

Full Length Research Paper

***Leonurus heterophyllus* sweet extract improves hemorheology and inhibits thrombosis in rat with induced myocardial ischemia**

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The effects of *Leonurus heterophyllus* sweet (*LHS*) extract on hemorheological variables, platelet aggregation and thrombus formation *ex vivo* in rat with induced myocardial ischemia were investigated. Sixty male Sprague-Dawley rats were randomly and equally divided into 6 groups. Rats in Group III to Group VI received intravenously *LHS* extract one time a day and continuously for 7 days, the dose of which was 20, 40, 80 and 100 mg/kg of *LHS* extract for each rat in Group III to Group VI respectively. While rats in Group I and Group II were administered with the same volume of 0.85% sodium chloride (NaCl) solution as control. After the 7th administration of NaCl/*LHS* extract, rat myocardial ischemia was accomplished by ligating the left coronary arteries of rats in Group II to Group VI, rats in Group I served as sham-operated control. After 3 h of myocardial ischemia, blood sample was collected from carotid artery for investigation. In addition, another sixty normal male Sprague-Dawley rats were grouped and treated as the same as mentioned above but no surgical preparation was performed, 0.5 ml blood was obtained from each rat for erythrocyte morphology analysis after 3 h of anoxia *ex vivo*. Results showed that after myocardial ischemia, blood viscosity, plasma viscosity, erythrocyte aggregation index, erythrocyte sedimentation rate (ESR) and plasma fibrinogen in rats all went up, platelet aggregation and thrombus formation *ex vivo* were both intensified ($p < 0.05$ and 0.001 compared with Group I). With the dose increased, however, *LHS* extract reduced blood viscosity, plasma viscosity, ESR, plasma fibrinogen that increased during myocardial ischemia, inhibited platelet aggregation rate induced by arachidonic acid, adenosine diphosphate and collagen respectively, and diminished the length, wet weight and dry weight of thrombus formed *ex vivo* ($p < 0.05$ and 0.01 compared with Group II). In addition, after anoxia *ex vivo*, the perimeter, perimeter/area ratio and form factor of erythrocyte increased significantly, and the area of erythrocyte reduced obviously ($p < 0.001$, compared with Group I), while in *LHS* extract treated groups, the changes in morphological parameters of erythrocyte were significantly improved with the dose of *LHS* extract increased ($p < 0.05$ and 0.01 , compared with Group II). Consequently, *LHS* extract improves hemorheological variables, inhibits platelet aggregation and thrombus formation *ex vivo* during myocardial ischemia in a dose-dependent manner. It is indicated that *LHS* is a kind of potential herbs in relieving myocardial ischemia.

Key words: *Leonurus heterophyllus* sweet, extract, hemorheological variables, platelet aggregation, thrombus

INTRODUCTION

Myocardial ischemia is a main pathophysiological process in ischemic heart disease, especially in coronary artery ischemia induced heart disease, which is a common illness with high incidence (Ostadal, 2009). The

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onset and progress of myocardial ischemia is often accompanied by hemorheologic disorders such as high blood viscosity, augmented erythrocyte aggregation and inclination of thrombogenesis (Froom, 2000; Becker et al., 1995). Therefore, it is an important strategy in preventing and curing myocardial ischemia to improve hemorheological variables, inhibit platelet activation and thrombogenesis (Sander et al., 2005).

The severe adverse reactions of current therapies to ischemic heart disease have been driving an increasing number of patients toward plants medicine. Traditional Chinese Medicine (TCM) has an over 5000-year history in preventing and curing thrombotic diseases. Many edible and medicinal plants described in the classic *Chinese Materia Medica* provide a potential resource for research and development of herbal agents removing blood stasis and inhibiting thrombosis.

Leonurus heterophyllus Sweet (LHS) is also called Chinese motherwort herb (Chinwala et al., 2003). In ancient China, *LHS* might have been used for promoting circulation, clearing clots, treating breast pain and swelling, as well as anticancer treatment (Liao, 2000). Accordingly, it is valuable to further test the effects of *LHS* on hemorheological variables and thrombus formation ex vivo in rat with myocardial ischemia so as to provide useful information for the application of *LHS* in clinic to prevent and cure ischemic heart disease.

MATERIALS AND METHODS

Subjects

All procedures and handling of animals were reviewed and approved by the Animal Care and Use Committee of Medical College of Shantou University and conformed with *the Guide for the Care and Use of Laboratory Animals* (No.2 Document from the Science and Technology Committee, the People's Republic of China, 1988). One hundred and twenty normal male Sprague-Dawley rats, weight 295 ± 42 g, were used.

Materials

Herb extract

The dried aerial part of *LHS* was used for the preparation of the extract.

LHS extract preparation

Dried *LHS* was extracted and subsequent dilutions prepared in distilled water as follows. Twenty grams of the dried herb (aerial part) was extracted with 250 ml of distilled water for a period of 1 h at room temperature. It was then heated at 70°C for 3 min and kept at room temperature or a period of 1 h. The extract was further filtered and collected. This extraction procedure was repeated once.

The filtrates obtained from both the extraction steps were combined and centrifuged at 4 000 rpm for 10 min. The supernatant of the extract was then collected, filtered, and freeze-dried. A stock solution of 100 mg raw material per milliliter was prepared from the freeze-dried powder of the herbal extract using distilled water. This solution was sterilized by filtering it through a 0.2 µm membrane and stored at 4°C. This solution had a pH value in the range of 7.35 to 7.45 (Chinwala et al., 2003).

Chromatography and mass spectrometry

Chemical fingerprint of *LHS* extract stock solution was conducted by using a Waters Alliance 2695 liquid chromatographic system (Waters Corp., Milford, MA, USA) with autosampler, vacuum degasser and column oven. Chromatographic separation was performed on an AichromBond-AQ-C18 column (5.0 µm, 250mm x 4.6 mm; Agilent Technologies, USA). The mobile phase consisted of 0.1 mol/L NaH₂PO₄ (pH was adjusted to 5.5 with 0.1 mol/L Na₂HPO₄). The flow rate was 1.0 ml/min. The detection wavelength was 242 nm, and the column temperature was set at 30°C (Tao et al., 2009).

Essential components of *LHS* extract were identified by using a Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer (ITMS, Thermo Finnigan, San Jose, CA, USA) with an electrospray ionization (ESI) interface. The acquisition parameters were collision gas, ultrahigh-purity helium (He); ion spray voltage 4.5 kV (positive mode), -4.5 kV (negative mode); sheath gas (N₂) 5 arbitrary units; auxiliary gas (N₂) 0 arbitrary units; capillary temperature 275°C; capillary voltage 19V (positive mode), -15V (negative mode); tube lens offset voltage 25V (positive mode), -30V (negative mode). All accurate masses were measured on an Apex III (7.0 Tesla) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker, Daltonics, Billerica, MA, USA). Both positive and negative ESI modes were employed. The solutions were infused from the ESI source at 3 l/min (Tao et al., 2009).

Experimental design, surgical preparation and blood sampling

Sixty normal male Sprague-Dawley rats were randomly divided into 6 groups, with 10 rats in each group. Rats in Group I and Group II were administrated intravenously with 0.85% sodium chloride (NaCl) solution (2.0 ml/kg) as control, one time a day, continuously for 7 days. While rats in Group III to Group VI received intravenously *LHS* extract solution (2.0ml/kg) also one time a day and continuously for 7 days, the dose of which was 20, 40, 80 and 100 mg/kg of *LHS* extract for each rat in Group III, Group IV, Group V and Group VI respectively. The time interval of NaCl/*LHS* extract administration in each rat was equal.

1 h after the 7th administration of NaCl/*LHS* extract solution, rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.), intubated and ventilated with room air. Simultaneously, continuous tracing of surface II-lead electrocardiogram (ECG) was obtained on a chart recorder. The heart of each rat in all groups was exposed through a left thoracotomy, a 4-0 silk was placed under the left coronary artery by briefly exteriorizing the heart, approximately 2 to 3 mm from its origin (Kim et al., 2009). After a 5 min stabilization, left coronary artery occlusion was accomplished by tying the silk under it in each rat in Group II to Group VI, and that ST-segment elevated over 0.3 millivolt from the baseline in ECG demonstrated the model of myocardial ischemia was successfully established

(Kléber, 2000). Group I served as sham-operated control group, rats in this group underwent the same surgical procedures as those in other groups except for that the silk under the left coronary artery was not tied.

After 3 h of myocardial ischemia, blood sample was collected from carotid artery for analysis, afterwards the rat was killed under 734 J. Med. Plant. Res.

deep anesthesia. Ethylenediaminetetraacetate (EDTA) served as anticoagulant in blood viscosity, plasma viscosity and hematocrit (Hct) measurement with the final concentration of 1.5 mg/ml. Erythrocyte sedimentation rate (ESR), plasma fibrinogen concentration and platelet aggregation rate were measured with anticoagulated blood by using sodium citrate as coagulant, the final concentration of which was 3.8 mg/ml except 7.6 mg/ml for ESR measurement. Blood dilution was no more than 10% after mixing of blood with anticoagulant for parameters mentioned above measurement. Blood sample without anticoagulant was used for thrombus formation *ex vivo* detection.

In addition, another sixty normal male Sprague-Dawley rats were grouped and treated as the same as mentioned above but no surgical preparation was performed. 1 h after the 7th administration of NaCl/LHS extract solution, 0.5 ml blood was obtained from each rat by arteriopuncture and collected into sodium citrate (3.8%, w/v) as anticoagulant (blood:citrate ratio, 10:1) for erythrocyte morphology analysis.

Blood sample analysis

Hemorheological variables

In the present study, we determined hemorheological variables such as blood viscosity, plasma viscosity, erythrocyte aggregation index, Hct, plasma fibrinogen, ESR and erythrocyte morphology as follows.

Blood viscosity, plasma viscosity and erythrocyte aggregation index

Blood viscosity and plasma viscosity were analyzed with LBY-N6A viscometer (Beijing Precil Instrument Co. Ltd, China) according to the manufacturer's instructions and new guidelines for hemorheological laboratory techniques (Baskurt et al., 2009). Before blood viscosity measurement, Hct was adjusted to 42% by adding or removing the calculated amounts of autologous plasma to/from the sample. Plasma was separated by centrifugation (1 500×g, 5 min at room temperature). The shear rate range was 1s⁻¹ to 200s⁻¹ for the viscometer, and viscosity measurements were made at 37°C. Blood viscosity at low shear rate of 10s⁻¹ and high shear rate of 120s⁻¹ were measured respectively. At low shear rate, erythrocytes are inclined to form aggregates, whereas at high shear rate, blood behaves like Newtonian fluid. Therefore, erythrocyte aggregation index was obtained by calculating the ratio between blood viscosity at low shear rate (10 s⁻¹) and high shear rate (120 s⁻¹) in the present study to indicate erythrocyte aggregation (Kirschenbaum, 2000).

Hct, plasma fibrinogen and ESR

Hct was determined with Wintrobe tube (Wintrobe, 1929), blood sample was carefully filled into the Wintrobe tube (DAIHAN Scientific Co., Ltd., Seoul, South Korea) to the 100 mark and centrifuged for 30 minutes at 3000 rpm, Hct was calculated by dividing the height of the column of the packed erythrocytes by the total height of the cell column and plasma.

Plasma fibrinogen was measured according to Clauss method (Clauss, 1957) with a STA automated coagulation analyzer (Diagnostica Stago, Gennevilliers, France), and reagents were also

from Diagnostica Stago (Diagnostica Stago, Gennevilliers, France). The clotting time in seconds was inversely proportional to the fibrinogen level. The assay was calibrated by using Unical (Diagnostica Stago, Asnieres, France).

ESR was determined by using the method of Westergren (International Committee for Standardization in Hematology,

1977). Blood sample was put into a Westergren tube (Ningbo Fuchun Co., Ltd. China) to the 200 mm mark. The tube is placed in a rack in a strictly vertical position for 1 h at room temperature, at which time the distance from the lowest point of the surface meniscus to the upper limit of the red cell sediment was measured. The distance of fall of erythrocytes, expressed as millimeters in 1 h, was ESR.

Erythrocyte morphology analysis

Erythrocytes were separated by centrifugation (3000 rpm for 5 min) from 0.5 ml blood obtained from rats in each group, and plasma and buffy coat were removed by aspiration. Erythrocytes were then washed two times with pH 7.2 phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM NaH₂PO₄). Erythrocyte suspensions were used at a final Hct of 35% in PBS. To induce anoxic stress, erythrocyte suspensions in Group II to Group VI were introduced into an anoxia chamber (Forma Scientific, Inc., Marietta, OH, USA). Erythrocytes in Group II to Group VI were maintained under anoxic stress at 37°C in a 0% O₂/5% CO₂~12% H₂/balance N₂ atmosphere within the anoxic chamber. The chamber environment was rendered anoxic by a palladium catalyst, and O₂ concentration was maintained at 0 PPM (Coy Laboratories products Inc., Grass Lake, MI, USA). The erythrocyte suspensions were removed from the anoxic chamber after 3 h. While erythrocyte suspensions in Group I were incubated in a 95% air/5% CO₂ incubator (normoxia) at 37°C for 3 h as control. Blood smears of erythrocytes in each group were obtained and air-dried. These were viewed by Leitz Dialux 22 (Leica, Wetzlar, Germany) transmission video microscopic system. A well-collimated beam of light from 12V/100W tungsten halogen lamp was used for viewing purposes. The erythrocyte images were obtained by video microscopic system at a magnification of 40. These images were recorded on videotape by a VHS video tape recorder (National NV-370, Japan). The recorded data of the selected erythrocytes was digitized for analysis (Babu, 2009).

During observation on erythrocyte images, the edges of the cell image often showed a sudden transition in the gray level intensity. These were enhanced by amplifying the gray level difference at these points and smoothing the areas of constant gray levels based on the computation of a local derivative operator. The pixel intensity at any point is replaced by the computed value of the intensity gradient around the neighborhood of the pixel under consideration (Babu, 2009).

The pixel gray levels of the image were compared with a selected threshold value and were classified based on whether the pixel value was greater than or less than the threshold value. If the pixel value is greater than the threshold value, a value of 1 is assigned to that pixel or 0 value otherwise. Usually a binary image of the erythrocyte obtained by this procedure had an extended boundary, with a noisy pattern of isolated intensity over the edges. In order to avoid the patches, the image was filtered by a median filter. Contour extraction was achieved by successive deletion of the outermost layer of the image until a connected unit pixel width framework or skeleton remained (Babu, 2009).

The thinned images of the erythrocytes obtained by the above procedures were used for erythrocyte shape analysis. The perimeter (P) of the erythrocyte was obtained from the thinned image by counting the number of pixels on the contour. The contour of the erythrocyte was then filled with pixels and the number of pixels in the filled region was counted as the area (A). Based on

these, perimeter to area ratio (P/A) and form factor ($FF=P^2/4\pi A$) were calculated. FF is a measure of the compactness or rudeness. Disc has the minimum FF of value equal to one. The FF of a normal erythrocyte is found to be 1. This confirms the accuracy of the method, as the normal erythrocytes are disc-shaped. The other conformity test is that the radii computed by the P and A are

comparable (Babu, 2009).

Platelet aggregation rate

Platelet aggregation rate was determined according to the method of Born (Born, 1962) with an aggregometer (model 530VS, Chrono-Log, Haverton, PA, USA). Arachidonic acid (AA), adenosine diphosphate (ADP) and collagen served respectively as aggregating agent, the final concentration of AA was 782.0 $\mu\text{mol/L}$, ADP 47.6 $\mu\text{mol/L}$ and collagen 4.8 mg/L, respectively.

Thrombus formation *ex vivo*

Thrombus formation *ex vivo* was made according to the method of Chandler (Chandler, 1958). Blood sample (1.0 ml) without anticoagulant was added immediately into the rotary circulus of MR-4 thrombometer (Shanghai Science and Tech University Gaoji Co. China) and spun for 10 min (under 37°C conditions) for thrombus formation *ex vivo*. Then the thrombus was poured out, rinsed with saline, desiccated with filter paper and measured for length and wet weight. While after being baked for 3 h at 80°C, the thrombus was determined for dry weight.

Statistical analysis

Results were expressed as mean \pm SD. Intergroup comparisons were performed with one-way ANOVA to test for difference among groups. Statistical Package for the Social Sciences software (SPSS Inc.) was used for all analyses. Multiple group comparison corrections were made by using Dunnett's T3 and Dunnett's C methods when the variances among groups were not equal. Differences were considered to be significant at a value of $P < 0.05$.

RESULTS

LHS extract fingerprint and identification of its essential components

LHS extract fingerprint was produced as described in the section of chromatography and mass spectrometry. The HPLC separation was followed by mass spectrometry analysis. Eight major components in LHS extract were identified to be rutin (1), leonurine (2), quercetin (3), stachydrine (4), kaempferol-7-O- α -L-rhamnoside (7), heteronone A (8), heterolignan (9) and kaempferol-3-O- β -D-glucopyranoside-7-O- α -L-rhamnoside (10). The fingerprint of LHS extract and the structures of its eight essential components were shown in Figure 1 A and B respectively (Figure 1 A and B).

Blood viscosity, plasma viscosity and erythrocyte aggregation index

Figure 2 summarized blood viscosity, plasma viscosity and erythrocyte aggregation index in the experiments. The results showed a significant increase in blood viscosity, plasma viscosity and erythrocyte aggregation index after myocardial ischemia ($p < 0.05$ and 0.001, Yin et al. 735

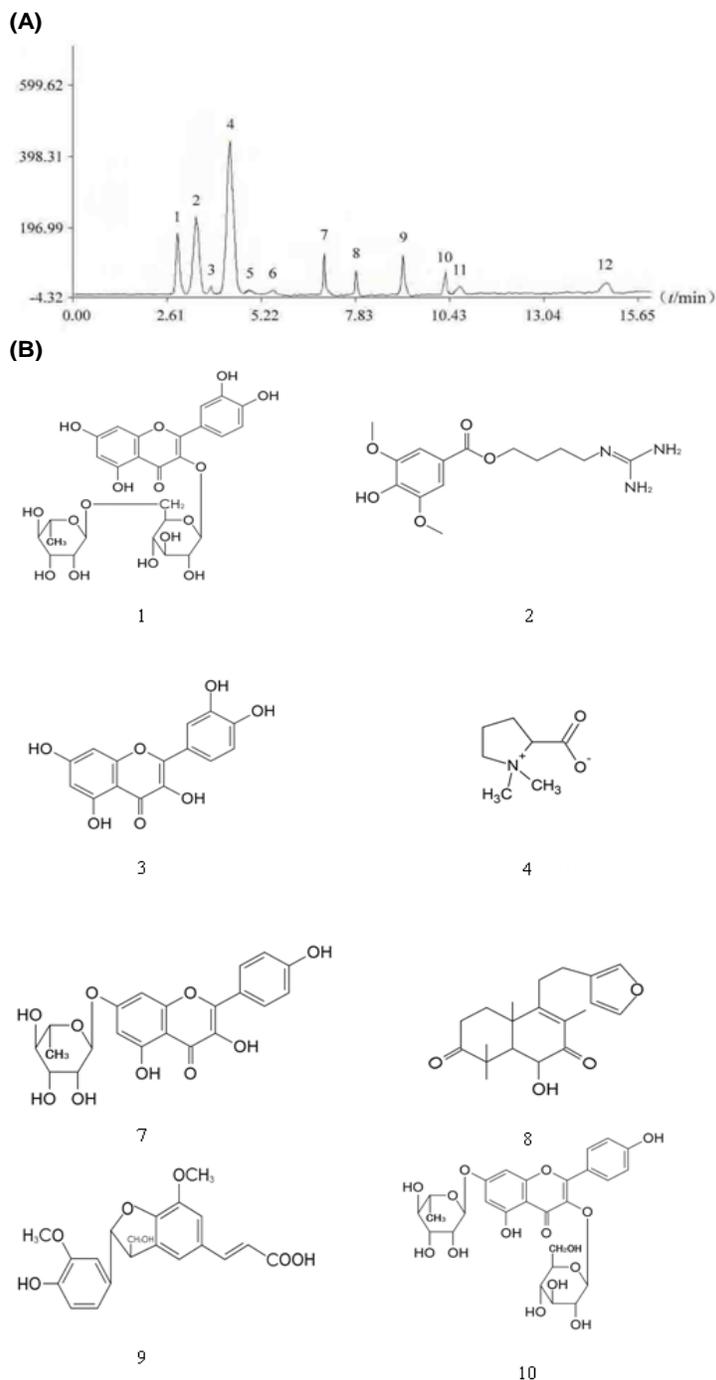


Figure 1. LHS extract fingerprint (A); Structures of eight essential components in LHS extract identified by mass spectrometry (B). The number (1, 2, 3, 4, 7, 8, 9 and 10) in B indicated the structure of the corresponding wave in A.

compared with Group I). However, *LHS* extract reduced the increased blood viscosity, plasma viscosity and erythrocyte aggregation index markedly with the dose 736 J. Med. Plant. Res.

increased ($p < 0.05$ and 0.01 , compared with Group II) (Figure 2).

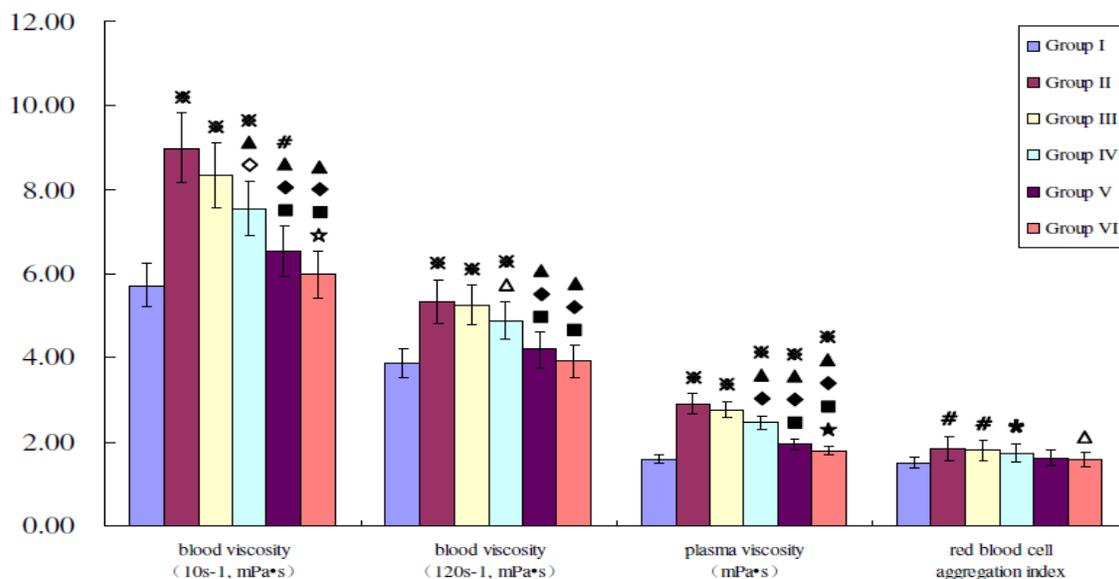


Figure 2. The effects of *LHS* extract on blood viscosity, plasma viscosity and erythrocyte aggregation index in rat with induced myocardial ischemia.

*: $p < 0.05$, #: $p < 0.01$, ✱: $p < 0.001$, compared with Group I; △: $p < 0.05$, ▲: $p < 0.01$, compared with Group II; ◇: $p < 0.05$, ◆: $p < 0.01$, compared with Group III; ▢: $p < 0.01$, compared with Group IV; ☆: $p < 0.05$, ★: $p < 0.01$, compared with Group V.

ESR, Hct and plasma fibrinogen

Table 1 showed the effects of *LHS* extract on ESR, Hct and plasma fibrinogen level. In Group II, ESR and plasma fibrinogen level increased significantly in rats after myocardial ischemia ($p < 0.001$, compared with Group I), while *LHS* extract significantly inhibited the up-regulation of ESR and plasma fibrinogen with the dose increased ($p < 0.05$ and 0.01 , compared with Group II). However, no statistical difference was displayed in Hct among the six groups (Table 1).

Erythrocyte morphology

Table 2 showed the effects of *LHS* extract on morphological changes of erythrocytes after 3 hours of anoxia. In Group II, P, P/A and FF of erythrocytes were significantly increased, and A was obviously decreased after anoxia ($p < 0.001$, compared with Group I), while the changes in morphological parameters of erythrocytes in *LHS* extract treated groups were significantly improved ($p < 0.05$ and 0.01 , compared with Group II). With the dose increased, the role of *LHS* extract was getting more effective (Table 2).

Platelet aggregation rate

Figure 3 demonstrated that there was a higher platelet aggregation rate in Group II. Table 1. Effects of *LHS* extract on ESR, Hct and plasma fibrinogen level in rat with induced myocardial ischemia.

Groups	ESR (mm/h)	Hct (%)	Fibrinogen (g/L)
Group I	9.81 ± 3.05	41.86 ± 3.38	1.98 ± 0.31
Group II	18.10 ± 5.79✱	43.14 ± 4.29	3.44 ± 0.78✱
Group III	17.73 ± 5.18✱	43.15 ± 4.31	3.13 ± 0.72✱
Group IV	15.55 ± 4.56#	42.96 ± 4.12	2.96 ± 0.65✱
Group V	12.64 ± 4.05△◇	42.57 ± 3.95	2.35 ± 0.49△◇□
Group VI	11.52 ± 3.37△◆□	42.44 ± 3.64	2.12 ± 0.35△◆□

#: $p < 0.01$, ✱: $p < 0.001$, compared with Group I; △: $p < 0.05$, ▲: $p < 0.01$, compared with Group II; ◇: $p < 0.05$, ◆: $p < 0.01$, compared with Group III; □: $p < 0.05$, ▣: $p < 0.01$, compared with Group IV;

aggregation rate with AA, ADP and collagen as aggregating agent respectively after myocardial ischemia in rats ($p < 0.05$ and 0.001 , compared with Group I), while in *LHS* extract treated groups, *LHS* extract reduced

obviously platelet aggregation rate induced by AA, ADP and collagen respectively ($p < 0.05$ and 0.01 , compared with Group II). With the dose increased, the effect of *LHS* extract was getting more pronounced (Figure 3).

Table 2. Effects of *LHS* extract on morphological changes of erythrocytes induced by anoxia.

Erythrocyte (n=10)	Perimeter (P) (pixels)	Area (A) (pixels)	P/A	Form factor (FF)
Group I	135.30 ± 15.11	1487.60 ± 122.80	0.0908 ± 0.0035	0.9810 ± 0.1403
Group II	175.00 ± 15.87*	1244.40 ± 79.81*	0.1404 ± 0.0045*	1.9613 ± 0.2378*
Group III	169.20 ± 14.06*	1283.90 ± 95.73 [#]	0.1317 ± 0.0025* [▲]	1.7759 ± 0.1684*
Group IV	162.40 ± 12.62 [#]	1310.00 ± 99.84 [#]	0.1240 ± 0.0014* ^{▲◆}	1.6031 ± 0.1294* ^{▲◇}
Group V	156.20 ± 13.36 ^{#△}	1341.50 ± 96.26* [△]	0.1163 ± 0.0021* ^{▲◆■}	1.4484 ± 0.1452* ^{▲◆◇}
Group VI	149.40 ± 13.05* ^{▲◆◇}	1371.70 ± 97.06* ^{△◇}	0.1088 ± 0.0021* ^{▲◆■☆}	1.2960 ± 0.1366* ^{▲◆■☆}

*: $p < 0.05$, #: $p < 0.01$, *: $p < 0.001$, compared with Group I;
 △: $p < 0.05$, ▲: $p < 0.01$, compared with Group II;
 ◇: $p < 0.05$, ◆: $p < 0.01$, compared with Group III;
 □: $p < 0.05$, ■: $p < 0.01$, compared with Group IV;
 ☆: $p < 0.05$, *☆: $p < 0.01$, compared with Group V.

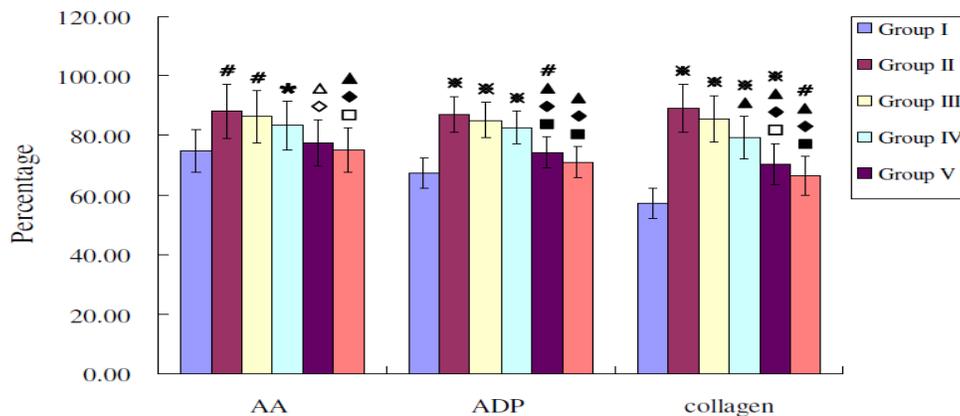


Figure 3. The effects of *LHS* extract on platelet aggregation rate with AA, ADP and collagen as aggregating agent respectively. *: $p < 0.05$, #: $p < 0.01$, *: $p < 0.001$, compared with Group I; △: $p < 0.05$, ▲: $p < 0.01$, compared with Group II; ◇: $p < 0.05$, ◆: $p < 0.01$, compared with Group III; □: $p < 0.05$, ■: $p < 0.01$, compared with Group IV.

Thrombus formed ex vivo

Table 3 displayed longer and heavier thrombus formed ex vivo after myocardial ischemia in rats ($p < 0.05$ and 0.001 , compared with Group I), however, in *LHS* extract treated groups, the length of thrombus was shorter, and the wet weight and dry weight were both lighter than those in Group II ($p < 0.05$ and 0.01 , compared with Group II). With the dose of *LHS* extract increased, the changes in thrombus formed ex vivo were more distinct (Table 3).

DISCUSSION

Myocardial ischemia is associated with up-regulated blood viscosity and plasma viscosity (Chien, 1986), which result from activated blood coagulation and other acute phase reaction (Vaziri et al., 1992). As showed in the **Table 3**. Effects of *LHS* extract on thrombus formation ex vivo in rat with induced myocardial ischemia.

Groups	Length (mm)	Wet weight (mg)	Dry weight (mg)
Group I	16.6 ± 1.3	99.5±9.8	19.2±2.9
Group II	23.9±2.0*	145.1±13.8*	28.6±4.9*
Group III	22.5±1.8*	138.5±13.1*	27.2±4.4*
Group IV	21.7±1.7* [△]	129.8±12.5* [△]	25.8±3.9*
Group V	19.2±1.5* ^{▲◆■}	118.2±11.3* ^{▲◆□}	23.3±3.6* ^{△◇}
Group VI	18.5±1.4* ^{▲◆■}	112.4±10.7* ^{▲◆■}	21.9±3.1* ^{▲◆□}

*: p<0.05, #: p<0.01, ✕: p<0.001, compared with Group I; [△]: p<0.05, [▲]: p<0.01, compared with Group II; [◇]: p<0.05, [◆]: p<0.01, compared with Group III; [□]: p<0.05, [■]: p<0.01, compared with Group IV

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erythrocyte aggregation index, ESR and fibrinogen level all went up during myocardial ischemia.

As the most important acute phase reactant, fibrinogen is a well-known consequence of acute tissue injury such as myocardial infarction, inflammation or trauma (Somer et al., 1993). Fibrinogen not only is the most important determinant of plasma viscosity, but also promotes erythrocyte aggregation (Abrignani et al., 1999). While erythrocyte aggregation plays an important role in blood viscosity, especially during low-flow and low-shear conditions, with the formation of erythrocyte aggregates tending to increase viscosity by increasing particle size and hence energy dissipation in the suspension (Chien, 1975; Schmid-Schönbein, 1988). Once erythrocyte aggregates form, the disturbance of blood flow streamlines becomes more pronounced, blood viscosity is significantly increased. Erythrocyte aggregation is thus the major determinant of blood viscosity under low shear conditions (Baskurt et al., 2003).

In addition, we also investigated morphological changes of erythrocyte after anoxia *ex vivo*. The results showed increased P and FF, and decreased A of erythrocyte induced by anoxia. It is indicated that erythrocyte deformability is impaired during anoxia. Erythrocyte deformability is closely related to blood viscosity (Baskurt et al., 2003) and has significant effects on blood flow resistance (Chien, 1987). Blood flow in vivo manifests phase separation, axial migration, and the formation of a marginal cell-poor fluid zone near the vessel wall. Axial migration is promoted by erythrocyte deformability and reduced as the cell becomes rigid (McKay et al., 1988). Deformable erythrocytes are easily oriented in the blood flow streamlines and thereby reduce blood viscosity; impaired erythrocyte deformability limits cell orientation in blood flow and thus increases blood viscosity. Normal erythrocytes are highly deformable bodies and tend to orient themselves with the flow streamlines, especially if the shear forces are high

enough to slightly deform these cells (Baskurt et al., 2003). It has been observed that deformable erythrocytes behave like fluid drops under most flow conditions (Schmid-Schönbein et al., 1971). Therefore erythrocyte deformability is the primary cellular factor affecting blood viscosity at high shear rates (Schmid-Schönbein et al., 1971; Wells et al., 1969).

High plasma fibrinogen level and impaired erythrocyte deformability induced by ischemia/anoxia facilitate hyperviscosity, which limits the fluidity of blood, reduces blood flow and thereby favors thrombogenesis (Lowe, 1998).

In general, hyperviscosity is classified into primary form, where hyperviscosity is the mechanism of disease, and secondary form, where hyperviscosity is actually caused by or at least associated with ischemia (Forconi et al., 1987). Primary hyperviscosity compromises the mechanisms responsible for the transduction of the endothelium-dependent vasodilative signals and causes

a worsened endothelial function (Gori et al., 2007). While, secondary blood hyperviscosity appears to be particularly REDOX sensitive (Baskurt et al., 1998), myocardial ischemia shows an increased blood viscosity, and this change of blood viscosity is associated with an increased production of reactive oxygen species (ROS) (Nemeth et al., 2006). ROS can rapidly react with nitric oxide (NO), which relaxes blood vessels (Schulz et al., 2004), to form the highly toxic peroxynitrite (Huie et al., 1993). Since ROS and peroxynitrite are potent toxics for endothelial structures due to their capacity to oxidize and damage or inactivate a variety of cellular structures, high concentrations of ROS and peroxynitrite may reduce the bioavailability of endothelium-derived NO, impairing its vasodilative activity, and possibly, directly counteract NO-induced protective effects, as ROS causes vasoconstriction and vascular damage (Elliott et al., 1998; Gori et al., 2007). Thus, it is commonly accepted that hyperviscosity is a negative event for cardiovascular system.

However, one has to see the other side of the coin: an increased viscosity might irritate sufficient NO release by endothelia for vasodilation. As well, by increasing the time of transit of blood through capillaries and venules, hyperviscosity might prolong the erythrocytes permanence within microvessels, and thereby increases oxygen extraction and favors oxygenation of ischemic tissues (Forconi et al., 2009). In other words, secondary hyperviscosity might be an important compensatory mechanism aimed at normalizing vascular homeostasis, this is an instinct protective response to adverse factors in the body. Certainly, an excess of this compensatory mechanism might produce the opposite effects, as persistent hyperviscosity will go awry and lead to impaired perfusion and further ischemia (Gori et al., 2007). Slower blood flow even blood stasis induced by sustained blood hyperviscosity (primary or secondary) contributes hypoxia injury to myocardial microcirculation

and destroys endothelia of small blood vessels, the impaired endothelial function causes fierce microcirculation disorder, which aggravates myocardial ischemia and hypoxia.

Our results also demonstrated the hyper-aggregation of platelets to AA, ADP and collagen *in vitro* during myocardial ischemia. Blood stasis is a precondition for local injury of coronary artery, following the arterial injury, platelets may be activated and adhere to the damaged vessel wall, release a variety of chemical mediators such as ADP and thromboxane A₂ (TXA₂), etc. which are also powerful vasoactive substances and produce vasoconstriction or even vasospasm apart from recruiting additional platelets from the circulation. Platelet-induced coronary vasoconstriction may therefore contribute to the progress of blood flow blockade and myocardial ischemia (Abrams, 1997; Köhler et al., 2009). In addition, our results also displayed an intensified thrombus formation *ex vivo* after myocardial ischemia. Increased blood

viscosity, augmented platelet aggregation and thrombogenesis result in microcirculation stagnation, which injures blood vessel endothelia and contracts capillaries. On the contrary, the contraction of microcirculation aggravates also blood stagnation, therefore a vicious circle emerges (Smith et al., 1982; Vulpis, 1999).

The present study showed that *LHS* extract retarded the increment of blood viscosity, plasma viscosity, fibrinogen level and ESR during myocardial ischemia, and ameliorated impaired erythrocytes deformability induced by anoxia. This implies that *LHS* extract improves hemorheological variables and microcirculation. The results also indicated that *LHS* extract inhibited platelet aggregation induced by AA, ADP and collagen, and prevented thrombus formation *ex vivo* after myocardial ischemia in a dose dependent manner. The interruption of platelet aggregation and thrombogenesis during myocardial ischemia can relax contracted coronary artery (Catella-Lawson, 2001). It is thus implied that *LHS* extract improves the supply of blood and oxygen in ischemic myocardium and thereby relieves myocardial injury.

While, the underlying mechanisms responsible for the beneficial changes induced by *LHS* extract in myocardial ischemia are under investigation. *LHS* extract is a mixture of many sorts of components including stachydrine, leonurine, rutin, quercetin, kaempferol-7-O- α -L-rhamnoside, heteronone A, heterolignan and kaempferol-3-O- β -D-glucopyranoside-7-O- α -L-rhamnoside, etc. Previous publication shows that *LHS* induces plasminogen activator release and thereafter leads to fibrinogen and fibrin degradation, namely fibrinolysis (Liu, 2002), which may be one of the possible mechanisms responsible for *LHS* extract induced changes in the increased plasma fibrinogen level during myocardial ischemia. As mentioned above, high level of fibrinogen leads to high plasma viscosity and facilitates the

increment of blood viscosity at low shear rate via promoting erythrocyte aggregation. Thus *LHS* extract impedes the augment in plasma viscosity and blood viscosity under low shear conditions by inhibiting high level of fibrinogen and subsequent erythrocyte aggregation. This may be one of the possible mechanisms responsible for the improvement in plasma viscosity and blood viscosity at low shear rate induced by *LHS* extract during myocardial ischemia. The results in our present study showed that *LHS* extract improved morphological changes and deformability of erythrocytes induced by anoxia. As mentioned above, impaired erythrocyte deformability manifested with increased FF results in high blood viscosity at high shear rate. *LHS* extract improves erythrocyte deformability and thereby ameliorates the increased blood viscosity at high shear rate during myocardial ischemia, this may be one of the possible mechanisms responsible for *LHS* extract induced changes in blood viscosity under high shear conditions. As to Hct, since *LHS* mainly focuses on improving the

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features of erythrocytes, and presents no effect on the quantity of erythrocytes during a short time, there was no statistical change in Hct induced by *LHS* extract during myocardial ischemia.

Previous data shows that one of the mechanisms of *LHS* inhibiting the activation and aggregation of platelets is that *LHS* antagonizes the receptor of platelet activating factor (PAF) (Lee et al., 1991). PAF causes the activation of mitogen-activated protein (MAP) kinases including p42/p44 MAP kinase (also called Erk2 and Erk1) and p38 MAP kinase by binding with specific transmembrane PAF receptors on platelets. MAP kinases are a set of important signaling molecules involved in platelet aggregation induced by aggregating agents such as AA, ADP and collagen, which can cultivate both G_q and G_i-protein linked pathways leading towards platelet aggregation (Shah et al., 2001). As an antagonist against the receptor of PAF, *LHS* interrupts the binding between PAF and its receptor on platelets, subsequently inhibits the activation of MAP kinases induced by PAF and weakens platelet aggregation. This may be one of the possible mechanisms responsible for the effect of *LHS* extract on platelet aggregation. In addition, our previous study showed that *LHS* inhibits tissue factor (TF) expression at both RNA and protein level (data not shown). Previous data shows that TF can also induce activation of MAP kinases (Poulsen et al., 1998). As mentioned above, MAP kinases play an essential role in platelet aggregation. Therefore, cutting off TF-MAP kinases pathway is another mechanism responsible for *LHS* induced changes in platelet aggregation.

TF is also the primary initiator of blood coagulation, reduced TF expression results in weakened blood coagulation. *LHS* interrupts blood coagulation via inhibiting TF expression. Thrombus formation is a process of blood coagulation, in which activation and aggregation of platelets aggravate the process. The mechanism responsible for the effect of *LHS* extract on

thrombus formation is not only ascribed to inhibition of TF expression and platelet aggregation, but also to fibrinogen reduction, for fibrinogen is a main component in thrombosis as coagulant factor I to form thrombus by converting itself into fibrin.

In conclusion, *LHS* extract improves hemorheological variables, inhibits platelet aggregation and thrombus formation *ex vivo* during myocardial ischemia in a dose dependent manner. Our findings suggest that *LHS* is a potential Chinese herb in relieving myocardial ischemia. Although some of the mechanisms responsible for the beneficial changes induced by *LHS* are being elucidated, the details of which still remain unclear and need to be further investigated.

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