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Effect of bioactivity of salvianolic acid B in rats

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Abstract

The present study was conducted to evaluate the effect of salvianolic acid B on hemorheological parameters in rats. The animals were orally administered with salvianolic acid B. The blood samples were collected. Various hemorheological parameters were estimated. Salvianolic acid B could significantly decreased whole blood viscosity, plasma viscosity, hematocrit, RBC aggregation index, maximum platelet aggregation ratio and Fib and increased APTT, TT and PT in rats comparing with those in normal rats. It can be concluded that salvianolic acid B improved hemorheological characteristics in rats.

Key words: Salvianolic acid B, hemorheology, whole blood viscosity, maximum platelet aggregation ratio.

Introduction

Salvia miltiorrhiza (Danshen) is one of the most common traditional Chinese medical herbs. It is listed in the Chinese Pharmacopoeia and is widely used in oriental medicine to treat heart and brain disease, hepatitis, hemorrhage and menstrual abnormalities. The aqueous extract of *S. miltiorrhiza* contains phenolic compounds, such as salvianolic acids A, B and C, and protocatechualdehyde, which are effective in protecting liver microsomes, hepatocytes and erythrocytes against oxidative damage [1-4]. Furthermore, it was demonstrated that salvianolic acid B protected endothelial cells from membrane hypermeability and injury induced by inflammatory factors such as vascular endothelial growth factor (VEGF), tumor necrosis factor a (TNF-a) and cholestantriol [5-7]. Salvianolic acid B suppressed lactate dehydrogenase leakage and malondialdehyde formation in the epithelial cells induced by cisplatin [8] and inhibited intracellular ROS generation in cultured mesangial cells induced by high glucose and hydrogen peroxide [9].

Blood stasis is an important underlying pathology of many disease processes according to traditional Chinese medicine. Described in TCM theory as a slowing or pooling of the blood due to disruption of heart Qi, it is often understood in biomedical terms in terms of hematological disorders such as hemorrhage, congestion, thrombosis, local ischemia (microclots) and tissue changes [10]. As soon as blood stasis developed, the blood circulation will further be affected and thus leads to new pathological changes. Pharmacokinetic characteristics could be affected by disease condition [11, 12]. The blood stasis model, which was obtained by placing the rats in ice-cold water during the time interval between two injections of epinephrine, has been widely used in China. As an acute-stress, injecting epinephrine could produce the hemorheological disorders in various forms, such as blood hypercoagulability and a rise of whole blood viscosity [13].

Therefore, it is very important to investigate the pharmacokinetics of drugs in animals with blood stasis syndrome, which may influence absorption, metabolism and elimination of drugs in blood. In recent years, some pharmacokinetic studies showed that salvianolic acid B possesses the effect of activating blood circulation to dissipate blood stasis. In the present, we investigate the effect of salvianolic acid B on hemorheological characteristics in rats with acute blood stasis.

Materials and Methods

Material: Salvianolic acid B (purity>98%) was purchased from Shanghai JIMI Biology Science-Technology Ltd.

Animals: Three-month-old Wistar rats, weighing 237±30 g, were given a normocaloric standard diet and water ad libitum, while being maintained in a controlled environment (12 h light and dark cycle, 21-23°C). The animals were acclimatized to the laboratory conditions for one week before the start of the experiment. All experiment protocol was approved by the Institutional Committee on Ethics of Animal Experimentation.

In vivo study: Animals were randomly divided into the following five groups (n = 8): normal control group (I), acute blood stasis model group (II) and three acute blood stasis groups administrated with salvianolic acid B (30, 50 and 80 mg/kg b.w.) (III, IV and V). Each rat was in an individual cage. The animals (groups III, IV, V) were orally administered with salvianolic acid B (30, 50 and 80 mg/kg b.w.) once daily for 10 days. The acute blood stasis (groups II-V) was induced by injecting adrenaline hydrochloride injection (0.7 mg/kg). After 4 h, those rats were injected with the same injection again, waiting for 2 h, the rats were soaked in ice-water for 5 min keeping their heads outside surface. The rats were put in metabolic cages, and fed freely. Normal control group was injected with the same volume of saline. Then, 1 ml blood samples were

collected in heparinized Eppendorf tube via the caudal vein. After centrifuging at 5000 rpm for 10 min, the plasma samples were obtained and frozen at -20°C until analysis.

Biochemical analysis:

Whole blood viscosity and plasma viscosity: Whole blood viscosity corrected at 43 ± 1% was measured by a tubby rotational viscometer (Chinese Academy of Sciences, Transduction Technology Co., Beijing, China) at two different points on shear rates (200, 100, 50, 1 s^{-1}) five times, respectively. The averages of the five values were then calculated. The same procedure was repeated in each blood sample. The final viscosity was estimated for each point by the average of the two repeated tests. Plasma viscosity was estimated at the highest shear rate of 50 s^{-1} by the average of five measurements.

Determination of RBC aggregation indexes: RBC aggregation was also quantified using the Couette system described above (LORCA,RR Mechatronics, Hoorn, The Netherlands). RBC suspensions in autologous plasma were sheared at 400 s^{-1} for 5 s for disaggregation and laser light reflection from the sample was recorded for 120 s, after a sudden stop. The obtained light reflection-time curve (i.e. sylectogram) was analyzed by a microcomputer and several parameters of RBC aggregation were calculated. The aggregation index "M" is equivalent to the "M index" of Myrenne aggregometer and corresponds to the area above the sylectogram. The aggregation index "A" is the ratio of this area to the sum of the areas under and above the sylectogram. The computer fits a double exponential equation to the sylectogram and calculates two time constants. The smaller time constant (tFast) has been used for comparisons in this study. Disaggregation shear rate (gTmin) was measured by shearing RBC suspensions at 11 separate levels of shear rate between 10 and 800 s^{-1}, and finding the shear rate at which the light reflection was maximum. The system and calculation of these parameters are described elsewhere in more detail [14]. RBC aggregation measurements were also conducted at temperature (37ºC).

Determination of hematocrit level: The hematocrit level was calculated from the measurement of red blood cells and either the calculated erythrocyte mean cell volume (Coulter counter; Coulter Diagnostics, Hialeah, Florida) or pattern of light scattering (Hemalog H-6000; Technicon Corporation, Tarrytown, New York).

Determination of platelet aggregation: Platelet aggregation was determined by turbidimetry [15]. Blood (9.0 ml) was collected into a plastic syringe containing 1.0 ml of 3.8% sodium citrate. The blood was then transferred into a test tube, the tube capped and mixed by gentle inversion. The volume of blood collected can be adjusted depending on the number of tests to be performed, but the blood:anticoagulant ratio must be maintained at 9:1. The anticoagulated blood was centrifuged (100 × *g*, 10 min) and the platelet rich plasma (PRP) carefully removed using a plastic pipette while avoiding red cell contamination. The PRP was transferred to a plastic container, which was capped and stored at room temperature. The remaining blood sample was centrifuged again (1500 × *g*, 20 min) and the platelet poor plasma (PPP) transferred to a plastic container with a plastic pipette, and the container capped and stored at room temperature. A platelet count was performed and the PRP adjusted to 200,000 ± 50,000 per µl platelets with the PPP as required. An aliquot of PRP was transferred to a small glass cuvette, which was maintained at body temperature (37°C). This was constantly stirred for 5 min and an aggregation agent added to clump the platelets together and measure their maximum aggregation ratio.

Plasma preparation and hemostatic coagulation tests: About 0.7 ml of venous blood was collected from each mouse by suctioning from the right ventricle using a syringe containing sodium citrate.

Coagulation was blocked in the blood sample with 10^9 mM sodium citrate (1:9 vol/vol), and the sample was then centrifuged at 2,400 × g for 15 min to get platelet-free plasma. In this experiment, only fresh blood samples were used. All tests were completed within 2 h at room temperature. The plasma was incubated with different concentrations of rofecoxib or 20-HETE ranging from 10^{-9} to 10^{-5} M for exactly 3 min at 37°C before being used for all measurements, which were performed by modifications of the conventional clinical procedures using a photoelectrical electromagnetism coagulometer (STEELLEX).

For testing PT, 50 µl of pretreated plasma was mixed with 100 µl of activating reagent at 37°C. The coagulation time was then measured, and the INR was calculated by the coagulometer automatically. To measure TT, 100 µl of plasma was pretreated for 3 min at 37°C; 100 µl of thrombin solution was added, and coagulation time was measured. For the measurement of APTT, 50 µl of plasma and 50 µl of APTT reagent, which contained cephalin and ellagic acid, were mixed. After incubating for 3 min at 37°C, 50 µl of 25 mM $CaCl_2$ solution (37°C) was added and coagulation time was measured. To measure the plasma FIB level, 90 µl of buffer and 10 µl of plasma were incubated for 3 min at 37°C; 50 µl of activating reagent was added, and the coagulation time was measured. The plasma FIB concentration (g/l) was calculated by the coagulometer automatically from a reference curve.

Statistical analysis: Values were expressed as mean ± S.E.M. for six rats in each group. Statistical significance of changes in different groups was evaluated by one-way ANOVA using SPSS software package for Windows. The statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ was considered to be significant.

Table 1. Effect of salvianolic acid B on the whole blood viscosity and plasma viscosity in normal and experimental animals.

Group	whole blood viscosity (mPa·s)				plasma viscosity (mPa·s)
	200	100	50	1 (s^{-1})	50 (s^{-1})
I	3.9±0.2	4.5±0.3	5.8±0.4	22.1±1.57	1.31±0.09
II	4.9±0.2 [a]	5.8±0.4 [b]	6.7±0.4 [b]	30.4±2.73 [b]	1.74±0.11 [b]
III	4.5±0.3	5.3±0.5	6.5±0.6	28.9±1.95	1.65±0.08 [c]
IV	4.2±0.4 [c]	5±0.4 [c]	6.4±0.6 [c]	27.1±1.83 [c]	1.52±0.09 [d]
V	4±0.2 [c]	4.8±0.3 [c]	6.1±0.5 [c]	24.7±1.79 [d]	1.4±0.07 [d]

[a] P<0.05, [b] P<0.01, compared with group I; [c] P<0.01, [d] P<0.01, compared with group II.

Results and Discussion

Hemorheology is the study on blood fluidity, deformability, coagulability, visco-elasticity and cardiovascular viscosity, and it is the main diagnostic index of blood stasis. Blood viscosity depends mainly on hematocrit and fibrinogen concentration [16, 17]. In this

article, all the parameters reflecting the hemorheology increased significantly in the model group.

Table 1 shows the whole blood viscosity and plasma viscosity in normal and experimental animals. There was a significant elevation in whole blood viscosity and plasma viscosity in group II compared to the corresponding control group (I). Administration of salvianolic acid B (30, 50 and 80 mg/kg b.w.) dose-dependently significantly decreased the level of whole blood viscosity and plasma viscosity in rats with acute blood stasis (groups III-V) compared to group II.

The hematocrit is a test that measures the percentage of blood that is comprised of red blood cells. The hematocrit is the most important determinant of blood viscosity [18,19]. Consequently, in patients with clinical signs of the hyperviscosity syndrome, hemodilution has proven to be a simple but effective method to improve tissue perfusion by lowering blood viscosity. Several studies have indicated that an optimal hematocrit for tissue perfusion exists [20, 21]. Furthermore, there is evidence that the hematocrit affects thrombosis. The aggregation and disaggregation of red blood cells (RBC) play an important role in the pathophysiological behavior of the blood circulation [22]. The aggregation of RBC is a reversible phenomenon that occurs with macromolecules bridging the membranes of adjacent cells [23, 24].

There was a significant elevation in hematocrit level and RBC aggregation index in group II compared to the corresponding control group (I). The level of hematocrit and RBC aggregation index was reduced significantly (Table 2) in group II, on oral administration of the salvianolic acid B, at a dose of 30, 50, and 80 mg/kg body weight for 10 days to the acute blood stasis groups (III-V) of rats compared to the group II.

Table 2. Effect of salvianolic acid B on the hematocrit and RBC aggregation index.

Group	Hematocrit (Hct)	RBC aggregation index
I	0.52±0.02	5.04±0.21
II	0.61±0.03 [b]	5.73±0.19 [b]
III	0.59±0.03	5.52±0.26 [c]
IV	0.55±0.02 [d]	5.27±0.22 [d]
V	0.53±0.02 [d]	5.11±0.27 [d]

[b] P<0.01, compared with group I; [c] P<0.01, [d] P<0.01, compared with group II.

Blood became viscous, thick and aggregated. The results indicated that salvianolic acid B can significantly decrease whole blood viscosity, plasma viscosity, RBC aggregation index and hematocrit, thereby significantly improving the hemorheology parameters of acute blood stasis rats and improving the pathological state of blood stasis. This helps to prevent the formation of highly viscous blood in acute myocardial infarction.

Platelet aggregation plays an important role in the pathogenesis of thromboembolic cerebrovascular disease. Platelet aggregation ratio (PAR) and its derivates have been used successfully to identify the effectiveness of antiplatelet agents and their optimum dosage in patients suffering from stroke. Normally, platelets do not aggregate spontaneously *in vivo*. However, if a suitable stimulus is present, aggregation can occur. Postulated mechanisms include exposure to collagen in atherosclerotic lesions[25, 26]; platelet damage secondary to shear stress [27]; postobstructive luminal turbulence resulting in increased platelet-platelet interactions [28]; and enhanced or abnormal responsiveness to circulating catecholamines [29].

In the present study, maximum platelet aggregation ratio was increased significantly in the group II compared to the corresponding control group (I). However, oral administration with the salvianolic acid B for 10 days at a dose of 30, 50, and 80 mg/kg body weight to the acute blood stasis groups (III-V) of rats, decreased the maximum platelet aggregation ratio compared to the group II (Table 3). Our result confirmed that salvianolic acid B has strong blood anticoagulation activity.

Table 3. Effect of salvianolic acid B on the maximum platelet aggregation ratio.

Group	Maximum platelet aggregation ratio
I	17.82±1.07
II	34.82±1.59 [b]
III	28.71±1.36 [c]
IV	24.15±1.52 [d]
V	21.69±1.48 [d]

[b] P<0.01, compared with group I; [c] P<0.01, [d] P<0.01, compared with group II.

Fibrinogen is a protein produced by the liver. This protein helps stop bleeding by helping blood clots to form. Prothrombin time (PT) is a blood test that measures the time it takes for the liquid portion (plasma) of your blood to clot. The partial thromboplastin time (PTT) or activated partial thromboplastin time (aPTT or APTT) is a performance indicator measuring the efficacy of both the "intrinsic" (now referred to as the contact activation pathway) and the common coagulation pathways. Apart from detecting abnormalities in blood clotting [30], it is also used to monitor the treatment effects with heparin, a major anticoagulant. The thrombin time (TT), is a blood test which measures the time it takes for a clot to form in the plasma of a blood sample anticoagulant to which an excess of thrombin has been added. This test is repeated with pooled plasma from normal patients. The difference in time between the test and the 'normal' indicates an abnormality in the conversion of fibrinogen (a soluble protein) to fibrin, an insoluble protein [31].

Adrenaline hydrochloride injection treatment caused a significant increase in the level of Fib, and decrease in APTT, TT and PT in group II when compared with control group (I) (Table 4). The treatment of salvianolic acid B at the doses of 30, 50 and 80 mg/kg bw/day resulted in a dose-dependent decrease of fib and increase of APTT, TT and PT in groups III-V when compared to group II rats. This results indicated that anticoagulated blood effect of salvianolic acid B was closely associated with coagulation factors of intrinsic and extrinsic system of blood clotting.

Table 4. Effect of salvianolic acid B on the Fib, APTT, TT and PT.

Group	Fib (g/L)	APTT (s)	TT (s)	PT (s)
I	2.04±0.13	22.19±2.76	31.12±1.27	16.08±1.21
II	5.15±0.33 [b]	15.37±1.83 [b]	25.16±2.41 [b]	14.63±1.09 [a]
III	4.65±0.31 [c]	18.98±1.66 [d]	28.59±1.79 [c]	15.82±1.14
IV	4.47±0.29 [c]	21.68±1.93 [d]	28.77±1.99 [c]	15.97±1.37 [c]
V	4.28±0.3 [d]	22.06±2.28 [d]	30.36±2.75 [d]	16.07±1.55 [c]

[a] P<0.05, [b] P<0.01, compared with group I; [c] P<0.01, [d] P<0.01, compared with group II.

Conclusions

The study confirms the effect of activating blood circulation to dissipate blood stasis of salvianolic acid B. Salvianolic acid B can improve hemorheological characteristics in rats with acute blood stasis, and further decrease hematocrit and platelet aggregation. Therefore, salvianolic acid B can prevent blood viscosity induced myocardial injury in heart ischemia-reperfusion rat.

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