

# Astragaloside IV protects endothelial progenitor cells from the damage of ox-LDL via the LOX-1/NLRP3 inflammasome pathway

This article was published in the following Dove Press journal:  
*Drug Design, Development and Therapy*

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**Purpose:** Functional impairment of endothelial progenitor cells (EPCs) is frequently observed in patients with diabetic vascular complications. Astragaloside IV (ASV) has a significant protective effect against vascular endothelial dysfunction. Thus, this study aimed to investigate the role of ASV on oxidized low-density lipoprotein (ox-LDL)-induced EPCs dysfunction and its potential mechanisms.

**Methods:** EPCs were isolated from the peripheral blood of mice and treated with different concentration of ASV (10, 20, 40, 60, 80, 100 and 200  $\mu$ M). ox-LDL was served as a stimulus for cell model. The proliferation and migration, and improved tube formation ability of EPCs were determined. Reactive oxygen species (ROS) production and the levels of inflammatory cytokines, including interleukin 1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-10 and tumor necrosis factor (TNF- $\alpha$ ) were measured. The expression of lectin-like oxidized LDL receptor (LOX-1) and Nod-like receptor nucleotide-binding domain leucine rich repeat containing protein 3 (NLRP3) inflammasome were detected by Western blot analysis.

**Results:** We found ASV treatment alleviated ox-LDL-induced cellular dysfunction, as evidenced by promoted proliferation and migration, and improved tube formation ability. Besides, ASV treatment significantly suppressed ox-LDL-induced ROS production and the levels of inflammatory cytokines. ASV inhibited ox-LDL-induced expression of LOX-1 in a concentration-dependent manner. Overexpression of LOX-1 in EPCs triggered NLRP3 inflammasome activation, while inhibition of LOX-1 or treatment with ASV suppressed ox-LDL-induced NLRP3 inflammasome activation. Furthermore, overexpression of LOX-1 in ox-LDL-induced EPCs furtherly impaired cellular function, which could be ameliorated by ASV treatment.

**Conclusion:** Our study showed that ASV may protect EPCs against ox-LDL-induced dysfunction via LOX-1/NLRP3 pathway.

**Keywords:** endothelial progenitor cells, astragaloside IV, lectin-like oxidized LDL receptor, NLRP3 inflammasome

## Introduction

Type 2 diabetes mellitus (T2DM), the major type of DM, has become a global emergency due to its rapidly rising morbidity and chronic microvascular and macrovascular complications. Patients with T2DM have risks of death and cardiovascular events that have 2–4 times the risks of the general population.<sup>1</sup> Diabetic macrovascular disease morphologically and functionally takes after atherosclerotic lesion, but diabetic microvascular disease manifests as retinopathy, nephropathy and vascular abnormalities in the lower extremities,<sup>2</sup> leading to visual impairment, kidney failure and lower

extremity dysfunction.<sup>3</sup> Endothelial dysfunction is a well-accepted central event that precedes the development of atherogenesis, which is the prominent pathophysiological disorder of diabetic cardiovascular disease.<sup>4</sup> Endothelial dysfunction is commonly observed in T2DM patients, which is resulting from accumulation of advanced glycated end products and oxidized low-density lipoprotein (ox-LDL), and recent evidence suggesting endothelial dysfunction might be associated with endothelial progenitor cells (EPCs) dysfunction.<sup>5</sup> EPCs, derived primarily from the bone marrow, are precursors to mature endothelial cells with distinctive characteristics. At present, ox-LDL plays a vital role in the development and progression of atherosclerosis, and it induces endothelial cells damage through increased oxidative stress, inflammatory response and secretion of adhesion molecules.<sup>6</sup> Studies found ox-LDL also influences the growth and bioactivity of EPCs, with its binding to the lectin-like oxidized LDL receptor (LOX-1).<sup>7</sup> It has been demonstrated that human EPCs express LOX-1 and its activation by ox-LDL induces cellular senescence<sup>8</sup> and apoptosis.<sup>9</sup> However, the exact mechanism through which LOX-1 exerts its role remains unclear.

Ox-LDL induces inflammation by stimulating the production of plenty of inflammatory cytokines, including interleukin (IL)-1 $\beta$ .<sup>10</sup> The level of IL-1 $\beta$  is remarkably increased in the serum of T2DM patients with macrovascular complications.<sup>11</sup> The maturation of IL-1 $\beta$  is mediated by NLR family pyrin domain containing 3 (NLRP3) inflammasome. Once NLRP3 inflammasome is activated, NLRP3 oligomerizes with the adaptor protein ASC (apoptosis-associated speck-like protein containing a card), leading to the cleavage and activation of caspase-1. Caspase-1 then cleaves precursors IL-1 $\beta$  into IL-1 $\beta$ , which contribute to the pathogenesis and progression of vascular complications in T2DM.<sup>12</sup> Recently, a study demonstrated that NLRP3 inflammasome can be activated by ox-LDL stimulation, subsequently causing the secretion of IL-1 $\beta$  in macrophage.<sup>13</sup> However, the role of ox-LDL on NLRP3 inflammasome activation and its underlying mechanisms in EPCs remains poorly understood.

Astragaloside IV (ASV) is a traditional Chinese medicine extract with various pharmacological effects.<sup>14</sup> It has also been suggested that ASV has protective effect on diabetic microvascular complications including diabetic retinopathy and diabetic nephropathy.<sup>15,16</sup> ASV also improved aortic endothelial function induced by hyperglycemia.<sup>17</sup> However, whether ASV has a protective effect on EPCs from ox-LDL-induced injury remains

unclear. In the present study, we therefore studied the potential protective effects of ASV on ox-LDL induced EPCs dysfunction and investigated whether LOX-1 and NLRP3 inflammasome activation are involved.

## Materials and methods

### Isolation and characterization of EPCs

Mononuclear cells were isolated from peripheral blood of normal rats by density gradient centrifugation.<sup>18</sup> Then, the cells were supplemented with endothelial growth medium-2 (EGM-2; Lonza, Basel, Switzerland) containing EGM-2 growth kit supplement (Lonza). After 7 days in the culture, EPCs were trypsinized and adjusted to  $3 \times 10^6$ /mL, followed by incubation for 30 mins at 4°C with fluorescent-labeled goat anti-rat antibodies against CD34 (1:50, ab81289, Abcam, Cambridge, USA), CD31 (1:50, ab216459, Abcam, Cambridge, USA), CD45 (1:20, ab214437, Abcam, Cambridge, USA) and CD133 (1:200, ab19898, Abcam, Cambridge, USA). After washed with PBS, the cells were measured using flow cytometry analysis. Immunophenotypic analysis was also performed by staining  $5 \times 10^5$  cells with monoclonal antibodies against 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labelled Ac-LDL (10  $\mu$ g/mL, Invitrogen, Carlsbad, USA) in cell medium for 4 hrs at 37°C. Cells were then incubated with FITC-labeled lectin (UEA-1, 10  $\mu$ g/mL, Invitrogen) for another 1 hr at 37°C. EPCs were used at passages 7–9 for all experiments. All of the animal procedures were approved by the Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine and the guidelines from the National Institutes of Health.

### Treatments

EPCs were treated with different concentrations (0.1, 1, 5, 10, 25 and 50  $\mu$ M) of ox-LDL (Sigma, Merck KGaA, Darmstadt, Germany) for 24 hrs and/or different concentrations (10, 20, 40, 60, 80, 100 and 200  $\mu$ M) of ASV (C19193, purity  $\geq 98\%$ , Xiya Reagent, Shandong, China),<sup>19</sup> as indicated. To identify whether LOX-1 is involved in the effects of ASV on EPCs, LOX-1 neutralizing antibody (R&D, 10  $\mu$ g/mL), or the chemical inhibitor polyinosinic acid (Poly(I), Sigma, 250  $\mu$ g/mL),<sup>20</sup> were added to cultural medium 2 hrs prior to treatment with ox-LDL.

## Cell proliferation assay

Cells were plated at a density of  $2 \times 10^3$  cells/well in 96-well microplates and after 48 hrs culture, the number of viable cells was evaluated by a CCK8 reagent (MedChem Express, Shanghai, China). Briefly, after the supernatants were discarded and CCK-8 solution was added to each culture well and incubated for 30 mins at  $37^\circ\text{C}$ . The number of viable cells was measured by evaluating absorbance at 450 nm on a microtiter plate reader (Bio-Rad Laboratories, Inc., Hercules, USA). The cell viability was calculated as the absorbance of treated cells/control cells  $\times 100\%$ .

## Cell migration assay

To determine cell migrative ability,  $1 \times 10^5$  cells were plated on the top side of polycarbonate Transwell filter (Cell Biolabs, Inc. Santiago, USA). Cells were suspended in the upper chamber in medium without serum, and EGM-2 medium containing 10% serum was used as a chemoattractant in the lower chamber, and then incubated at  $37^\circ\text{C}$  for 24 hrs. The non-migrative cells in the upper chambers were gently removed and cells on the bottom membrane surface were fixed with 4% paraformaldehyde for 15 mins, followed by stained with 0.5% crystal violet solution (Beyotime), and counted under a microscope (Leica DMI8, Leica Company, Germany). Experiments were repeated three times independently.

## Tube formation

Tube formation was evaluated with an Angiogenesis (Tube Formation) Assay Kit (BioVision, Inc. Milpitas, CA, USA). Extracellular Matrix Solution was mixed with ECMatrix diluent buffer and maintained in a  $\mu$ -Slide plate at  $37^\circ\text{C}$  for 60 mins to allow the matrix solution to solidify. About  $1 \times 10^4$  EPCs in 100  $\mu\text{L}$  EGM-2 media were seeded into the wells. After 4 hrs, capillary-like structures were imaged under the microscope at  $\times 100$  magnification.

## ROS staining

Intracellular ROS generation was determined using peroxide-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFHDA, Molecular Probes). The cells were cultured into 6-well plates followed by exposure to the indicated experimental conditions, and the cells were incubated with 5 mM DCFHDA for 20 mins at  $37^\circ\text{C}$  in the dark. Subsequently, the cells were rinsed with serum-free EGM-2 twice, and the representative images of ROS generation were captured using a Nikon Eclipse Ti-U epifluorescence microscope or a flow cytometry (FACS Calibur, Bio-Rad Laboratories, Inc., USA).

## Enzyme-linked immunosorbent assay (ELISA)

After experimental treatments, cell membranes were disrupted and the cell lysates centrifuged at  $10,000g$  at  $4^\circ\text{C}$  for 15 mins. The supernatant was separated from cell debris by a tissue tearor (Biospec, Bartlesville, OK, USA) and used for subsequent measurements. The concentrations of IL- $1\beta$ , IL-6, IL-10 and TNF- $\alpha$  were measured using the corresponding ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

## Western blot

Total protein was extracted from treated cells using 1% RIPA Lysis Buffer (Beyotime, Jiangsu, China) with a phosphorylation inhibitor (Roche, Indianapolis, USA). The total protein concentration for each sample was measured with a bicinchoninic acid (BCA) kit (Beyotime). Equal amounts of protein samples were then subjected to SDS-PAGE and proteins were then transferred to PVDF membranes (Beyotime). The membranes were blocked with 5% skim milk at room temperature for 1 hr and incubated with the primary antibodies against LOX-1 (1:1,000, ab60178, Abcam), NLRP3 (1:1,000, ab4207, Abcam), ASC (1:5,000, ab127537, Abcam), caspase1 (1:1,000, ab1872, Abcam), mature IL- $1\beta$  (1:1,000, ab200478, Abcam) and  $\beta$ -actin (1:1,000, ab8226, Abcam). Subsequently, the membranes were incubated in anti-rabbit IgG horseradish peroxidase conjugated antibody (1:5,000; Abcam) for 1 hr at room temperature. The immune complexes were detected using the SuperSignal west pico kit (Shanghai solarbio Bioscience & Technology, China). The intensities of the signals were quantified using ImageJ software 6.0.

## Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed to detect the LOX-1 mRNA expression levels in EPCs. Firstly, RNA was reverse transcribed into cDNA and the resulting cDNA was used as templates for PCR amplification. The primer sequences used were: LOX-1 forward, 5'-AAAAAGTCGGGAGAATTGCCTATC-3'; reverse, 5'-CCGGGTTTTTGTCTTCTGGTCTT-3';  $\beta$ -actin forward, 5'-GAGGGGAGAGCGGGTAAGA-3' reverse, 5'-TCGGGGTCCGACAAAACCC-3'. All reactions were performed in triplicates and each experiment was repeated three times in LightCycler480 PCR system (Roche Diagnostics, Rotkreuz, Switzerland), with the SYBR Green (Takara, Dalian, China)

as fluorescent dye, and normalized towards  $\beta$ -actin mRNA levels. The conditions were as follows: at 94°C for 10 mins, followed by 40 cycles (94°C for 10 s; 60°C for 45 s; 72°C for 60 s). The gene expression level was calculated by  $2^{-\Delta\Delta CT}$  methods.<sup>21</sup>

## Statistical analysis

Data are presented as mean  $\pm$  S.D. All statistical analyses were performed by SPSS statistics v20.0 (IBM Corp., Armonk, NY, USA). Multiple comparisons were performed using one-way ANOVA followed by Tukey–Kramer as a post-hoc test, as appropriate.  $P < 0.05$  indicated statistical significance. All experiments were conducted at least three separate times.

## Results

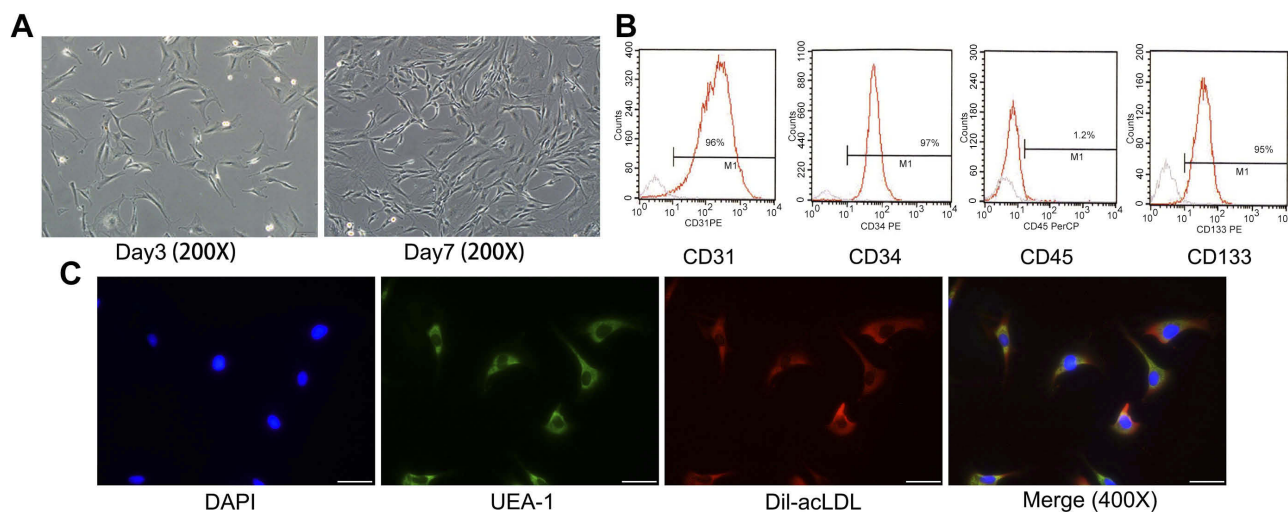
### ASV protected ox-LDL-induced cell damage on EPCs function

EPCs were isolated from rat peripheral blood cells, and the isolated cells displayed a characteristic spindle morphology 7 days later (Figure 1A). The surface markers of cells were additionally tested by flow cytometry and the percentage of CD31 was 95% $\pm$ 4%, the percentage of CD34 was 97% $\pm$ 6%, the percentage of CD133 was 95% $\pm$ 3%. The expression levels of CD45 were very low (Figure 1B). In addition, the characterized EPCs were proved by double staining with scavenger receptor of Dil-acLDL and the ligand of UEA-1, which two are hallmarks of EPCs (Figure 1C). To evaluate the effect of ASV on EPCs, cell

viability was measured by CCK8 assay. As illustrated in Figure 2A, low concentration of (10, 20, 40, 60 and 80  $\mu$ M) ASV exposure for 24 hrs had no influence on EPCs viability, indicating no toxicity on EPCs at this dose range. However, compared with normal control group, a high concentration of (100 and 200  $\mu$ M) ASV presented statistical differences on cell viability of EPCs ( $P < 0.05$  or  $P < 0.01$ ). According to the above results, 10, 20, 40  $\mu$ M ASV were chosen for the subsequent experiments. As depicted in Figure 2B, when compared with the control group, ASV with concentration range from 10 to 40  $\mu$ M exerted protective effects on ox-LDL-induced cell viability in a dose-dependent manner. In addition, we found that ASV promoted cell migration and improved impaired tube formation upon stimulation of ox-LDL (Figure 2C–E). Taken together, above results revealed that ASV attenuates ox-LDL-induced dysfunction of EPCs.

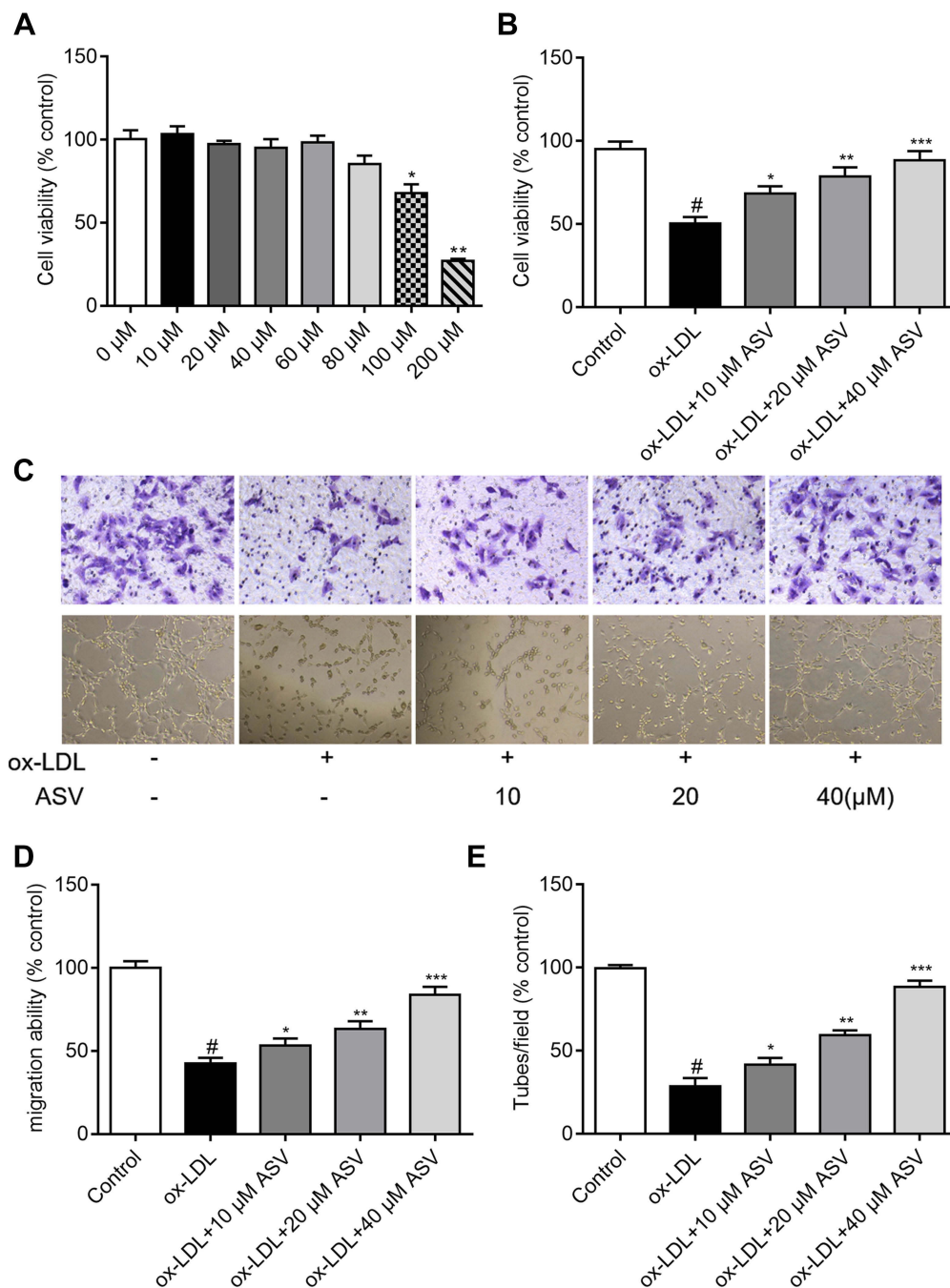
### ASV suppress oxidative stress and inflammation of EPCs induced by ox-LDL via inhibition of LOX-1

The intracellular ROS was detected by fluorescein-labeled dye, DCFH-DA. EPCs stimulated with ox-LDL led to a significant increase in intracellular ROS generation. Nevertheless, pretreatment with ASV for 24 hrs reduced the intracellular ROS levels induced by ox-LDL in a dose-dependent manner (Figure 3A). To reveal the mechanisms of the antioxidative effects of ASV, we investigated whether LOX-1, the receptor of ox-LDL, was involved in ox-LDL induced



**Figure 1** Cultivation and identification of EPCs. (A) Morphology of EPCs in culture 3 and 7 days later. Scale bar=100  $\mu$ m. (B) Flow cytometry results of peripheral blood mononuclear cell-derived endothelial progenitor cells' FACS analysis showed that EPCs highly expressed CD31, CD34 and CD133 and rarely expressed CD45. (C) Adherent cells showing double-positive of UEA-1 (green, excitation wavelength 488 nm) and Dil-acLDL (red, excitation wavelength 549 nm) were differentiated EPCs. These data indicated that the cells we used were outgrowth endothelial progenitor cells. Scale bar=50  $\mu$ m.

**Abbreviation:** EPCs, endothelial progenitor cells.

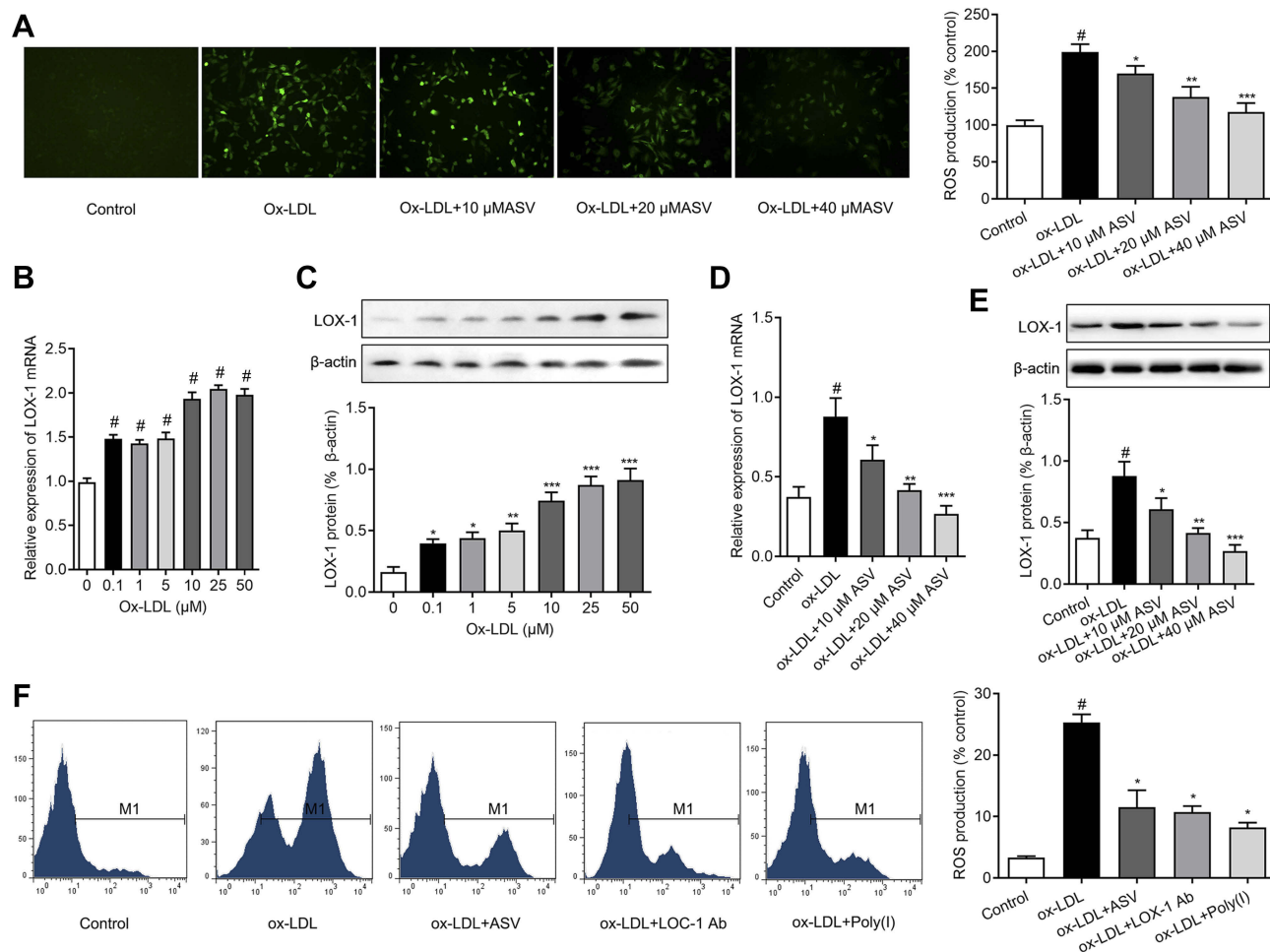


**Figure 2** ASV attenuates ox-LDL-induced EPCs dysfunction. **(A)** Cell Counting Kit-8 results of EPCs treated with different concentration of ASV for 48 hrs. **(B)** Cell Counting Kit-8 results of EPCs treated with treated with 50 μM ox-LDL and different concentration of ASV for 48 hrs. **(C and D)** A Transwell migration assay was used to study the effects of ASV on EPC migration. Scale bar=100 μm. **(C and E)** A tube formation assay was used to investigate the effects of ASV on EPC neovascularization. Scale bar=100 μm. #*P*<0.05 compared with control, \**P*<0.01 compared with ox-LDL group, \*\**P*<0.01 compared with ox-LDL+10 μM ASV group, \*\*\**P*<0.01 compared with ox-LDL group+20 μM ASV.

**Abbreviations:** EPCs, endothelial progenitor cells; ASV, astragaloside IV.

cell damage. We found the expression levels of LOX-1 mRNA and protein were both enhanced by ox-LDL treatment in a concentration-dependent manner (Figure 3B and C). To the contrary, ASV treatment reduced LOX-1 mRNA and protein expression levels induced by 50 μM ox-LDL exposure in a dose-dependent manner (Figure 3D and E). Subsequently, a

neutralizing antibody and chemical inhibitor to downregulate LOX-1 in EPCs were used to determine the role of LOX-1 in ROS production in response to ox-LDL. The intracellular ROS quantified by flow cytometer showed that similar with ASV, treatment with LOX-1 neutralizing antibody or inhibitor also reduced intracellular ROS levels (Figure 3F).



**Figure 3** ASV inhibits ox-LDL-induced oxidative stress of EPCs via LOX-1. **(A)** ROS levels of EPCs were determined by H2DCF-DA staining and expressed as % to control group. **(B)** Relative expression of LOX-1 protein in EPCs induced by different concentration of ox-LDL for 24 hrs. **(C)** Effect of different concentration of ASV on the expression of LOX-1 in EPCs induced by 50 μM ox-LDL for 24 hrs. **(D)** Effect of ASV and a LOX-1 neutralizing antibody (LOX-1 Ab) and a chemical inhibitor (Poly (I)) on ROS levels detected by flow cytometry. #P<0.05 compared with control, \*P<0.01 compared with ox-LDL group, \*\*P<0.01 compared with ox-LDL+10 μM ASV group, \*\*\*P<0.01 compared with ox-LDL group+20 μM ASV.

**Abbreviations:** EPCs, endothelial progenitor cells; ASV, astragaloside IV.

**Table 1** Effect of astragaloside IV on release of proinflammatory cytokines triggered by ox-LDL in EPCs (n=5, mean±SD)

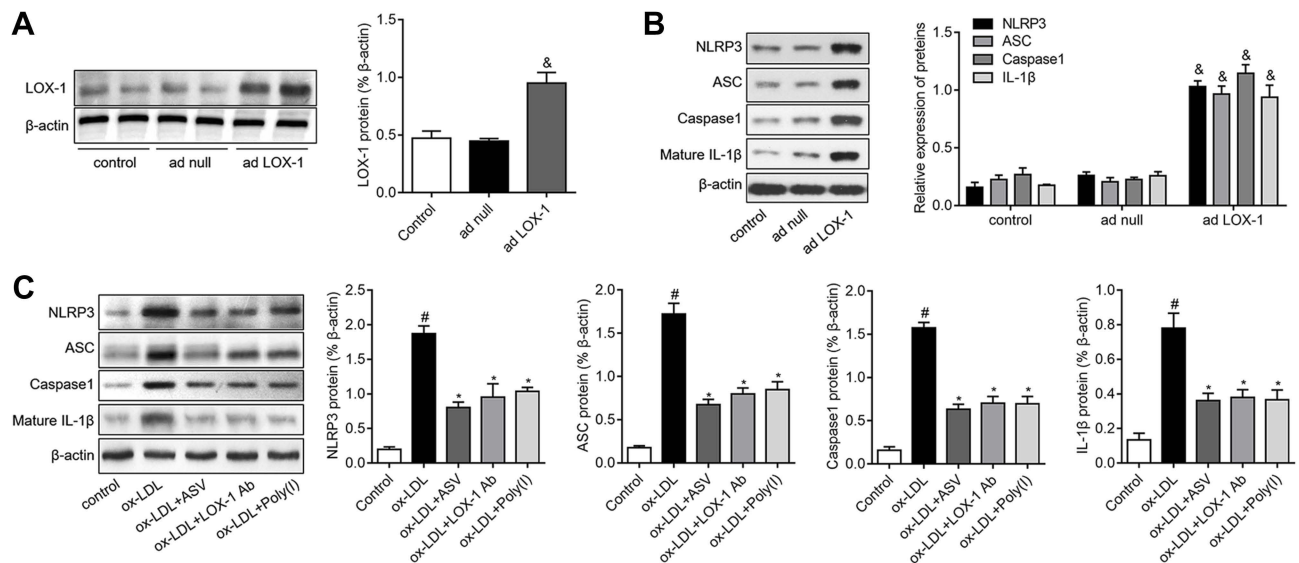
| Cytokines       | IL-1β (pg/mL) | IL-6 (pg/mL) | IL-10 (pg/mL) | TNF-α (pg/mL) |
|-----------------|---------------|--------------|---------------|---------------|
| Control         | 0.73±0.09     | 2.18±0.18    | 9.79±0.82     | 1.39±0.26     |
| Ox-LDL          | 2.61±0.18#    | 11.25±0.53#  | 2.02±0.13#    | 12.77±1.57#   |
| Ox-LDL+ASV      | 1.32±0.12*    | 5.46±0.28*   | 6.68±0.45*    | 4.93±0.76*    |
| Ox-LDL+LOX-1 Ab | 1.46±0.13*    | 5.88±0.42*   | 6.29±0.42*    | 5.65±0.84*    |
| Ox-LDL+Ploy(I)  | 1.55±0.10*    | 5.96±0.26*   | 6.53±0.76*    | 5.29±0.94*    |

**Notes:** #P<0.05 vs control group; \*P<0.05 vs ox-LDL group.

**Abbreviations:** Ox-LDL, oxidized low-density lipoprotein; EPCs, endothelial progenitor cells; ASV, astragaloside IV.

Furthermore, our ELISA assay results showed that the level of inflammatory cytokines including IL-1β, IL-6, IL-10 and TNF-α were significantly increased in the ox-LDL-stimulated EPCs compared to that in the control EPCs. ASV, LOX-1

neutralizing antibody or inhibitor treatment all reduced above inflammatory cytokines significantly (Table 1). These results indicated that ASV inhibits ox-LDL-induced oxidative stress and inflammation through inhibition of LOX-1.



**Figure 4** ASV inhibited LOX-1/NLRP3 inflammasome pathway in ox-LDL-induced EPCs. **(A)** Western blot analysis of LOX-1 in EPCs transfected with adenovirus containing the LOX-1 gene (ad LOX-1) or control adenovirus (ad null). **(B)** EPCs were transfected with ad null or ad LOX-1 and the expression levels of NLRP3, ASC, caspase1 and mature IL-1β were detected by Western blot. **(C)** EPCs were treated with 50 μM ox-LDL in the presence or absence of 40 μM ASC, LOX-1 neutralizing antibody (LOX-1 Ab), LOX-1 inhibitor Poly(I) for 24 hrs. The expression levels of NLRP3, ASC, caspase1 and mature IL-1β were detected by Western blot. <sup>#</sup>*P*<0.05 vs control group; <sup>\*</sup>*P*<0.05 vs ox-LDL group; <sup>&</sup>*P*<0.05 vs ad null group.

**Abbreviations:** EPCs, endothelial progenitor cells; ASV, astragaloside IV.

## ASV inhibits ox-LDL-induced NLRP3 inflammasome activation via LOX-1

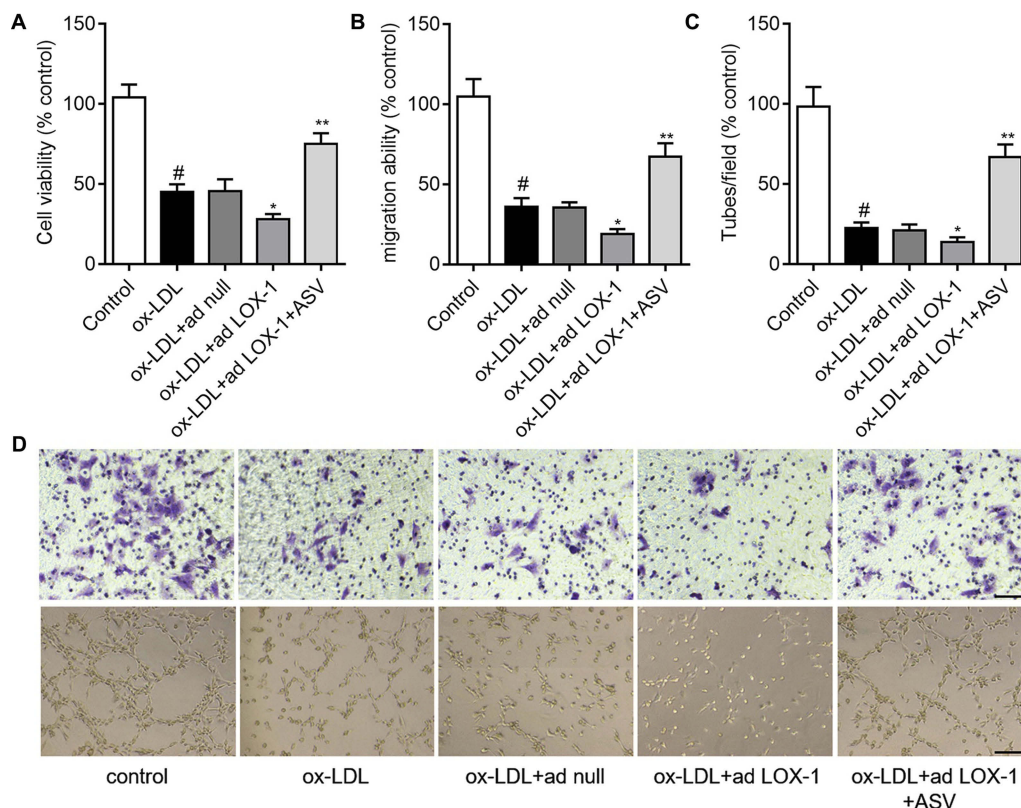
To reveal the further mechanisms of how LOX-1 regulates ox-LDL-induced inflammation in EPCs, we investigated the activation of NLRP3 inflammasome, which plays a key role in inflammation. As illustrated in Figure 4A, EPCs transfected with adenovirus containing the LOX-1 gene (ad LOX-1) showed higher LOX-1 expressions compared to the cells transfected with control adenovirus (as null). Interestingly, the results of Western blot showed that upregulation of LOX-1 in EPCs resulted in the activation of NLRP3 inflammasome, as evidenced by the great increase in expression of NLRP3, ASC, caspase1 and mature IL-1β proteins (Figure 4B). We then explored the effects of ASV on ox-LDL-induced NLRP3 inflammasome activation. As depicted in Figure 4C, ox-LDL stimulation caused an increase in NLRP3, ASC, caspase1 and mature IL-1β proteins. However, the stimulatory effect of ox-LDL on EPCs was partly abolished by pretreatment with ASV, LOX-1 neutralizing antibody or inhibitor, corroborating the notion that ASV inhibited ox-LDL-induced NLRP3 inflammasome activation via regulation of LOX-1 in EPCs.

## ASV alleviates ox-LDL-induced EPCs dysfunction via LOX-1 dependent NLRP3 signaling pathway

To validate the role of LOX-1/NLRP3 pathway in the protective effects of ASV on EPCs proliferation, migration and tube formation were detected. We found that over-expression of LOX-1 in ox-LDL-induced EPCs showed decreased cell viability than the control, which could be revised by ASV treatment (Figure 5A). Similarly, the results of cell migration and tube formation assay showed that LOX-1-upregulated EPCs had worse migrative and angiogenic ability than the control. And as expected, ASV treatment could improve LOX-1-upregulation-induced EPCs dysfunction (Figure 5B–D). The data suggested that ASV protects EPCs from ox-LDL-induced damage through LOX-1/NLRP3 pathway.

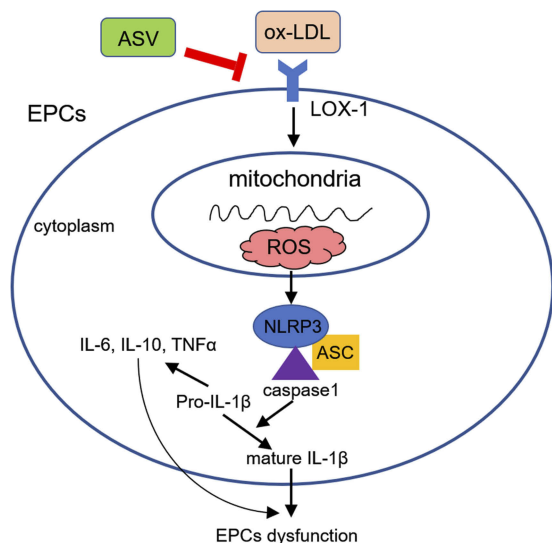
## Discussion

The results of the present study revealed that ox-LDL stimulation increased the expression of LOX-1 and subsequent NLRP3 inflammasome activation, resulted in reduced cell proliferation and migration, and impaired tube formation in EPCs. Moreover, we found that ASV could attenuate ox-LDL-



**Figure 5** ASV inhibited LOX-1/NLRP3 inflammasome pathway in ox-LDL-induced EPCs. (A) Western blot analysis of LOX-1 in EPCs transfected with adenovirus containing the LOX-1 gene (ad LOX-1) or control adenovirus (ad null). (B) EPCs were transfected with ad null or ad LOX-1 and the expression levels of NLRP3, ASC, caspase1 and mature IL-1 $\beta$  were detected by Western blot. (C) EPCs were treated with 50  $\mu$ M ox-LDL in the presence or absence of 40  $\mu$ M ASC, LOX-1 neutralizing antibody (LOX-1 Ab), LOX-1 inhibitor Poly(I) for 24 hrs. The expression levels of NLRP3, ASC, caspase1 and mature IL-1 $\beta$  were detected by Western blot. <sup>#</sup>*P*<0.05 vs control group; <sup>\*</sup>*P*<0.05 vs ox-LDL group; <sup>\*\*</sup>*P*<0.01 compared with ox-LDL+10  $\mu$ M ASV group.

**Abbreviations:** EPCs, endothelial progenitor cells; ASV, astragaloside IV.



**Figure 6** Schematic diagram showing the protective effects of ASV in ox-LDL-induced EPCs dysfunction.

**Abbreviations:** EPCs, endothelial progenitor cells; ASV, astragaloside IV.

induced EPCs dysfunction and the protective effect of ASV on EPCs function is dependent on the LOX-1/NLRP3 pathway (Figure 6).

EPCs are a population of cells with the inherent capacity to differentiate into mature endothelial cells. They are under investigation due to their association with vascular injury in response to trauma or ischemia.<sup>22</sup> A variety of disease conditions including T2DM negatively affect EPCs.<sup>23</sup> Ox-LDL induced reduction and dysfunction of EPCs has been shown in many studies. Hamed et al found ox-LDL exerted a deleterious effect on migrative ability of EPCs.<sup>24</sup> Similarly, other researchers identified that ox-LDL-affected EPC tube formation by activating eNOS mechanisms.<sup>25</sup> Consistently, we also found that ox-LDL reduced proliferation and migration, and impaired tube formation of EPCs. Therefore, the number and function of both cultured and circulating EPCs are profoundly altered in patients with T2DM, contributing to both the

microvascular and macrovascular complications of diabetic disease.<sup>26</sup>

Plenty of studies were taken to find solutions to improve EPCs function. Anti-inflammatory agents, anti-hypertensive agents, as well as vitamin D supplementation are all considered as therapeutic strategies.<sup>22</sup> Recently, an increasing number of studies focused on traditional Chinese medicine.<sup>27</sup> Yang et al demonstrated that the expression of VCAM-1/ICAM-1 in EPCs were promoted by TNF- $\alpha$ , which were reduced by Tanshinone IIA.<sup>28</sup> Salvianolic acid and Salidroside B were also found to promote cell migration and capillary tube formation of EPCs.<sup>29</sup> In addition, icariin could exert proangiogenic effects by promoting cell viability, migration, cell-matrix adhesion and enhancing capillary tube formation of EPCs.<sup>30</sup> In our previous published papers, we have demonstrated that ASV inhibited TGF $\beta$ 1-induced epithelial-mesenchymal transition of alveolar epithelial cells.<sup>31</sup> And studies have found that ASV has certain endothelial cell protective function.<sup>32</sup> However, whether ASV could protect EPCs from ox-LDL-induced injury is unclear. Here, we showed for the first time that ASV prevented ox-LDL-induced EPCs dysfunction, which was evidenced by enhanced cell proliferation, migration and improved tube formation capacity.

To figure out how ASV exerts its protective effects in EPCs, we tested the influence of ASV on LOX-1 expression in EPCs under ox-LDL stimulation. Interestingly, we found ASV significantly inhibited ox-LDL-induced upregulation of LOX-1 expression in EPCs in a concentration-dependent manner. LOX-1 is the major receptor for binding and uptake of ox-LDL in endothelial cells. Activation of LOX-1 promotes secretion of pro-inflammatory cytokines like IL-6, IL-8 and TNF $\alpha$  connecting LOX-1 to the development of atherosclerosis.<sup>33</sup> Knockdown of LOX-1 prevented ox-LDL-induced endothelial dysfunction and blocking the LOX-1 receptor preserved nitric oxide synthase and decreased superoxide anion radical formation.<sup>34,35</sup> We also found that inhibition of LOX-1 attenuated ox-LDL-induced EPCs dysfunction in the current study. Therefore, LOX-1 is a potential target for prevention of ox-LDL-mediated EPCs dysfunction and ASV could be served as an agent targeting LOX-1 in EPCs.

It was reported that ASV could decrease the release of IL-1 $\beta$ , TNF $\alpha$  and IL-6 in diabetic rats.<sup>36</sup> ASV treatment also reduced the level of ROS and apoptosis of renal tubular epithelial cells under free fatty acids stimulation.<sup>37</sup> Furthermore, administration of ASV ameliorates diabetic nephropathy by inhibition of ERK1/2 signaling,<sup>15</sup> indicating that ASV has the

ability to prevent diabetic vascular complications. In this study, we found that the levels of IL-1 $\beta$ , IL-6, IL-10 and TNF $\alpha$  were inhibited by ASV treatment. We also identified that ASV treatment suppressed LOX-1 expression in ox-LDL induced EPCs. Previous studies have demonstrated that NLRP3 inflammasome played an important role in inflammatory disease, including diabetes and diabetic complications.<sup>38</sup> NLRP3 inflammasome has been reported to be related to LOX-1.<sup>39</sup> Knockdown of LOX-1 decreased ox-LDL or xanthine oxidase-induced NLRP3 inflammasome in endothelial cells<sup>40</sup> and vascular smooth muscle cells.<sup>41</sup> Blockade LOX-1 with neutralizing antibody also inhibited electronegative LDL-induced activation of caspase-1 and NF- $\kappa$ B in human macrophages.<sup>42</sup> Consistent with these studies, we found depletion of LOX-1 using chemical inhibitor or LOX-1 neutralizing antibody in EPCs resulted in significant inhibition of NLRP3 inflammasome activation. All these data suggested that the LOX-1 and NLRP3 inflammasome pathways formed a signaling axis and this axis is a novel mechanism through which ASV exerts its anti-inflammation and antioxidant property in EPCs.

In summary, our results demonstrated that ASV may suppress EPCs dysfunction, which was relating to LOX-1/NLRP3 signaling pathway disturbed by ox-LDL. The data suggested that ASV may be considered as a strategy for T2DM vascular complications treatment.

## Abbreviation list

EPCs, endothelial progenitor cells; T2DM, Type 2 diabetes mellitus; ASV, astragaloside IV; ox-LDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; TNF- $\alpha$ , tumor necrosis factor; LOX-1, lectin-like oxidized LDL receptor; NLRP3, Nod-like receptor nucleotide-binding domain leucine-rich repeat containing protein 3.

## Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant no. 81704071), Key Research and Development Plan of Shandong province (Grant no. 2018GSF119027), the Taishan Scholars Youth Expert Program of Shandong Province in China (tsqn201812146), Young Elite Scientists Sponsorship Program by CAST (CACM-2018-QNRC2-B01), the Natural Science Foundation of Shandong Province (Grant nos. ZR2017BH027, ZR2016HB19 and ZR2012HM093), the Project of Scientific and Technological Development Program of Shandong Province (Grant no. 2010GSF10242), Project of Scientific and Technological Development Program

of Traditional Chinese Medicine of Shandong Province (Grant nos. 2017-180, 2011-038, 2009Z004-1), the Project of Scientific and Technological Development Program of Jinan (Grant nos. 201805081, 201805009).

## Author contributions

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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