

## Original Article

# Acetylshikonin suppresses atherogenesis by attenuating vascular inflammation in apolipoprotein E-deficient mice

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**Abstract:** Atherosclerosis is a chronic inflammatory disease and underlies cause of death worldwide. Acetylshikonin (AS), as the main ingredient of Zicao, is proposed to have capacity to suppress inflammation. However, the effects and related mechanisms of AS on atherosclerosis are unclear. 8-week-old apolipoprotein E deficient (apoE<sup>-/-</sup>) mice were received 12-week high fat diet (HFD), and were randomly given either AS (100 mg/kg body weight, once daily) or vehicle i.p. for 4 weeks. Compared with HFD-fed apoE<sup>-/-</sup> mice, administration of AS resulted not only in suppressed body weight gain, but also in decreased triglyceride (TC) level. However, there was no effect on total cholesterol (TC), low density lipoprotein cholesterol (LDL) and high density lipoprotein cholesterol (HDL) levels. Further investigation of lipid accumulation using oil red O staining showed that the atherosclerotic lesions were significantly decreased in aortic sinus and arch after AS treatment. Moreover, immunohistochemistry staining exhibited that the infiltration of inflammatory cells (e.g. T-lymphocytes, neutrophils and macrophages) in plaque was markedly attenuated by AS administration. AS-treated mice displayed reduced intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in aorta, concomitantly with decreased serum interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, tumor necrosis factor alpha- $\alpha$  (TNF- $\alpha$ ) and monocyte chemoattractant protein-1 (MCP-1) levels. In addition, AS treatment suppressed nuclear factor kappa B (NF- $\kappa$ B) activation in aorta of HFD-fed apoE<sup>-/-</sup> mice, indicated by attenuated p65 and p50 nuclear translocation. Taken together, these results demonstrate that AS ameliorates vascular inflammation and atherogenesis at least partially via inhibiting NF- $\kappa$ B signaling. Our findings suggest that AS may be a useful therapeutic agent for the treatment of atherosclerosis.

**Keywords:** Atherosclerosis, blood lipid, vascular inflammation, nuclear factor kappa B, acetylshikonin

## Introduction

Atherosclerosis is an inflammatory progressive disease of blood vessels as well as a disorder of lipid metabolism [1]. Its sequelae such as ischemic stroke, myocardial infarction and coronary heart disease represent the major cause of death worldwide [2]. Increasing lines of evidences suggest that inflammation is one of the most important factors for the initiation and development of atherosclerosis [3, 4]. Lipid over-loaded macrophage foam cells can secrete inflammatory cytokines (e.g. interleukin-1 $\beta$ , interleukin-6, tumor necrosis factor alpha- $\alpha$  and monocyte chemoattractant protein-1) that trigger a series of acute inflammatory reactions [5,

6]. The upregulation of these inflammatory factors induces inflammatory cell infiltration into plaque, which in turn decreases the stability of plaque and accelerates the pathological changes of atherosclerosis [4, 7, 8]. It has been also demonstrated that these inflammatory cytokines promote atherosclerosis development by activating nuclear factor kappa B (NF- $\kappa$ B) signaling pathways [5, 9]. Although numerous experimental researches have indicated the inflammatory nature of atherosclerosis, the treatment options for inflammation are still lacking beyond statins [10].

Zicao is a traditional Chinese medicine widely used for thousands of years in China [11]. Its

primary active ingredients contain 5 naphthoquinone derivatives: shikonin, acetylshikonin (AS),  $\beta$ ,  $\beta$ -dimethylacrylshikonin, isovalerylal-kannin and  $\beta$ -hydroxyisovalerylshikonin [12]. They have been found to reveal important pharmacological properties, including anti-inflammatory, anti-bacterial, anti-cancer and anti-viral [13-15]. Among them, AS is the major ingredient of Zicao [11]. It is worthy to note that AS could prevent obesity in high fat diet (HFD)-fed rats by inducing lipolysis [16]. Moreover, AS was found to exert protective effect on hepatic steatosis through inhibiting inflammation [11]. AS also attenuated pancreatic cancer cell proliferation by suppressing NF- $\kappa$ B activity [17]. On the basis of the previous studies, we hypothesized that AS might protect against atherosclerosis in which was associated with inflammation inhibition. Therefore, the present study aims to explore the potential role of AS in anti-atherosclerosis, and to clarify the underlying mechanism for the pharmacological action of AS.

## Materials and methods

### Chemicals and reagents

Acetylshikonin (purity >98%, HPLC) was purchased from Biopurify phytochemicals Ltd (Chengdu, China) and dissolved in dimethylsulfoxide (DMSO). CD3, CD68, intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), p65 and p50 antibodies were obtained from Cell Signaling Technology (MA, USA). Ly-6G, GAPDH and Histone H3 were from Santa Cruz (CA, USA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and donkey anti-goat IgG secondary antibodies were provided by Abcam (MA, USA). TRITC-labeled goat anti-rabbit and FITC-labeled donkey anti-goat were obtained from Boster (Wuhan, China). Triton X100, PBS and all other chemicals if not specified otherwise were from Sigma-Aldrich (MO, USA).

### Animals

Eighty male apolipoprotein E deficient (apoE<sup>-/-</sup>) mice (8-week-old, 20 $\pm$ 2 g) bred from a C57BL/6J background were purchased from Jackson Laboratory (ME, USA) and fed with a normal diet (ND) (n=20) or a HFD (60% kcal fat; D12-492, Research Diets, NJ, USA) (n=60) from 8 to 20 weeks of age. All mice were housed in microisolator cages and maintained at 20-24°C and 45-55% humidity with free access to water and food. At 20 weeks of age, HFD-treated

apoE<sup>-/-</sup> mice were randomized to treatment with either AS (100 mg/kg body weight, once daily) (n=20) or a vehicle (0.1%, DMSO) (n=20) i.p. for 4 weeks. The concentrations of AS in this study were selected according to a previous publication [16]. Mice were weighted at the end of the experimental period. Systolic blood pressure and heart rate were noninvasively measured using an occlusion tail-cuff plethysmography (BP-98A, Softron Co, Tokyo). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Hengyang Central Hospital and approved by the Institutional Animal Care and Use Committee of Hengyang Central Hospital. Aortic sinus and aortas were isolated, cleaned of fat, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

### Biochemical analysis

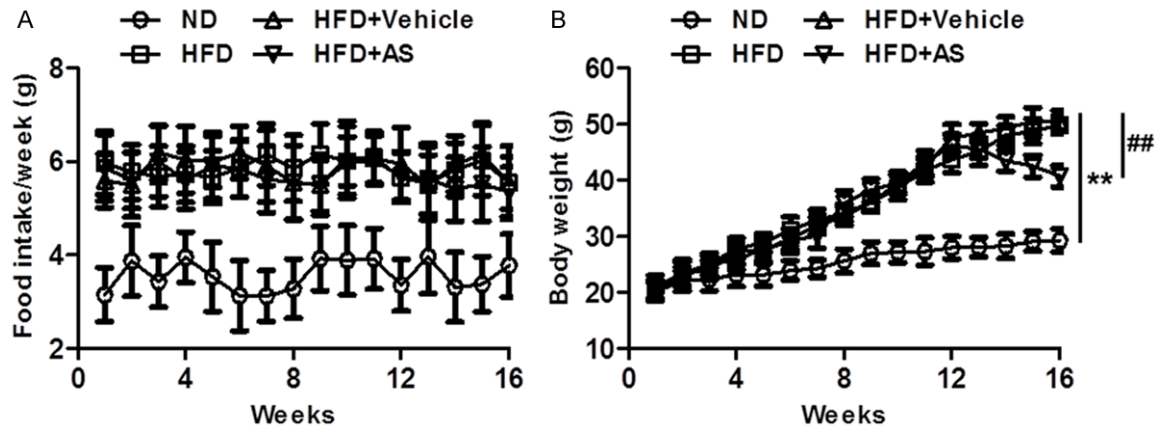
Mice were anesthetized and sacrificed after 24 weeks on the respective diets. Blood was collected from the abdominal vena cava and serum were harvest by centrifugation at 1000 $\times$  g for 10 min. Serum total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglyceride (TG), levels were measured using enzymatic methods by a Model 7180 automated biochemical analyzer (Hitachi, Tokyo, Japan) [18, 19].

### Oil Red O staining

The thoracic aortas were removed from each mouse and cut opened longitudinally. The aortic roots were harvested, then fixed in ice-cold 4% paraformaldehyde (PFA), dehydrated in 30% sucrose, embedded in the optimal cutting temperature (OCT) compound (Tissue-Tek®, Sakura, CA, USA), and snap-frozen. The frozen aortic sinuses were cut into 8  $\mu$ m. The atherosclerotic lesion in aortic sinus sections and aortic arches were stained with oil red O. Pro PlusImage analysis software (Media Cybernetics Company, MD, USA) was used to quantify the lesion area of aortic sinus and aortic arch [4].

### Immunohistochemistry

The frozen sections were rehydrated in PBS and added with 3% hydrogen peroxide aqueous solution to block endogenous peroxidase activity. Nonspecific binding was blocked by using 2% nonimmune serum solution at room temperature. The sections were then incubated with primary antibodies against CD3 (1:100),



**Figure 1.** AS reduced weight gain in HFD-treated apoE<sup>-/-</sup> mice. (A and B) 8-week-old apoE<sup>-/-</sup> mice were fed with a normal diet (ND) or a high fat diet (HFD) for 12 weeks, and then were treated with acetylshikonin (AS, 100 mg/kg body weight) or DMSO (vehicle) i.p. once daily for 4 weeks. Food intake (A) and change in body weight (B) were measured weekly. \*\*P<0.01 vs. ND; ##P<0.01 vs. HFD, n=20 mice in each group.

**Table 1.** Comparison of physiological parameters of mice at the end of experiment

	ND	HFD	HFD+Vehicle	HFD+AS
SBP (mmHg)	111±8	132±7**	129±6**	127±8**
HR (bpm)	566±84	524±65	531±74	542±62
TC (mM)	1.05±0.14	1.64±0.11**	1.72±0.15**	1.60±0.17**
LDL (mM)	0.17±0.03	0.48±0.19**	0.52±0.11**	0.45±0.05**
HDL (mM)	0.56±0.03	0.45±0.04**	0.42±0.07**	0.46±0.02**
TG (mM)	0.64±0.06	1.34±0.13**	1.41±0.09**	0.81±0.11##

SBP, systolic blood pressure; HR, heart rate; TC, total cholesterol; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; TG, triglyceride. Data were expressed as mean ± SEM of 14 mice. \*\*P<0.01 vs. ND; ##P<0.01 vs. HFD, n=10 mice in each group.

Ly-6G (1:50) and CD68 (1:100) overnight at 4°C. After incubation, sections were washed 3 times with PBS, incubated with biotinylated secondary antibody (Zhongshan Jinqiao Bio-Technology Co. Ltd., Beijing, China), and visualized with the streptavidin-peroxidase reaction using 3,3'-diaminobenzidine (DAB). The sections were dehydrated and examined under a light microscope (magnification, ×400) (IX70, Olympus, Tokyo, Japan). Quantitative analysis of the CD3-, Ly-6G- and CD68-positive areas in the lesions was performed using Pro PlusImage analysis software.

#### Western blotting analysis

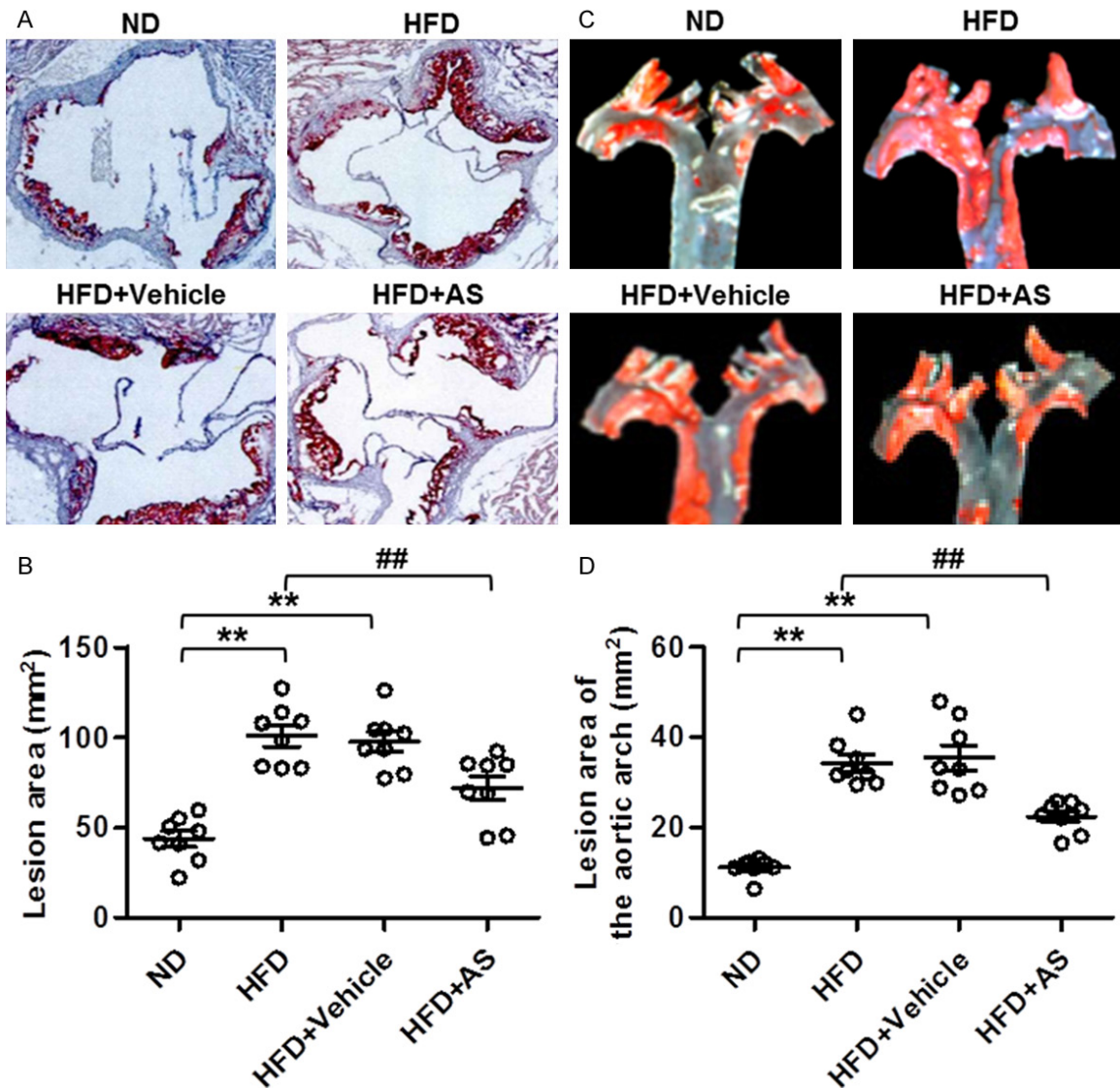
The whole aorta was retrieved and lysed by an ultrasonic homogenizer in a lysis buffer containing 50 mM HEPES (Promocell, Heidelberg, Germany), 1% Triton X100, and protease and phosphatase inhibitors (Pierce Biotechnology,

IL, USA). Cytoplasmic and nuclear proteins of aortas were extracted using a Nuclear/Cytosol Fractionation Kit (BiCA, USA) according to the manufacturer's protocols. Protein concentrations were quantified using a bicinchoninic acid kit (BioRad, CA, USA). Western blotting was performed as described previously [4]. Equal amount of protein was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins

were transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA), followed by incubation with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies against the following proteins: ICAM-1 (1:1000), VCAM-1 (1:1000), p65 (1:500), p50 (1:500), GAPDH (1:2000) and Histone H3 (1:1000). After washing 3 times with TBST, membranes were incubated with HRP-conjugated goat anti-rabbit IgG or donkey anti-goat IgG secondary antibody (1:1000) for 1 h at room temperature. Blots were exposed to ECL Kit (Beyotime, Jiangsu, China) and quantified with Pro PlusImage analysis software.

#### Immunofluorescence

After blocking with 2% nonimmune serum solution, the sections were incubated with primary



**Figure 2.** AS treatment inhibited atherosclerotic lesion formation. A: Representative oil red O staining of atherosclerotic lesions in aortic sinus. B: Quantification of atherosclerotic lesion formation within aortic sinus. \*\* $P < 0.01$  vs. ND; ## $P < 0.01$  vs. HFD,  $n = 8$  mice in each group. C: Representative oil red O staining of atherosclerotic lesions in aortic arch. D: Quantification of atherosclerotic plaque areas in aortic arch. \*\* $P < 0.01$  vs. ND; ## $P < 0.01$  vs. HFD,  $n = 8$  mice in each group.

antibodies against ICAM-1 (1:50) and VCAM-1 (1:100) overnight at 4°C. This was followed by incubation with TRITC- or FITC-labeled secondary antibodies for 1 h at room temperature. The sections were analyzed by a confocal microscopy (Olympus IX70, Olympus, Tokyo, Japan) with 400× magnifications. Staining intensity was quantified using Pro PlusImage analysis software.

#### Enzyme linked immunosorbent assay (ELISA)

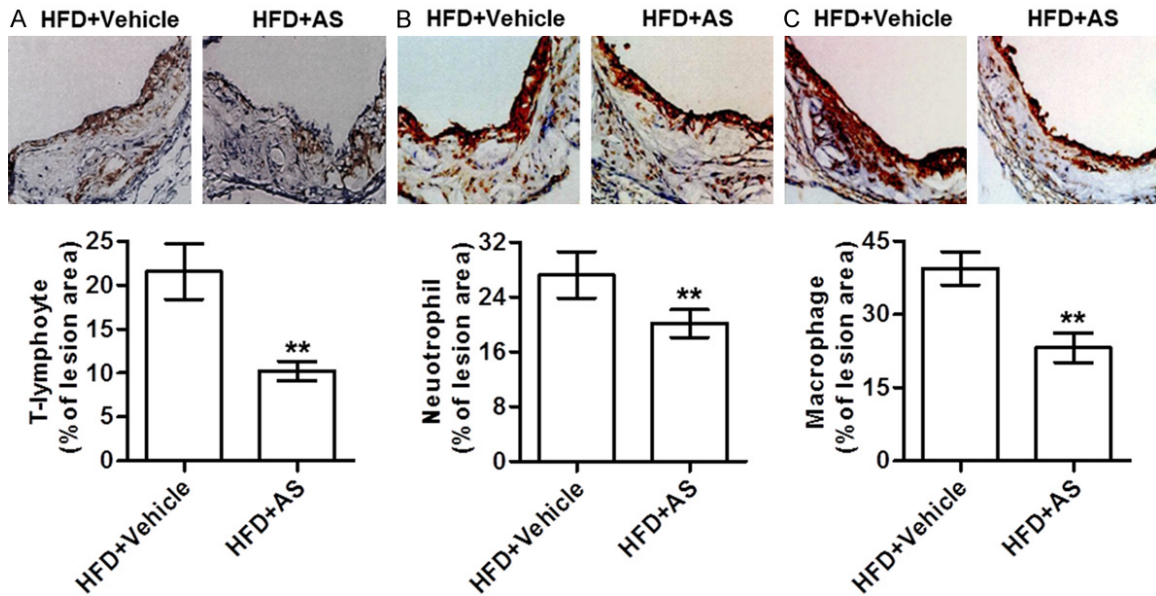
The serum levels of interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, tumor necrosis factor alpha- $\alpha$  (TNF- $\alpha$ ) and mo-

nocyte chemoattractant protein-1 (MCP-1) were determined using immunoassay kits (R&D System, MN, USA). Procedures were performed according to the instructions provided by manufacturer. All samples were assayed and absorbance was read using a luminometer (AutoLumat Plus, Berthold Technology, Calmbacher, Germany).

#### Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was performed using Graph Pad Prism v.5.0 (GraphPad,





**Figure 3.** AS administration inhibited inflammatory cells recruitment in atherosclerotic plaque. (A-C) Cross-sections of aortic sinus plaques were stained with CD3 for T-lymphocytes (A), Ly-6G for neutrophils (B) and CD68 for macrophages (C), respectively (400× magnifications). Quantification of the positive staining area was shown in the lower panel. \*\* $P < 0.01$  vs. HFD,  $n = 5-7$  mice in each group.

CA, USA). Student's paired  $t$  test or one-way analysis of variance, followed by a post hoc comparison with the Bonferroni test was used to analyze the differences between groups.  $P$  values below 0.05 were considered as statistically significant.

## Results

### Effect of AS on physiological parameters

As shown in **Figure 1A**, weekly food intake per mouse remained stable and was not different among 4 groups throughout the experiment. HFD showed remarkably increased body weight compared with ND-fed apoE<sup>-/-</sup> mice. 4-week administration of AS suppressed the average weight gain by 40.7% compared with HFD-fed apoE<sup>-/-</sup> mice without treatment (**Figure 1B**). Moreover, other physiological parameters of 24-week-old apoE<sup>-/-</sup> mice were presented in **Table 1**. Systolic blood pressure was higher in HFD-fed than in ND-fed apoE<sup>-/-</sup> mice, and it did not differ among HFD groups during the experimental period. Furthermore, there was no difference in heart rate observed among all groups. Moreover, blood lipid levels were also significantly increased in HFD-fed apoE<sup>-/-</sup> mice than that of ND-fed apoE<sup>-/-</sup> mice. TG level was notably reduced by AS administration, while no significant difference in TC, LDL and HDL was

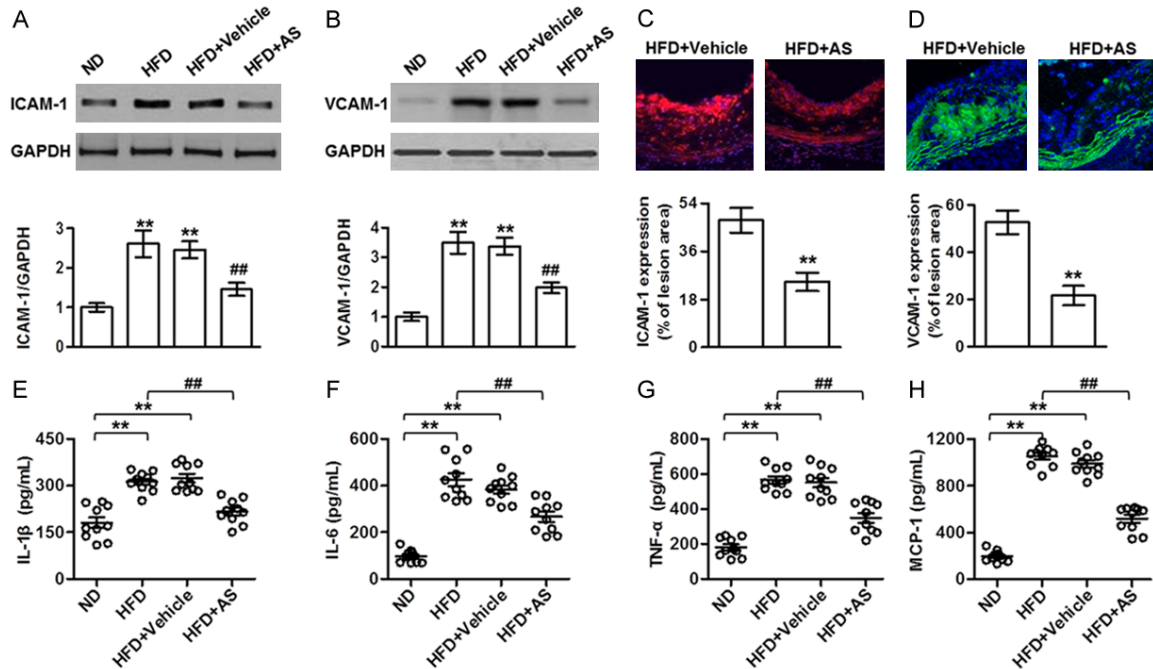
found in HFD-fed apoE<sup>-/-</sup> mice before and after AS treatment.

### AS treatment attenuated the development of atherosclerosis

To further confirm the protective effect of AS on atherosclerosis, atherosclerotic lesions in the aortic sinus and arch were examined. As illustrated in **Figure 2A**, the atherosclerotic lesion areas in aortic sinus were significantly blunted in AS-treated mice compared with HFD mice without treatment. Accordingly, the areas covered by atherosclerotic lesions in aortic sinus were reduced by 31.8% after AS treatment (**Figure 2B**). In addition, the inhibitory effect of AS on lesion formation was further supported in thoracic aortic arch (**Figure 2C**). Quantitative analysis revealed that the lesion areas in aortic arch were significantly smaller in AS-treated HFD-fed mice than in HFD-fed mice without treatment (**Figure 2D**). The above results indicate that AS inhibited atherosclerotic lesion formation in apoE<sup>-/-</sup> mice.

### AS administration inhibited the infiltration of inflammatory cells into atherosclerotic plaque

Infiltration of inflammatory cells, such as T-lymphocytes, neutrophils, and monocyte-derived macrophages, into the arterial vessels is an



**Figure 4.** AS treatment limited vascular inflammation in HFD-treated apoE<sup>-/-</sup> mice. (A and B) Western blotting analysis for the protein expression of ICAM-1 (A) and VCAM-1 (B) in aorta from HFD mice treated with or without AS. \*\*P<0.01 vs. ND; ##P<0.01 vs. HFD, n=6 mice in each group. (C and D) Representative immunofluorescence staining of ICAM-1 (red) (C) and VCAM-1 (green) (D) in aortic sinus from HFD mice treated with or without AS (400× magnifications). Quantification of ICAM-1 and VCAM-1 staining intensity was shown in the lower panel. \*\*P<0.01 vs. HFD, n=6 mice in each group. (E-H) The serum levels of IL-1β (E), IL-6 (F), TNF-α (G) and MCP-1 (H) were determined by ELISA. \*\*P<0.01 vs. ND; ##P<0.01 vs. HFD, n=10 mice in each group.

essential step in atherogenesis [4]. Immunohistochemistry staining was performed to evaluate the inflammatory cell infiltration in aortic sinus. As shown in **Figure 3A**, infiltration of T-lymphocytes, indicated by CD3-positive area, was significantly less in AS-treated HFD-fed mice than in HFD-fed mice alone. Moreover, AS-treated mice revealed remarkably lower abundance of Ly-6G-positive neutrophils (**Figure 3B**). Finally, infiltration of macrophages was also decreased in the plaques of AS-treated HFD-fed mice than in HFD-fed mice, evident by decreased CD68-positive area (**Figure 3C**).

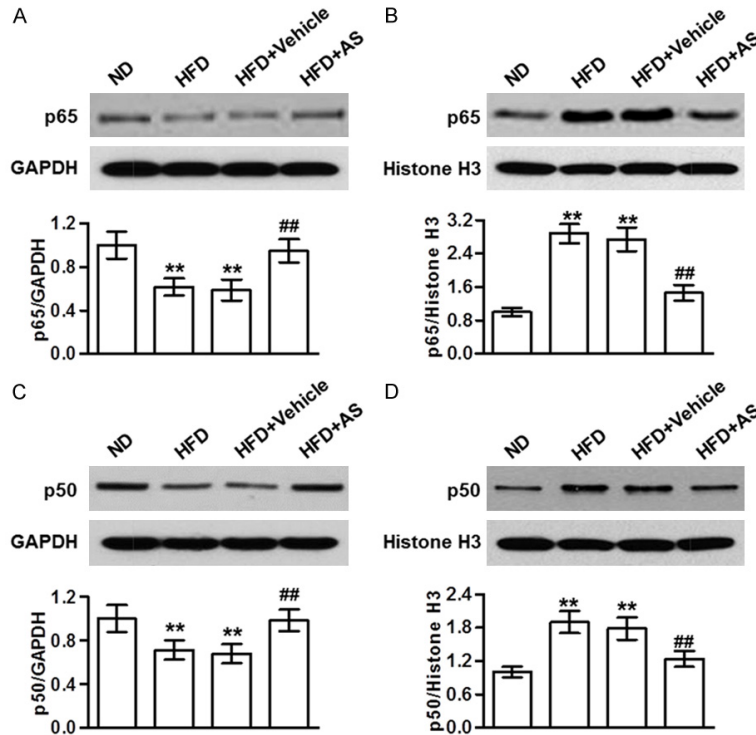
#### AS treatment ameliorated vascular inflammation

Inflammation plays a critical role during all stages of atherosclerosis [5, 7]. Therefore, we quantified the expression levels of ICAM-1 and VCAM-1 in aortas and atherosclerotic lesions. Western blotting results showed that HFD resulted in a marked increase of ICAM-1 and VCAM-1 expressions in aortas, which was notably attenuated after AS administration (**Figure 4A** and **4B**). Similarly, immunofluorescence staining revealed that the staining intensity of

ICAM-1 and VCAM-1 in aortic sinus was less pronounced in AS-treated HFD-fed mice than in those of HFD-fed mice (**Figure 4C** and **4D**). Moreover, serum levels of IL-1β, IL-6, TNF-α and MCP-1 were dramatically decreased after AS treatment in HFD-fed apoE<sup>-/-</sup> mice (**Figure 4E-H**).

#### AS treatment abrogated NF-κB activation in aortas of atherosclerotic model mice

It has been documented that the NF-κB signaling plays an important role in regulating inflammation in the development of atherosclerosis [9, 20]. To investigate the mechanism that allows AS treatment to inhibit vascular inflammation during atherogenesis, we determined the effect of AS on NF-κB signaling pathway. Western blotting results showed that the translocation of p65 from cytoplasm to nucleus was significantly increased in HFD-fed apoE<sup>-/-</sup> mice compared with ND-fed apoE<sup>-/-</sup> mice. However, this effect was abolished after AS treatment (**Figure 5A** and **5B**). Moreover, we also examined the nuclear accumulation of another subunit of NF-κB p50. As expected, HFD-fed apoE<sup>-/-</sup> mice showed markedly enhanced nuclear accumulation of p50. Following AS treatment,



**Figure 5.** AS treatment inhibited NF- $\kappa$ B activation in aortas of atherosclerotic model mice. (A and B) Cytoplasmic and nuclear proteins of aortas were extracted using a Nuclear/Cytosol Fractionation Kit. Cytosol (A) and nuclear (B) amount of p65 were detected by western blotting using p65 antibody. (C and D) AS also inhibited p50 translocation from cytoplasm (C) to nuclei (D). \*\* $P < 0.01$  vs. ND; ## $P < 0.01$  vs. HFD,  $n = 6$  mice in each group.

the ability of HFD to induced p50 nuclear translocation was abrogated (Figure 5C and 5D). These data suggest that AS treatment ameliorates vascular inflammation via inhibiting NF- $\kappa$ B activation.

## Discussion

Zicao is widely used in China to treat inflammatory and infectious diseases [11, 13]. In addition, recent study has shown that AS, the main ingredient of Zicao, can inhibit lipid accumulation [16, 21]. However, it is unclear whether AS affects the development of atherosclerosis. In the current study, we demonstrate for the first time that AS can significantly ameliorate atherosclerosis in apoE<sup>-/-</sup> mice fed with HFD by decreasing blood lipid levels and plaque areas. The anti-atherosclerotic effects of AS was associated with inhibition of vascular inflammation. Moreover, NF- $\kappa$ B signaling pathway may be involved in this process. Collectively, our study reveals another critical pharmacologic effect of Zicao.

Here, we found that 4-week administration of AS dramatically decreased body weight gain. This was consistent with another study demonstrating that AS prevented excessive body weight gain and obesity in HFD-fed rats [16]. Next, we investigated the effects of AS on blood lipid levels using bioassays. Our results showed that AS administration markedly lowered serum TG level compared to those of HFD-fed apoE<sup>-/-</sup> mice, whereas there was no significant effect on TC, LDL and HDL. These results were also consistent with previous studies that AS treatment significantly reduced serum level of TG without changing TC, LDL and HDL in HFD-fed rats and spontaneous obese db/db mice [11, 16]. Given that these previous studies also reported that AS treatment could reduce free fatty acid level, we hypothesize that the reduction in TG accumulation following AS treatment is partially mediated by reduced fatty acid synthesis. Of course, the mechanism by which AS treatment only affects TG level remains to be further illustrated.

Upregulation of ICAM-1, VCAM-1 and inflammatory cytokines are important events in atherosclerosis, which recruits inflammatory cells into atherosclerotic lesions and contributes to the initiation of atherosclerosis [1, 4, 7]. It has been known that Zicao attenuated lupus nephritis-induced ICAM-1 and VCAM-1 expression and kidney injury [22]. Moreover, AS treatment exerted anti-nonalcoholic fatty liver disease through anti-inflammation effects [11]. Consistent with the inhibitory effect of AS on inflammation, our data revealed that AS also affects vascular inflammation and atherogenesis. Western blotting and immunofluorescence staining showed that AS treatment significantly decreased ICAM-1 and VCAM-1 expression in the aorta and aortic sinus of HFD-treated apoE<sup>-/-</sup> mice, concomitantly with decreased serum levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and MCP-1. Furthermore, our results consistently re-

aled that AS treatment reduced the infiltration of inflammatory cells, such as T-lymphocytes, neutrophils and macrophages, indicating AS can increase plaque stability. In line with suppressed vascular inflammation by AS administration, the decrease in atherosclerotic lesion formation using oil red O staining in aortic sinus and arch was observed in AS-treated HFD-fed apoE<sup>-/-</sup> mice. Hence, these data indicate that AS treatment ameliorates vascular inflammation and in turn limits the progression of atherosclerosis.

Because various adhesion molecules and inflammatory cytokines have been known to be direct targets of NF- $\kappa$ B, this signaling is suggested to play a vital role in regulating inflammation [8, 23]. It has been reported that AS can suppress TNF- $\alpha$ -induced NF- $\kappa$ B activity in pancreatic cancer cells [17]. However, it is yet unknown whether AS treatment regulates vascular inflammation and atherosclerosis in apoE<sup>-/-</sup> mice through NF- $\kappa$ B signaling. Therefore, to further explore the mechanism for the action of AS, we investigated the involvement of NF- $\kappa$ B signaling. The results of this study revealed that AS treatment significantly abolished NF- $\kappa$ B activation in HFD-fed apoE<sup>-/-</sup> mice. The inhibition of NF- $\kappa$ B was mediated by blocking p65 and p50 nuclear translocation. These findings suggest that AS treatment attenuates vascular inflammation in atherosclerotic mice partly via negative regulation of NF- $\kappa$ B signaling.

In conclusion, the results of the current study demonstrate that AS limits the development of atherosclerosis and attenuates blood lipid level and vascular inflammation. Furthermore, the suppressive effects of AS are potentially mediated by inhibiting NF- $\kappa$ B activation. Thus, AS may be a therapeutic agent for the treatment of atherosclerosis.

## Disclosure of conflict of interest

None.

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