

ANTICOAGULANT EFFECT OF HUISHENG ORAL SOLUTION IN A RAT MODEL OF THROMBOSIS

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Abstract – The aim of this study was to investigate whether Huisheng Oral Solution has an anticoagulant effect in a rat model of thrombosis. Forty male SD rats were equally and randomly divided into four groups: blank group, model group, and two treatment groups (A and B). Rats were subcutaneously injected with carrageenan to induce thrombosis. Rats in treatment group A were intragastrically administered with Huisheng Oral Solution in a dose of 2 mL/100 g body weight (once per 8 h) 72 h after carrageenan injection, and those in treatment group B both 72 h before and after the induction of thrombosis. Blood samples were collected 24, 48, and 72 h after carrageenan injection for measurements of prothrombin time (PT), activated partial thromboplastin time (APTT), international normalized ratio (INR), fibrinogen (FIB), prothrombin activity (PTA), platelets (PLT), fibrin degradation products (FDPs), and D-dimer. Lung, liver and mesentery samples were taken 72 h after carrageenan injection for histopathological analysis. The number of microthrombi in sections of different tissue samples were counted under a microscope. Blood parameters among each group were compared using the Welch test, Kruskal-Wallis test or SNK test after testing for normality, while the number of microthrombi was compared using the Bonferroni test. Compared to the model group, PT, APTT, INR, and FIB levels at the majority of time points were significantly shorter or lower in the two treatment groups ($P < 0.05$ for all), but the PTA was not significantly improved in the treatment groups. The level of FDPs and D-dimer and PLT counts at the majority of time points were significantly lower ($P < 0.05$ for all), and the number of microthrombi in the lung, liver and mesentery samples were significantly decreased ($P < 0.05$ for all) in the two treatment groups. The above parameters at the majority of time points showed no significant differences between the two treatment groups. We conclude that Huisheng Oral Solution can significantly improve coagulation parameters, fibrinolysis parameters, and PLT count, and reduce blood hypercoagulability and microthrombosis suggesting that Huisheng Oral Solution has an anticoagulant effect in a rat model of thrombosis.

Key words: Huisheng Oral Solution, hypercoagulable state, microthrombus, fibrinolysis, thrombosis

INTRODUCTION

Environmental pollution and population aging in modern society have greatly increased the incidence of malignant tumors and other diseases. Many patients with malignant tumors are in a hypercoagulable state. Venous thromboembolism, pulmonary embolism, and mild disseminated intravascular co-

agulation (DIC) are common manifestations of a hypercoagulable state (Rickles et al., 1992; Buller et al., 2007). Studies have shown that the incidence of venous thromboembolism and pulmonary embolism in patients with malignant tumors is approximately 1.5% (Andreasen et al., 2012). About 25% of patients with thromboembolic diseases have malignant tumors (Lee and Levine, 2003).

Huisheng Oral Solution was developed based on a classical recipe called Huazheng Huisheng Dan that was recorded in the Detailed Analysis of Epidemic Warm Diseases, a medical book written in the Qing Dynasty. Its main ingredients include ginseng, rhizoma cyperi, angelica, motherwort, rhizoma sparganii, trogopteris, turtle shell, frankincense, saffron, *Ligusticum wallichii*, peach kernel, rhubarb, leech, clove, and *Ferula asafoetida* (Fu, 2011). Being able to promote the flow of Qi and blood circulation, eliminate phlegm and dampness, warm Yang and dredge meridians, and replenish Qi and blood, Huisheng Oral Solution is effective and safe in the treatment of tumors.

Treatment with Huisheng Oral Solution combined with radiotherapy and/or chemotherapy can improve the quality of life and prolong survival in patients with advanced cancers. Coagulation disorders are common complications of malignant tumors, mainly manifesting as thrombosis caused by hypercoagulability (Gupta et al., 2005). Huisheng Oral Solution contains ingredients that are able to promote the flow of Qi and blood circulation and can therefore be used to treat coagulation disorders in clinical practice. In this study, we investigated the effect of Huisheng Oral Solution on coagulation function in a rat model of venous thrombosis.

MATERIALS AND METHODS

Animals

Forty 7-9-week-old male Sprague-Dawley (SD) rats, weighing 180-220 g, were provided by the Laboratory Animal Center of Chinese Academy of Medical Sciences. The animals were raised in a temperature-controlled room ($24 \pm 1^\circ\text{C}$; humidity $\geq 40\%$) on a 12-hour light:dark cycle, with free access to food and water.

Reagents and equipment

Huisheng Oral Solution (batch No. 110203) was provided by the Chengdu Diao Group Tianfu Pharmaceutical Co., Ltd. Carrageenan was purchased from

Sigma-Aldrich. The main equipment used in this study included an electronic balance (BS 423S, Sartorius), microscope, automatic blood analyzer (LH750, Beckman, USA), and automated coagulation analyzer (Acl-Top, IL, USA).

Animal groups

Forty SD rats were equally and randomly divided into four groups: blank group, model group, and two treatment groups (A and B). Rats in the treatment group A were given Huisheng Oral Solution 72 h after the induction of thrombosis, while those in group B were given Huisheng Oral Solution both 72 h before and after the induction of thrombosis.

Model of thrombosis

The rats were subcutaneously injected in the sole of the rear foot with 2% carrageenan (20 mg/mL, in normal saline) at a dose of 20 mg/kg of body weight. The rats were placed at room temperature after injection. A dark red thrombus formation area appeared in the tail tip of the majority of animals and gradually expanded toward the tail root. Approximately 48-72 h later, the sites of thrombus formation became black, and the tail was shed. Pathological evaluation revealed the presence of great amounts of fiber and few white blood cells and platelets in the small tail veins and capillaries, nuclear condensation in vascular endothelial cells (cube-shaped), and adherence of neutrophils to the inner walls of the blood vessels 24 h after carrageenan injection. At 72 h, inflammatory changes along with mixed thrombus formation were visible in bigger blood vessels.

Treatments, measurements and histopathology

All the rats were observed for 72 h. Those in group A were intragastrically administered with Huisheng Oral Solution at a dose of 2 mL/100 g body weight (once per 8 h) 72 h after carrageenan injection, while those in treatment group B were initially given Huisheng Oral Solution at the same dose 72 h before carrageenan injection, followed by injection 72 h after carrageenan injection. Blood samples were col-

lected 24, 48, and 72 h after carrageenan injection for routine blood tests, coagulation tests (PT, APTT, INR, FIB, and PTA), and measurements of D-dimer and fibrin degradation products (FDPs). Lung, liver and mesentery samples were taken 72 h after carrageenan injection, fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections (4 μm) were then cut using tissue samples from the upper lobe of the left lung, right liver lobe and middle part of the mesentery, stained with hematoxylin and eosin, and observed under a microscope. After choosing the field that contained the maximum number of microthrombi under low magnification ($\times 10$), the number of microthrombi was counted under high magnification ($\times 40$). Due to specimen problems or technical reasons, some specimens could not meet the requirements of testing and some data were missing. In this case, new data were obtained by repeating experiments in a random manner.

Statistical analysis

Numerical data are expressed as mean \pm standard deviation (SD). Statistical analysis was performed using SAS 9.1 (for analysis of blood parameters) and SPSS 9.1 (for analysis of the number of microthrombi). Blood parameters in each group were compared using the Welch test, Kruskal-Wallis test, or SNK test after testing for normality. The number of microthrombi was compared using the Bonferroni test. P -values < 0.05 were considered statistically significant.

RESULTS

Coagulation parameters

At all three time points, PT and APTT were significantly shorter and PTA was significantly longer in the model group than in the blank group ($P_s < 0.05$ for all, Table 1). There was no significant difference in INR between the blank group and the model group. At 48 and 72 h, FIB was significantly higher in the model group than in the blank group ($P_s < .005$ for both, Table 1). These data suggest that rats in the model group were in a hypercoagulable state.

Compared to the blank group, PT showed no significant differences at 24 and 48 h but was significantly shorter at 72 h in the two treatment groups. However, PT was significantly longer at all three time points in the two treatment groups than in the model group ($P_s < 0.05$ for both, Table 1). There was no significant difference in PT between the two treatment groups. These data suggest that hypercoagulable states were significantly improved in the two treatment groups compared to the model group.

Compared to the blank group, APTT did not differ significantly at 72 h in treatment group B ($P > 0.05$, Table 1) but was significantly different in the two treatment groups at all other time points. APTT at 48 and 72 h were significantly longer in the two treatment groups than in the model group ($P_s < 0.05$ for both, Table 1). In addition, APTT at 72 h were significantly longer in group A than in the group B.

Compared to the model group, INR did not differ significantly at 24 h in the treatment group A ($P > 0.05$, Table 1), but was significantly higher in the two treatment groups at all other time points ($P_s < 0.05$ for all, Table 1). Compared to the blank group, INR was significantly higher in the two treatment

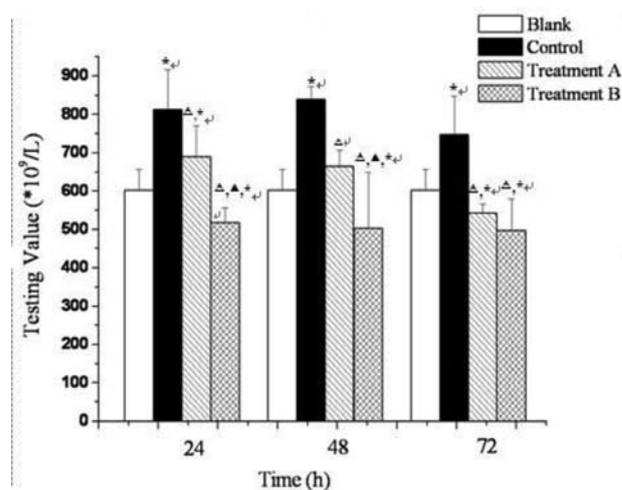


Fig. 1. Platelet counts in rats of each group. * $P < 0.05$ vs. the blank group; # $P < 0.05$ vs. the control group; $\Delta P < 0.05$ vs. the treatment group A.

Table 1. Coagulation parameters in rats of each group (mean \pm SD).

Groups	Time	PT	APTT	INR	FIB	PTA (%)
Blank		9.14 \pm 0.30	16.09 \pm 0.87	0.87 \pm 0.02	2.81 \pm 0.72	131.40 \pm 7.24
	24h	7.97 \pm 0.75*	13.74 \pm 1.30*	0.84 \pm 0.03	3.51 \pm 0.82	147.70 \pm 7.44*
Control	48h	7.31 \pm 0.22*	12.04 \pm 0.34*	0.79 \pm 0.05	4.29 \pm 0.75*	144.90 \pm 2.23*
	72h	7.17 \pm 0.76*	10.96 \pm 0.71*	0.79 \pm 0.05	4.16 \pm 0.53*	150.40 \pm 29.17*
Treatment A	24h	8.88 \pm 0.56 \square	13.93 \pm 0.94*	1.16 \pm 0.45	2.63 \pm 0.57 \square	159.10 \pm 20.29*
	48h	8.76 \pm 0.36 \square	14.36 \pm 0.79 \square *	1.42 \pm 0.50 \square *	2.79 \pm 0.52 \square	155.80 \pm 6.12 \square *
	72h	8.53 \pm 0.41 \square *	15.91 \pm 3.55 \square *	1.40 \pm 0.46 \square *	3.39 \pm 0.37	144.30 \pm 8.07*
Treatment B	24h	9.00 \pm 0.44 \square	14.11 \pm 0.93*	1.48 \pm 0.42 \square *	2.89 \pm 0.64	159.20 \pm 11.58*
	48h	8.98 \pm 0.49 \square	14.52 \pm 0.54 \square *	1.38 \pm 0.44 \square *	2.59 \pm 0.28 \square	144.90 \pm 7.36 \square *
	72h	8.49 \pm 0.66 \square *	15.84 \pm 1.20 \square \blacksquare	1.15 \pm 0.23 \square *	2.53 \pm 0.39 \square \blacksquare	153.60 \pm 12.30*

PT: prothrombin time; APTT: activated partial thromboplastin time; INR: international normalized ratio; FIB: fibrinogen; PTA: prothrombin activity. * P < 0.05 vs. the blank group; \square P < 0.05 vs. the control group; \blacksquare P < 0.05 vs. the treatment group A.

Table 2. Fibrinolysis parameters in rats of each group (mean \pm SD).

Groups	Time	FDPs	D-Dimer
Blank		3.51 \pm 0.26	90.90 \pm 20.12
	24h	4.93 \pm 0.61*	147.30 \pm 27.82*
Control	48h	5.50 \pm 0.34*	181.00 \pm 19.15*
	72h	5.48 \pm 0.36*	199.10 \pm 27.12*
Treatment A	24h	4.40 \pm 0.37 \square *	133.60 \pm 18.00*
	48h	4.68 \pm 0.28 \square *	148.00 \pm 7.20 \square *
	72h	3.22 \pm 0.78 \square	137.00 \pm 22.36 \square *
Treatment B	24h	3.74 \pm 0.39 \square \blacksquare	124.60 \pm 16.13*
	48h	3.11 \pm 0.53 \square \blacksquare	139.00 \pm 9.39 \square *
	72h	3.09 \pm 0.43 \square	118.70 \pm 9.78 \square *

FDPs: fibrin degradation products. * P < 0.05 vs. the blank group; \square P < 0.05 vs. the control group; \blacksquare P < 0.05 vs. the treatment group A.

groups at all other time points except at 24 h in treatment group A. There was no significant difference in INR between the two treatment groups.

Compared to the model group, FIB did not differ significantly at 72 h in the treatment group A and at 24 h in group B, but was significantly higher in the two treatment groups at all other time points (P s < 0.05 for all, Table 1). There was no significant difference in INR between the blank group and the two treatment groups. INR was significantly lower at 72 h in treatment group B than in treatment group A but showed no significant difference at other time points between the two treatment groups.

Compared to the model group, FIB was significantly higher at 48 h in group A, but showed no significant differences at other time points in the two treatment groups (P s > 0.05 for all, Table 1). FIB was significantly higher at all time points in the two treatment groups than in the blank group. PTA was significantly lower at 48 h in group A than in group B, but showed no significant difference at other time points between the two treatment groups.

The above results suggest that the majority of blood coagulation parameters were significantly improved in the two treatment groups compared to the model group.

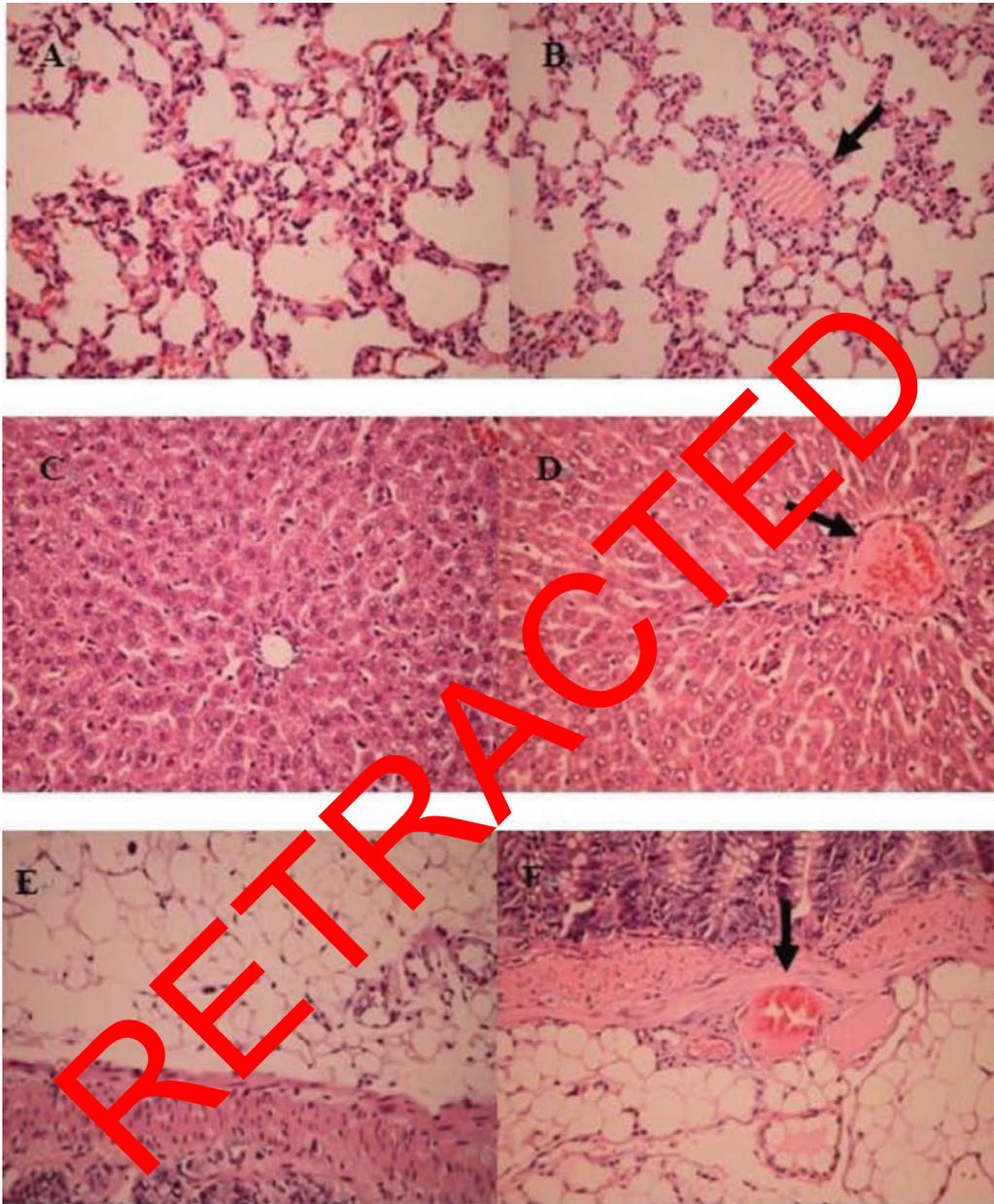


Fig. 2. Histopathological changes in the lung, liver and mesentery

Fibrinolysis parameters

At all three time points, the levels of FDPs and D-dimer were significantly higher in the model group than in the blank group ($P_s < 0.05$ for all, Table 2), indicating that the fibrinolytic activity was high in rats in the model group. This result indirectly sug-

gests that rats of the model group were in a hypercoagulable state.

At all three time points, the levels of FDPs were significantly lower in the two treatment groups than in the model group ($P_s < 0.05$ for all, Table 2). Compared to the blank group, the levels of FDPs were

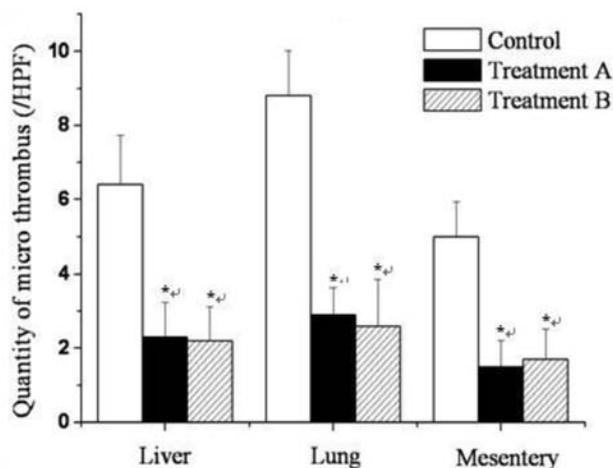


Fig. 3. Comparison of the numbers of microthrombi in the liver, lung and mesentery of rats in each group. * $P < 0.05$ vs. the control group

significantly higher at 24 and 48 h in the treatment group A ($P_s < 0.05$ for both, Table 2), but showed no significant difference at other time points in the two treatment groups. The levels of FDPs were significantly lower at 24 and 48 h in group B than in group A ($P_s < 0.05$ for both, Table 2).

At 48 and 72 h, the levels of D-dimer were significantly lower in the two treatment groups than in the model group ($P_s < 0.05$ for all, Table 2). Compared to the blank group, the levels of D-dimer were significantly higher at all time points in the two treatment groups ($P_s < 0.05$ for all, Table 2). There was no significant difference in the levels of D-dimer between the two treatment groups.

The above results suggest that the majority of fibrinolysis parameters were significantly improved in the two treatment groups compared to the model group.

Platelets

At all three time points, platelet counts were significantly higher in the model group than in the blank group and two treatment groups ($P_s < 0.05$ for all, Fig. 1). Compared to the blank control group, platelet counts were significantly higher at 24 h,

showed no significant difference at 48 h, and were significantly lower at 72 h in group A. At all three time points, platelet counts were significantly lower in group B than in the blank control group. Additionally, platelet counts were significantly lower at 24 and 48 h in group B than in group A.

Histopathology

Seventy-two hours after carrageenan injection, lung, liver and mesentery tissue samples were taken, stained with hematoxylin and eosin, and examined by microscopy. Extensive microthrombus formation was visible in the lung, liver and mesentery of rats in the model group. Additionally, capillary hemorrhaging and interstitial neutrophil infiltration in the lungs, cloudy swelling, degeneration and necrosis of hepatocytes in local areas in the liver, as well as local congestion and hemorrhage in the mesentery, were seen in rats in the model group (Fig. 2).

We next examined the field that contained the maximum number of microthrombi under low magnification ($\times 10$) and then counted and compared the number of microthrombi under high magnification ($\times 40$) among each group. The numbers of microthrombi in the lung, liver and mesentery were significantly lower in the two treatment groups than in the model group. There were no significant differences in the numbers of microthrombi in the lung, liver and mesentery between the two treatment groups (Fig. 3).

Tissue samples were taken 72 h after carrageenan injection, fixed, dehydrated, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by microscopy. Thrombus formation in the small blood vessels was indicated (arrows): A, lung of a normal rat; B, lung of a rat in the model group; C, liver of a normal rat; D, liver of a rat in the model group; E, mesentery of a normal rat; F, mesentery of a rat in the model group (magnification $\times 40$).

DISCUSSION

Hypercoagulable states in patients with malignant

tumors have become a hot research topic in the medical field. Many factors may contribute to hypercoagulable states, including increased levels of physiological coagulation factors, increased activity of pathological coagulation factors, elevated fibrin monomer levels in blood, coagulation factor transfusion, decreased ability to inhibit and eliminate activated coagulation factors, diminished fibrinolytic activity, platelet factors, and other genetic factors (Geng and Zhang, 2008). Hypercoagulable states can be congenital, acquired, or both (Crowther and Kelton, 2003). For cancer patients, central venous catheterization, surgery or chemotherapy during cancer treatment may cause vascular endothelial cell injury, increased levels of adhesion molecules, adhesion of blood cells to each other and the release of procoagulants, thereby resulting in the formation of hypercoagulable states (Van Marion et al., 2005). Chemotherapy-induced reactive thrombocytosis is also a cause of hypercoagulable states in cancer patients (Zecchina et al., 2007). A previous study has shown that increased blood concentrations of tissue factor-positive microparticles are associated with the pathogenesis of hypercoagulable states in cancer patients (Hron et al., 2007).

More than 90% of patients with malignant tumors have subclinical coagulation disorders, and many of them are in a hypercoagulable state, which manifests as elevated levels of FIB, PT, coagulation factors V, VIII, IX and X, FDPs and plasminogen activator inhibitor 1, and decreased levels of coagulation factor inhibitors (antithrombin, protein C and protein S) (Rickles et al., 1992; Edwards et al., 1987; Sun et al., 1979). In the present study, we chose PT, APTT, INR, FIB and PTA to assess comprehensively the coagulation function changes in the subject rats. PT is a sensitive screening test for the extrinsic coagulation pathway. APTT reflects the integrity of the endogenous pathways of the procoagulant cascade (VIII, IX, XI). The INR is calculated by normalizing the PT ratio to the power of the international sensitivity index (ISI). It standardizes the PT across different reagents of varying sensitivity and can better reflect the true nature of coagulation disorders. FIB positively correlates with whole blood viscosity,

plasma viscosity, erythrocyte sedimentation rate and platelet aggregation. Elevated blood levels of FIB can increase blood viscosity, promote red blood cell aggregation and platelet aggregation, and thereby result in the formation of hypercoagulable states and thrombosis.

In the five coagulation parameters detected in this study, approximately 66.7% (20/30) of data values in the two treatment groups were significantly different from those in the model group. In contrast, only about 20% (4/15) of data values were significantly different between the two treatment groups.

Platelet counts are closely associated with the coagulation system and has been used as an important parameter to assess coagulation function in many studies (Vilar Saavedra et al., 2011; Wang et al., 2011; Dutoy-Bouthers, 2010; Machida et al., 2010). In this study, we found that platelet counts were significantly reduced in the treatment groups compared to the model group. In addition, a significant difference in platelet count between the two treatment groups was observed only at 72 h.

A hypercoagulable state is often associated with hyperfibrinolysis, which manifests as increased levels of FDPs and D-dimer. In the present study, we found that approximately 83.3% (10/12) of data values in the two treatment groups were significantly different from those in the model group. In contrast, only 1/3 (2/6) of data values were significantly different between the two treatment groups.

Histopathological analysis revealed obvious vascular microthrombosis in both the model group and the two treatment groups. However, the numbers of microthrombi in the lung, liver and mesentery were significantly lower in the two treatment groups than in the model group, but showed no significant difference between them.

Huisheng Oral Solution was traditionally used as an anticancer drug in clinical practice. There has been no evidence so far about whether it can improve coagulation or not. In this study, we found that the ma-

majority of coagulation parameters (66.7%), fibrinolysis parameters (83.3%) and platelet counts (100%) in the two treatment groups were significantly improved compared to the model group. In contrast, there were no significant differences in these parameters between the two treatment groups. These findings were also confirmed by histopathological analysis.

Huisheng Oral Solution is derived from a variety of Chinese herbs that have antitumor effects. A previous study has found that Huisheng Oral Solution has significant anti-cancer effects in mice bearing transplanted Lewis lung carcinoma (Huang et al., 1998). Ma et al. (2005) found that Huisheng Oral Solution is beneficial in improving cellular immune function in elderly cancer patients. We surmise that the anticoagulant effect of Huisheng Oral Solution observed in this study may be because it contains many blood-invigorating ingredients. Yin et al. (1980) found that angelica and its active ingredient, ferulic acid, could significantly inhibit thrombin-induced platelet aggregation. Zhang et al. (1980) investigated the effect of motherwort on mouse platelet cAMP and cGMP and PGI-like substance in the rat carotid artery wall and found that it could significantly inhibit platelet aggregation. In addition, rhizoma polygoni, saffron, rhubarb and leech can suppress platelet aggregation, prolong thrombosis time, reduce blood viscosity, and improve microcirculation. Hirudin, the main active ingredient of leech, is by far the strongest natural thrombin inhibitor in the world and has strong anticoagulant effect (Mao et al., 1998; Zou, 1994; Zhao et al., 2009).

In this study, we found that Huisheng Oral Solution could significantly reduce blood hypercoagulability, microthrombosis and secondary fibrinolysis in a rat model of thrombosis. This may be because it contains a variety of ingredients that have anti-clotting effects. However, we did not find that Huisheng Oral Solution had preventive effects against the pathogenesis of hypercoagulable states.

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