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Article

The Protective Effects of the Active Fraction of Shaofu Zhuyu Decoction on Hydrogen Peroxide-Induced Oxidative Injury in Vascular Smooth Muscle Cells

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Abstract: In this paper, the protective effects of the active fraction (SF-7) from Shaofu Zhuyu decoction (SFZYD) were tested on a hydrogen peroxide (H_2O_2)-induced rat vascular smooth muscle cells (VSMCs) oxidative injury model. This active fraction (SF-7) shows potent antioxidant properties. The cell viability and oxidative damage markers of VSMCs were determined after exposure to H_2O_2 for 16 hours. It was observed that SF-7 significantly increased cell survival and reduced apoptosis of H_2O_2-injured VSMCs. Moreover, SF-7 could markedly increase intracellular superoxide dismutase (SOD) activity and decrease the malondialdehyde (MDA) level in H_2O_2-injured VSMCs, and suppress the generation of intracellular reactive oxygen species (ROS), as well as intracellular Ca^{2+} concentration. Thus, SF-7 exhibits protective effects against H_2O_2-injury on VSMCs, which may be associated with its antioxidant properties. It is suggested that SF may be useful in the treatment of blood stasis syndrome in which oxidative injury is mainly implicated.

Keywords: blood stasis syndrome; oxidative damage; reactive oxygen species; apoptosis; vascular smooth muscle cells

1. Introduction

Shaofu Zhuyu decoction (SFZYD), created by Qingren Wang in the Qing Dynasty of China is a Traditional Chinese Medicine formula for treating blood stasis syndrome. SFZYD consists of ten crude herbs: *Angelica sinensis*, *Ligusticum chuanxiong*, *Paeonia lactiflora*, *Zingiber officinale*, *Cinnamomum cassia*, *Foeniculum vulgare*, *Commiphora myrrha*, *Trogopterus xanthipes*, *Typha angustifolia* and *Corydalis yanhusuo*. This ancient prescription has been used in the clinical treatment of gynecology diseases in China, such as primary dysmenorrheal, menoxenia, pelvic inflammation, etc [1]. A recent study showed that SFZYD could significantly inhibit the constriction of uterine smooth muscle and possess anti-inflammatory activity [2]. A recent study also showed that SFZYD could significantly improve hemorheological indexes of rats in the model of blood stasis and regulation for the function on rat ovary [3].

In our previous study, using the *in vitro* uterine smooth muscle contraction model, an active fraction (SF-7) with obvious inhibitory activity on mouse uterus contraction of SFZYD was isolated [4,5]. It was also found that SF-7 showed other significant activities, such as inhibiting platelet aggregation, promoting rat ovarian granulosa cells proliferation and inhibiting NO of rat peritoneal macrophages. Therefore, the results above implied that SF-7 might be one of the active fractions and contribute to the efficacy of the whole-formula SFZYD.

In blood stasis syndrome, uterine smooth-muscle contraction leads to vascular pressure and the transient ischemic/reperfusion of myometrium and endometrium. As a result, uterine tissue cells generated more reactive oxygen species (ROS). Excessive ROS are able to produce cellular membrane lipid peroxidation, lipid–protein interaction alteration, enzyme inactivation and DNA breakage, and in the end, to cause cell injury (e.g. vascular endothelial cells and vascular smooth cells), apoptosis or necrosis. These detrimental effects are attributed to enhancing intracellular ROS and Ca^{2+} concentration and to activating inflammatory reactions and apoptotic pathway [6, 7].

The anti-oxidative and anti-inflammatory actions of the herbs and some compounds in SFZYD had been reported previously [8-15]. In addition, we investigated the protective effects of SF-7 on human umbilical vein endothelial cell (HUVEC) damage induced by adrenaline. The results showed that SF-7 could significantly inhibit the ET release, reverse the NO secretion and promote the PGI_2 release of HUVEC [16]. In blood stasis syndrome, besides to endothelial cells, vascular smooth muscle cells (VSMCs) are one of the main constituents of the blood vessel wall, and are involved in the maintenance of vessel structure and function. Nowadays, more abundant vascular damages are evidenced by the oxygenation of endothelial cells and smooth muscle cells. Oxygen free-radical attack seems to greatly contribute dominant pathogenesis of vascular disease [17].

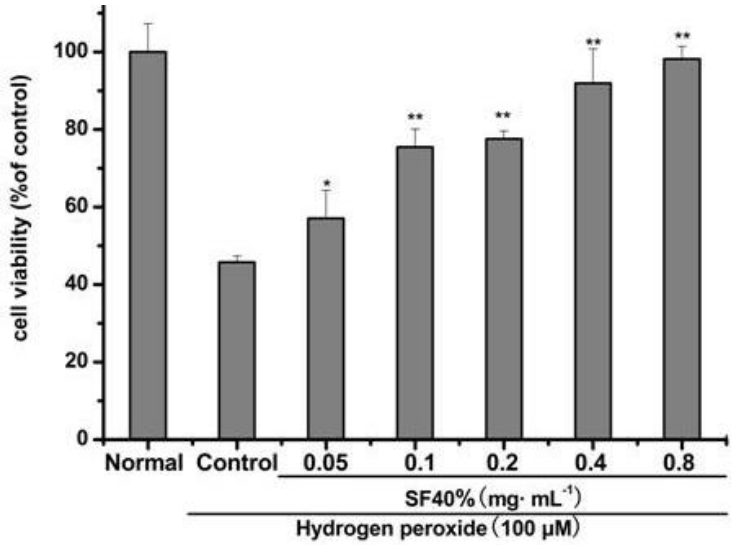
Therefore, in the present study, using the oxidative injury model of rat VSMCs induced by hydrogen peroxide (H_2O_2), which was widely used as exogenous ROS to produce oxidative stress *in vitro* study [18-23]. This paper evaluated the protective effects of SF-7. The aim of this study may provide scientific information to further understanding of the mechanism of action of this formula in blood stasis syndrome.

2. Results and Discussion

2.1. Effect of SF-7 on viability of H_2O_2-injured VSMCs

To determine the effect of SF-7 on H_2O_2-induced injury, VSMCs were treated with different concentrations of SF-7 and 100 μM H_2O_2 for 16 h. Cell viability was then examined using an MTT mitochondrial function assay. As shown in Figure 1, 100 μM H_2O_2 significantly decreased cell viability, which was concentration-dependent attenuated by SF-7 treatment.

Figure 1. Effect of SF-7 on viability of H_2O_2-injured VSMCs.



Cell viability was examined by the MTT assay. Data are presented as mean ± SD (n = 6) of three independent experiments. (*P < 0.05 or **P < 0.01 *vs.* H_2O_2 group).

2.2. Effects of SF-7 on LDH leakage, MDA level and SOD activity in H_2O_2-injured VSMCs

A significant increase in LDH release reflecting injury was also observed. The LDH activity was significantly induced by 100 μM H_2O_2. As shown in Table 1, the activity of LDH increased to 148.97 ± 18.88% of the normal value. Different concentrations of SF-7 could inhibit the LDH leakage.

Table 1. Effects of SF-7 on LDH leakage, intracellular MDA level and SOD activity in H_2O_2-injured VSMCs.

Group	LDH	MDA	SOD
	(% of normal)		
Normal	100.00 ± 1.70	100.21 ± 4.65	99.36 ± 4.24
Control	148.97 ± 18.88	146.77 ± 4.23	46.28 ± 4.35
0.1 mg·mL^{-1}	145.62 ± 4.88	127.83 ± 16.40*	65.57 ± 2.22**
0.2 mg·mL^{-1}	124.00 ± 2.85**	123.09 ± 12.53**	76.48 ± 4.43**
0.4 mg·mL^{-1}	107.08 ± 6.29**	119.94 ± 7.23**	96.31 ± 3.81**

The culture medium from each treatment was collected and the LDH activity was analyzed. The intracellular MDA level and SOD activity were determined with spectrophotometry. Data were presented as mean ± SD (n = 6) of three independent experiments. (*P < 0.05 or **P < 0.01 *vs.* H_2O_2 group.)

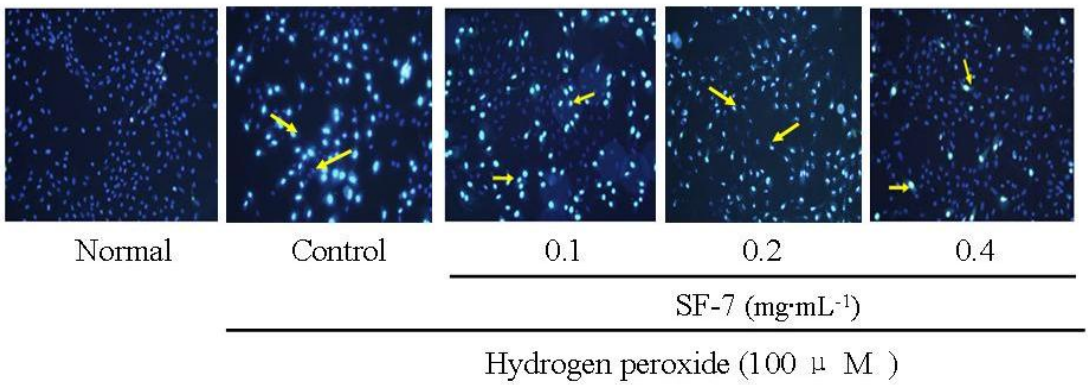
After exposure to 100 μM H_2O_2 for 16 h, the intracellular MDA level increased to 146.7 ± 4.23% and SOD activity reduced to 46.28 ± 4.35% of the normal value, suggesting that H_2O_2 induced production of thiobarbituric acid reactive substances and inhibited SOD activity. Treatment of the cells with different concentrations of SF-7 lowered MDA level and reversed the decreased SOD activity induced by H_2O_2.

2.3. Effect of SF-7 on apoptosis in H_2O_2-injured VSMCs

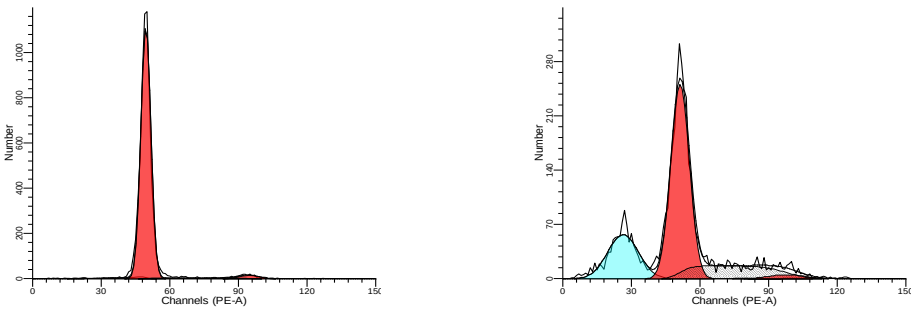
To observe the effects of SF-7 on H_2O_2-induced apoptosis, the cells were stained with Hoechst33342. As shown in Figure 2A, the normal group appeared homogeneous blue fluorescence. But the cells exposed to 100 μM H_2O_2 for 16 h displayed blazing blue fluorescence compared to the normal group.SF-7 treatment obviously attenuated VSMCs apoptosis induced by H_2O_2. Similarly, as shown in Figure 2B, using flow cytometry, a significant induction of cell apoptosis by H_2O_2 were detected, increasing the percentage of apoptotic cells to 21.84% as compared to normal cells. Cell apoptosis induced by H_2O_2 was decreased with different concentrations of SF-7.

Figure 2. Effect of SF-7 on apoptosis in H_2O_2-injured VSMCs. (A) Morphological apoptosis was determined by staining with Hoechst 33342. Arrowheads indicted apoptosis cells. (B) The apoptosis were determined by flow cytometry. Data were presented of three independent experiments.

(A)



(B)

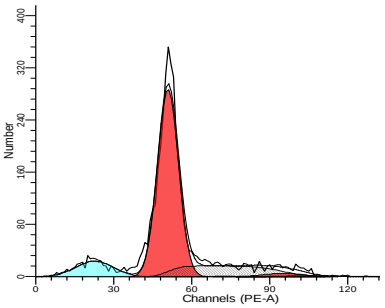


Normal: Apoptosis: 0.00%

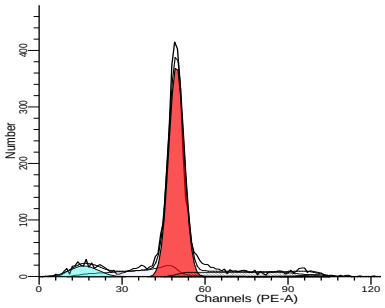
Control: Apoptosis: 21.84% ± 4.12 (n = 3)

Hydrogen peroxide (100 μM)

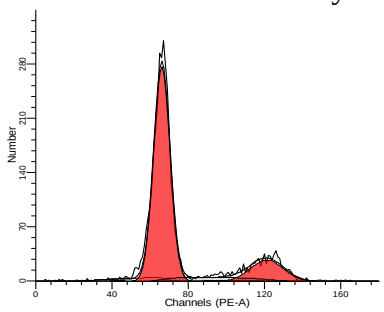
Figure 2. *Cont.*



0.1 mg·mL^{-1}SF:
Apoptosis: 10.35% ± 3.57 (n = 3):



0.2 mg·mL^{-1}SF:
Apoptosis: 7.56% ± 5.92 (n = 3)
Hydrogen peroxide (100 μM)



0.4 mg·mL^{-1}SF: Apoptosis: 0.21% ± 2.48 (n = 3)
Hydrogen peroxide (100 μM)

2.4. Effect of SF-7 on intracellular ROS concentration in H_2O_2-injured VSMCs

ROS were considered to play an important role in H_2O_2-dependent cell death. To elucidate the effect of SF-7 on H_2O_2-induced oxidative stress, levels of ROS production in cells were measured using the fluorescence probe DCF. As shown in Figure 3A, and Figure 3B the cells exposed to 100 μM H_2O_2 for 16 h displayed increased intensity of DCF-labeled cells. SF-7 treatment could attenuate the increase in fluorescent intensity. SF-7 showed significant inhibition of H_2O_2-induced intracellular accumulation of ROS.

Figure 3. Effect of SF-7 on intracellular ROS in H_2O_2-injured VSMCs.

(A)

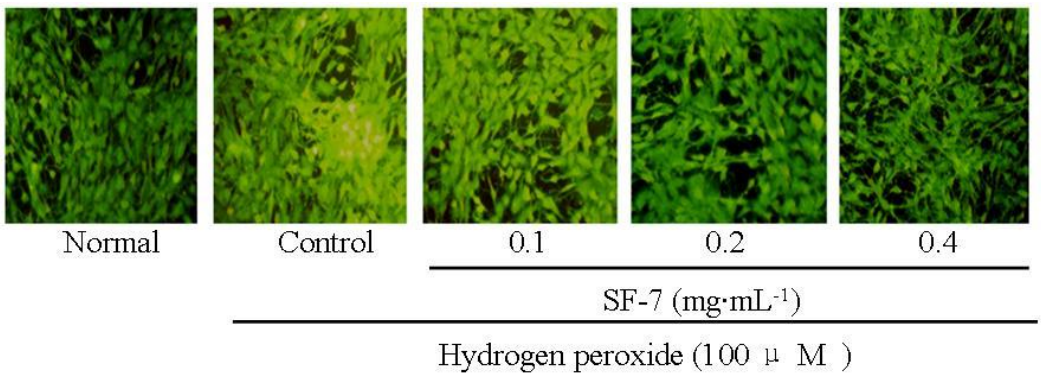
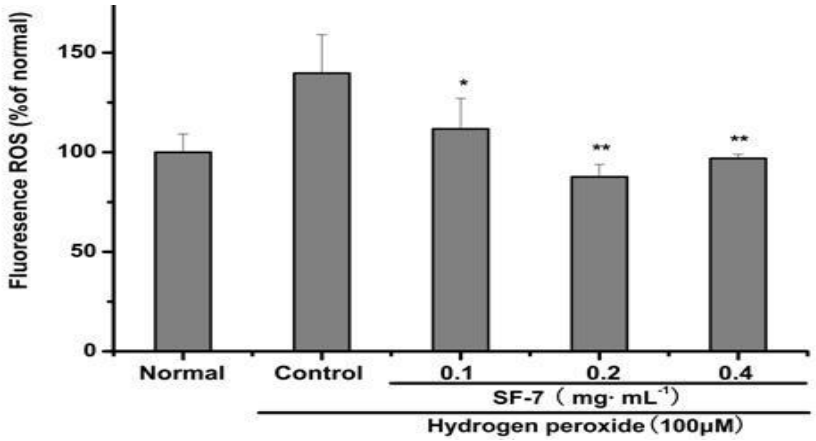


Figure 3. *Cont.*

(B)

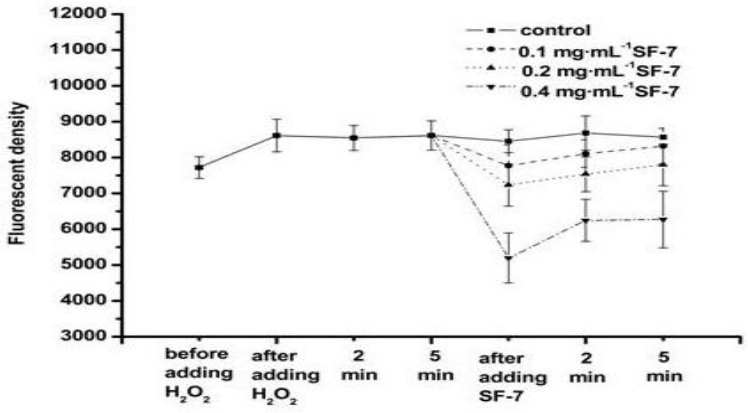


Intracellular ROS level was determined based on peroxide-sensitive DCF fluorescence. The results were expressed as the original figures of fluorescent pictures by observation under fluorescence microscope (A) and percentage of ROS fluorescent density of the normal cells was detected (B). Data were presented as mean ± SD (n = 6) of three independent experiments. (*P < 0.05 or **P < 0.01 *vs.* H_2O_2 group.)

2.5. Effect of SF-7 on intracellular Ca^{2+} concentration in H_2O_2-injured VSMCs

As shown in Figure 4, the intracellular Ca^{2+} concentration of the cells increased after exposure to 100 μM H_2O_2. Treatment of the cells with SF-7 decreased the intracellular Ca^{2+} concentration especially the high dose group. The result suggested that SF-7 could reduce the intracellular Ca^{2+} concentration in H_2O_2-injured VSMCs.

Figure 4. Effect of SF-7 on intracellular Ca^{2+} in H_2O_2-injured VSMCs.



Intracellular Ca^{2+} levels were determined based on fluorescence density. Data were presented of three independent experiments.

In this study, time-dependent and concentration dependent studies of viability losses in VSMCs induced byH_2O_2. SF-7 (0.05–0.8 mg·mL^{-1}) showed the most markedly preventive effects on cell injury

induced by H_2O_2 at 100 μM as compared to other concentrations tested. The magnitude of cell injury peaked at 16 h after H_2O_2 exposure. Based on these results, VSMCs were treated with 100 μM of H_2O_2 for 16 h.

The present results provide direct and visible evidence of SF-7 for protection against H_2O_2-injured VSMCs using the MTT method and LDH leakage assays. In this study, we first demonstrated that the active fraction (SF-7) from an aqueous extract of SFZYD could significantly protect the VSMCs against H_2O_2 induced injury. SF-7 significantly prevented the decrease in cell viability and LDH leakage in H_2O_2-injured VSMCs. With increasing concentrations of SF-7, the cell viability approached the normal level.

In the VSMC oxidative injury model, MDA is produced under oxidative stress and reflects oxidative damage of cell membrane and resultant thiobarbituric acid reactive substances, which are proportional to lipid peroxidation and oxidant stress. However, the activity of antioxidant enzymes in cells, such as SOD, for scavenging reactive oxygen species to prevent cell damage, is reduced in oxidative damage. In this study, treatment of VSMCs with H_2O_2 caused the decline of SOD activity and increase of MDA level, while incubation of the cells with SF-7 reversed the changes. It could be suggested that the protective effect of SF-7 was related to its antioxidant ability.

Exposure of VSMCs to H_2O_2 also followed an increased VSMCs apoptosis which was attenuated by SF-7, suggesting that SF-7 could act by reduction of apoptosis. Therefore, these results clearly demonstrated that SF-7 exerted protective effects on oxidative damages.

In the vascular system, ischemia injury including inflammation, thrombosis, and angioplasty are accompanied by excessive productions of ROS [24-26]. In order to further investigate any relationship between SF-7 inhibition of oxidative damages and the antioxidant properties, ROS generation was assessed in VSMCs treated with SF-7. Addition of SF-7 strongly suppressed the increase in H_2O_2 stimulated DCF fluorescence, which indicated strong suppression of intracellular ROS generation. In the present report, after exposure to H_2O_2, there was a marked increase of intracellular ROS formation accompanying elevation of intracellular free Ca^{2+} level. It was considered as the result of membrane depolarization leading to the opening of ion channels and increasing Ca^{2+} influx through Ca^{2+} channels. But treatment with SF-7 could significantly reduce the intracellular ROS formation and block H_2O_2-induced Ca^{2+} influx. This indicated that SF-7 could attenuate intracellular ROS and Ca^{2+} level [27, 28]. Since ROS have been implicated in many pathologic states, it seems possible to speculate that SF-7 might reduce the intracellular Ca^{2+} level via the antioxidant reaction, or thereby modulating the cellular responses to oxidative injury, but the exact mechanism is not yet clear.

In summary, the results demonstrated that SF-7 inhibited H_2O_2 induced injury in VSMCs. These protective effects may be attributed to anti-oxidative actions associated with inhibition of intracellular ROS generation and Ca^{2+} influx. It was reported that SF-7 contained many compounds including paeoniflorin, ferulic acid, typhaneoside, isorhamnetin-3-O-neohesperidin, senkyunolide I and senkyunolide H and quercetin. The preliminary study showed that several compounds may be responsible for its activity [29-31]. Therefore, it is necessary to study the antioxidant ability and mechanisms of these compounds in the future. In conclusion, the findings in this study suggested that SF-7 may be used as a feasible alternative therapeutic agent for oxidative damage of the transient ischemic/reperfusion in blood stasis syndrome.

3. Experimental

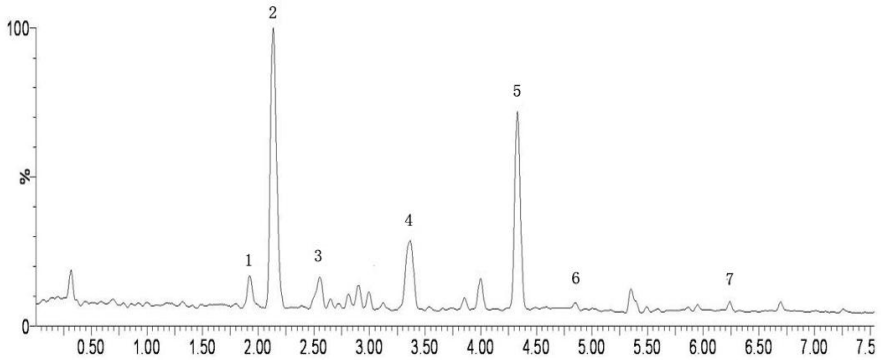
3.1. Materials

Dulbecco's Modified Eagles Medium (DMEM), trypsin, and 3-(4,5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hangzhou Sijiqing Bioengineering Institute (Hangzhou, Zhejiang, China). Lactate dehydrogenase (LDH), malondialdehyde (MDA), and superoxide dismutase (SOD). Hoechst33342, propidium iodide (PI) and 6-carboxy-2-7-dichlorofluorescein diacetate (DCFH-DA) were provided by Haimen Biyuntian Bioengineering Institute (Haimen, Jiangsu, China). Fluo-4-AM was got Molecular Probe (Eugene, OR, USA). Albiflorin, paeoniflorin, ferulic acid and quercetin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Isorhamnetin-3-*O*-neohesperidin, senkyunolide I, senkyunolide H, with 98% purity was provided by Jiangsu Key Laboratory for TCM Formulae Research, Nanjing University of Chinese Medicine, China. SF-7 was freshly prepared as stock solutions in dimethylsulfoxide (DMSO) and diluted with cell culture medium before the experiment. 0.1% (v/v) DMSO had no protective or toxic effect by itself.

3.2. Preparation for active fraction SF-7

The mixed crude herbs, *Angelica sinensis, Ligusticum chuanxiong, Paeonia lactiflora, Cinnamomum cassia, Foeniculum vulgare, Zingiber officinale, Commiphora myrrha, Trogopterus xanthipes, Typha angustifolia, and Corydalis yanhusuo* the weight ratio of 3:1:2:1:0.5:1:1:2:3:1(1080, 360, 720, 360, 180, 360, 360, 720, 1080 and 360 g, 5.58 kg total weight) were crushed into small pieces. The mixture was refluxed with water (55.8 L) for 2 h. The filtrates were collected and the residues were then refluxed twice in water (55.8 L) for 1.5 h. Two batches of filtrates were combined.

Figure 5. The active fraction of SF-7 in effect UPLC-MS/MS total ion flow diagram (positive ion detection mode). Compounds: 1 = albiflorin, 2 = paeoniflorin, 3 = ferulic acid, 4 = isorhamnetin-3-*O*-neohesperidin, 5 = senkyunolide I, 6 = senkyunolide H, 7 = quercetin).



The solvent was removed below 70 ºC to a certain volume at the ratio of 1:1 (w/w, weight of all constituting herbs and the extract filtrates) under vacuum; 95% ethanol was added to the extract filtrates until the concentration of ethanol had been adjusted to 80%. The ethanol solvent was removed below 70 ºC to a certain volume of filtrates. The final filtrates were separated by gradient elution with different concentrations of ethanol from macroporous adsorptive resins and then the different fractions obtained. The active fraction, the 40% ethanol elution fraction, which contained many compounds such as albiflorin, paeoniflorin, ferulic acid, isorhamnetin-3-*O*-neohesperidin, senkyunolide I, senkyunolide H, quercetin [32,33] as shown in Figure 5.

3.3. Cell culture and drug treatment

Rat VSMCs line was obtained from Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in high glucose DMEM supplemented with 15% FBS, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin at 37 ºC under an atmosphere of 95% air and 5% CO_2. The cells were passaged every three days. To study the effects of SF-7 on VSMCs, cells were incubated with SF-7 and H_2O_2 at the indicated concentrations for 16 h. The cells underwent the same procedures except SF-7 treatment in H_2O_2 group and without both H_2O_2 and SF-7 treatments in the normal group. After the cells were cultured for 16 h at 37 ºC, the following experiments were performed.

3.4. Assay of cell viability with the MTT method

VSMCs (10^4cells/well in 100 μL medium) were treated with the different concentrations (0.05, 0.1, 0.2 0.4, 0.8 $mg \cdot mL^{-1}$) of SF-7 and 100 μM H_2O_2 for 16 h at 37 ºC. Then, MTT (20 μL, 0.5 $g \cdot L^{-1}$) was added to each culture well for further incubation. After 4 h, the culture medium was removed and the formazan crystal was dissolved by addition of 150 μL DMSO to each well with vigorously shaking the plate to ensure complete solubilization. Finally, formazan absorbance was assessed by a Multi-detection Microplate Reader (Bio-Tek, USA) at 490 nm.

3.5. Detection of LDH leakage, intracellular MDA level and SOD activity with spectrophotometry

After cells were exposed to 100 μM H_2O_2 in the presence of the different concentrations (0.1, 0.2, 0.4 $mg \cdot mL^{-1}$) of SF-7 for 16 h, the medium was collected, and the amount of LDH released by cells was determined using an assay kit (Nanjing Jiancheng Co., China) according to the manufacturer's instruction. The absorbance of samples was read at 440 nm.

Cells were exposed to 100 μM H_2O_2 in the presence of the different concentrations (0.1, 0.2, 0.4 $mg \cdot mL^{-1}$) of SF-7 for 16 h. Then the plate was by three freeze/thaw cycles with sonication (10 s, 25 ºC) between the cycles [21]. The homogenate was centrifuged at 4,000 rpm at 4 ºC for 15 min. Then, SOD activity and MDA level were determined according to the direction of the assay kit (Nanjing Jiancheng Co., China). Thiobarbituric acid reactive substances were assessed by measuring the MDA concentration at 532 nm with the thiobarbituric acid method, which was based on the reaction of MDA with thiobarbituric acid to form a stable chromophoric production. SOD activity was

assayed at 550 nm on the basis of its ability to inhibit the oxidation of hydroxylamine by superoxide anion from xanthine-xanthine oxidase system [34,35].

3.6. Observation of VSMCs apoptosis by nuclear staining with Hoechst33342

VSMCs (2×10^5 cells/well in 1000 μL medium) were seeded on poly-L-lysine coated glass cover slips (25 × 25 mm) in six well plates. The cells were exposed to 100 μM H_2O_2 and different concentrations (0.1, 0.2, 0.4 mg·mL^{-1}) of SF-7 for 16 h and 10 μg·mL^{-1} Hoechst33342 for further 15 min at room temperature. Then the cells were rinsed twice with phosphate-buffered saline (PBS) and imaged with a Fluorescent Microscope (Olympus, JAPAN) in a dark environment within 5 min after the specimen was mounted on the object stage.

3.7. Detection of apoptotic cells

Apoptosis was assayed by PI staining followed by analysis with fluorescence-activated cell sorting. The cultured VSMCs cells (10^6cells/mL) were harvested, washed and fixed with ice-cold alcohol (75%) for more than 24h. After two additional washing, cells were incubated with PBS (pH 7.4) containing RNase (5 U) and PI (50 μg·mL^{-1}) for 15 min at 37 ºC. Flow cytometry was performed using a FACS vantage SE Flow Cytometer (FACS, Becton Dickinson, USA).

3.8. Assay for intracellular ROS

Accumulation of intracellular ROS can be detected using DCFH-DA which crosses cell membranes and is hydrolyzed enzymatically ally by intracellular esterases to non-fluorescent DCFH [35]. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF), which is readily detected by a fluorescent microplate reader. VSMCs (2×10^4 cells/well in 100 μL medium) were grown on a black plate for 24 h, and then the cells were exposed to H_2O_2 in the presence of the different concentrations (0.1, 0.2, 0.4 mg·mL^{-1}) of SF-7. After 16 h, the cells were incubated with 10 μM DCFH-DA in the loading medium in 95% air and 5% CO_2 for 30 min. DCFH-DA was removed and the cells were rinsed with PBS. The fluorescence was observed by a Fluorescent Microscope (Olympus, Japan). And the fluorescence from each well was captured using a Fluorescent Microplate Spectrophotometer, Spectrum MAX190 (AD, USA) with an excitation wavelength of 488 nm and emission wavelength of 525 nm at 37 ºC.

3.9. Determination of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration was determined using fluo-4-AM. The Ca^{2+}-sensitive dye fluo-4-AM and pluronic F-127 were separately dissolved in DMSO, and then mixed before the use. VSMCs (2×10^4 cells/well in 100 μL medium) were grown on a black plate. After 24 h, the cells were incubated with 2.5 μM fluo-4-AM in the loading medium in 95% air and 5% CO_2 for 30 min. Fluo-4-AM was removed and the cells were rinsed with Hank's solution. The fluorescence from each well was captured using a Fluorescent Microplate Spectrophotometer, Spectrum MAX190 (AD, USA) with an excitation wavelength of 494 nm and emission wavelength of 516 nm at 37 ºC. The change of

intracellular Ca^{2+} concentration was determined by time course and observed after adding 100 μM H_2O_2 and the different concentrations (0.1, 0.2, 0.4 mg·mL^{-1}) of SF-7.

3.10. Statistical analysis

Statistical Analysis SPSS 12.0 software and Origin 7.0 software were applied to analyze experimental data and results were expressed as means ± S.D. All data were evaluated with analysis of variance (ANOVA) following by Student's t-test for multiple comparisons and $P < 0.05$ indicates that the difference was statistically significant.

4. Conclusions

SF-7 exhibits protective effect against H_2O_2-injury on VSMCs, which may be associated with its antioxidant properties. It is suggested that SF may be useful in the treatment of blood stasis syndrome in which oxidative injury are mainly implicated.

Acknowledgements

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Sample Availability: Samples of the compounds. are available from the authors.