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**Original Research Article**

# Puerarin modulates apoptosis of MC3T3-E1 and ATDC5 cells by attenuating the expression levels of endoplasmic reticulum stress markers

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## Abstract

***Purpose:*** *To investigate the protective effect of puerarin against apoptosis of osteoblasts and chondrocytes.*
***Methods:*** *Osteoblast-like MC3T3-E1 cells and chondrocyte ATDC5 were used as cellular models. Dexamethasone (Dex) was used to induce cellular stress. Cell viability was determined with cell counting kit-8 (CCK-8) assay. Cell apoptosis and the level of reactive oxygen species (ROS) were also measured. Changes in ERS markers were measured with quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting.*
***Results:*** *Treatment with puerarin reduced cell viability and increased apoptosis, and inhibited the production of ROS. Furthermore, the expression levels of ER stress markers (ATF6, IRE1α, GRP78, XBP1, and eIF2α) were decreased after treatment with puerarin.*
***Conclusion:*** *Puerarin protected MC3T3-E1 and ATDC5 cells from apoptosis via attenuation of the expression levels of ER stress markers. Thus, puerarin may be a potential drug for treatment of ONFH.*

***Keywords:*** *Osteoblasts; Chondrocytes; Puerarin; Apoptosis; Endoplasmic reticulum; Osteonecrosis*

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## INTRODUCTION

Osteonecrosis of the femoral head (ONFH) is a common orthopaedic disease characterized by structural changes, collapse, joint dysfunction, and persistent pain in the femoral head. Total hip replacement (THR) is still the most reliable long-term therapy for patients with ONFH, although it imposes a heavy financial burden on patients' families and society [1]. In China, the use of steroids remains an important risk factor for ONFH [2]. Therefore, investigations aimed at developing a new therapy for steroid-induced ONFH are of great significance for early prevention and slowing down of the disease, and may even lead to avoidance of hip replacement. Puerarin (Figure 1 A), a flavonoid glycoside extracted from the leguminous plant Pueraria lobata, is one of the effective bioactive agents for the treatment of cardiovascular and cerebrovascular diseases. A previous study demonstrated that puerarin inhibited activation

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of amyloid β-induced NLRP3 inflammasomes in retinal pigment epithelial cells by suppressing ROS-dependent oxidation and endoplasmic reticulum stress (ERS) [3]. Moreover, puerarin suppressed the adipogenesis of marrow stroma cells (MSCs) and prevented alcohol-induced osteonecrosis [4]. However, the possible inhibitory effect of puerarin on steroid-induced ONFH has not been investigated. Endoplasmic reticulum (ER) is known to modify, fold, and transport a variety of proteins [5]. It is involved in a series of stress responses of cells in conditions of oxidative stress, ischemia, anoxia, viral infection and nutritional deficiency; it decreases stress, which may induce unfolded protein response (UPR), thereby restoring ER homeostasis, as well as protecting cells [6]. Studies have demonstrated that ERS plays a substantial role in the pathogenesis of several diseases such as tumors, neurodegenerative diseases, obesity, diabetes, and hypertension. Short-term and moderate ERS restore ER-associated protein homeostasis via a series of regulations, while long-term or severe ERS may disrupt this homeostasis, and lead to cell apoptosis. Apoptosis of a large number of osteoblasts and chondrocytes is significant for suppression of steroid-induced ONFH [7]. A previous research found that when ERS was suppressed, the symptoms of ONFH, such as osteonecrosis, bone loss, decreased vascular perfusion, and overproduction of osteoclasts, were markedly alleviated, while angiogenesis and osteogenesis were increased [8]. Therefore, the present study was designed to investigate the biological effect of puerarin on steroid-induced ONFH, and the specific mechanism involved.

## EXPERIMENTAL

### Cell culture and treatment

Mouse MC3T3-E1 osteoblasts and mouse ATDC5 chondrocytes were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The MC3T3-E1 osteoblasts were cultured in Alpha Minimum Essential Medium (α-MEM; Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 10 % fetal bovine serum (FBS; Shanghai ExCell Biology, Inc., Shanghai, China); penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mL L-glutamine. The ATDC5 cells were incubated in Dulbecco Modified Eagle Medium/Ham Nutrient Mixture F12 (DMEM/F12; Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % FBS (Shanghai ExCell Biology, Inc., Shanghai, China), penicillin (100 U/mL) and streptomycin (100 μg/mL). The two cell lines were cultured in a humidified atmosphere of 5 % $CO_2$ at 37 °C. The cells were treated with Dex (Sigma-Aldrich, St. Louis, MO, USA), puerarin, or both in order to investigate the possible protective role of puerarin. The concentration of Dex was set at 1 μM, as reported previously [12]. Puerarin was purchased from the National Pharmaceutical Engineering Centre, Jiangxi, China. Cell assays were carried out after 12 h of treatment.

### Cell viability assay

The appropriate concentration of puerarin used to treat osteoblasts and chondrocytes was determined using Cell Counting Kit-8 (CCK-8) assay. The MC3T3-E1 and ATDC5 cells were suspended in complete medium at a cell concentration of 5 × 106 cells/mL before incubation in a 96-well plate (100 μL for each well). After 24 h of incubation at 37 ℃, the cells were treated with puerarin at different concentrations (up to 120 μM). Cell viability was measured on a microplate reader, following 24 h of incubation.

### Assay of cell apoptosis and production of ROS in cells

Apoptosis was determined using Annexin V-FITC Apoptosis Detection Kit (BestBio, China). After treatment with Dex and puerarin at different concentrations, the cells were incubated for 24 h at 37 °C and suspended in 1× Annexin V binding buffer at a concentration of 1 × $10^6$ cells/mL. Then, the cells were incubated with 5 μL Annexin V-FITC/400 μL solution for 14 min. Thereafter, 10 μL propidium iodide (PI) was added, followed by incubation for 5 min at 4 °C in the dark. The percentage of apoptotic cells was analyzed in a flow cytometer and FlowJo software (Becton Dickinson, Franklin Lakes, NJ, USA).

The levels of ROS in cells were measured using dichlorodihydrofluorescein diacetate (DCFH-DA; Life Technologies Corp., Carlsbad, CA, USA). After treatment with different concentrations of Dex and puerarin, the cells were incubated for 24 h at 37 °C, followed by staining with DCFH-DA (10 μM) for 15 min at 37 °C according to the manufacturer's instructions. The levels of ROS were determined using flow cytometry.

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, 3

**Table 1:** Primers used and their sequences

| Gene name | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| ATF6 | GCCTTTATTGCTTCCAGCAG | TGAGACAGCAAAACCGTCTG |
| IRE1α | GTTTGTGTCAACGCTGGATGG | TTCCACATGTGTTGGGACCTG |
| GRP78 | CATCACGCCGTCCTATGTCG | CGTCAAAGACCGTGTTCTCG |
| XBP1 | CCGCAGCAGGTGCAGG | GAGTCAATACCGCCAGAATCCA |
| eIF2α | CTCCTGAAAGCAGCAACCTC | GACCGAGATGAAGCATCGTG |
| GAPDH | GAGTCAACGGATTTGGTCGT | TTGATTTTGGAGGGATCTCG |

mg of RNA was reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) in line with the manufacturer's instructions. Specific transcripts were detected with qRT-PCR using QuantiTect SYBR Green PCR Kit (Takara Bio Inc., Tokyo, Japan), and analyzed with ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sequences used are shown in Table 1.

### Western blot assay

Protein was extracted from cells with Total Exosome RNA & Protein Isolation Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were rinsed twice in phosphate-buffered saline (PBS), followed by addition of radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease inhibitor (Roche, Basel, Switzerland). After heating at 95 ℃ for 10 min, centrifugation was performed and the protein content of the supernatant was measured. The proteins were separated with 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked by incubation with 5 % skim milk powder solution at ambient temperature for 1 h. Then, the membrane was incubated overnight at 4 ºC with the following primary antibodies: rabbit anti-ATF6 antibody (ab203119; 1:300; Sigma-Aldrich, St. Louis, MO, USA); anti-IRE1α antibody (#3294; 1:1000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA); anti-GRP78 antibody (ab198787; 1:200; Sigma-Aldrich, St. Louis, MO, USA); anti-XBP1 (ab109221; 1:1000 dilution; Sigma-Aldrich, St. Louis, MO, USA); anti-eIF2α antibody (#5324; 1:4000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-GAPDH antibody (#5174; 1:2000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA). Next, the membranes were rinsed thrice with Tris-buffered saline (TBST, containing 0.1 % Tween 20; 5 min each time), followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (diluted 1:1000) for 1 h at 37 ℃. Thereafter, the membrane was rinsed thrice with TBST (5 min for each rinse). After rinsing, the membrane loaded with protein molecular imprints and antibodies was transferred to Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and scanned with Tanon-5200 imaging system (Shanghai, China) after introduction of 200 μL chemiluminescent solution (Millipore, Billerica, MA, USA).

### Statistical analysis

Student's t-test and one-way analysis of variance (ANOVA) were used to perform statistical analysis with GraphPad Prism 7.0 software. Each experiment was carried out in triplicate. The data are expressed as mean ± standard deviation (SD). Significance level was set at $p < 0.05$.

## RESULTS

### Effect of puerarin on viability of MC3T3-E1 and ATDC5 cells

Compared with the control group, puerarin at concentrations of ≤ 60 μM had no significant influence on the viabilities of MC3T3-E1 and ATDC5 cells (Figure 1 B and C). The viabilities of MC3T3-E1 and ATDC5 cells were significantly reduced after treatment with Dex ($p < 0.001$; Figure 1 D and E). However, treatment of MC3T3-E1 and ATDC5 cells with 15 or 30 μM puerarin before treatment with Dex resulted in suppression of Dex-induced loss of cell viability ($p < 0.05$; Figure 1 D and E). Comparison of the three different concentrations of puerarin used revealed that 15 μM puerarin was best for maintaining the viabilities of MC3T3-E1 and ATDC5 cells.

### Effect of puerarin on the apoptosis of MC3T3-E1 and ATDC5 cells

Results from flow cytometry indicated that reduction in apoptosis of MC3T3-E1 and ATDC5

cells treated with 15 or 30 μM puerarin, when compared with the control group ($p < 0.05$; Figures 2 A and B). Treatment with Dex markedly increased percentage apoptosis of MC3T3-E1 and ATDC5 cells ($p < 0.001$; Figures 2 C and 2 D). It was observed that treatment with 15 μM puerarin significantly counteracted the Dex-induced apoptosis of MC3T3-E1 and ATDC5 cells ($p < 0.01$; Figure 2 C and D). Therefore, this concentration of puerarin (15 μM) was used in subsequent experiments.

## Effect of puerarin on Dex-induced ROS production

Treatment of MC3T3-E1 and ATDC5 cells with Dex resulted in increased levels of ROS, when compared with the control group ($p < 0.001$; Figure 3 A and B). However, treatment of the cells with 15 μM puerarin reversed the Dex-induced increases in ROS generation (Figure 3 C and D, $p < 0.01$). Therefore, it can be concluded that puerarin inhibited Dex-mediated production of ROS in MC3T3-E1 and ATDC5 cells.

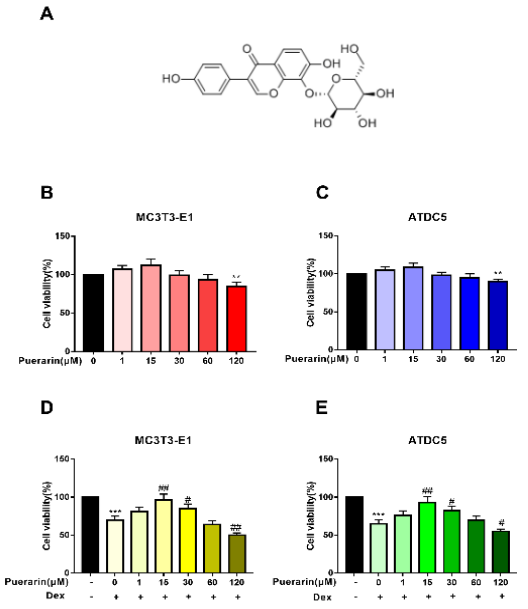


**Figure 1:** Effect of puerarin on the viabilities of MC3T3-E1 and ATDC5 cells. (A) Chemical structure of puerarin. B & C: Viabilities of MC3T3-E1 and ATDC5 cells treated with 1, 15, 30, 60, and 120 μM puerarin for 24 h, as measured using CCK-8 assay. D & E: Viabilities of MC3T3-E1 and ATDC5 cells treated with 1 μM Dex for 6 h prior to incubation with different concentrations of puerarin for 24 h. Cell viability was determined with CCK-8 assay. $^{**}P < 0.01$, $^{***}p < 0.001$, vs. control group (without any treatment); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. Dex group

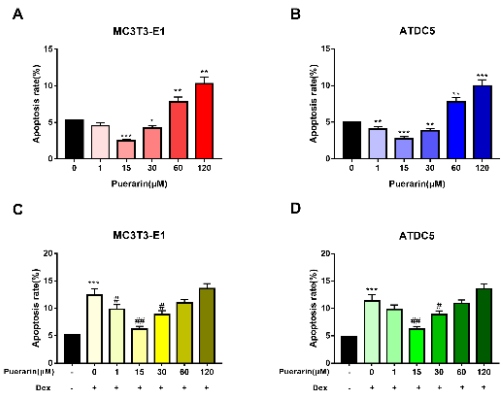


**Figure 2:** Effect of puerarin on the apoptosis of MC3T3-E1 and ATDC5 cells. A & B: Apoptosis in MC3T3-E1 and ATDC5 cells treated with different concentrations of puerarin for 24 h, as measured with flow cytometry. C & D: Apoptosis of MC3T3-E1 and ATDC5 cells treated with 1 μM Dex for 6 h prior to incubation with different concentrations of puerarin for 24 h. Cell viability was tested using CCK-8 assay. $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. control group (without any treatment); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. Dex group

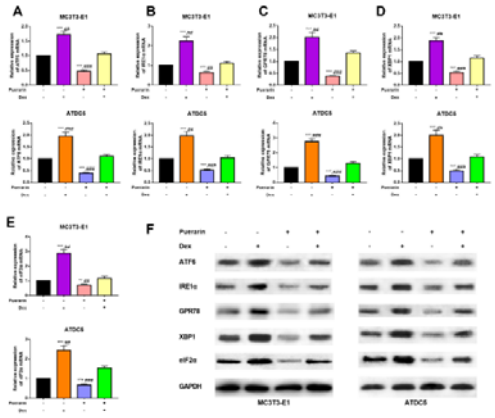


**Figure 3:** Effect of puerarin on Dex-induced ERS. A - E: mRNA expression levels of ATF6, IRE1α, GRP78, XBP1, and eIF2α in MC3T3-E1 and ATDC5 cells, as determined using RT-qPCR. $^{**}P < 0.01$, $^{***}P < 0.001$, vs control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs puerarin + Dex group. F: protein expression levels of ATF6, IRE1α, GRP78, XBP1, and eIF2α in MC3T3-E1 and ATDC5 cells, as measured using Western blotting

## Effect of puerarin on Dex-induced ERS

The expression levels of ATF6, IRE1α, GRP78, XBP1, and eIF2α in MC3T3-E1 and ATDC5 cells were significantly decreased after treatment with 15 μM puerarin, when compared with the control group ($p < 0.001$). These results are presented in Figure 4 A - F. Following Dex treatment, ERS increased, while ERS in the group treated with Dex + puerarin was significantly reduced ($p < 0.01$). These findings

indicate that puerarin inhibited ERS in MC3T3-E1 and ATDC5 cells.

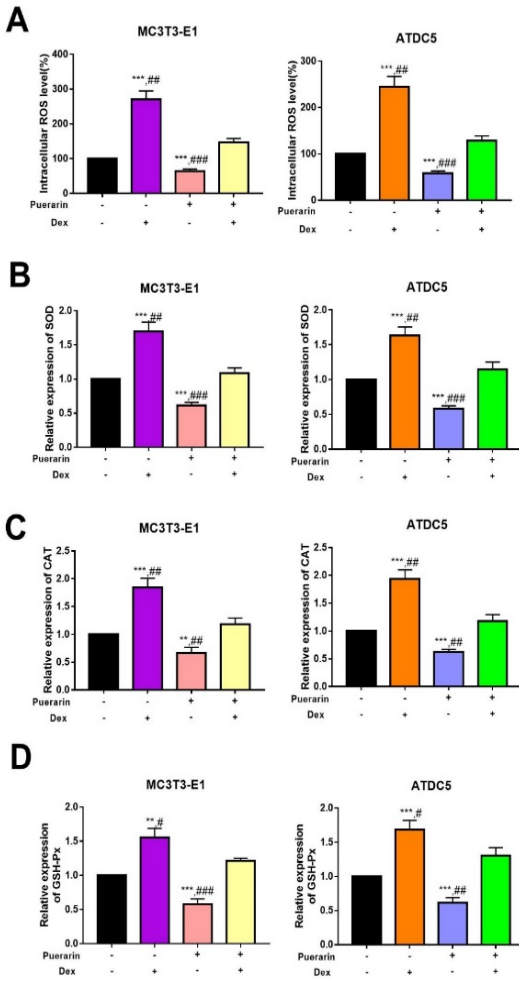


**Figure 4:** Effect of puerarin on ROS levels. A – D: levels of ROS in MC3T3-E1 and ATDC5 cells, as measured using DCFH-DA and flow cytometry. ***$P$ < 0.001, vs. control group; ## $p$ < 0.01, ### $p$ < 0.001, vs. puerarin + Dex group

## DISCUSSION

Osteonecrosis of the femoral head is a metabolic bone disease associated with a high degree of disability [9]. Osteoblasts play a significant role in bone repair, while apoptosis and degradation of chondrocytes are of great importance in ONFH [10]. The use of glucocorticoids may trigger inflammation and therefore promote ONFH by inducing apoptosis of osteocytes and chondrocytes [11]. Therefore, in the present study, an *in vitro* model of ONFH was established in MC3T3-E1 osteoblasts and ATDC5 chondrocytes using Dex, and the effect of puerarin on the steroid-induced ONFH was investigated, as well as the specific mechanism involved.

Puerarin, a major bioactive component of Chinese herb *Pueraria lobata,* has a wide range of therapeutic effects such as heart protection, nerve protection, anti-inflammation, anti-tumor, anti-oxidation, and enhancement of bone formation [12]. Puerarin has been widely used in the treatment of cardiovascular and cerebrovascular diseases, diabetes and diabetic complications; osteonecrosis, Parkinson's disease, Alzheimer's disease (AD), endometriosis, and tumors [13]. Previous studies showed that puerarin enhanced osteogenesis. In this study, it was observed that pre-treatment of cells with puerarin suppressed Dex-induced decreases in cell viability, and decreased Dex-indued increases in apoptosis.

The endoplasmic reticulum (ER) is an important organelle that participates in the synthesis, folding, and transport of at least one-third of proteins in eukaryotes. Endoplasmic reticulum stress (ERS) plays a pivotal role in several ischemic diseases such as cerebral ischemia, myocardial ischemia, and ischemia-reperfusion injury [14-16]. In ONFH, excessive ERS may induce apoptosis of human MC3T3-E1 cells and chondrocytes [17,18]. Therefore, it can be concluded that ERS is implicated in ONFH. In the *in vitro* model of ONFH, Dex-induced ERS was suppressed by puerarin, indicating that puerarin exerted a protective effect by inhibiting ERS.

Reactive oxygen species (ROS) are chemically reactive entities containing oxygen, and they include hydrogen peroxide, hydroxyl radical, and superoxide anion. They are derivatives of aerobic metabolism, and they oxidize proteins, lipids, and DNA, eventually leading to changes in cellular function. Under normal physiological conditions, the antioxidant system and ROS generation in cells are in dynamic equilibrium, thereby regulating osteoblasts differentiation [19]. The production of ROS is associated with ERS and UPR, and ERS and oxidative stress aggravate each other in a positive feed-forward loop, thereby interfering with cellular function, as well as activating pro-apoptotic signaling [20]. It has been earlier reported that ROS also play pivotal roles in the pathogenesis of ONFH [21]. A previous study demonstrated that Dex induced apoptosis of osteoblasts via the AKT/GSK3β signaling pathway [22]. In the *in vitro* model of ONFH used in the present study, puerarin significantly inhibited Dex-induced ROS generation, suggesting that it performed an antioxidant function in steroid-induced ONFH.

## CONCLUSION

The present study has demonstrated that puerarin protects osteoblasts and chondrocytes in steroid-induced ONFH by inhibiting ERS and ROS production. This finding indicates that puerarin may be used as an adjuvant therapy for ONFH.

## DECLARATIONS

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this manuscript, and all liabilities pertaining to claims relating to the content of this manuscript will be borne by the authors. Xin Li conceived and designed the experiments; Shuqin Zhang performed the experiments; Yating Bai, Lixin Fu and Yanjiang Cui did statistical analysis, while Shuqin Zhang wrote the manuscript. All authors read and approved the final manuscript.

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