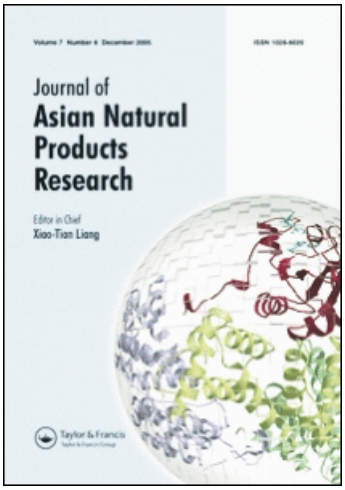

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Schisanhenol attenuated ox-LDL-induced apoptosis and reactive oxygen species generation in bovine aorta endothelial cells *in vitro*

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Schisanhenol attenuated ox-LDL-induced apoptosis and reactive oxygen species generation in bovine aorta endothelial cells *in vitro*

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The aim of this paper was to investigate the protective effect of schisanhenol (Sal) isolated from *Schisandra rubriflora* Rhed, on human ox-LDL-induced bovine aorta endothelial cells (BAECs) apoptosis and intracellular reactive oxygen species (ROS) production *in vitro*. The BAECs were cultured with ox-LDL (200 μg/ml) in the presence and absence of Sal (10 and 50 μmol L^{-1}) for 24 h. The cytotoxicity of ox-LDL was evaluated by LDH leakage, cell viability and morphological change. Cell apoptosis was estimated by DNA ladder, chromatin condensation, and flow cytometry assay. The intracellular ROS production was detected by using DCF, a ROS probe, with laser confocal microscopy and flow cytometry. Sal was shown to reduce LDH leakage and increase cell viability. Sal also attenuated ox-LDL-induced BAECs apoptosis as indicated in typical internucleosomal DNA degradation (DNA ladder), condensed chromatin, and the sub-G1 peak appearance in flow cytometry assay. Furthermore, Sal was shown to inhibit ROS generation in BAECs with stimulation of ox-LDL. The results indicated that the anti-apoptosis effect of Sal on BACSs might be related to its inhibition of ROS generation.

Keywords: oxidized LDL; schisanhenol; vascular endothelium cells; apoptosis; reactive oxygen species

1. Introduction

Atherosclerosis (AS) has been considered to be the principal cause of heart attack, stroke, and gangrene of the extremities. The oxidized low density lipoprotein (ox-LDL) is believed to play an important role in different stage of atherosclerosis [1]. The oxidation of LDL caused the formation of lipid hydroxyperoxides, reactive aldehydes, and oxysterols. ox-LDL is a chemotactic factor for monocytes, and blocks egress of monocytes from the vessel wall, which results in accumulation of inflammatory cells and generation of oxygen-derived free radicals [2]. The end result is growth of the atherosclerotic plaque and a propensity of the diseased vessel toward vasospasm. It was also reported that ox-LDL and some of its reactive constituents have cytotoxicity to vascular cells including endothelial cells, and induce both necrosis and apoptosis of these cells [3,4]. One of the mechanisms involved in apoptosis is the production of free radicals. The treatment with anti-oxidants may decrease vascular injury induced by ox-LDL [5].

In our previous study, eight dibenzocyclooctene lignans (DCLs) isolated from *Fructus Schizandracea* were shown to have anti-oxidant activity. Among eight DCLs, the anti-oxidant action of schisanhenol (Sal, Figure 1) was more potent than others [6]. Further study demonstrated that Sal significantly inhibited human serum LDL oxidation induced by copper ion and mouse peritoneal macrophages

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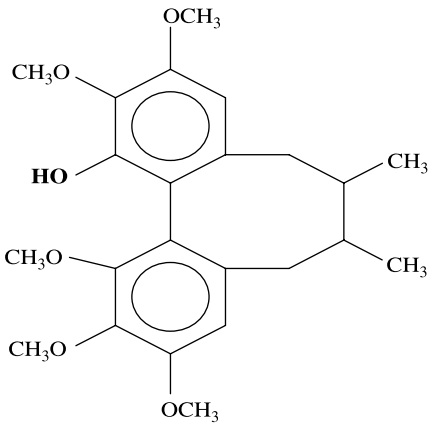

Figure 1. Chemical structure of schisanhenol.

[7]. The purpose of this research was to investigate whether Sal can prevent endothelium cells injury and apoptosis induced by ox-LDL, and its relation to intracellular ROS production.

2. Results

2.1. Protective effect of Sal on ox-LDL-induced cytotoxicity of BAECs

After exposing to ox-LDL 200 μg ml^{-1}, the BAECs became contracted and smaller, and appeared spherical, star-like or elongation, while native (n-LDL) had no such effect on BAECs. Besides this, ox-LDL 200 μg ml^{-1} caused approximate 40% decrease of cell survival rate and remarkable increase of LDH release from BAECs. Sal at 50 and 10 μmol L^{-1} maintained the normal appearance of cell morphology, increased cell viability, and inhibited the leakage of LDH from cells, indicating that the cell injury was well improved (Figure 2 and Table 1).

2.2. Protective effect of Sal on ox-LDL-induced BAECs apoptosis

The staining of cells with Hoechst33342, DNA fragmentation detected by agarose gel electrophoreses and ratio of apoptosis cells assayed by flow cytometry were used as hallmarks of apoptosis. As shown in results, after intoxication with 200 μg ml^{-1} ox-LDL for 24 h, the cell chromatin became condensed and formed apoptosis bodies (Figure 3), and the pattern of agarose gel electrophoreses also showed typical DNA ladder (Figure 4). The results of flow cytometric analysis of the sub-G1 cell cycle phase also indicated that ox-LDL increased the percentage of cells in sub-G1 phase from 13.2% for the control group to 45.4% for the ox-LDL 200 μg ml^{-1}

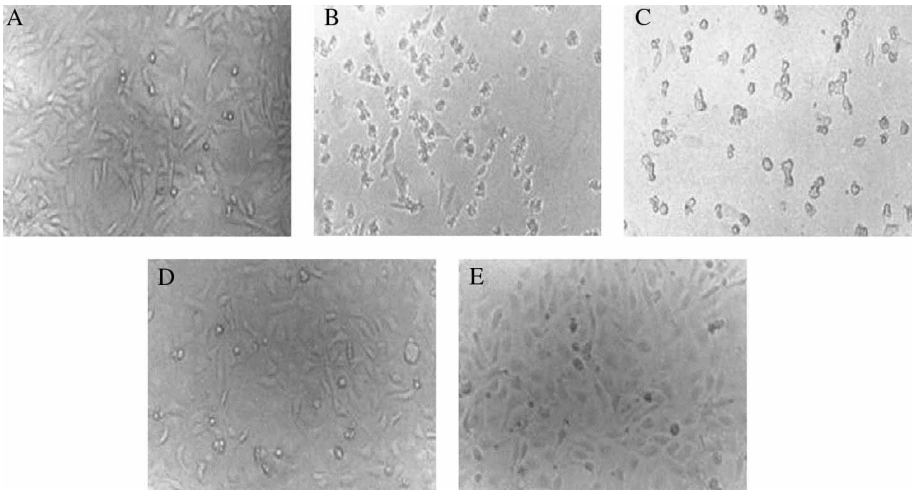

Figure 2. The protective effect of Sal on ox-LDL-induced BAECs morphological changes. The BAECs were treated with 200 μg ml^{-1} ox-LDL in the presence or absence of Sal for 24 h. The morphologic change was observed under inverted microscope (× 20). (A) Control, (B) n-LDL 200 μg ml^{-1}, (C) ox-LDL 200 μg ml^{-1}, (D) Sal 50 μmol L^{-1} + ox-LDL 200 μg ml^{-1}, and (E) Sal 10 μmol L^{-1} + ox-LDL 200 μg ml^{-1}.

Table 1. Protective effect of Sal on ox-LDL-induced cytotoxicity (MTT and LDH)to BAECs.

Group	Cell survival (OD570 nm)	LDH (U 100 ml^{-1})
Control	0.779 ± 0.050**	1092.5 ± 85.2*
ox-LDL 200 μg ml^{-1}	0.472 ± 0.053	1432.5 ± 114.1
ox-LDL 200 μg ml^{-1} + Sal 50 μmol L^{-1}	0.754 ± 0.083**	1142.5 ± 54.4*
ox-LDL 200 μg ml^{-1} + Sal 10 μmol L^{-1}	0.595 ± 0.027**	1467.5 ± 146.3
ox-LDL 200 μg ml^{-1} + Sal 5 μmol L^{-1}	0.576 ± 0.076	1512.5 ± 135.3

$n = 4$, mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* ox-LDL group.

group (Figure 5). Pretreatment with Sal (50 and 10 μmol L^{-1}) decreased the above three hallmarks of cell apoptosis in a dose-dependent manner. Particularly, the protective effects of Sal at 10 μmol L^{-1} was more pronounced.

2.3. Inhibitory effect of Sal on intracellular ROS production induced by ox-LDL

The stimulation of BAECs with ox-LDL 200 μg ml^{-1} for 15 min increased the intracellular fluorescence-intensity of DCF

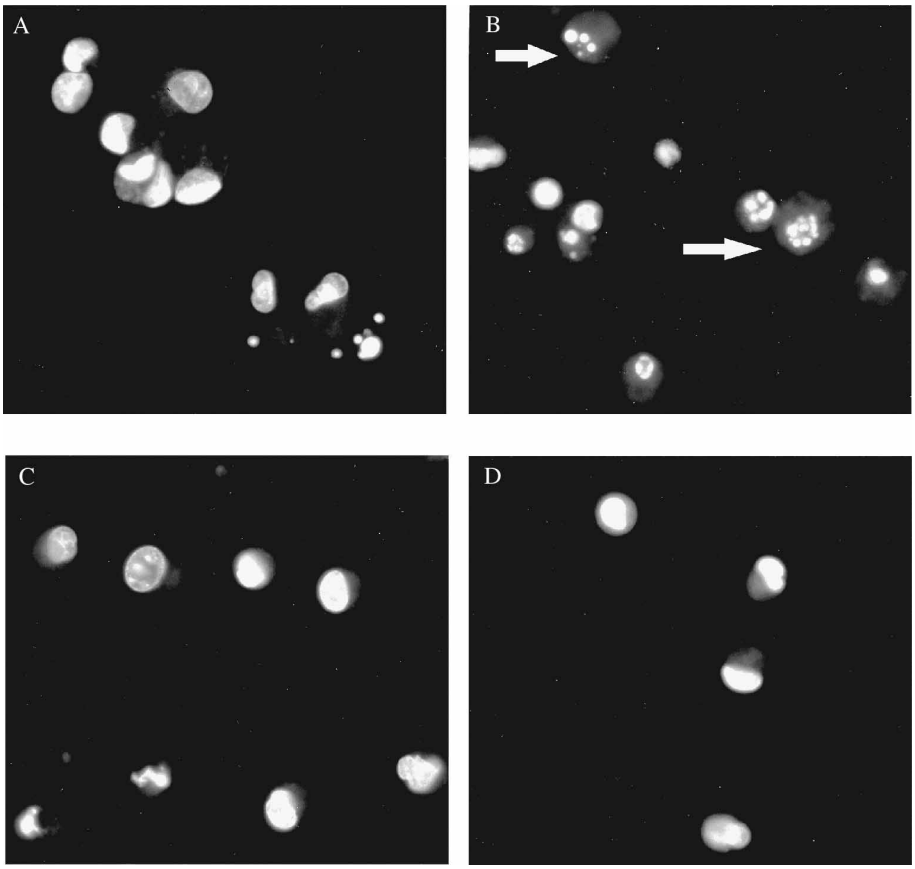

Figure 3. Protective effect of Sal on ox-LDL-induced BAECs apoptosis. The cells were stimulated with ox-LDL 200 μg ml^{-1} in the presence or absence of Sal at indicated concentrations for 24 h. Chromatin condensation (indicated by arrow) was observed by fluorescent microscopy after staining with Hoechst 33324. (A) Control, (B) ox-LDL 200 μg ml^{-1}, (C) Sal 50 μmol L^{-1} + ox-LDL 200 μg ml^{-1}, and (D) Sal 10 μmol L^{-1} + ox-LDL 200 μg ml^{-1}.

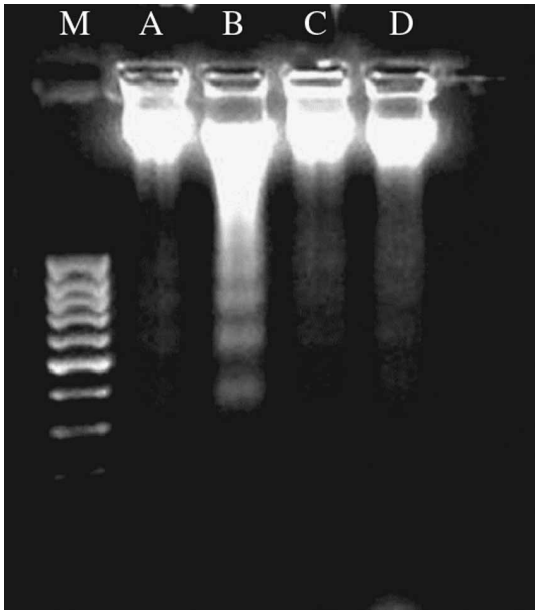

Figure 4. Effect of Sal on ox-LDL-induced DNA fragmentation of BAECs. The cells were stimulated with ox-LDL 200 $\mu g\,ml^{-1}$ in the presence or absence of Sal at indicated concentrations for 24 M. After that, internucleosomal DNA was extracted and analyzed by 1.5% agarose gel electrophoresis. (M) DNA ladder marker, (A) Control, (B) ox-LDL 200 $\mu g\,ml^{-1}$, (C) Sal 50 $\mu mol\,L^{-1}$ + ox-LDL 200 $\mu g\,ml^{-1}$, and (D) Sal 10 $\mu mol\,L^{-1}$ + ox-LDL 200 $\mu g\,ml^{-1}$.

(Figures 6 and 7), indicating that the production of intracellular ROS was increased. But the n-LDL at 200 $\mu g\ ml^{-1}$ had no influence on intracellular ROS production. Pre-incubation of Sal 10 $\mu mol\ L^{-1}$ for 30 min, the level of ROS production in BAECs decreased to normal level (Figure 6). Similarly, the laser confocal microscope also detected that addition of ox-LDL to the cultured BAECs immediately increased the fluorescence intensity of DCF (Figure 7). Pre-treatment with Sal significantly reduced DCF

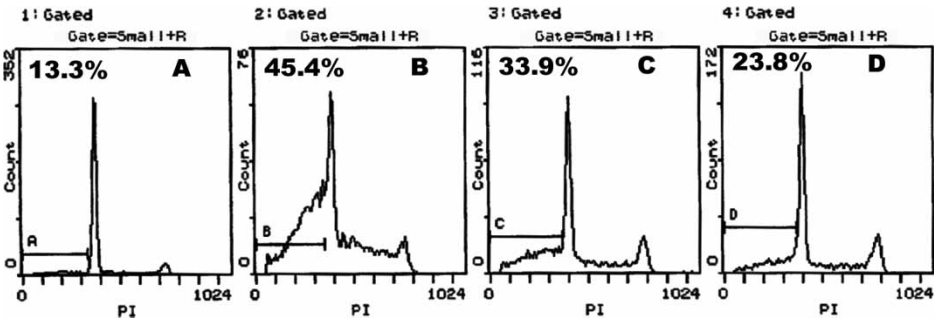

Figure 5. Inhibitory effect of Sal on ox-LDL-induced BAECs apoptosis detected by flow cytometry. The cells were cultured with ox-LDL 200 $\mu g\,ml^{-1}$ in the presence or absence of Sal at indicated concentrations for 24 h, then collected and fixed with 70% ethanol. After staining with PI, 10,000 cells were analyzed by flow cytometry for each sample and the percentage of apoptotic cells was calculated. (A) control (B) ox-LDL 200 $\mu g\,ml^{-1}$, (C) Sal 50 $\mu mol\,L^{-1}$ + ox-LDL 200 $\mu g\,ml^{-1}$, and (D) Sal 10 $\mu mol\,L^{-1}$ + ox-LDL 200 $\mu g\,ml^{-1}$.

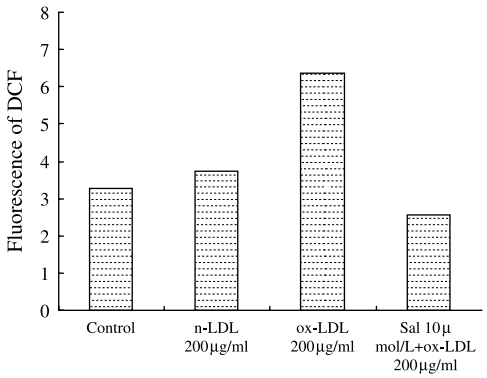

Figure 6. Inhibitory effect of Sal on ox-LDL-induced intracellular ROS production in BAECs by flow cytometry The BAECs were incubated with DCFH-DA in the presence or absence of Sal for 30 min. After renewed the medium, 200 $\mu g\,ml^{-1}$ of ox-LDL or n-LDL was added to stimulate the cells for 15 min. ROS production in 10,000 cells/sample was detected by flow cytometry. This experiment was repeated triplicate, similar results were obtained.

fluorescence intensity, indicating that ROS generation was inhibited.

3. Discussion

A number of articles reported that ox-LDL plays a crucial role in the pathogenesis of atherosclerosis [17,18]. n-LDL can be oxidized to ox-LDL by a complex process both *in vivo* and *in vitro*. The process of oxidation is thought to bring a number of alternations in the n-LDL molecule, and may stimulate a number of important processes in the pathogenesis of atherosclerosis.

Endothelial cells (ECs) form the lining of blood vessels. They regulate the permeability of blood vessels to leukocytes and inflammatory mediators. During inflammation, the expression of certain adhesion molecules and chemoattractants by the endothelium cells is necessary for localization and infiltration of blood leukocytes and macrophages into the insulted area.

Endothelial cell injury mainly by ox-LDL is commonly considered to play a pivotal role in atherosclerosis both in the early stages of lesion formation and later in the process of disease development by inducing atherosclerotic plaque instability [19,20]. Thus, inhibition of vascular endothelial cell apoptosis might prevent or reduce thrombosis.

The natural compound Sal has been shown to have prominent anti-oxidant activity including scavenging superoxide anion in our previous study [6]. In the present study, the authors found that Sal protected against ox-LDL-induced cytotoxicity and apoptosis of BAECs, and also inhibited ROS generation in BAECs *in vitro*. As mentioned in the results, the treatment of BAECs with ox-LDL for 24 h induced remarkable morphology injury, and significant decrease of cell viability, and increase of LDH leakage, which reflected the damage of plasma membrane integrity of BAECs. Pre-incubation with Sal 50 μmol/L for 1 h significantly protected against ox-LDL cytotoxicity to BAECs as expressed in inhibition of LDH release, increase of cell viability and preservation of normal cellular morphology of BAECs. Furthermore, Pre-incubation with different concentrations of Sal markedly inhibited the hallmarks of BAECs apoptosis induced by ox-LDL as indicated in the decrease of typical internucleosomal DNA degradation (DNA ladder), condensed DNA chromatin stained with Hoechst 33342, and the sub-G1 peak appearance in flow cytometry assay.

Reactive oxygen species (ROS) are involved in various biological processes such as cell activation, proliferation, survival, and apoptosis by plenty of signaling pathways, namely MAPK (ERK1/2, SAPK, and JNK), NFκB, Akt, caspases, and calcium [21], and ROS also participate in ox-LDL-induced cytotoxicity through activation of the caspase cascade and apoptosis. It was reported that ox-LDL induces the intracellular ROS generation by the binding of LDL to a lectin-like oxLDL receptor (LOX-1) [22] in different vascular cell types. Ox-LDL may also induce a rise of ROS levels by decrease of ROS degradation, since ox-LDL results a decrease of

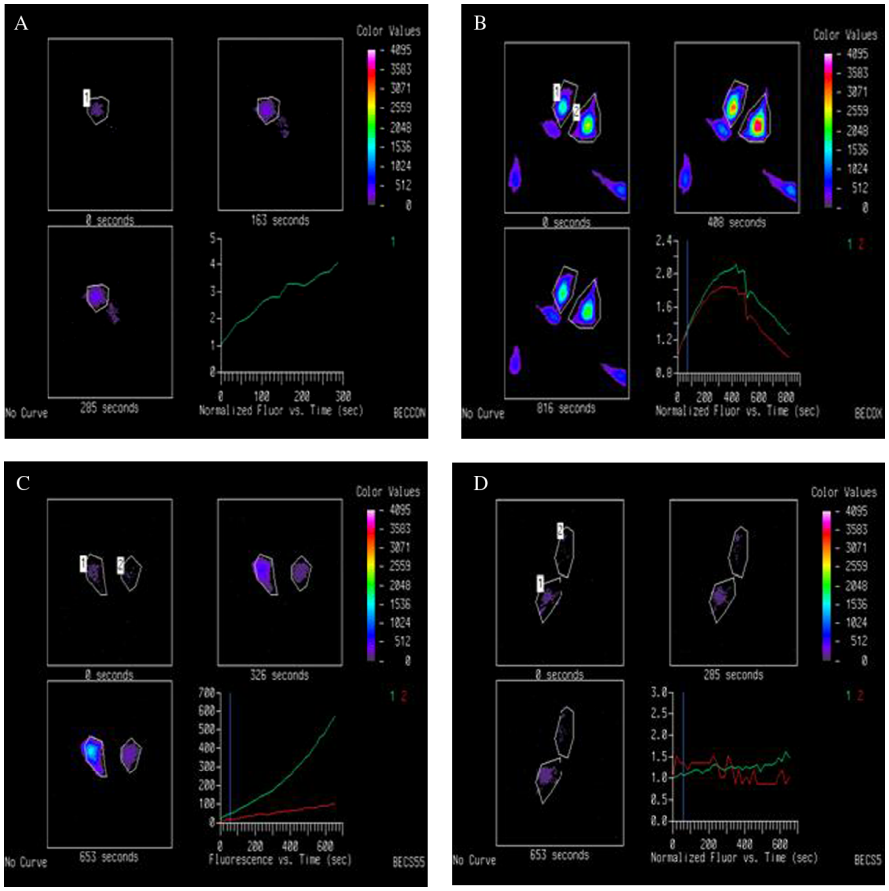

Figure 7. Inhibitory effect of Sal on ox-LDL-induced ROS production in BAECs detected by laser confocal microscopy. After pre-incubated with DCFH-DA in the presence or absence of Sal for 30 min, the BAECs were stimulated with ox-LDL 200 $\mu g\ m^{-1}$, the intensity of intracellular fluorescence was observed immediately by laser confocal microscopy and recorded at different time point. The brilliance reflected the amount of intracellular ROS production. (A) Control, (B) ox-LDL 200 $\mu g\ ml^{-1}$, (C) Sal 50 $\mu mol\ L^{-1}$ + ox-LDL 200 $\mu g\ ml^{-1}$, and (D) Sal 10 $\mu mol\ L^{-1}$ + ox-LDL 200 $\mu g\ ml^{-1}$.

anti-oxidant enzymes such as Cu/Zn superoxide dismutase and glutathione peroxidase [23].

In the present study, the intracellular ROS generation under stimulation of ox-LDL was confirmed by laser confocal microscopy and flow cytometry assay with the ROS probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The intensity of DCF fluorescent in the cultured BAECs increased immediately after the addition of ox-LDL both in laser confocal microscopic and flow cytometry assay. Pre-incubation of Sal markedly inhibited the intracellular ROS production, which may contribute to its protective action against cytotoxicity and cell apoptosis induced by ox-LDL.

In summary, Sal has protective effect on vascular endothelium cell injury and apoptosis induced by ox-LDL. Combining with our previous findings that Sal inhibited oxidation of n-LDL to ox-LDL, it is worthy to further study the protective effect on the development of atherosclerosis in animals.

4. Experimental

4.1. Compound and reagents

Sal was kindly provided by Professors YanYong Chen and Lianniang Li in our institute. It is a white crystal with purity over 98%. Sal was dissolved in dimethylene formamide (DMF) before use. Human blood serum was obtained from Beijing friendship hospital. Dialysis pocket (M_W: 8000–10,000) was purchased from Tianxiangren Biochemistry Company. DCFH-DA was purchased from Sigma Chemical Co. (USA). RPMI1640 medium was obtained from Gibco-BRL. Fetal bovine serum (FBS) was purchased from Hyclone Ltd. Other reagents were all chemical pure grade from local market.

4.2. ox-LDL preparation

LDL was separated from human blood serum by discontinuous density gradient ultracentrifugation.[8] The separated LDL was dialyzed against phosphate-buffered saline containing 0.01% EDTA (pH 7.4), then sterile-filtered and stored at 4°C. Before the incubation of oxidation, LDL was separated from EDTA by dialyzed against PBS for 40 h. LDL (1.0 mg of LDL protein/ml) oxidation was performed by exposure of LDL to 10 μM $CuSO_4$ for 20 h at 37°C, and this reaction was stopped by addition of 200 μmol EDTA. The extent of LDL oxidation was determined by evaluating the level of thiobarbituric acid-reactive substances (TBARSs) [9]. Protein was measured by method of Lowry [10].

4.3. Cell culture

BAECs were separated from newborn bovine thoracic aorta. The luminal surface of bovine aorta vessels was digested by 0.1% Type IV collagenase for 15 min at 37°C, and ended by addition of RPMI1640 supplemented with 10% heat-inactivated FBS. The digested cells were collected and cultured in complete medium in an atmosphere of 95% air, 5% CO_2 at 37°C. Cells were passed 3:1 at confluence and were used at passages 4–9 for all experiments. BAECs were identified by their non-overlapping, cobblestone-like morphology [11].

4.4. Cytotoxicity measurement

BAECs were seeded at 5000 cells/well (96-well plates) and cultured in 10% FBS RPMI1640 for 48 h. Then, the medium was renewed by fresh serum-free medium containing 200 μg ml^{-1} ox-LDL and cultured for up to 24 h. After treatment, the cell morphological change was observed under light microscope. LDH release was determined by LDH diagnostic kit. Cell viability was assayed by thiazolyl blue (MTT) method [12]. To study the protective effect of Sal, BAECs were pre-incubated with various concentration of Sal for 1 h and then co-cultured with ox-LDL for 24 h.

4.5. Apoptosis assay

BAECs were exposed to 200 μg ml^{-1} ox-LDL with or without Sal for 24 h. After treatment, different group of cells were digested and collected by centrifugation at 1000 rpm for 5 min, then divided into two parts. One part of cells were resuspended in 1 ml Dulbaco and stained with 10 μg/ml Hoechst 33324 for 30 min at 37°C, and then the chromatin condensation was observed by fluorescent microscopy [13]. The other part of cells were lysed with 400 μl lysis buffer (10 mmol/L Tris–HCl, pH 8.0, 10 mmol/L EDTA, 15 mmol/L NaCl, and 0.5% SDS), and digested by protease K for 3 h at 50°C. The DNA in lysates was extracted with equal volumes of phenol and phenol–chloroform–isoamyl alcohol (25:24:1) sequentially. After precipitated in absolute alcohol and ammonium acetate, the DNA pellet was resuspended in 10 mM Tris–EDTA buffer, then separated on 1.5% agarose gels, and visualized under ultraviolet light [14].

The percentage of apoptosis cell was determined by flow cytometry assay [15]. After treatment with ox-LDL and Sal as described above, cells were collected and fixed with 1 ml of 70% ethanol for over 12 h

at 4°C. The fixed cells were collected and washed with PBS buffer, and resuspended with 0.5 ml culture medium containing 50 $\mu g\,ml^{-1}$ RNase A to hydrolyze RNA at 37°C for 1 h. After staining with PI 50 μg/ml at 4°C for 1 h, cells were analyzed with 10,000 cells/sample by flow cytometry (Becton–Dickinson, Heidelberg, Germany).

4.6. Measurement of intracellular ROS production

The formation of intracellular ROS was measured using DCFH-DA as probe. In the presence of ROS, DCFH-DA is oxidized to fluorescent compound DCF which can be measured by fluorescent microscopy and flow cytometry [16]. To quantify the intracellular ROS production, the cells were incubated with 10 $\mu mol\,L^{-1}$ DCFH-DA in the presence or absence of Sal for 30 min. Then, the medium was renewed, and 200 $\mu g\ ml^{-1}$ ox-LDL was added to stimulate the cells for 15 min and then ROS production in cells was detected by flow cytometry.

To viewing the ROS production by laser confocal microscopy, confluent BAECs in 35 mm plates were incubated with 10 $\mu mol\,L^{-1}$ DCFH-DA in the presence or absence of Sal for 30 min. After renewed medium, 200 $\mu g\,ml^{-1}$ ox-LDL was added to stimulate the cells and the change of intracellular fluorescence intensity was examined immediately by laser confocal microscopy and lasting for 15 min. The intensity of intracellular fluorescence of DCF indicates the degree of intracellular ROS production, the higher the intensity of fluorescence, the more production of ROS.

4.7. Statistical analysis

All data were expressed as means ± SD. Student's *t*-test was used to analyze the statistical difference between groups. The $P < 0.05$ was considered significant statistically.

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