

Paeoniflorin inhibits IL-1 β -induced chondrocyte apoptosis by regulating the Bax/Bcl-2/caspase-3 signaling pathway

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Abstract. Apoptosis serves a pivotal role in the pathogenesis of osteoarthritis (OA). Increasing evidence has demonstrated that paeoniflorin exerts key properties (including anticancer, anti-inflammation and neuroprotective) for clinical applications. However, the precise role of paeoniflorin in articular cartilage apoptosis remains unknown. The present study explored the effects and potential molecular mechanism of paeoniflorin on rat chondrocyte apoptosis. Rat articular chondrocytes were cultured in monolayers. The lactate dehydrogenase (LDH) release rate of cells was determined by an LDH release assay. Annexin V-fluorescein isothiocyanate and propidium iodide staining were performed to detect early and advanced apoptotic cells by flow cytometry. The activity of caspase-3 in chondrocytes was determined using a caspase-3 activity assay. The expression of B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) was examined by reverse transcription-quantitative polymerase chain and western blotting. The present study also examined the protein kinase B (Akt) signaling pathway by western blotting. Treatment with 25 or 50 μ M paeoniflorin markedly decreased the release of LDH and the ratio of apoptotic cells in interleukin (IL)-1 β -induced rat chondrocytes. Paeoniflorin treatment decreased the mRNA and protein levels of Bax, and increased the level of Bcl-2. Paeoniflorin also reduced the activity of caspase-3 in chondrocytes. Furthermore, paeoniflorin was determined to regulate the Akt signaling pathway by increasing Akt phosphorylation. Therefore, paeoniflorin may exert its protective effect by inhibiting apoptosis in IL-1 β -induced rat chondrocytes and

thus, may be an effective agent in the prevention and treatment of OA.

Introduction

Osteoarthritis (OA) is a common degenerative disorder of human articular cartilage characterized by the destruction of articular cartilage and osteophyte formation (1). Chondrocytes are the only cell type present in articular cartilage and show little metabolic activity. Recent studies have suggested that chondrocyte apoptosis is related to extracellular matrix remodeling (2). In the progression of OA, the imbalance between apoptosis and the proliferation of chondrocytes causes chondrocyte cytokine production and matrix degeneration (3). Therefore, one method to prevent articular cartilage degeneration is to inhibit apoptosis-related signaling molecules.

Paeoniflorin, a major pharmacological pinane monoterpene glucoside, was first isolated from the Ranunculaceae plant in 1963. It is widely accepted that paeoniflorin has antioxidant, anti-inflammation, hepatoprotective and neuroprotective effects (4-7). In an adjuvant-induced arthritis model, paeoniflorin inhibited the expression of IL-1 β , IL-6, IL-17, and TNF- α and upregulated the production of TGF- β 1 (8). In other musculoskeletal systems, Chen *et al* (9) demonstrated that paeoniflorin could block the apoptosis of fiber ring cells by reducing the expression of Fas and caspase-3 proteins via regulation of Fas-FasL signaling. Moreover, in internal disc disruption disease, paeoniflorin was also reported to decrease the percentage of dead nucleus pulposus cells by inhibiting the activation of caspase-3 and -9 and increasing Bcl-2 family protein expression (10).

We previously reported that treatment with paeoniflorin downregulated the expression of matrix metalloproteinase (MMP)-1, -3 and -13, and increased the expression of TIMP-1 at both the mRNA and protein levels in a dose-dependent manner in rat articular chondrocytes stimulated by IL-1 β . Hui *et al* (11) found that increased levels of MMP-13 were closely related to the destruction of cartilage matrix and chondrocyte apoptosis. Nevertheless, little is known about the effect of paeoniflorin in chondrocyte apoptosis (11). Therefore, the present study evaluated the effects of paeoniflorin on IL-1 β -induced chondrocyte

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Table I. Reverse transcription-quantitative polymerase chain reaction primers and cycling conditions.

Gene	GenBank accession no.	Primer sequence (5'-3')	Size (bp)	Annealing temperature (°C)
Bax	NM_017059	F: CATGGGCTGGACACTGGACTT R: TGGTGAGTGAGGCAGTGAGGA	152	60
Bcl-2	L14680	F: GGATTGTGGCCTTCTTTGAGTTCG R: GGCATCCCAGCCTCCGTTATC	155	60
GAPDH	NM_017008.4	F: GAAGGTCGGTGTGAACGGATTTG R: CATGTAGACCATGTAGTTGAGGTCA	127	60

Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; bp, base pairs; F, forward; R, reverse.

apoptosis and determined the associated mechanism by examining Bcl-2, Bax and caspase-3.

Materials and methods

Reagents. Paeoniflorin (purity $\geq 98\%$) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant IL-1 β was purchased from PeproTech (Rocky Hill, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, fetal bovine serum (FBS), 0.05% trypsin, and collagenase II were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Primary cultures of normal rat articular chondrocytes. Rat articular chondrocytes for primary culture were obtained from the tibial plateau and femoral condyle of a 4-week-old Sprague-Dawley rat (The Animal Center of Zhejiang University, Hangzhou, China). In brief, cartilage was rinsed in phosphate-buffered saline (PBS) three times and finely cut into pieces of 1-3 mm³, digested with 0.2% pronase for 0.5 h, and then digested with 0.1% collagenase for 4 h at 37°C. Cells were cultured in complete DMEM containing antibiotic-antimycotic solution and 10% FBS at 37°C under a humidified 5% CO₂ atmosphere. The medium was replaced every 2 days. The animal experiments performed in the present study were approved by the University of Zhejiang Institutional Animal Care and Use Committee, Hang Zhou, China.

Lactate dehydrogenase cytotoxicity assay. According to our previous MTT assay, paeoniflorin concentrations ranging from 12.5 to 100 μ M did not show any significant toxicity to chondrocytes (Fig. 1A). Therefore, concentrations from 25 to 50 μ M were used in subsequent experiments. The lactate dehydrogenase (LDH) cytotoxicity assay was performed using the LDH Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China) (12). In brief, chondrocytes were cultured in 96-well plates. Cells were pretreated with 25 or 50 μ M paeoniflorin for 3 h and then incubated with IL-1 β (10 ng/ml) for 24 h. The release of LDH was measured according to the manufacturer's instructions.

Analysis of apoptotic cells by flow cytometry. Annexin V-fluorescein isothiocyanate (FITC) antibody immunofluorescence combined with propidium iodide (PI)/DNA binding was

used to analyze apoptosis. Chondrocytes were pretreated in growth medium supplemented with paeoniflorin for 3 h and then incubated in the absence or presence of rat recombinant IL-1 β (10 ng/ml) for 24 h. Next, according to the instructions with the Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology), 1×10^5 cells were treated with Annexin V-FITC and PI in the provided binding buffer for 0.5 h in the dark at 4°C. Cells were then subjected to flow cytometry at the emission wavelength of 488 nm.

Caspase-3 activity. Caspase-3 activity was determined using a Caspase-3 Cellular Activity Assay Kit (Cell Signaling Technology, Inc., Danvers, MA, USA). During the assay, activated caspase-3 cleaves the fluorogenic substrate (N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin [Ac-DEVDAMC]) between DEVD and AMC. Thus, we can determine highly fluorescent AMC concentrations using a fluorescence reader with excitation at 380 nm. Cells were pre-incubated in growth medium supplemented with different concentrations of paeoniflorin for 3 h, and then incubated with rat recombinant IL-1 β (10 ng/ml) for 24 h. According to the manufacturer's protocol, chondrocytes were collected and lysed using cell lysis buffer in the presence or absence of 5 μ l DEVD-pNA for 1 h at 37°C. Caspase-3 activity was measured at 405 nm on a microplate reader. Experiments were performed in triplicate.

Paeoniflorin treatment and mRNA expression analysis of Bcl-2 and Bax by reverse transcription-quantitative polymerase chain reaction (PCR). Chondrocytes were incubated in growth medium supplemented with 25 or 50 μ M paeoniflorin for 3 h and then incubated in the absence or presence of rat recombinant IL-1 β (10 ng/ml) for 24 h. Total RNA was isolated using TRIzol reagent (Sigma-Aldrich). Briefly, 1 μ g of total RNA after genomic DNA deletion by DNase I was reverse transcribed in 10 pmol of random hexanucleotide primers (Promega, Madison, WI, USA), 0.5 mM dNTPs, and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega). Then, the Bcl-2 and Bax mRNA levels were quantified by RT-qPCR, using the iQTM SYBR-Green SuperMix PCR Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol with 5 ng of template cDNA, 45 cycles: 95°C/15 sec, 60°C/15 sec with the primers listed in Table I. Rat-GAPDH (NM_017008) was used as a parallel amplification to normalize the expression data of the targeted

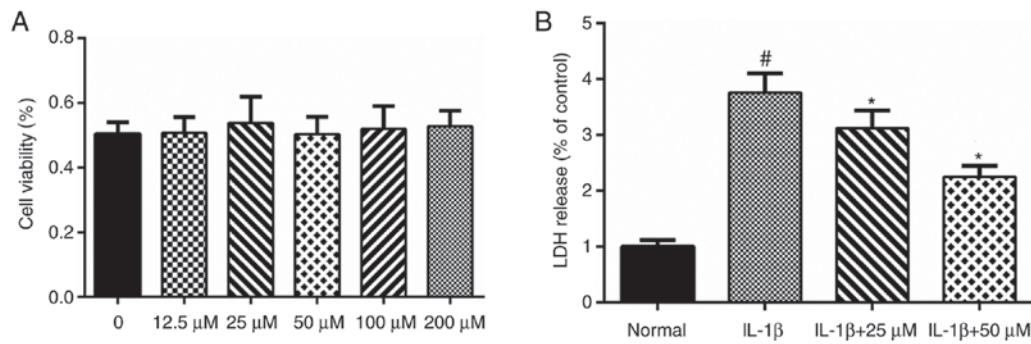


Figure 1. Effects of paeoniflorin on cell viability and LDH release. (A) Chondrocytes were treated with various concentrations of paeoniflorin (12.5-200 μM) for 72 h and examined with an MTT assay. Chondrocytes incubated in medium were used as a control and considered 100% viable. (B) Chondrocytes were treated with various concentrations of paeoniflorin for 3 h and then incubated with IL-1β (10 ng/ml) for 24 h. Chondrocytes incubated in medium without paeoniflorin were viewed as a control and considered 100% viable. Data are presented as the mean ± standard deviation. [#]P<0.05 vs. Normal group; ^{*}P<0.05 vs. IL-1β group. LDH, lactate dehydrogenase; IL, interleukin.

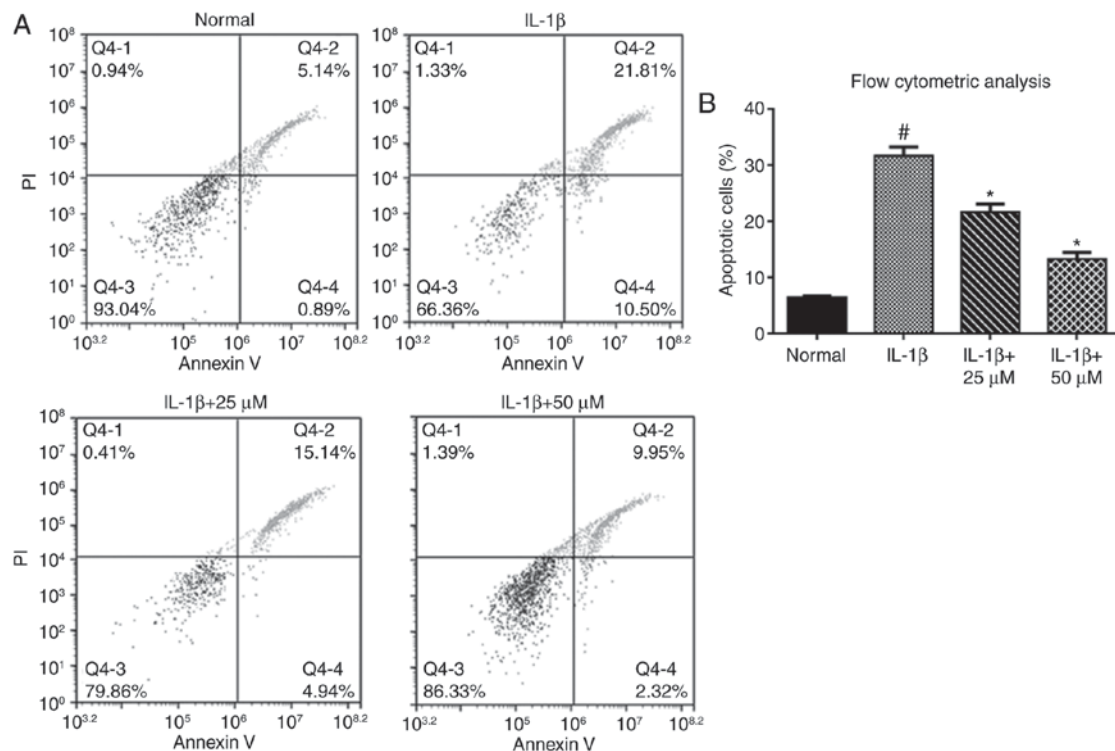


Figure 2. Determination of the rate of apoptosis using flow cytometry. (A) Annexin V/PI double staining assays of cells treated with or without paeoniflorin. (B) Untreated cells displayed a low background of staining with either Annexin V or PI. IL-1β-stimulation alone significantly increased the percentage of apoptotic chondrocytes. Annexin V- and PI-positive cells were markedly decreased in a dose-dependent manner following pre-treatment with 25 or 50 μM paeoniflorin. Data are presented as the mean ± standard deviation. [#]P<0.05 vs. Normal group; ^{*}P<0.05 vs. IL-1β group. PI, propidium iodide; IL, interleukin.

genes. The relative gene expression was calculated using the formula: $n = 100 \times 2^{-(\Delta Cq \text{ targeted gene} - \Delta Cq \text{ GAPDH})}$.

Western blot analyses of Bcl-2, Bax, Akt and phosphorylated Akt. Rat articular chondrocytes were plated onto 6-well plates at a density of 5×10^4 cells/cm². Then, the cells were treated using the same settings for RT-qPCR. After rinsing with ice-cold PBS, the cells were lysed using cell lysis buffer and boiled at 100°C for 10 min. Western blotting was carried out following our reported protocol. Targeted protein was probed with primary antibodies against Bax (Cell Signaling Technology, Inc.), Bcl-2, protein kinase B (Akt), and phosphorylated Akt

(p-Akt; Abcam, Cambridge, UK). After incubation with horse radish peroxidase (HRP)-labeled secondary antibodies, the blots were detected using enhanced chemiluminescent (ECL) substrate and exposure to Kodak X-Omat film.

Statistical analysis. All experiments were performed in triplicate. Results are expressed as the mean ± standard deviation (SD) of three experiments. The data were evaluated using one-way ANOVA and followed by Dunnett's analysis. Statistical significance was set at P<0.05. The statistical analyses were performed with SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL, USA).

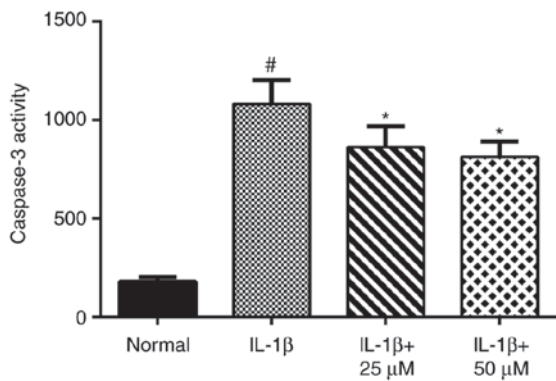


Figure 3. Effects of paeoniflorin on caspase-3 activity. Without pre-treatment with paeoniflorin, caspase-3 activity was significantly increased in IL-1 β -stimulated cells. Paeoniflorin (25 and 50 μ M) significantly decreased IL-1 β -induced caspase-3 activity. Data are presented as the mean \pm standard deviation. [#] P <0.05 vs. Normal group; ^{*} P <0.05 vs. IL-1 β group. IL, interleukin.

Results

Effects of paeoniflorin on LDH release. In our previous study of paeoniflorin and chondrocytes (unpublished data), we found that paeoniflorin concentrations from 12.5 to 100 μ M caused no significant toxicity to chondrocytes (Fig. 1A). Therefore, in the present study, we used paeoniflorin concentrations from 25 to 50 μ M. According to the LDH release assay, IL-1 β significantly increased the levels of LDH release. Paeoniflorin (25-50 μ M) suppressed the LDH release induced by IL-1 β in a dose-dependent manner and showed a protective effect *in vitro* (Fig. 1B).

Paeoniflorin suppresses IL-1 β -induced chondrocyte apoptosis. We assessed chondrocyte apoptosis by flow cytometric analysis. The percentage of apoptotic chondrocytes was significantly increased in the IL-1 β group compared with the controls. When chondrocytes were pretreated with paeoniflorin for 3 h, a decrease in the percentage of apoptotic chondrocytes was observed compared with the IL-1 β alone group (Fig. 2A and B).

Paeoniflorin inhibits IL-1 β -induced apoptosis by suppressing caspase-3 activity. After treatment of chondrocytes with IL-1 β for 24 h, caspase-3 activity increased significantly. However, chondrocytes treated with paeoniflorin exhibited markedly decreased caspase-3 activity caused by IL-1 β stimulation (Fig. 3).

Paeoniflorin suppresses the apoptotic pathway mediated by Bcl-2 and Bax. Using RT-qPCR (Fig. 4) and western blot analyses (Fig. 5), IL-1 β -stimulation alone significantly increased the level of Bax and decreased the level of Bcl-2. Moreover, the production of Bax in the low- and high-dose paeoniflorin pretreated groups was significantly decreased compared with the IL-1 β group (P <0.05). However, the transcript and protein levels of Bcl-2 in the paeoniflorin groups were significantly increased compared with the IL-1 β group (P <0.05).

Effects of paeoniflorin on the Akt pathway in IL-1 β -treated chondrocytes. In the present study, we also examined Akt and p-Akt to evaluate the involvement of the Akt pathway. As expected, the western blot assay showed that IL-1 β -stimulation alone decreased the phosphorylation level of Akt, which was consistent with another study (24286347). Different concentrations of paeoniflorin increased the IL-1 β -induced activation of Akt, as shown in Fig. 6 (P <0.05 vs. IL-1 β cells).

Discussion

Paeoniflorin not only has various pharmacological effects, it also exhibits low toxicity and few side-effects against different cell types (13). In an experimental model of intervertebral disc degeneration, paeoniflorin was shown to hinder the Bcl-2/caspase-9 pathway, which resulted in the inhibition of nucleus pulposus cell apoptosis. This demonstrates the close link between paeoniflorin and the musculoskeletal system. To date, little has been reported on the effects of paeoniflorin on chondrocytes (10). In the present study, we investigated the anti-apoptotic effects of paeoniflorin *in vitro*. Treatment of IL-1 β -induced rat articular chondrocytes

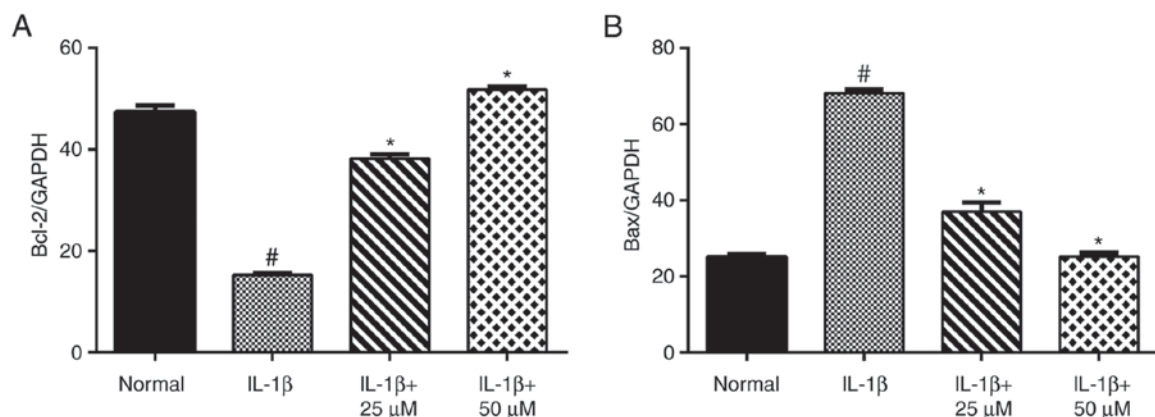


Figure 4. Effects of paeoniflorin on the mRNA expression of (A) Bcl-2 and (B) Bax *in vivo*. The mRNA expression of Bax was significantly increased, while Bcl-2 expression was decreased in IL-1 β -stimulated cells. Paeoniflorin significantly decreased the production of Bax and increased the expression of Bcl-2 induced by IL-1 β . Data are presented as the mean \pm standard deviation. [#] P <0.05 vs. Normal group; ^{*} P <0.05 vs. IL-1 β group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; IL, interleukin.

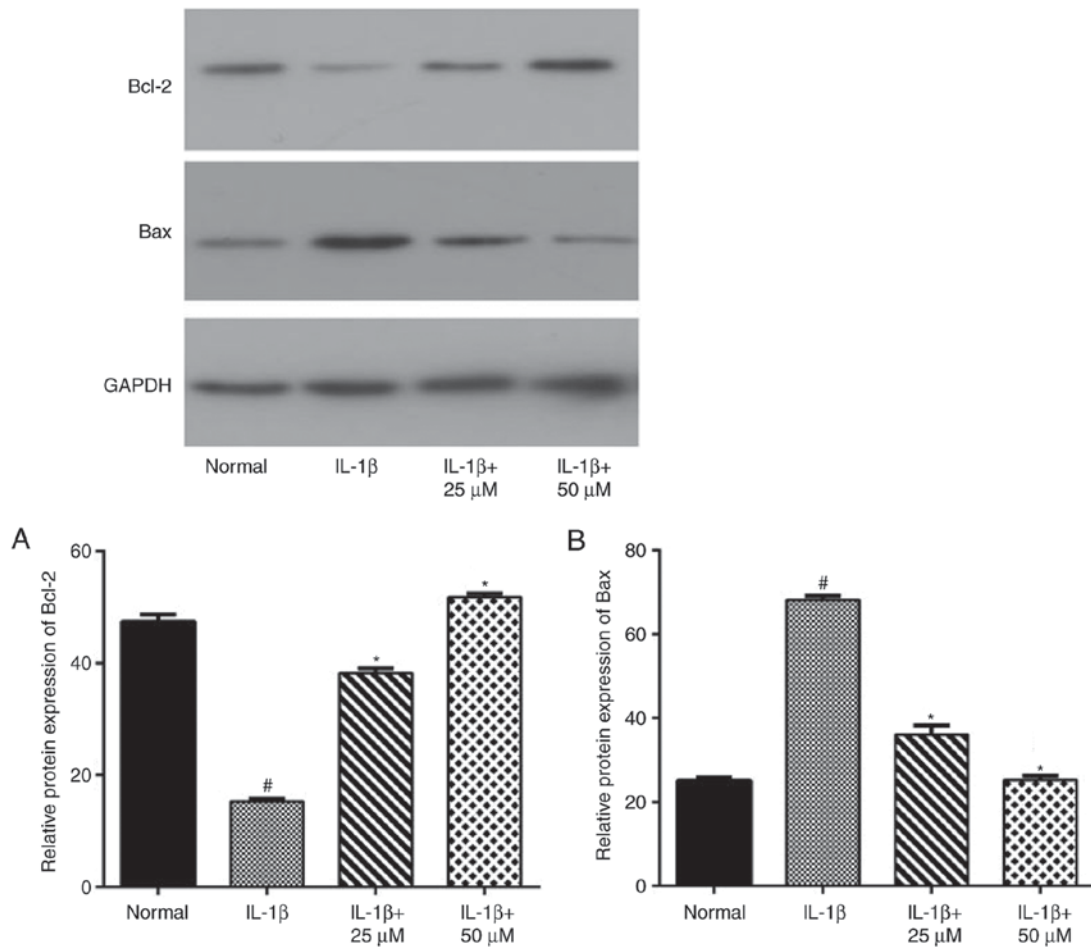


Figure 5. Protein synthesis of Bcl-2 and Bax in IL-1 β -treated chondrocytes. Following treatment with paeoniflorin, the protein level of (A) Bcl-2 significantly increased and (B) Bax decreased. Data are presented as the mean \pm standard deviation. [#]P<0.05 vs. Normal group; ^{*}P<0.05 vs. IL-1 β group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; IL, interleukin.

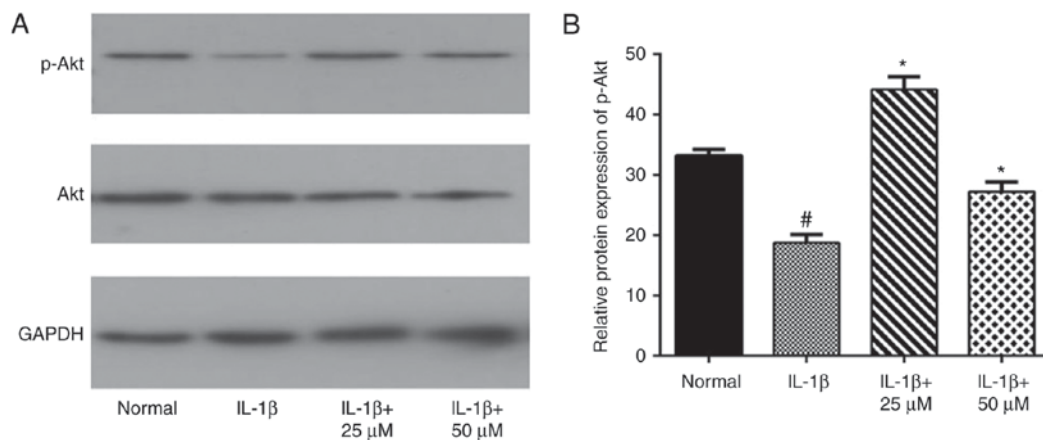


Figure 6. Effects of paeoniflorin on the Akt signaling pathway in IL-1 β -treated chondrocytes. (A) Image and (B) quantification of western blot analysis, which indicated that paeoniflorin increased the IL-1 β -induced activation of Akt. Data are presented as the mean \pm standard deviation. [#]P<0.05 vs. Normal group; ^{*}P<0.05 vs. IL-1 β group. Akt, protein kinase B; p-, phosphorylated; IL, interleukin.

with paeoniflorin decreased the rate of apoptosis by regulating the production of Bcl-2 family proteins. Moreover, IL-1 β -induced caspase-3 activity was abolished by high-dose paeoniflorin.

Apoptosis is a normal physiological process that is a critical step in the progression of osteoarthritis (OA) (14). In several

immunohistochemical studies of cartilage specimens obtained from OA patients, the results indicated that apoptosis-positive cells were closely related to the process of OA (15). Bcl-2 family proteins are key factors in the apoptotic process (16). Bcl-2 family proteins can be divided into an anti-apoptotic group (e.g., Bcl-2 and Bcl-2-like 1 protein extra-large) and

a pro-apoptotic group (e.g., Bax, and Bcl-2-like protein 11) (17). As a classical anti-apoptotic protein, Bcl-2 mainly inhibits the release of cytochrome c and blocks the activation of caspase-9 (18). Bax, a bcl-2-like protein 4, was found in the cytosol and is involved in the initiation of apoptosis (19). The ratio of Bax/Bcl-2 protein determines whether the cell will survive or undergo apoptosis (20). Karaliotas *et al* (21) found that the level of Bax transcripts in the OA group was significantly higher than that in the control group, while the Bcl-2/Bax was significantly decreased in the OA group. Studies have also shown that IL-1 β induces chondrocyte apoptosis by regulating the expression of Bcl-2/Bax (22). In the present study, we found that IL-1 β (10 ng/ml) significantly inhibited both the protein and gene expression of Bcl-2 and increased the level of Bax, which was consistent with the previous results.

The caspase family also plays an essential role in chondrocyte apoptosis (23). Sharif *et al* (24) demonstrated that the expression of apoptosis-related mediators such as caspase-3 was higher in OA cartilage compared with non-arthritic controls, as analyzed with TUNEL assay and immunohistochemistry. Without pretreatment with paeoniflorin, IL-1 β significantly increased caspase-3 activity compared with the normal group. Treatment with 25 or 50 μ M paeoniflorin decreased caspase-3 activity, demonstrating that paeoniflorin exerts an anti-apoptotic effect by blocking the activation of caspase-3 (50 μ M paeoniflorin was the optimum concentration).

Akt, also called protein kinase B, has several important physiological functions and is involved in cell survival (25). Specifically, the activated PI3K/Akt pathway has been implicated in chondrocyte survival (26). A previous study that focused on the role of Akt in paeoniflorin-induced gastric carcinoma suggested that paeoniflorin induces apoptosis by suppressing PI3K/Akt signaling (27). In our research, it was clear that Akt activated by paeoniflorin was involved in the chondroprotective effect of paeoniflorin on IL-1 β -induced apoptosis. However, the precise mechanism by which Akt controls this process is not entirely understood and further studies are needed.

In summary, we determined that paeoniflorin blocked IL-1 β -induced LDH release and decreased the percentage of apoptotic cells. Paeoniflorin also exhibited a chondroprotective effect by downregulating both the mRNA and protein expression of Bax and increasing the level of Bcl-2. Paeoniflorin also reduced the activity of caspase-3 in chondrocytes. Furthermore, paeoniflorin regulates the Akt signaling pathway by increasing the phosphorylation of Akt. These results demonstrate that paeoniflorin plays an anti-apoptotic role in the progression of OA and may be useful in the treatment of OA.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

PFH and LDW conceived and designed the study. PFH, WPC and JPB performed the experiments, and PFH and LDW wrote the present study. PFH, WPC, JPB and LDW reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The animal experiments performed in this study were approved by the University of Zhejiang Institutional Animal Care and Use Committee (Hangzhou, Zhejiang, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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