## Original Article
# Sodium paeonolsilate inhibits ox-LDL induced macrophage foam cell formation and inflammation in atherosclerosis

Wenbo Fu[1*], Wenlan Fu[2*], Shifang Ding[1], Zhinan Chen[1], Juquan Jiang[1], Zhigang Gong[1], Zhigang Li[1], Qing Lu[1]

[1]Department of Cardiology, Wuhan General Hospital of Guangzhou Military Region, Wuhan 430070, P. R. China; [2]Department of Obstetrics and Gynecology, The Third People's Hospital of Hubei Province, Wuhan 430033, P. R. China. *Equal contributors.

**Abstract:** Atherosclerosis, formerly considered as a bland arterial collection of cholesterol, characterized by the accumulation of lipids and fibrous elements in smooth muscle cell, actually involves an ongoing inflammatory response. According to the concept, inflammation and lipid metabolism disorder are key regulatory processes to atherosclerosis and its complications. In the present study, we found that SPae (Sodium Paeonolsilate) potently inhibited ox-LDL-induced NF-κB activation and oxidative stress response, which were critical processes during inflammation. It was also revealed that SPae mediated macrophage-derived foam cell formation. Our data showed that SPae potently inhibited ox-LDL-induced up-regulation of the SRA and CD36 expression via JKN/P38 pathway. Meanwhile it promotes reverse cholesterol transport via PPARγ/ABCA1 pathway. And, atherosclerotic plaques, intracellular cholesterol and uptake of lipid were assayed by enzymatic, Red oil O staining, fluorometric method and DiI-labeled Ox-LDL. Further, we investigated the effect of SPae on atherosclerosis in apolipoprotein E deficient (ApoE$^{-/-}$) mice, SPae significantly suppressed the atherosclerotic plaque areas growth and inflammatory reaction and inhibit the progression of atherosclerotic lesions. Collectively, the data suggested that sodium paeonolsilate played as an effective anti-AS agent, probably via anti-inflammatory pathway and inhibition of foam cell formation.

**Keywords:** Sodium paeonolsilate, atherosclerosis, inflammatory, macrophage, apoe knockout mice

## Introduction

Inflammation and macrophage-derived foam cell formation plays a major role in the developing of atherosclerosis. Generally, inflammatory factors activate oxidative stress response in vivo, sustained by vascular endothelial cell injury. The endothelial dysfunction is considered to be the initiating factor of AS, and disorder of lipid metabolism is the key link in the development of AS [1]. Endothelial cell injure not only lead to the further release of inflammatory cytokines which exacerbate the damage of endothelial cells, but also cause the release of cell adhesion factors and chemokines, resulting in the invading of monocyte at endothelial layer and the formation of macrophages [2]. Further with imbalance between reverse cholesterol transport and uptake of lipid, especially the oxidized low density lipoprotein (oxidized

low density lipoprotein, ox-LDL), which lead to the formation of foam cells [3]. As we know, overload of lipid do harm to cells and could lead to cell death, resulting in lipid releasing and formation of the early fatty streak. So the binding and uptake of ox-LDL is the central part in the formation of foam cells, by activating inflammatory events. And phagocytosis of ox-LDL was mainly controlled by the scavenger receptors (SRs). Among them, SR-A and CD36 are main receptors that mediate the uptake of various modified low density lipoprotein, especially ox-LDL [4].

Paeonol extracted from the moutan bark turned to be sodium paeonolsilate after sulfonation and salification. It was found to have multiple effects in anti-inflammatory, analgesic, anti-tumor, anti-lipid peroxidation and immune regulation. Our previous study found that sodium

paeonolsilate has an important role in the anti-oxidative stress induced by TNF-α and anti blood vassal inflammatory. At the same time, many studies have shown that the formation of foam cells is accompanied by inflammation and continued oxidative stress. In the present study, we further observed function of sodium paeonolsilate in inhibition of ox-LDL induced macrophage foam cells and the formation of atherosclerosis, and further investigated its potential mechanisms.

## Materials and methods

### Reagents and antibodies

Sodium Paeonolsilate powder was provided by Shandong Fangming Pharmaceutical Group and the powder was dissolved in DD $H_2O$. (The chemical structure of Sodium Paeonolsilate is shown in (**Figure 1A**). Antibodies of p38, phos-pho-p38, JNK, phospho-JNK, IKKβ, p-IKKβ, IκBα, p-IκBα, PPARγ were all purchased from Cell Signaling Technology (Beverly, MA). Antibodies of SRA, CD36 and ABCA1 were purchased from Santa Cruz Biotech (Santa Cruz, CA). Ox-LDL was purchased from Guangzhou Ladder Biotechnology (Guangzhou, China).

### Cell culture and treatment

Human monocyte cell line THP-1 was kindly provided by Dr. Chuanming Luo (Sun Yat-sen University) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, HyClone, UT, USA), 5 U/ml heparin, 100 U/ml penicillin, and 100 U/ml streptomycin in a humidified atmosphere of 5% $CO_2$ at 37°C.

Human monocyte cell line THP-1 were plated in 60 mm dishes and induced to differentiate into macrophages by pretreated with 100 ng/ml PMA for 48 h, and then treated with or without 10 (low), 20 (Mid), 40 (High) μM SPae for 2 h, followed by incubation with or without 80 μg/mL ox-LDL for 24 h.

### Cell viability assay

CCK-8 assay was used to assess the cytotoxicity of SPae on macrophages. Briefly, Human monocyte cell line THP-1 cells were seeded in 96-well plates with a density of $1×10^5$/well. After treated as method "Cell culture and treatment", CCK-8 reagent was given and incubated

for 2 h; absorbance at wavelength of 450 nm was measured using microplate reader (Bio-Tek, Winooski, VT, USA).

### Lipid accumulation by Oil red O staining

To determine whether SPae displays an effect in inhibiting of foam cell formation, we assayed foam cell formation by Oil red O staining. Cells treated as method "Cell culture and treatment", the cells were washed with PBS for 3 times, fixed with 60% isopropanol for 1 min, and then stained with Oil-Red O for 30 min. Finally, examined and photographed with ×400 magnification.

### Measurements of intracellular cholesterol, NO, SOD and ROS

The cells treated as method "Cell culture and treatment", cells were measured of intracellular total cholesterol and cholesterol ester according to the kit manual. NO, SOD and ROS were measured according to the manuals of kits respectively.

### Binding and uptake assay of Dil-ox-LDL

Ox-LDL was labeled with fluorescent probe, l,l'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocy-anine perchlorate (Dil) for assaying the binding and uptake of Ox-LDL by macrophages via related receptor. Dil-ox-LDL binding and uptake was used to assess the formation of foam cells and the function of scavenger receptor. After treated as method "Cell culture and treatment", cells were washed with PBS, and switched into serum-free RPMI 1640 medium containing 10 μg/ml (w/v) Dil-ox-LDL and incubated at 37°C for another 2-4 h. After removal the medium, the cells were washed 3 times with distilled water and taken photos randomly with laser scanning confocal microscopy (Olympus FV500-1×81, Japan, 400×) with the excitation wavelength and emission wavelength of 520 nm and 580 nm, respectively. The fluorescence density was analyzed by Image-Pro Plus 5.0 fluorescence View software.

### Western blot analysis

The cells were treated according to the above. Moreover, protein isolation and Western blot analysis of the cell lysates were performed as previously described [3]. The primary antibod-
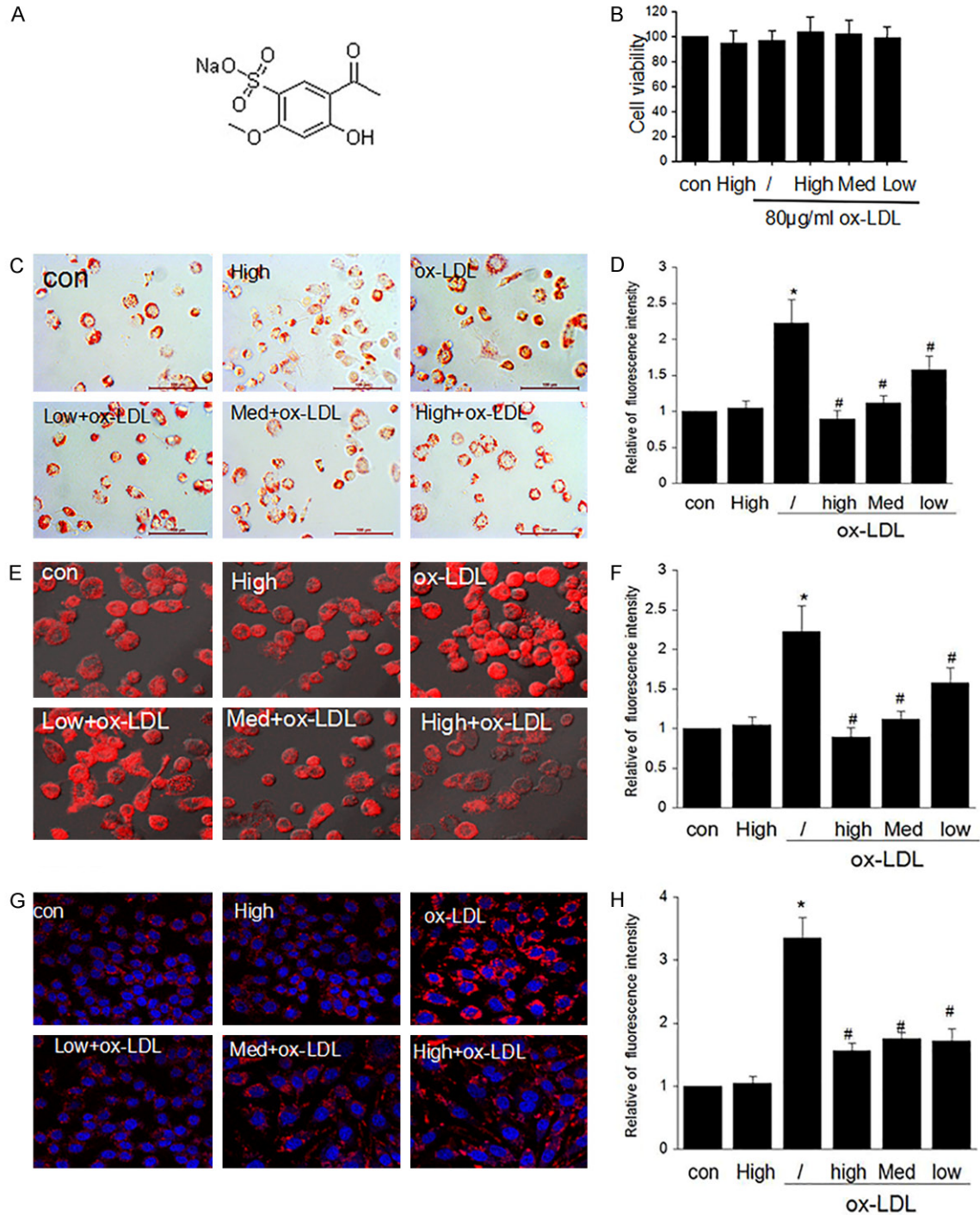
Figure 1. SPae inhibits ox-LDL-induced macrophage-derived foam cell formation. A. The chemical structure of SPae. B. Cell viability after treated with SPae and/or ox-LDL. C. Representative images of Oil Red O staining were measured with Inverted Microscopy. D. Statistical date of Oil Red O staining indicated the changes of foam cell formation. E. Representative images of Dil-ox-LDL staining to analyze the capacity of uptake measured with confocal. F. Statistical date of Dil-ox-LDL staining indicated the changes of Dil-ox-LDL uptake. G. Representative images of Dil-ox-LDL staining to analyze the capacity of binding measured with confocal. H. Statistical date of Dil-ox-LDL staining indicated of the changes of Dil-ox-LDL binding. Bars represent mean ± SD (n=3) *P<0.05 vs. Control, #P<0.05 vs. Ox-LDL only.

ies used in the present study were: Antibodies of ABCA1 (1:1000), p38 (1:4000), phospho-p38 (1:1000), JNK (1:4000), phospho-JNK (1:1000), IKKβ (1:4000), p-IKKβ (1:1000), IκBα (1:4000), p-IκBα (1:1000), PPARγ (1:1000), SRA (1:5000) and CD36 (1:2000).

**Table 1.** Effect of SPae on cholesterol in THP-1 macrophage foam cell

| Group | TC (µg/mg protein) | FC (µg/mg protein) | CE (µg/mg protein) | CE/TC (%) |
|---|---|---|---|---|
| Con | 70.85±5.34 | 57.46±4.2 | 11.23±1.12 | 15.85±1.34 |
| High | 74.82±5.34 | 67.47±5.56 | 10.53±1.34 | 14.07±1.94 |
| ox-LDL | 159.94±13.53* | 105.73±5.35* | 69.42±8.62* | 43.41±3.12* |
| Low+ox-LDL | 140.43±10.33# | 89.52±3.72# | 54.15±7.21# | 38.56±3.51# |
| Med+ox-LDL | 132.43±9.33# | 80.46±4.15# | 48.16±5.28# | 36.37±2.13# |
| High+ox-LDL | 100.18±8.79# | 64.65±5.89# | 28.75±7.16# | 28.71±1.64# |

*$P<0.05$ vs. Con; #$P<0.05$ vs. ox-LDL, n=5.

*Mouse model of atherosclerosis*

Male apoE$^{-/-}$ mice (age 7 weeks, 16-18 g) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice were specific pathogen-free, and maintained in static microisolator cages. Mice were randomly divided into five group: a control group, a model group and three SPae treatment group (high: 200 mg/kg/d SPae, i.p.; med: 100 mg/kg/d SPae, i.p. and low: 50 mg/kg/d SPae, i.p.). Three mice in each group. The control group was given normal diet, and rest of the mice with a high-fat diet containing 20% fat and 0.15% cholesterol for 8 weeks. The SPae group mice received the intraperitoneal injection of SPae for 12 weeks, and the model group mice received the intraperitoneal injective of the same volume of $H_2O$ for 12 weeks. Blood and tissues were collected at the age of 18 weeks for further analysis. All procedures were performed in adherence with the National Institutes of Health Guidelines.

At the end of experiment, all mice were anaesthetized by diethylether. Whole blood was collected from the retroorbital venous plexus for measurement of serum cholesterol and pro-inflammatory cytokine.

*Quantitation of arterial atherosclerotic lesions*

The heart and aorta of apoE-/- mice were perfused with 20 ml PBS (mM: NaCl 130.0, KCl 2.5, $Na_2HPO_4$ 10.0, $KH_2PO_4$ 1.5, pH 7.4) at a rate of 2 ml/min. The aorta was dissected from the heart to the iliac bifurcation; cleaned the adventitial tissue carefully, and fixed in 10% buffered formalin for 24 h. The fixed aorta was opened longitudinally and stained with 0.3% oil red O for 2 h. Images were captured using an Olympus digital camera with macro conversion lens. The positive straining area was determined using Image-Pro plus 5.0 software.

The proximal aortas of apoE$^{-/-}$ mice were immersed in ice-cold 4% paraformaldehyde for 30 min. Then transferred to 30% sucrose-PBS solution at 4°C for 24 h. After that aortas were embedded in tissue freezing medium and snap frozen in liquid nitrogen. Cryosections (5 µm thick) were cut from the proximal aortas beginning at the end of the aortic sinus, and stained with oil red O. Arterial atherosclerotic lesions were also measured by enface oil red O staining. Quantitative analysis of the lesions was performed using Image Pro Plus software on at least 15 sections from each animal by an operator who was blinded to group assignment.

*Statistical analysis*

All data are expressed as mean ± SD and analyzed by Student's *t* test or ANOVA using SPSS for windows 13.0. A value of $P<0.05$ is considered as statistically significant.

**Results**

*Sodium paeonolsilate inhibits ox-LDL-stimulated macrophage-derived foam cell formation*

In cultured THP-1 cells, pre-incubation with 0-40 µM SPae for 2 h obviously decreasd the ox-LDL-induced lipid accumulation in a concentration dependent manner (**Figure 1C** and **1D**). These results hint that SPae may inhibit ox-LDL-stimulated macrophage-derived foam cell formation. During the course of treatment with SPae, no significant changes were observed of cell viability among all groups (**Figure 1B**), which suggests that SPae was toxicity free to THP-1.

We further observed the effect of SPae on macrophage binding and uptake of Dil-oxLDL. The results showed that, compared with the control group, after treated with ox-LDL for 24 h, the ability of macrophage binding (**Figure 1E** and **1F**) and uptake (**Figure 1G** and **1H**) of Dil-oxLDL
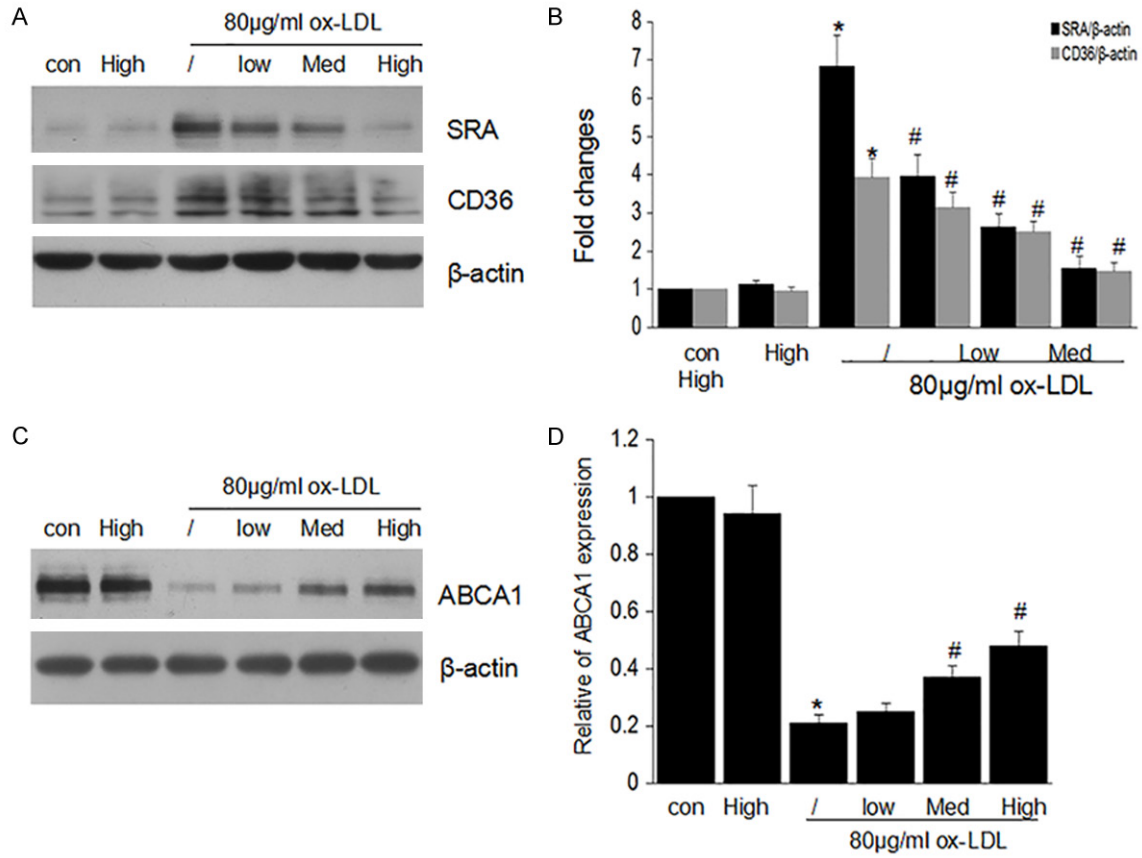
**Figure 2.** SPae regulates ox-LDL-challenged scavenger receptors and ABCA1 in THP-1 macrophages. A and B. Representative Images and Statistical date for the effect of SPae on SRA and CD-36 expression. C and D. Representative Images and Statistical date of ABCA1 expression. Bars represent mean ± SD (n=3) *P<0.05 vs. Control, #P<0.05 vs. Ox-LDL only.

increased obviously. While pre-treated with SPae for 2 hours could significantly inhibit the capacity of macrophage binding and uptake ox-LDL.

As the diffusion of cholesterol is also important in the formation of foam cells and the measurement of intracellular cholesterol showed the same result that SPae decreased ox-LDL-induced high concentration of intracellular cholesterol (**Table 1**). Research showed that PPARγ/ABCA1 has a major role in mediating cholesterol efflux from macrophages [5], and we detected the expression of ABCA1 and PPARγ. As shown in **Figure 2C** and **2D**, 80 μg/mL ox-LDL greatly decreased the expression of ABCA1, and SPae could reverse this effect. While expression of PPARγ was increased after ox-LDL treatment and SPae has a reduction effect on PPARγ expression in higher concentration (**Figure 3A** and **3B**). It means that SPae promotes reverse cholesterol transport via PPARγ/ABCA1 pathway.

*Effect of sodium paeonolsilate on the scavenger receptor*

Because the uptake of lipid was mainly mediated by the scavenger receptor, we analyzed the effect of SPae on the expression of SR-A and CD36 in macrophages. As shown in **Figure 2A** and **2B**, with the increase of SPae concentration, the expression of SR-A and CD36 gradually decreased. And the SPae not only inhibits the expression of SR-A and CD36 in basic condition, but also reverse ox-LDL induced upregulation of SR-A and CD36 protein.

MAP kinases signaling pathway was reported involved in the regulation of scavenger receptor and be regulated by ox-LDL [6-9]. So we detected the changes of activity of JNK and p38 during ox-LDL-induced macrophage-derived foam cell formation process. The results are shown in **Figure 3C** and **3E**, cells were treated with 80 μg/ml ox-LDL for 60 min. and the phosphoryla-
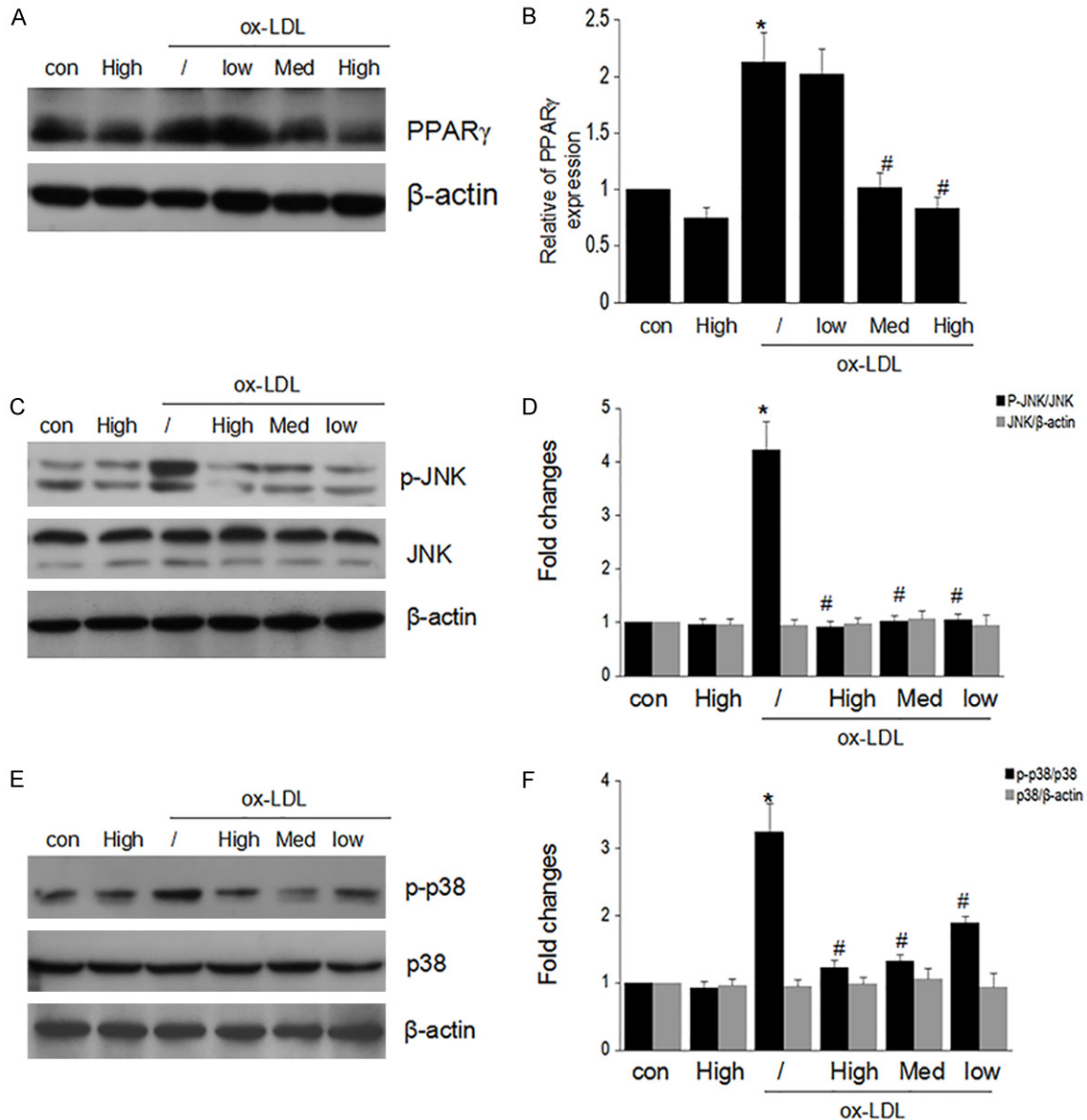
**Figure 3.** SPae regulates ox-LDL-challenged the activity of PPARγ/JNK/p38. A and B. Representative Images and Statistical date of PPARγ expression. C and D. Representative images and Statistical date for the effect of ox-LDL to the activity of JNK. E and F. Representative images and Statistical date for the effect of SPae to the ox-LDL-induced phosphorylation of p38.

tion levels of JNK and p38 were maximized in about 15 minutes, then slowly recovering, and at the end of 60 min, JNK and p38 have been restored to normal levels.The effect of SPae on JNK and p38 were also analyzed, we found that SPae inhibited JNK and p38 phosphorylation, but has no effection on JNK and p38 expression (**Figure 3C** and **3E**). And these results hints that SPae inhibits the expression of SR-A and CD36 by JNK/p38 pathway, which further affect lipid binding and uptake.

*Effect of sodium paeonolsilate to the ox-LDL-induced inflammation*

Throughout the process of AS accompanied with inflammation, we further examined the anti-inflammation effect of SPae in AS. Oxidative stress response accelerates the formation of foam cells in atherosclerotic initiation stage by exacerbating inflammatory response and ox-LDL uptake. And in its late stage, ROS and SOD makes the atherosclerotic plaque
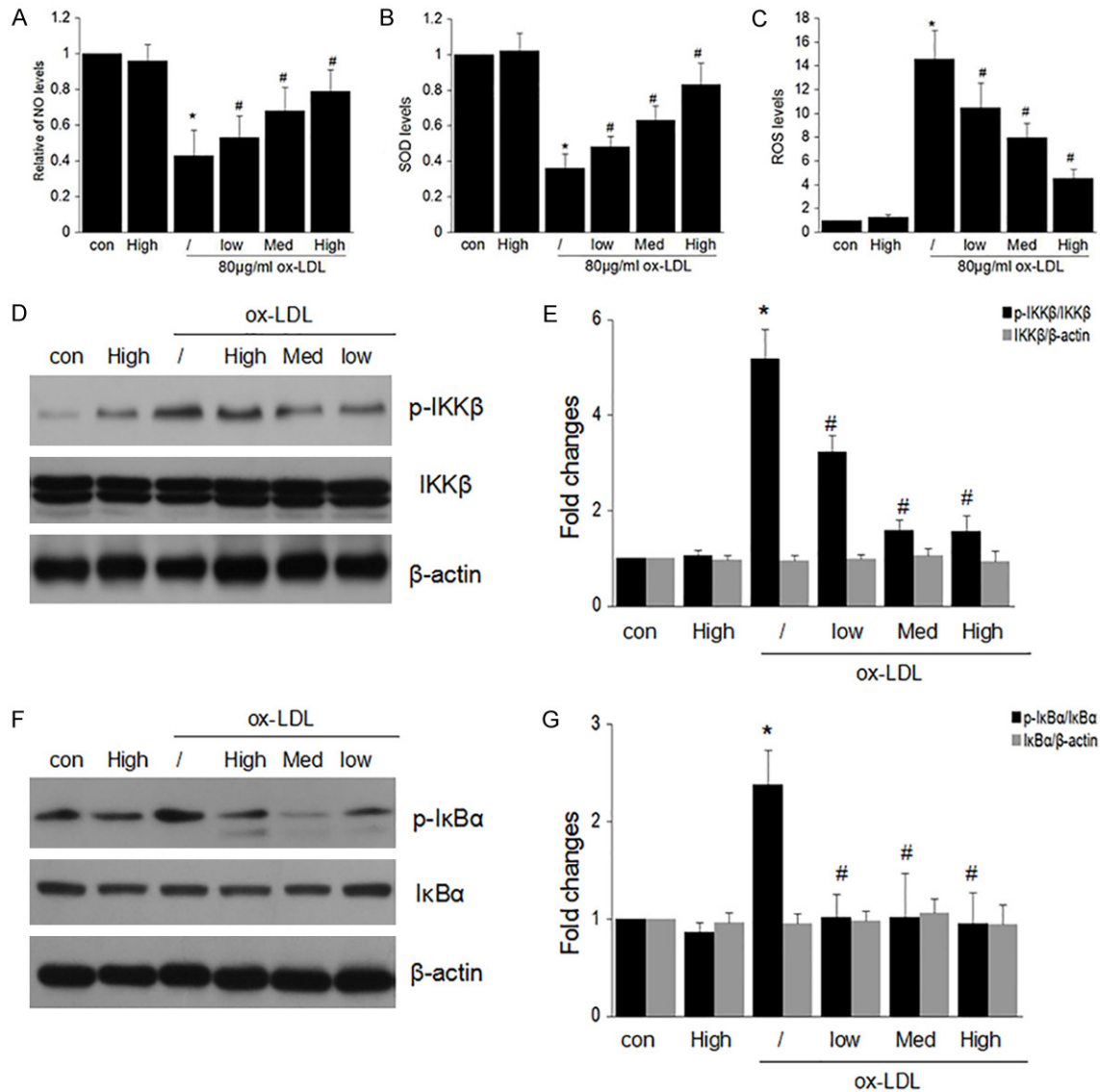
**Figure 4.** SPae regulates the ox-LDL-induced oxidative stress response by mediating the NF-κB pathway in THP-1 macrophages. A-C. Levels of intracellular NO, SOD and ROS. D-G. Representative images and Statistical date of the activity of NF-κB pathway. Bars represent mean ± SD (n=3) *P<0.05 vs. Control, #P<0.05 vs. Ox-LDL only.

instability, leading to the occurrence of acute cardiovascular events. So in our experiment we evaluated effect of SPae to ox-LDL-induced oxidative stress response. Our results showed that SPae could reverse ox-LDL-induced decreasing of NO (**Figure 4A**) and SOD (**Figure 4B**), with a supressed activation of ROS (**Figure 4C**).

NF-κB pathway was considered to play an important role in regulating inflammatory response [10-12]. Therefore, we investigated if SPae acts as an anti-inflammatory agent and

mediated macrophage-derived foam cell formation by blocked the NF-κB pathway. Our results showed that cells treated with 80 μg/mL ox-LDL after 24 h, IKKβ and IκBα phosphorylation level increased. Pre-incubation with SPae for 2 h, the phosphorylation level of IKKβ and IκBα were reduced compared with the model group (**Figure 4D-G**).

*Sodium paeonolsilate inhibits mouse atherosclerotic plaque area in aorta and aortic sinus*

The oil red O staining results as shown in **Figure 5A**, aortic plaque area of control group, model
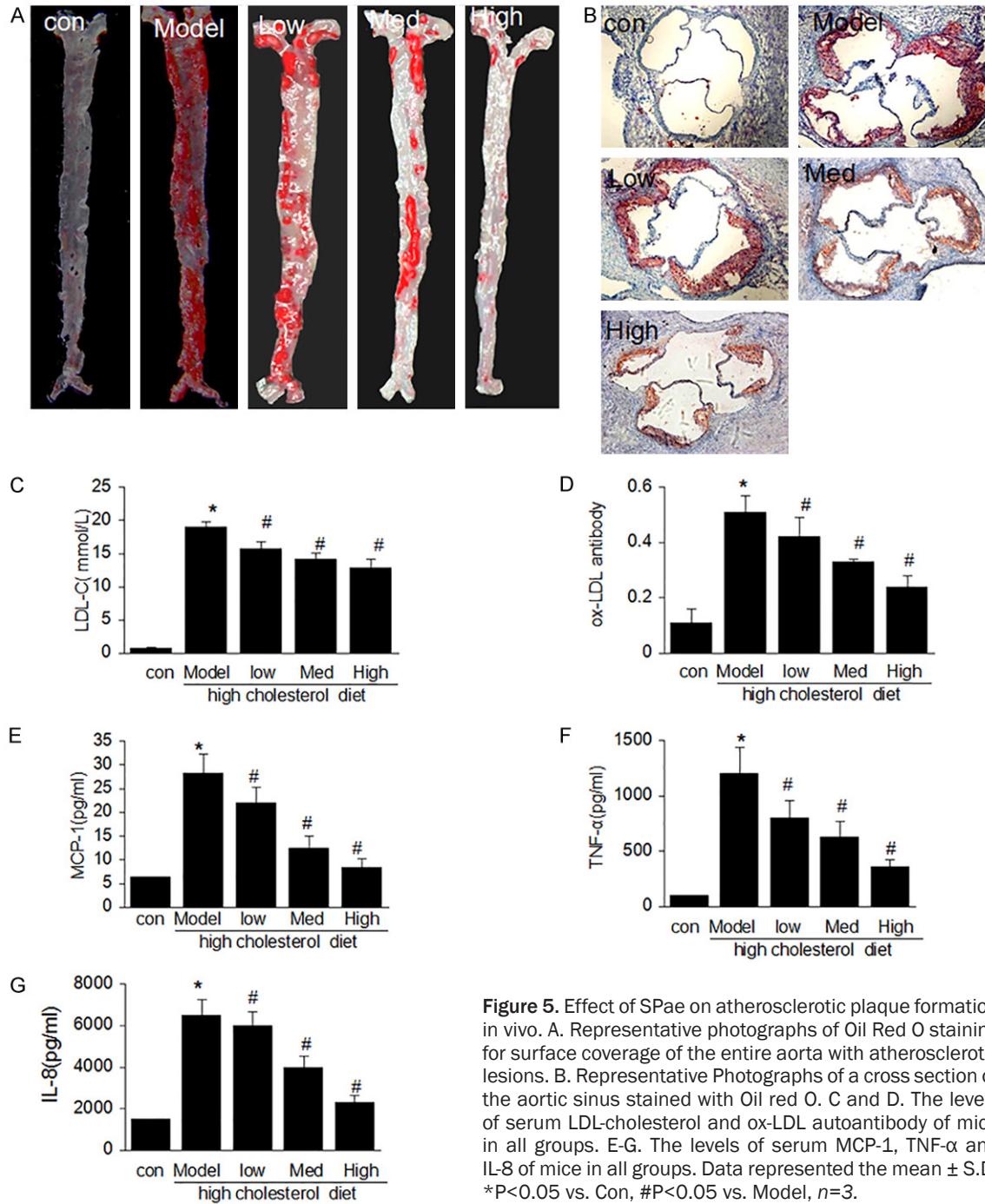
**Figure 5.** Effect of SPae on atherosclerotic plaque formation in vivo. A. Representative photographs of Oil Red O staining for surface coverage of the entire aorta with atherosclerotic lesions. B. Representative Photographs of a cross section of the aortic sinus stained with Oil red O. C and D. The levels of serum LDL-cholesterol and ox-LDL autoantibody of mice in all groups. E-G. The levels of serum MCP-1, TNF-α and IL-8 of mice in all groups. Data represented the mean ± S.D. *P<0.05 vs. Con, #P<0.05 vs. Model, *n=3*.

group, low dosage group, middle dosage group and high dosage group were: 0, 20.1±1.5%, 4.7±0.7%, 9.8±1.8% and 15.4±2.0%. And the results suggest, atherosclerotic model was successfully established, SPae can inhibit the formation of atherosclerosis plaque.

Quantitative analysis of lesions in the aortic sinus was shown in **Figure 5B**. The high dose

group, middle dose group, low dose treatment group ApoE$^{-/-}$ mice aortic wall plaque area percentage of total lumen area was 9.85±3.2%, 15.39±3.4% and 18.21±4.2%, significantly lower than that in the model group (25.38±4.83%, P<0.05, n=3); at the same time, aortic plaque area was 0.287±0.065 mm², 0.321±0.075 mm² and 0.423±0.065 mm², were also significantly lower than that in the
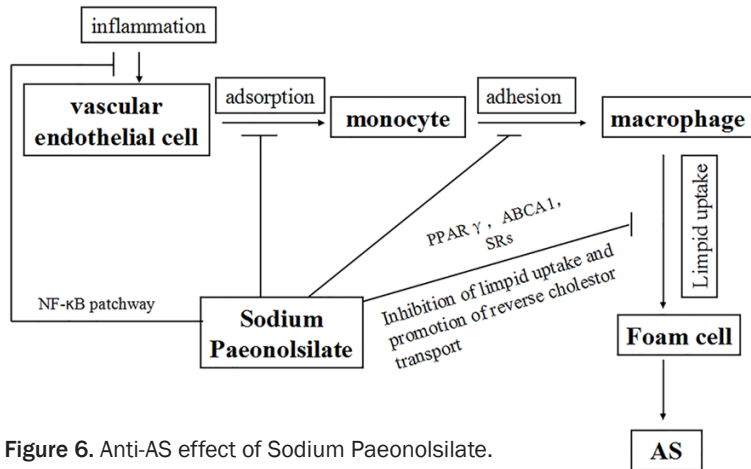
**Figure 6.** Anti-AS effect of Sodium Paeonolsilate.

model group ($0.468\pm0.079$ mm$^2$, $P<0.05$, n=3). Our animal experimental results show that SPae has the ability to inhibit the atherosclerotic plaque formation.

*Analysis of LDL and OX-LDL in the serum*

The LDL and Ox-LDL level in the serum were analysis by enzymatic procedures with an automatic biochemical analyzer (Hitachi 7170A, Tokyo, Japan). As shown in **Figure 5C** and **5D**, The level of LDL and anti ox-LDL antibody in Model group were significantly increased. Compared with Model group, the LDL and anti ox-LDL antibody level of SPae group significantly decreased. The results suggested that, SPae can reduce the level of LDL and anti ox-LDL antibody in serum.

*Analysis of pro-inflammatory cytokine in the serum*

The IL-8, MCP-1 and TNF-α level in the serum were analysis by ELISA method. As showed in **Figure 5E-G**, the level of IL-8, MCP-1 and TNF-α in model group was significantly increased. Compared with Model group, the level of IL-8, MCP-1 and TNF-α in SPae treatment group decreased obviously. The results suggested that, SPae can reduce the levels of IL-8, MCP-1 and TNF-α in serum.

**Discussions**

SPae was found to have multiple effects in anti-inflammatory, anti-lipid peroxidation and immune regulation. As infiltration and oxidation of lipids are signs of AS, we conjecture that SPae has an anti-AS effect. And early studies

have found that ox-LDL is an important pathogenic factor of AS [9], especially ox-LDL-induced macrophage-derived foam cell formation is main reason for AS. So we detected the possible effect of SPae on ox-LDL-induced macrophage-derived foam cell formation and its potential mechanisms.

Our results showed that SPae decreasd the ox-LDL-induced lipid accumulation in THP-1 cells. As we know, intracellular lipid metabolism mainly include two aspects. On the one hand, bind and uptake ox-LDL via scavenger receptor. This process is not affected by cholesterol content in the cytoplasm, eventually led to the formation of foam cells [13-15]. In our experiment, Oil red O stain assay showed that SPae could significantly inhibit the capacity of macrophage binding and uptake ox-LDL, and the expression of SRA and CD36 was also restrained. On the other hand, the intracellular free cholesterol could efflux from macrophages by interaction of ABCA1 and HDL [16]. SPae could reverse the intracellular cholesterol reduction and the downregulation of ABCA1 induced by ox-LDL. All these result means that SPae could inhibit ox-LDL-induced macrophage-derived foam cell formation via these two aspects. Furthermore, we investigated its potential mechanisms. Mietus discovered that the promoter of the scavenger receptor SRA has binding site for transcription factor AP-1 and CEBPβ in vascular smooth muscle cells, and these two transcription factor can be activated by JNK and P38 [17]. Research also demonstrated that, in mouse macrophages treated with ox-LDL, phosphorylation of p38 and JNK kinase was enhanced [18-20]. Therefore, we investigated the effects of JNK and p38 kinase, on macrophage-derived foam cell formation. The results showed that SPae inhibited JNK and p38 phosphorylation, but has no effection on JNK and p38 expression. This means, SPae mediated the CD36 and SRA expression through JNK and p38 signaling pathway and affect the formation of foam cell.

Inflammation also plays a major role in the developing of atherosclerosis. And in our exper-

iment we evaluated effect of SPae to ox-LDL-induced inflammation response. Our results showed that SPae could reverse ox-LDL-induced increase of ICAM-1 and VCAM-1, namely SPae inhibit ox-LDL-induced inflammation in THP-1. Further detection of NO, SOD and ROS show SPae could inhibit ox-LDL-induced oxidative stress response in THP-1. NF-κB plays an important role in regulating inflammatory response [6, 7, 21]. Our results showed that SPae could reduce the phosphorylation level of IKKβ and IκBα compared with the model group. So SPae could have an anti-Inflammation effect by mediating the NF-κB pathway.

In addition, we further observed the effect of SPae on atherosclerosis using apoE$^{-/-}$ gene knockout mice and found that SPae inhibits mouse atherosclerotic plaque area in aorta and aortic sinus. Meanwhile, SPae can reduce the level of LDL, anti Ox-LDL antibody and some pro-inflammatory cytokine in serum.

In summary (**Figure 6**), the role SPae played in arteriosclerosis is mainly in two aspects. On the one hand, SPae could inhibit synthesis of ROS and inflammatory cytokines ox-LDL induced and excessive production of NO, which is helpful in maintaining endothelial structure integrity and reducing vascular inflammation. On the other hand, SPae inhibits ox-LDL-induced macrophage-derived foam cell formation via inhibiting the capacity of macrophage uptake ox-LDL and promoting reverse cholesterol transport.

### Acknowledgements

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Shifang Ding, Department of Cardiology, Wuhan General Hospital of Guangzhou Military Region, 627 Wuluo Road, Wuhan 430070, Hubei, P. R. China. Tel: +86-27-50772018; Fax: +86-27-50772018; E-mail: dingshifang@126.com

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