SPANDIDOS PUBLICATIONS

INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 43: 2241-2251, 2019

Peimine suppresses interleukin-1β-induced inflammation via MAPK downregulation in chondrocytes

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Received August 24, 2018; Accepted March 13, 2019

DOI: 10.3892/ijmm.2019.4141

Abstract. Osteoarthritis (OA) is the most common type of degenerative joint disease and secreted inflammatory molecules serve a pivotal role in it. Peimine has been reported to have anti-inflammatory activity. In order to investigate the potential therapeutic role of Peimine in OA, mouse articular chondrocytes were treated with IL-1β and different doses of Peimine *in vitro*. The data revealed that Peimine not only suppressed IL-1β-induced production of nitric oxide (NO) and prostaglandin E2, but also reduced the protein levels of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2). In addition, Peimine inhibited the IL-1β-induced mRNA expression of matrix metalloproteinase (MMP)-1, MMP-3, MMP-9, MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5. Furthermore, Peimine inhibited IL-1β-induced activation of the mitogen-activated protein kinase (MAPK) pathway. The protective effect of Peimine on IL-1β-treated chondrocytes was attenuated following activation of the MAPK pathway, as demonstrated by the increased expression levels of MMP-3, MMP-13, ADAMTS-5, iNOS and COX-2 compared with the Peimine group. The *in vivo* data suggested that Peimine limited the development of OA in the mouse model. In general, the data indicate that Peimine suppresses IL-1β-induced inflammation in mouse chondrocytes by inhibiting the MAPK pathway, suggesting a promising therapeutic role for Peimine in the treatment of OA.

Introduction

Osteoarthritis (OA), which is characterized by progressive joint dysfunction and cartilage degradation, is the most prevalent joint disease and is considered as one of the major health problems, particularly for middle-aged and elderly people (1-3). Although the pathophysiology of OA remains poorly understood, an inflammatory component, which is marked by symptoms including joint pain and stiffness, is involved in OA development and progression (4,5).

IL-1β, which is significantly increased in chondrocytes as well as synovial cells of patients with OA, is known to serve a pivotal role in the pathogenesis of OA (6). Numerous studies have demonstrated that IL-1β can not only increase chondrocyte production of inflammatory mediators, including inducible nitric oxide (NO) synthase (iNOS), cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2), but also enhance chondrocyte expression of cartilage catabolic enzymes, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (7-9). IL-1β is considered to be one of the most promising targets for treating OA and IL-1β stimulation is used as a conventional *in vitro* model to study OA (10-14).

Peimine is the main compound extracted from *Bulbus Fritillariae* (BF). BF is a traditional Chinese medicine that has been used as an antitussive and antiasthma drug for >2,000 years due to its high therapeutic effects, low toxicity

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Abbreviations: OA, osteoarthritis; IL-1β, interleukin-1β; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; MAPK, mitogen-activated protein kinase; BF, Bulbus *Fritillariae*; MMT, medial meniscal tear

Key words: osteoarthritis, chondrocytes, interleukin-1β, mitogen-activated protein kinase, inflammation

and few side effects (15). Peimine has been reported to have antioxidant, anti-inflammatory and pain suppressing effects (16-19). However, to the best of our knowledge, the effects of Peimine on OA have not been studied. Therefore, the aim of the present study was to investigate the potential beneficial effects and the underlying mechanism of Peimine on OA using both *in vivo* and *in vitro* models.

Materials and methods

Medium and reagents. Peimine was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; cat. no. SMB00446), dissolved in DMSO and stored at -20˚C until use. Anisomycin (cat. no. SC-3524) and U-46619 (cat. no. SC-201242) were gained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). IL-1β was obtained from R&D Systems, Inc. (Minneapolis, MN, USA; cat. no. 401-ML), dissolved in 0.1% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and stored at -80˚C prior to use. A PGE2 ELISA kit was purchased from R&D Systems, Inc., (cat. no. KGE004B), the Nitric Oxide Assay kit was acquired from Abcam (Cambridge, UK; cat. no. ab65328) and the Cell Counting Kit-8 (CCK)-8 kit was purchased from Sigma-Aldrich (Merck KGaA; cat. no. 96992). The Prime Script reverse transcription (RT) reagent kit and SYBR for the RT-quantitative polymerase chain reaction (PCR) experiments were obtained from Takara Bio, Inc. (Otsu, Japan). Primary antibodies of iNOS (cat. no. 13120), COX-2 (cat. no. 4842), P38 (cat. no. 9212), phosphorylated (P)-P38 (Thr180/Tyr192; cat. no. 4511), ERK (cat. no. 4695), P-ERK (Thr202/Tyr204; cat. no. 4370), JNK (cat. no. 9252) and P-JNK (Thr183/Tyr185; cat. no. 4668) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies of MMP-3 (cat. no. 1908-1) and MMP-13 (cat. no. 1923-1) were acquired from Epitomics, Inc., (Abcam), the primary antibody for ADAMTS-5 (cat. no. PA5-14350) was purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA) and the primary antibody of GAPDH was obtained from Wuhan Boster Biological Technology, Ltd., (Wuhan, China; cat. no. BM3876). Horseradish peroxidase-conjugated secondary antibodies, including anti-rabbit immunoglobulin (Ig)G (cat. no. W4011) and anti-mouse IgG (cat. no. W4021), were acquired from Promega Corporation (Madison, WI, USA). The basic medium consisted of low glucose Dulbecco's modified Eagles medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine, 50 U/ml penicillin and 0.05 mg/ml streptomycin.

Primary chondrocyte isolation and culture. Primary chondrocytes were isolated from the femoral heads, femoral condyles and the tibial plateau of 4-day-old C57BL/6J mice as described previously (20). Briefly, the cartilage was minced into 1-mm^3 pieces and digested in 3 mg/ml collagenase D solution for 45 min at 37˚C twice. Subsequently, the cartilage pieces were retrieved and digested in 0.5 mg/ml collagenase D solution overnight. The digestion solution was then retrieved and centrifuged at 400 x g at room temperature for 5 min to obtain the chondrocytes. The isolated chondrocytes were placed on a treated plate and cultured in the basic medium at 37˚C with 5% CO_2. The second passage was used for the experiments.

Cell viability assay. The chondrocytes were seeded in 96-well plates at the same density (1x10^4 cells/well) and cultured in the basic medium for 24 h. Subsequently, the cells were treated with the vehicle (DMSO) or different concentrations of Peimine (5, 10 and 20 μg/ml) with or without IL-1β (10 ng/ml) for a further 24 h. The CCK-8 was used to quantify cell viability according to the manufacturer's protocol.

NO and PGE2 measurement. The chondrocytes were seeded in 6-well plates at the same density (3x10^5 cells/ml) and cultured in the basic medium for 24 h. Subsequently, the cells were treated with the vehicle (DMSO) or different doses of Peimine (5, 10 and 20 μg/ml) with or without IL-1β (10 ng/ml) for a further 24 h. The culture medium supernatant from each sample was harvested and kept for further analysis. The fresh culture medium was used as a blank control. The NO concentration was measured using the Nitric Oxide Assay kit and the PGE2 concentration was measured using the PGE2 ELISA kit according to the respective manufacturer's protocol. All assays were performed in duplicate.

Western blot analysis. The western blot analysis was performed as previously described (21). The chondrocytes were seeded in 6-well plates at a density of 3x10^5 cells/ml and cultured for 24 h in the basic medium. Subsequently, the cells were treated with the vehicle (DMSO) or different doses of Peimine (5, 10 and 20 μg/ml) with or without IL-1β (10 ng/ml). The total protein from the chondrocytes was collected using radioimmunoprecipitation assay lysis buffer acquired from Sigma-Aldrich (Merck KGaA). A bicinchoninic acid protein assay kit obtained from Thermo Fisher Scientific, Inc., (cat. no. 23225) was used to measure the concentrations of the proteins. A total of 40 μg protein from each sample was loaded on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After blocking in 5% non-fat milk for 1 h at room temperature, the membranes were incubated with the aforementioned primary antibodies at 4˚C overnight (GAPDH primary antibody was diluted 1:400, all the other primary antibodies were diluted 1:1,000). The membranes were washed twice and then incubated with the corresponding secondary antibodies for 1 h (diluted 1:4,000). Subsequently, the ECL reagent from Millipore (Billerica, MA, USA; cat no. 345818-100ML) was applied to the membranes and the immunoreactive proteins were detected using premium autoradiography films (Denville Scientific, Holliston, MA, USA; cat. no. E3218). Finally, the grey value of the bands was quantified using ImageJ2 software (National Institute of Health, Bethesda, MD, USA) (22).

RNA isolation and RT-qPCR. The chondrocytes were placed in 6-well plates at the same density (3x10^5 cells/ml) and cultured in the basic medium for 24 h. Subsequently, the cells were treated with the vehicle (DMSO) or different concentrations of Peimine (5, 10 and 20 μg/ml) with or without IL-1β (10 ng/ml) for the following 24 h. Total RNA from each well was isolated using a RNAeasy Mini kit from Qiagen, Inc., (Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using the Prime Script RT reagent kit. qPCR was performed using SYBR and the thermocycling condition was as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 30 sec, and a final extension step at 72˚C for 3 min. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (23). GAPDH was used as the housekeeping gene to normalize the expression of the target genes. The primer sequences were as follows: GAPDH forward 5'-TGCACCACCAACTGCTTAG-3' and reverse 5'-GGATGCAGGGATGATGTTC-3' (24); MMP-1 forward 5'-AACTACATTTAGGGGAGAGGTGT-3', and reverse 5'-GCAGCGTCAAGTTTAACTGGAA-3' (25); MMP-3 forward 5'-TCCTGATGTTGGTGGCTTCAG-3', and reverse 5'-TGTCTTGGCAAATCCGGTGTA-3' (26); MMP-9 forward 5'-ACCACATCGAACTTCGA-3', and reverse 5'-CGACCATACAGATACTG-3' (27); MMP-13 forward 5'-TGATGGACCTTCTGGTCTTCTGG-3', and reverse 5'-CATCCACATGGTTGGGAAGTTCT-3' (27); ADAMDTS-4 forward 5'-CATCCGAAACCCTGTCAACTTG-3', and reverse 5'-GCCCATCATCTTCCACAATAGC-3' (27); ADAMDTS-5 forward 5'-GCCATTGTAATAACCCTGCACC-3', and reverse 5'-TCAGTCCCATCCGTAACCTTTG-3' (27).

Establishment of the murine OA model. The animal experiment was designed and conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health, and was approved by the Ethics Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The mice were housed under standard laboratory conditions (20-25˚C; humidity, 40-70%; 16/8-h light/dark cycle), and provided with food and water *ad libitum*. A total of 15, 12-week-old C57BL/6 male mice were purchased from the Experimental Animal Center of Tongji Medical College and were divided randomly into the following three groups (n=5): Sham/Vehicle group (sham operation + vehicle treatment), OA/Vehicle group (OA operation + vehicle treatment) and OA/Peimine group (OA operation + Peimine treatment). The right knee of each mouse was used for the experiments. Intraperitoneal injections of xylazine (10 mg/kg) and ketamine (100 mg/kg) were performed to anesthetize mice. For the mice in the OA/Vehicle and OA/Peimine groups, OA was induced using the medial meniscal tear (MMT) model, in which the medial collateral ligament and medial meniscus of the knee were identified and excised, and the other structures were preserved. In the Sham/Vehicle group, the mice underwent sham operations; the joint cavity was opened without transection of the medial meniscus ligament and medial meniscus. Following surgery, the mice in the OA/Peimine group were administered Peimine (20 mg/kg) 5 days a week for 8 weeks. The Peimine was first dissolved in 20% ethanol to produce the 5 mg/ml working solution and then the solution was administered to mice by oral gavage at a dose of 4 ml/kg. For the mice in the Sham/Vehicle and OA/Vehicle groups, a vehicle solution (20% ethanol in distilled water) was administered by oral gavage at a dose of 4 ml/kg for 5 days a week. After a total period of 8 weeks, all mice were sacrificed with an overdose injection of pentobarbital (180 mg/kg) and the right knee joints of each mouse were collected for further evaluation.

Histological analysis. The right knee joints from the mice were dissected and fixed in 4% paraformaldehyde overnight at 4˚. Subsequently, the samples were decalcified in 10% EDTA at 4˚C for 4 weeks and embedded in paraffin. The specimens were cut into 5-μm-thick sections along the sagittal plane. The tissue sections were stained with Safranin O/Fast Green at room temperature for 45 min using Safranin O/Fast Green staining kit (ScienCell Research Laboratories lnc., Carlsbad, CA, USA; cat. no. 8348) according to the manufacturer's protocol. The severity of OA-associated alterations were assessed under a microscope using the Osteoarthritis Research Society International (OARSI) histopathology scoring system (28). Two pathologists (K. Chen and W. Huang) independently reviewed the severity of OA and their results were then compared. In cases of a discrepancy between the two reviewers, a third investigator (P. Cheng) was consulted until a mutual consensus was reached.

Statistical analysis. All experiments were repeated at least three times. All data are expressed as the mean ± standard deviation. Differences between groups were analyzed by one-way analysis of variance with the Tukey-Kramer honest significant difference test using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). The unpaired t-test was used for comparisons between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of Peimine on chondrocyte viability. The potential cytotoxicity of Peimine on mouse chondrocytes was tested using a CCK-8 kit. The chondrocytes were treated with a vehicle or different doses of Peimine (5, 10 and 20 μg/ml) in the presence or absence of IL-1β for 24 h. As presented in Fig. 1A and B, the different doses of Peimine exhibited no cytotoxicity on the mouse chondrocytes. Therefore, these concentrations of Peimine (5, 10 and 20 μg/ml) were used in the subsequent experiments (Fig. 1).

Effect of Peimine on IL-1β-induced NO and PGE2 production in chondrocytes. It has been reported that IL-1β can induce production of inflammatory mediators such as NO and PGE2 in chondrocytes (29,30). In order to evaluate the effect of Peimine on IL-1β-induced NO and PGE2 production, mouse chondrocytes were stimulated with IL-1β (10 ng/ml) and treated with different doses of Peimine (0, 5, 10 and 20 μg/ml) for 24 h. The NO concentration was measured using a Nitric Oxide Assay kit and the PGE2 concentration was measured using a PGE2 ELISA kit. As expected, the concentrations of NO and PGE2 were significantly elevated following treatment with IL-1β compared with the control group ($P<0.01$; Fig. 2). Furthermore, treatment with Peimine significantly reduced IL-1β-induced NO and PGE2 production in a dose-dependent manner ($P<0.05$; Fig. 2).

Effect of Peimine on IL-1β-induced protein expression of iNOS and COX-2 in chondrocytes. The effect of Peimine on IL-1β-induced protein expression of inflammatory mediators, including iNOS and COX-2, was evaluated using western blot analysis. As expected, following stimulation with IL-1β (10 ng/ml, 24 h), the protein expression levels of iNOS and COX-2 were significantly increased compared with the control ($P<0.01$; Fig. 3). However, when the chondrocytes

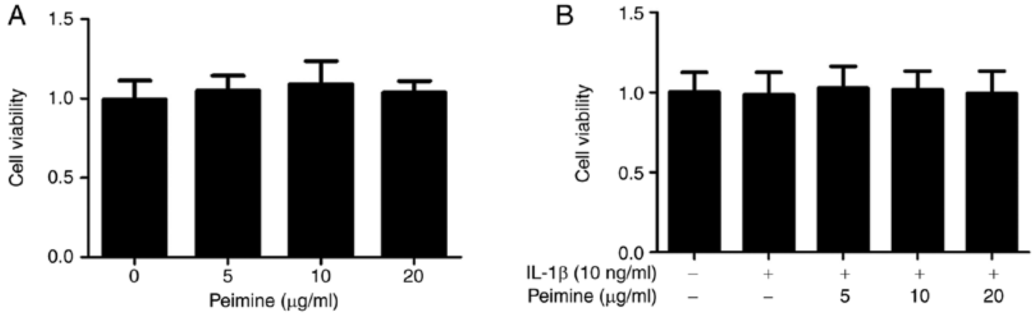

Figure 1. Effect of Peimine on chondrocyte viability. Cells were treated with Peimine (5, 10, 20 µg/ml) or a vehicle in the absence or presence of IL-1β (10 ng/ml) for 24 h. Cell viability was measured using Cell Counting Kit-8. (A) Effect of Peimine on chondrocyte viability without IL-1β stimulation. (B) Effect of Peimine on chondrocyte viability with the stimulation of IL-1β (10 ng/ml). The experiments were repeated 3 times separately. IL, interleukin.

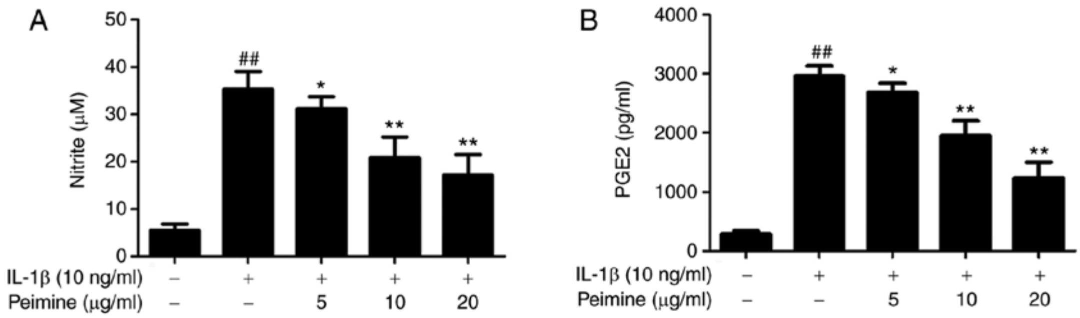

Figure 2. Effect of Peimine on IL-1β-induced NO and PGE2 production in chondrocytes. Chondrocytes were treated with different doses of Peimine (5, 10, 20 µg/ml) or a vehicle in the absence or presence of IL-1β (10 ng/ml) for 24 h. The culture medium was collected. (A) NO concentrations were measured using a Nitric Oxide Assay kit. (B) PGE2 concentrations were determined by a PGE2 ELISA kit. The experiments were repeated 3 times separately. $^{\#\#}P<0.01$ vs. the control group; $^{*}P<0.05$ and $^{**}P<0.01$ vs. the IL-1β group. IL, interleukin; PGE2, prostaglandin E2; NO, nitric oxide.

were treated with Peimine together with IL-1β, the protein expression of iNOS and COX-2 was significantly inhibited in a dose-dependent manner (P<0.05), indicating that Peimine prohibits IL-1β-induced protein expression of inflammatory mediators, namely iNOS and COX-2 (Fig. 3).

Effect of Peimine on IL-1β-induced expression of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4 and ADAMTS-5 in chondrocytes. A number of studies have demonstrated that IL-1β stimulation can induce the expression of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4 and ADAMTS-5, which are major cartilage degradation enzymes in OA (4,5). To investigate whether Peimine can inhibit the IL-1β-induced overexpression of these enzymes, mouse chondrocytes were treated with different doses of Peimine in the presence or absence of IL-1β for 24 h. According to the RT-quantitative PCR results, the mRNA overexpression of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4 and ADAMTS-5 induced by IL-1β was inhibited by Peimine in a dose-dependent manner (Fig. 4).

Effect of Peimine on IL-1β-induced MAPK activation in chondrocytes. Given the result that Peimine can inhibit IL-1β-induced inflammation in mouse chondrocytes, the underlying mechanism of this inhibitory effect was subsequently investigated. MAPK signaling pathways, which serve a significant role in inflammation, are considered as promising therapeutic targets for inflammatory diseases and OA (31,32). Furthermore, it has been reported that Peimine can inhibit MAPK signaling pathways in lipopolysaccharide (LPS)-induced macrophages (15). Therefore, the effects of Peimine on IL-1β-induced MAPK activation in chondrocytes were evaluated. Cells were treated with different concentrations of Peimine (0, 5, 10 and 20 µg/ml) in the presence or absence of IL-1β (10 ng/ml) for 24 h, and then the total protein was isolated and western blot analysis was performed. As expected, IL-1β stimulation significantly increased the phosphorylation of ERK, JNK and p38 (P<0.01; Fig. 5). Peimine treatment inhibited this phosphorylation in a dose-dependent manner (Fig. 5).

In addition, anisomycin, a p38 and JNK activator, or U-46619, a p38 and ERK activator were used together with Peimine (20 µg/ml) on IL-1β-treated chondrocytes. As expected, the phosphorylation levels of p38 and JNK increased in the Anisomycin + Peimine group compared with the Peimine group, and the phosphorylation levels of p38 and ERK increased in the U-46619 + Peimine group compared with the Peimine group (Fig. 6A and B). Furthermore, the protein levels of MMP-3, MMP-13, ADAMTS-5, iNOS and COX-2 significantly increased following the activation of MAPK signaling compared with the Peimine group (P<0.05; Fig. 6C and D). These results indicate that the protective effect of Peimine on IL-1β-treated chondrocytes was attenuated following activation of MAPK signaling. Therefore, the data indicate that Peimine inhibits IL-1β-induced inflammation in mouse chondrocytes via inhibition of MAPK signaling pathways.

Effect of Peimine on cartilage degradation in an OA model. A mouse OA model was established to evaluate whether Peimine

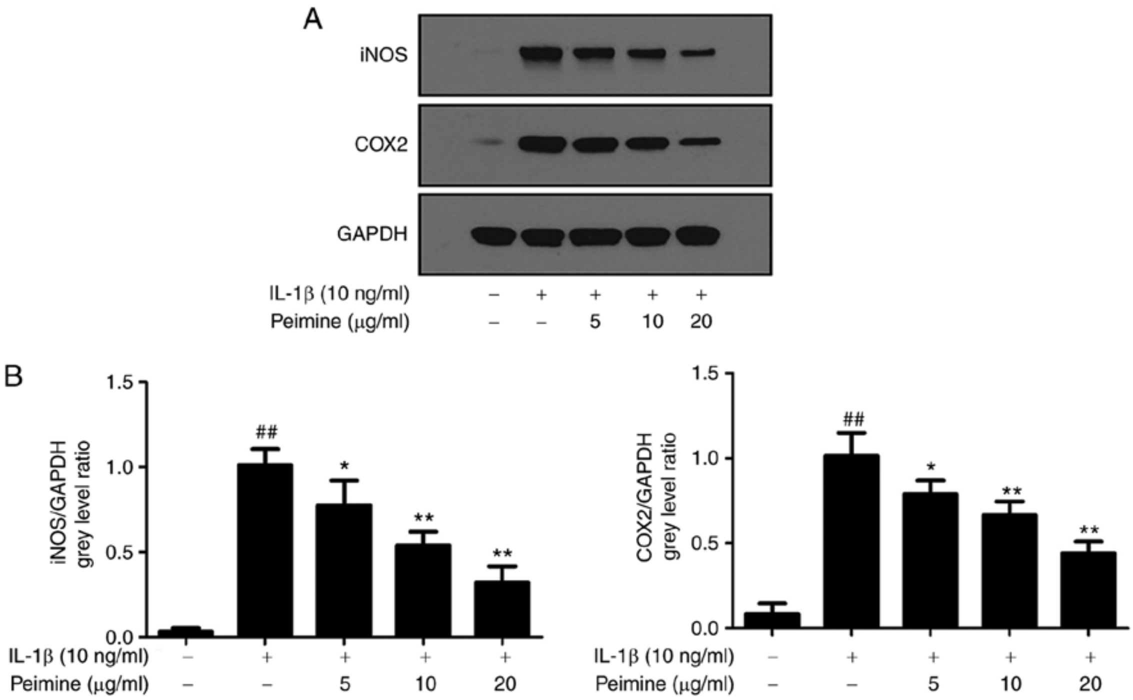

Figure 3. Effect of Peimine on IL-1β-induced protein expression of iNOS, COX-2 in chondrocytes. Chondrocytes were treated with Peimine (5, 10, 20 μg/ml) or a vehicle in the absence or presence of IL-1β (10 ng/ml) for 24 h. Total proteins from chondrocytes in each group were isolated, protein expression levels were determined using western blotting. (A) Representative images of the western blot analysis for iNOS, COX-2 and GAPDH. (B) Relative protein expression was quantified by ImageJ software. The experiments were repeated 3 times separately. $^{\#\#}$P<0.01 vs. the control group; *P<0.05 and **P<0.01 vs. the IL-1β group. IL, interleukin; iNOS, inducible nitric oxide synthase; COX-2, cyclic oxide synthase-2.

has a protective effect on OA *in vivo*. The mice were randomly divided into the following three groups: Sham/Vehicle group (sham operation + vehicle treatment), OA/Vehicle group (OA operation + vehicle treatment) and OA/Peimine group (OA operation + Peimine treatment). Following surgery, the vehicle or Peimine (20 mg/kg) was administered to the mice by oral gavage 5 days a week for a total period of 8 weeks. Histological analysis was performed using Safranin O/Fast Green staining and the severity of OA-associated changes was assessed using the OARSI scoring system. As presented in Fig. 7A, the joint surface was intact and almost no cartilage destruction was observed in the mice of the Sham/Vehicle group. As expected, the joint surface was discontinued and severe cartilage destruction was observed in the mice of the OA/Vehicle group. However, the morphology of the joint surface in the OA/Peimine group was much better than that of the OA/Vehicle group. Furthermore, the OARSI scores were significantly increased in the OA/Peimine group compared with the OA/Vehicle group, indicating that Peimine exerts protective effects against the development of OA (P<0.05; Fig. 7B).

Discussion

OA, which is characterized by progressive articular cartilage destruction, is the most common type of joint disease (33). It is estimated that 25% of the adult population in the US will be affected by OA by the year 2020 and OA will become one of the major health problems (34,35). However, there are no effective interventions to restore degraded cartilage or decelerate disease progression. Therefore, there is an urgent requirement to identify novel drugs to treat OA efficiently.

An accumulating number of studies have reported that compounds extracted from plants have the potential to protect against OA (7,36,37). Peimine, the main compound extracted from BF, has been demonstrated to have antioxidant, anti-inflammatory and pain suppressing effects (16-19). In the present study, the effects of Peimine on IL-1β-induced inflammation were evaluated in mouse chondrocytes *in vitro* and the potential beneficial effects of Peimine on OA were also investigated in a mouse model *in vivo*.

The results of the present study revealed the following observations that are, to the best of our knowledge, novel: i) Peimine reduces IL-1β-induced production of the inflammatory mediators iNOS, COX-2 and PGE2 in mouse chondrocytes; ii) Peimine suppresses IL-1β-induced chondrocyte expression of the cartilage catabolic enzymes MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4 and ADAMTS-5 in mice; iii) Peimine inhibits the MAPK signaling pathway in a dose-dependent manner in mouse chondrocytes; iv) Peimine has the potential to slow the progression of OA in a mouse model of OA.

Inflammatory cytokines including IL-1β and tumor necrosis factor-α serve a pivotal role in the pathogenesis of OA (5). The levels of IL-1β are significantly increased in articular chondrocytes from patients with OA (38). The increased levels of IL-1β stimulate chondrocytes to produce more inflammatory mediators, including iNOS, NO, COX-2 and PGE2 (39,40). IL-1β also enhances the degradation of the extracellular matrix via increased chondrocyte expression of cartilage catabolic enzymes, including MMPs and ADAMTS (41,42). As a result of its important role in the progression of OA, IL-1β stimulation is used as a conventional *in vitro* model to study OA and was used in the present study.

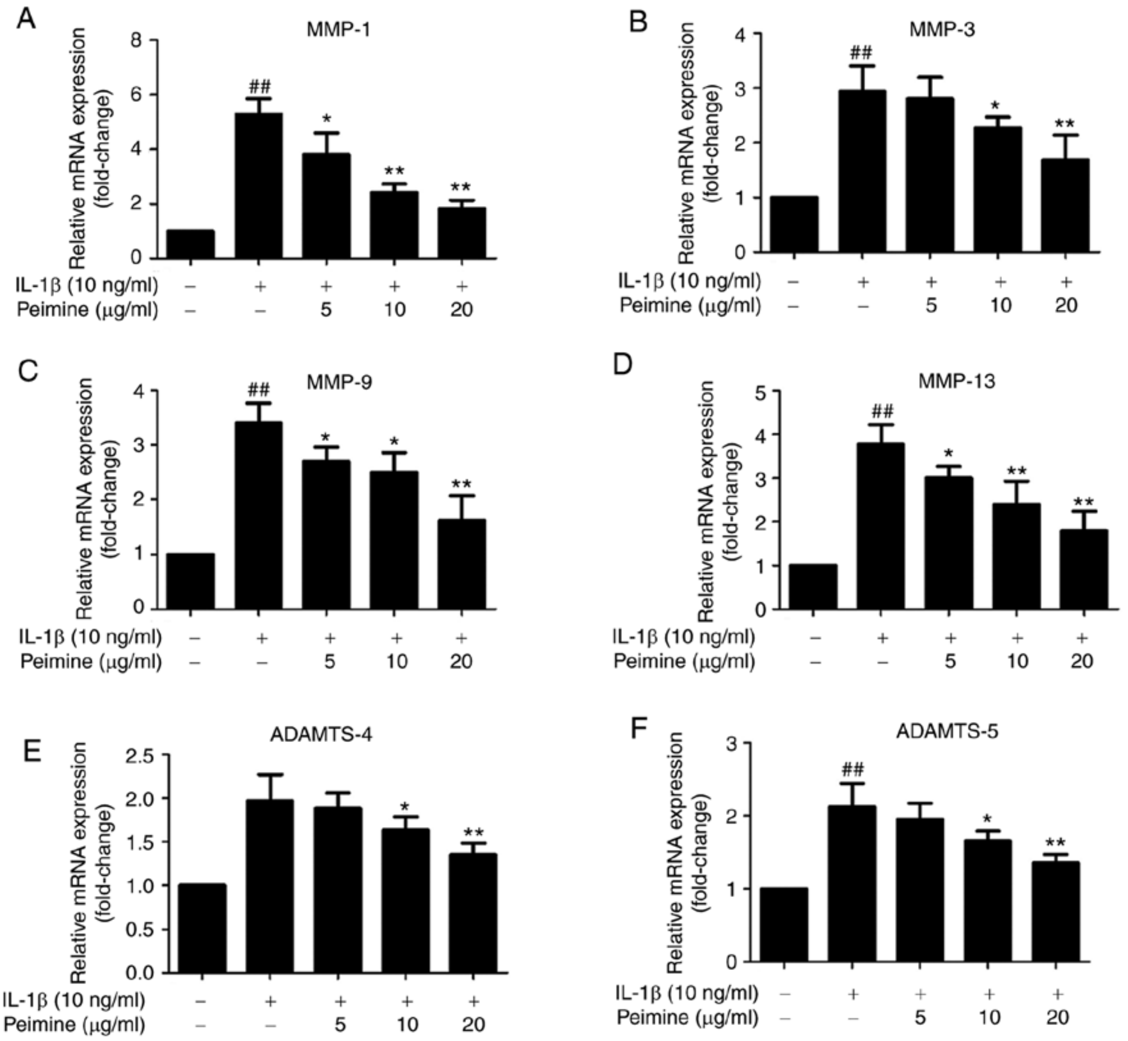

Figure 4. Effect of Peimine on IL-1β-induced expression of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4 and ADAMTS-5 in chondrocytes. Chondrocytes were treated with Peimine (5, 10, 20 ug/ml) or vehicle in the absence or presence of IL-1β (10 ng/ml) for 24 h. Total RNA from each group was isolated. mRNA expressions of (A) MMP-1, (B) MMP-3, (C) MMP-9, (D) MMP-13, (E) ADAMTS-4 and (F) ADAMTS-5 in chondrocytes was determined using reverse transcription-polymerase chain reaction. The experiments were repeated 3 times separately. #P<0.05 and ##P<0.01 vs. the control group; *P<0.05 and **P<0.01 vs. IL-1β group. MMP, matrix metalloproteinase; IL, interleukin; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs.

In the present study, mouse chondrocytes were stimulated with IL-1β (10 ng/ml) for 24 h. The data demonstrated that the concentrations of NO and PGE2 were upregulated and the protein expression levels of iNOS and COX-2 were elevated in the chondrocyte culture medium supernatant. These results are in accordance with a previous study, suggesting that IL-1β stimulates the OA microenvironment *in vitro* (37). iNOS, a member of the NO synthase family of enzymes, is used to synthesized NO (39). Another inflammatory mediator, PGE2, is the predominant product of COX-2 (43). It has been established that PGE2 and COX-2 serve key roles in the pathogenesis of OA, including inhibition of matrix synthesis and promotion of cartilage degradation (39). Targeting of PGE2 and COX-2 is considered a promising approach for the therapeutic intervention of OA (44). The data of the present study demonstrate that Peimine treatment alleviates IL-1β-induced expression of iNOS, NO, COX-2 and PGE2, indicating that Peimine inhibits IL-1β-induced inflammation in mouse chondrocytes.

The progressive cartilage destruction is the most characteristic change in OA (45). IL-1β stimulates chondrocytes to produce proteolytic enzymes, including aggrecanases and MMPs, which contribute to the destruction of the cartilage matrix (46). Among the various chondrocyte-secreted enzymes, MMP-13 and ADAMTS-5 are considered as the pivotal factors that accelerate the cartilage destruction in OA (47). Other enzymes, including MMP-1, MMP-3, MMP-9 and ADAMTS-4, also participate in the progression of OA (48). In the present study, IL-1β stimulation (10 ng/ml, 24 h) increased the mRNA expression levels of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4 and ADAMTS-5 in the mouse chondrocytes compared with the control group, which is in accordance with previous studies (7,36,37). Furthermore, Peimine treatment inhibited the elevated mRNA expression levels in a dose-dependent manner. These data suggest that Peimine ameliorates the increased expression levels of catabolic enzymes caused by IL-1β and protects cartilage from IL-1β-induced destruction.

MAPK signaling pathways are involved in multiple cellular activities, including cell survival, proliferation and inflammation (49). Accumulating evidence has suggested that MAPK signaling serves a significant role in the progression of OA (4).

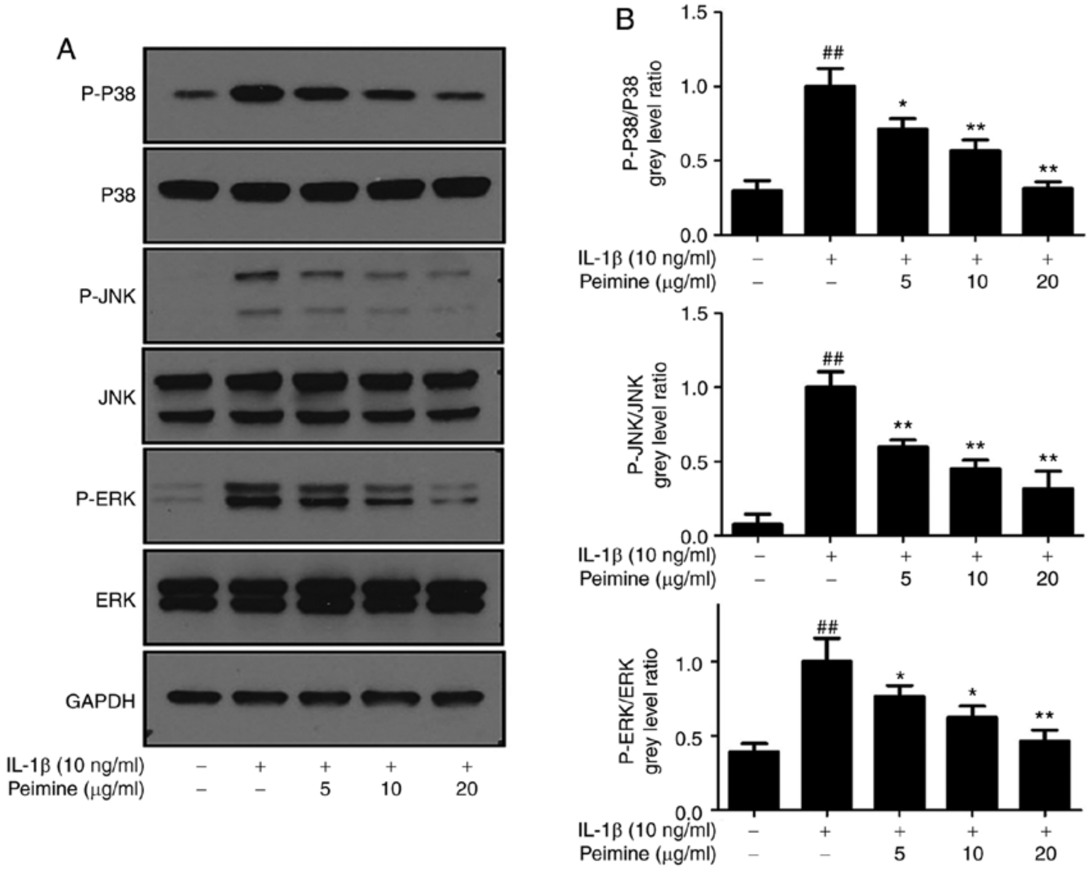

Figure 5. Effect of Peimine on mitogen-activated protein kinase signaling. Chondrocytes were treated with Peimine (5, 10, 20 μg/ml) or vehicle in the absence or presence of IL-1β (10 ng/ml) for 24 h. Total proteins from chondrocytes in each group were isolated, protein expression levels were determined using western blot analysis. (A) Representative images of western blotting for P-P38, P38, P-JNK, JNK, P-ERK, ERK and GAPDH. (B) Relative protein expression was quantified using Image-J software. The experiments were repeated 3 times separately. $^{\#\#}$P<0.01 vs. the control group; *P<0.05 and **P<0.01 vs. the IL-1β group. IL, interleukin; JNK, Janus kinase; P-ERK, phosphorylated extracellular signal regulated kinase.

Furthermore, a previous study reported that Peimine can inhibit MAPK signaling pathways in LPS-induced macrophages (15). In order to elucidate the anti-inflammatory mechanisms of Peimine in chondrocytes, MAPK signaling pathway was evaluated in the present study. Following stimulation with IL-1β for 24 h, MAPK signaling pathway in the mouse chondrocyte cells was activated, as revealed by the significantly increased phosphorylation levels of p38, ERK and JNK. However, the Peimine treatment reduced the expression levels of P-p38, P-ERK and P-JNK in a dose-dependent manner. These data suggest that Peimine inhibits IL-1β-induced inflammation in mouse chondrocytes via inhibition of the MAPK signaling pathway. However, this is just one of the associated signaling pathways and there is a high possibility that a number of other mechanisms are involved in the anti-inflammation effects of Peimine on mouse chondrocytes.

To investigate the effects of Peimine on OA *in vivo*, a mouse OA model based on MMT was established in the present study. After the surgery and indicated treatments, the severity of OA-associated changes was assessed using the OARSI scoring system. The mice in the OA/Vehicle group (MMT operation + vehicle treatment) exhibited increased OARSI scores compared with the Sham/Vehicle group (sham operation + vehicle treatment). This result suggests that the mouse OA model was established successfully. The OARSI scores of the mice in the OA/Peimine group (OA operation + Peimine treatment) were significantly reduced compared with the OA/Vehicle group, indicating that Peimine exerts protective effects against the development of OA.

It is important to note the limitations within the present study that represent the future directions for the present study. First, the data suggest that the MAPK signaling pathway is involved in the anti-inflammation effects of Peimine on mouse chondrocytes. However, there is a high possibility that a number of other mechanisms are also involved. Second, the *in vivo* and *in vitro* experimental models are both based on mice, and it remains to be verified whether Peimine exhibits the same effects on humans. Third, Peimine is the main compound extracted from BF. Although BF has been used in Chinese traditional medicine for >2,000 years due to its low toxicity and few side effects, it remains unclear whether Peimine has side effects when used for a long period of time to treat OA.

In conclusion, the results of the present study report the anti-inflammatory effects of Peimine in OA for the first time to the best of our knowledge. Peimine inhibits IL-1β-induced iNOS and COX-2 production and expression of MMPs and ADAMTs in a dose-dependent manner via blockage of IL-1β-induced MAPK signaling activation. Furthermore, Peimine has protective effects against the development of OA in a mouse model of OA. These results indicate that Peimine is a promising therapeutic agent for OA.

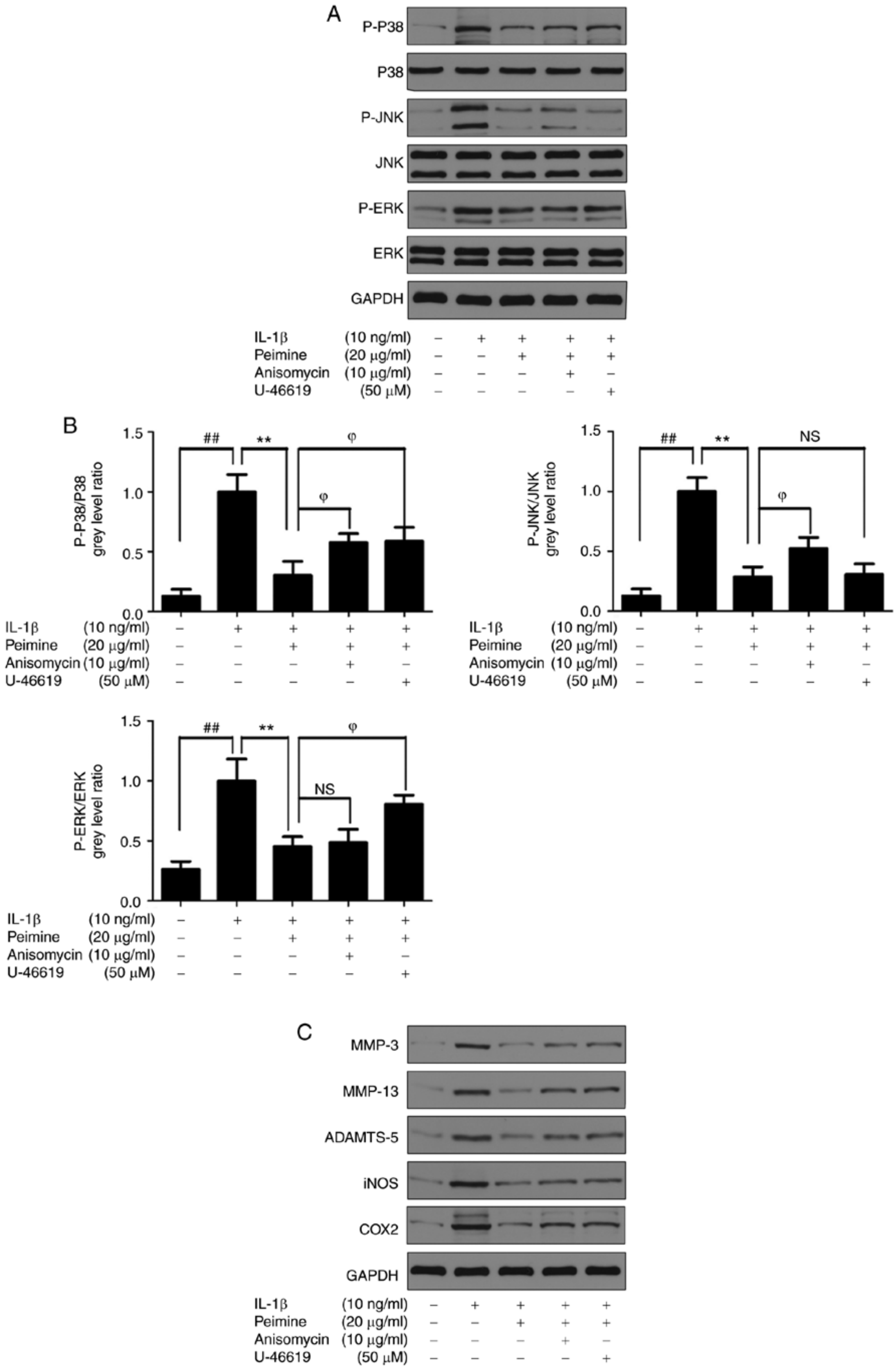

Figure 6. Effect of Peimine on IL-1β treated chondrocytes is alleviated following the activation of mitogen-activated protein kinase signaling. Chondrocytes were treated with Peimine (20 μg/ml) or vehicle in the absence or presence of IL-1β (10 ng/ml) for 24 h. In 'Peimine + Anisomycin' group and 'Peimine + U-46619', 10 μg/ml Anisomycin or 50 μM was treated together with Peimine (20 μg/ml) and IL-1β (10 ng/ml) for 24 h. Total proteins from chondrocytes in each group were isolated, protein expression levels were determined using western blotting. (A) Representative images of western blotting for P-P38, P38, P-JNK, JNK, P-ERK, ERK and GAPDH. (B) Relative protein expression was quantified using Image-J software. (C) Representative images of western blotting for COX2, iNOS, MMP-3, MMP-13, ADAMTS-5 and GAPDH.

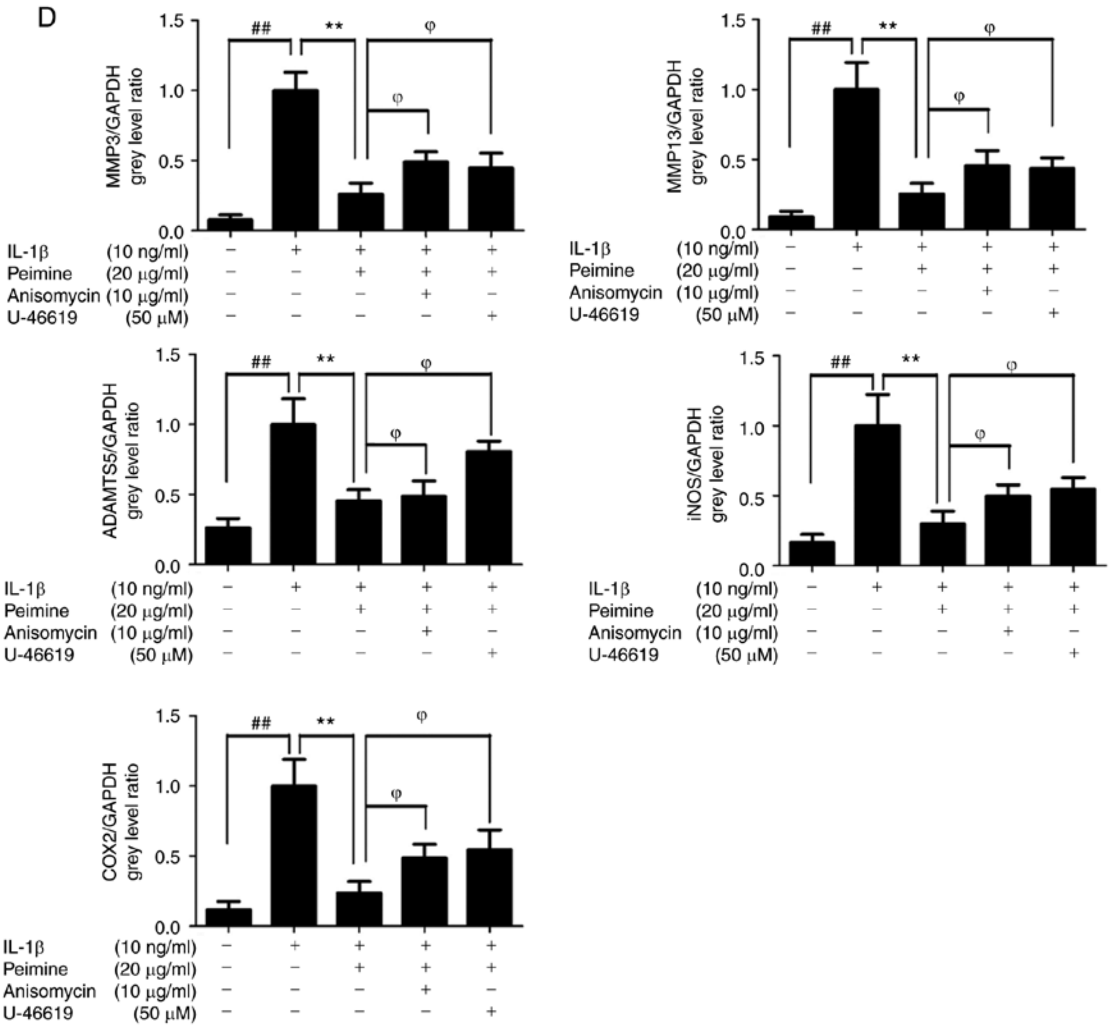

Figure 6. Continued. (D) Relative protein expression was quantified using Image-J software. ##P<0.01 vs. the control group; **P<0.01 vs. the IL-1β group; φP<0.05 vs. 'IL-1β+ Peimine' group. IL, interleukin; JNK, Janus kinase; P-ERK, phosphorylated extracellular signal regulated kinase; MMP, matrix metalloproteinase; COX-2, cyclo-oxygenase-2; iNOS, inducible nitric oxide synthase; ADAMSTS, a disintegrin and metalloproteinase with thrombospondin motifs; NS, not significant.

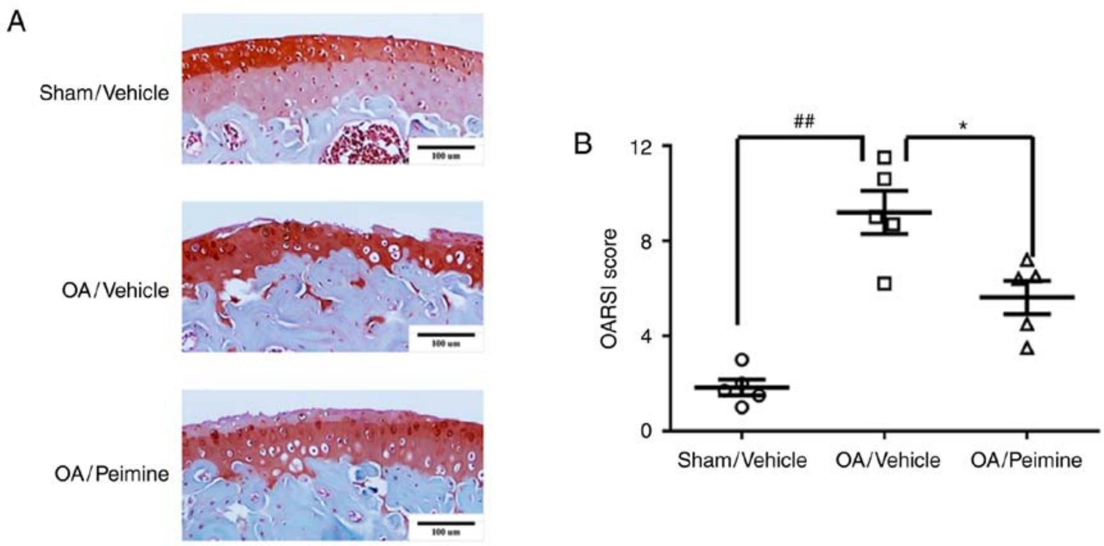

Figure 7. Effect of Peimine on cartilage degradation in a mice model of OA. A total of 15, 12-weeks-old mice were divided randomly into three groups (n=5), namely the Sham/Vehicle group (Sham operation + vehicle treatment), OA/Vehicle group (OA operation + vehicle treatment) and OA/Peimine group (OA operation + Peimine treatment). After the surgery, mice were treated with vehicle or Peimine (20 mg/kg) 5 days a week for 8 weeks. (A) Representative Safranin O/Fast Green staining pictures from each group were present. (B) The OARSI score was calculated for each group. ##P<0.01 vs. Sham/Vehicle group; *P<0.05 vs. OA/Vehicle group. OA, osteoarthritis; OARSI, Osteoarthritis Research Society International.

Acknowledgements

Not applicable.

Funding

The present study was supported by research grants from the National Natural Science Foundation of China (grant nos. 81672168 and 81472082, awarded to AC), the Natural Science Foundation of Hubei Province of China (grant no. 2018CFB714, awarded to WZ), and the Hubei Province Health and Family Planning Scientific Research Project (grant no. WJ2019Q028, awarded to PC).

Availability of data and materials

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

Authors' contributions

PC and AC designed the experiments. KC, ZL, WH, SL, ZW, XJ, CZ, FG, WZ and HL performed the experiments. ZW, XJ, KC and ZL performed the measurements and analysis. FG, KC and PC drafted the manuscript. WZ and ZL revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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