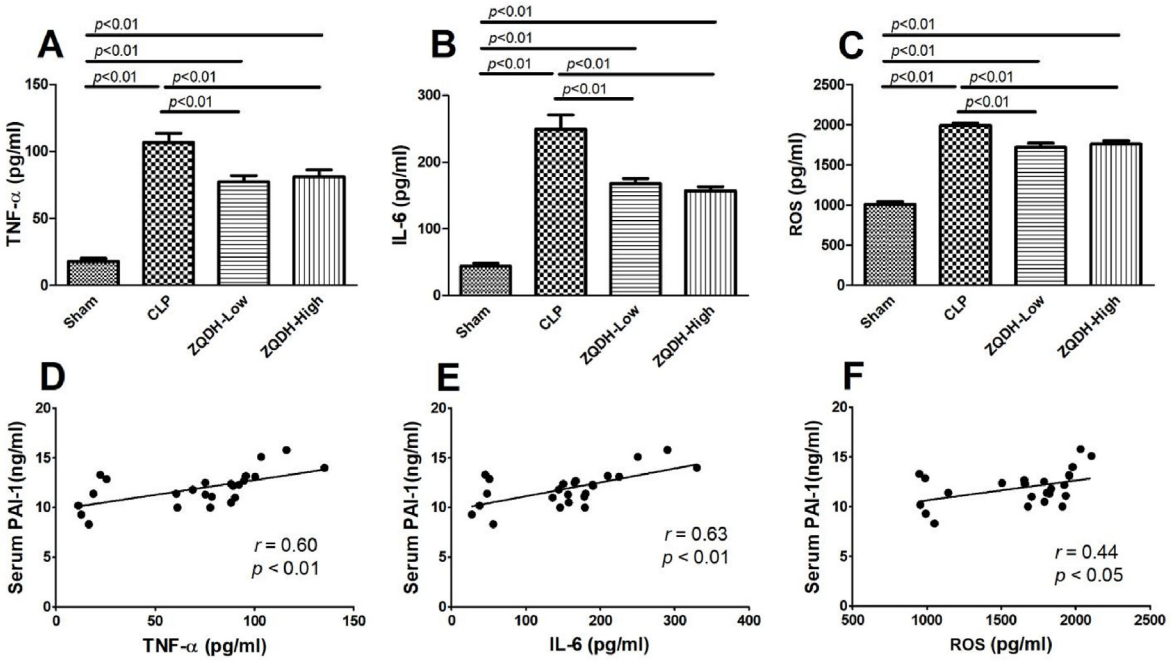

Fig. 7. Serum tumor necrosis factor (TNF-α, Fig. 7A), interleukin-6 (IL-6, Fig. 7B) and reactive oxygen species (ROS, Fig. 7C) in rats, and their correlation with serum plasminogen activator inhibitor-1 (PAI-1, Fig. 7D, E, 7F).

which limits the efficacy and safety of the study drug; Second, the administration of ZQDH decoction before CLP, rather than after CLP, is inconsistent with clinical management of sepsis; Third, the effect of ZQDH decoction on non-CLP rats was not clear; Finally, PAI-1, as an important key molecule, was not detected at different time points after CLP to observe its dynamic changes.

5. Conclusion

According to the findings obtained in this study, ZQDH decoction could inhibit systemic inflammatory injury, reduce fibrin deposition and microthrombosis, and regulate fibrinolytic system in a dose-dependent manner. This work demonstrates the protective role of ZQDH decoction in septic rats, and opens a broad application prospect for its use in clinical practice. However, further studies are needed to explore the underlying molecular mechanisms and other key targets.

Authors' contributions

GYX drafted the manuscript. FSY conducted animal experiment. PYH and CQH collected and analyzed data. FSY and WJ conducted

laboratory tests. JH designed the study and reviewed the final manuscript. All authors approved the final manuscript.

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Declaration of competing interest

There are no conflicts to declare.

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