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# Puerarin alleviates LPS-induced endothelial cells injury via SIRT1-mediated mitochondrial homeostasis signaling

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ABSTRACT

Endothelial inflammation injury is a key mechanism that occurs in the pathological processes of various cardiovascular diseases. Puerarin (PUE) is an isoflavone compound with strong antioxidant properties and the main active component isolated from the rhizome of *Pueraria lobata*. PUE exhibits a good anti-atherosclerotic pharmacological effect, but there are few studies on the mechanism of its protective effect on endothelial cells. This study found that PUE could regulate to some extent the mitochondrial function of human umbilical vein endothelial cells (HUVECs) and reduce or inhibit lipopolysaccharide-induced inflammatory reactions and oxidative stress injury in HUVECs. Furthermore, the protective effect of PUE on HUVECs was closely related to the SIRT-1 signaling pathway. PUE increased the level of mitophagy and the activity of mitochondrial antioxidant enzymes by increasing SIRT-1 expression, reducing excessive production of ROS, and inhibiting expression of inflammatory factors and oxidative stress injury. Therefore, PUE may improve mitochondrial respiratory function and energy metabolism and increase the activity of HUVECs in the inflammatory state.

## 1. Introduction

Inflammation induced myocardial or vascular injury is a protective response to injury of arterial wall endothelium and smooth muscle.[1] When the inflammatory response is excessive, it may lead to vascular endothelial cell damage and plaque formation.[2,3] The structure and function of endothelial cells play important roles in maintaining balance of the micro-circulation.[4,5] Endothelial cells are the main cells constituting the intima of the artery wall. As the barrier between blood and external tissues, endothelial cells function to regulate blood flow and participating in lipoprotein metabolism.[6,7] Furthermore, endothelial cells can secrete bioactive substances, such as nitric oxide (NO) and endothelin, and affect the function of smooth muscle cells, platelets, and white blood cells.[8] During the development of inflammatory injury, inflammatory reactions are often related to reactive oxygen species (ROS)-mediated oxidative stress.[9] With the occurrence and development of inflammation, with the demands of increased activated infiltrating immune cells and inflammatory resident cells, mitochondrial energy gradually increases, which further leads to hypoxia and Mitochondrial homeostasis disorder. Furthermore, ROS are overproduced and endothelial cells exhibit more serious oxidative damage.[10,11]

Mitochondria are the main sites of oxidative phosphorylation in eukaryotes and are where carbohydrates, fats, and proteins are oxidized and decomposed to produce energy.[12] It is hydrolyzed to form triacyl acid and pyruvic acid in the mitochondria. Ultimately, $H_2O$ and $CO_2$ are generated and adenosine triphosphate (ATP) is released and used to maintain the normal physiological function of cells.[13] During inflammatory injury progression, mitochondrial energy metabolism disorder mainly manifests as respiratory dysfunction and decreased expression of energy metabolism-related genes and proteins.[14] Mitochondria are important mediators in cells and mitochondrial dysfunction can indirectly activate a variety of inflammatory signal transduction pathways, leading to tissue and cell damage. ROS-mediated oxidative stress may result in Mitochondrial homeostasis disorder through direct cytotoxicity and can promote the occurrence and development of local inflammatory responses.[15]

Oxidative stress and inflammation are interdependent, especially in mitochondria.[16] A study by Chen also revealed that damaged mitochondria activate inflammatory bodies of NLR family pyrin domain containing 3 (NLRP3).[17] Furthermore, activation of the NLRP3 inflammasome is inhibited when mitochondrial autophagy clears abnormal mitochondria and damaged proteins.[18] The oxidative effect of ROS on

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mitochondrial DNA during activation of NLRP3 leads to a partial inflammatory potential of free circulating mitochondrial DNA. This shows that oxidative stress and the mitochondrial pathway can affect Mitochondrial homeostasis and endothelial cell inflammatory responses in an interdependent manner.

Puerarin (PUE) a flavonoid glycoside extracted from the dried roots of *Pueraria lobata*.[19,20] Because of its obvious estrogen-like effects, PUE can play an important regulatory role in the treatment of atherosclerotic diseases and help protect endothelial cell function.[21] PUE also significantly reduces lipopolysaccharide (LPS)-induced p-NF-κb-p65 and Bax expression and increases the expression levels of Bcl-2. Furthermore, PUE can inhibit the release of inflammatory cytokines and protect umbilical vein endothelial cells.[22] Other studies have shown that PUE is able to reduce vascular endothelium injury and the expression of IL-1β, IL-8, ICAM-1, and PAI-1 in the supernatant of human umbilical vein endothelial cells (HUVECs) stimulated with LPS. It can also reduce LPS-induced neutrophil adhesion to HUVECs, inhibiting LPS-induced endothelial injury.[23]

Although experimental studies have confirmed that PUE has a certain protective effect on endothelial cells, the specific mechanism remains unclear. In the current study, we investigated whether the inhibitory effect of PUE on LPS-induced endothelial cell inflammation and oxidative stress injury was mediated by mitochondria.

## 2. Materials and methods

### 2.1. Cell culture and treatment

HUVECs were purchased from the Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). LPS was acquired from Sigma. SRT1720 and EX527 were purchased from MedChemExpress (USA). PUE (purity ≥ 98%) was purchased from Chinese Medicine Resource Center, Chinese Academy of Traditional Chinese Medicine (Beijing, China).

The cells were cultured in Dulbecco's modified Eagle' s medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 μg/mL penicillin streptomycin (Gibco). Cells were passaged using Trypsin-EDTA (Gibco). The cells were incubated at 37 °C, 95% humidity, and 5% $CO_2$. The medium was renewed every 2 days. HUVECs were used up to passage five.[24] The HUVECs were activated with 10 μg/mL LPS for 24 h[25] and accordingly pretreated with 10, 20, 50, 100, or 150 mg/L PUE for 24 h before LPS induction. As indicated, HUVECs were incubated with EX527 (Catalog No: HY-15452; MedChemExpress) for 6 h to inhibit SIRT-1 activity or incubated with SRT1720 (Catalog No.: HY-10532; MedChemExpress) for 6 h to activate SIRT-1 activity.

### 2.2. CCK-8 assays

The HUVECs were determined to be in good condition with the total cell coverage rate being more than 90%. The medium was discarded and the cells washed with phosphate-buffered saline (PBS) and then digested with trypsin. The digestion was terminated by adding fresh complete DMEM medium and the cells then counted. The cells were seeded into 12-well plates (50,000 cells/well) and incubated 12 h. The medium was discarded, the cells rinsed twice with PBS and the adherent cells then observed under an inverted microscope. Cellular metabolic activity as an indicator of cell viability was measured by the CCK-8 method.

### 2.3. Mitochondrial membrane potential

Mitochondrial membrane potential of the HUVECs was measured using JC-1 Dye (CAT No: HY-15534; MedChemExpress, USA). HUVECs were washed three times with PBS and then stained with JC-1 Dye for 30 min in a dark room. The HUVECs were then washed three times with PBS and the mitochondrial membrane potential images captured using a Nikon A1 laser confocal microscope.

### 2.4. Immunofluorescence staining

HUVECs were fixed with 4% paraformaldehyde for 10 min, washed with cold PBS three times, and blocked on ice with PBS containing 5% BSA for 30 min. After washing with PBS three times, the cells were stained with Alexa fluor-594-conjugated goat anti-mouse secondary antibody in 1% BSA/PBS at 4 °C for 1 h. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and image were captured using the Nikon A1 confocal microscope.

### 2.5. ELISA quantitative analysis

The cells were digested with 0.25% trypsin in PBS according to the manufacturer's instructions. The Complexes I/III, SOD, GSH, GPX, IL-10, IL-18, and TNF-α contents were detected using a total assay kit.

### 2.6. Quantitative real time PCR

Total RNA was isolated from HUVECs using a Quick-RNA Microprep Kit. An iScript cDNA Synthesis Kit (Bio RAD) was used to reverse transcribe 150–250 ng total RNA into complementary DNA (cDNA). The cDNA samples were diluted 10-fold with $ddH_2O$ and real-time quantitative PCR (qPCR) was performed on a LightCycler 480 Instrument (Roche) using 2 μL cDNA. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with 18S RNA used as an internal control.

### 2.7. Cellular respiration assays

An XFp Extracellular Flux Analyzer (Seahorse Biosciences) was used according to the manufacturer's instructions to analyze the oxygen consumption rate (OCR) of intact cells in real time. Briefly, HUVECs were inoculated at $5 \times 10^5$ cells/well. The results were normalized to the actual cell count determined immediately after obtaining the OCR recording.

### 2.8. Statistical analysis

All statistical analyses were performed using the Statistical Product and Service Solutions (SPSS) 22.0 software package (SPSS, Chicago, IL, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, United States). All data are expressed as mean ± SEM and evaluated by analysis of variance (ANOVA).

## 3. Results

### 3.1. PUE improved HUVEC activity in LPS-induced inflammation

A HUVEC inflammatory model was established by stimulating HUVECs with LPS to preliminarily confirm the effect of PUE on the function of HUVECs in a LPS-mediated inflammatory state. Different concentrations of PUE (10, 20, 50, 100, 150 mg/L) were then used as intervention treatments. HUVEC activity was determined using CCK-8 assays. CCK-8 analysis showed that LPS decreased the activity of HUVECs compared with that of the control group, as shown in Fig. 1A. Pretreatment with different concentrations of PUE improved HUVEC vitality after LPS treatment with the cell activity being most significant after pretreatment with 100 mg/L PUE (Fig. 1B). Accordingly, 100 mg/L PUE was chosen as the optimal drug concentration to treat HUVECs in subsequent experiments. Analysis also showed that LPS increased apoptosis levels of HUVECs, but 100 mg/L PUE inhibited the apoptosis (Fig. 1B–D). These results indicated PUE was able to maintain the activity of HUVECs and inhibit cell apoptosis under a LPS-induced inflammatory state. At the same time, we further conducted network pharmacology analysis on the mechanism of PUE's intervention in vascular inflammatory injury. The experimental results showed that the enriched target genes were mainly closely related to inflammation and apoptosis

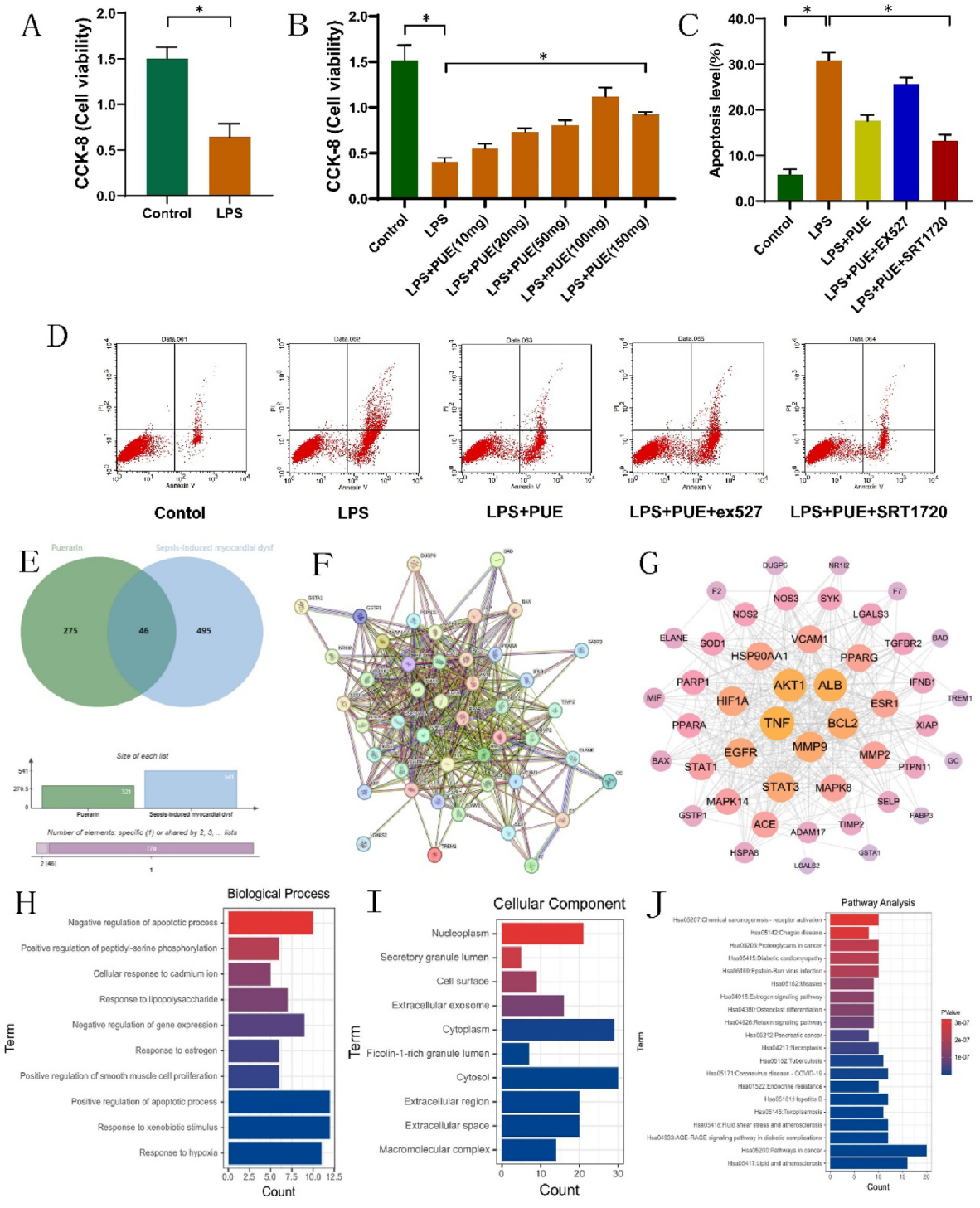


**Fig. 1.** PUE improves the activity of human umbilical vein endothelial cells (HUVECs) under LPS-induced inflammation. (A) Cell viability in a LPS-induced inflammatory state was determined using the CCK-8 method. After LPS stimulation, HUVECs viability was severely reduced. (B) Treatment of HUVECs with different concentrations of PUE (10, 20, 50, 100, and 150 mg/L). HUVECs viability under the different concentrations of drug was determined using the CCK-8 method. (C–D) The level of HUVEC apoptosis was analyzed before and after administration of PUE. (E-J) Network pharmacology analysis of intervention targets of puerarin (PUE). Experiments were repeated at least three times and the data are shown as mean ± SEM. *$P < 0.05$. EX527: the SIRT1 blocker; SRT1720: the SIRT-1 activator.

mediating genes such as *TNF-α/MMP9* and *BCL2* (Fig. 1E–J).

### 3.2. PUE inhibited LPS-induced inflammatory responses and oxidative stress damage in HUVECs

We found PUE could also reduce LPS-induced inflammatory injury. Compared with that of the control group, the expression level of the inflammatory factor IL-10 was significantly reduced (Fig. 2A), and the expression of inflammatory factors TNF-α and IL-18 were significantly increased in HUVECs treated with LPS (Fig. 2B and C). Fig. 2 Pretreatment of HUVECs with PUE reversed the LPS-induced expression of IL-10, TNF-α and IL-18(Fig. 2A−C). The results confirmed that PUE intervention could improve the HUVEC inflammatory response induced by LPS.

We further investigate the protective mechanism of PUE in improving

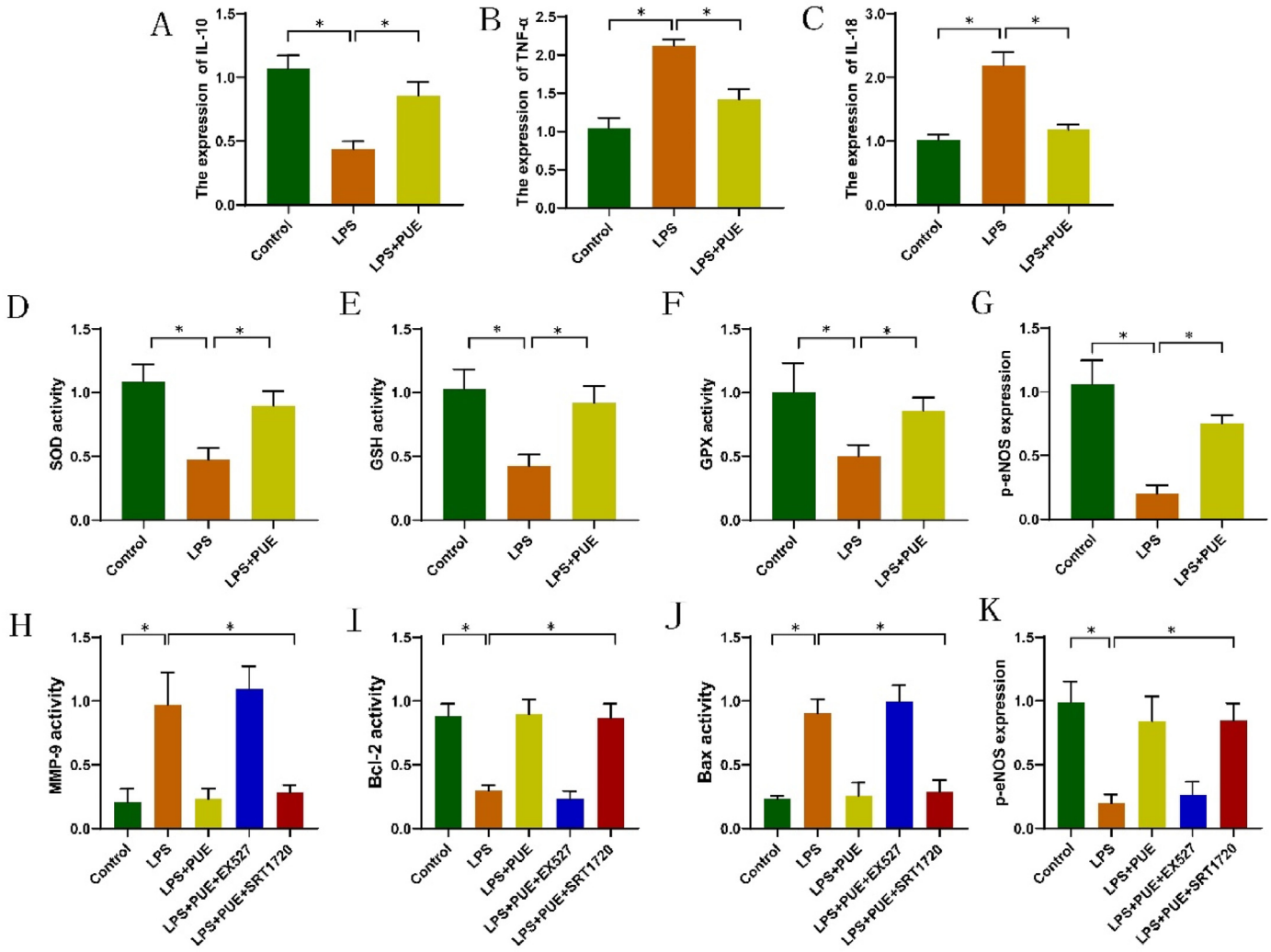


**Fig. 2.** PUE inhibits LPS-induced inflammatory responses and oxidative stress damage in human umbilical vein endothelial cells (HUVECs). Pro-inflammatory and anti-inflammatory factors and antioxidant enzymes were evaluated using ELISA. (A) Detection of anti-inflammatory factor IL-10 levels after LPS stimulation. (B) Levels of pro-inflammatory factor TNF-α after LPS stimulation. (C) Levels of pro-inflammatory factor IL-18 levels after LPS stimulation. (D) Levels of SOD after PUE treatment. (E) Levels of GSH after PUE treatment. (F) Levels of GPX after PUE treatment. (G) Levels of p-eNOS after PUE treatment. (H) Levels of MMP-9. (H) Levels of BcL-2. (I) Levels of Bax. (K) Levels of p-eNOS. EX527: the SIRT1 blocker; SRT1720: the SIRT-1 activator.
Experiments were repeated at least three times and the data are shown as mean ± SEM. $^{*}P < 0.05$.

the vitality and reducing vulnerability of HUVECs under LPS-induced inflammation. To explore the protective effect of PUE on the redox state of endothelial cells and mitochondrial oxidative stress damage, enzyme-linked immunosorbent assays (ELISAs) were used to evaluate the activity of antioxidant enzymes, such as glutathione (GSH), superoxide dismutase (SOD), and glutathione peroxidase (GPX). We found that the inflammatory state induced by LPS exhibited reduced GSH, SOD, and GPX activity in HUVECs (Fig. 2D−F). Pretreatment of the cells with PUE resulted in increased GSH, SOD, and GPX activity (Fig. 2D-F). These results suggested LPS-induced inflammation could induce oxidative stress damage by inhibiting the activity of antioxidant enzymes, including GSH, SOD, and GPX. To verify the accuracy of network pharmacology results, we further experimentally validated the enriched genes related to cell apoptosis and inflammation (MMP-9/Bcl-2 and Bax). The experimental results showed that PUE can increase the expression level of Bcl-2 and p-eNOS in endothelial cells after inflammatory injury, and reduce the expression levels of MMP-9 and Bax (Fig. 2G−K). However, the SIRT1 blocker (EX527) blocked the therapeutic effect of PUE (Fig. 2G−K). The experimental results suggest that puerarin can regulate endothelial cell inflammatory response and block the apoptotic pathway through SIRT1. The results of this study are consistent with those of network pharmacology research.

### 3.3. *PUE regulated HUVEC mitochondrial homeostasis under LPS-induced inflammation*

Mitochondria are the main sites of ATP production that is required for cell energy metabolism. Studies have demonstrated that LPS-induced inflammatory responses can cause serious damage to the structure and function of mitochondria.[26,27] In the current study, we evaluated HUVECs for the level of ROS generation, mitochondrial structure and function, and mitochondrial membrane potential. Compared with those in the control group, the level of ROS generation was increased (Fig. 3A and B), the number and activity of HUVEC mitochondria in the LPS group were significantly reduced, $P < 0.05$ (Fig. 3C and D), Fig. 3 and the mitochondrial membrane potential was significantly reduced (Fig. 3E and F). PUE treatment of HUVECs resulted in significantly increased membrane potential levels (Fig. 3E and F), while the levels of ROS generation were significantly reduced (Fig. 3A and B).

Mitochondria are the main sites of oxygen consumption in cells, as well as the main source of ROS and main target of ROS attack. Excessive production of ROS results in the release of pro-inflammatory factors IL-18

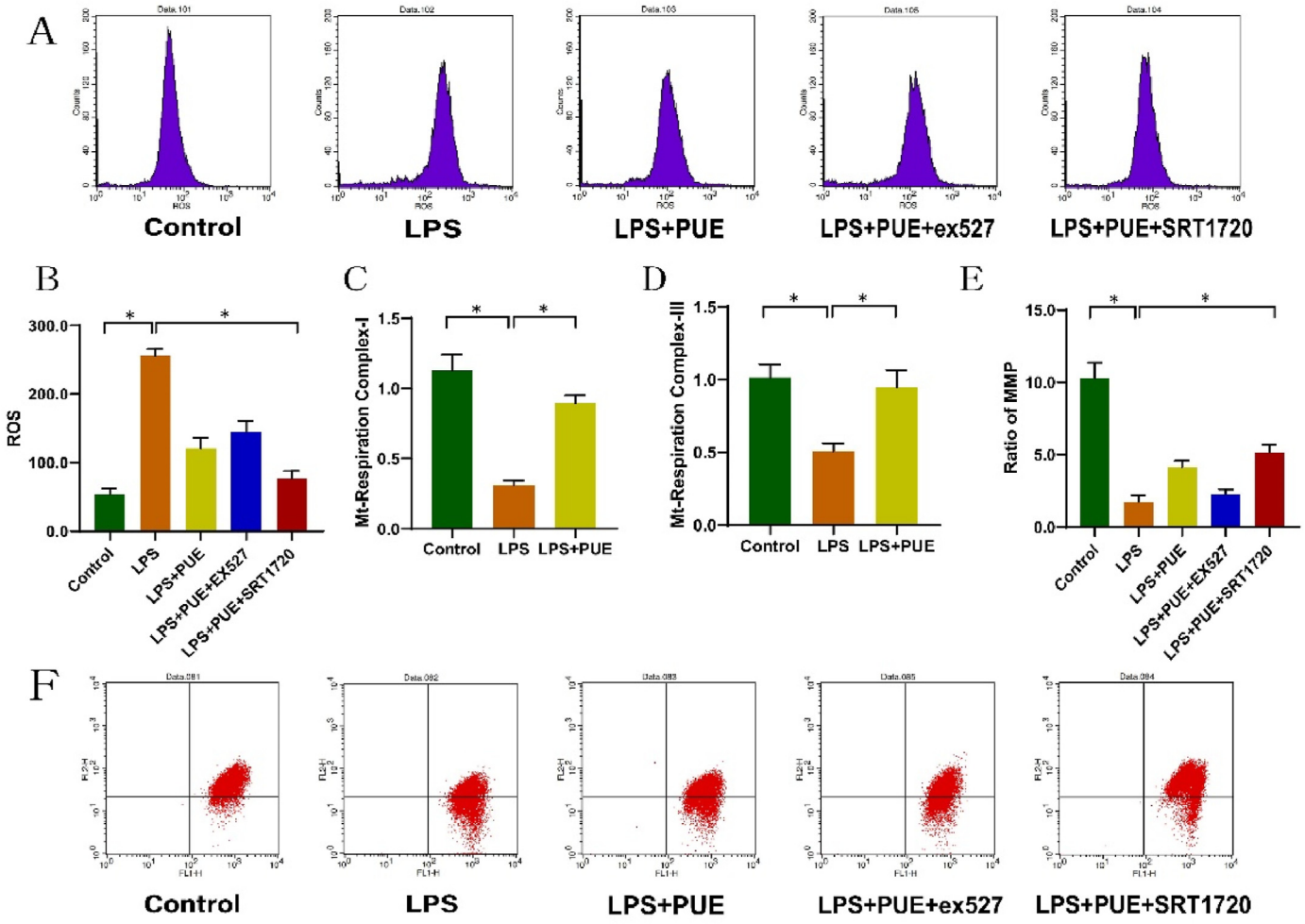


**Fig. 3.** PUE improves mitochondrial activity under LPS-induced inflammation and oxidative stress damage. (A, B) Immunofluorescence detection of HUVEC ROS expression. (C, D) ELISA detection of mitochondrial respiratory complex I and III activity. (E) Detection of mitochondrial membrane potential. (F) Differences in fluorescence intensity were used to determine mitochondrial membrane potential. Experiments were repeated at least three times and the data are shown as mean ± SEM. *$P < 0.05$. EX527: the SIRT1 blocker; SRT1720: the SIRT-1 activator.

and TNF-α and can directly affect mitochondrial structure and function.[28–30] LPS-induced HUVEC inflammatory responses and oxidative stress damage changed the structure and function of mitochondria compared with those of the control, and destroyed the mitochondrial membrane potential. However, PUE intervention significantly increased the activity and number of mitochondria, restored the mitochondrial membrane potential, and inhibited the generation of ROS. We also evaluated the activity of mitochondrial respiratory complexes I and III by ELISA (Fig. 3B and C). Mitochondrial respiratory complexes I and III showed reduced expression under LPS-induced inflammatory injury, but their activities were restored with PUE intervention.

### *3.4. PUE promoted HUVEC mitochondrial energy metabolism under LPS-induced inflammation*

Abnormal mitochondrial energy metabolism and respiratory function are closely related to mitochondrial dysfunction.[31–33] In the current study, we investigated whether PUE under inflammatory conditions could improve mitochondrial energy metabolism levels and respiratory function in HUVECs using a mitochondrial energy metabolism test. Compared with those of the control group, HUVECs treated with LPS exhibited significant reductions in mitochondrial respiration levels (Fig. 4A), maximum respiration capacities (Fig. 4B), respiration reserve values (Fig. 4C), ATP production capacities (Fig. 4D), and exhibited significant increase in non mitochondrial respiratory expenditure levels (Fig. 4E), and proton leakage values (Fig. 4F). However, PUE pretreatment reversed all these effects, resulting in significant increases in the level of mitochondrial energy metabolism and respiratory function (Fig. 4A–F).

To investigate the mechanism by which PUE improved the mitochondrial respiratory function of HUVECs in an inflammatory state, EX527 was used, which is an effective and specific inhibitor of sirtuin-1 (SIRT-1). The results showed that mitochondrial respiratory function of HUVECs was significantly inhibited in the EX527 + LPS + PUE group compared to that of the LPS + PUE group (Fig. 4A–F). This demonstrated the effect of PUE on improving mitochondrial respiratory function was eliminated by the SIRT-1 inhibitor EX527. These findings suggest that PUE was able to improve mitochondrial respiratory function of LPS-treated HUVECs. According to the results, the protective mechanism of PUE may have been mediated through SIRT-1.

### *3.5. PUE regulated HUVEC autophagy in an inflammatory state through SIRT-1*

The protective mechanism of PUE against oxidative stress injury and mitochondrial function damage in HUVECs was further explored. Expression of select mRNAs were detected by real-time quantitative polymerase chain reaction (qPCR). The qPCR analysis showed the mRNA

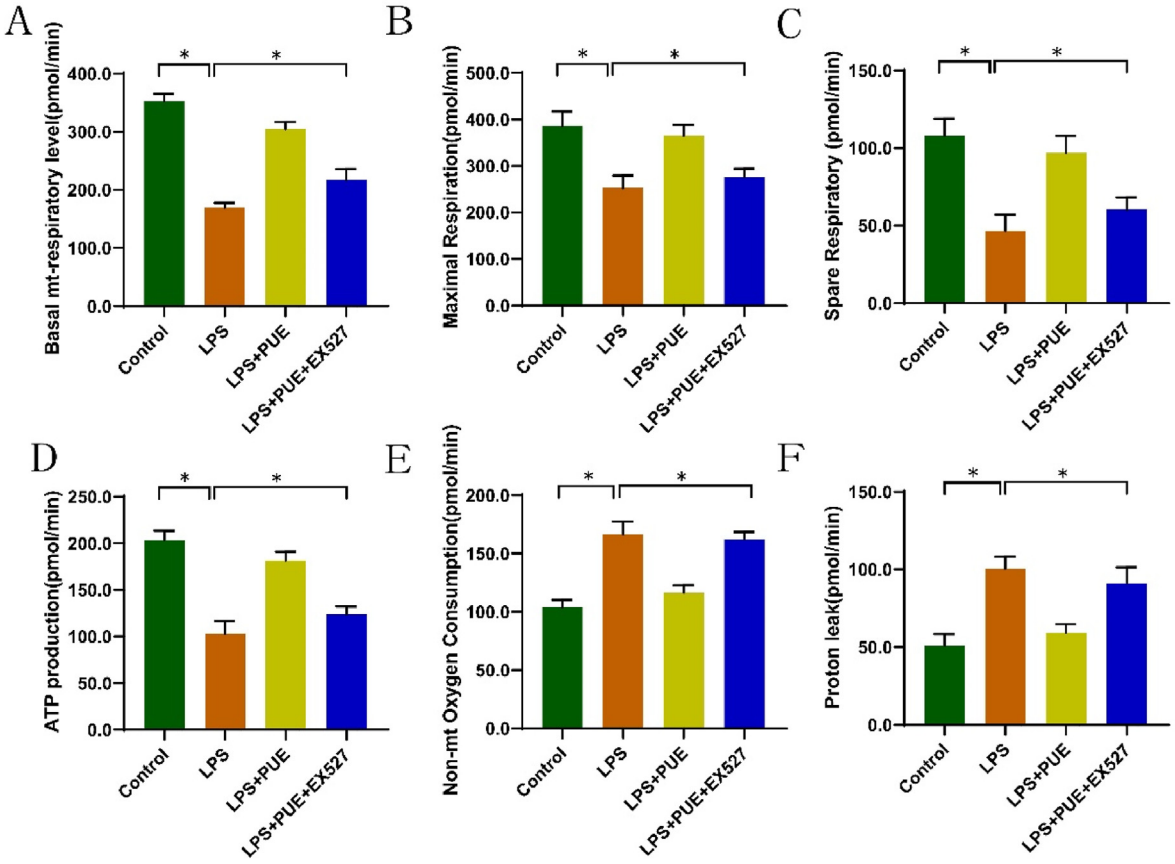


**Fig. 4.** Pue promotes mitochondrial energy metabolism in human umbilical vein endothelial cells (HUVECs) induced with LPS. (A) Basic mitochondrial respiratory level values. (B) Maximum respiratory capacity. (C) Respiratory reserve value. (D) ATP production capacity. (E) non mitochondrial respiratory expenditure levels. (F) Proton leakage level. Experiments were repeated at least three times and the data are shown as mean ± SEM. *$P < 0.05$. EX527: the SIRT1 blocker; SRT1720: the SIRT-1 activator.

levels of atg5, atg7, sirt-1, and PINK1/parkin were significantly lower in the LPS group compared to those in the control group, but they were significantly higher in PUE + LPS-treated HUVECs (Fig. 5A–E). The mRNA levels of atg5, atg7, and PINK1/parkin in HUVECs treated with EX527 + PUE + LPS were also significantly reduced (Fig. 5A–E). However, the mRNA levels of atg5, atg7, sirt-1, and parkin were significantly increased in HUVECs treated with the SIRT-1 activator SRT1720 (SRT1720 + PUE + LPS) (Fig. 5A–E). Further experimental results showed that the protein expression level of SIRT1 was significantly reduced after inflammatory injury, while PUE could significantly increase the protein expression level of SIRT1. The activators and inhibitors of SIRT1 would affect the therapeutic effect of PUE to varying degrees (Fig. 5F). In addition, we also found that PUE can protect vascular endothelial cells after LPS injury through SIRT1, maintaining the integrity of mitochondrial quantity and morphological structure of vascular endothelial cells (Fig. 5G). These results suggested PUE regulated autophagy through the SIRT-1 signaling pathway and that EX527 could eliminate the regulation of autophagy by PUE.

### *3.6. Inhibition of the SIRT-1 signaling pathway abolished PUE-mediated protection*

To further determine whether PUE could induce mitochondrial and HUVEC protection through the SIRT-1 signaling pathway, we used EX527 and SRT1720 to intervene in PUE + LPS-treated HUVECs. As activators (SRT1720) and inhibitors (EX527) of SIRT1, EX527 and SRT1720 can block or activate the SIRT1 pathway to varying degrees, affecting the regulatory effect of SIRT1 on mitochondrial and vascular function. The specific regulatory mechanism of PUE was verified through evaluation of cell viability, apoptosis levels, antioxidant enzyme activity, anti-inflammatory ability, and mitochondrial function in the various groups of HUVECs. As shown in Fig. 6A–J, CCK-8 and flow cytometry analyses showed that EX527 treatment significantly reduced the activity of HUVECs treated with PUE + LPS and increased the level of cell apoptosis. SRT1720 did not affect the targeted therapeutic effect of PUE (Fig. 6A–J). In addition, EX527 treatment eliminated the protective effect of PUE on the HUVECs treated with LPS, as well as the inhibitory effect of PUE on the inflammatory response and oxidative stress injury (Fig. 6A–J). Myosin, as a molecular motor of the cytoskeleton, is a multifunctional protein that plays a crucial regulatory role in regulating cellular homeostasis and physiological/pathological activities. To further confirm the regulatory effect of PUE on the stability of endothelial cytoskeleton proteins, we detected the fluorescence expression levels of endothelial cytoskeleton proteins after LPS treatment. The results show that PUE improved the destruction of myosin after LPS injury and maintained the stability of myosin. However, EX527 blocked the improvement effect of PUE on myosin, while SRT1720 did not affect the therapeutic effect of PUE (Fig. 6K). These results further confirm that the improvement effect of PUE on HUVECs is related to SIRT1.

## 4. Discussion

Inflammation and oxidative stress are the main risk factors of cardiovascular damage. PUE, a natural antioxidant, can play a key role in the regulation of inflammatory responses and oxidative stress injury. However, few studies have aimed to reveal the underlying mechanism by which PUE reduces endothelial cell vulnerability in inflammatory conditions. In the current study, we found that a LPS-induced inflammatory

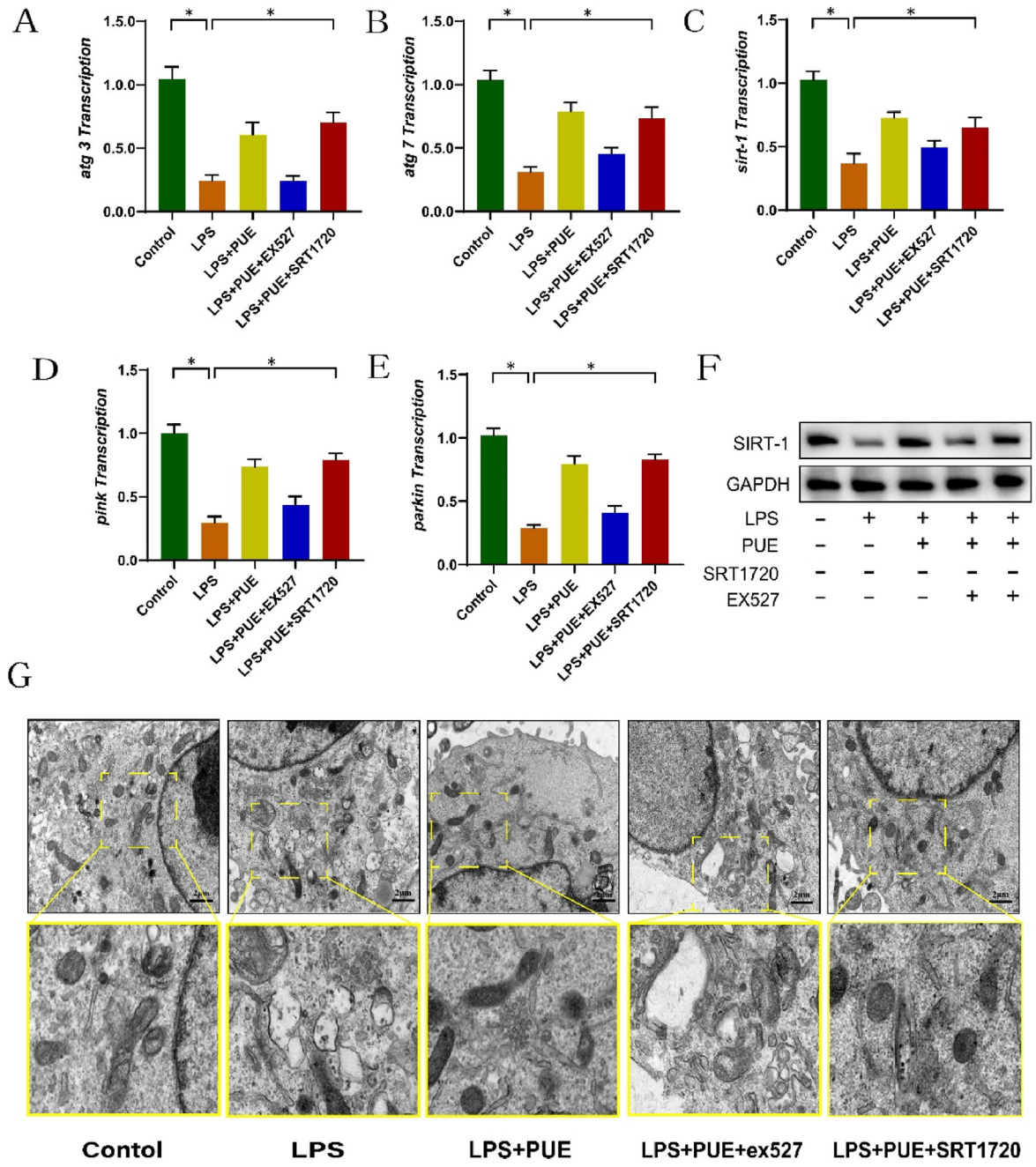


**Fig. 5.** Mechanism of Pue regulating mitochondrial autophagy through SIRT-1. (A–E) Changes in mRNA levels of atg5, atg7, sirt-1, and PINK1/Parkin were determined by qPCR and Westernblot. (G)Transmission electron microscopy detection of morphological structure; Experiments were repeated at least three times and the data are shown as mean ± SEM. *$P < 0.05$. EX527: the SIRT1 blocker; SRT1720: the SIRT-1 activator.

response in HUVECs led to increased ROS production in mitochondria, greater Mitochondrial homeostasis disorder, and further activated damage caused by oxidative stress. PUE was able to regulate Mitochondrial homeostasis, enhance mitophagy, and inhibit LPS-induced inflammation and oxidative stress injury in the HUVECs. PUE was also able to increase the mitochondrial membrane potential and level of energy metabolism of the HUVECs, increase the activity of SOD and other antioxidant kinases, inhibit excessive production of ROS and oxidative stress damage induced by inflammation, weaken the inflammatory response induced by LPS, and reduce the vulnerability of HUVECs in the inflammatory state.

From a mechanistic perspective, the data suggests that LPS can significantly reduce the expression level of SIRT1 in endothelial cells and further activate mitochondrial oxidative stress and inflammatory damage pathways. Studies have shown that myocardial remodeling and endothelial mesenchymal transition are accompanied by a decrease in SIRT1 ($NAD^+$ - dependent deacetylase) protein expression, and treatment with dapagliflozin can significantly reverse SIRT1 inhibition, promote SIRT1 nuclear displacement, and enhance the binding of SIRT1 to Notch1 (NICD) active intracellular domains, thereby alleviating myocardial injury.[34] Coincidentally, further research has confirmed that dapagliflozin reverses $H_2O_2$ mediated eNOS serine phosphorylation and reduced SIRT1 expression levels in endothelial cells. Dapagliflozin can restore eNOS activity and NO bioavailability by activating SIRT1, inhibit ROS mediated oxidative stress damage in endothelial cells, and alleviate endothelial dysfunction.[35] There are also studies showing that exposure to PM2.5 can lead to an increase in the activity of age-related β - galactosidase (SA - β - gal) and the expression of cell cycle blocking proteins P53/P21 and P16 in HUVECs. The cellular aging induced by PM2.5 is

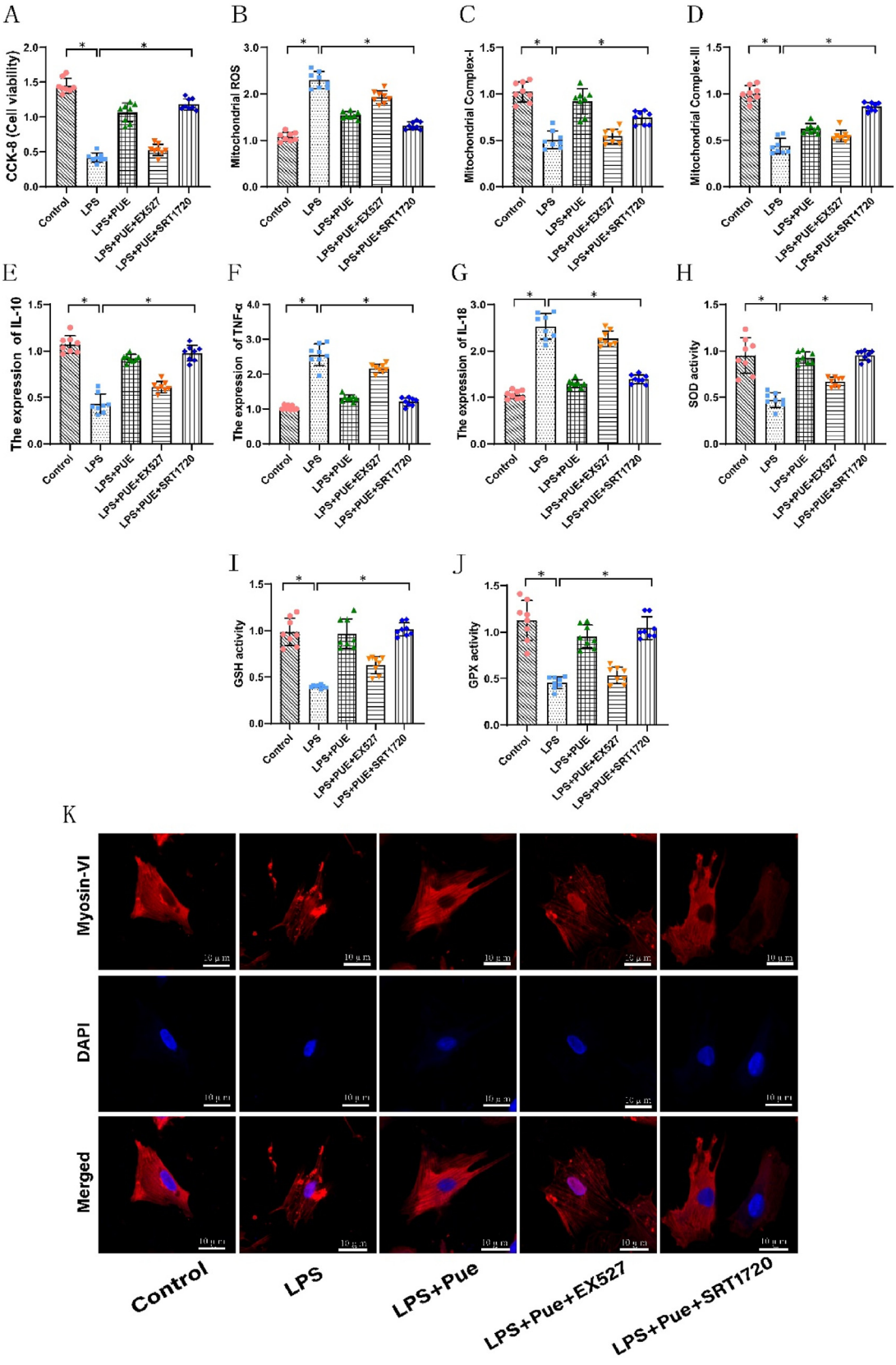


**Fig. 6.** Inhibition of the SIRT-1 signaling pathway abolishes Pue-mediated protection. (A) CCK-8 analysis and flow cytometry show the activity and apoptosis in different groups of human umbilical vein endothelial cells (HUVECs). (B) The expression level of ROS. (C, D) The activity of mitochondrial respiratory chain complexes. (E-G)The expression levels of anti-inflammatory and inflammatory factors (IL-10, TNF-α and IL18). (H-J) The activity of antioxidant enzymes (SOD/GSH/GPX). (K) The expression level of Myosin-VI. Experiments were repeated at least three times and the data are shown as mean ± SEM. *$P < 0.05$. EX527: the SIRT1 blocker; SRT1720: the SIRT-1 activator.

attributed to the disruption of cellular antioxidant defense system through the SIRT1/PGC-1 α/SIRT3 signaling pathway. Treatment with SIRT1 specific activator SRT1720 upregulated the SIRT1/PGC-1 α/SIRT3 signaling pathway, restored the antioxidant system, and reduced the expression of cellular aging markers.[36] The above results are consistent with our experimental findings, indicating the important regulatory role of SIRT1 in endothelial cell inflammation and oxidative stress damage. We further confirmed the targeted protective effect of PUE on SIRT1 based on the previous research. It was found that the intervention with EX527, a selective inhibitor of SIRT-1, counteracted the regulation of PUE on Mitochondrial homeostasis and the protective effect of PUE on HUVECs. However, intervention with the SIRT-1 activator SRT1720 resulted in the restoration of the protective effect of PUE to HUVECs. Accordingly, we conclude that PUE protected HUVECs from inflammation through SIRT-1. These results indicate that PUE, as a natural antioxidant, regulated Mitochondrial homeostasis through the SIRT-1 signaling pathway and reduced the vulnerability of HUVECs to the inflammatory state.

Damage to endothelial cells due to changes in membrane structure leads to the production of anti-arterial antibodies and the activation of the complement system, which aggravates vascular endothelial damage and promotes the development of AS.[37] Cytokinins, inflammatory factors, and mitochondrial dysfunction in the environment can all regulate the activity and function of endothelial cells by changing the extracellular concentration of oxidative stress products.[38,39] A large number of aging vascular endothelial cells are present in advanced arterial plaques. In the process of cell aging, mitochondria dysfunction intensifies and the level of intracellular ROS significantly increases. The abnormally elevated ROS in the cell further induces vascular damage during cell aging and aggravates the lesions.[40]

In the current study, we found that markers related to oxidative stress were significantly upregulated in HUVECs treated with LPS, while the expression of autophagy-related genes and antioxidant stress kinases were significantly downregulated. This indicated that inflammation was able to increase oxidative stress injury and inhibit autophagy. Under oxidative stress injury, mitochondrial folding of proteins and useless organelles could not be cleared in a timely manner due to autophagy being inhibited and the normal level of energy metabolism and respiratory chain function of the mitochondria may be disrupted, resulting in a rapid decrease of mitochondrial activity and Mitochondrial homeostasis disorder. Mitochondrial homeostasis is an important mechanism for eukaryotes to maintain a relatively stable number and function of mitochondria.[41] Mitochondrial homeostasis ensures the normal operation of the mitochondrial network, further regulates the timely updating of mitochondria, and maintains the relative stability of the quantity and quality of mitochondria in endothelial cells or it may participate in the occurrence and development of myocarditis injury.[42]

The distribution of mitochondria in endothelial cells can also affect cell signal transduction. Under physiological conditions, the mitochondrial dynamics of endothelial cells are in a state of stable dynamic equilibrium.[43,44] Inflammation-induced perinuclear aggregation of mitochondria can lead to the accumulation of ROS and affect the transcription level of the vascular endothelial growth factor (VEGF) gene.[42] Main functions of mitochondria in endothelial cells are to transmit cell response to environmental signals and to participate in the protective mechanism of endothelial cells under oxidative stress and inflammation.[45–47]

Many natural products of plants can protect endothelial cells by regulating mitochondrial function and reducing the vulnerability of endothelial cells under stress.[48,49] Our study directly confirmed the protective mechanism of natural antioxidants on endothelial cells through Mitochondrial homeostasis. We also found that PUE could regulate the autophagy flux of endothelial cells and maintain normal cell activity. Mitophagy not only controls the homeostasis of blood vessels, but also functions in mitochondria energy metabolism and the mitochondrial antioxidant system to maintain the basic physiological functions of mitochondria.[50] Further research is needed to determine the mechanistic interaction between autophagy and oxidative stress in the inflammatory state. In addition, the mechanism by which PUE affects mitochondrial quality by regulating mitophagy levels remains unclear.

SIRT-1 is a highly conserved $NAD^+$ -dependent histone deacetylase. In recent years, SIRT-1 has been shown to play important roles in many biological processes, including cell differentiation, aging, apoptosis, physiological rhythm, metabolic regulation, transcription regulation, signal transduction, and oxidative stress.[51,52] SIRT-1 exists in the nucleus and is highly expressed in vascular endothelial cells. Moreover, it has been found that the level of serum SIRT-1 in patients with cardiovascular disease is significantly higher than that in healthy individuals.[53,54] EX527 is an effective selective inhibitor of SIRT-1 and is often used to block the regulation of SIRT-1. We found in the current study that EX527 could counteract the protective effect of PUE in HUVECs. However, with intervention using SRT1720, the protective effect of PUE on HUVECs was restored, as well as its regulatory effect on Mitochondrial homeostasis. These results indirectly confirmed that PUE regulated mitophagy in HUVECs through SIRT-1 and may further protect HUVECs from inflammatory responses and oxidative stress injury. However, animal studies will be needed to confirm whether PUE directly effects blood flow by regulating Mitochondrial homeostasis and endothelial cells through SIRT-1.

In conclusion, we found that cell viability and Mitochondrial homeostasis of HUVECs were regulated by PUE through the SIRT-1 signaling pathway. LPS treatment reduced SIRT-1 signaling and induced oxidative stress injury and apoptosis. PUE was able to regulate Mitochondrial homeostasis by upregulating SIRT-1 signaling, improving the level of autophagy in HUVECs, and further reducing the vulnerability and oxidative stress injury of HUVECs under inflammatory conditions. As a natural antioxidant, PUE may be developed in the future as a candidate drug for the clinical treatment of AS. We expected PUE to become a treatment strategy that potentially is widely used in the treatment of a variety of cardiovascular diseases.

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## Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

## CRediT authorship contribution statement

Xing Chang: conceived, directed, and supervised the study and critically revised and approved the manuscript. Meng Cheng and Ying Li: performed the experiment, analyzed the data, and wrote the manuscript. Xing Chang and XiuTeng Zhou: participated in the experiments and analyzed the data. All authors read and approved the final manuscript.

## Declaration of competing interest

The authors have no conflicts of interest to declare.

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## References


1. Kobiyama K, Ley K. Atherosclerosis. *Circ Res*. 2018;123(10):1118–1120.
2. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352(16):1685–1695.
3. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420(6917):868–874.
4. Zhu H, Li Y, Wang MX, et al. Analysis of cardiovascular disease-related NF-kappaB-regulated genes and microRNAs in TNFalpha-treated primary mouse vascular endothelial cells. *J Zhejiang Univ - Sci B*. 2019;20(10):803–815.
5. Haybar H, Shahrabi S, Rezaeeyan H, et al. Endothelial cells: from dysfunction mechanism to pharmacological effect in cardiovascular disease. *Cardiovasc Toxicol*. 2019;19(1):13–22.
6. Sturtzel C. Endothelial cells. *Adv Exp Med Biol*. 2017;1003:71–91.
7. Falkenberg KD, Rohlenova K, Luo Y, et al. The metabolic engine of endothelial cells. *Nat Metab*. 2019;1(10):937–946.
8. Sabbatinelli J, Prattichizzo F, Olivieri F, et al. Where metabolism meets senescence: focus on endothelial cells. *Front Physiol*. 2019;10:1523.
9. Marchio P, Guerra-Ojeda S, Vila JM, Aldasoro M, Victor VM, Mauricio MD. Targeting early atherosclerosis: a focus on oxidative stress and inflammation. *Oxid Med Cell Longev*. 2019;2019:8563845.
10. Yuan T, Yang T, Chen H, et al. New insights into oxidative stress and inflammation during diabetes mellitus-accelerated atherosclerosis. *Redox Biol*. 2019;20:247–260.
11. Lahera V, Goicoechea M, de Vinuesa SG, et al. Endothelial dysfunction, oxidative stress and inflammation in atherosclerosis: beneficial effects of statins. *Curr Med Chem*. 2007;14(2):243–248.
12. van der Bliek AM, Sedensky MM, Morgan PG. Cell biology of the mitochondrion. *Genetics*. 2017;207(3):843–871.
13. Voos W, Jaworek W, Wilkening A, et al. Protein quality control at the mitochondrion. *Essays Biochem*. 2016;60(2):213–225.
14. Dorighello GG, Paim BA, Kiihl SF, et al. Correlation between mitochondrial reactive oxygen and severity of atherosclerosis. *Oxid Med Cell Longev*. 2016;2016:7843685.
15. Smith PM, Ferguson AV. Recent advances in central cardiovascular control: sex, ROS, gas and inflammation. *F1000Res*. 2016;5(F1000 Faculty Rev):420.
16. Manfredi AA, Rovere-Querini P. The mitochondrion-a Trojan horse that kicks off inflammation? *N Engl J Med*. 2010;362(22):2132–2134.
17. Chen J, Chen ZJ. PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. *Nature*. 2018;564(7734):71–76.
18. Zhong Z, Liang S, Sanchez-Lopez E, et al. New mitochondrial DNA synthesis enables NLRP3 inflammasome activation. *Nature*. 2018;560(7717):198–203.
19. Zhang L. Pharmacokinetics and drug delivery systems for puerarin, a bioactive flavone from traditional Chinese medicine. *Drug Deliv*. 2019;26(1):860–869.
20. Zhou YX, Zhang H, Peng C. Puerarin: a review of pharmacological effects. *Phytother Res*. 2014;28(7):961–975.
21. Ji L, Du Q, Li Y, et al. Puerarin inhibits the inflammatory response in atherosclerosis via modulation of the NF-kappaB pathway in a rabbit model. *Pharmacol Rep*. 2016;68(5):1054–1059.
22. Yuan Y, Zhou H, Wu QQ, et al. Puerarin attenuates the inflammatory response and apoptosis in LPS-stimulated cardiomyocytes. *Exp Ther Med*. 2016;11(2):415–420.
23. Deng HF, Wang S, Li L, et al. Puerarin prevents vascular endothelial injury through suppression of NF-kappaB activation in LPS-challenged human umbilical vein endothelial cells. *Biomed Pharmacother*. 2018;104:261–267.
24. Li C, Tan Y, Wu J, et al. Resveratrol improves bnip3-related mitophagy and attenuates high-fat-induced endothelial dysfunction. *Front Cell Dev Biol*. 2020;8:796.
25. Tian Y, Song H, Qin W, et al. Mammalian STE20-like kinase 2 promotes lipopolysaccharides-mediated cardiomyocyte inflammation and apoptosis by enhancing mitochondrial fission. *Front Physiol*. 2020;11:897.
26. Cao S, Zhang Q, Wang C, et al. LPS challenge increased intestinal permeability, disrupted mitochondrial function and triggered mitophagy of piglets. *Innate Immun*. 2018;24(4):221–230.
27. Lv Z, Song X, Xu J, et al. The modulation of Smac/DIABLO on mitochondrial apoptosis induced by LPS in Crassostrea gigas. *Fish Shellfish Immunol*. 2019;84:587–598.
28. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev*. 2014;94(3):909–950.
29. Kim YM, Kim SJ, Tatsunami R, et al. ROS-induced ROS release orchestrated by Nox4, Nox2, and mitochondria in VEGF signaling and angiogenesis. *Am J Physiol Cell Physiol*. 2017;312(6):C749–C764.
30. Zandalinas SI, Mittler R. ROS-induced ROS release in plant and animal cells. *Free Radic Biol Med*. 2018;122:21–27.
31. Melser S, Lavie J, Benard G. Mitochondrial degradation and energy metabolism. *Biochim Biophys Acta*. 2015;1853(10 Pt B):2812–2821.
32. Dolinsky VW, Cole LK, Sparagna GC, et al. Cardiac mitochondrial energy metabolism in heart failure: role of cardiolipin and sirtuins. *Biochim Biophys Acta*. 2016;1861(10):1544–1554.
33. Karwi QG, Jorg AR, Lopaschuk GD. Allosteric, transcriptional and post-translational control of mitochondrial energy metabolism. *Biochem J*. 2019;476(12):1695–1712.
34. Wang W, Li Y, Zhang Y, et al. SIRT1 mediates the inhibitory effect of Dapagliflozin on EndMT by inhibiting the acetylation of endothelium Notch1. *Cardiovasc Diabetol*. 2023;22(1):331.
35. Zhou Y, Tai S, Zhang N, et al. Dapagliflozin prevents oxidative stress-induced endothelial dysfunction via sirtuin 1 activation. *Biomed Pharmacother*. 2023;165:115213.
36. Yan Q, Zheng R, Li Y, et al. PM(2.5)-induced premature senescence in HUVECs through the SIRT1/PGC-1alpha/SIRT3 pathway. *Sci. Total Environ*. 2024;921:171177.
37. Wang T, Sun C, Hu L, et al. Sirt6 stabilizes atherosclerosis plaques by promoting macrophage autophagy and reducing contact with endothelial cells. *Biochem Cell Biol*. 2020;98(2):120–129.
38. Song CL, Wang JP, Xue X, et al. Effect of circular ANRIL on the inflammatory response of vascular endothelial cells in a rat model of coronary atherosclerosis. *Cell Physiol Biochem*. 2017;42(3):1202–1212.
39. Wang L, Qiu XM, Hao Q, et al. Anti-inflammatory effects of a Chinese herbal medicine in atherosclerosis via estrogen receptor beta mediating nitric oxide production and NF-kappaB suppression in endothelial cells. *Cell Death Dis*. 2013;4:e551.
40. Childs BG, Baker DJ, Wijshake T, et al. Senescent intimal foam cells are deleterious at all stages of atherosclerosis. *Science*. 2016;354(6311):472–477.
41. Wang J, Toan S, Zhou H. New insights into the role of mitochondria in cardiac microvascular ischemia/reperfusion injury. *Angiogenesis*. 2020;23(3):299–314.
42. Zhou H, Toan S. Pathological roles of mitochondrial oxidative stress and mitochondrial dynamics in cardiac microvascular ischemia/reperfusion injury. *Biomolecules*. 2020;10(1):85.
43. Wen Q, Fan TJ, Tian CL. Cytotoxicity of atropine to human corneal endothelial cells by inducing mitochondrion-dependent apoptosis. *Exp Biol Med*. 2016;241(13):1457–1465.
44. Zhao W, Feng H, Sun W, et al. Tert-butyl hydroperoxide (t-BHP) induced apoptosis and necroptosis in endothelial cells: roles of NOX4 and mitochondrion. *Redox Biol*. 2017;11:524–534.
45. Davidson SM. Endothelial mitochondria and heart disease. *Cardiovasc Res*. 2010;88(1):58–66.
46. Tang X, Luo YX, Chen HZ, et al. Mitochondria, endothelial cell function, and vascular diseases. *Front Physiol*. 2014;5:175.
47. Puhm F, Afonyushkin T, Resch U, et al. Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce type I IFN and TNF responses in endothelial cells. *Circ Res*. 2019;125(1):43–52.
48. Chang X, Zhang T, Zhang W, et al. Natural drugs as a treatment strategy for cardiovascular disease through the regulation of oxidative stress. *Oxid Med Cell Longev*. 2020;2020:5430407.
49. Song N, Jia L, Cao H, et al. Gypenoside inhibits endothelial cell apoptosis in atherosclerosis by modulating mitochondria through PI3K/Akt/Bad pathway. *BioMed Res Int*. 2020;2020:2819658.
50. Hughes WE, Beyer AM, Gutterman DD. Vascular autophagy in health and disease. *Basic Res Cardiol*. 2020;115(4):41.
51. Savran M, Asci H, Ozmen O, et al. Melatonin protects the heart and endothelium against high fructose corn syrup consumption-induced cardiovascular toxicity via SIRT-1 signaling. *Hum Exp Toxicol*. 2019;38(10):1212–1223.
52. Yamac AH, Huyut MA, Yilmaz E, et al. MicroRNA 199a is downregulated in patients after coronary artery bypass graft surgery and is associated with increased levels of sirtuin 1 (SIRT 1) protein and major adverse cardiovascular events at 3-year follow-up. *Med Sci Mon Int Med J Exp Clin Res*. 2018;24:6245–6254.
53. Sosnowska B, Mazidi M, Penson P, et al. The sirtuin family members SIRT1, SIRT3 and SIRT6: their role in vascular biology and atherogenesis. *Atherosclerosis*. 2017;265:275–282.
54. Kilic U, Gok O, Bacaksiz A, et al. SIRT1 gene polymorphisms affect the protein expression in cardiovascular diseases. *PLoS One*. 2014;9(2):e90428.