

Puerarin rescued Dex-induced Osteoblast Apoptosis via AIF-mediated Caspase-independent pathway

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Abstract

GIOP is the most common secondary osteoporosis, and the increased apoptosis of osteoblasts is thought to be the main cause of GIOP. *Puerarin* is the major isoflavone extracted from *Pueraria lobata*. Our previous study indicated that *Puerarin* protects osteoblast apoptosis partly through the caspase-dependent pathway. So, we concluded that there existed other anti-apoptotic mechanisms in *Puerarin*. Therefore, in the current study, we focused on the role of the caspase-independent pathway, specifically the apoptosis-inducing factor (AIF) pathway. MTS assay, flow cytometry, TUNEL staining, Western blotting, and immunofluorescence staining were used in this study. Caspase inhibitor Z-VAD-FMK and RNA silencing (siRNA) AIF were used to assist *Puerarin* in detecting the anti-apoptosis mechanisms. The results showed that *Puerarin* significantly alleviated Dex-induced osteoblast cell proliferation inhibition and cell cycle arrest at G0/G1 phase. *Puerarin* can restore the protein expression of cell cycle-related proteins (cyclin D1) and cyclin-dependent kinase (CDK4). Flow cytometry and TUNEL staining revealed that the pan-caspase inhibitor Z-VAD-FMK and siRNA AIF partially inhibited Dex-induced osteoblast apoptosis, while *Puerarin* significantly reduced Dex-induced osteoblast cell apoptosis. Immunofluorescence and Western blotting results showed that nucleus translocation of AIF from mitochondria in Dex-treated cells also inhibited by *Puerarin*. Our results suggest that *Puerarin* can restore Dex-induced cell cycle inhibition and apoptosis of osteoblasts through the AIF-regulated caspase-independent pathway.

1. Introduction

Glucocorticoids (GCs) are widely used in clinics, especially in the treatment of immune system diseases. Studies have found that long-term or short-term use of high doses of GCs can induce the development of glucocorticoid-induced osteoporosis (GIOP) (1). GIOP is the most common secondary osteoporosis in the clinic, and its risks are reduced bone density, and increased risk of bone fragility and fracture, which seriously affect patients' quality of life (2).

To date, the pathogenesis of GIOP is still not fully known. However, it is widely accepted that GCs inhibit the proliferation of osteoblasts, enhance their apoptosis, and disrupt the osteoblast–osteoclast balance by inhibiting the differentiation of bone marrow mesenchymal stem cells (BMSCs) into osteoblasts and promoting their differentiation into adipocytes (3, 4). In this process, the regulatory mechanisms may include changes in the pro-apoptotic and anti-apoptotic genes of osteoblasts, changes in the expression of cyclin-dependent kinases, and changes in the regulatory proteins of osteogenic and lipogenic differentiation pathways of BMSCs (5–7). It has been observed that GCs can promote the apoptosis of osteoblasts by activating caspase-3, inhibit the differentiation of osteoblasts by down-regulating BMP and Wnt pathways, and stimulate the differentiation of BMSCs into adipocytes rather than osteoblasts through PPAR γ 2 (8, 9).

The cell cycle plays an important role in the regulation of cell proliferation and apoptosis (10). The cells' proliferation and division are regulated by cyclins, cyclin-dependent kinases, and cyclin-dependent kinase

inhibitors. The balance between apoptosis and cell proliferation is essential for maintaining a healthy cell population. However, this balance is out of control in the GIOP population. Many studies have found that apoptosis was caused by cell cycle arrest. Magnolol and miR-22 had a significant inhibition effect on the growth of Osteosarcoma cells and induced its apoptosis and cell cycle arrest at G0/G1 phase (11, 12, 13). It was also found that 6 α -methylprednisolone sodium succinate (MPSL) induced apoptosis and G0/G1 block in MC3T3-E1 osteoblasts under hypoxia conditions. Correspondingly, MPSL up-regulated levels of cleaved caspase 3 and down-regulated levels of cyclin D1 during hypoxia (14). The inhibitory effects of dexamethasone on murine osteoblastic MC3T3-E1 cells were largely attributed to apoptosis and G1 phase arrest (15, 16). Our previous study has shown that *Puerarin* prevented the Dex-induced apoptosis of hFOB1.19 cells via inhibition of the JNK pathway and activation of the PI3K/Akt signalling pathway in hFOB1.19 cells. Whether *Puerarin* exerts its pharmacological effect on osteoblast apoptosis by regulating cell cycle recovery remains to be further studied.

Apoptosis is one of the most common programmed cell deaths (PCD) (17, 18, 19). In the caspase-dependent pathway, pro-apoptotic proteins of the Bcl-2 family cause changes in mitochondrial membrane permeability, ultimately leading to the release of cytochrome c and cell death (20, 21). During the caspase-independent pathway, apoptosis-inducing factor (AIF), a protein that regulates caspase-independent apoptosis, is released from mitochondria and transported to the nucleus to participate in DNA degradation (22–25).

Many studies have found that AIF mediates nuclear apoptosis (26). AIF is thought to play a central role in caspase-independent apoptotic pathways. The pro-apoptotic effect of AIF is also reflected in its own positive feedback, that is, AIF released into the cytoplasm can act on other mitochondria, increase their permeability, and thus promote the release of AIF (27). However, investigations have reported that the apoptotic effect of AIF is dependent on caspase; AIF exerts an apoptotic effect after the activation of caspase (28, 29). After apoptotic activation, AIF interacts in the cytoplasm with the heat shock 70-kDa protein (HSP70) or with the endonuclease cyclotriptin A (CypA). After entering the nucleus, the AIF:CypA complex recruits H2AX and assembles it into the degradation complex AIF:CypA:H2AX (30), which binds DNA and promotes its degradation into 20–50 kb fragments, leading to apoptosis (31).

Puerarin, an isoflavone compound extracted from *pueraria* root, has protective properties for heart and liver cells, as well as anti-inflammatory and anti-apoptotic effects (32, 33, 34). Recent studies have reported that it can promote osteoblast differentiation and mineralization, inhibit osteoblast apoptosis, and prevent bone loss (35–38).

In our previous study, *Puerarin* significantly decreased the mRNA and protein expression of cleaved caspase-3, suggesting the involvement of the caspase-dependent mitochondrial apoptosis pathway in this process. In the present study, it was determined that activated caspase-3 was highly expressed in Dex-treated cells, and the caspase-3 specific inhibitor Z-VAD-FMK could not completely reverse cell death. Therefore, it was hypothesized that another type of apoptosis may be involved in Dex-induced cell death.

Therefore, this study focused on whether *Puerarin* can restore the cell cycle during the treatment of Dex-induced osteoblast apoptosis, and whether it exerts pharmacological effects by regulating the caspase-independent apoptosis pathway, specifically the AIF pathway.

2. Materials and methods

Dexamethasone (Dex) was purchased from sigma (Sigma Chemical Co.St.Louis, MO, USA). Puerarin was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dex and Puerarin were used at a concentration of 10^{-5} M and 10^{-8} M. The final concentration of Dex (10^{-5} M) was dissolved in ethanol and Puerarin (10^{-5} M) was dissolved in DMSO (Sigma Chemical Co.St.Louis, MO, USA). Dex and Puerarin were stored at -20°C.

2.1 Cell lines and cell culture

The cells of conditionally immortalized human fetal osteoblastic cell line hFOB1.19 were cultured according to the ATCC protocol and as described (22). For *in vitro* proliferation, hFOB1.19 cells were maintained in a non-differentiation medium consisting of 1:1 DMEM/F-12 (HyClone, Logan, Utah, USA) medium with 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), and 0.3 g/L G418 (Sigma Chemical Co.St.Louis, MO, USA) and were cultured in a humidified incubator with 5% CO₂ at 33.4°C. The medium was changed twice per week and cells were subcultured using 0.25% trypsin with 0.02% EDTA (Sigma Chemical Co.St.Louis, MO, USA).

2.2 Cell proliferation assay

The hFOB1.19 cells were inoculated at 3×10^3 cells/well in 96-well plates and the number of cells were quantified using an MTS assay, according to the manufacturer's instructions (Promega (Beijing) Biotech Co.,Ltd, Beijing, China). Briefly, 20 µl MTS solution was added to 100 µl culture medium in each well. Following incubation for 2 h at 37°C, the absorbance was read at a wave-length of 490 nm on a microplate reader (Thermo Fisher Scientific, Massachusetts, USA), with measurements presented as the mean of at least three independent experiments, with each data point based upon three replicates.

2.3 Cell cycle analysis

The cell cycle distribution of hFOB1.19 cells was determined via flow cytometric analysis. Briefly, hFOB1.19 cells were incubated at a density of 1×10^5 cells/well. After exposure to Dex (10^{-5} M, 24h), both with and without Puerarin pretreatment

(10^{-8} M, 3 h), cells were washed twice with PBS and fixed with ice-cold 70% ethanol at 4°C overnight. The fixed cells were washed twice with PBS followed by incubation with at 37°C for 30 min (each specimen contained Dye buffer 0.5 ml, RNase A 10 µl, and PI solution 25 µl). Then, cell cycle analysis was performed via the FACS Calibur flow cytometry system (BD Biosciences, San Jose, CA, USA). A total of

20,000 cells per sample were recorded for analysis. Results were processed via FCS Software (BD Accuri Software, Los Angeles, CA, USA).

2.4 Analysis of apoptosis via flow cytometry

Annexin V-FITC and PI staining were conducted according to the recommended protocol and cells were analyzed via flow cytometry (Beyotime Biotechnology Co., LTD, Shanghai, China). Briefly, cells were harvested and washed in PBS, 1×10^5 of resuspended cells were collected, and 195 μ l binding buffer was added. The cell solution (195 μ l 1×10^5 cells) was transferred to a 5 ml culture tube, and incubated with 5 μ l Annexin V-FITC and 10 μ l PI for 15 min at 25°C in the dark. After incubation, 300 μ l of binding buffer was added to each tube and cells were analyzed via flow cytometry within 1 h.

2.5 Gene silencing

AIF siRNA (AAGAGAAACAGAGAAGAGCCA) and nonfunctional control siRNA (mut siRNA, 5NA, 5utCAGAGAAGAGCCA) and nonfunctional conform TaKaRa (TaKaRa Corporation, Dalian, China). Cells were cultured to 70% confluence in serum-free mediums. Lipofectamine 2000 was used to transfect cells with either AIF siRNA or with non-specific siRNA as negative control. In brief, Opti-MEM (Invitrogen, California, USA) was used to dilute Lipofectamine 2000 and siRNA, and this diluted Lipofectamine 2000 was then added to the diluted siRNA. Following incubation for 20 min at room temperature, the mixture was added to the cells. Both the cells and transfection reagents were incubated at 37°C in an atmosphere of 5% CO₂ for 6 h before the medium was replaced by the normal serum-containing medium. After transfection for 48 h, the following assays were performed (23).

2.6 Caspase activity measurements

The cells were cultivated in six-well plates (at a density of 10^5) and were lysed in 100 μ l lysis buffer for 15min (Beyotime Biotechnology Co., LTD, Shanghai, China). The lysis solution was centrifuged at 20 000g, 15 min, and the 50 μ l supernatant were incubated with 40 μ l detection buffer (Beyotime Biotechnology Co., LTD, Shanghai, China) and Ac-DEVD-pNA (2 mM) (Beyotime Biotechnology Co., LTD, Shanghai, China). The absorbance was read at a wavelength of 490 nm on a microplate reader. The measurements are represented by the means of at least three independent experiments, with each data point based on three replicates. Caspase-3 activity was inhibited via the potent general caspase inhibitor Z-VAD-FMK (R&D Systems, Wiesbaden, Germany). Z-VAD-FMK (50 μ M) was applied 1 h before Dex exposure.

2.7 AIF immunofluorescence staining

Briefly, hFOB1.19 cells were plated onto coverslips and cultured overnight. Cells were cultured in medium with Puerarin (10^{-8} M, 3 h) and AIF siRNA (40 nM, 48 h) was applied, then treated with Dex (10^{-5} M, 24 h). Afterwards, cells were fixed in immune stationary liquid (Beyotime Biotechnology Co., LTD, Shanghai, China) for 15 min at room temperature and washed with immune washing liquid (contains 0.1% Triton X-100) for 3 min on ice. The cells were incubated with AIF antibody (diluted at a ratio of 1:400) overnight

and DAPI (5 min) was used to stain nuclei. Cells were observed using a fluorescence microscope (at a minimum slit width of 200 μ l).

2.8 Western Blot Analysis

Total protein was extracted in RIPA lysis buffer (Beyotime Biotechnology Co., LTD, Shanghai, China) and quantified via BCA protein assay (Beyotime Biotechnology Co., LTD, Shanghai, China). Then, equal amounts of protein were separated via SDS-PAGE and transferred to PVDF membranes (Millipore, Massachusetts, USA). The membranes were blocked with 5% nonfat milk in PBS for 1 h at room temperature and then incubated with Cyclin D1, Cdk4, and AIF antibodies (Cell Signaling Technology, Boston, USA) overnight at 4°C. After extensive washing, membranes were re-probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Boston, USA). The blots were then washed, and the signal was visualized via HRP chemiluminescent substrate reagent kit (Invitrogen, California, USA) according to the manufacturer's protocol. The band intensity was quantified via densitometric analysis using Image J 1.36 software (National Institutes of Health, Bethesda, USA). The absorbance ratio of each protein to internal reference is represented as the relative amount of target protein.

2.9 Statistical Analysis

The Statistical Package for the Social Sciences version 17.0 software package

(SPSS Inc., Chicago, IL, USA) was used for all analyses. All data are expressed as means $\pm$ standard deviation (SD). Statistical analyses of the data were performed via one-way analysis of variance (ANOVA) and $P < 0.05$ was considered as statistical significant difference. All experiments were repeated at least three times.

3. Results

3.1 Puerarin rescued the proliferation inhibition of Dex in hFOB1.19 cells

Our previous studies have shown that Dex (10^{-5} M) can inhibit osteoblast proliferation activity and induce it apoptosis significantly, while Puerarin (10^{-8} M) can protect Dex-induced osteoblast apoptosis. However, the optimal action time of Puerarin and Dex on the osteoblasts proliferation and apoptosis is still unclear. So, the

hFOB1.19 cells were treated with Puerarin (10^{-8} M) at different times (0, 3, 12, 24, 48 and 72 h). The results showed that Puerarin could improve cell proliferation at different times (*all $P < 0.05$ compared to control*). Notably, the optimal time for Puerarin promote proliferation rate (10^{-8} M) was 3h after treatment. The 72h exposure of hFOB1.19 cells to Dex (10^{-5} M) at different time points (0, 3, 12, 24, 48, and 72 h) decreased the cell proliferation in a time-dependent manner (*all $P < 0.01$ compared to control*).

Pre-treatment with Puerarin (10^{-8} M, 3 h) followed by Dex (10^{-5} M, 0–72 h) showed that Puerarin can rescue the proliferation inhibition induced by Dex (*all P < 0.05 compared to the Dex treatment group*). Puerarin pretreatment had a good protective effect for Dex in 12h and 24h, especially at 24h. Therefore, in this study, Puerarin was pretreated for 3 hours, followed by Dex for 24 hours, as the medication time in this experiment (Table 1, Fig. 1). (* P < 0.05 vs. control, ** P < 0.01 vs. control. N > 3).

Table 1
The MTS was used to analyze the Cell proliferation

	<i>Control</i>	<i>Dex</i>	<i>Puerarin</i>	<i>Dex + Puerarin</i>
<i>0h</i>	<i>0.96 ± 0.01</i>	<i>0.95 ± 0.02</i>	<i>0.96 ± 0.02</i>	<i>0.97 ± 0.01</i>
<i>3h</i>	<i>0.96 ± 0.01</i>	<i>1.00 ± 0.01</i>	<i>1.70 ± 0.02**</i>	<i>0.96 ± 0.02*</i>
<i>12h</i>	<i>1.18 ± 0.02</i>	<i>0.81 ± 0.02*</i>	<i>1.54 ± 0.01**</i>	<i>1.13 ± 0.01*</i>
<i>24h</i>	<i>1.51 ± 0.03</i>	<i>0.61 ± 0.01**</i>	<i>1.45 ± 0.01**</i>	<i>1.23 ± 0.02**</i>
<i>48h</i>	<i>1.35 ± 0.02</i>	<i>0.51 ± 0.02**</i>	<i>1.30 ± 0.02*</i>	<i>1.10 ± 0.03*</i>
<i>72h</i>	<i>1.15 ± 0.01</i>	<i>0.50 ± 0.03**</i>	<i>1.10 ± 0.02</i>	<i>0.95 ± 0.02</i>

3.2 Puerarin rescued Dex-induced cell G0/G1 arrest

The flow cytometry was used to analyze the cell cycle of hFOB1.19 affected by Dex. The percentage of cells in the G0/G1 phase increased significantly to $85.79 \pm 1.21\%$ in the Dex group, as compared to $26.59 \pm 1.55\%$ in the control group, and $75.96 \pm 2.14\%$ in the Dex + Puerarin group. The percentage of cells in the S phase decreased significantly to $6.18 \pm 1.11\%$ in the Dex group compared to $44.83 \pm 2.21\%$ in the control group, and $16.04 \pm 0.81\%$ in the Dex + Puerarin group, respectively. The G2/M phase decreased to $20.11 \pm 1.33\%$ in the Dex group compared to $48.13 \pm 1.22\%$ in the control group, and $30.14 \pm 1.61\%$ in the Dex + Puerarin group.

The result reminded us that the cells arrested at G0/G1 phase after Dex treatment, with the corresponding proportion of cells decreasing at the S and G2/M phase, and Puerarin rescued the cell cycle arrest at three stages (Table 2, Fig. 2). (* P < 0.05 vs. control, ** P < 0.01 vs. control. N > 3).

Table 2
The flow cytometry analyze the cell cycle of hFOB1.19

	<i>Control</i>	<i>Dex</i>	<i>Dex + Puerarin</i>
<i>G0/G1</i>	<i>26.59 ± 1.55%</i>	<i>85.79 ± 1.21%**</i>	<i>75.96 ± 2.14%*</i>
<i>S</i>	<i>44.83 ± 2.21%</i>	<i>6.18 ± 1.11%**</i>	<i>16.04 ± 0.81%*</i>
<i>G2/M</i>	<i>48.13 ± 1.22%</i>	<i>20.11 ± 1.33%**</i>	<i>30.14 ± 1.61%*</i>

To further elucidate the possible mechanism of the cell cycle arrest at the G0/G1 phase induced by Dex, the expression of cell cycle regulatory proteins (cyclins) and cyclin-dependent kinases (CDKs) was determined via Western blot analysis. Western blot analyses further confirmed that the inhibitory effect of Dex was possibly caused by the downregulation of cyclin D1 and CDK4. Pre-treatment with Puerarin can relieve this inhibition effect, and the expression of cyclin D1 and CDK4 proteins was restored (Fig. 3).

3.3 Caspase inhibitor Z-VAD-FMK inhibited Dex-induced cell death partly

After apoptotic activation, the subsequent increase in mitochondrial membrane permeability can trigger caspase-dependent and caspase-independent cell death through the release of cytochrome c and AIF. TUNEL method was used to detect apoptosis of hFOB1.19 cells after exposure to Dex, the AI (apoptosis index) is about $15 \pm 0.85\%$ in Dex treated group compared to $4 \pm 0.41\%$ in the control group. The caspase inhibitor Z-VAD-FMK inhibits the activity of Caspase-3; however, this treatment partly inhibited Dex-induced cell death (the AI is about $11 \pm 1.21\%$). Therefore, we concluded that the caspase-independent apoptotic pathway was involved in Dex-induced apoptosis in osteoblasts (six fields were randomly selected and the percentage of positive cells was calculated as the apoptosis index (AI) using the following equation: $AI = (\text{number of positive cells} / \text{total number of cells}) \times 100\%$) (21). Our previous studies found that Puerarin inhibited Dex-induced apoptosis in osteoblasts and inhibited the release of caspase-3 through the form of caspase-dependent. Therefore, Puerarin plays a pharmacological role similar to caspase inhibitor (Fig. 4).

3.4 AIF siRNA inhibited Dex-induced cell death partly

Two pathways linking mitochondrial and cell apoptosis have been found: one is the caspase-dependent pathway and caspase-3 is considered to be the most important protein in this apoptosis pathway because it plays a key role in the initiation of apoptosis; The second pathway is the caspase-independent pathway, that is AIF pathway. AIF plays a central role in the regulation of caspase-independent apoptosis, which causes large DNA fragments to break (26–28). TUNEL assay indicated that siRNA AIF decreases Dex-induced apoptosis in hOB1.19 cells. Briefly, hFOB1.19 cells were treated with Dex in either the absence or presence of AIF siRNA. The percentage of apoptotic cells exhibiting green condensed or

fragmented nuclei was identified via TUNEL staining. The ratio of apoptotic cells to total cells was ($15 \pm 0.75\%$) in the Dex group compared to $4 \pm 0.41\%$ in the control group and $12 \pm 0.52\%$ in the siRNA AIF group. The results suggest that AIF is involved in Dex-induced apoptosis (Fig. 5).

3.5 Puerarin inhibited Dex-induced cell apoptosis

The morphological changes of apoptotic cells were observed by inverted microscope, and Flow Cytometry was used to detect the cells apoptosis according to protocol. The hFOB1.19 cells were treated with culture medium (control group), Dex (10^{-5} M, 24 h), Dex (10^{-5} M, 24 h) after Puerarin (10^{-8} M, 3 h) pretreatment. We can see that apoptotic osteoblasts atrophy, lose their adhesion characteristics, and float in the medium. The ratio of apoptotic cells to total cells was significantly higher in the Dex group ($15 \pm 0.85\%$) compared to $4 \pm 0.21\%$ in the control group and $7 \pm 0.19\%$ in the Dex + Puerarin group (Fig. 6).

3.6 Puerarin inhibits mitochondrial translocation of AIF to the nucleus

Firstly, the translocation of AIF from mitochondria to nucleus was analyzed by immunofluorescence. The current result of AIF siRNA partially preventing cell death further supports the role of AIF in Dex-induced cell death. We analyzed the translocation of AIF with or without Puerarin. AIF appeared as red fluorescence and mainly distributed in mitochondria when it was in a non-activated state. When AIF was activated by Dex, AIF translocated to the nucleus and initiated the apoptosis process. After pretreatment with Puerarin, the translocation of AIF was inhibited and the apoptosis of osteoblasts were decreased (Fig. 7).

Secondly, we used western blotting to detect two forms of AIF expression in mitochondria and nucleus respectively. As shown, in the control group, the expression of AIF was higher in mitochondria and lower in nucleus. In the Dex-treated group, when AIF was activated, a large number of AIF were translocated from mitochondria to nucleus, resulting in a sharp decrease of AIF in mitochondria and a significant increase of AIF in nucleus. After pretreatment with Puerarin, the transfer of AIF into the nucleus was inhibited. ImageJ software detects the fluorescence intensity in the nucleus, the Mean fluorescence intensity in the nucleus = the sum of the fluorescence intensity in the region (IntDen, Integrated Density)/the Area of the region (Area) (Fig. 8)

In summary, these findings demonstrate that Puerarin exerts pharmacological effects similar to Z-VAD-FMK and AIF siRNA, Puerarin inhibited mitochondrial AIF release and translocate to the nucleus, and inhibited Dex-induced hFOB1.19 cell death via a caspase-independent pathway.

4. Discussion

Epidemiological studies have found that approximately 1–3% of patients treated with GCs, especially older adults, postmenopausal women, and older men, experience bone loss. Bone loss in GIOP generally

occurs in two phases, beginning with a rapid bone loss phase (6–12% decrease in lumbar bone density during the first year of treatment) followed by a slow but sustained decline in bone density (41).

Osteoblasts are derived from mesenchymal progenitor cells and are primarily responsible for bone formation and development (42). GCs inhibit osteoblast activity and induce apoptosis during the formation and development of GIOP (43). Studies have shown that high doses ($\geq 10^{-6}$ M) of dexamethasone inhibit the proliferative activity and function of osteoblasts by accelerating the rate of apoptosis (44).

It is well known that the cell cycle is involved in cell proliferation and apoptosis, and cell cycle arrest can induce apoptosis (10). Based on this, we used flow cytometry to obtain the percentage of cells at different periods after Dex treatment. The results showed that Dex significantly induced G0/G1 cell cycle inhibition, that is, Dex significantly increased the proportion of G0/G1 phase cells, while decreasing the proportion of G2/M and S phase cells. In order to further elucidate the mechanism of Dex-induced osteoblast cycle arrest, we detected the expressions of Cyclins and CDKs by Western blotting.

Cyclin D1 and CDK4 are considered to be the key regulators of G1(14). Previous studies have shown that Dex-induced G1 phase arrest and apoptosis of osteoblasts (15). Our present results further support the notion that the cell cycle inhibition effect of Dex is closely related to the downregulation of cyclin D1 and CDK4. When the cells are pretreated with *Puerarin*, the cells proceed into the S phase and G0/G1 inhibition decreases, while the levels of cyclin D1 and CDK4 increase. These results suggested that the potential mechanism of Dex inhibiting osteoblast proliferation and promoting osteoblast apoptosis may be related to G0/G1 cell cycle arrest, which may also be an important pharmacological mechanism of *Puerarin* regulating osteoblast proliferation and apoptosis.

Our previous studies have shown that *Puerarin* protects Dex-induced osteoblast apoptosis through the caspase-dependent mitochondrial apoptosis pathway, and whether there are other apoptosis mechanisms remains unclear. In our study, the apoptosis rate of osteoblasts in Dex group was about 15%, and was about 7% in *Puerarin* + Dex group, so we can assume that *Puerarin* can inhibit nearly 50% of cell apoptosis. We also found that a caspase inhibitor (Z-VAD-FMK) can inhibit about 30% of osteoblast apoptosis, suggesting that there are other apoptotic regulatory mechanisms besides caspase-dependent pathways. Therefore, we investigated the AIF pathway which independent of caspase. After RNA silencing AIF, the apoptotic effect of Dex was partially inhibited by about 20%. AIF is recognized as a novel death effector induced by several death stimuli and released from mitochondria during apoptosis. The AIF expression has been found in several organs and tissues of mammals. The findings suggested that AIF release by attacking the mitochondrial membrane and translocates into the nucleus to destroy cellular DNA, resulting in apoptosis (45, 46, 47).

In this study, the translocation of AIF from mitochondria to the nucleus was analyzed by immunofluorescence first. The result of AIF siRNA partially prevent cells from death further supports the role of AIF in Dex-induced cell death. In order to analyze the specific role of *Puerarin* in mitochondrial AIF

release and subsequent nucleus translocation, we analyzed the translocation of AIF with or without *Puerarin*. Fluorescence micrographs showed that AIF mainly distributed in mitochondria when it was in a non-activated state, it translocated to the nucleus and initiated the apoptosis process after activating by Dex. *Puerarin* could inhibit the translocation of AIF, and then inhibit the apoptosis of osteoblasts. Secondly, Western blotting further demonstrated *Puerarin* could inhibit Dex-induced AIF transfer from mitochondria to the nucleus. These results suggest that the anti-apoptotic effects of *Puerarin* include both caspase-dependent and caspase-independent mechanisms.

Conclusions

Our results demonstrate that Dex significantly induces G0/G1 phase arrest in osteoblasts, leading to the down-regulation of cyclin D1 and CDK4. Following *Puerarin* treatment, the cells entered the S phase, weakening the inhibition of G0/G1, and increasing cyclin D1 and CDK4 levels. Our previous studies have demonstrated that *Puerarin* protects Dex-induced osteoblast apoptosis through the caspase-dependent mitochondrial apoptosis pathway. *Puerarin* significantly reduces cleaved caspase-3 mRNA and protein expression. In this study, we observed that the caspase-3 specific inhibitor Z-VAD-FMK could not completely reverse Dex-induced cell death. AIF siRNA can partially prevent osteoblast death. *Puerarin* inhibits Dex-induced AIF transfer from mitochondria to the nucleus. These results suggest that *Puerarin* protects Dex-induced osteoblast apoptosis through the AIF-mediated apoptosis pathway.

Declarations

Conflicts of interest

The authors declare that they have no competing interests.

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Author Contribution

YU wrote the main manuscript text and QIAO Long, QIAO Ye prepared figures and GU, CHEN done this experiment. All authors reviewed the manuscript.

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Figures

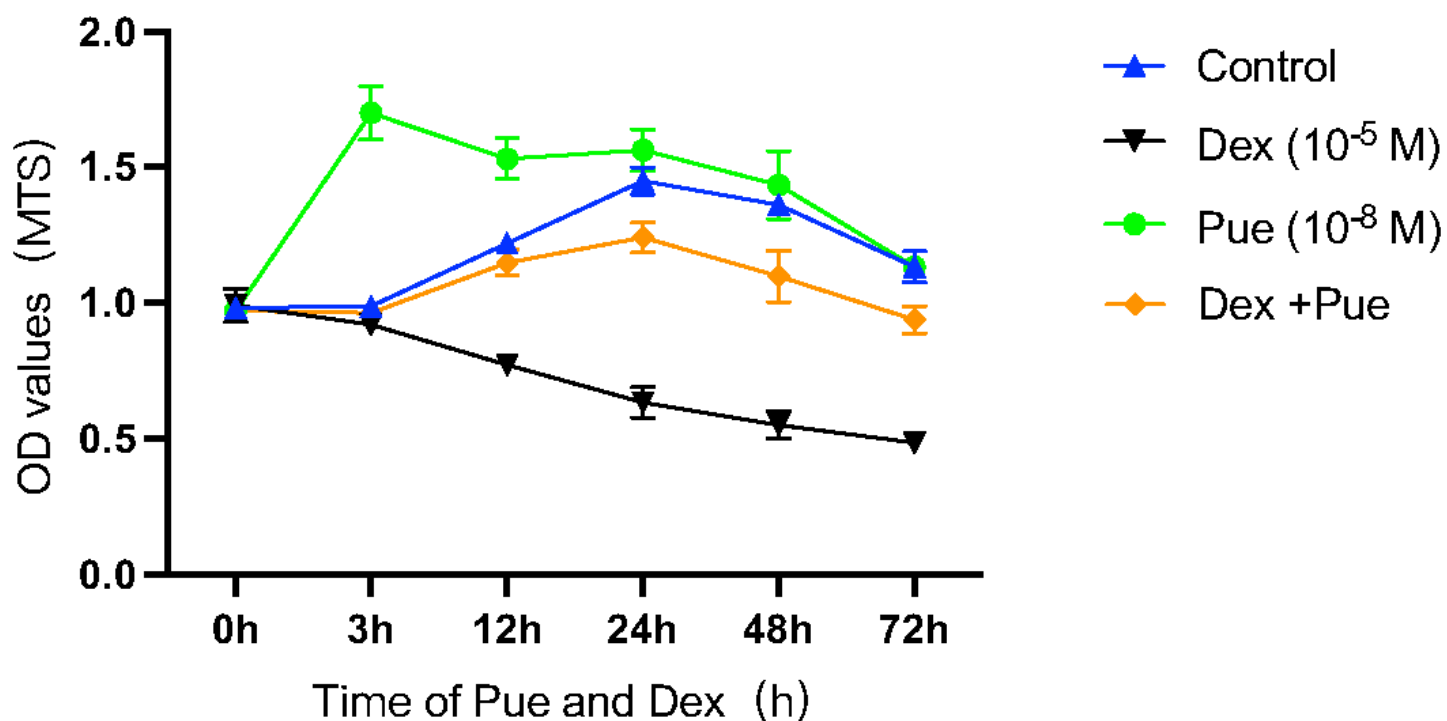


Figure 1

Puerarin rescued the proliferation inhibition of Dex in hFOB1.19 cells. Dex reduced the viability of hFOB1.19 cells in a time-dependent manner as evaluated via MTS assay. *Puerarin* promoted the viability of hFOB1.19 cells at different time points; notably, hFOB1.19 cells treated with *Puerarin* (10⁻⁸ M, 3 h) had the highest proliferation rate. Pretreatment with *Puerarin* rescued the Dex-induced proliferation inhibition at 12, 24, 48h ($P < 0.05$ vs. control. $N > 3$).

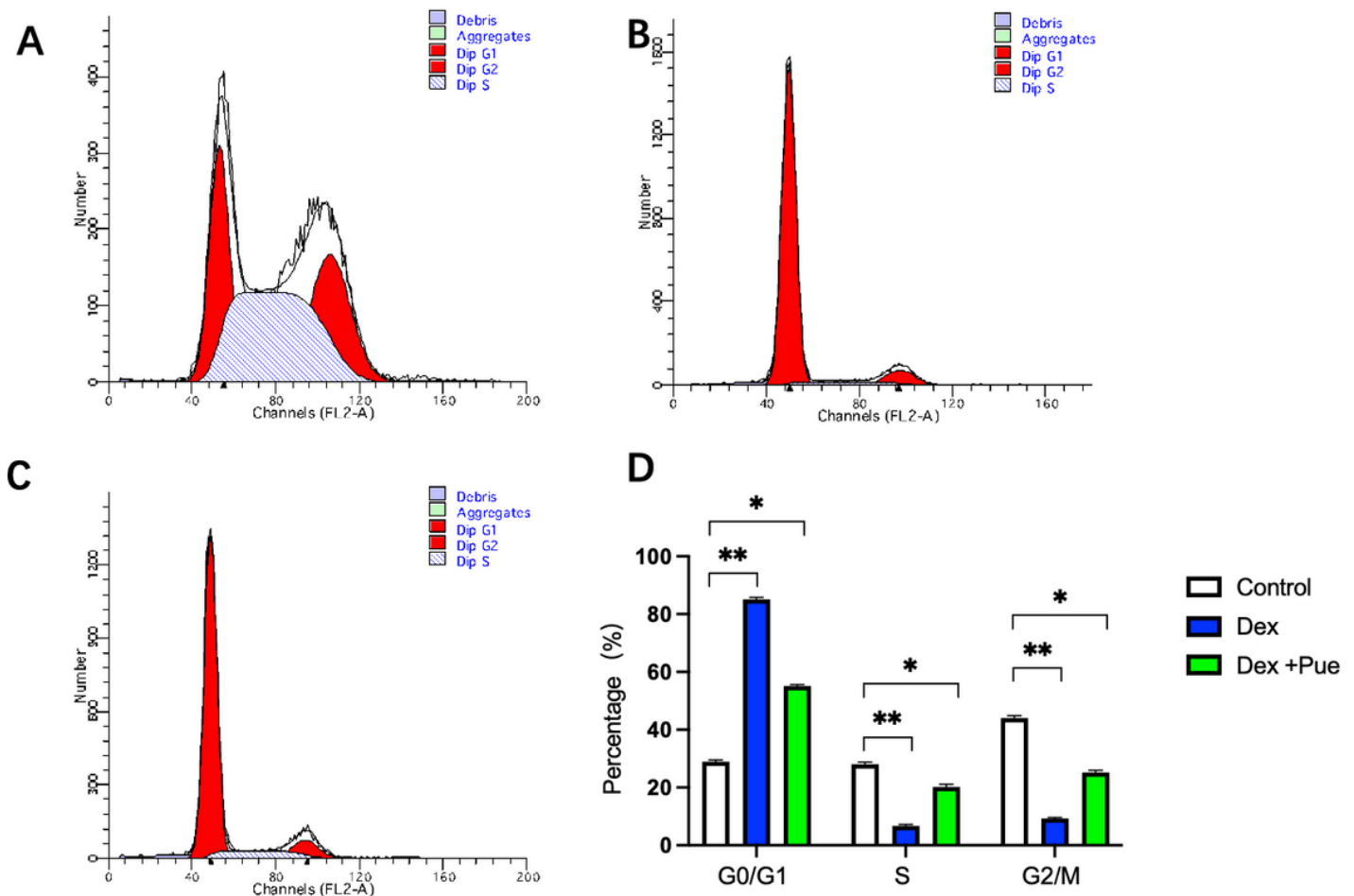


Figure 2

Puerarin relieved the cell cycle arrest at G0/G1 induced by Dex. The flow cytometry was used to analyze the cell cycle of hFOB1.19. The percentage of cells at G0/G1 phase was significantly increased in the Dex-group. *Puerarin* pretreatment alleviated the cell cycle arrest at G0/G1 induced by Dex. (b) The statistical analysis chart. (* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. $N > 3$).

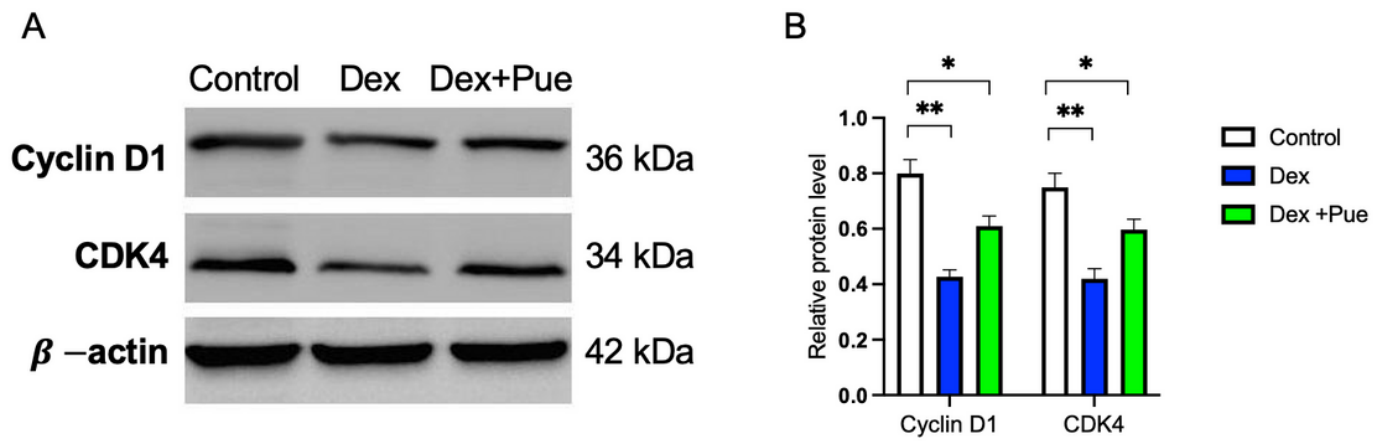


Figure 3

Western blotting was used to analysis the levels of cyclin D1 and CDK4. (a) The protein express of cyclin D1 and CDK4 related to G0/G1 phase were inhibited by Dex and *Puerarin* relieved this inhibition effect. (b) The relative quantification of the protein levels was analyzed by ImageJ software. (* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. $N > 3$).

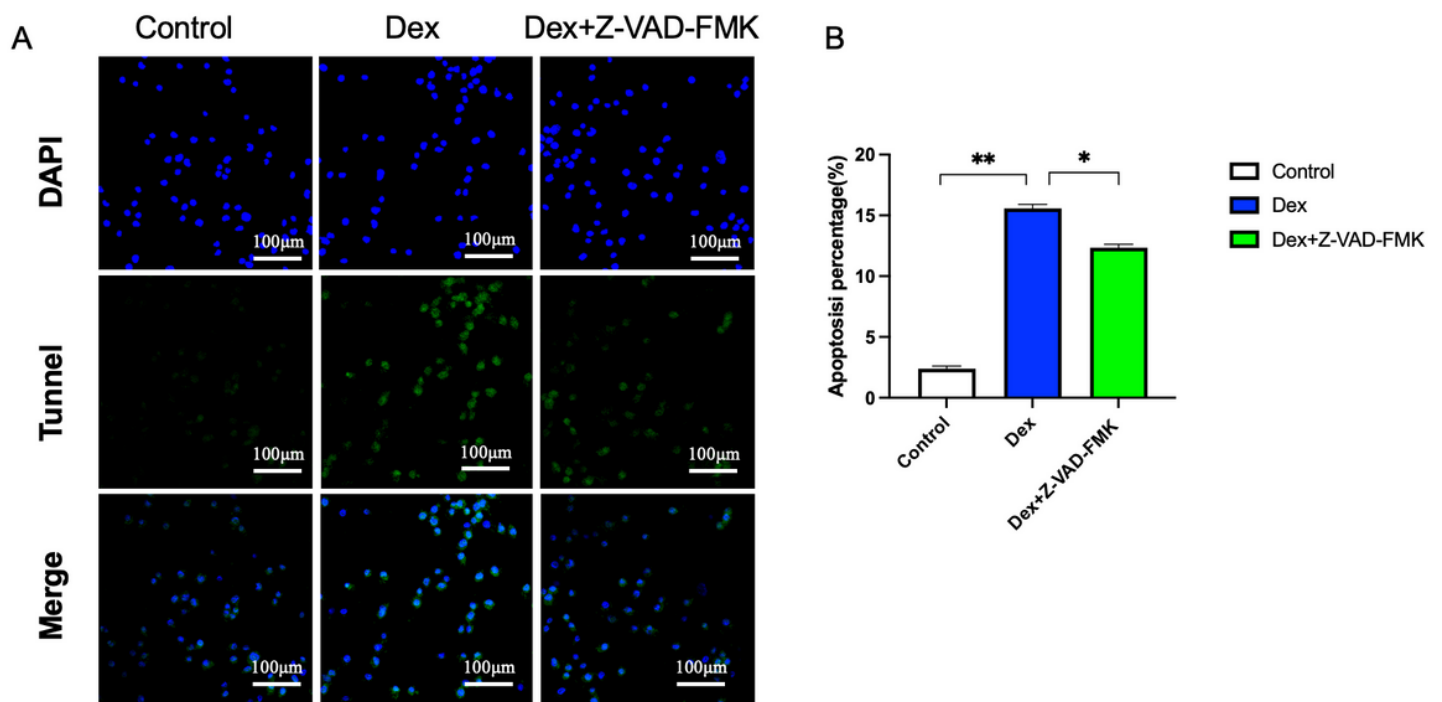


Figure 4

Z-VAD-FMK partially inhibited Dex-induced cell apoptosis. (a) TUNEL was used to detect the apoptosis of hFOB1.19 cells,Z-VAD-FMK could protect hFOB1.19 cells from Dex-induced cell death partly. (b) The

statistical analysis chart. apoptosis index (AI) using the following equation: AI = (number of positive cells / total number of cells) x 100%) (* P < 0.05 vs. control, ** P < 0.01 vs. control. N > 3).

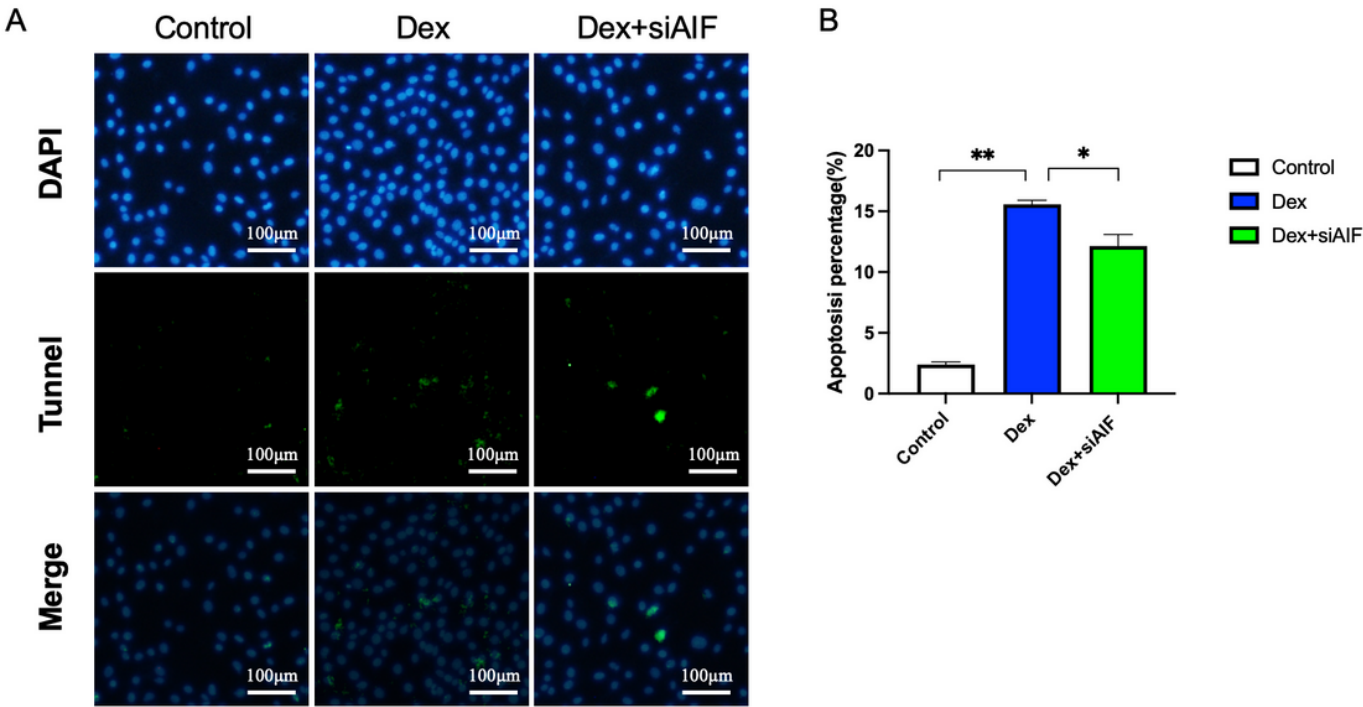


Figure 5

AIF siRNA inhibited Dex-nduced cell deathpartly. (a)TUNEL assay indicated that siRNA AIF decreased Dex-induced apoptosis in hOB1.19 cells. (b) The statistical analysis chart. (* P < 0.05 vs. control, ** P < 0.01 vs. control. N > 3).

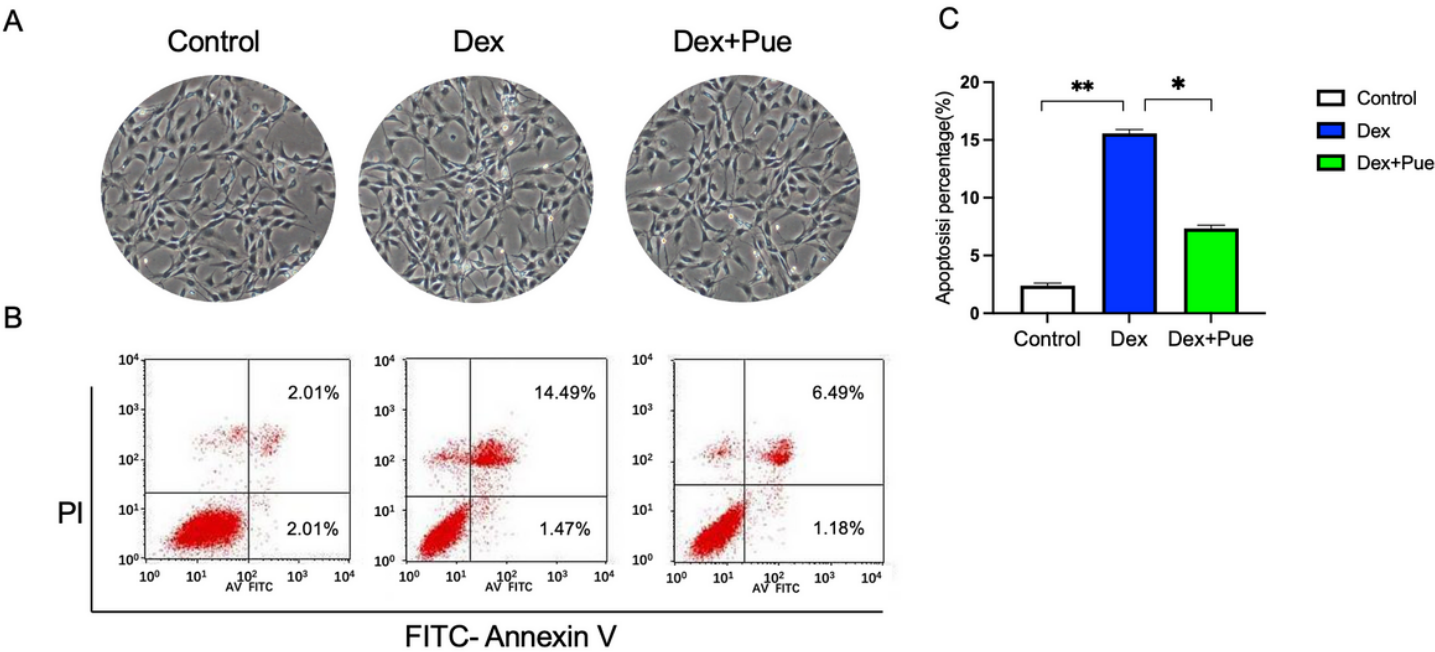


Figure 6

Puerarin protects the cells against Dex-induced apoptosis. (a) Inverted microscope ($\times 100$) showed that the Dex-treated cells lost their spindle shape and floated in the medium. (b) The FITC-annexin-V labeled cells were analyzed by flow cytometry to detect apoptotic cells. *Puerarin* significantly reduced Dex-induced osteoblasts apoptosis. (c) The statistical analysis chart. (* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. $N > 3$).

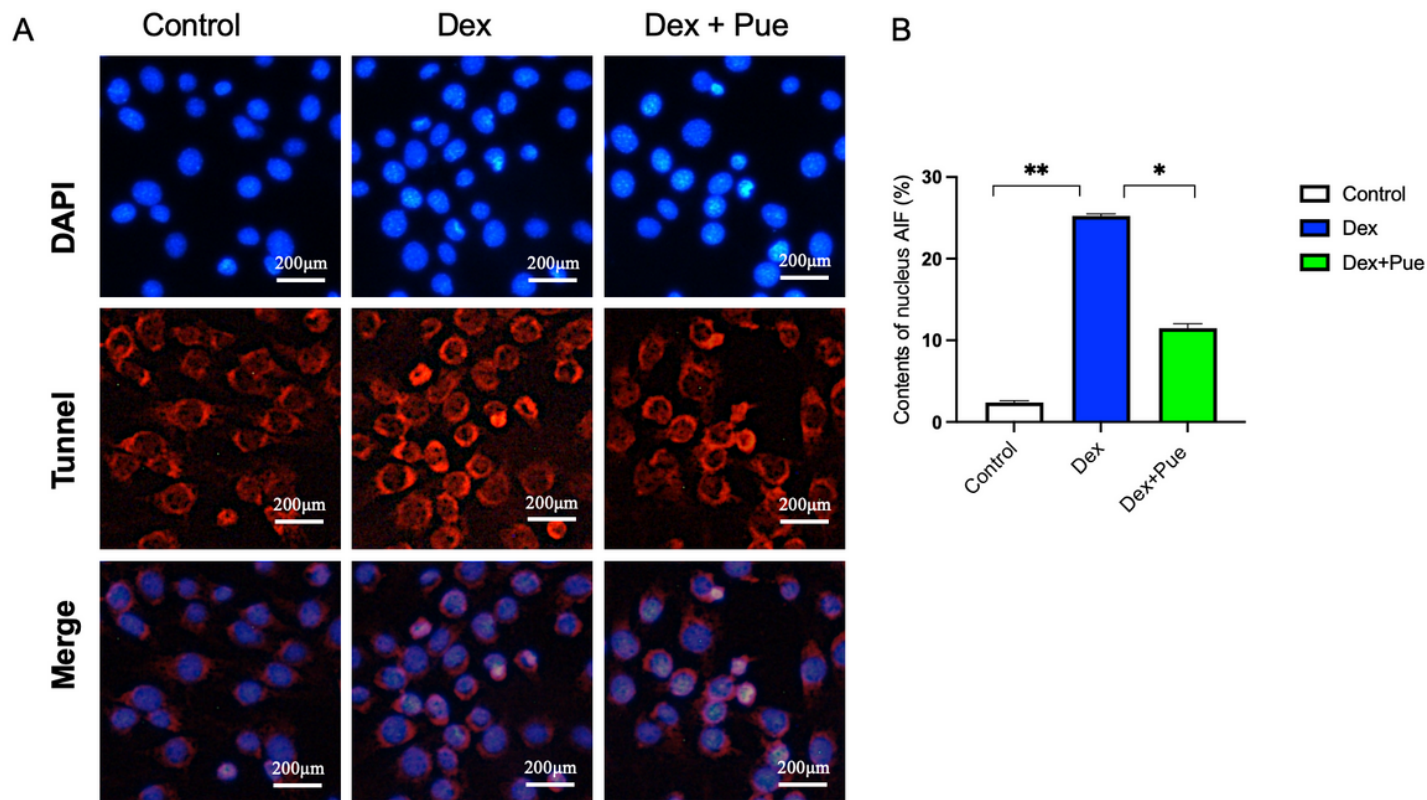


Figure 7

Confocal microscopy detected the translocation of AIF. (a) The AIF accumulates around the nucleus in mitochondria and was rapidly released into the nucleus after activation. *Puerarin* decreased translocation of AIF induced by Dex. (b) The relative quantification of the protein levels was analyzed by ImageJ software. (* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. $N > 3$).

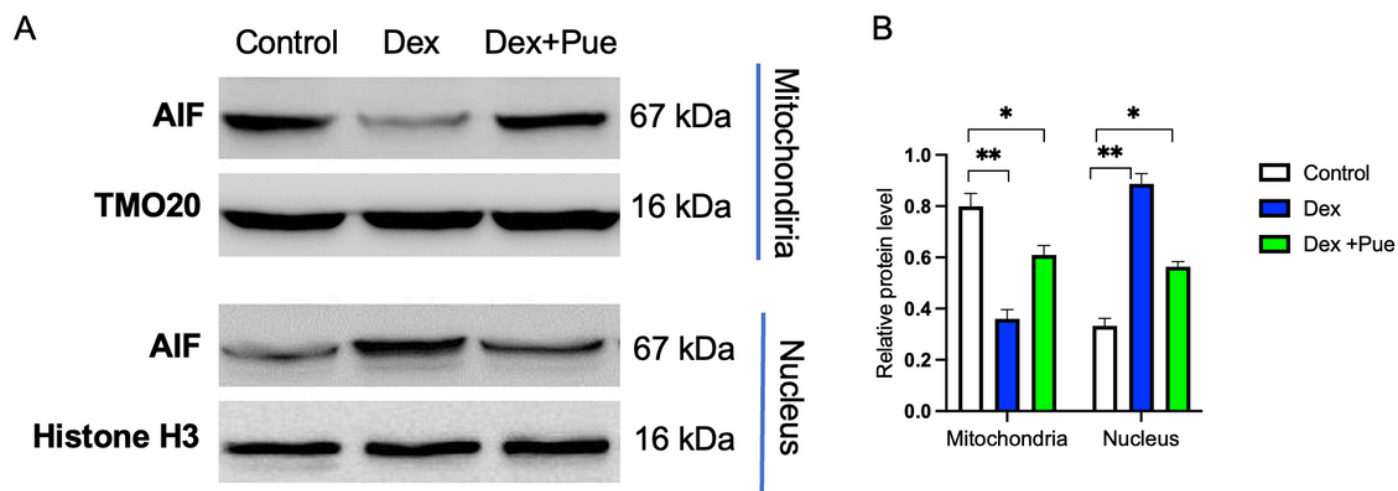


Figure 8

Western blotting detected the translocation of AIF. (a) The results shown that AIF transferred from mitochondria to the nucleus in Dex-treated group. After treatment with *Puerarin*, the translocation of AIF was inhibited. (b) The relative quantification of the protein levels was analyzed by ImageJ software. (* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. $N > 3$).