Improving abnormal hemorheological parameters in aging guinea pigs by water-soluble extracts of Salvia miltiorrhiza Bunge

Wen-Chi Hou\textsuperscript{a}, Hsin-Sheng Tsay\textsuperscript{b}, Hong-Jen Liang\textsuperscript{c}, Tzung-Yan Lee\textsuperscript{d}, Guei-Jane Wang\textsuperscript{e}, Der-Zen Liu\textsuperscript{f,}\textsuperscript{*}

\textsuperscript{a} Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan
\textsuperscript{b} Graduate Institute of Biotechnology, Chaoyang University of Technology, Taichung, Taiwan
\textsuperscript{c} Department of Food Science, Yuanpei University of Science and Technology, Hsinchu, Taiwan
\textsuperscript{d} Graduate Institute of Traditional Chinese Medicine, Chang Gung University, Taoyuan, Taiwan
\textsuperscript{e} National Research Institute of Chinese Medicine, Taipei, Taiwan
\textsuperscript{f} Graduate Institute of Biomedical Materials and Engineering, Taipei Medical University, 250 Wu-Hsing Street, Taipei, Taiwan

Abstract

Salvia miltiorrhiza Bunge, known as Danshen in Chinese traditional medicine is effective at promoting blood circulation and removing (or decreasing) blood stasis. In the present study, we selected aging, 24-month-old guinea pigs as the animal experimental models and fed them a diet containing 75, 100 or 150 mg/(kg day) of water-soluble extract components of Salvia miltiorrhiza Bunge (WSm) for 28 days, respectively, in order to evaluate the effects of WSm on their abnormal hemorheological parameters.

The results showed that the blood biochemical parameters of the aging guinea pigs remained unaffected by orally given WSm compared to the controls, except that the fibrinogen levels of the group fed the high dose of WSm (150 mg/(kg day)) decreased. Aging guinea pigs fed a low dose of WSm (75 mg/(kg day)) showed no significant difference in hemorheological parameters. However, feeding of WSm at 100 mg/(kg day) (medium dose), significantly reduced erythrocyte membrane MDA levels, which probably increased erythrocyte deformability and decreased erythrocyte flow resistance, though no improvement in erythrocyte aggregation, blood viscosity, and blood viscoelasticity could be observed. Furthermore, when the dose reached 150 mg/(kg day) of WSm (high dose), a significant decrease in whole blood viscosity was observed at high, medium and low shear rates. Blood viscosity and viscoelasticity exhibited significant improvement in oscillatory measurements. Also, we found that the oxygen transport efficiency of whole blood increased.

Keywords: Salvia miltiorrhiza; Danshen; Aging; Blood viscosity; Erythrocyte deformability

1. Introduction

Hemorheology is the study of the flow of blood in relation to the pressure, flow volume, and resistance in blood vessel and includes blood viscosity, erythrocyte deformability, erythrocyte aggregability, and blood platelet aggregation. Over the past three decades, hemorheological impairment, in such forms as a rise in blood viscosity, plasma viscosity, fibrinogen levels, erythrocyte aggregation, and impaired erythrocyte deformability, has been observed in patients with cardiovascular diseases (Lowe et al., 2002; Lipowsky, 2005; Steiner et al., 2005), stroke (Ratnayake et al., 2000; Grasso, 2004), and hypertension (Sandhagen, 1999; Lip et al., 2001). Patients with neuropathies such glaucoma, Alzheimer’s disease, and even hearing impairment show a marked association with hemorheological abnormalities (Hamard et al., 1992; Wen et al., 2000; Solert et al., 2000). We do not intend to discuss the extent of association between hemorheological abnormalities and diseases in this study. However, it is known that such abnormalities generally reflect the physiological stress leading to disease occurrence.

Danshen (Salvia miltiorrhiza, Sm) is widely used as a traditional Chinese medicine with a number of physiological benefits. Zhao et al. (1996) found that a Salvia miltiorrhiza injection could...

2. Materials and methods

2.1. Animals and treatment

After 1 week of acclimation, 48 guinea pigs, 24-month-old, were randomized and divided into four groups: a control group (n = 12) received a normal diet and sterile water, and three experimental groups representing low, medium, and high doses of WSm (n = 12, respectively for each group), respectively, received an equivalent diet and water with different amounts of WSm dried powder, i.e. low dose: 75 mg/(kg day); medium dose: 100 mg/(kg day) and high dose: 150 mg/(kg day). The experiment lasted 28 days after oral administration of WSm. At experiment end, all animals were sacrificed to collect blood samples from their hearts for subsequent hemorheological measurements. The animal experimental protocols were approved by the Animal Ethics Committee of the Taipei Medical University (no: LAC-94-0010).

2.2. Preparation of WSm

The aqueous extract of Sm (WSm) was prepared as described in our earlier report (Lee et al., 2003a,b) with some modification. The powder of Sm (400 g) root was mixed with three volumes of distilled water at room temperature with continuous shaking overnight. After filtration, the WSm was concentrated and lyophilized with an approximate yield of 51%. The WSm was stored at −20°C until further use. The 20 µL of WSm (20 mg/mL, dissolved in distilled water) was analyzed by Hitachi HPLC system equipped with a photodiode array detector (L-2450) and a BioSil Aqu-ODS-W column (4.6 mm i.d. × 250 mm). The mobile phase was composed of distilled water (solvent A) and methanol (solvent B) with linear gradient elution from 0% solvent B (0 min) to 100% solvent B (50 min) and hold for 10 min. The flow rate was 1.0 mL/min. The wavelength was set at 290 nm.

2.3. Analysis of WSm constituents

Twenty microliters of WSm (20 mg/mL in distilled water) was analyzed by Hitachi HPLC (high performance liquid chromatography) system equipped with a photodiode array detector (L-2450) and a BioSil Aqu-ODS-W column (4.6 mm i.d. × 250 mm). The mobile phase was composed of distilled water (solvent A) and methanol (solvent B) with linear gradient elution from 0% solvent B (0 min) to 100% solvent B (50 min) and hold for 10 min. The flow rate was 1.0 mL/min. The wavelength was set at 290 nm. Analysis of the standard compounds of danshensu (β-3,4-dihydroxyphenol lactic acid) and salvianolic acid B was carried out under the same conditions.

2.4. Hematological measurements

Fresh blood samples from guinea pigs were collected into plastic test tubes containing EDTA (1.5 mg/mL) as an anticoag-
ulat. Blood cell counts and other hematological data such as mean cell volume (MCV) and hematocrit (Hct) were determined by an automatic cell counter (SYSMEX NE-800, TOA Medical Electronic Co., Kobe, Japan). Plasma was separated from blood by centrifugation at 1500 × g for 10 min. Plasma fibrinogen was determined by the thrombin clot technique (Rampling and Gaffney, 1976).

2.5. Plasma and blood viscosity

Plasma and blood viscosity were measured using a Rheostress 1 double cone viscometer (HAAKE Mess-Technik, Karlsruhe, Germany), with a cone angle of 1° at 310 K. The serial blood viscosities at different shear rates were determined via a testing program. Shear rates of 500, 250, and 5 s⁻¹, reflecting high, medium, and low shear rates were estimated. The viscoelasticity of blood was tested in an oscillatory mode. The oscillatory shear strain was set at a constant 5% at frequencies ranging from 0.3 to 0.1 Hz (Liu et al., 2004).

2.6. Erythrocyte membrane MDA analysis

To measure the oxidative stress of erythrocyte membranes, the level of malondialdehyde (MDA), a product of lipid peroxidation which reacts with thiobarbituratic acid (TBA), was examined by estimating the quantity of MDA–TBA complex at 532 nm with a spectrophotometer (Hitachi U2000, Hitachi Corp., Japan) (Stocks and Dormandy, 1971). The detailed preparation procedure for measuring the MDA–TBA complex was followed as per an earlier report (Jain et al., 1989). Quantities of MDA presented in the results are based on 10¹⁰ erythrocytes (Jain et al., 1989).

2.7. Flow resistance of erythrocytes

For the flow resistance of erythrocytes, we used constant flow rate filtration method for the preparation of erythrocyte suspensions (Huang et al., 2004). After separation from plasma by centrifuging the sample at 1500 × g for 10 min, the erythrocytes were washed thrice in PBS followed by suspension in 5% hematocrit. The leukocytes (concentration usually less than 100 cells mm⁻³) were filtered through a 5-μm pore size Nuclepore® membrane (13 mm diameter and an effective area of 0.8 cm² at a constant flow rate of 1.5 mL/min). The pressure–time data were measured with a pressure transducer (Model DP45, Valdyne Engineering Corp., Northridge, USA) connected to a Validyne digital transducer indicator (Model CD-23). The continuous output data of the indicator were digitized and recorded on a computer. Recorded data were played back off-line, and P₀ values for ringer solutions and Pᵢ values for erythrocyte suspensions were determined as in a previous report (Schonbein et al., 1996). The β values were calculated using the data of Pᵢ/P₀ and were indexed as the flow resistance of erythrocytes when flowing through the pores (Schmaizer et al., 1983).

2.8. The deformability and aggregability of erythrocytes

Erythrocyte deformability and aggregability were measured by laser diffraction meter. The deformability measurement is based on a laser diffraction method in which the laser beam traverses the diluted blood suspension (0.8 mL) and is diffraacted by RBC. The diffraction pattern was projected on a screen monitored by the photoelectric sensors, linked to a frame grabber integrated in the computer. The best fitting ellipse represented the deformed erythrocyte. The long and short axes, A and B, respectively, of this ellipse were used to calculate the deformation index: DI = (A − B)/(A + B). Prior to measurement, the erythrocyte suspension was suspended in a phosphate-buffer saline solution with 5.5% polyvinylpyrrolidone (PVP). The details of this procedure are described elsewhere (Schonbein et al., 1996). For the erythrocyte aggregation test, an 0.8-mL EDTA-blood sample was placed into the plate that had been used for the aggregation degree estimation. The signal obtained from the intensity of the back-scattered light measured by the photoelectric sensors was further processed by the computer to estimate the aggregation degree. In our model, the optional disaggregation shear rate and its duration prior to stopping the motor were generally set to 600 s⁻¹ and 15 s, respectively. Using syllectogram analysis to measure erythrocyte aggregation, we were able to obtain an aggregation index (AI) of erythrocytes. A detailed description of the syllectogram can be referenced in a separate report (Hardeman et al., 1994).

2.9. Erythrocyte rigidity and oxygen transport efficiency of the blood

Erythrocyte rigidity (T_K) at a shear rate of 500 and 250 s⁻¹ was calculated by the equation of Dintenfass (1975). Oxygen transport efficiency (T_E) of the blood was calculated as the ratio of the Hct to blood viscosity (Messmer et al., 1972) at a fixed shear rate.

2.10. Statistical analysis

Statistical analysis of data were performed with the SAS package (8.1, SAS Institute, Cary, NC, USA), and was performed using a one-way analysis of variance (ANOVA) followed by Duncan’s post hoc test to compare the experimental groups and control group. A value of P < 0.05 was selected as indicating statistical significance. Variability was expressed as the mean ± S.D. Linear regressions exceeding a 95% confidence level were also calculated by SigmaStat® Statistical Software (Jandel Scientific, San Rafael, CA, USA).

3. Results

Table 1 shows the comparison of blood biochemical parameters in the 24-month-old guinea pigs, 28 days after oral administration of WSm. We observed no significant change among experimental groups and the control group with respect to erythrocyte counts, leucocyte counts, and hematocrit levels. However, the fibrinogen level in the aging guinea pigs of the
Table 1
Comparisons of blood biochemical parameters in the experimental groups of aging guinea pigs orally administrated with different doses of WSm (low-dose group: 75 mg/(kg day); medium-dose group: 100 mg/(kg day); high-dose group: 150 mg/(kg day)) and the control group (no fed in WSm)

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<th>Parameters</th>
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<tr>
<td>MCV (fL)</td>
<td>81.8 ± 1.66 a</td>
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<td>81.9 ± 1.94 a</td>
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<td>81.4 ± 1.90 a</td>
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<td>Hgb (g/dL)</td>
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<td>14.4 ± 1.67 a</td>
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<td>14.5 ± 1.68 a</td>
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<td>14.5 ± 1.56 a</td>
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<td>Hct (%)</td>
<td>43.9 ± 3.4 a</td>
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<td>43.9 ± 3.3 a</td>
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<td>43.8 ± 3.2 a</td>
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<td>RBC (×10^{12} dL^{-1})</td>
<td>5.36 ± 0.32 a</td>
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<td>5.34 ± 0.29 a</td>
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<td>5.36 ± 0.30 a</td>
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<td>5.37 ± 0.27 a</td>
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<td>Fibrinogen (mg/dL)</td>
<td>326 ± 44 a</td>
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<td>337 ± 47 a</td>
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<td>318 ± 32 a</td>
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<td>285 ± 42 b’</td>
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<td>Albumin (g/dL)</td>
<td>4.72 ± 0.39 a</td>
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<td>4.65 ± 0.23 a</td>
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<td>4.78 ± 0.45 a</td>
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<td>4.68 ± 0.29 a</td>
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Table 2
Comparisons of hemorheological parameters (including the viscosity of plasma, the viscosity of whole blood on 500, 250, 5 s^{-1}, the viscoelasticity of whole blood and Aggregate index of erythrocyte) in the experimental groups of aging guinea pigs orally administrated with different doses of WSm (low-dose group: 75 mg/(kg day); medium-dose group: 100 mg/(kg day); high-dose group: 150 mg/(kg day)) and the control group (no fed in WSm)

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<td>High-dose group</td>
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<tr>
<td>η plasma (cP)</td>
<td>1.37 ± 0.12 a</td>
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<tr>
<td></td>
<td>1.37 ± 0.11 a</td>
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<td>1.36 ± 0.11 a</td>
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<td>1.28 ± 0.08 b’</td>
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<tr>
<td>η_{500} blood (cP)</td>
<td>2.63 ± 0.23 a</td>
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<td>2.59 ± 0.24 a</td>
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<td></td>
<td>2.41 ± 0.22 b’</td>
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<td></td>
<td>2.37 ± 0.23 b**</td>
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<tr>
<td>η_{250} blood (cP)</td>
<td>4.16 ± 0.23 a</td>
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<td>4.15 ± 0.29 a</td>
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<td></td>
<td>4.11 ± 0.30 ab</td>
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<td>3.88 ± 0.29 b**</td>
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<td>ηS blood (cP)</td>
<td>14.38 ± 1.92 a</td>
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<td>13.21 ± 1.89 a</td>
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<td>12.66 ± 1.88 ab</td>
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<td>11.24 ± 1.66 b**</td>
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<td>η’ blood (cp)</td>
<td>11.85 ± 1.97 a</td>
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<td>11.28 ± 2.24 a</td>
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<td>10.75 ± 2.54 a</td>
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<td>9.02 ± 1.98 b**</td>
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<td>η” blood (cP)</td>
<td>1.49 ± 0.26 a</td>
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<td></td>
<td>1.45 ± 0.33 ab</td>
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<td>1.44 ± 0.36 ab</td>
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<td>1.21 ± 0.15 b**</td>
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<tr>
<td>AI</td>
<td>2.64 ± 0.20 a</td>
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<td>2.57 ± 0.20 a</td>
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<td>2.51 ± 0.17 a</td>
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<td>2.16 ± 0.26 b**</td>
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Table 3
Comparisons of hemorheological parameters (including erythrocyte rigidity, deformability index of erythrocyte, flow resistance of erythrocytes, MDA of erythrocyte membranes) in the experimental groups of aging guinea pigs orally administrated with different doses of WSm (low-dose group: 75 mg/(kg day); medium-dose group: 100 mg/(kg day); high-dose group: 150 mg/(kg day)) and the control group (no fed in WSm)

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<td>High-dose group</td>
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<tr>
<td>T_{K50}</td>
<td>0.54 ± 0.11 a</td>
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<td>0.55 ± 0.13 a</td>
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<td>0.53 ± 0.11 a</td>
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<td></td>
<td>0.52 ± 0.12 a</td>
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<tr>
<td>T_{K250}</td>
<td>0.84 ± 0.12 a</td>
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<td></td>
<td>0.83 ± 0.09 a</td>
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<td>0.82 ± 0.14 a</td>
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<td></td>
<td>0.82 ± 0.15 a</td>
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<tr>
<td>MDA</td>
<td>5.81 ± 0.12 a</td>
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<td>5.59 ± 0.46 ab</td>
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<td></td>
<td>5.40 ± 0.32 b’</td>
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<td>4.79 ± 0.32 c’**</td>
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<td>DI_{500}</td>
<td>0.31 ± 0.02 a</td>
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<td>0.31 ± 0.02 a</td>
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<td></td>
<td>0.34 ± 0.03 b’</td>
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<td></td>
<td>0.38 ± 0.03 c’**</td>
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<tr>
<td>DI_{250}</td>
<td>0.26 ± 0.03 a</td>
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<td></td>
<td>0.26 ± 0.04 a</td>
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<td></td>
<td>0.28 ± 0.03 a</td>
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<td></td>
<td>0.31 ± 0.02 b**</td>
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<tr>
<td>β</td>
<td>9.89 ± 1.45 a</td>
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<td>9.76 ± 1.72 a</td>
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<td>9.57 ± 1.45 a</td>
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<td>8.48 ± 0.63 b**</td>
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Significant differences were observed between high-dose group and another groups only in fibrinogen level. However, there were no significant differences among various dose groups for another parameters including the MCV, Hgb, Hct and albumin level. MCV: mean corpuscular cell volume of erythrocytes; Hgb: hemoglobin; Hct: hematocrit; RBC: erythrocyte.

* P < 0.05.
** P < 0.01.

flow resistance of erythrocytes ($P < 0.05$) (Fig. 2b). Furthermore, our HPLC analysis showed that WSm contained danshensu (3,4-dihydroxyphenol lactic acid) and salvianolic acid B as compared to nil in a pure standard (Fig. 3).

4. Discussion and conclusion

In the present study, aging guinea pigs 24-months old were selected as experimental models because aging subjects are susceptible to hemorheological and biochemical abnormalities like elevations in blood viscosity (Ajmani et al., 2000), fibrinogen levels (Papet et al., 2003), erythrocyte rigidity (Tozzi-Ciancarelli et al., 1989), impaired erythrocyte deformability (Kameneva et al., 1998), lowered MCV (Yamarat et al., 2000) and lower erythrocyte counts (Coppola et al., 2000). Therefore, it is of interest to investigate changes in the hemorheological and blood biochemical parameters of aging guinea pigs after oral administration of WSm for 28 days.

Erythrocyte deformability is considered to have vital influence on peripheral microcirculation or on the blood's oxygen transport efficiency. Tillmann et al. (1980) reported that the aging process may induce an increase in blood internal viscosity and erythrocyte rigidity leading to impaired erythrocyte deformability. The previous studies of Kameneva et al. (1998) and Terranova et al. (1985) also elucidated that the aging process significantly decreases erythrocyte deformability, which was greatly affected by the changes in the membrane's mechanical properties like erythrocyte internal viscosity and rigidity. In this present work, we used MDA as an indirect indicator of erythrocyte membrane properties and expressed erythrocyte internal viscosity by the Dintenfass equation (Dintenfass, 1975). Our results also show that the increased MDA levels of the erythrocyte membrane result in more impairment to erythrocyte deformability (Fig. 2). Although oral administration of WSE for 28 days effected no significant improvement in erythrocyte internal viscosity, it decreased the MDA of the erythrocyte membrane, which is evident from the improvement in erythrocyte deformability. MDA is known as an end-product of lipid peroxidation which is mainly produced under oxidative stress. Our present results showing a decrease in the MDA level of aging guinea pigs by oral administration of WSm for 28 days is consistent with our previous findings (Lee et al., 2003a,b). We believe that this decrease was probably due to the fact that danshensu (3,4-dihydroxyphenyl-nylactic acid) (Fig. 3) acted as an antioxidant by trapping free radicals and inhibiting lipid peroxidation. Also, the contribution of salvianolic acid B, another active component of WSm, cannot be neglected (Fig. 3).

Lishnevskaia suggested that the aging process might enhance the activity of erythrocyte aggregation (Abe et al., 1984). In fact, increased erythrocyte aggregability not only raises the blood viscosity under high shear rate, it is also a risk factor for the formation of blood clots. The mechanism of erythrocyte aggregation is extremely complicated and depends on the interaction energy (attractive force) between fibrinogen and erythrocyte, which has a correlation with the erythrocyte surface charge distribution, surface geometry shape, and fibrinogen level. Therefore, we believe that the decreased erythrocyte aggregability of aging rats effected by the oral administration of WSm for 28 days is associated with the reduced fibrinogen level.

Numerous studies have documented that aging may lead to rises in blood viscosity. Abe et al. (1984) using 60-, 150-, 320- and 710-day-old rats as animal modes, thought that their impaired erythrocyte deformability might have resulted from an increase in blood viscosity. Kameneva et al. (1998) theorized that aging led to decreased erythrocyte deformability and an increase in both erythrocyte aggregation and blood viscosity. Ajmani and Rifkind (1998) emphasized that the aging process raised erythrocyte rigidity, plasma viscosity and blood viscosity. Oder et al. (1991) demonstrated that erythrocyte aggregation at a low shear rate was increased by aging and resulted in a rise in blood elasticity. In the present study, we used two different flow fields of hemodynamics to simulate blood flow in vitro and measured the blood viscosity and viscoelasticity. In steady flow conditions, shear rates at 500, 250 and 5 s$^{-1}$ represented high, medium and low shear rates, respectively. We found that oral feeding of WSm for 28 days improved the blood viscosity of aging guinea pigs at all of these shear rates. It is generally known that decreased blood viscosity under a high shear flow field results in a rise in erythrocyte deformability while under a low shear rate flow field because blood viscosity is decreased by the reduced erythrocyte aggregation. Thus, our results on blood viscosity are in good agreement with finding on erythrocyte deformability and erythrocyte aggregability. The unsteady oscillatory flow, in fact, displays the closest simulation to human blood flow. In an oscillatory model shear stress force is influenced by a frequency-dependent sinusoidal flow instead of a continuous flow. Owing to the low elasticity of blood, the measurement was performed with controlled strain and frequency. As flow proceeds, the sliding of the internal cellular structure requires a continuous input of energy, which is dissipated through viscous friction. These effects make blood a viscoelastic fluid, exhibiting both viscous and elastic properties. In the present study, oral feeding of WSm for 28 days significantly reduced blood viscosity and elasticity in aging guinea pigs. This change, we believe is caused by decreased erythrocyte aggregability. Furthermore, the improved plasma viscosity in these animals may be correlated with decreased fibrinogen levels, perhaps, partially contributed to by decreased peroxidative content in the blood plasma.

Also, we took account of the oxygen transport efficiency of blood, which reflects an inverse relation to blood viscosity, but a direct relation to hematocrit levels, that is, Hct/$\eta$ (Messmer et al., 1972). Our results demonstrated that the hematocrit levels of aging guinea pigs after 28 days of oral administration of WSm did not exhibit any significant change, but the oxygen transport efficiency of the blood increased owing to decreased blood viscosity (Fig. 1). This indicates an important means of maintaining healthy peripheral microcirculation. Thus, the above results indicate that Danshen is effective at restoring blood circulation following impairment, especially for impairment due to age-induced hemorheological abnormalities.

In conclusion, our study demonstrates a clear recovery from the hemorheological impairment of aging. 24-month-old aging guinea pigs when WSm was orally administered for 28 days. Also, WSm effected a significant reduction in erythrocyte mem-


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brane MDA levels due to its strong antioxidant property, and thus improved blood viscosity and viscoelasticity. Furthermore, blood viscosity decreased at the high shear rate. Additionally, WSm significantly reduced fibrinogen concentration in aging guinea pigs leading to decreased erythrocyte aggregation and blood viscosity at the low shear rate. In the oscillation flow mode also, WSm showed a significant decrease in blood viscosity and elasticity in aging guinea pigs. We further affirm that oxygen transport efficiency of blood in aging guinea pigs was enhanced by orally administered WSm.

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