Inhibitory Effect of the Water-Soluble Extract of *Salvia miltiorrhiza* on Neutrophil-Endothelial Adhesion

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ABSTRACT—The effect of the water-soluble extract (WSE) of *Salvia miltiorrhiza* on neutrophil-endothelial cell adhesion was investigated. Cell adhesion was evaluated by testing neutrophil myeloperoxidase activity: expression of adhesion molecules in human umbilical vein endothelial cells (HUVEC) was measured by ELISA: the neutrophil activation rate induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP) was tested by the method of nitroblue tetrazolium (NBT) reduction. The results showed that the adhesion rate of neutrophils to unstimulated HUVEC was very low. TNFα (50–800 U/ml) increased the adhesion of neutrophils to TNFα-stimulated HUVEC in a concentration- and time-dependent manner. The WSE of *Salvia miltiorrhiza* (0.01–1 mg/ml) dose-dependently inhibited the adhesion of neutrophils. The inhibitory rate of the WSE of *Salvia miltiorrhiza* at 0.01, 0.1 and 1 mg/ml was 6.2%, 17.0% and 28.0%, respectively. fMLP (10^-9 – 10^-5 M) increased the activation rate of neutrophils concentration-dependently. The WSE of *Salvia miltiorrhiza* also concentration-dependently inhibited the adhesion of fMLP-activated neutrophils to HUVEC. The inhibitory rate of the WSE of *Salvia miltiorrhiza* at 0.001, 0.01 and 0.1 mg/ml was 5.3%, 26.3% and 28.9%, respectively. Moreover, TNFα upregulated expression of adhesion molecule E-selectin, ICAM-1 and VCAM-1. The WSE of *Salvia miltiorrhiza* had an inhibitory effect on TNFα-induced expression of these molecules. These results indicated that the WSE of *Salvia miltiorrhiza* inhibited neutrophil-endothelial adhesion. The action mechanism of the WSE of *Salvia miltiorrhiza* was partly related to suppressing the expression of adhesion molecules.

Keywords: *Salvia miltiorrhiza* (water-soluble extract), Endothelial cell, Neutrophil, Cell adhesion, Adhesion molecule

The adhesion of neutrophils to endothelium is an important stage in some pathological conditions such as hypoxia, acute inflammation ischemia-reperfusion, etc. (1, 2). The adhesion was mediated by adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Many cytokines (e.g., TNFα and IL-1) could regulate adhesion molecules expression in endothelial cells (3). In vitro, adhesive neutrophils could cause endothelial cell injury mediated by neutrophil-driven elastase (4). In addition, activation and migration of neutrophils into lung or other organs contributed to inflammatory tissue injury and remodeling of tissue architecture (5).

*Salvia miltiorrhiza* BUNGE is a Chinese herb widely used for the treatment of cardiovascular disorders. The water-soluble extract (WSE) of *Salvia miltiorrhiza*, isolated from the roots of this plant, contained salvianolic acid A, salvianolic acid B, rosmarinic acid, etc. (6). Our previous studies showed that salvianolic acid B inhibited ischemia-reperfusion injury, lipid peroxidation, scavenged hydroxyl radical, and so on (7). Salvianolic acid B was also found to inhibit the expressions of adhesion molecules, reducing the binding of U937 to endothelial cells (8). However, whether the WSE of *Salvia miltiorrhiza* had some effect on adhesion of neutrophil to endothelial cell was not known. In the present experiments, the effect of the WSE of *Salvia miltiorrhiza* on neutrophil-endothelial cell adhesion was investigated.

MATERIALS AND METHODS

The anti-human E-selectin (1 2B6), ICAM-1 (W-CAM-1) and VCAM-1 (1.G11B1) monoclonal antibody were ob-
tained from NeoMarkers Co., Ltd. (Fremont, CA, USA). The goat anti-mouse Ig G labeled with horseradish peroxidase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rhTNFα was obtained from Promega (Madison, WI, USA). O-Phenylenediamine (OPD), nitroblue tetrazolium (NBT) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) were from Sigma Chemical Co. (St. Louis, MO, USA). Dextran T-500 was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Lymphocytes separation medium was from Beijing Chemical Factory (Beijing, China). The WSE of Salvia miltiorrhiza was provided by Professor Lian-niang Li (Department of Phytochemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences). Both sexes of Wistar rats (200 – 300 g) were purchased from the Experimental Animal Center of Chinese Academy of Medical Sciences.

**Cell culture**

Human umbilical vein endothelial cells (HUVEC) were cultered in M199 medium supplemented with 15% heat-inactivated fetal calf serum, 20 μg/ml endothelial cell growth factor, 20 U/ml penicillin, 100 μg/ml streptomycin, 20 U/ml heparin and 20 mM l-glutamine at 37°C and 5% CO2. Cells were used within the first 3 passages.

**Neutrophil preparation**

Rat neutrophils were isolated and purified from blood taken from Wistar rat according to the reference (9). In brief, 40 ml of blood were anticoagulated with 4 ml 3.8% sodium citrate. One milliliter of a 6% dextran T-500 solution was added to 9 ml anticoagulated blood. After the bulk of the erythrocytes had sedimented, the leukocyte-rich upper phase was added to 3 ml lymphocytes separation-medium (density 1.077) and centrifuged at 300 × g for 20 min at 4°C. The neutrophil-rich phase was obtained from the bottom. The contaminating red blood cells were removed by hypotonic lysis with distilled water for 30 s. The medium was rapidly returned to isotonicity by the addition of 2 × PBS, and centrifuged at 100 × g for 10 min. The neutrophils were washed three times with PBS and suspended in M199 medium.

**Adhesion assay**

HUVEC were seeded at 2 × 10⁴/well in 96-well microplate. Confluent HUVEC were washed two times with PBS and received fresh medium. TNFα stimulated HUVEC for 8 h, and then the medium was removed and 100 μl/well of neutrophils (5 × 10⁴/ml) were added. After incubation for 30 min, monolayers were washed three times carefully with PBS and then the neutrophil myeloperoxidase activity was tested as described in the reference (10). The absorbance at 460 nm was determined in a BioTek 2000 microplate reader. The WSE of Salvia miltiorrhiza was added 30 min prior to TNFα stimulation.

**Neutrophil activation rate determined by NBT reduction**

Neutrophils suspension (5 × 10⁴/ml) incubated with the same volume of 10⁻⁸ M fMLP for 20 min at 37°C. After incubation, 200 μl activated neutrophils was added to same volume of 0.1% NBT solution and incubated for a further 20 min at 37°C. The neutrophils were then left to stand for 10 min at room temperature. The activated neutrophils were analyzed by Wright’s staining. The neutrophils with formazan in the cytoplasm were NBT-positive cells.

**Adhesion of activated neutrophils induced by fMLP to endothelial cells**

HUVEC were preincubated with the WSE of Salvia miltiorrhiza for 30 min at 37°C before the addition of 150 μl activated neutrophils (5 × 10⁴/ml) for a further 30 min. The adhesive neutrophils were determined by adhesion assay as described as above.

**Determination of endothelial cell adhesion molecules expression by ELISA**

The expressions of E-selectin, ICAM-1 and VCAM-1 in HUVEC were analyzed by ELISA after cell fixation. For these experiments, cells were seeded into a 96-well microplate in 100 μl M199 medium. After confluence, 400 U/ml TNFα was added. Expression levels of E-selectin, ICAM-1 and VCAM-1 were determined after 4 h or 24 h. Cell monolayers were washed three times with PBS, fixed for 15 min with 2% paraformaldehyde, and 100 μl/well of primary anti-E-selectin, anti-ICAM-1 and anti-VCAM-1 antibody (1:1000 diluted in PBS containing 5% goat serum) was added, followed by incubation for 1 h at room temperature. Monolayers were then washed three times with PBS, and 50 μl/well of secondary antibody (1:500 diluted in PBS containing 5% goat serum) was added, followed by incubation for 1 h at room temperature. Monolayers were again washed three times with PBS, and 50 μl of OPD (0.4 mg/ml in citrate buffer) was added. After 15 min, the color development was stopped by the addition of 50 μl 2 M sulfuric acid. Absorbance at 490 nm was determined in a Biomek 2000 microplate reader. Cells were treated with the WSE of Salvia miltiorrhiza for 30 min prior to TNFα stimulation.

**Data analyses and statistics**

Data were given as the mean ± S.D. and the differences were calculated with Student’s two-tail t-test. A value of P<0.05 was considered to be significant statistically.
RESULTS

Adhesion of neutrophils to HUVEC stimulated by TNFα
As shown in Fig. 1, the adhesion of neutrophils to unstimulated HUVEC was low. TNFα increased the adhesion of neutrophil to HUVEC in a concentration- and time-dependent manner. The number of adhesive neutrophils was about fourfold increased compared with the control after 8-h incubation with 400 U/ml TNFα. As shown in Fig. 2, the adhesion increase was observed as early as 2 h and the peak was at 8 h. The adhesive rate increased by 180.5% ($P<0.001$). After 24 h, the adhesion declined but was still significantly increased above the control.

Effect of the WSE of Salvia miltiorrhiza on adhesion of neutrophils to TNFα-stimulated endothelial cells
The WSE of Salvia miltiorrhiza inhibited adhesion of neutrophils to TNFα-stimulated endothelial cells concentration-dependently. The inhibitory rate for the WSE of Salvia miltiorrhiza at 0.01, 0.1 and 1 mg/ml was 6.2%, 17.0% ($P<0.001$) and 28.0% ($P<0.001$), respectively (as shown in Fig. 3).

Effect of fMLP on neutrophil activation
The fMLP activated neutrophils when neutrophils were incubated with fMLP for 20 min. The fMLP effect on the activation of neutrophils was concentration-dependent as

![Figure 1](image1.png)

**Fig. 1.** Effect of TNFα at different concentrations on endothelial-neutrophil adhesion. HUVEC were stimulated with different concentration of TNFα for 8 h and then incubated with neutrophils. Adhesive neutrophils were measured by testing neutrophil myeloperoxidase activity. Data were expressed as the mean ± S.D. of 8 determinations. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control.

![Figure 2](image2.png)

**Fig. 2.** Time course of TNFα-induced endothelial-neutrophil adhesion. HUVEC were stimulated with 400 U/ml TNFα for different periods of time and then incubated with neutrophils for 30 min. Adhesive neutrophils were measured by testing neutrophil myeloperoxidase activity. Data were expressed as the mean ± S.D. of 8 determinations. ***$P<0.001$ vs control.

![Figure 3](image3.png)

**Fig. 3.** Effect of the WSE of Salvia miltiorrhiza on the adhesion of neutrophils to TNFα-stimulated endothelial cells. HUVEC were treated with or without different concentrations of the WSE of Salvia miltiorrhiza for 30 min, then stimulated with TNFα 400 U/ml for 8 h, and finally incubated with neutrophils. Adhesive neutrophils were measured by testing neutrophil myeloperoxidase activity. Data were expressed as the mean ± S.D. of 8 determinations. ###$P<0.001$ vs blank; **$P<0.01$, ***$P<0.001$, vs TNFα.

![Figure 4](image4.png)

**Fig. 4.** Effect of fMLP on neutrophil activation. Neutrophils incubated with different concentrations of fMLP for 20 min at 37°C and then the neutrophil activation rate was measured by NBT reduction. Data were expressed as the mean ± S.D. of 6 determinations. ***$P<0.001$ vs control.
shown in Fig. 4. At the concentration of 10⁻⁹ M fMLP, the neutrophil activation rate increased by 204.7% (P<0.001).

Effect of the WSE of Salvia miltiorrhiza on adhesion of fMLP-activated neutrophils to endothelial cells

The WSE of Salvia miltiorrhiza decreased the adhesion of fMLP-activated neutrophils to endothelial cells in a dose-dependent manner. The inhibitory rate for the WSE of Salvia miltiorrhiza at 0.001, 0.01 and 0.1 mg/ml was 5.3%, 26.3% (P<0.01) and 28.9% (P<0.001), respectively (as shown in Table 2).

Effect of the WSE of Salvia miltiorrhiza on the expression of adhesion molecules in TNFα-stimulated endothelial cells

In the basal condition, HUVEC expressed a low level of ICAM-1 and nearly had no expression of E-selectin and ICAM-1 in TNFα-stimulated HUVEC in a concentration-and time-dependent manner. The result was in part due to increasing expression of adhesion molecules in endothelial cells, which was in accordance with those of others (13, 14). TNFα regulated adhesion molecule expression through different signal pathways. The protein tyrosine kinase (PTK) inhibitor genistein inhibited TNFα-induced E-selectin and VCAM-1 expression, but not ICAM-1 expression (15). In contrast, PKC inhibitor H-7 prevented TNFα-induced E-selectin and ICAM-1 expressions (16).

In addition, the chemotactic peptide fMLP activated neutrophils and increased neutrophil-endothelial adhesion. FMLP could induce neutrophils shape change and increase the adhesive functions of LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (17). ICAM-1 was an important complementary endothelial ligand for Mac-1. After stimulation, Mac-1 could be rapidly mobilized to the PMN surface (18). Binding of Mac-1 to ICAM-1 led to firm adhesion of neutrophils to endothelial cells. Therefore, fMLP increased adhesion of neutrophils might be related to increasing Mac-1 expression in neutrophils.

Our results also showed that the WSE of Salvia miltior-

DISCUSSION

In this study, TNFα increased the adhesion of neutrophils to TNFα-stimulated HUVEC in a concentration-and time-dependent manner. The result was in part due to increasing expression of adhesion molecules in endothelial cells, which was in accordance with those of others (13, 14). TNFα regulated adhesion molecule expression through different signal pathways. The protein tyrosine kinase (PTK) inhibitor genistein inhibited TNFα-induced E-selectin and VCAM-1 expression, but not ICAM-1 expression (15). In contrast, PKC inhibitor H-7 prevented TNFα-induced E-selectin and ICAM-1 expressions (16).

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Our results also showed that the WSE of Salvia miltior-

Table 1. Effect of the WSE of Salvia miltiorrhiza on adhesion of fMLP-activated neutrophils to endothelial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>fMLP</td>
<td>10⁻⁹ M</td>
<td>0.38 ± 0.03***</td>
</tr>
<tr>
<td>WSE 0.001 mg/ml</td>
<td>0.36 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>WSE 0.01 mg/ml</td>
<td>0.28 ± 0.02**</td>
<td></td>
</tr>
<tr>
<td>WSE 0.1 mg/ml</td>
<td>0.27 ± 0.03***</td>
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</tbody>
</table>

Neutrophils were stimulated with 10⁻⁹ M fMLP for 20 min. HUVEC were pretreated with or without the WSE of Salvia miltiorrhiza for 30 min and then added with fMLP-stimulated neutrophils and incubated for 30 min. The adhesion of neutrophils to endothelial cells was tested by testing neutrophil myeloperoxidase activity. Data were expressed as the mean ± S.D. of 8 determinations. ***P<0.001 vs blank; **P<0.01, ***P<0.001 vs fMLP.

Table 2. Effect of the WSE of Salvia miltiorrhiza on the expression of adhesion molecules in TNFα-stimulated endothelial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose</th>
<th>E-selectin</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>0.06 ± 0.04</td>
<td>0.15 ± 0.03</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>TNFα 400 U/ml</td>
<td>0.62 ± 0.09***</td>
<td>0.59 ± 0.05***</td>
<td>0.69 ± 0.01***</td>
<td></td>
</tr>
<tr>
<td>WSE 0.001 mg/ml</td>
<td>0.54 ± 0.09</td>
<td>0.45 ± 0.03**</td>
<td>0.68 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>WSE 0.01 mg/ml</td>
<td>0.50 ± 0.04**</td>
<td>0.37 ± 0.02***</td>
<td>0.62 ± 0.02*</td>
<td></td>
</tr>
<tr>
<td>WSE 0.1 mg/ml</td>
<td>0.47 ± 0.05***</td>
<td>0.32 ± 0.04***</td>
<td>0.56 ± 0.02**</td>
<td></td>
</tr>
</tbody>
</table>

HUVEC were treated with or without the WSE of Salvia miltiorrhiza for 30 min and then stimulated with 400 U/ml TNFα for a further 4 h (E-selectin) or 24 h (ICAM-1 and VCAM-1). The expressions of adhesion molecules in HUVEC were measured by ELISA. Data were expressed as the mean ± S.D. of 8 determinations. ***P<0.001 vs blank; *P<0.05, **P<0.01, ***P<0.001 vs TNFα.
rhiza inhibited adhesion of neutrophils to TNF α-stimulated endothelial cells and adhesion of IMLP-activated neutrophils to endothelial cells. These effects were partly associated with the inhibitory effect of the WSE of Salvia miltiorrhiza on expression of adhesion molecules. The results indicated that the WSE of Salvia miltiorrhiza inhibited neutrophil-endothelial cell adhesion by suppressing E-selectin, ICAM-1 and VCAM-1 expressions. These actions of the WSE of Salvia miltiorrhiza was different from those of salvianolic acid B. Salvianolic acid B did not affect the expression of E-selectin in endothelial cells (8). Another component of the WSE of Salvia miltiorrhiza may account for the difference. These results demonstrated that the WSE of Salvia miltiorrhiza had anti-inflammatory properties and might explain their anti-cardiovascular disorders. The mechanism of action of the WSE of Salvia miltiorrhiza might help explain its efficacy in the treatment of cardiovascular diseases.

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