Paeoniflorin inhibits IL-1β-induced MMP secretion via the NF-κB pathway in chondrocytes

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Abstract. Paeoniflorin serves important cellular roles, exerting anti-cancer, anti-inflammatory and anti-pulmonary fibrosis effects and possesses immune-modulatory properties. However, the exact role of paeoniflorin in the pathogenesis of osteoarthritis (OA) remains unclear. The aim of the present study was to investigate the effects of paeoniflorin on articular surfaces *in vitro*. Rat chondrocytes were cultured *in vitro* and an MTT assay was performed to assess chondrocyte survival. Following treatment with interleukin (IL)-1β and paeoniflorin, the production of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases-1 (TIMP-1) was examined using reverse transcription-quantitative polymerase chain reaction and western blotting. The interleukin (IL)-1β-induced nuclear factor (NF)-κB pathway activation was also investigated. The results demonstrated that paeoniflorin was able to down-regulate the expression of MMP and increase the expression of TIMP-1ntmRNA and protein in IL-1β-induced rat chondrocytes. Furthermore, treating chondrocytes with paeoniflorin blocked the activation of NF-κB. These results suggest that paeoniflorin may serve am anti-catabolic role in the progression of OA and may be an effective preventative treatment for OA.

Introduction

Osteoarthritis (OA), which is the most common joint disorder, is characterized by the destruction of articular cartilage and osteophyte formation (1). OA has been recognized as a slowly progressing whole-joint soft tissue disease, characterized by synovial inflammation and subchondral bone degradation (2). During the development of OA, the imbalance between synthesis and degradation of extracellular matrix remodeling may lead to erosion of articular cartilage (3). Matrix metalloproteinases (MMPs) are considered to be the most important catabolic enzymes associated with the pathogenesis of OA due to their ability to digest collagen fibers and proteoglycans (4). Tissue inhibitors of metalloproteinases (TIMPs), which inhibit MMP activity, also serve an important role in the catabolic and anabolic processes of cartilage matrix maintenance (5). Additionally, pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6, are able to stimulate the expression of MMP and the subsequent development of OA (6,7).

Paeoniflorin, a pinane monoterpene glucoside, was first isolated from plants in the Ranunculaceae family in the 1960s (8). Recent studies have reported that paeoniflorin exhibits a number of pharmacological activities, including anti-cancer, anti-inflammation, anti-pulmonary fibrosis and anti-spasmodic effects (9-11). In nucleus pulposus cells, paeoniflorin serves an anti-apoptotic role via regulating B-cell lymphoma-2 family and caspase expression (12). At present, the association between paeoniflorin and chondrocytes remains unclear. A recent study demonstrated that paeoniflorin-6'-O-benzene (CP-25), a derivative of paeoniflorin, is able to decrease the production of ILs and TNF-α whilst increasing the expression of transforming growth factor-β in an adjuvant-induced arthritis model (13). Furthermore, CP-25 decreased the expression of receptor activator of nuclear factor (NF)-κB ligand and MMP-9, which suggests that it may have an anti-arthritic effect and so may be used in the treatment of human rheumatoid arthritis (RA). Although MMPs are considered to be important for the pathophysiology of OA, the role of paeoniflorin in OA remains unclear.

The aim of the present study was to assess the protective effects of paeoniflorin on IL-1β-induced chondrocytes and measure the levels of MMP-1, MMP-3, MMP-13 and tissue TIMP-1 *in vitro*. In addition, the activation of the NF-κB pathway was measured using western blotting.

Materials and methods

Primary cultures of normal rat articular chondrocytes. A total of 24 Sprague-Dawley rats (male:female, 1:1; 4-week

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old; Animal Center of Zhejiang University, Hangzhou, China) were housed at 25˚C with 45-75% relative humidity and food and water at regular intervals with a 12 h light/dark cycle. Rat articular chondrocytes were isolated as previously described (14). Briefly, the rats were sacrificed by intraperitoneal injection with 10% chloral hydrate (4 ml/kg; Sigma Aldrich; Merck KGaA, Darmstadt, Germany). Rat knees were then disinfected with 75% alcohol and transferred into a sterile bench. Following this, knee joints were opened and the articular cartilage were carefully isolated, rinsed three times in PBS, cut into 1-3 mm^3 pieces and digested with 0.2% pronase (Sigma Aldrich; Merck KGaA) for 30 min at 37˚C followed by 0.1% collagenase (Sigma Aldrich; Merck KGaA) for 4 h at 37˚C. Cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing antibiotic-antimycotic solution and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 2 days. The present study was approved by the University of Zhejiang Institutional Animal Care and Use Committee, Zhejiang University.

Assessment of cell viability. The effect of paeoniflorin on chondrocyte proliferation was assessed using an MTT assay. Chondrocytes were seeded in 96-well plates at a density of $5x10^3$ cells/well and incubated with 0, 12.5, 25, 50, 100 and 200 μM paeoniflorin (Sigma Aldrich; Merck KGaA; Fig. 1) for 72 h at 37˚C. The absorbance at 570 nm was subsequently measured using a microplate reader and the cell viability was calculated. Paeoniflorin concentrations that did not induce significant cytotoxicity were considered non-cytotoxic and were selected for using in the following experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Isolated articular chondrocytes were seeded in a 6-well plate at a density of $5x10^5$ cells/well. Following 2 days of cultivation at 37˚C, cells were cultured in DMEM with 25 or 50 μM of paeoniflorin for 3 h at 37˚C, followed by co-incubation with rat recombinant IL-1β (10 ng/ml; Sigma Aldrich; Merck KGaA) for 24 h at 37˚C. The monolayer of chondrocytes was harvested and stored at -80˚C until use. Total RNA was extracted from chondrocytes using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (15). Briefly, 1 μg of total RNA was reverse transcribed in a system containing 10 pmol of random hexanucleotidic primers (Promega Corporation, Madison, WI, USA), 0.5 mM dNTPs and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega Corporation) at 37˚C for 1 h. qPCR was performed using the iQTM SYBR Green SuperMix PCR kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Thermocycling conditions included 45 cycles of 95˚C for 15 sec and 60˚C for 30 sec. Annealing temperatures and primers are listed in Table I. GAPDH was used as an internal control and the relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Western blotting. Rat articular chondrocytes were seeded on a 6-well plate at a density of $5x10^4$ cells/cm^2 for 2 days at 37˚C. The cells were cultured in DMEM with 25 or 50 μM of paeoniflorin for 3 h at 37˚C and followed by co-incubation

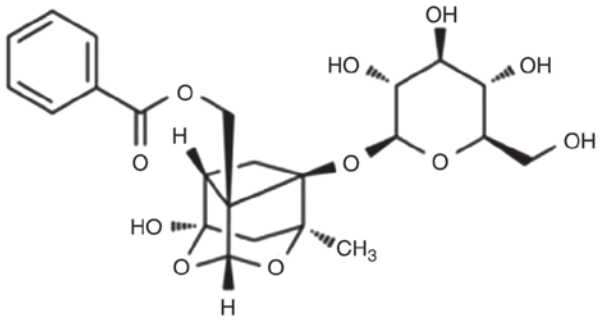

Figure 1. Chemical structure of paeoniflorin.

with 10 ng/ml rat recombinant IL-1β for 24 h at 37˚C. Cells were rinsed with ice-cold PBS, lysed using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Protein concentrations were determined by a BCA kit and the protein was boiled at 100˚C for 10 min. Western blotting was performed as previously described (17). Briefly, a total of 50 μg protein was loaded per lane. The samples were then separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. After blocking with 5% bovine serum albumin. (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, proteins were probed using primary antibodies against MMP-1, MMP-3, MMP-13, TIMP-1 (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), inhibitor of NF-κB (IκB-α), NF-κB p65 and β-actin (all Cell Signaling Technology, Danvers, MA, USA) at 4˚C overnight (Table II). Blots were subsequently incubated with horseradish peroxide-labeled secondary antibodies for 1 h at 37˚C, blots were visualized using an enhanced chemiluminescence substrate (Fude Biological Technology, Hangzhou, China) and exposure to Kodak X-Omat film (Kodak, Rochester, NY, USA) according to the manufacturer's protocol. The densitometry of the bands was quantified using the ImageJ software (version 1.45s; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experiments were performed in triplicate. Data are presented as the mean + standard deviation and were analyzed using one-way analysis of variance. Statistical analyses were performed using SPSS for Windows software (v19.0; IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of paeoniflorin on cell viability. To investigate whether paeoniflorin is cytotoxic to chondrocytes, cells were treated with varying concentrations of paeoniflorin and an MTT assay was performed. The results revealed that treatment with 12.5-200 μM paeoniflorin was not cytotoxic to chondrocytes (Fig. 2). Referring to previous results from Pubmed, concentrations ranging from 25 to 50 μM were used in the following experiments (18,19).

Effect of paeoniflorin on MMP-1, MMP-3, MMP-13 and TIMP-1 mRNA expression. Following treatment with rat recombinant IL-1β (10 ng/ml) and paeoniflorin (25 and 50 μM), the expression of MMP-1, MMP-3, MMP-13 and TIMP-1 were

Table I. Primers and conditions for reverse transcription-quantitative polymerase chain reaction.

Gene	Genbank accession	Direction	Primer sequences (5' to 3')	Size (bp)	Annealing temp (°C)
MMP-1	NM_001134530.1	Forward	GCTTAGCCTTCCTTTGCTGTTGC	136	60
		Reverse	GACGTCTTCACCCAAGTTGTAGTAG		
MMP3	NM_133523	Forward	CTGGGCTATCCGAGGTCATG	77	60
		Reverse	TGGACGGTTTCAGGGAGGC		
MMP13	NM_133530	Forward	CAACCCTGTTTACCTACCCACTTAT	85	60
		Reverse	CTATGTCTGCCTTAGCTCCTGTC		
TIMP-1	NM_053819	Forward	TCCCTGTTCAGCCATCCCTTG	96	60
		Reverse	TCGCTCTGGTAGCCCTTCTC		
GAPDH	NM_017008.4	Forward	GAAGGTCGGTGTGAACGGATTTG	127	60
		Reverse	CATGTAGACCATGTAGTTGAGGTCA		

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMPs.

Table II. Information of antibodies.

Name	Catalogue numbers	Dilution
MMP-1	sc-21731	1:1,000
MMP-3	sc-21732	1:1,000
MMP-13	sc-515284	1:1,000
TIMP-1	sc-21734	1:1,000
IκB-α	#9242	1:3,000
p65	#6956	1:500
β-actin	#3700	1:1,000
Goat anti-Mouse IgG	#31160	1:5,000
Goat anti-Rabbit IgG	#31210	1:5,000

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMPs; IκB-α, inhibitor of NF-κB; Ig, immunoglobulin.

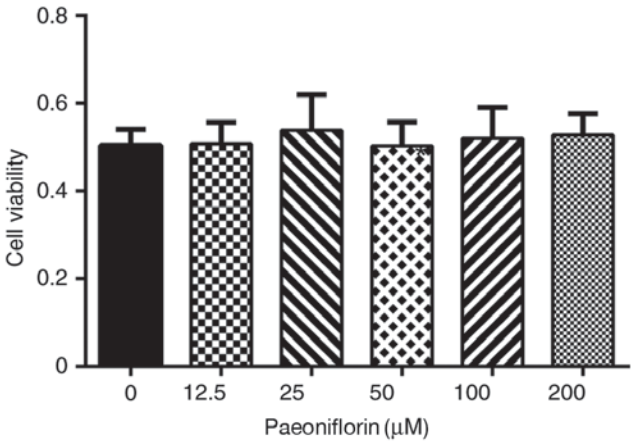

Figure 2. Effects of paeoniflorin on cell viability. Chondrocytes were treated with 0-200 μM paeoniflorin for 72 h and examined using the MTT assay.

examined using RT-qPCR. Stimulation with IL-1β resulted in a significant upregulation in MMP-1, MMP-3 and MMP-13 expression and a significant decrease in TIMP-1 expression compared with the negative control (P<0.05; Fig. 3). Treatment with 25 or 50 μM paeoniflorin significantly reversed the IL-1β-induced upregulation of MMP-1, MMP-3 and MMP-13 and downregulation of TIMP-1 (P<0.05; Fig. 3).

Effect of paeoniflorin on protein synthesis of MMP-1, MMP-3, MMP-13 and TIMP-1. Following treatment with IL-1β, the expression of MMP-1, MMP-3 and MMP-13 was significantly upregulated (P<0.05; Fig. 4A and B), whereas the expression of TIMP-1 was significantly decreased compared with the negative control group (P<0.05; Fig. 4A and B). Compared with IL-1β treatment alone, the expression of MMP-1, MMP-3 and MMP-13 was significantly decreased (P<0.05; Fig. 4A and B) and the expression of TIMP-1 was significant increased in cells treated with 25 or 50 μM paeoniflorin (P<0.05; Fig. 4A and B).

Effect of paeoniflorin on IκB-α degradation and NF-κB activation in IL-1β-treated chondrocytes. Activation of the NF-κB pathway was assessed using western blotting. NF-κB p65 levels were significantly increased and IκB-α levels were significantly decreased in chondrocytes treated with IL-1β alone (P<0.05; Fig. 5A and B). The effects of IL-1β were significantly reversed by treatment with 25 or 50 μM paeoniflorin (P<0.05; Fig. 5A and B).

Discussion

Paeoniflorin is commonly used as a Chinese medicine anti-inflammatory treatment for autoimmune diseases (20-22). At present, little is known about the effects of paeoniflorin on OA. In the present study it was demonstrated that paeoniflorin exhibits a significant chondroprotective effect *in vitro*.

Previous studies have reported an association between paeoniflorin and arthritis (23,24). Paeoniflorin has been demonstrated to exhibit a potential protective effect against RA in rat models by decreasing the production of IL-1β and TNF-α, thus impeding the progression of arthritis and bone erosion (25). Jia and He (24) reported that paeoniflorin ameliorated RA in rats by decreasing the activity of the NF-κB p65 unit, TNF-α, IL-1β and IL-6, as well as reducing cyclooxygenase-2 protein expression. In human pancreatic

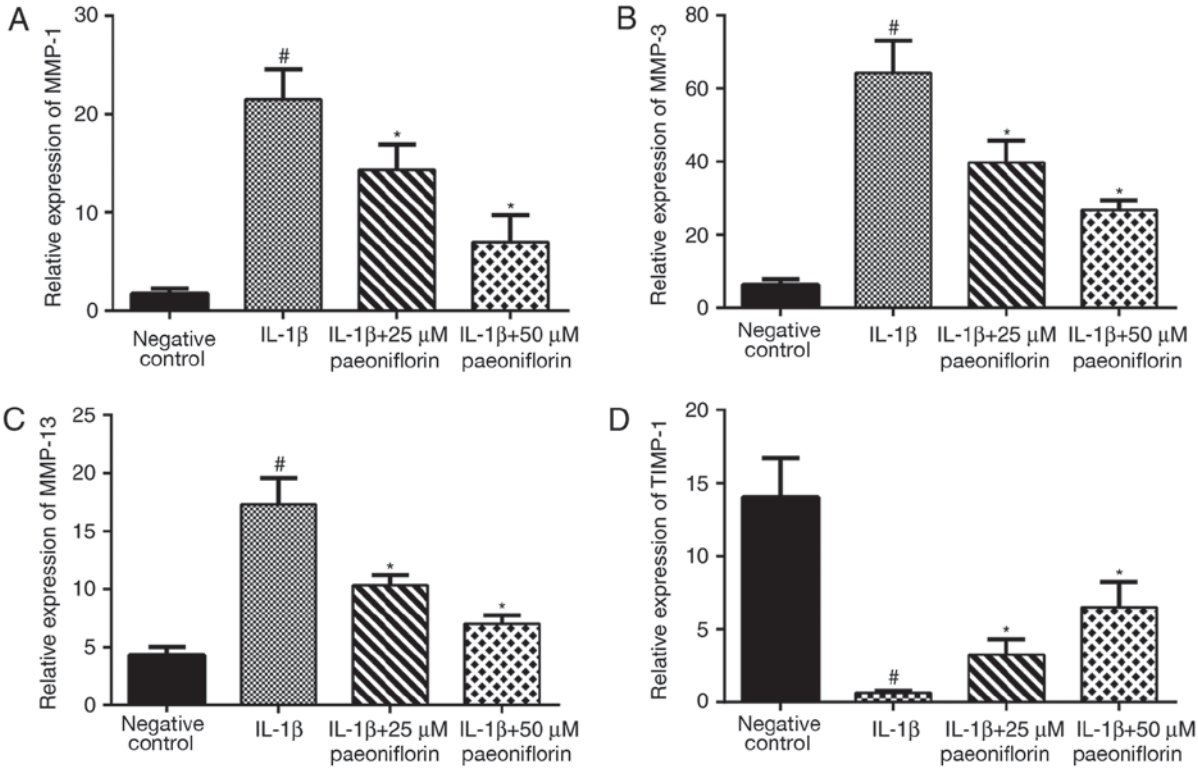

Figure 3. Effects of paeoniflorin on the expression of (A) MMP-1, (B) MMP-3, (C) MMP-13 and (D) TIMP-1. Chondrocytes were pretreated with 0, 25 or 50 μM paeoniflorin for 3 h, followed by stimulation with IL-1β (10 ng/ml) for 24 h. Gene expression was assessed using reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. IL-1β alone and #P<0.05 vs. negative control. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; IL, interleukin.

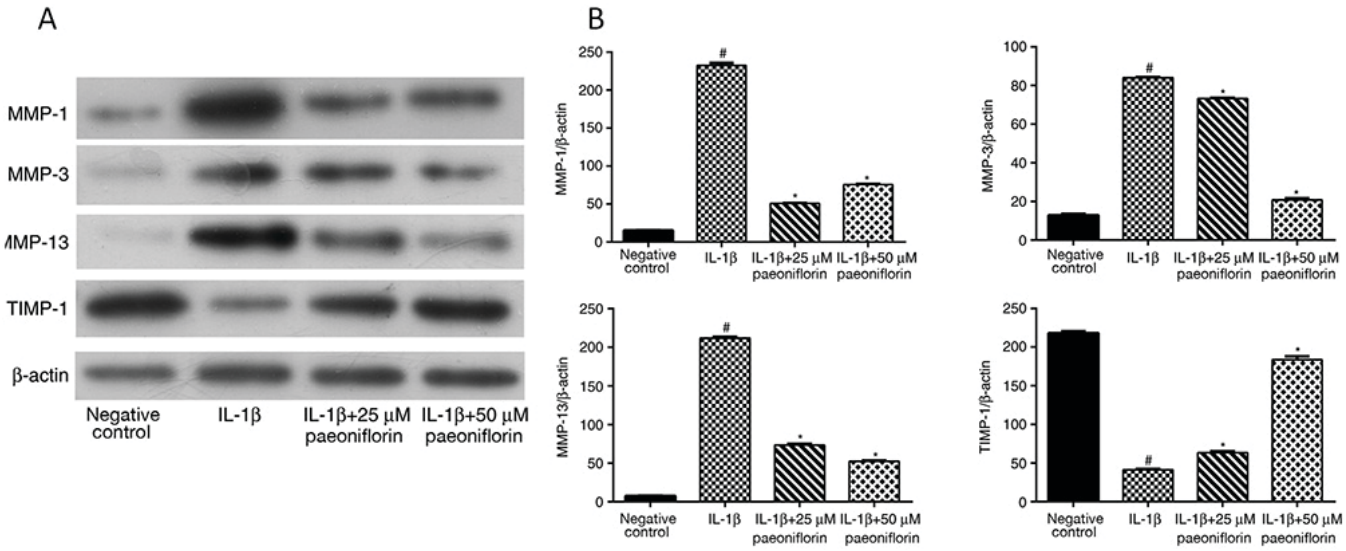

Figure 4. Effects of paeoniflorin on the expression of MMPs and TIMP-1. (A) Western blotting and (B) quantified western blotting results for MMP-1, MMP-3, MMP-13 and TIMP-1. Chondrocytes were pretreated with 0, 25 or 50 μM paeoniflorin for 3 h, followed by stimulation with IL-1β (10 ng/ml) for 24 h. Protein expression was assessed using western blotting with β-actin as a control. *P<0.05 vs. IL-1β alone and #P<0.05 vs. negative control. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; IL, interleukin.

cancer cells, paeoniflorin has potential anti-tumor functions, enhancing apoptosis via the inhibition of MMP-9 and extracellular-related kinase signaling (26). A recent study by Zhao *et al* (27) reported that paeoniflorin is able to reduce the IL-1β-induced upregulation of inflammatory mediators and MMPs in human chondrocytes, which is similar to the results of the present study.

The MMP family, in particular MMP-1, MMP-3 and MMP-13, is known to serve a role in the degeneration of articular cartilage matrix components (28). MMP-1 predominantly destroys fibrillar collagens, while MMP-3 decays extracellular cartilage matrix substrates (29,30). MMP-13, also referred to as collagenase-3, is an enzyme that serves a role in the degradation of type II collagen and is considered to

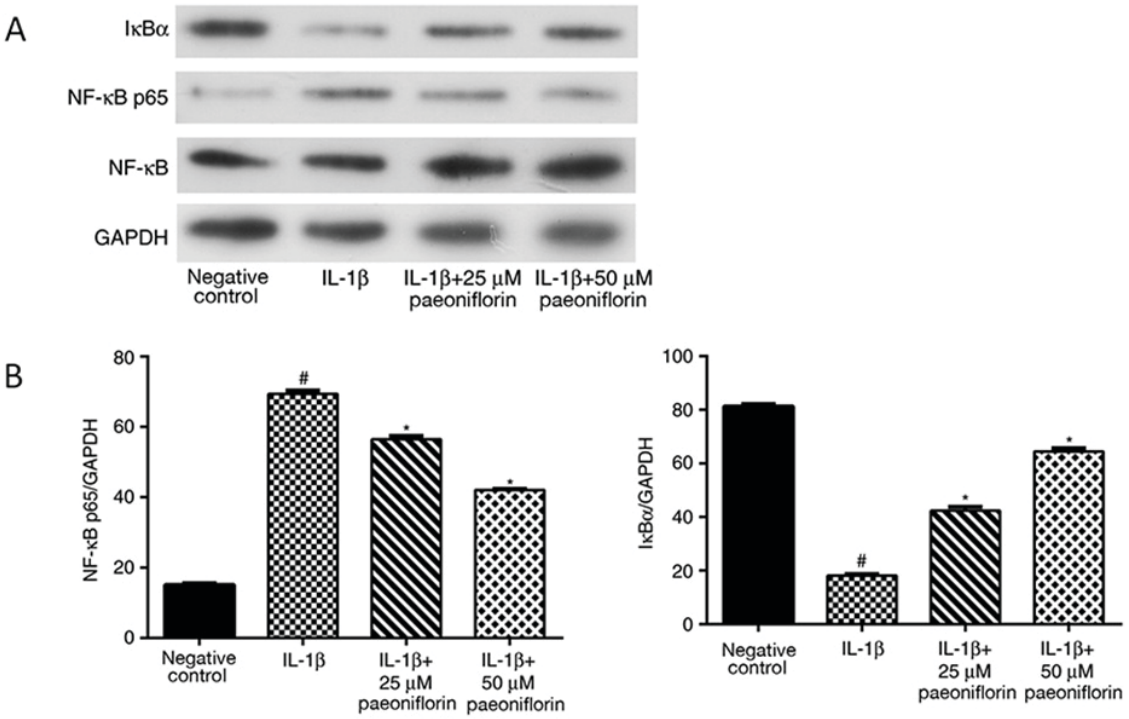

Figure 5. Effects of paeoniflorin on NF-κB and IκB-α expression. (A) Western blotting and (B) quantified western blotting results for NF-κB p65 and IκB-α. Chondrocytes were pretreated with 0, 25 or 50 μM paeoniflorin for 3 h, followed by stimulation with IL-1β (10 ng/ml) for 24 h. Protein expression was assessed using western blotting with GAPDH as a control. *P<0.05 vs. IL-1β alone and #P<0.05 vs. negative control. NF-κB, nuclear factor-κB; IκB-α, inhibitor of NF-κB; IL, interleukin.

be the principal collagenase associated with restructuring of the collagen matrix (31-33). However, MMP-induced cartilage disintegration may be ameliorated by reducing the expression of endogenous TIMPs (4). As MMPs and TIMP are essential for the pathophysiological progression of OA, the aim of the present study was to assess whether paeoniflorin serves an important role in OA by regulating the expression of MMPs. An *in vitro* model was produced by cultivating a monolayer of primary rat chondrocytes in a medium containing IL-1β, which is one of the most important pro-inflammatory cytokines released by chondrocytes (34,35). When cells were pre-treated with IL-1β, the expression of MMPs was significantly upregulated at the mRNA and protein levels, whereas TIMP-1 expression decreased compared with the control group. These results were consistent with a previous report (36). However, the IL-1β-induced changes in expression may be attenuated by pretreatment with paeoniflorin (25 and 50 μM), which exerts a protective effect by downregulating the expression of MMPs while upregulating TIMP-1. It is therefore possible that paeoniflorin is able to balance the MMP/TIMP ratio and may be used as a therapeutic treatment for OA.

The results of the present study revealed that NF-κB is associated with paeoniflorin-mediated MMP/TIMP system regulation. The NF-κB signaling pathway regulates the expression of several genes (37,38), including MMP-1, MMP-3 and MMP-13. NF-κB in the cytoplasm maintains an inactive form via binding with IκB (39). When stimulated with IL-1β, NF-κB dimers are activated via a series of signaling pathways, resulting in the phosphorylation and degradation of IκB (37). In the present study, paeoniflorin blocked the activation of NF-κB p65 by protecting IκB-α against degradation. It has previously been reported that paeoniflorin is able to inhibit the nuclear translocation of NF-κB by preventing IκBα phosphorylation in gastric carcinoma cells, which is in agreement with a previous *in vitro* study by our group (18,40).

The underlying mechanism of inflammation in chondrocytes exposed to paeoniflorin remains unclear. Further studies are required to elucidate the precise signal transduction pathway underlying paeoniflorin regulation in inflammatory processes. The present study is limited as rat articular chondrocytes were cultured in a monolayer only. In future studies, a dynamic three-dimensional chondrocyte culture system is strongly recommended (41). In addition, a number of additional catabolic enzymes and inflammatory factors serve key roles in the pathophysiology of OA, including a disintegrin and metalloproteinase with thrombospondin motifs and inducible nitric oxide synthase, which should be investigated in future studies.

In summary, the results of the present study demonstrate that paeoniflorin has a chondroprotective effect in an *in vitro* model via decreasing the expression of MMP-1, MMP-3 and MMP-13 whilst upregulating TIMP-1. Furthermore, it was revealed that this anti-catabolic effect was exerted by inhibiting the NF-κB pathway. These results suggest that paeoniflorin may be applied as an effective therapeutic for the treatment of OA.

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Availability of data and materials

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

Authors' contributions

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

Ethics approval and consent to participate

The present study was approved by the University of Zhejiang Institutional Animal Care and Use Committee, Zhejiang University (Zhejiang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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