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# Inhibitory effect of puerarin on vascular smooth muscle cells proliferation induced by oxidised low-density lipoprotein via suppressing ERK 1/2 phosphorylation and PCNA expression

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Puerarin, an isoflavonoid isolated from the traditional Chinese herbal medicine *Pueraria lobata* (Wild.) Ohwi, has been shown to process antioxidant, anti-inflammatory, anti-cancer, anti-hypercholesterolemic, and anti-hyperglycemic activities *in vivo* and *in vitro*. The aim of the present study was to investigate the antiproliferative effects and the possible mechanisms of puerarin in vascular smooth muscle cells (VSMCs) stimulated with oxidised low-density lipoprotein (ox-LDL). VSMCs were cultured and pretreated with different concentrations of puerarin (0, 1, 10, 50 $\mu$M) before stimulated by ox-LDL (50 $\mu$g/mL). Cell proliferation was evaluated by MTT assay. Flow cytometry was used to study the influence of puerarin on cell cycle. Proliferating cell nuclear antigen (PCNA) expression and phosphorylation levels of extracellular signal-regulated kinase (ERK) 1/2 were detected by western blotting analysis. The results indicated that puerarin significantly inhibited VSMCs proliferation induced by ox-LDL and phosphorylation of ERK 1/2. Furthermore, puerarin also blocked the ox-LDL-induced cell-cycle progression at G1/S-interphase and down-regulated the expression of PCNA of VSMCs. The results suggest puerarin inhibits ox-LDL-induced proliferation of VSMCs by suppressing ERK 1/2 phosphorylation and PCNA expression.

## 1. Introduction

Atherosclerosis is a chronic and progressive vascular inflammatory disease, characterized with the process of lumen narrowing and rigid, as a result of cholesterol and lipid accumulation (Dell'omo et al. 2006; Liu et al. 2014b; Zhu et al. 2014a). It is widely accepted that abnormal proliferation of vascular smooth muscle cells (VSMCs) located in the arterial intima leads to intimal thickening of the aorta, plays a crucial role in initiation and progression of atherosclerosis (Daemen et al. 1991; Ross 1993). Consequently, inhibition of VSMCs proliferation represents a potentially important therapeutic strategy for the treatment of atherosclerosis.

*Pueraria lobata* (Wild.) Ohwi, one of the most popular Chinese traditional medicines, has been widely used to treat hypertension, diabetes and angina pectoris in China (Zhu et al. 2014b; Fu et al. 2015). Puerarin (4'-7-dihydroxy-8-β-D-glucosylisoflavone, $C_{21}H_{20}O_{10}$, Fig. 1), an isoflavonoid isolated from the root of *Pueraria lobata*, is considered as one of the major pharmacological active constituents (Bao et al. 2014) and has been reported to possess various pharmacological activities, including antioxidant (Meng et al. 2009), anti-inflammatory (Yang et al. 2010), anti-cancer (Hien et al. 2010), anti-hypercholesterolemic (Yan et al. 2006), and anti-hyperglycemic activities (Meng et al. 2009), etc. In addition, puerarin has been shown to have beneficial effects on cardiovascular diseases such as atherosclerosis (Bao et al. 2014, 2015; Fu et al. 2014). However, it is still unclear whether puerarin prevents oxidized low-density lipoprotein (ox-LDL)-induced proliferation of VSMCs. Since VSMCs proliferation induced by ox-LDL in arterial wall play a critical role in the progress of atherosclerosis (Chang et al. 2008), we therefore studied the potential protective effects of puerarin on ox-LDL-induced proliferation of VSMCs and to further illuminate the underlying mechanisms.

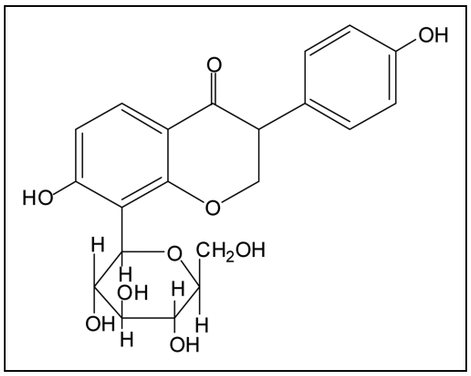


Fig. 1: Chemical structure of puerarin.

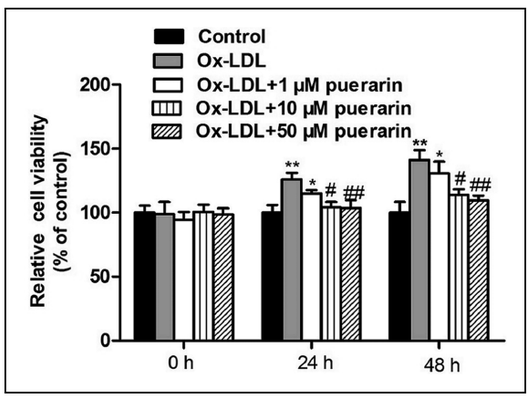


Fig. 2: Effect of puerarin on ox-LDL-induced proliferation of VSMCs. VSMCs were cultured with puerarin at different doses (0, 1, 10, 50 μM) 24 h before ox-LDL stimulation. Cell proliferation was assessed at 24 h or 48 h after ox-LDL stimulation by the MTT method. Puerarin pretreatment significantly increased the cell viability. Data are expressed as mean ± SEM. Compared with control, $^{*}P<0.05$, $^{**}P<0.01$; compared with ox-LDL, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

## 2. Investigations and results

### *2.1. Effect of puerarin on ox-LDL-induced proliferation of VSMCs*

To evaluate the effect of puerarin on ox-LDL-induced cell proliferation, MTT cell proliferation assay was performed. As shown in Fig. 2, after incubation with ox-LDL for 24 h or 48 h, a significant increase in cell viability was observed as compared to the controls. However, puerarin was able to dose-dependently inhibit the effect of ox-LDL, with higher doses having a greater effect. These findings suggested that puerarin had available antiproliferative effects in VSMCs stimulated with ox-LDL.

### *2.2. Effect of puerarin on ox-LDL-induced cell cycle progression and PCNA expression of VSMCs*

To investigate the mechanism underlying the inhibitory effect of puerarin on ox-LDL-induced cell proliferation, the effect of puerarin on cell cycle progression was determined by flow cytometric analysis of cellular DNA content. As shown in Fig. 3, after incubation with ox-LDL for 24 h, the percentages of cells in S and and G2/M phase were markedly increased, and the percentages in G0/G1 phase were correspondingly reduced. However, pretreatment with puerarin significantly reversed these effects in a concentration-dependent manner, with higher doses having a greater effect. To demonstrate the mechanism underlying the effect of puerarin on the cell cycle, we next analyzed the expression of proliferating cell nuclear antigen (PCNA) using western blotting. As shown in Fig. 4, the protein expression patterns were consistent with the cell cycle analysis data. Ox-LDL arrested the cell cycle at S phase; this effect was accompanied by increasing the expression of PCNA. However, puerarin was able to dose-dependently reverse this effect. These findings suggested that puerarin inhibits ox-LDL induced VSMCs proliferation via blocking cell cycle progression.

### *2.3. Effect of puerarin on ox-LDL-induced phosphorylation of ERK 1/2 of VSMCs*

To elucidate whether puerarin inhibited ox-LDL-induced cells proliferation by inhibiting the activation of extracellular signal-

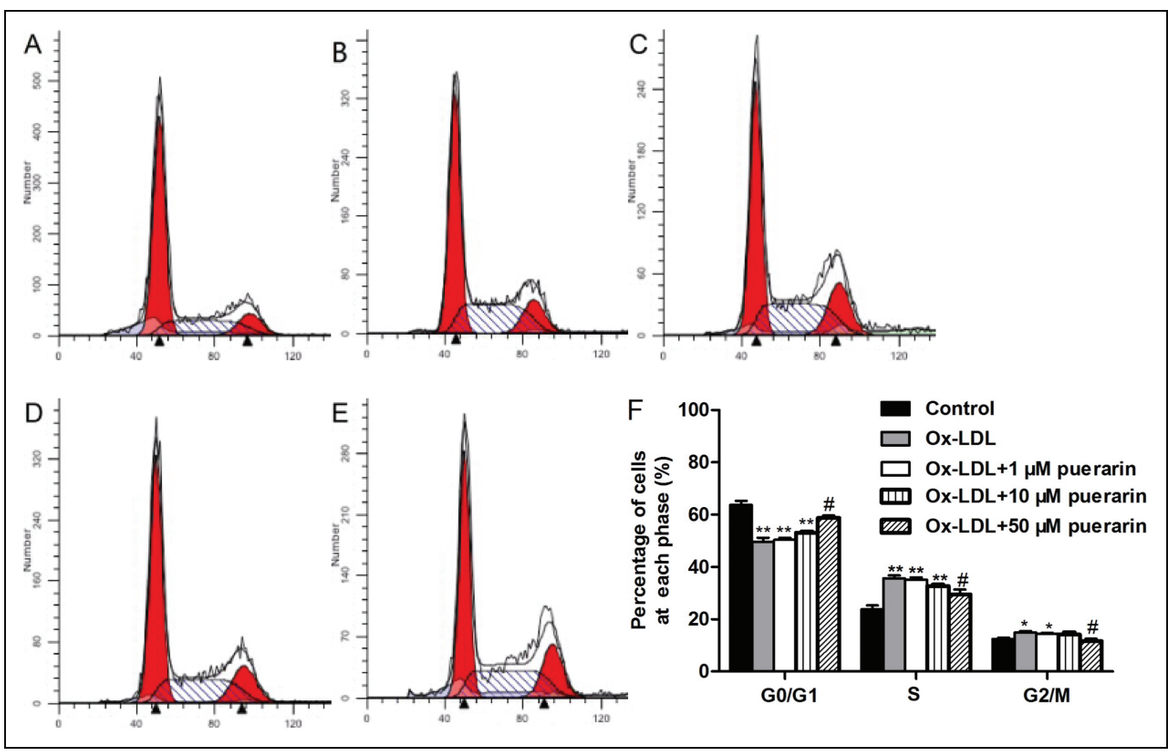


Fig. 3: Effect of puerarin on VSMCs cell cycle induced by ox-LDL. VSMCs were cultured with puerarin at different doses (0, 1, 10, 50 μM) 24 h before ox-LDL stimulation. Then cell cycle was determined at 24 h after ox-LDL stimulation by individual nuclear DNA content reflected by fluorescence intensity of incorporated propidium iodide (PI). Percentage of cells in G0/G1, S and G2/M phases were calculated using computer software. (A) Representative flow cytometric analysis of VSMCs without any treatment (control). (B) Representative flow cytometric analysis of VSMCs induced by ox-LDL. (C-E) Representative flow cytometric analysis of VSMCs induced by ox-LDL following pretreatment with puerarin (1 10, 50 μM). (F) The percentage of cell cycle distribution after the indicated treatment. Data are expressed as mean ± SEM. Compared with control, $^{*}P<0.05$, $^{***}P<0.001$; compared with ox-LDL, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

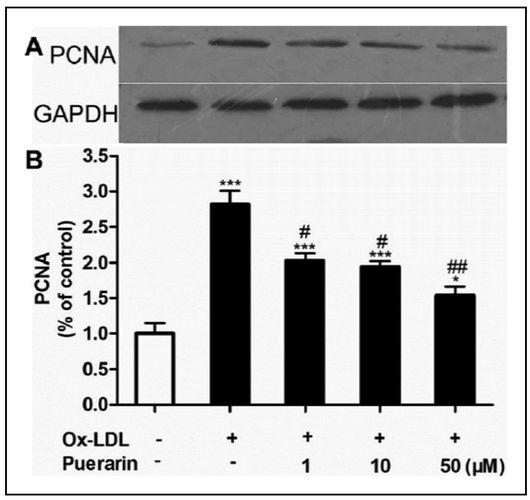


Fig. 4: Effect of puerarin on ox-LDL-induced PCNA expression of VSMCs. VSMCs were cultured with puerarin at different doses (0, 1, 10, 50 μM) for 24 h, and stimulated with ox-LDL (50 $\mu$g/mL) for 24 h consequently, Then proteins were extracted from the cells and d by western blotting. GAPDH was served as the loading control. (A) Western blotting assays for PCNA expression. (B) PCNA expression as a ratio to the control. Data are expressed as mean ± SEM. Compared with control, $^{**}P<0.01$; compared with ox-LDL, $^{\#\#}P<0.01$.

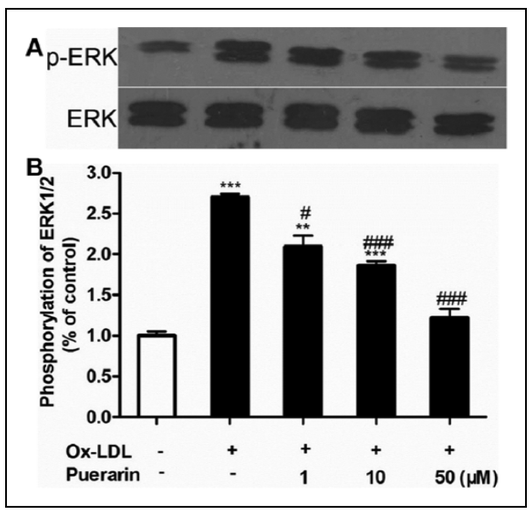


Fig. 5: Effect of puerarin on ox-LDL-induced ERK 1/2 phosphorylation of VSMCs. VSMCs were cultured with puerarin at different doses (0, 1, 10, 50 μM) for 24 h, and stimulated with ox-LDL (50 $\mu$g/mL) for 24 h consequently, Then proteins were extracted from the cells and determined by western blotting analysis. The phospho-antibody to ERK 1/2 was served as loading controls. (A) Western blotting analysis for ERK 1/2, p-ERK 1/2 expression. (B) Protein expression ratio of p-ERK 1/2/ERK 1/2. Data are expressed as mean ± SEM. Compared with control, $^{**}P<0.01$, $^{***}P<0.001$; compared with ox-LDL, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$.

regulated kinase (ERK) 1/2 signaling pathway, we detected the phosphorylation levels of ERK 1/2 by western blotting. As shown in Fig. 5, ox-LDL and puerarin had no effect on the total level of ERK 1/2, but ERK1/2 phosphorylation was significantly upregulated after VSMCs was incubated with ox-LDL (50 $\mu$g/mL) for 24 h as compared to the controls. Puerarin was able to dose-dependently reduce ERK1/2 phosphorylation in ox-LDL-induced VSMCs. These findings suggested that down-regulation of phosphorylated ERK 1/2 was also involved in the inhibitory effect of puerarin on ox-LDL induced cell proliferation.

## 3. Discussion

Ox-LDL, one of the most important risk factors for atherosclerosis, is involved in the generation of atherosclerotic lesions (Liu et al. 2014a). Recent studies have indicated that ox-LDL is a mitogen for VSMCs (Boehm and Nabel 2001), which stimulates the proliferation of VSMCs and the activation of the ERK 1/2 signaling pathway (Guo et al. 2012). Therefore, the ox-LDL-induced proliferation of VSMCs in the intima of the arterial wall is considered to be an important factor in atherosclerotic plaque development (Chang et al. 2008). In the present study, we found that ox-LDL induced VSMCs proliferation, and provided the first evidence that puerarin significantly inhibited ox-LDL-induced proliferation of VSMCs. In our study, the antiproliferative effect of puerarin was associated with an accumulation of cells in G0/G1 phase of the cycle as revealed by flow cytometry. Treatment with ox-LDL markly decreased the percent of VSMCs at G0/G1 phase and correspondingly increased their percentages at the S phase (DNA synthesis phase) and G2/M phase (mitosis). Puerarin significantly reversed ox-LDL mediated effects in VSMCs. The results suggested a dose-dependent accumulation of puerarin-treated cells at the G1/S-interphase.

How does puerarin inhibit VSMCs proliferation? To use puerarin for clinical development effectively, it is indispensable to understand its mechanism. We focused on the protein levels of PCNA and the ERK1/2 signaling pathway in VSMCs. PCNA is an acidic nuclear protein that has been implicated in a number of essential cellular processes, including DNA replication, DNA repair, and cell-cycle regulation (Simons et al. 1994; He et al. 2001), which is regulated by a combination of mechanisms that act at both transcriptional and post-transcriptional levels (Li et al. 2003). It has also been reported that PCNA synthesis is strictly regulated during the cell cycle, and its protein is necessary for the transition of cells from G0/G1 phase to S phase (Chang et al. 2008). It is evident that PCNA represents a key protein necessary for the transition of cells from G0/G1 phase to S phase. In the present study, it was found that the percentage of VSMCs in S and and G2/M phase increased after treatment with ox-LDL but decreased after pretreatment with puerarin (Fig. 3). The amount of PCNA protein was consistent with the cell-cycle analysis data (Fig. 4). It is therefore likely that puerarin inhibited the ox-LDL-induced proliferation of VSMCs through suppression of the expression of PCNA.

ERK1/2 signaling pathway is a mitogen-activated protein kinase (MAPK) pathway that was considered as a central regulator of growth and proliferation in many cell types (Roskoski 2012; Funcke et al. 2015; Kim et al. 2015), and ox-LDL could induce VSMCs proliferation through activation of the ERK1/2 signaling pathway (Yang et al. 2001; Zhang et al. 2013; Farrokhi et al. 2015). We confirmed the effect of ox-LDL on ERK1/2 signaling pathway by activating phosphorylation of ERK 1/2. In an additional western blot analysis, we found that the phosphorylation of ERK 1/2 was reduced when puerarin was added to ox-LDL-induced VSMCs (Fig. 5). The results indicate that puerarin significantly inhibited ox-LDL-induced proliferation of VSMCs perhaps by inhibiting the activation of the ERK1/2 signaling pathway.

Taken together, our study demonstrated that puerarin is able to inhibit VSMCs proliferation induced by ox-LDL for the first time. The antiproliferative effects at least partly are involved in inactivating the ERK 1/2 signaling pathway and blocking G0/G1 phase to S phase transition, which is mediated via by suppressing the expression of PCNA. These results may provide additional evidence that puerarin can reduce the development of atherosclerosis. However, to investigate the biological activity of puerarin, additional studies such as the effects of puerarin

on vascular endothelial cells or foam cells are needed in further work.

## 4. Experimental

### *4.1. Materials*

Human aortic vascular smooth muscle cells (HA-VSMCs) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Puerarin (4.16 mg; Formula weight: 416.38 g/mol; purity (GC): $\geq$ 98%, Vic's biological technology Co., Ltd, Sichuan, China) was dissolved in 50 $\mu$L DMSO and diluted in 20 mL medium. The concentrations ultimately chosen for the following experiments were 1, 10, and 50 $\mu$M. The concentration of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in the subsequent experiments was lower than 0.1 % and had no effect on cell viability. After pretreatment with different concentrations of puerarin for 24 h, the cells were incubated with or without ox-LDL (50 $\mu$g/mL, Yiyuan Biotechnologies Co., Ltd, Guangzhou, China) for another 24 h. Cell viability and proliferation assays were conducted using methyl thiazolyl tetrazolium (MTT) and the phases of cell cycle were analyzed using flow cytometry. In addition, western blotting analysis was used to detect the expression of proliferating cell nuclear antigen (PCNA) and the phosphorylation levels of extracellular signal-regulated kinase (ERK) 1/2 involved in the impact of puerarin on ox-LDL-stimulated VSMCs. The specific procedures and materials for each assay are described below.

Cell culture: VSMCs were cultured in 100 mm dishes in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Company USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sijiqing Bioengineering Material Co., Ltd. Hangzhou, China) and 1% penicilin/streptomycin (Sigma-Aldrich) in a humidified atmosphere at 37 °C with 5 % $CO_2$.

### *4.2. Cell viability assay*

VSMCs in the logarithmic growth phase were seeded into 96-well plates. Each well contained $1 \times 10^4$ cells in suspension. After being initially cultured in DMEM supplemented with 10 % heat-inactivated FBS for 12 h, the cells were placed in serum-free medium for another 24 h to facilitate cell cycle synchronization. After pretreatment with the different concentrations of puerarin described above for 24 h, the cells were stimulated with or without ox-LDL (50 $\mu$g/mL) for 24 h or 48 h. MTT solution (5 mg/ml) was added to each well. Following incubation at 37 °C for 4 h, the liquid in the wells was removed gently and 150 $\mu$L of DMSO was added to each well for 15 min incubation. The absorbance of each sample was measured using a microplate reader (Thermo Scientific, Rockford, IL, USA) at a detection wave length of 490 nm. The initial absorbance before puerarin treatment was also measured.

### *4.3. Cell cycle analysis*

VSMCs in the logarithmic growth phase were seeded into 6-well plates. Each well contained $1 \times 10^4$ cells in suspension. After being initially cultured in DMEM supplemented with 10 % heat-inactivated FBS for 12 h, the cells were placed in serum-free medium for another 24 h to facilitate cell cycle synchronization. After pretreatment with the different concentrations of puerarin described above for 24 h, the cells were stimulated with or without ox-LDL (50 $\mu$g/mL) for 24 h. Then the cells were trypsinized, collected, and washed twice with ice-cold PBS before fixing in 70% cold ethanol at 4 °C overnight. Next, the fixed cells were resuspended in phopshate-buffered saline (PBS) containing 100 $\mu$g/mL RNase A (Sigma-Aldrich) and 50 $\mu$g/mL propidium iodide (PI; Sigma-Aldrich) for 30 min at room temperature. Cells were then analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). The rates of G0/G1, S and G2/M phases were determined using the computer program (ModFit LT V3.3.11).

### *4.4. Western blotting analysis*

VSMCs in the logarithmic growth phase were seeded into 6-well plates. Each well contained $1 \times 10^4$ cells in suspension. After being initially cultured in DMEM supplemented with 10 % heat-inactivated FBS for 12 h, the cells were placed in serum-free medium for another 24 h to facilitate cell cycle synchronization. After pretreatment with the different concentrations of puerarin described above for 24 h, the cells were stimulated with or without ox-LDL (50 $\mu$g/mL) for 24 h. Then the cells were scraped in ice cold PBS and lysed in cold lysis buffer (Beyotime, Jiangsu, China). After centrifugation at 13 000 g, the supernatant (total protein extract) was separated and quantified by the BCA protein assay (Beyotime Biotech, Shanghai, China). Equal amounts of protein samples were electrophoresed on 10 % SDS-PAGE gels at 120 V for 1.5 h and then electrotransfered onto PVDF membranes at 100 V for 1 h. The membranes were blocked with 5% non-fat dry milk in TBST (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20) for 1 h at room temperature, then the membranes were incubated with specific primary antibodies (anti-ERK 1/2, anti-p-ERK 1/2 anti-PCNA, diluted 1:1000 in 5% non-fat dry milk/TBST, incubated for 24 h at 4 °C; Abcam), followed by incubation with secondary antibody coupled to horseradish peroxidase for 1 h at room temperature and detection with ECL/X-ray films (Amersham). GAPDH (Sigma) was used for the protein loading control.

### *4.5. Statistical analysis*

The statistics were performed by the SPSS 19.0 program (Chicago, IL, USA). Each independent experiment had been repeated at least three times, and the data were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test for multiple comparisons. Results are represented as mean $\pm$ SEM, and the criterion for statistical was $P < 0.05$.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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