Proliferative inhibition of danxiongfang and its active ingredients on rat vascular smooth muscle cell and protective effect on the VSMC damage induced by hydrogen peroxide

Li Wu, Xiaorong Li, Yuhang Li, Lijuan Wang, Yu Tang, Ming Xue*
Department of Pharmacology, School of Chemical Biology & Pharmaceutical Sciences, Capital Medical University, Beijing 100069, China

Abstract
Aim of the study: Danxiongfang (DF) is a new Chinese medicine formula used to treat atherosclerosis and vascular restenosis. The active ingredients in DF are danshensu (DSS), tanshinones (cryptotanshinone, CT) and ferulic acid (FA). The aim of present study was to evaluate the inhibitory effects of DF and its active ingredients on cell proliferation and protection against hydrogen peroxide (H2O2)-induced injury in rat vascular smooth muscle cells (VSMC) in vitro.

Methods: VSMC proliferation was assayed by cell counting and measurement of cell viability using the 3-(4, 5-dimethylthiazol -2yl)-2, 5-diphenyltetrazolium bromide (MTT) method and protein content was measured by the Bradford method. The nitric oxide (NO) level was detected by an assay kit. The endothelin-1 (ET-1) level was measured by ELISA. The protective effects of DF and its active ingredients on H2O2-induced cell injury was evaluated in terms of cell viability (MTT assay), superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels. Hydroxyl free radicals generated by the Fenton reaction was detected with the spin-trapping technique on an electron spin resonance spectrometer.

Results: The results suggest that DSS, CT, FA and DF inhibited VSMC proliferation by increasing the NO level and decreasing the ET-1 content. In rat VSMCs exposed to H2O2, FA, DSS, CT and the six formulations of DF increased cell viability and SOD activity, and reduced the levels of MDA and hydroxyl free radicals. These effects of FA, DSS and CT occurred in a dose-dependent manner. Of the six formulas, DF 4 and DF 5 had the more significant activities. The effects of DF were much greater than those of the individual ingredients, even though the concentrations of these ingredients in the DF formulas were much lower than the doses of the individual ingredients used in each study, indicating markedly synergistic effects of DSS, CT and FA in DF on rat VSMCs.

Conclusions: these findings provide a pharmacological foundation for the clinical use of DF in the prevention and treatment of hyperlipidemia and atherosclerosis relevant to endothelial cell proliferation and damage.

1. Introduction
The remarkable efficacies of traditional Chinese medicine (TCM) prescriptions are gradually being recognized and accepted by an increasing number of people worldwide. In fact, most TCMs are usually prescribed in combination to obtain synergistic effects and diminish adverse drug reactions. The root of Salvia miltiorrhiza Bunge (SMB) is a well-known TCM, “Danshen”, which is widely used to treat cardiovascular diseases such as angina pectoris, myocardial infarction and atherosclerosis, anti-inflammatory, hepatitis and liver fibrosis, and has antineoplastic, antioxidant and antiplatelet aggregation activities (Gao et al., 1979; Zhu et al., 1986; Wang et al., 1996; Lin et al., 2006). The major bioactive constituents of danshen are the lipid soluble diterpenoid quinone pigments (phenanthrofuranequinones generally known as tanshinones) and the water-soluble phenolic acid compounds (e.g., danshensu (DSS), rosmarinic acid and salvianolic acids) (Kong, 1989; Xu, 1990; Don et al., 2006). Ligusticum chuanxiong Hort (LCH), another well-known TCM, “Chuanxiong”, has similar therapeutic effects to SMB.

To achieve greater therapeutic effects, SMB and LCH are commonly combined to form new preparations. There are currently several TCM preparations that contain these two herbs, such as guanxinning tablets and danxiong tongmai pellets, which are used to treat hyperlipidemia and atherosclerosis (Shi et al., 2004; Chen et al., 2005). Based on pharmacological and clinical studies, three bioactive ingredients (DSS, the main water-soluble phenolic component; cryptotanshinone (CT), the main lipophilic component...
extracted from the roots of SMB; and ferulic acid (FA), the main component extracted from LCH (Yang et al., 2002; Song et al., 2004) are combined to create danxiongfang formula (DF), which is used to treat atherosclerosis and vascular restenosis. This is a new compound preparation that has been manufactured by our laboratory. The three active ingredients are also used as biomarkers in quality control of the manufacturing process (Li et al., 2007a,b). The chemical structures of the three compounds are shown in Fig. 1.

Atherosclerosis is currently one of the most prevalent and critical diseases worldwide. Lipid peroxidation and death of endothelial cells may contribute to the initial endothelial injury, which promotes atherosclerotic lesion formation (Muzykantov, 2001). Vascular smooth muscle cells (VSMC) are one of the main constituents of the blood vessel wall, and are involved in the maintenance of vessel structure and function. The proliferation of VSMCs plays a key role in the formation and progression of atherosclerosis and vascular restenosis (Ross, 1993, 1999; Schwartz et al., 1995). Proliferation and damage of VSMCs with thickening of the intima and narrowing of the vessel lumen are hallmarks of vascular stenotic disease (Zhang and Tenne, 1999; Wang et al., 2002). Antioxidants can inhibit VSMC proliferation, and thus exhibit cardiovascular protection and anti-atherosclerotic activities (Thiemermann and Vane, 1990).

The inhibition of VSMC proliferation and the protection against VSMC damage induced by hydrogen peroxide (H2O2) of some of the constituents of tanshinones and DSS from SMB and FA from LCH have been reported on an individual basis (Hou et al., 2004a,b, 2005; Wang et al., 2004, 2005); however, the synergistic effects of DSS, tanshine (CT) and FA on rat VSMCs have not yet been reported. Therefore, it is important to investigate the inhibitory effects of DF and the major active ingredients on rat VSMC proliferation and the protective effects of these compounds on VSMC injury induced by oxidative stress. Therefore, in this study, we investigated the inhibited effect of these compounds individually and in various formulations on rat VSMC proliferation and the protective effects on H2O2-induced rat VSMC injury. These findings provide the pharmacological basis for DF in the prevention and treatment of hyperlipidemia and atherosclerosis, focusing on endothelial cell proliferation and damage.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Constitute</th>
<th>Ferulic acid (µg/ml)</th>
<th>Danshensu (µg/ml)</th>
<th>Cryptotanshinone (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.022</td>
<td>0.185</td>
<td>0.361</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.013</td>
<td>0.533</td>
<td>0.743</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.047</td>
<td>0.132</td>
<td>1.512</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.065</td>
<td>0.377</td>
<td>0.253</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.094</td>
<td>0.091</td>
<td>0.519</td>
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<td>6</td>
<td></td>
<td>0.098</td>
<td>0.265</td>
<td>1.058</td>
</tr>
</tbody>
</table>

2. Materials and methods

2.1. Reagents and chemicals

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco (Gibco Industries Inc.). Fetal bovine serum (FBS) was purchased from Hyclone (Perbio Scientific Company). N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, and trypsin were purchased from Amresco Chemical Co. Ltd. (±)-α-tocopherol and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Sigma. Nitric oxide (NO), superoxide dismutase (SOD) and malondialdehyde (MDA) assay kits were purchased from the Institute of Jiancheng Biology Engineering (Nanjing, Jiangsu, China). Endothelin-1 (ET-1) assay kit was purchased from Shanghai Xitang Biotechnological Company (Shanghai, China). H2O2 was purchased from Tianjin Chemical Engineering (Nanjing, Jiangsu, China). H2O2 and DSS were dissolved in distilled water and tanshinones (containing 90% CT) were purchased from Xi’an Honson Biotechnology Co. Ltd. (Xi’an, Shanxi, China). All other reagents and chemicals used were of the highest grade available.

2.2. Preparation and determination of danxiongfang

FA and DSS were dissolved in distilled water and tanshinones was dissolved in distilled water containing 0.1% DMSO (v/v). DF, comprising FA, DSS and tanshinones, was manufactured with defined proportions of each compound according to a Latin square design optimized. Six DF were prepared and dissolved in DMEM and sterilized by filtration to form a stock concentration. Additional concentrations of the test compounds were prepared with DMEM as the diluent. The determination of the concentration of DF and its active ingredients was performed by reverse phase–high performance liquid chromatography (RP-HPLC) according to a method previously used in our laboratory (Li et al., 2007a). The experimental conditions of the RP-HPLC method were as follows: Agilent HC-C18 column (150 mm × 4.6 mm; 5 μm), methanol–water mobile phase (from 20:80 to 80:20, v/v) containing 0.5% (v/v) glacial acid, with gradient elution at a flow rate of

Fig. 1. Chemical structures of ferulic acid, danshensu and cryptotanshinone (I.S.). (A) Ferulic acid; (B) danshensu; (C) cryptotanshinone.
Fig. 2. Effects of danxiongfang (DF) and its active ingredients on rat VSMC proliferation. (A) Ferulic acid (FA); (B) danshensu (DSS); (C) cryptotanshinone (CT); (D) different groups of DF (1–6). The data were presented as means ± S.E.M. (n=6–8). * P<0.05 and ** P<0.01, compared with normal control group. C stands for normal control group.

Fig. 3. Effects of danxiongfang (DF) and its active ingredients on rat VSMC number. (A) Ferulic acid (FA); (B) danshensu (DSS); (C) cryptotanshinone (CT); (D) different groups of DF (1–6). The data were presented as means ± S.E.M. (n=7). * P<0.05 and ** P<0.01, compared with normal control group. C stands for normal control group.
Fig. 4. Effects of danxiongfang (DF) and its active ingredients on NO production of rat VSMC. (A) Ferulic acid (FA); (B) danshensu (DSS); (C) cryptotanshinone (CT); (D) different groups of DF (1–6). The data were presented as means ± S.E.M. (n = 4–7). *P < 0.05 and **P < 0.01, compared with normal control group. C stands for normal control group.

1.0 ml/min. The detection wavelength was 281 nm for DSS and FA, and 254 nm for tanshinones. The final concentrations of FA, DSS and CT in each DF are shown in Table 1.

2.3. Cell culture

VSMC were prepared by the explant method (Skalli, 1986) from the thoracic aorta of male Sprague-Dawley rats (150 ± 30 g) obtained from the Laboratory Animal Center of the Capital Medical University (LAC, CCMU, Beijing, China). The animal experimental protocols were approved by the Animal Center of Capital Medical University. First, the thoracic aorta was freed of connective tissue and adherent fat, and the extima and the intima of the aortas were carefully removed with a mechanical method. The remainder of the aorta was rinsed and cut into about 1-mm cubes. Then, they were inoculated and placed in a culture flask containing DMEM supplemented with 20% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C until the VSMCs exhibited a typical “hill and valley” growth pattern. Finally, the identity of the VSMCs was confirmed by morphological examination and staining for α-actin. The cells became confluent and subcultured by trypsinization. Confluent cells at passages 3–6 were used in the experiments.

2.4. Effect of danxiongfang and its active ingredients on VSMC proliferation

2.4.1. Cell viability assay

Cells were counted and seeded into 96-well culture plates at a density of 1 × 10⁵ cells/well. VSMCs (100 µl of VSMC suspension per well) were cultured in DMEM containing 10% FBS. VSMCs were quiesced for 24 h and stimulated with 10% FBS in the absence or presence of various concentrations of DF and its active ingredients. The cells were treated with FA (0.5, 5, 40 or 100 µg/ml), DSS (0.4, 2, 10 or 100 µg/ml), CT (0.2, 1, 5 or 10 µg/ml) or six formulations of DF for 24 h. Then, 10 µl of MTT (5 mg/ml) was added to each well and incubated for 4h. After the culture medium was removed, 200 µl of DMSO was added to each well. Cell proliferation was assayed by the MTT method (Takahashi and Abe, 2002). The optical density (OD) of each well was immediately measured on an ELISA microplate reader (Bio-Rad 680) at 570 nm to assess cellular viability.

2.4.2. Cell number assay

Cells were seeded in 96-well culture plates at a density of 2 × 10⁵ cells/well and 100 µl of the VSMC suspension per well was cultured at 37 °C for 24 h. The cells were then incubated with FA (0.5, 5, 40 or 100 µg/ml), DSS (0.4, 2, 10 or 100 µg/ml), CT (0.2, 1, 5 or 10 µg/ml) or six formulations of DF for 24 h, trypsinized, and counted with a hemocytometer under a microscope.

2.4.3. Protein assay

Cells were seeded into 96-well culture plates at a density of 1 × 10⁵ cells/well. The cells were then pretreated with FA (20, 40, 80 or 200 µg/ml), DSS (0.4, 2, 10 or 100 µg/ml), CT (0.2, 1, 5 or 10 µg/ml) or six formulations of DF for 24 h. Each well was then washed twice with phosphate-buffered saline (200 µl) and 100 µl of cell lysis buffer was added to each well to lyse the VSMCs. The protein content was assayed by the Bradford method (Bradford, 1976).
2.4.4. Measurement of NO and ET-1 levels
After pretreatment with the different concentrations of FA, DSS, CT and DF for 24 h, the culture medium was collected to measure the levels of NO and ET-1. NO content was determined by the nitrate reductase method (Zhu et al., 2001) and the ET-1 level was measured by an ELISA assay kit.

2.5. Effect of danxiongfang and its active ingredients on 
H2O2-induced VSMC injury

2.5.1. Oxidative damage induced by H2O2 and measurement of cell viability
VSMCs (100 µl/well) were seeded into 96-well plates at a density of 1 × 10^5 cells/ml and cultured for 24 h, and were allowed to grow to the desired confluence. Different concentrations of FA (20, 80, 200 or 400 µg/ml), DSS (0.4, 2, 10 or 50 µg/ml), CT (0.2, 1 or 5 µg/ml) or six formulations of DF were added to the test wells and α-tocopherol (5 µg/ml) was added to the control wells. The cells were then incubated for 24 h, after which the cells were exposed to H2O2 for 2 h (final concentration, 2 mmol/l), except for control wells.

The viability of the rat VSMCs was measured by the MTT assay, as described in section 2.4.1. The OD of formazan that formed in the control cells was considered to indicate 100% viability.

2.5.2. Determination of SOD activity and the level of MDA
After VSMCs were exposed to the different concentrations of FA, DSS, CT and DF for 24 h, they were incubated with H2O2 for another 2 h. Samples of the supernatant were collected from each well to measure SOD activity and the level of MDA using a xanthine oxidase assay (Spitz and Oberley, 1989) and a thiobarbituric acid assay (Uchiyama and Mihara, 1978), respectively.

2.6. Effect of danxiongfang and its active ingredients on levels of hydroxyl free radicals

Hydroxyl free radicals were detected by the spin-trapping technique on a JES-FA 300 electron spin resonance (ESR) spectrometer (JEOL, Tokyo, Japan) (Zhou et al., 2003a,b). The experimental settings were as follows: central magnetic field 324.53 ± 5.00 mT; modulation frequency 100 kHz; modulation width 0.35 mT; modulation amplitude 200; microwave power 0.998 mW; time constant 0.03 s and sweep time 30 s. The reaction mixtures described below were added to a quartz capillary tube and placed in a flat cell. The ESR signals were recorded as the peak height produced by the DMPO-OH conjugate. The experiments were performed at room temperature.

2.6.1. Measurement of hydroxyl free radical in the Fenton system
The reaction mixtures consisted of 10 µl of FeSO4·7H2O, 10 µl of H2O2 (1%), 20 µl of DMPO (0.1 mol/l) and 25 µl of phosphate buffer (0.1 mol/l). Using the spin-trapping technique, the scavenging effects of the test compounds on hydroxyl radicals in molecular bone were calculated with the following formula:

\[
\text{Elimination rate} (\%) = \left[ \frac{h_0 - h_x}{h_0} \right] \times 100
\]

where \(h_0\) and \(h_x\) are the height of the second peak of the ESR signals for the test samples in the absence and the presence of the experimental compounds, respectively.
2.6.2. Scavenging effect of danxiongfang and its active ingredients on hydroxyl free radicals

Different concentrations of FA (0.2, 1, 5 or 25 µg/ml), DSS (0.6, 3, 15 or 75 µg/ml), CT (0.08, 0.192, 0.962 or 2 µg/ml) or six formulations of DF were added to the Fenton system to replace the phosphate buffer. The final concentrations of FA, DSS and CT in the formulations of DF in this experiment were only 50% of the corresponding concentrations of these compounds used individually, as shown in Table 1.

2.7. Statistical analysis

Statistical analyses for three or more groups were done using one-way analysis of variance followed by a modified least significant difference procedure (SPSS Software version 13.0, Chicago, IL, USA). Data are presented as means ± standard error of the mean and values of P<0.05 were considered statistically significant.

3. Results

3.1. Effects of danxiongfang and its active ingredients on VSMC proliferation

The effects of DF and its active ingredients on rat VSMC viability are shown in Fig. 2. Compared with the control group, FA (200 and 400 µg/ml), DSS (2, 10 and 100 µg/ml) and CT (5 and 10 µg/ml) significantly inhibited rat VSMC viability in a dose-dependent manner (P<0.05). Of the six DF formulations tested, DFs 1, 2, 5 and 6 inhibited cell viability but not statistically significantly. DF 4 significantly inhibited rat VSMC viability (P<0.01). The effect of DF 4 (9.55%) on cell viability was much greater than that associated with FA (20 µg/ml, 1.31%), DSS (0.4 µg/ml, 1.60%) and CT (1 µg/ml, 2.23%) individually, even though the concentrations of the three ingredients (FA, DSS and CT: 0.065 µg/ml, 0.377 µg/ml and 0.253 µg/ml) in DF 4 were much lower than the concentrations of the individual compound. These data indicate that DF effectively inhibits proliferation of rat VSMCs in vitro, and FA, DSS and CT act synergistically to inhibit VSMC proliferation.

All concentrations of FA and DSS and CT at 0.2–5 µg/ml significantly reduced the OD value but there was no obvious change in growth of rat VSMCs in these experiments. CT at 10 µg/ml caused cell death, suggesting that 10 µg/ml CT is cytotoxic. Therefore, the maximum concentration of CT used in the following experiments did not exceed 10 µg/ml.

3.2. Effects of danxiongfang and its active ingredients on VSMC number

The effects of DF and its active ingredients on VSMC number are shown in Fig. 3. Compared with the control group, FA (40 and 100 µg/ml), DSS (2, 10 and 100 µg/ml) and CT (1 and 5 µg/ml) significantly decreased the cell number in a dose-dependent manner (P<0.05 or 0.01). All formulations of DF, except DF 4, significantly decreased the cell number (P<0.01). DF 1 and DF 3 elicited the most marked effects on cell number. The effect of DF 1 (33.22%) on cell number was much greater than that associated with FA (20 µg/ml, 1.31%), DSS (0.4 µg/ml, 1.60%) and CT (1 µg/ml, 2.23%) individually, even though the concentrations of the three ingredients (FA, DSS and CT: 0.065 µg/ml, 0.377 µg/ml and 0.253 µg/ml) in DF 4 were much lower than the concentrations of the individual compound. These data indicate that DF effectively inhibits proliferation of rat VSMCs in vitro, and FA, DSS and CT act synergistically to inhibit VSMC proliferation.

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and FA, DSS and CT had a synergistic effect on reducing the number of VSMCs.

3.3. Effects of danxiongfang and its active ingredients on protein synthesis in VSMCs

Overall, there were no statistically significant differences between the test compounds (FA, DSS, CT and DF) and the control group. Although the protein content tended to be lower in the experimental groups, there were no obvious differences between the six formulations of DF and the active ingredients in terms of protein content. However, DF 4 seemed to have the greatest effect and decreased protein synthesis by 1.43%, which was greater than that of FA (20 μg/ml, 0.58%), DSS (0.4 μg/ml, 0.7%) and CT (5 μg/ml, 0.64%) individually. The concentrations of these ingredients in DF 4 were 0.065 μg/ml, 0.377 μg/ml and 0.253 μg/ml, respectively. Accordingly, FA, DSS and CT elicited a synergistic effect on the inhibition of VSMC protein synthesis, although the effect was very weak.

3.4. Effects of danxiongfang and its active ingredients on NO production and the level of ET-1

The effects of DF and its active ingredients on NO production are shown in Fig. 4. Compared with the control group, FA (100 μg/ml), DSS (50 and 200 μg/ml) and CT (0.2 and 1 μg/ml) significantly increased the level of NO in a dose-dependent manner (P < 0.05). CT (5 μg/ml) reduced the level of NO in rat VSMCs, perhaps due to the inhibitory effect of CT on VSMC proliferation. All formulations of DF, except DF 2, significantly increased the level of NO (P < 0.05), and DF 4 showed the most pronounced effect. DF 4 increased the level of NO by 72.2%, which was greater than that of FA (40 μg/ml, 6.10%), DSS (10 μg/ml, 10.30%) and CT (1 μg/ml, 31.56%) individually. The concentrations of these three ingredients in DF 4 were 0.065 μg/ml, 0.377 μg/ml and 0.253 μg/ml, respectively. Accordingly, most of the DFs promoted NO production in rat VSMCs, and FA, DSS and CT had a pronounced synergistic effect on the promotion of NO release in VSMCs.

We found that FA, DSS, CT and DF slightly decreased the production of ET-1, but there was no significant difference between the experimental groups and the control group. Furthermore, FA, DSS and CT did not exhibit a synergistic effect on ET-1 levels in rat VSMCs.

3.5. Effects of danxiongfang and its active ingredients on rat VSMCs exposed to H2O2

H2O2 markedly decreased rat VSMC viability. Compared with the model group, FA (200 and 400 μg/ml), DSS (10 and 50 μg/ml) and CT (1 and 5 μg/ml) significantly increased cell viability in a dose-dependent manner (shown in Fig. 5). By contrast, CT at 10 μg/ml markedly decreased VSMC viability, with severe cytotoxicity. All six formulations of DF increased cell viability, and the effect of DF 5 (113.72%) was much greater than that of FA (40 μg/ml, 6.10%), DSS (50 μg/ml, 30.90%) and CT (1 μg/ml, 31.56%) individually. The concentrations of these three ingredients in DF 5 were 0.065 μg/ml, 0.091 μg/ml and 0.519 μg/ml, respectively. Accordingly, these results indicate that DF protected rat VSMCs from oxidative stress-induced cellular injury, and FA, DSS and CT had a marked synergistic effect.
3.6. Effects of danxiongfang and its active ingredients on SOD activity and the level of MDA in rat VSMCs exposed to H$_2$O$_2$

As shown in Fig. 6, H$_2$O$_2$ significantly decreased SOD activity. Compared with the model group, FA (200 and 400 μg/ml), DSS (10, 50 and 200 μg/ml) and CT (1 and 5 μg/ml) significantly increased the SOD activity in a dose-dependent manner (P<0.05). All six formulations of DF increased SOD activity, and the effect was greatest with DF 5.

As shown in Fig. 7, H$_2$O$_2$ significantly increased the level of MDA in the VSMCs. Compared with the model group, FA (80, 200 and 400 μg/ml), DSS (50 and 100 μg/ml) and CT (5 μg/ml) significantly reduced the MDA level in a dose-dependent manner (P<0.05). All formulations of DF, except for DF 6, reduced the level of MDA, and the effect was greatest with DF 5.

DF 5 increased SOD activity by 45.76%, which was much higher than that with FA (200 μg/ml, 11.77%), DSS (10 μg/ml, 13.96%) or CT 5 μg/ml (15.01%) individually, and reduced the MDA level by 70.73%, which was greater than that with FA (80 μg/ml, 35.32%), DSS (50 μg/ml, 24.15%) and CT (5 μg/ml, 16.28%) individually. The concentration of these three ingredients in DF 5 were 0.094 μg/ml, 0.091 μg/ml and 0.519 μg/ml, respectively. Accordingly, these data indicate that DF enhanced the activity of anti-oxidative enzymes and reduced the production of thiobarbituric acid reactive substances in rat VSMCs. FA, DSS and CT do not have a synergistic effect on SOD activity and MDA levels.

3.7. Effects of danxiongfang and its active ingredients on scavenging hydroxyl free radicals

FA, DSS, CT and DF markedly decreased the peak heights of the ESR signals produced by DMPO-OH conjugates, indicating that these compounds could scavenge hydroxyl free radicals in vitro in a dose-dependent manner. DF 3 showed the greatest scavenging activity (shown in Fig. 8). The data indicated that DF and its active ingredients significantly reduce the levels of hydroxyl free radicals in vitro. The scavenging activity of DF 3 on hydroxyl free radicals was greater than that of FA and DSS, but was less than that elicited by CT at the same concentration. Therefore, it seems that FA, DSS and CT do not have a synergistic effect on hydroxyl free radical scavenging in vitro.

4. Discussion and conclusion

Abnormal proliferation of VSMCs is one of the critical stages in the pathological processes involved in vascular proliferative diseases such as atherosclerosis, hypertension and coronary heart disease (Lu et al., 2006). Therefore, inhibition of VSMC proliferation is a fundamental pathway to treat vascular proliferative diseases. It has been reported that FA and LCH have inhibitory effects on VSMC proliferation (Hou et al., 2004a, 2005) and tanshinones were protective against endothelial cell damage caused by H$_2$O$_2$ by decreasing the level of MDA (Lin et al., 2006; Wang et al., 2006). However, until now, no studies have investigated the synergistic effect of FA, DSS and tanshinone (CT) on VSMC proliferation and H$_2$O$_2$-induced cell injury. Therefore, it was important to investigate the synergistic effects of these active ingredients to develop novel anti-atherosclerotic drugs, to better understand the mechanism of action of these active ingredients and to establish more effective and safer formulations for use in clinical practice. Our data indicate that both DF, and its active ingredients, inhibit the proliferation of VSMCs by inhibiting protein synthesis and decreasing cell viability, and thus decreasing cell number. Aberrant VSMC proliferation might, in part, be due to an imbalance between NO and ET-1 expres-
sion. DF and its active ingredients seemed to play an inhibitory role on VSMC proliferation by promoting NO production and decreasing the level of ET-1. DSS, CT and FA stimulated the release of NO in a dose-dependent manner, suggesting that the anti-proliferative effects of DF and its active ingredients on rat VSMC may be associated with NO production.

There is considerable evidence that oxidative damage contributes to the development of atherosclerosis through the formation of reactive oxygen species (ROS) and lipid peroxidation. Hydrogen peroxide can decrease cell viability and induce cell apoptosis (Stridh et al., 1998). Some antioxidant enzymes such as SOD are thought to be effective in the augmentation of antioxidant defenses in VSMCs (Hermann et al., 1997). Our present study was performed to assess the possible synergistic effects of the ingredients of DF on H$_2$O$_2$-induced oxidative damage of rat VSMCs, which are involved in atherosclerosis. We found that DSS, FA and CT significantly increased SOD activity and reduced the level of lipid peroxidation metabolites in cells exposed to H$_2$O$_2$ in a dose-dependent manner, indicating that these compounds protected rat VSMCs from H$_2$O$_2$-induced injury via an anti-oxidative approach. DF 4 and DF 5 showed the greatest effect on inhibiting rat VSMC proliferation and on protecting against H$_2$O$_2$-induced injury of rat VSMCs. FA, DSS and CT showed potent synergistic effects on most of the targets examined in this study.

The in vitro scavenging effect of DF on hydroxyl free radicals was much greater than that of FA and DSS at similar concentrations. Tanshinone IIA was shown to directly transfer electrons to oxygen and to protect against ischemia–reperfusion injury via an electron transfer reaction in mitochondria, and thus prevent the formation of reactive oxygen radicals (Zhou et al., 2003a,b). Many important chemotherapeutic drugs have a quinone fragment in their molecular structure. Redox cycling of quinones, catalyzed by one-electron transfer enzymes, leads to the production of ROS. The hydroxyl free radical can be eliminated by a dismutation reaction to form H$_2$O$_2$ and O$_2$, and H$_2$O$_2$ can be further converted to H$_2$O. CT is an abietane-type diterpenoid quinone analog with an ortho-quinone chromophore in its aromatic ring C and undergoes rapid biotransformation to tanshinone IIA, which can accept an electron from the complex in system. In turn, tanshinone IIA can mediate an electron transfer reaction to form simequinone free radicals, and scavenges hydroxyl free radicals via a currently unknown reaction mechanism. Therefore, more studies of the electron transfer mechanism of tanshinones are needed to better understand this pathway. DF 3 had a more potent scavenging effect on hydroxyl free radicals compared with the other DFs evaluated, but its effects on VSMC proliferation and H$_2$O$_2$-induced VSMC injury were relatively weak, phenomena that warrant further investigation of the mechanism of action of DF.

Lipid peroxidation in endothelial cells and inflammation are now recognized as key factors involved in atherosclerosis (Ross, 1993, 1999). Our previous investigations revealed that DF had powerful lipid regulating actions, credible anti-inflammatory effects (Li et al., 2008a,b; Wang et al., 2008), and protected against hypoxia in the brain (Chen et al., 2008) and liver injury induced by CCL$_4$ (Li et al., 2007c). These data indicate that there is relationship between the pharmacological effects observed in vivo and those observed in vitro. These results provide convincing evidence that DF could be used in the future for the prevention or treatment of hyperlipidemia and atherosclerosis in clinical practice.

In conclusion, we have demonstrated that the ingredients of DF have a significant synergistic effect on the inhibition of rat VSMC proliferation and protection against cell injury induced by H$_2$O$_2$ via anti-oxidative and anti-inflammatory pathways. DF and its active ingredients increased the level of NO and decreased the ET-1 content. In H$_2$O$_2$-induced VSMC injury, FA, DSS, CT and the six DF formulas increased cell viability and SOD activity, and reduced the level of MDA. Furthermore, these compounds reduced the levels of hydroxyl free radicals. Of the six formulations tests, DF 4 and 5 had the most marked activities. Accordingly, these findings provide a pharmacological foundation for the clinical use of DF in the prevention and treatment of hyperlipidemia and atherosclerosis relevant to endothelial cell proliferation and damage.

Acknowledgements

The authors thank the National Foundation of Natural Sciences of China (Grant No. 30472057), National Natural Science Foundation Program (Grant No. 7052007) and Scientific Program of Beijing Municipal Commission of Education (Grant No. KM200410025003) for their financial supporting.

References


