

# Sacubitril/valsartan inhibits ox-LDL-induced MALAT1 expression, inflammation and apoptosis by suppressing the TLR4/NF- $\kappa$ B signaling pathway in HUVECs

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**Abstract.** The therapeutic effect of sacubitril/valsartan (S/V) on heart failure has been confirmed, while its role in atherosclerosis remains largely unexplored. The present study aimed to investigate the effects of S/V on the expression of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), inflammation and apoptosis in human umbilical vein endothelial cells (HUVECs) induced by oxidized low-density lipoprotein (ox-LDL) and to elucidate its possible mechanism. Cell Counting Kit-8 assay was used to detect cell viability. Reverse transcription-quantitative PCR was performed to detect the MALAT1 expression. ELISA was performed to detect the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . Flow cytometry was conducted to detect the apoptotic rate of cells. A nitric oxide (NO) detection kit was used to determine the concentration of NO. Western blotting analysis was performed to determine the levels of intercellular cell adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, endothelin-1, caspase-3, Bax, Bcl-2, Toll-like receptor 4 (TLR4), p65 and p-p65. Compared with the ox-LDL group, S/V treatment significantly increased the cell viability, NO concentration and Bcl-2 expression, decreased the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  and reduced the expressions of MALAT1, ICAM-1, VCAM-1, cleaved-caspase-3, Bax, TLR4 and p-p65. Overall, the findings suggested that S/V could downregulate the expression of MALAT1, inhibit inflammation and apoptosis and improve endothelial function in ox-LDL-induced HUVECs via inactivating the TLR4/NF- $\kappa$ B signaling pathway. Therefore,

S/V might be utilized as a promising therapeutic strategy for the prevention and treatment of atherosclerosis.

## Introduction

Atherosclerosis has become a common disease, which increasingly threatens human health. According to the World Health Organization, an estimated 17.5 million people died of cardiovascular disease in 2012, of which 7.4 million died of ischemic heart disease and 6.7 million died of stroke (1). Atherosclerosis is the fundamental cause of a series of diseases, such as myocardial infarction, stroke and gangrene (2). Its pathogenesis is complex. With the development of research, the currently recognized mechanisms include lipid metabolism disorder, inflammatory response, oxidative stress and so on (3,4). Vascular endothelial cells serve a key role in the pathogenesis of atherosclerosis by regulating vascular tension, platelet adhesion, inflammation and fibrinolysis (5). Oxidized low-density lipoprotein (ox-LDL) is widely recognized to participate in the occurrence and development of atherosclerosis through inducing oxidative chain reaction and endothelial dysfunction (6). At the onset of atherosclerosis, LDL is deposited on vascular endothelial cells to be oxidized to form ox-LDL, which is then absorbed by macrophages to form foam cells. During this process, many inflammatory factors are released, leading to aggravated progression of atherosclerosis and the formation of atherosclerotic plaques (7). Therefore, the study of ox-LDL-mediated endothelial cell dysfunction can further clarify the pathogenesis of atherosclerosis and provide a more theoretical basis for the treatment and prevention of atherosclerosis.

Long non-coding RNAs (lncRNAs) belong to non-coding RNAs without protein-coding function and comprise >200 nucleotides (8). Although they cannot encode proteins, they are involved in many biological processes as regulators, such as proliferation, apoptosis, migration and invasion (9). The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was initially identified as a tumor-related lncRNA, which can control the proliferation and metastasis of lung adenocarcinoma (10). Previous studies have shown that the expression of MALAT1 is increased in endothelial cells

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induced by ox-LDL and its expression level can reflect the damage degree of endothelial cells (11,12).

Sacubitril/valsartan (S/V) is the first angiotensin receptor neprilysin inhibitor drug, which comprises two components, neprilysin inhibitor sacubitril and angiotensin II receptor antagonist (ARB) valsartan (13). S/V is more effective than the classic renin-angiotensin system blockers (including ARBs and angiotensin-converting-enzyme inhibitors in the treatment of congestive heart failure (14). Myocardial ischemia caused by coronary atherosclerosis is an important cause of heart failure (15). However, whether S/V has an anti-atherosclerotic effect remains controversial. The protective effect of S/V on endothelial cells induced by ox-LDL has not been studied. Therefore, the present study first established an ox-LDL-induced injury model of human umbilical vein endothelial cells (HUVECs) and then explored the effects of S/V on MALAT1 expression, inflammation, apoptosis and other indicators and clarified the protective effect of S/V on endothelial cells and its underlying mechanism.

## Materials and methods

**Cell culture and intervention.** HUVECs were purchased from Shanghai Zhongqiaoxin Zhou Biotechnology Co., Ltd. and grown in endothelial cell culture medium supplemented with 1% endothelial growth factor and 5% fetal bovine serum (Shanghai Zhongqiaoxin Zhou Biotechnology Co., Ltd). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Subsequently, the cells were exposed to 80 µg/ml ox-LDL (Peking Union-Biology Co., Ltd.) for 72 h or pretreated with S/V (Novartis International AG) for 2 h and then exposed to 80 µg/ml ox-LDL for another 72 h.

**Cell Counting Kit-8 (CCK-8) assay.** Cell viability was determined using a CCK-8 assay (Wanleibio Co., Ltd.). The cells were prepared as a single-cell suspension (3×10<sup>4</sup> cells/ml) and seeded into 96-well plates. After 72 h of incubation under different conditions, 10 µl of CCK-8 solution was added into each well, followed by the incubation at 37°C for another 2 h. Subsequently, 10 µl termination solution was added into each well. The absorbance at a wavelength of 450 nm was determined by a microplate reader (BioTek Instruments, Inc.).

**RNA extraction and reverse transcription-quantitative (RT-q) PCR.** Total RNA was extracted from cells using TriPure reagent (BioTeke Corporation) according to the manufacturer's protocols. The number of cells in each group was ~1×10<sup>6</sup>. Purified RNA was reversely transcribed into cDNA using super M-MLV reverse transcriptase kit (BioTeke Corporation) according to the manufacturer's protocols. RT-qPCR was conducted on an Exicycler 96 (Bioneer Corporation) using 2X Power Taq PCR MasterMix kit (BioTeke Corporation) according to the manufacturer's protocols. The reaction volume was 20 µl. RT was performed as follows: 70°C for 5 min, 42°C for 60 min and 80°C for 10 min. The thermocycling conditions of PCR amplification consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec. GAPDH was used as a housekeeping gene. The relative expression of the target gene were calculated with the 2<sup>-ΔΔC<sub>q</sub></sup> method (16). Each experiment

was replicated three times. The primer sequences were as follows: MALAT1 forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'; GAPDH forward, 5'-TCAAGAAGGTGGTGAAGCAGG-3' and reverse, 5'-TCAAAGGTGGAGGAGTGGGT-3'.

**ELISA.** The levels of interleukin IL-1β (cat. no. WLE03), IL-6 (cat. no. WLE04) and TNF-α (cat. no. WLE05) were determined by ELISA. Following cell stimulation, the supernatant was collected and tested according to the manufacturer's instructions (Wanleibio Co., Ltd.).

**Flow cytometry.** After the cells were harvested and resuspended, cell apoptosis was determined with the Annexin V-FITC/PI Apoptosis Detection kit (Wanleibio Co., Ltd.). The cells were incubated with 5 µl Annexin V-FITC and 10 µl PI in the dark for 15 min at room temperature. The apoptotic rate was analyzed by a flow cytometer (NovoCyt; ACEA Bioscience, Inc.; Agilent Technologies, Inc.). NovoExpress 13.0 software (Agilent Technologies, Inc.) was used for analysis. The percentage of early + late apoptotic cells was the apoptotic rate.

**Measurement of nitric oxide (NO) production.** HUVECs were divided into various groups according to different treatments and the supernatant of each group was collected. The content of NO in the supernatant was detected by the NO detection kit (Nanjing Jiancheng Bioengineering Inc.) according to the manufacturer's instructions.

**Western blotting analysis.** Total proteins were extracted from HUVECs of different groups using a total protein extraction kit (Wanleibio Co., Ltd.) according to the manufacturer's instructions and the protein concentration was determined using a BCA kit (Wanleibio Co., Ltd.). Briefly, equal amounts of proteins (40 µg) were subjected to SDS-PAGE on 10% gels and transferred onto a PVDF membrane (EMD Millipore). The membranes were blocked with 5% skimmed milk in TBST with 0.15% Tween-20 at room temperature for 1 h, followed by the incubation with different primary antibodies as follows: Intercellular cell adhesion molecule (ICAM)-1 (cat. no. WL02268; 1:500; Wanleibio Co., Ltd.), vascular cell adhesion molecule (VCAM)-1 (cat. no. A0279; 1:2,000; ABclonal Biotech Co., Ltd.), endothelin (ET)-1 (cat. no. WL07780; 1:500; Wanleibio Co., Ltd.), p65 (cat. no. WL01980; 1:500; Wanleibio Co., Ltd.), p-p65 (cat. no. WL01980; 1:500; Wanleibio Co., Ltd.), Toll-like receptor 4 (TLR4; cat. no. WL00196; 1:500; Wanleibio Co., Ltd.), caspase-3 (cat. no. WL02117; 1:500; Wanleibio Co., Ltd.), Bcl-2 (cat. no. WL01556; 1:500; Wanleibio Co., Ltd.), Bax (cat. no. WL01637; 1:500; Wanleibio Co., Ltd.) and GAPDH (cat. no. WL01114; 1:500; Wanleibio Co., Ltd.) at 4°C overnight. Membranes were washed with TBST three times (10 min x3). Subsequently, the membranes were incubated with HRP-conjugated secondary antibody (cat. no. WLA023; 1:5,000; Wanleibio Co., Ltd.) at room temperature for 1 h and washed with TBST three times (10 min x3). Immunoreactive bands were visualized using an ECL detection system (Wanleibio Co., Ltd.) and quantified by Gel-Pro analyzer software (Media Cybernetics, Inc.).

**Statistical analysis.** All data were expressed as mean  $\pm$  standard deviation. SPSS 21.0 software (IBM Corp.) was used for statistics. Groups of data were compared by one-way ANOVA with post hoc analysis using Tukey's test for pairwise comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**S/V increases the viability of HUVECs induced by ox-LDL.** The present study first used a CCK-8 assay to assess the effect of S/V on the viability of HUVECs induced by ox-LDL. Compared with the control group, the cell viability of HUVECs exposed to 80  $\mu\text{g/ml}$  ox-LDL alone was significantly decreased, while pretreatments with different concentrations ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  and  $10^{-8}$  mmol/l) of S/V increased the cell viability and the most significant increase was found in the group of  $10^{-4}$  mmol/l S/V (Fig. 1). Therefore, cells were pretreated with  $10^{-4}$  mmol/l S/V in subsequent experiments.

**S/V reduces the expression of MALAT1 in HUVECs induced by ox-LDL.** The expression of MALAT1 was detected by RT-qPCR. Compared with the control group, the expression of MALAT1 in the ox-LDL group was significantly increased ( $P < 0.05$ ). Pretreatment with S/V significantly reduced the expression of MALAT1 in HUVECs induced by ox-LDL ( $P < 0.05$ ; Fig. 2). However, treatment with S/V alone did not affect the expression of MALAT1.

**S/V alleviates ox-LDL-induced inflammation of HUVECs.** The levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were detected by ELISA. Compared with the control group, the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the ox-LDL group were significantly increased ( $P < 0.05$ ), while the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the ox-LDL+S/V group were significantly decreased compared with the ox-LDL group ( $P < 0.05$ ) (Fig. 3A-C). The expressions of adhesion molecules at the protein level were detected using western blotting analysis. Compared with the control group, the expressions of VCAM-1 and ICAM-1 in the ox-LDL group were significantly increased ( $P < 0.05$ ), while the expressions of VCAM-1 and ICAM-1 in the ox-LDL+S/V group were significantly decreased compared with the ox-LDL group ( $P < 0.05$ ; Fig. 3D). These results indicated that S/V alleviated ox-LDL-induced inflammation of HUVECs.

**S/V inhibits ox-LDL-induced apoptosis of HUVECs.** The apoptotic rate of each group was detected by flow cytometry. Compared with the control group, the apoptotic rate of the ox-LDL group was significantly increased ( $P < 0.05$ ), while the apoptotic rate of the ox-LDL+S/V group was significantly decreased compared with the ox-LDL group ( $P < 0.05$ ; Fig. 4A). The expression levels of apoptosis-related proteins, caspase-3, Bax and Bcl-2 were detected at the protein level using western blotting analysis. Compared with the control group, the expressions of pro-apoptotic proteins, cleaved-caspase-3 and Bax in the ox-LDL group were significantly increased, while the expression of anti-apoptotic protein Bcl-2 was significantly decreased ( $P < 0.05$ ). Furthermore, compared with the ox-LDL group, the expressions of cleaved-caspase-3 and Bax were

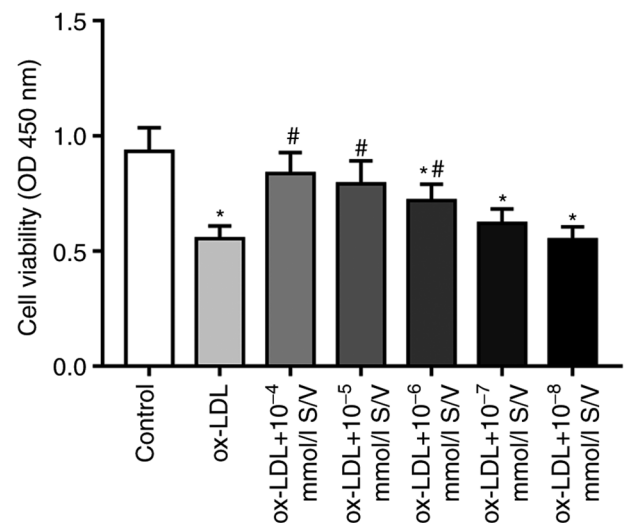


Figure 1. S/V increases the viability of HUVECs induced by ox-LDL. HUVECs were pretreated with or without different doses of S/V ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  and  $10^{-8}$  mmol/l) for 2 h, followed by the incubation with ox-LDL (80  $\mu\text{g/ml}$ ) for 72 h. The cell viability was tested by CCK-8 assay. Data are reported as mean  $\pm$  standard deviation. \* $P < 0.05$  vs. the control group; # $P < 0.05$  vs. the ox-LDL group. S/V, sacubitril/valsartan; HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein; OD, optical density.

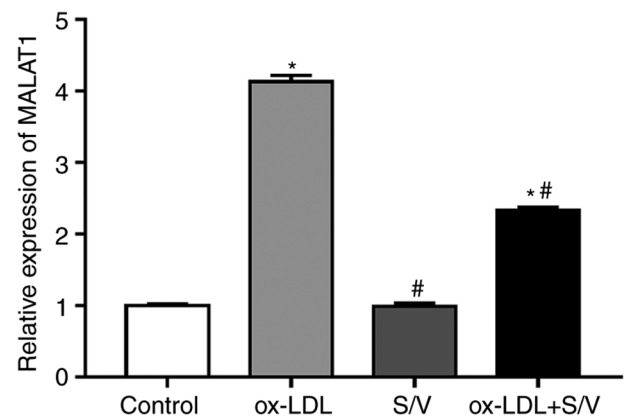


Figure 2. S/V reduces the expression of MALAT1 in HUVECs induced by ox-LDL. HUVECs were pretreated with or without S/V ( $10^{-4}$  mmol/l) for 2 h, followed by the incubation with ox-LDL (80  $\mu\text{g/ml}$ ) for 72 h. The expression of MALAT1 was detected using reverse transcription-quantitative PCR. The values are the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. the control group; # $P < 0.05$  vs. the ox-LDL group. S/V, sacubitril/valsartan; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein.

significantly decreased and the expression of Bcl-2 was significantly increased in the ox-LDL+S/V group ( $P < 0.05$ ; Fig. 4B). These results suggested that S/V could inhibit the apoptosis of HUVECs induced by ox-LDL.

**S/V increases NO release and decreases endothelin 1 (ET-1) expression in ox-LDL-induced HUVECs.** To examine whether S/V could promote the production and release of NO in HUVECs, the content of NO was measured under different treatment conditions. Compared with the control group, the NO concentration of the ox-LDL group was significantly decreased ( $P < 0.05$ ), while the NO concentration of the ox-LDL+S/V

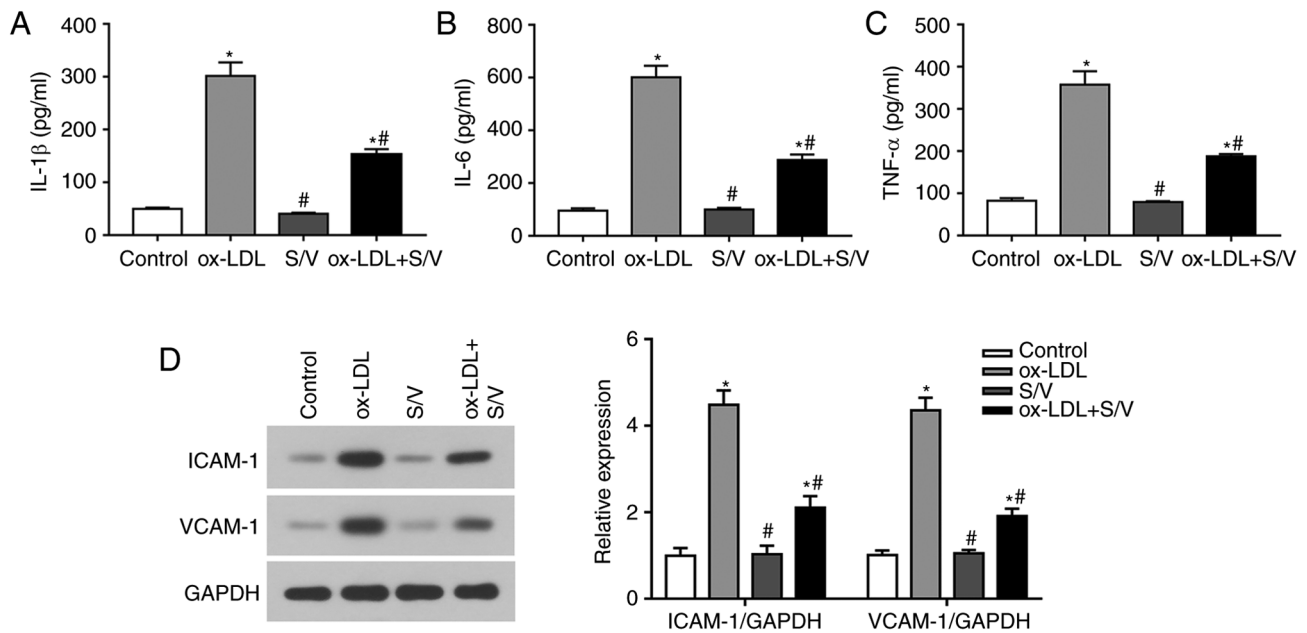


Figure 3. S/V alleviates ox-LDL-induced inflammation of HUVECs. HUVECs were pretreated with or without S/V ( $10^{-4}$  mmol/l) for 2 h, followed by the incubation with ox-LDL (80  $\mu$ g/ml) for 72 h. The secreted levels of (A) IL-1 $\beta$ , (B) IL-6 and (C) TNF- $\alpha$  were detected by ELISA. (D) The protein expression levels of VCAM-1 and ICAM-1 were detected by western blotting assay. The values are the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. the control group; # $P < 0.05$  vs. the ox-LDL group. S/V, sacubitril/valsartan; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; VCAM, vascular cell adhesion molecule; ICAM, intercellular cell adhesion molecule.

group was significantly increased compared with the ox-LDL group ( $P < 0.05$ ; Fig. 5A). ET-1 expression was detected by western blotting analysis. Compared with the control group, the expression of ET-1 in the ox-LDL group was significantly increased, while the expression of ET-1 in the ox-LDL+S/V group was significantly decreased compared with the ox-LDL group ( $P < 0.05$ ; Fig. 5B). These results indicated that S/V alleviated ox-LDL-induced endothelial dysfunction.

**S/V inhibits TLR4/NF- $\kappa$ B signaling pathway in ox-LDL-induced HUVECs.** The present study found that S/V alleviated the inflammation and apoptosis of HUVECs induced by ox-LDL, while the specific mechanism remained unclear. TLR4/NF- $\kappa$ B signaling pathway has been found to regulate endothelial cell inflammation and apoptosis (17,18). Based on these studies, the present study examined whether S/V alleviated inflammation and apoptosis by inhibiting the TLR4/NF- $\kappa$ B signaling pathway. Compared with the control group, the expressions of TLR4 and p-p65 in the ox-LDL group were significantly increased ( $P < 0.05$ ), while the expressions of TLR4 and p-p65 in the ox-LDL+S/V group were significantly decreased compared with the ox-LDL group ( $P < 0.05$ ; Fig. 6). These results suggested that S/V reduced ox-LDL-induced endothelial cell injury by suppressing the TLR4/NF- $\kappa$ B signaling pathway.

## Discussion

As a drug with dual targets, S/V has been fully affirmed in the treatment of heart failure and is considered a major breakthrough in the field of heart failure treatment in recent years (19). The PARADIGM-HF study has shown that compared with enalapril, S/V significantly reduces the major

composite endpoint of hospitalization or cardiovascular mortality due to heart failure, as well as cardiovascular mortality and the hospitalization rate of heart failure (14). S/V also shows great potential in the treatment of hypertension, diabetes and other diseases (20,21). Endothelial dysfunction serves a key role in the formation of early atherosclerosis, including many complex processes, such as inflammatory response and cell apoptosis (5). Seki *et al* (22) found that S/V can improve endothelial dysfunction in spontaneously hypertensive rats. The present study investigated the role of S/V in ox-LDL-induced endothelial cell injury and elucidated its possible mechanism.

In general, the stated doses of S/V in patients is 100 mg twice daily and the target maintenance dose is 200 mg twice daily (14,23). The absorption and metabolism of drugs in the body is a very complicated process. In *in vitro* experiments, there is no influence from neuroendocrine, immune and other complex factors. Therefore, the drug concentration *in vitro* does not correspond to the drug concentration *in vivo* or in patients. In the present study, in order to select the appropriate concentration, the drug was first made into different concentration gradients ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  and  $10^{-8}$  mmol/l). Then CCK-8 was used to assess the effect of S/V on the viability of HUVECs induced by ox-LDL. The results showed that  $10^{-4}$  mmol/l S/V had the best intervention effect. Therefore, cells were pretreated with  $10^{-4}$  mmol/l S/V in subsequent experiments.

A number of studies have confirmed that lncRNA MALAT1 is upregulated in ox-LDL-induced endothelial cells and participates in ox-LDL-induced endothelial dysfunction. Wang *et al* (11) reported that MALAT1 enhances the expression of Beclin-1 by combining miR-216a-5p and promotes autophagy to protect the endothelial cells. Tang *et al* (12)



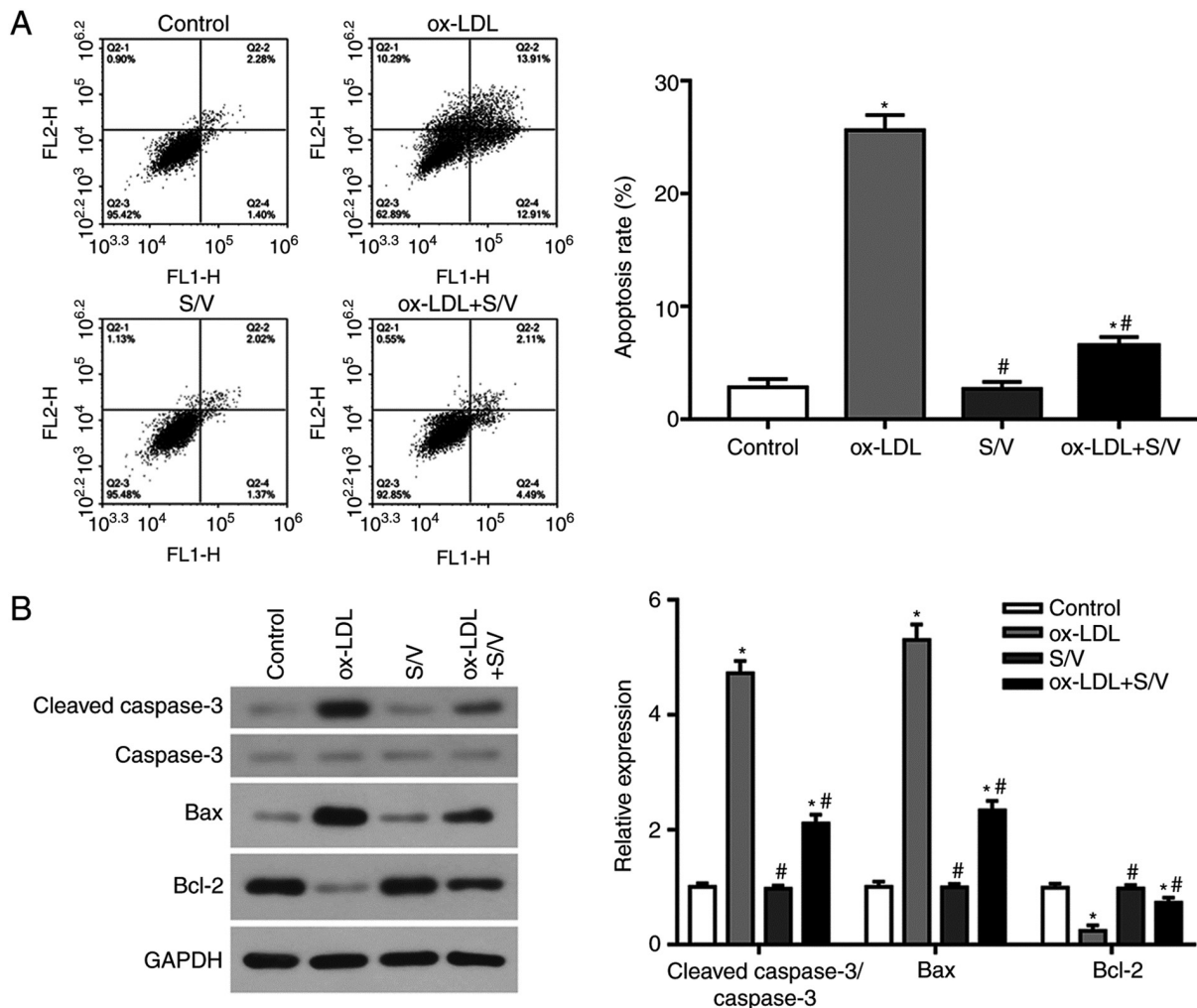


Figure 4. S/V inhibits ox-LDL-induced apoptosis of HUVECs. HUVECs were pretreated with or without S/V ( $10^{-4}$  mmol/l) for 2 h, followed by the incubation with ox-LDL (80  $\mu$ g/ml) for 72 h. (A) Flow cytometry was used to evaluate cell apoptosis in HUVECs. (B) The protein expression levels of cleaved-caspase-3, caspase-3, Bax and Bcl-2 were detected by western blotting analysis. The values are the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. the control group; # $P < 0.05$  vs. the ox-LDL group. S/V, sacubitril/valsartan; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells.

demonstrated that MALAT1 protects the endothelial cells from ox-LDL-induced endothelial dysfunction partly through competing with miR-22-3p for endogenous RNA. Based on the above-mentioned studies, it was hypothesized that MALAT1 could be used as an indicator of endothelial function. The present study found that the expression of MALAT1 was increased in ox-LDL-induced HUVECs, which was consistent with previous findings. In addition, compared with the ox-LDL group, pretreatment with S/V significantly reduced the expression of MALAT1 in HUVECs induced by ox-LDL. Taken together, it was confirmed that S/V could improve endothelial function.

Ox-LDL is a key component of hyperlipidemia, which can induce endothelial inflammation and apoptosis by enhancing the oxidative stress of endothelial cells (24). Ox-LDL-induced inflammation is the leading cause of endothelial dysfunction (25). Atherosclerosis is a persistent inflammatory response, which can be activated by ox-LDL aggregation on the arterial wall (26). The increased expressions of inflammatory cytokines, including inflammatory factors and cell adhesion molecules, can advance the adhesion between monocytes

and vascular endothelial cells (27). Macrophages are subsequently activated and these macrophages absorb lipoproteins, leading to foam cell formation (28). The present study found that the levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , VCAM-1 and ICAM-1 were increased in endothelial cells after ox-LDL stimulation. When HUVECs were pretreated with S/V, the levels of these pro-inflammatory factors were decreased compared with the ox-LDL group, indicating that S/V could alleviate the inflammatory response induced by ox-LDL.

Endothelial cell apoptosis can increase the permeability of endothelial monolayer by reducing the number of endothelial cells, thus promoting lipid migration and deposition (29). Then, monocytes and smooth muscle cells migrate to the endothelium, engulf large amounts of lipids, form foam cells, further damage blood vessels and promote plaque formation (29). Meanwhile, the growth factors and cytokines secreted by infiltrating white cells also affect the proliferation of smooth muscle cells (30). Ox-LDL is a carrier of oxygen-free radicals, which can produce toxic effects on vascular cells, promote their apoptosis and cause vascular endothelial damage (31). In the present study, the results of

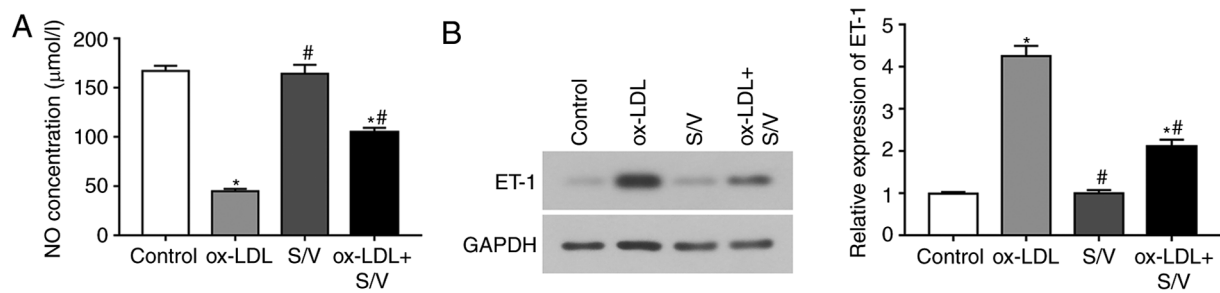


Figure 5. S/V increases NO release and decreases ET-1 expression in ox-LDL-induced HUVECs. HUVECs were pretreated with or without S/V ( $10^{-4}$  mmol/l) for 2 h, followed by the incubation with ox-LDL ( $80 \mu\text{g/ml}$ ) for 72 h. (A) The content of NO in the supernatant was detected by an NO detection kit. (B) The protein expression level of ET-1 was detected by western blotting assay. The values are the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. the control group; # $P < 0.05$  vs. the ox-LDL group. S/V, sacubitril/valsartan; NO, nitric oxide; ET-1, endothelin 1; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells.

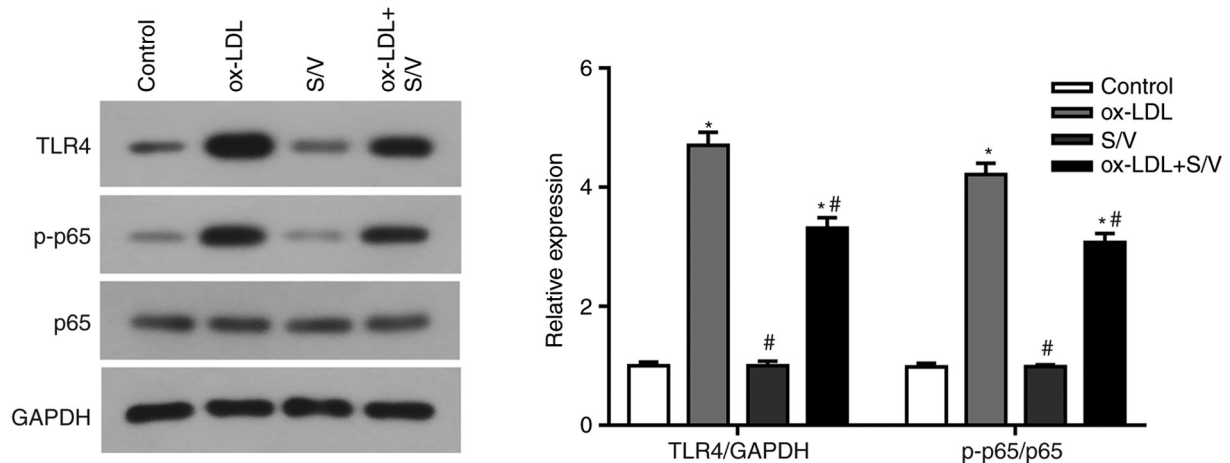


Figure 6. S/V inhibits TLR4/NF- $\kappa$ B signaling pathway in ox-LDL-induced HUVECs. HUVECs were pretreated with or without S/V ( $10^{-4}$  mmol/l) for 2 h, followed by the incubation with ox-LDL ( $80 \mu\text{g/ml}$ ) for 72 h. The protein expression levels of TLR4, p-p65 and p65 were detected by western blotting analysis. The values are the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. the control group; # $P < 0.05$  vs. the ox-LDL group. S/V, sacubitril/valsartan; TLR4, Toll-like receptor 4; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; p-, phosphorylated.

flow cytometry showed that the apoptotic rate of the ox-LDL group was significantly increased compared with the control group and S/V pretreatment could significantly reduce the apoptotic rate. Western blotting analysis showed that S/V pretreatment could reverse the upregulation of pro-apoptotic proteins, cleaved-caspase-3 and Bax and promoted the expression of anti-apoptotic protein Bcl-2. This finding was consistent with the results of flow cytometry. These results suggested that S/V could inhibit the apoptosis of HUVECs induced by ox-LDL.

Endothelium-derived NO is an important regulator of endothelial function, which serves an important role in the regulation of vascular homeostasis. Its regulatory role is mainly achieved by regulating vascular tension and blood pressure, inhibiting vascular smooth muscle proliferation and migration, suppressing platelet aggregation and constraining monocyte and platelet adhesion (32). ET-1 is a factor secreted by the vascular endothelium that has the opposite effect of NO (33). ET-1 can activate the exchange of  $\text{Na}^+/\text{H}^+$  and  $\text{Na}^+/\text{Ca}^{2+}$  in vascular smooth muscle fibers, increase intracellular  $\text{Ca}^{2+}$  concentration, induce vascular smooth muscle contraction and cause ischemia and hypoxia (34). As important indicators of endothelial function, NO and ET-1 serve an important role

in maintaining vascular tension and cardiovascular system homeostasis. In the present study, HUVECs exposed to ox-LDL showed increased expression of ET-1 and decreased level of NO. However, pretreatment with S/V could reduce the ET-1 expression and increase the NO level, indicating that S/V could reduce the injury of endothelial cells induced by ox-LDL.

The present study identified the role of S/V in ox-LDL-induced inflammation and apoptosis in HUVECs and further studied its underlying mechanism. TLRs are the most important pattern recognition receptors in the natural immune system and TLR4 is an important member of the TLR family (35). After binding to its ligand, TLR4 can promote the expressions of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , ICAM-1 and other inflammatory factors through NF- $\kappa$ B and other signal transduction pathways, enhance the immune-inflammatory response and induce the apoptosis of target cells (36). NF- $\kappa$ B is an important downstream signaling molecule of TLR4 (24,37), which exists in the cytoplasm as a dimer (p65/p50) and usually binds to its inhibitory protein I $\kappa$ B. When ox-LDL binds to TLR4 and activates downstream signaling molecules, I $\kappa$ B kinase is activated (38). Consequently, I $\kappa$ B protein is phosphorylated, ubiquitinated and then degraded and cytoplasmic p65 is released (39). Then phosphorylated

p65 enters the nucleus and combines with target genes on the nucleus to generate a large number of inflammatory factors, which in turn act on the receptors of endothelial cells to induce apoptosis (40). In the present study, compared with the ox-LDL group, S/V pretreatment reduced the expressions of TLR4 and p-p65 in HUVECs induced by ox-LDL, indicating that S/V could suppress the TLR4/NF- $\kappa$ B signaling pathway. However, there are several limitations to the present study. S/V is a new drug composed of two drugs, sacubitril and valsartan. At present, there are few studies on the effect of S/V on endothelial cells. The purpose of the present study was only to initially explore whether S/V had beneficial effects on endothelial cells, so groups of each drug alone were not added to the study. Therefore, it is unclear whether the beneficial effects of the drug were caused by sacubitril, valsartan, or both. The present study was conducted *in vitro* only and it is unclear whether S/V has the same beneficial effect on endothelial cells *in vivo*. Thus, further research is still required.

In conclusion, the present study found that S/V could downregulate the expression of MALAT1, inhibit inflammation and apoptosis and improve endothelial function in ox-LDL-induced HUVECs by suppressing the TLR4/NF- $\kappa$ B signaling pathway. Therefore, S/V might be used as a promising therapeutic strategy for the prevention and treatment of atherosclerosis.

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#### Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

#### Authors' contributions

WB and XQ designed the study, analyzed the data and wrote the manuscript. TH, XC, and XS performed the experiments and prepared the figures. CM, YD, CR and LD prepared the figures and analyzed the data. WL and XQ performed critical revision of the manuscript and supervised the study. WB and XQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### References

- Mendis S, Davis S and Norrving B: Organizational update: the world health organization global status report on noncommunicable diseases 2014; one more landmark step in the combat against stroke and vascular disease. *Stroke* 46: e121-122, 2015.
- Hansson GK: Inflammatory mechanisms in atherosclerosis. *J Thromb Haemost* 7 (Suppl 1): S328-S331, 2009.
- Moore KJ and Tabas I: Macrophages in the pathogenesis of atherosclerosis. *Cell* 145: 341-355, 2011.
- Förstermann U, Xia N and Li H: Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. *Circ Res* 120: 713-735, 2017.
- Gimbrone MA and García-Cardeña G: Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circ Res* 118: 620-636, 2016.
- Han QA, Yan C, Wang L, Li G, Xu Y and Xia X: Urolithin A attenuates ox-LDL-induced endothelial dysfunction partly by modulating microRNA-27 and ERK/PPAR- $\gamma$  pathway. *Mol Nutr Food Res* 60: 1933-1943, 2016.
- Fan X, Wang J, Hou J, Lin C, Bensoussan A, Chang D, Liu J and Wang B: Berberine alleviates ox-LDL induced inflammatory factors by up-regulation of autophagy via AMPK/mTOR signaling pathway. *J Transl Med* 13: 92, 2015.
- Mercer TR, Dinger ME and Mattick JS: Long non-coding RNAs: Insights into functions. *Nat Rev Genet* 10: 155-159, 2009.
- Boon RA, Jaé N, Holdt L and Dimmeler S: Long noncoding RNAs: From clinical genetics to therapeutic targets. *J Am Coll Cardiol* 67: 1214-1226, 2016.
- Tano K, Mizuno R, Okada T, Rakwal R, Shibato J, Masuo Y, Ijiri K and Akimitsu N: MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. *FEBS Lett* 584: 4575-4580, 2010.
- Wang K, Yang C, Shi J and Gao T: Ox-LDL-induced lncRNA MALAT1 promotes autophagy in human umbilical vein endothelial cells by sponging miR-216a-5p and regulating Beclin-1 expression. *Eur J Pharmacol* 858: 172338, 2019.
- Tang Y, Jin X, Xiang Y, Chen Y, Shen CX, Zhang YC and Li YG: The lncRNA MALAT1 protects the endothelium against ox-LDL-induced dysfunction via upregulating the expression of the miR-22-3p target genes CXCR2 and AKT. *FEBS Lett* 589: 3189-3196, 2015.
- Gu J, Noe A, Chandra P, Al-Fayoumi S, Ligueros-Saylan M, Sarangapani R, Maahs S, Ksander G, Rigel DF, Jeng AY, *et al*: Pharmacokinetics and pharmacodynamics of LCZ696, a novel dual-acting angiotensin receptor-neprilysin inhibitor (ARNi). *J Clin Pharmacol* 50: 401-414, 2010.
- McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, *et al*: Angiotensin-neprilysin inhibition versus enalapril in heart failure. *N Engl J Med* 371: 993-1004, 2014.
- Pagliaro BR, Cannata F, Stefanini GG and Bolognese L: Myocardial ischemia and coronary disease in heart failure. *Heart Fail Rev* 25: 53-65, 2020.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Cheng J, Liu Q, Hu N, Zheng F, Zhang X, Ni Y and Liu J: Downregulation of hsa\_circ\_0068087 ameliorates TLR4/NF- $\kappa$ B/NLRP3 inflammasome-mediated inflammation and endothelial cell dysfunction in high glucose conditioned by sponging miR-197. *Gene* 709: 1-7, 2019.
- Wan CX, Xu M, Huang SH, Wu QQ, Yuan Y, Deng W and Tang QZ: Baicalein protects against endothelial cell injury by inhibiting the TLR4/NF- $\kappa$ B signaling pathway. *Mol Med Rep* 17: 3085-3091, 2018.
- Proudfoot C, Studer R, Rajput T, Jindal R, Agrawal R, Corda S and Senni M: Real-world effectiveness and safety of sacubitril/valsartan in heart failure: A systematic review. *Int J Cardiol*: Feb 3, 2021 (Epub ahead of print).
- Ruilope LM, Dukat A, Böhm M, Lacourcière Y, Gong J and Lefkowitz MP: Blood-pressure reduction with LCZ696, a novel dual-acting inhibitor of the angiotensin II receptor and neprilysin: A randomised, double-blind, placebo-controlled, active comparator study. *Lancet* 375: 1255-1266, 2010.
- Seferovic JP, Claggett B, Seidemann SB, Seely EW, Packer M, Zile MR, Rouleau JL, Swedberg K, Lefkowitz M, Shi VC, *et al*: Effect of sacubitril/valsartan versus enalapril on glycaemic control in patients with heart failure and diabetes: A post-hoc analysis from the PARADIGM-HF trial. *Lancet Diabetes Endocrinol* 5: 333-340, 2017.

22. Seki T, Goto K, Kansui Y, Ohtsubo T, Matsumura K and Kitazono T: Angiotensin II receptor-neprilysin inhibitor sacubitril/valsartan improves endothelial dysfunction in spontaneously hypertensive rats. *J Am Heart Assoc* 6: e006617, 2017.
23. Kido K, Bianco C, Caccamo M, Fang W and Sokos G: Evaluating sacubitril/valsartan dose dependence on clinical outcomes in patients with heart failure with reduced ejection fraction. *Ann Pharmacother*: Dec 31, 2020 (Epub ahead of print).
24. Zhu L, Gong X, Gong J, Xuan Y, Fu T, Ni S, Xu L and Ji N: Notoinsenoside R1 upregulates miR-221-3p expression to alleviate ox-LDL-induced apoptosis, inflammation, and oxidative stress by inhibiting the TLR4/NF- $\kappa$ B pathway in HUVECs. *Braz J Med Biol Res* 53: e9346, 2020.
25. Li W, Li Y, Zhao Y and Ren L: The protective effects of alopurinol against ox-LDL-induced endothelial dysfunction and inflammation in HUVECs. *Artif Cells Nanomed Biotechnol* 48: 107-115, 2020.
26. García de Tena J: Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 353: 429-430, 2005.
27. Yang X, Wan M, Cheng Z, Wang Z and Wu Q: Tofacitinib inhibits ox-LDL-induced adhesion of THP-1 monocytes to endothelial cells. *Artif Cells Nanomed Biotechnol* 47: 2775-2782, 2019.
28. Feng C, Chen Q, Fan M, Guo J, Liu Y, Ji T, Zhu J and Zhao X: Platelet-derived microparticles promote phagocytosis of oxidized low-density lipoprotein by macrophages, potentially enhancing foam cell formation. *Ann Transl Med* 7: 477, 2019.
29. Choy JC, Granville DJ, Hunt DW and McManus BM: Endothelial cell apoptosis: Biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* 33: 1673-1690, 2001.
30. Qin C and Liu Z: In atherogenesis, the apoptosis of endothelial cell itself could directly induce over-proliferation of smooth muscle cells. *Med Hypotheses* 68: 275-277, 2007.
31. Zhang Y, Wang L, Xu J, Kong X and Zou L: Up-regulated miR-106b inhibits ox-LDL-induced endothelial cell apoptosis in atherosclerosis. *Braz J Med Biol Res* 53: e8960, 2020.
32. Moncada S and Higgs EA: The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol* 147 (Suppl 1): S193-S201, 2006.
33. Bourque SL, Davidge ST and Adams MA: The interaction between endothelin-1 and nitric oxide in the vasculature: New perspectives. *Am J Physiol Regul Integr Comp Physiol* 300: R1288-R1295, 2011.
34. Penna C, Rastaldo R, Mancardi D, Cappello S, Pagliaro P, Westerhof N and Losano G: Effect of endothelins on the cardiovascular system. *J Cardiovasc Med (Hagerstown)* 7: 645-652, 2006.
35. Moghimpour Bijani F, Vallejo JG and Rezaei N: Toll-like receptor signaling pathways in cardiovascular diseases: Challenges and opportunities. *Int Rev Immunol* 31: 379-395, 2012.
36. Zhao L, Li M, Sun K, Su S, Geng T and Sun H: Hippophae rhamnoides polysaccharides protect IPEC-J2 cells from LPS-induced inflammation, apoptosis and barrier dysfunction in vitro via inhibiting TLR4/NF- $\kappa$ B signaling pathway. *Int J Biol Macromol* 155: 1202-1215, 2020.
37. Zhang G and Ghosh S: Toll-like receptor-mediated NF-kappaB activation: A phylogenetically conserved paradigm in innate immunity. *J Clin Invest* 107: 13-19, 2001.
38. Zhang M, Xue Y, Chen H, Meng L, Chen B, Gong H, Zhao Y and Qi R: Resveratrol Inhibits MMP3 and MMP9 Expression and Secretion by Suppressing TLR4/NF- $\kappa$ B/STAT3 Activation in Ox-LDL-Treated HUVECs. *Oxid Med Cell Longev* 2019: 9013169, 2019.
39. Yu XH, Zheng XL and Tang CK: Nuclear Factor- $\kappa$ B activation as a pathological mechanism of lipid metabolism and atherosclerosis. *Adv Clin Chem* 70: 1-30, 2015.
40. Zhong X, Zhang L, Li Y, Li P, Li J and Cheng G: Kaempferol alleviates ox-LDL-induced apoptosis by up-regulation of miR-26a-5p via inhibiting TLR4/NF- $\kappa$ B pathway in human endothelial cells. *Biomed Pharmacother* 108: 1783-1789, 2018.