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Astragaloside IV Protects Against Oxidized Low-Density Lipoprotein (ox-LDL)-Induced Endothelial Cell Injury by Reducing Oxidative Stress and Inflammation

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Background: Endothelial injury is the main mechanism of atherosclerosis, and is caused by oxidized low-density lipoprotein (ox-LDL). Astragaloside IV (AS-IV) is the primary active ingredient of the Chinese herb Huangqi, and exhibits antioxidant and anti-inflammatory properties in cardiovascular diseases. This study investigated the protective effect of AS-IV in human umbilical vein endothelial cells (HUVECs).

Material/Methods: HUVEC cells were induced with ox-LDL to establish an *in vitro* atherosclerosis model. Then HUVECs were pretreated for 1 h with AS-IV at different concentrations (10, 20, and 50 μM) and then exposed to ox-LDL (100 μg/mL) for 48 h. The cell viability, lactate dehydrogenase (LDH) release, apoptosis, migration, intracellular reactive oxygen species (ROS), and NADPH oxidase activity of HUVECs were measured. qRT-PCR was performed to measure the mRNA expressions of Nrf2, HO-1, TNFα, and IL-6. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the supernatant contents of TNFα and IL-6.

Results: Exposure of HUVECs to ox-LDL reduced cell viability and migration, induced apoptosis, and increased intracellular ROS production and NADPH oxidase. Pretreatment with AS-IV (10, 20, and 50 μM) significantly enhanced the cell viability and migration, suppressed LDH release, apoptosis, ROS production, and NADPH oxidase in HUVECs, in a concentration-dependent manner. The AS-IV (50 μM) alone did not show significant differences from control. AS-IV increased mRNA expressions of Nrf2 and HO-1 and decreased mRNA expressions of TNFα and IL-6 in the ox-LDL-HUEVC cells. Furthermore, AS-IV reduced supernatant contents of TNFα and IL-6.

Conclusions: Astragaloside IV prevents ox-LDL-induced endothelial cell injury by reducing apoptosis, oxidative stress, and inflammatory response.

MeSH Keywords: **Atherosclerosis • Endothelial Cells • Inflammation • Oxidative Stress • Receptors, Oxidized LDL**

Abbreviations: **AGEs** – advanced glycation end-products; **AS-IV** – Astragaloside IV; **ELISA** – enzyme-linked immunosorbent assay; **CCK-8** – Cell Counting Kit-8; **FBS** – fetal bovine serum; **HO-1** – heme oxygenase-1; **HUVECs** – human umbilical vein endothelial cells; **IL-6** – interleukin-6; **LDH** – lactate dehydrogenase; **Nrf2** – nuclear factor erythroid 2-related factor 2; **ox-LDL** – oxidized low-density lipoprotein; **qRT-PCR** – quantitative real-time polymerase chain reaction; **ROS** – reactive oxygen species; **TNFα** – tumor necrosis factor-α; **VSMCs** – vascular smooth muscle cells

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Background

Atherosclerosis is a chronic inflammatory disease of the arterial wall and is involved in the pathogenesis of many cardiovascular diseases, thus leading to accumulation of lipid plaques and aorta and coronary artery stenosis [1]. Endothelial injury and apoptosis are the main pathological processes of atherosclerosis, and trigger the thrombosis process and accelerates the formation of atherosclerotic plaque [2], thus increasing susceptibility to ischemic events and acute cardiovascular symptoms [3]. Endothelial injury can be caused by a variety of factors, such as oxidized low-density lipoprotein (ox-LDL), advanced glycation end-products (AGEs), and angiotensin [4]. Increased levels of reactive oxygen species (ROS), such as superoxide anion, when exceeding the antioxidant capacity, can induce oxidative stress and promote vascular wall LDL oxidation, resulting in a high level of ox-LDL, leading to apoptosis of vascular endothelial cells and subsequent endothelial dysfunction [5,6]. Endothelial dysfunction is the initial reversible step in the formation of atherosclerosis. Therefore, treating endothelial dysfunction has become an important therapeutic strategy for preventing or reversing atherosclerosis.

Astragaloside IV (AS-IV) is a major compound extracted from the plant *Astragalus membranaceus* and has recently been shown to have multiple pharmacological effects on the liver, nervous system, hematopoietic system, endocrine system, cardiac function, collagen metabolism, and organ immune system [7]. In addition, AS-IV has been shown to promote proliferation of human umbilical vein endothelial cells and the formation of tubular structures *in vitro* [8]. AS-IV also stimulates angiogenesis via phosphoinositide 3-kinase/Akt, STAT3, and ERK1/2 signaling pathways [9,10]. These observations suggest that AS-IV may have important effects on cardiovascular disease. However, the role of AS-IV in the cellular model of atherosclerosis is unclear and related mechanisms are still unknown.

In the present study, we constructed an *in vitro* atherosclerosis model by exposing endothelial cells to ox-LDL. Effects of AS-IV on ox-LDL-induced endothelial injury, apoptosis, migration, oxidative stress, and inflammation were investigated. We also quantitatively analyzed mRNA expressions of antioxidant and inflammatory pathways such as nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6). This study will add AS-IV as a potential candidate in the treatment of endothelial injury in atherosclerosis and other cardiovascular diseases.

Material and Methods

Reagents and chemicals

Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Astragaloside IV (purity >98%, Cat. No. 84687-43-4) was purchased from Nanjing Spring & Autumn Biological Engineering Co. (Nanjing, China), dissolved in DMSO, and frozen at –80°C. Ox-LDL (oxidized low-density lipoprotein) was purchased from Beijing Solarbio Life Science Company (Cat. No. H7950, Beijing, China). Low-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and AnnexinV-FITC kits were purchased from Invitrogen (Cat. No. V13241, Carlsbad, CA). Cell counting kit-8 (CCK-8) kits were obtained from Beyotime Institute of Biotechnology (Cat. No. C0037, Shanghai, China). LDH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Cat. No. A020-1, Nanjing, China). The Transwell chambers (8-μm diameter, 24 wells) were purchased from Corning, Inc. (Corning, NY). Matrigel matrix was purchased from BD System (Cat. No. 356234, Franklin Lakes, NJ). DCFH-DA (2',7'-dichlorofluorescin diacetate) was obtained from Sigma Chemical Co. (Cat. No. D6883, St. Louis, MO). NADPH oxidase kits were purchased from Genmed Scientific, Inc. (Cat. No. GMS50067, Shanghai, China). ELISA kits for TNFα (Cat. No. 88-7346-77) and IL-6 (Cat. No. 88-7066-77) were purchased from Invitrogen (Carlsbad, CA).

Cell culture and treatment

Endothelial cell line HUVECs were cultured in DMEM (low-glucose) supplemented with 10% FBS, and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was refreshed every 2–3 days after cells had reached confluence, and experiments were performed in cells at passages 3 to 8. HUVECs were pretreated for 1 h with AS-IV at different concentrations (10, 20, and 50 μM) and then exposed to ox-LDL (100 μg/mL) for 48 h. The control group received 0.1% DMSO as vehicle. Our pilot study found that this concentration of DMSO had no cytotoxic effect on the HUVEC cells in the presence or absence of ox-LDL.

Cell viability assay

The Cell Counting Kit-8 (CCK-8) was used to measure cell viability. HUVECs were cultured in 96-well plates (1×10^5 cells/mL, 100 μL/well). HUVEC cells were pretreated with AS-IV (10, 20, and 50 μM) and then exposed to ox-LDL (100 μg/mL) for 48 h. After washing 3 times with PBS, CCK-8 solution (1: 10 dilution) was added to the medium and incubated at 37°C for 1 h. The absorbance was measured at 450 nm by a microplate reader.

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LDH release assay

Lactate dehydrogenase (LDH) release assay was used to evaluate LDH content in culture medium to quantify the protective effect of AS-IV on endothelial cells from ox-LDL-induced injuries. HUVECs were cultured in 96-well plates at a density of 1×10^4 per well (100 µL DMEM). After 48 h of various treatments, the LDH content in the media was assessed using an LDH activity kit according to the manufacturer's instructions.

Apoptosis assay

Flow cytometry was applied to determine the percentage of apoptotic cells using the Annexin V-FITC kit following manufacturer's instructions. HUVECs were cultured in 24-well plates (2×10^5 cells/well, 1 mL) and treated with AS-IV (10, 20, and 50 µM) for 1 h, followed by 48 h of ox-LDL exposure. Then, the HEVUC cells were harvested and washed 3 times with PBS and resuspended at a density of 5×10^5 cells/mL. Then, cells were added to 5 µL of Annexin V-FITC and 5 µL of PI solution in 100 µL 1×binding buffer, and incubated for 15 min in the dark. The percentage of Annexin V-FITC-positive/PI-negative cells and Annexin V-FITC-positive/PI-positive cells was analyzed by flow cytometry (FACSCalibur, BD).

Migration assay

HUVECs were seeded into the upper chamber of Transwell chambers (8-µm pore size diameter and 24 wells) in 100 µL DMEM medium supplemented with 0.1% FBS at a density of at 1×10^5 cells/mL. The lower chamber contained 600 µL DMEM medium supplemented with 10% FBS. After 48-h incubation at 37°C of various treatments, cells in the upper chamber were wiped away a cotton swab. The migrated cells were fixed with 95% ethanol for 10 min at room temperature, and then stained with 0.1% crystal violet at 22°C for 30 min. The stained migrated cells were counted under a light microscope (magnification, ×100) from 10 fields to calculate the percentage of migrated cells.

Measurement of intracellular ROS

HUVECs were cultured in 24-well plates (2×10^5 cells/well, 1 mL) and treated with AS-IV (10, 20, and 50 µM) for 1 h, followed by 48 h of ox-LDL exposure. Then, HUVEC cells were incubated with DCFH-DA (1: 1000 v/v) at 37°C for 45 min in the dark and the samples were washed 3 times with PBS. The fluorescence was measured at 485 nm (excitation) and 535 nm (emission) using a microplate reader (F200, Tecan) [11].

Determination of NADPH oxidase activity

NADPH oxidase activity was assessed by colorimetric method. HUVECs were plated (5×10^6/mL, 2.5 mL) in 6-well plates (9.6 cm^2) with DMEM medium containing 1% FBS. After 24 h, cells were pretreated with AS-IV (10, 20, and 50 µM) for 1 h, followed by an additional 48 h of ox-LDL exposure. Then, cells were harvested, protein was extracted, and NADPH oxidase activity was evaluated by use of an NADPH oxidase kit according to the manufacturer's instruction [12].

Enzyme-linked immunosorbent assay (ELISA)

HUVECs were cultured in 24-well plates (2×10^5 cells/well, 1 mL) and treated with AS-IV (10, 20, and 50 µM) for 1 h, followed by 48 h of ox-LDL exposure. Then, the cellular supernatant was collected. The levels of the proinflammatory cytokines of TNFα and interleukin-6 (IL-6) were analyzed using an ELISA kit, measuring optical density at 450 nm using a microplate reader (Ricso RK201, Shenzhen Ricso Technology Co., Shenzhen, Guangdong, China). All ELISA assays were performed 3 times, according to the manufacturer's instructions. The serum TNFα and IL-6 concentrations of each group was determined according to the standard curve established by recombinant TNFα and IL-6, respectively.

Quantitative real-time PCR

Total RNA was extracted from HUVEC cells of different groups with Trizol to obtain cDNA by reverse transcription. qRT-PCR was performed to determine the expression levels of Nrf2, HO-1, TNFα, and IL-6 using SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) in an ABI PRISM 7500 Fast Real-time PCR instrument (Applied Biosystems, Foster City, CA). I Amplification conditions were set as follows: pre-denaturation at 95°C for 2 min, then 40 cycles of 95°C for 20 s and 58°C for 20 s. Human GAPDH gene was used as an internal reference. Specific primers were shown below: Nrf2: forward, 5'-TCC GGG TGT GTT TGT TCC AA-3', reverse, 5'-CGC CCG CGA GAT AAA GAG TT-3'; HO-1: forward, 5'-CAG GCA ATG GCC TAA ACT TC-3', reverse, 5'-GCT GCC ACA TTA GGG TGT CT-3'; TNFα: forward, 5'-GAG GCC AAG CCC TGG TAT G-3', reverse, 5'-CGG GCC GAT TGA TCT CAG C-3'; IL-6: forward, 5'-CCT GAA CCT TCC AAA GAT GGC-3', reverse, 5'-TTC ACC AGG CAA GTC TCC TCA-3'; GAPDH: forward, 5'-CCT CAA GAT CAT CAG CAA TG-3', reverse, 5'-CCA TCC ACA GTC TTC TGG GT-3'. The data was analyzed according to the $2-\Delta\Delta Ct$ method. All reactions were performed at least 3 times.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 19.0 software (IBM, USA). Differences between groups were calculated using analysis of variance (ANOVA), followed by the Student-Newman-Keuls (SNK) test. Power analysis of the difference in cell viability was performed. The parameters needed for the

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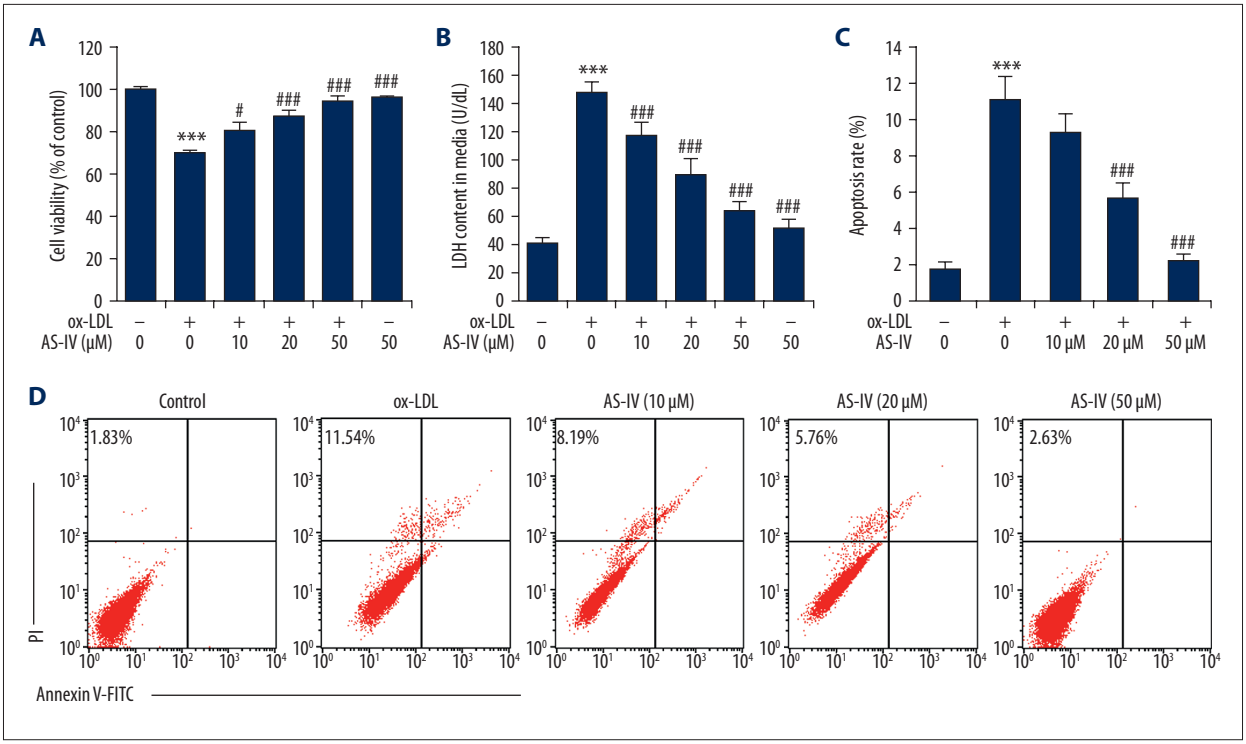

Figure 1. Effects of AS-IV on cell viability and LDH release in ox-LDL-induced HUVECs. HUVECs were pretreated for 1 h with AS-IV at different concentrations (10, 20, and 50 μM), and then exposed to ox-LDL (100 μg/mL) for 48 h. (**A**) Cell viability was evaluated by the CKK-8 method, normalized to the control group. (**B**) LDH content in media was measured by use of an assay kit. (**C**) Apoptosis was determined through Annexin V-FITC and PI double-staining using flow cytometry after AS-IV treatment for 48 h. The apoptotic rate was calculated from the sum of early apoptosis (**right lower quadrant**) and late apoptosis (**right upper quadrant**). (**D**) Quantitative data show that ox-LDL increased the apoptotic rate in HUVEC cells, which was significantly reversed by AS-IV, in a concentration-dependent manner. Values are means ±SD from 3 independent experiments. *** P<0.001 *vs.* control; # P<0.05, ### P<0.001 *vs.* ox-LDL. AS10 – AS-IV 10 μM; AS20 – AS-IV 20 μM; AS50 – AS-IV 50 μM.

power analysis (the number of reactions, mean values, and mean standard deviations) were estimated from the sample. A power of 95% was attained when cell viability change was used as a response. P<0.05 is defined as statistical significance.

Results

AS-IV prevented ox-LDL-induced endothelial injury

Cell viability was performed to assess the protective effect of AS-IV on endothelial injuries induced by ox-LDL. After 48-h exposure, ox-LDL (100 μg/mL) significantly reduced cell viability and enhanced LDH release in HUVEC cells, compared with normal control (P<0.05) (Figure 1A, 1B). The pretreatment with AS-IV (10, 20, and 50 μM) significantly attenuated the decrease in cell viability and increase in LDH release in HUVEC cells with ox-LDL, in a concentration-dependent manner. However, AS-IV alone (50 μM) did not significant influence cell viability or LDH release in HUVECs. Annexin V-FITC/PI staining was performed to measure apoptotic rate. ox-LDL significantly increased the apoptotic rate at 48 h, and AS-IV (at 10, 20, and 50 μM) significantly attenuated the apoptotic rate induced by ox-LDL (Figure 1C, 1D).

AS-IV inhibited ox-LDL-induced migration in HUVEC cells

To explore the migration ability of ox-LDL-induced HUVECs by AS-IV, we performed Transwell assay. Cells were treated with ox-LDL (100 μg/mL) or ox-LDL plus various concentrations of AS-IV (10, 20, and 50 μM) for 48 h, then the migrated cells were stained with crystal violet. Representative images showed that, compared with control group (Figure 2A), the ox-LDL group showed fewer stained cells (Figure 2B). AS-IV treatment increased the number of stained cells at 10 μM (Figure 2C), 20 μM (Figure 2D), and 50 μM (Figure 2E). Quantitative analysis showed AS-IV treatment significantly increased the numbers of migrated HUVEC cells exposed to ox-LDL, in a concentration-dependent manner (P<0.05) (Figure 2F).

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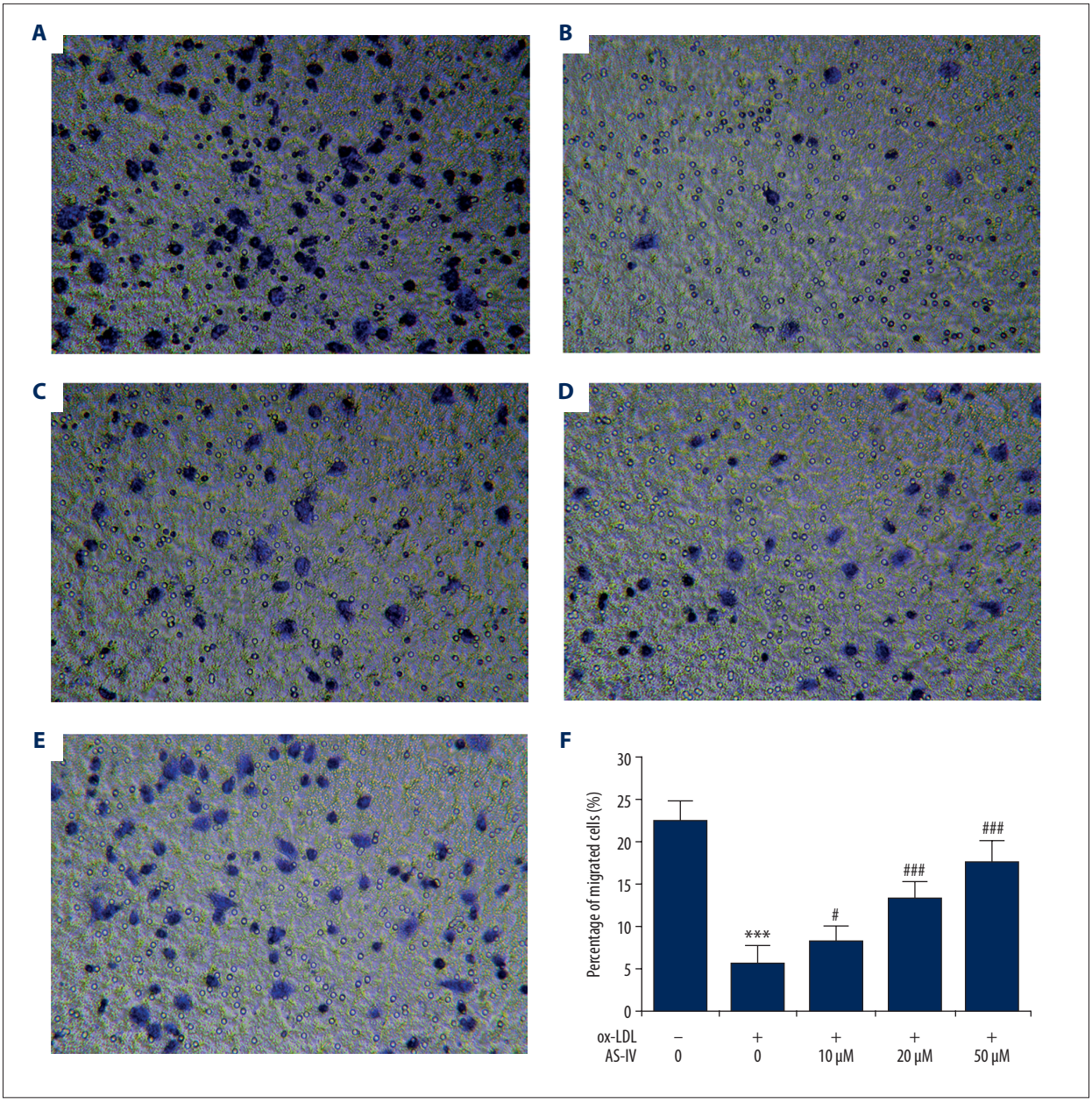

Figure 2. Effect of AS-IV on the migration ability of HUVECs. Transwell assay was used to evaluate the migration ability of cells. The migrated cells in the lower chamber were stained with 0.1% crystal violet, and their representative images are shown for the control group (**A**), ox-LDL group (**B**), cells treated with ox-LDL and AS-IV at different concentrations (10, 20, and 50 μM) (**C–E**) (magnification ×100). (**F**) Quantitative data show that ox-LDL significantly reduced the numbers of migrated HUVEC cells, but AS-IV significantly increased the numbers of migrated cells, in a concentration-dependent manner. Values are means ±SD from 3 independent experiments. *** P<0.001 *vs.* control; # P<0.05, ### P<0.001 *vs.* ox-LDL.

AS-IV inhibited ROS production and activated the Nrf2-HO-1 pathway in ox-LDL-induced HUVEC cells

To investigate the effect of AS-IV on ROS production, we measured intracellular ROS by fluorescent probe DCFH-DA and NADPH oxidase activity by colorimetric analysis. ox-LDL significantly increased the intracellular ROS (Figure 3A) and NADPH oxidase activity (Figure 3B) of HUVEC cells. However, AS-IV decreased the intracellular ROS and NADPH oxidase activity in ox-LDL-induced HUVEC cells (P<0.05). Our results show that ox-LDL promoted oxidative stress and AS-IV exerted an antioxidant effect on ox-LDL-induced HUVEC cells. To explore the molecular mechanism of AS-IV in oxidative stress, we then performed qRT-PCR to measure the mRNA levels of 2 antioxidant

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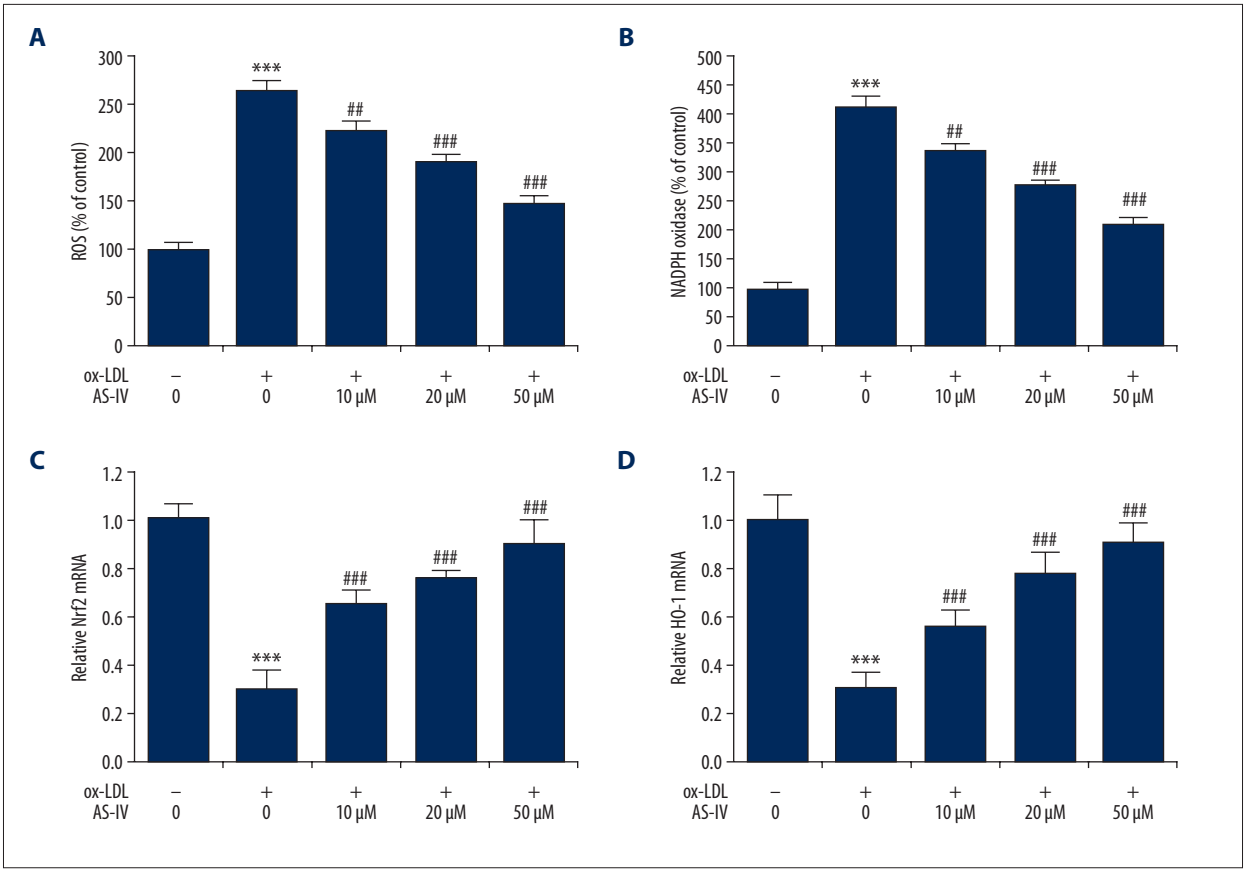

Figure 3. Inhibitory effect of AS-IV on oxidative stress of ox-LDL-induced HUVECs. After pretreated with AS-IV (10, 20, and 50 μM) for 1 h, cells were exposed to ox-LDL (100 μg/mL) and incubated for 48 h. (**A**) AS-IV attenuates intracellular ROS overproduction, as detected by incubation with fluorescent probe DCFH-DA (1: 1000) at 37°C for 45 min. Fluorescence intensity of cells was measured with a fluorescence microplate reader to evaluate intracellular ROS overproduction. (**B**) AS-IV attenuated the level of NADPH oxidase activation in HUVECs exposed to ox-LDL (100 μg/mL). NADPH oxidase activity was determined by colorimetric analysis. qRT-PCR shows that AS-IV activated the Nrf2 signaling pathway, as evidenced by increased mRNA levels of the Nrf2 (**C**) and HO-1 (**D**). GAPDH was used as a housekeeping gene and for normalization. Values are means ±SD from 3 independent experiments, and are all normalized to that of the control group. *** P<0.001 *vs.* control; ## P<0.01, ### P<0.001 *vs.* ox-LDL.

genes: Nrf2 and HO-1. After exposure to ox-LDL, the mRNA expressions of Nrf2 and HO-1 were significantly decreased. Treatment with AS-IV significantly increased Nrf2 and HO-1 mRNA expressions in a concentration-dependent manner (P<0.05) (Figure 3C, 3D).

AS-IV inhibited inflammatory response in ox-LDL-induced HUVEC cells

To determine whether AS-IV regulates inflammatory response in ox-LDL-induced HUVEC cells, we measured TNFα and IL-6 contents in supernatant media of cultured cells by use of ELISA. ox-LDL significantly increased the supernatant levels of TNFα and IL-6 in HUVEC cells, which was significantly attenuated by AS-IV, in a concentration-dependent manner (P<0.05) (Figure 4A, 4B). We further performed qRT-PCR to measure the mRNA levels. The mRNA expressions of TNFα and IL-6 were significantly decreased after exposure to ox-LDL, and were significantly increased by AS-IV treatment, in a concentration-dependent manner (P<0.05) (Figure 4C, 4D).

Discussion

In this study we evaluated the effect of AS-IV on ox-LDL-induced HUVECs injury. ox-LDL (100 μg/mL) exposure reduced cell viability and increased LDH release in supernatant media, which was reversed by treatment with AS-IV (10, 20, and 50 μM) for 48 h. In HUVEC cells exposed to ox-LDL, AS-IV reduced apoptosis, promoted cell migration, and suppressed oxidative stress and inflammatory cytokine production. The mechanisms might be related to increased mRNA expression of Nrf2 and HO-1 and decreased mRNA expression of TNFα and IL-6. Therefore, AS-IV protects HUVEC cells against ox-LDL-induced

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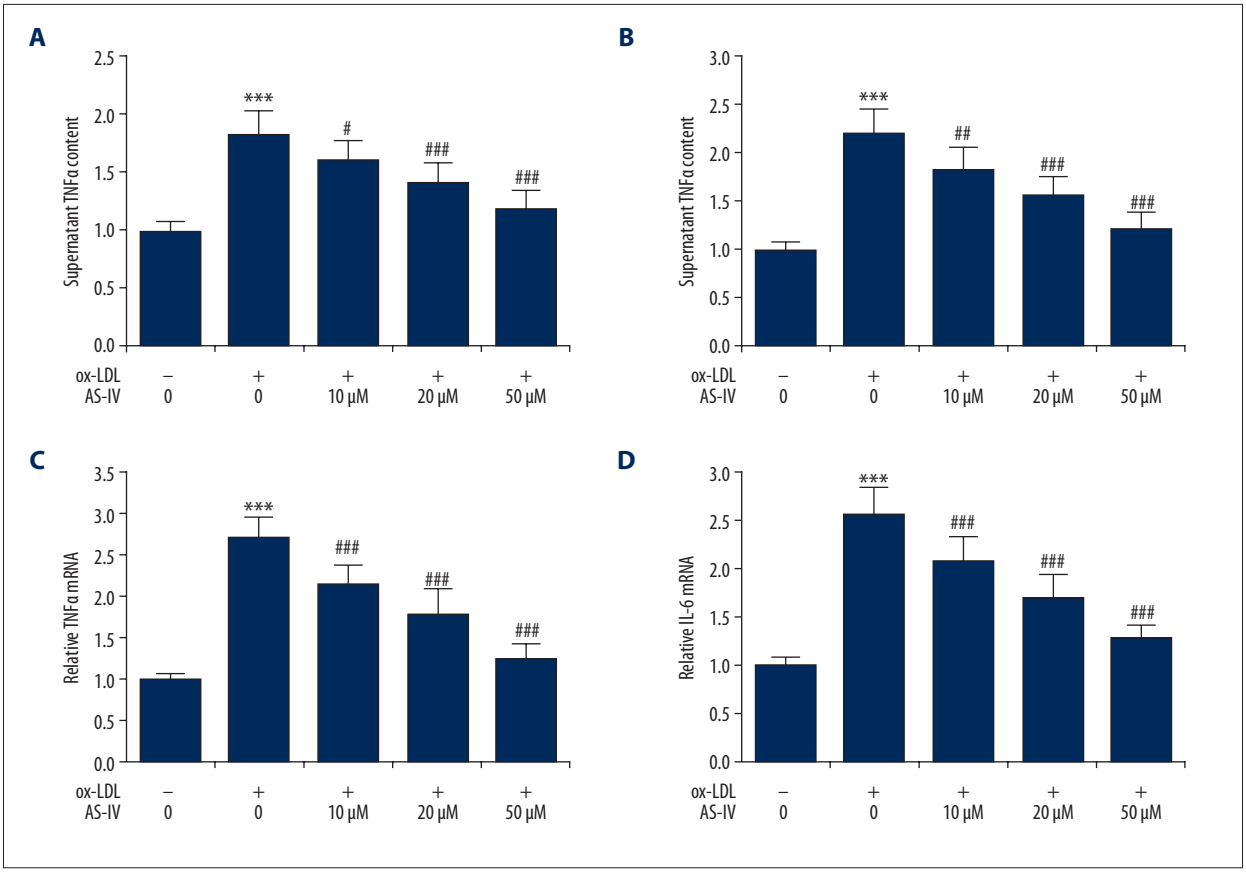

Figure 4. Inhibitory effect of AS-IV on inflammatory response of ox-LDL-induced HUVECs. After pretreated with AS-IV (10, 20, and 50 μM) for 1 h, cells were exposed to ox-LDL (100 μg/mL) and incubated for 48 h. Then, cells and supernatant media were collected. ELISA was performed to determine the production of proinflammatory cytokines, and showed AS-IV decreases TNFα (**A**) and IL-6 (**B**) contents in supernatant media of ox-LDL-induced HUVECs. qRT-PCR shows that ox-LDL reduced mRNA levels of the TNFα (**C**) and IL-6 (**D**), which was attenuated by AS-IV. *** P<0.001 *vs.* control; # P<0.05; ## P<0.01, ### P<0.001 *vs.* ox-LDL.

injury through modulation of anti-oxidative and anti-inflammatory response pathways.

Atherosclerosis is a contributing factor of many cardiac and cerebral vascular diseases, and can be initiated and promoted by several important processes, include oxidative stress [13], vascular endothelial injury [14], and release of inflammatory mediators [15]. Ox-LDL contributes to the formation and progression of atherosclerotic plaque through several mechanisms, including the induction of endothelial injury, endothelial apoptosis, foam cell formation from macrophages, and disruption of antioxidant capabilities [16]. Therefore, we used ox-LDL as an agent to induce endothelial injury of HUVECs as an *in vitro* model to investigate the protective effect of AS-IV.

The present study shows that AS-IV promoted proliferation and migration of HUVECs exposed to ox-LDL. Endothelial cells play key roles in maintaining physiological function of the cardiovascular system through regulating blood circulation, coagulation, angiogenesis, and inflammatory responses, and proliferation and migration of endothelial cells contribute to angiogenesis and vessel sprouting [17]. Our study shows that AS-IV protects against endothelial injury in prevention and therapy of atherosclerosis, which is in accordance with an *in vivo* study that reported AS-IV inhibited atherosclerotic lesions in hyperlipidemia-induced atherosclerotic ApoE(–/–) mice [18]. However, our results are also contradictory to previous reports on regulation of AS-IV in endothelial proliferation and migration. For example, AS-IV inhibited proliferation and migration of vascular smooth muscle cells (VSMCs) stimulated by platelet-derived growth factor-BB and Angiotensin II, respectively [19,20]. This inconsistency can be explained by the different roles in atherosclerosis of 2 major type of cells in blood vessel walls: endothelial cells and VSMCs. The proliferation and migration of VSMCs promote atherosclerosis development [21]. Therefore, our study was supported by another study reporting that AS-IV exerts pro-angiogenic effects through stimulating the proliferation and migration of HUVECs [22].

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This study showed that AS-IV suppressed oxidative stress and activated the Nrf2 antioxidant pathway in the protective effect on ox-LDL-induced endothelial injury. Oxidative stress is induced by excessive ROS, and acts as a critical mechanism in vascular homeostasis and atherosclerosis [13]. Intracellular ROS production promotes endothelial cell apoptosis in the development of atherosclerosis [23], while NADPH oxidase can generate vascular ROS and is involved in endothelial apoptosis [24]. Uncontrolled production of ROS leads to vascular injury, which is in accordance with our results showing that ox-LDL enhanced production of ROS and NADPH oxidase activity, with reduced cell viability. Nrf2 is an endogenous antioxidant defense system that is activated under stress conditions, thereby maintaining cellular redox homeostasis. Activation of the Nrf2/HO-1 signaling pathway protects endothelial cells from ox-LDL-induced injury [25]. Our results showed that ox-LDL promoted oxidative stress and decreased the mRNA expression of Nrf2, which is inconsistent with previous reports. In human umbilical vein endothelial cells, ox-LDL treatment increased Nrf2 protein expression [26] and nuclear Nrf2 translocation [27]. This contradiction can be explained by the fact that ox-LDL can induce endothelial senescence [28], accompanied by increased NADPH oxidases and NADPH oxidase-derived ROS [29]. In HUVEC, endothelial senescence was caused by suppressed expression of Nrf2 at the transcriptional level [30], and this decreased expression was modulated by direct targeting of Nrf2 mRNA by several microRNAs (miRNAs) [31,32]. AS-IV demonstrates a broad range of anti-oxidative stress by activating the Nrf2 antioxidant pathway, thereby protecting cortical neurons against ischemia/reperfusion injury [33], inhibiting hepatic stellate cell activation [34], and protecting lipopolysaccharide-induced microglia [35]. In particular, activation of Nrf2 by AS-IV protects the integrity of the blood-brain barrier, which was destroyed by LPS [36]. Since endothelial barrier disruption is an important mechanism by which macrophage-derived foam cells promote the development of atherosclerosis [37], Nrf2 activation may be an important mechanism of AS-IV against atherosclerosis, but this has not been reported in the literature. In addition, the potential regulation of AS-IV on cytoplasmic and nuclear Nrf2 protein and endothelial senescence-associated microRNAs deserve further investigation.

This study showed that AS-IV suppressed inflammatory response, as evidenced by decreased proinflammatory cytokines TNFα and IL-6 in supernatant media and decreased mRNA expression of TNFα and IL-6 in HUVEC cells. Atherosclerosis is an inflammatory disease with infiltration of monocytes and macrophages in the vessel wall [38]. ox-LDL is a potential source of inflammation in atherosclerosis, as ox-LDL promoted secretion and expression of several proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and MCP-1 in injured HUVECs [39,40]. In addition to being a proinflammatory cytokine, TNF-α is also a promotor of endothelial cell apoptosis in atherosclerosis [41]. AS-IV exhibits wide anti-inflammatory properties and reduces production of various cytokines in acute kidney injury and atherosclerosis, such as TNF-α, IL-1β, IL-6, and IL-18 [42,43], which is consistent with our *in vitro* results. Furthermore, the downstream pathway of proinflammatory cytokines can be regulated by Nrf2. For example, expression of Nrf2 suppressed TNFα-induced MCP-1 and VCAM mRNA and protein expressions in human aortic endothelial cells [44]. This indicates that activation of Nrf2 also has anti-inflammatory effects in ox-LDL-induced HUVECs, which is supported by a recent study showing that Nrf2 inhibited transcription of proinflammatory cytokine genes and thus suppressed the macrophage inflammatory response [45].

This study has some limitations. Firstly, the *in vitro* model of HUVECs was induced by only ox-LDL, and the protective effect of AS-IV on endothelial injury should be also confirmed in HUVECs induced by TNFα, LPS, and homocysteine. Secondly, this was only an *in vitro* study, and detailed mechanism should be further investigated in animal models of atherosclerosis.

Conclusions

The present study provides evidence that astragaloside IV treatment can protect against ox-LDL-induced endothelial injury, with suppressed oxidative stress and inflammatory response, which is related to enhanced expression of Nrf2 and HO-1 signaling pathways. These findings suggest that astragaloside IV has clinical potential in prevention of atherosclerosis.

Conflict of interest

None.

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