

Changes of type II collagenase biomarkers on IL-1 β -induced rat articular chondrocytes

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Abstract. Osteoarthritis (OA) is characterized by progressive degeneration of cartilage, formation of cartilage at the cartilage edge, and remodeling of the subchondral bone. Pro-inflammatory cytokines [e.g., interleukin (IL)-1 β] that induce inflammation and promote chondrocyte damage induce OA. Currently, the diagnosis of OA is commonly based on imaging examinations (e.g., X-ray) and evaluations of clinical symptoms; however, biomarkers that can effectively diagnose OA are currently not available. By studying the mechanism underlying OA cartilage injury and changes in the concentrations of the biomarkers procollagen type II carboxy-terminal propeptide (PIICP), collagen type-II C-telopeptide fragments (CTX-II), and type II collagen cleavage neoepitope (C2C) during pathogenesis, the present study established a theoretical basis for the evaluation and early diagnosis of OA. In an experiment, 10 ng/ml IL-1 β was used to treat chondrocyte-induced OA models *in vitro* for 0, 12, 24 and 48 h. Western blotting was used to detect the expression levels of matrix metalloproteinase (MMP)-3, MMP-13, and inducible nitric oxide synthase (iNOS) protein at each time-point. The concentrations of CTX-II, C2C, and PIICP in the cell culture supernatant were detected by ELISA kit. A biochemical kit was used to detect changes of nitric oxide (NO) in the cell culture supernatant. In addition, chondrocytes were treated with 10 ng/ml IL-1 β for 0, 30, 60 and 90 min and the translocation and phosphorylation of the NF- κ B pathway were investigated by western blotting. Following IL-1 β stimulation, the NF- κ B pathway was activated to increase the expression levels of MMPs and iNOS synthesis downstream of the pathway, resulting in an increased degradation of type II collagen (Col II). To sum up, pro-inflammatory IL-1 β induced

an OA chondrocyte model. During the development of OA, the expression of MMPs and NO increased and Col II was degraded.

Introduction

Osteoarthritis (OA) is one of the most common arthritic diseases (1) and a major cause of chronic pain and dysfunction in patients. OA is a chronic degenerative disease that is characterized by joint disease, cartilage degeneration and erosion, fibrosis, and osteophyte formation. In the worst cases, OA leads to pain and disability and severely affects the quality of life of patients (2-4). However, the origins and causes of OA have not been fully elucidated to date.

Chondrocytes are the only type of cell present in articular cartilage, where they are responsible for maintaining the normal synthesis and renewal of the cartilage matrix (4). A previous study associated pro-inflammatory cytokines [e.g., interleukin (IL)-1 β] with OA pathogenesis and revealed that it plays a pivotal role in OA (3). The activation of inflammation-related signaling pathways was revealed to be initiated by IL-1 β . Then, IL-1 β stimulated the expression of matrix metalloproteinases (MMPs) and nitric oxide (NO), which resulted in the destruction of the cartilage matrix (5). MMP-3 and MMP-13 have been recognized as the two most important collagenases that are involved in the degradation of the cartilage matrix; these are also crucial factors in OA (6,7). It has been revealed that the expression levels of MMP-3 and MMP-13 are increased in synovial fluid from patients with OA (8). The overexpression of metalloproteinases promotes degradation of type II collagen (Col II) (4).

Col II is the main component of the extracellular matrix (ECM) (4). The fibrillar Col II is the major collagen of articular cartilage, and includes both procollagen with both amino (N)-terminal (PIINP and PIIANP) and carboxy (C)-terminal propeptide (PIICP) (9). The PIICP levels have been correlated with the progression of OA in the knee (10). When Col II breaks down and produces fragments, such as collagenase, type II collagen cleavage neoepitope (C2C) and collagen type-II C-telopeptide fragments (CTX-II) are generated (11,12). C2C is a useful biomarker that reflects the cleavage of Col II in OA and the levels of CTX-II have been demonstrated to be associated with the total damage of osteophytes (13).

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Numerous studies have revealed that IL-1 β can activate the nuclear factor- κ B (NF- κ B) pathway (14-16). NF- κ B has been identified as an inducible transcription factor (17) and the NF- κ B pathway has been reported to regulate the balance in the catabolism that controls numerous inflammation-related genes (18). When NF- κ B has been stimulated by IL-1 β , inactive NF- κ B becomes active and translocates from the cytoplasm to the nucleus; moreover, the phosphorylation of p65 is translated into the nucleus and the expression of downstream genes is increased (14,19). Therefore, OA is associated with inflammation, and reducing activation of the NF- κ B pathway may be a therapeutic strategy for the treatment of OA.

As a class of molecules with high sensitivity, biomarkers associated with OA have been extensively studied and have broad application prospects (20). However, in previous studies, chondrocytes induced with 1 ng/ml IL-1 α or 10 ng/ml IL-1 β to simulate an *in vitro* OA model mostly focused on a single marker at a certain time-point (21,22), while not studying the change trend of these markers in OA, and thus, not reflecting OA progression. In the present study, the variation trend of biomarkers was observed at four different time-points. Currently, advanced OA can only be alleviated through analgesia or joint replacement, both of which are expensive (23). Therefore, clarifying the pathogenesis as well as the early diagnosis of OA is important for the prevention and treatment of OA. The present study explored the optimal conditions for IL-1 β -induced OA in a rat articular chondrocyte cell model. Additionally, the expression of MMPs and subsequent degradation of type II collagen in the course of OA were analyzed. The presented variation trend of the concentration of molecular markers provides a theoretical basis for the diagnosis and screening of OA molecular markers.

Materials and methods

Chondrocyte isolation and culture. Chondrocytes were isolated from male Sprague-Dawley (SD) rats aged 14-24 days, weight 30-50 g. SD rats were bought from the Animal Experimental Base of Heilongjiang University of Traditional Chinese Medicine. All the animals were kept separately in the animal room. The room was well ventilated, clean and comfortable: The temperature was 21 \pm 3 $^{\circ}$ C, the humidity was 50 \pm 15%, the light time was 12 h per day, the food and water supply was sufficient, and the bedding material was changed regularly. The use of rats in the present study was approved by the Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University (Harbin, China). Chondrocytes from different rats were pooled. Animals were euthanized by placing in a clean, fluoroscopically closed box and CO₂ was perfused at a rate of 10-30% of the replacement volume/min into the box. Animal sacrifice was ascertained when the animal was motionless, not breathing, and the pupils became dilated; then CO₂ was turned off. The animals were observed for an additional 2 min to confirm sacrifice. In a sterile environment, chondrocytes were isolated from articular cartilage and digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) for 30 min at 37 $^{\circ}$ C. Subsequently, DMEM/F12 containing 10% FBS (Corning, Inc.) and penicillin-streptomycin (Corning, Inc.) containing 0.2% collagenase type II (Biofrox) were mixed for 4 h at 37 $^{\circ}$ C in a shaker to isolate chondrocytes. The

cell suspension was centrifuged (400 x g; 7 min) to harvest primary chondrocytes at room temperature. Primary chondrocytes were cultured in DMEM/F12 at 37 $^{\circ}$ C with 5% CO₂ in growth medium. The medium was changed at 24-h intervals. When the cells reached 80% confluence, they were passaged, and the chondrocytes of passage 2 were used for all subsequent experiments.

Cell model. Passage 2 cells were washed three times with serum-starved medium (0.5% FBS) and incubated with DMEM/F12 for 12 h at 37 $^{\circ}$ C. Chondrocytes were treated with IL-1 β (10 ng/ml) (PeproTech, Inc.) for 0, 12, 24 and 48 h at 37 $^{\circ}$ C. OA is a pathologic process with varying degrees of severity (24). Chondrocytes treated with IL-1 β at different time-points were used to simulate an *in vitro* model of OA. The variation trend of biomarkers at different time-points was observed with the development of OA (14).

Toluidine blue staining. Chondrocytes were washed three times in phosphate-buffered saline (PBS) and fixed with 10% buffered formalin for 30 h at 4 $^{\circ}$ C. After washing for 5 min with distilled water, chondrocytes were gently washed for 15 min under running water, and then evenly dyed with 1% toluidine blue dye (Gibco; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. Finally, the samples were washed with distilled water and examined under a laser confocal optical microscope.

ELISA. According to the manufacturer's instructions, the concentrations of PIICP (cat. no. 88-1052), C2C (cat. no. EHJ-96082r), and CTX-II (cat. no. EHJ-96093r) were measured by ELISA kits (Xiamen Huijia Biological Technology Co., Ltd.). The expression of NO was measured by NO assay kit (cat. no. A013-2; NanJing JianCheng Bioengineering Institute). All assays were repeated three times. All kits were operated according to the manufacturers' instructions.

Western blot analysis. The protein concentrations were determined using the bicinchoninic acid assay. Cells were rinsed with ice-cold PBS and harvested in lysis buffer (Beyotime Institute of Biotechnology) to obtain total cellular protein, nuclear, or cytoplasmic fractions. Equal quantities (30 μ g/lane) of proteins were subjected to 8-12% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to nitrocellulose filter (NC) membranes (Pall Corporation). Then, the membranes were blocked in PBS containing 20% Tween-20 (PBST; Beyotime Biotechnology, Shanghai, China) plus 5% non-fat dry milk for 1 h at room temperature, and were incubated with primary antibodies overnight at 4 $^{\circ}$ C. The membranes were washed three times in Tris-buffered saline (TBS) with 20% Tween-20. The blots were then incubated with the secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Finally, the western blots were developed using ECL reagent (Beyotime Institute of Biotechnology) through a western blotting detection system.

Primary antibodies included mouse anti-GAPDH (1:3,000, cat. no. TA-08; ZSGB-BIO; OriGene Technologies, Inc.), mouse anti-MMP-13 (1:1,000; cat. no. NB100-91878V; Novus Biologicals, Inc.), rabbit anti-MMP-3 (1:1,000; cat. no. 69926), NF- κ B p65 (1:1,000; cat. no. 8242), phospho-NF- κ B p65

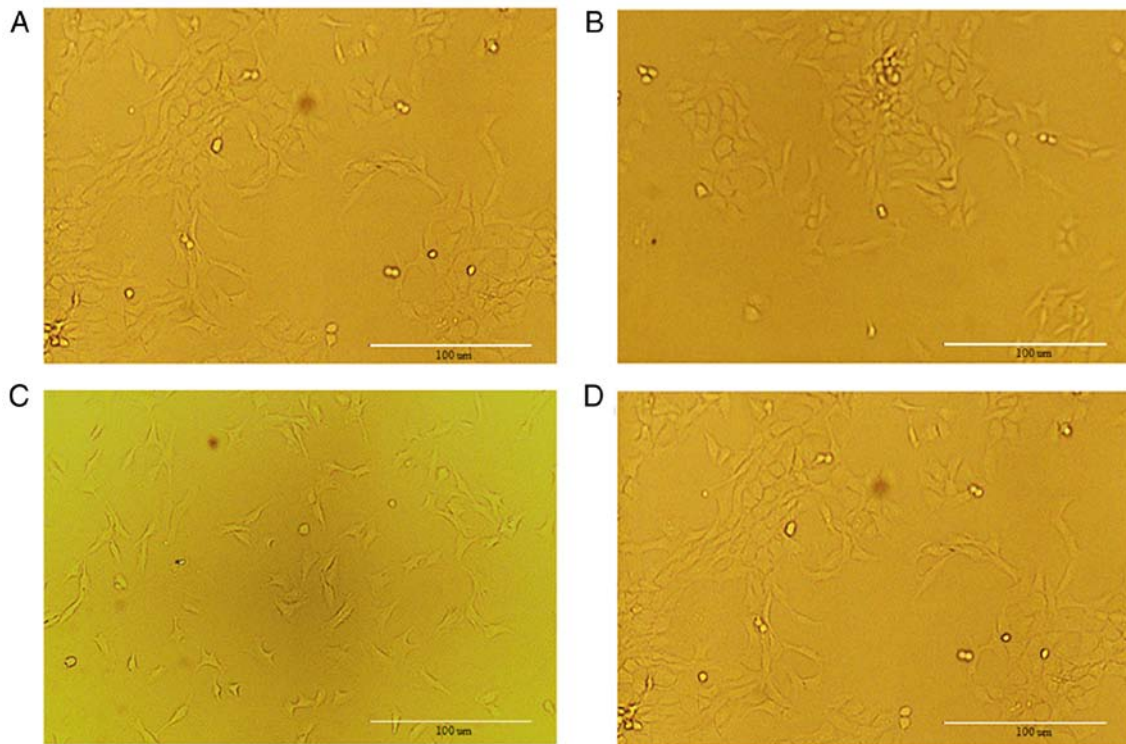


Figure 1. Morphological observation of chondrocytes (x100). (A) Primary cultures. (B) Chondrocytes of passage 2. (C) Chondrocytes of passage 3. (D) Chondrocytes of passage 4.

(1:1,000, cat. no. 3033) (all from Cell Signalling Technology, Inc.) and polyclonal antibody rabbit anti-iNOS (1:500; cat. no. NB300-605AF405; Novus Biologicals, Inc.). Secondary antibodies included goat anti-rabbit-IgG and goat anti-mouse-IgG (1:1,000; cat. no. ZB2305/ZB-2301, ZSGB-BIO; OriGene Technologies, Inc.).

Statistical analysis. All experimental data was analysed using SPSS software (version 19.0 for Windows; SPSS, Inc.). Values are expressed as the mean \pm standard deviation (SD). Statistical comparisons between multiple groups were conducted by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Results of chondrocytes. When the chondrocytes cover 80% of the culture bottle, primary cells can be sub-cultured. After more than three generations, the cells undergo a process known as 'dedifferentiation' (25). In the present study, undifferentiated second-generation chondrocytes were used and the cell morphology is shown in Fig. 1.

Results of toluidine blue staining. After dyeing, proteoglycans and the nucleus separated in chondrocytes, where proteoglycans were blue-purple and the nucleus was mazarine. Fig. 2 clearly revealed that the cultured cells were chondrocytes.

Effect of the expression levels of MMP-3 and MMP-13 in IL-1 β -induced chondrocytes. The expression levels of

MMP-3 and MMP-13 are presented in Fig. 3. The expression levels of MMP-3 increased in a time-dependent manner in IL-1 β -induced chondrocytes at 12, 24 and 48 h. MMP-13 expression levels were significantly increased at 12, 24 and 48 h, and were highest at 24 h.

Effect of iNOS and NO expression in IL-1 β -induced chondrocytes. Western blot results for the expression level of iNOS are presented in Fig. 4. After IL-1 β stimulation, the iNOS expression levels at 12, 24 and 48 h were significantly increased compared to those at 0 h, and reached the maximum value at 24 h (Fig. 4A). As revealed in Fig. 4B, the NO levels differed in culture supernatants at 12, 24, and 48 h compared with the 0-h time-point. In addition, the level of NO was significantly increased at 12, 24 and 48 h, and reached the maximum value at 24 h.

Effects of p65 on the nucleus translocation in IL-1 β -induced chondrocytes. Western blot results of the expression levels of p65 and phosphorylated (p)-p65 after chondrocyte stimulation with IL-1 β at 0, 30, 60 and 90 min in both the cytoplasm and nucleus are presented in Figs. 5 and 6. Firstly, cytoplasmic extracts exhibited a significantly reduced level of p65 upon IL-1 β stimulation. However, the cytoplasmic extracts exhibited the opposite result for p-p65. Secondly, the levels of p65 and p-p65 in chondrocyte nuclei after IL-1 β stimulation for 30, 60 and 90 min were increased compared to the group without stimulation (0 min). p65 levels first increased at 30 min and then decreased at 60 and 90 min. p-p65 levels increased and reached the maximum value at 60 min.

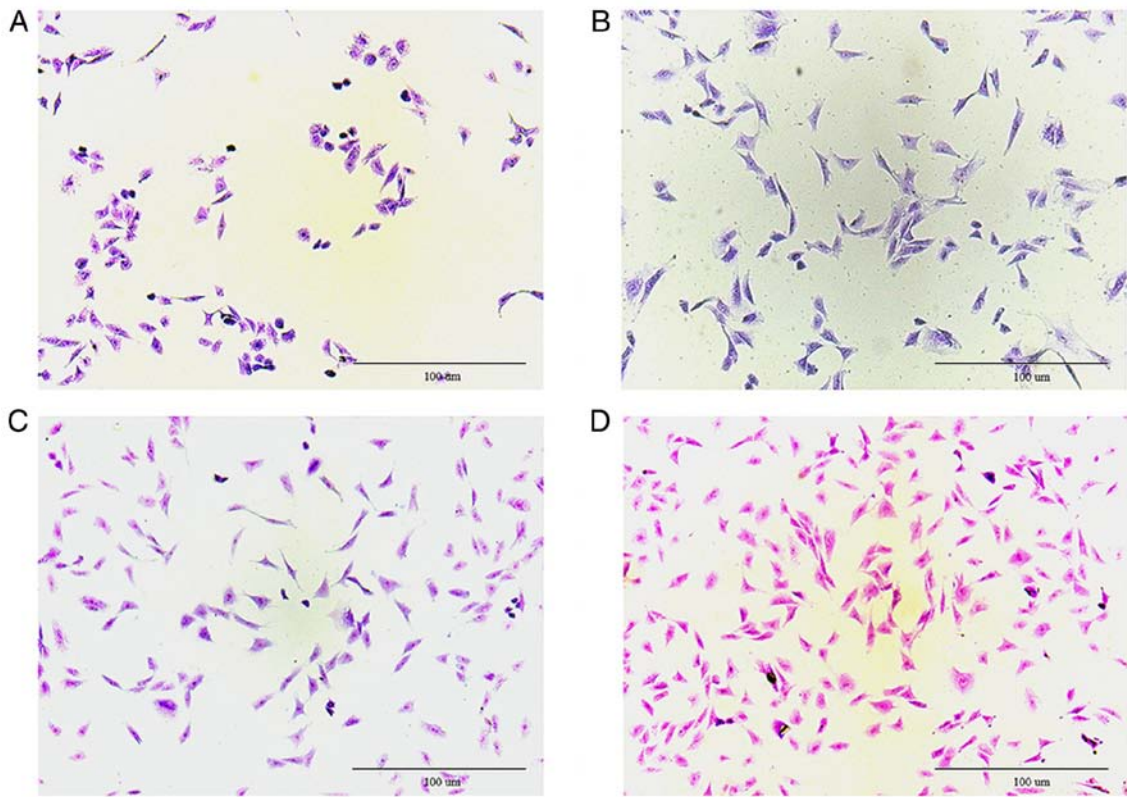


Figure 2. Toluidine blue staining of chondrocytes (x100). (A) Primary cultures. (B) Chondrocytes of passage 2. (C) Chondrocytes of passage 3. (D) Chondrocytes of passage 4. Proteoglycans are presented in blue-purple and the nucleus is presented in mazarine.

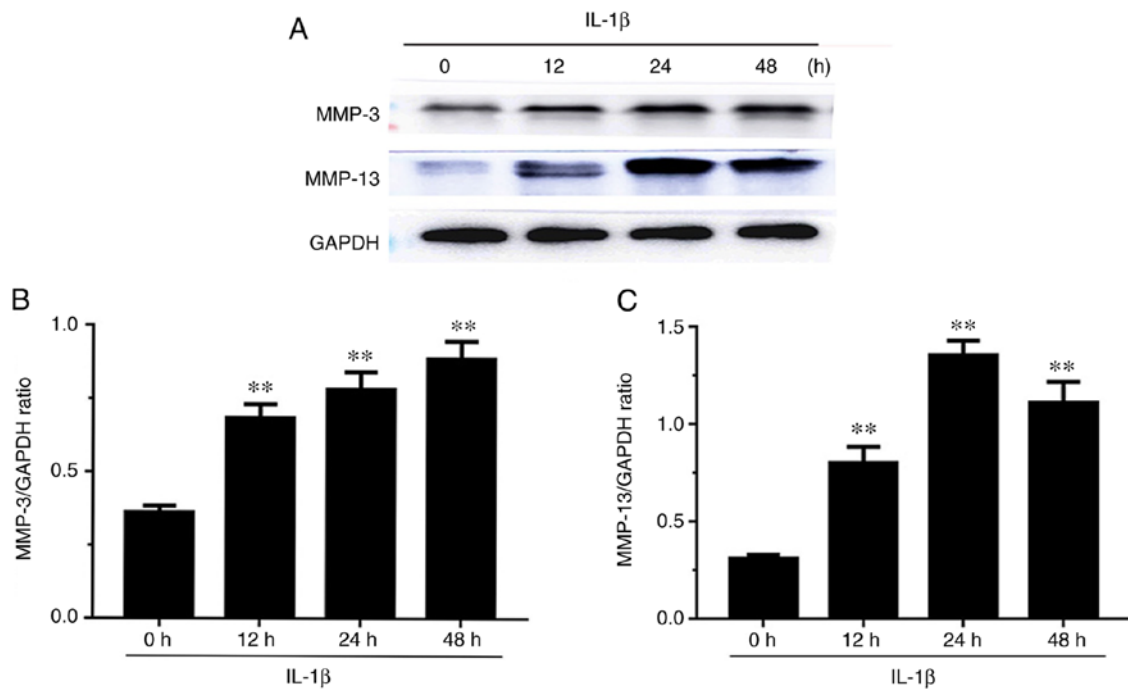


Figure 3. Effect of MMP-3 and MMP-13 expression levels in IL-1 β -induced chondrocytes. (A) Western blotting was applied to assess the protein levels of MMP-3 and MMP-13 in chondrocytes. Densitometry data of (B) MMP-3 and (C) MMP-13 was analyzed. **P<0.01. MMP, matrix metalloproteinase; IL, interleukin.

IL-1 β -induced chondrocyte effect on the levels of CTX-II, C2C and PIICP. ELISA assays revealed that the levels of CTX-II and C2C were significantly increased in IL-1 β -induced culture

supernatants, and reached a maximum value at 48 h (Fig. 7). However, no significant change was observed in the levels of PIICP after IL-1 β treatment.

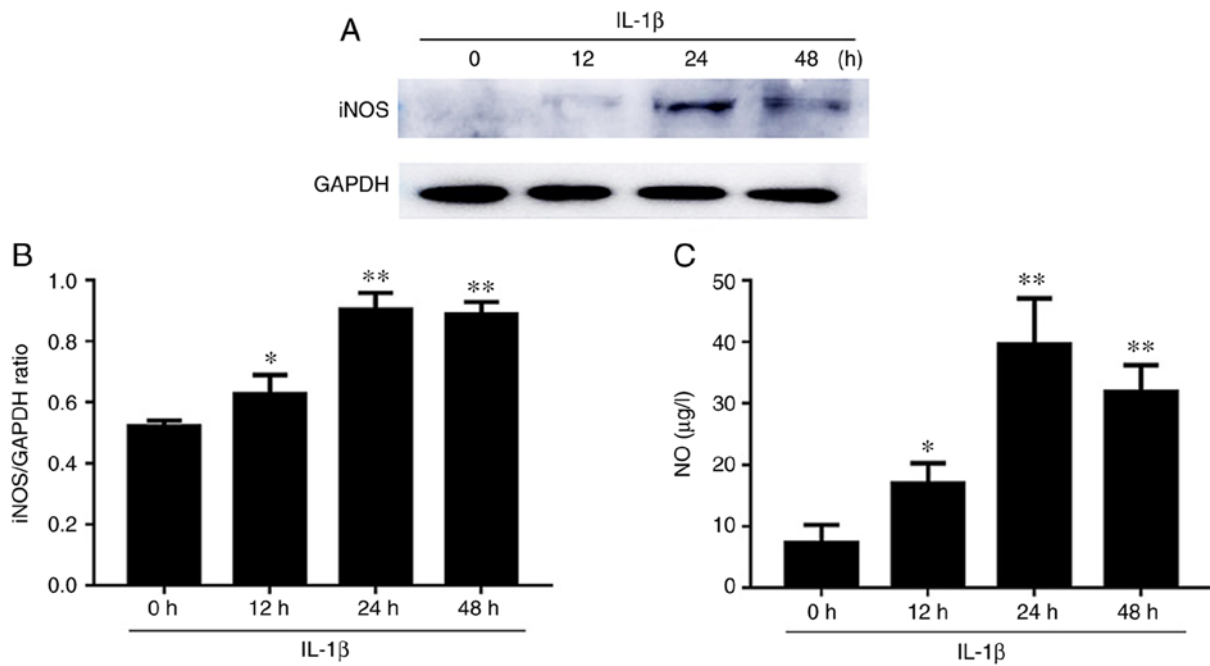


Figure 4. Effect of iNOS and NO expression levels in IL-1 β -induced chondrocytes. (A) Western blotting was applied to assess the protein levels of iNOS in chondrocytes. Densitometry data of (B) iNOS and (C) NO was analysed. *P<0.05 and **P<0.01. iNOS, inducible nitric oxide synthase; NO, nitric oxide; IL, interleukin.

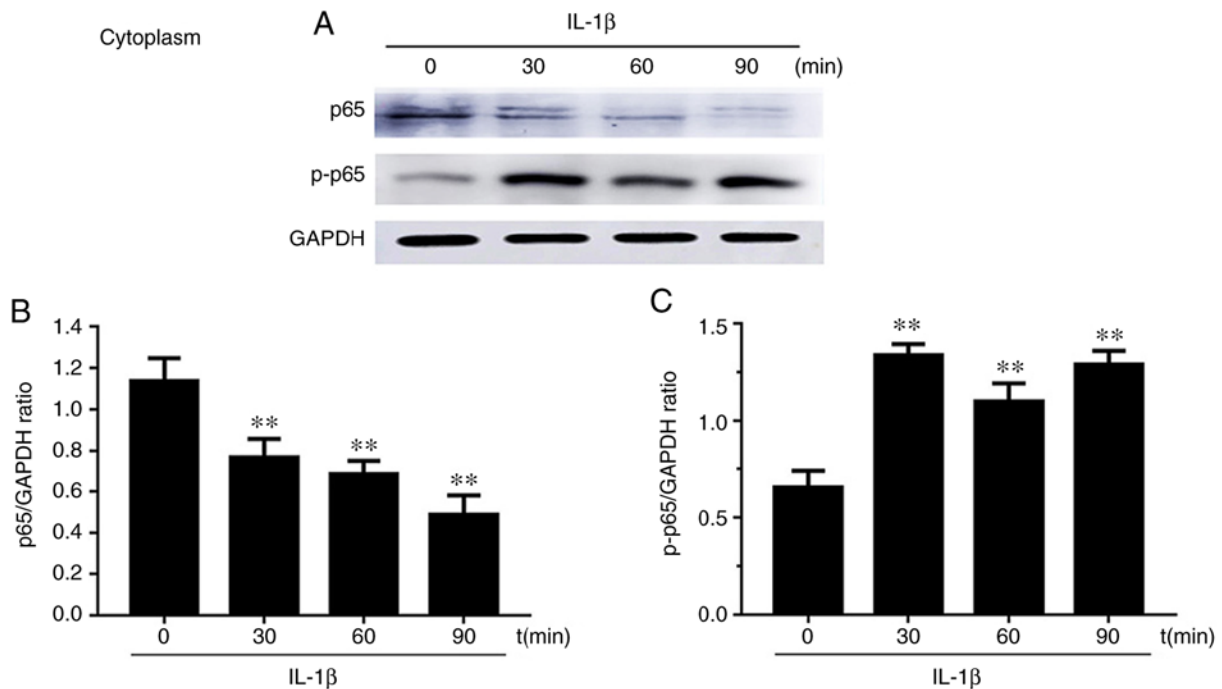


Figure 5. Effect of p65 and phosphorylated p65 expression levels in the cytoplasm. (A) Western blotting was applied to assess the protein levels of p65 and phosphorylated p65 in cytoplasm. Densitometry data of (B) p65 and (C) phosphorylated p65 was analysed. **P<0.01.

Discussion

Biomarkers are a class of highly sensitive molecules, and have been extensively studied in OA (9). It is important to study the mechanisms and molecular biomarkers of OA cartilage. Articular cartilage can be damaged by abnormal wear or pathological processes. At the early stage of OA, the ECM changes

and the cartilage surface remains intact (25). This change leads to the expression of markers such as Runx2, ColX, and MMP-13 (26). As OA further develops, the composition and structure of articular cartilage changes, the proteoglycan and collagen networks break down, the cartilage degrades, and the integrity of the cartilage is lost (27,28). The chondrocytes undergo apoptosis and eventually, the articular cartilage

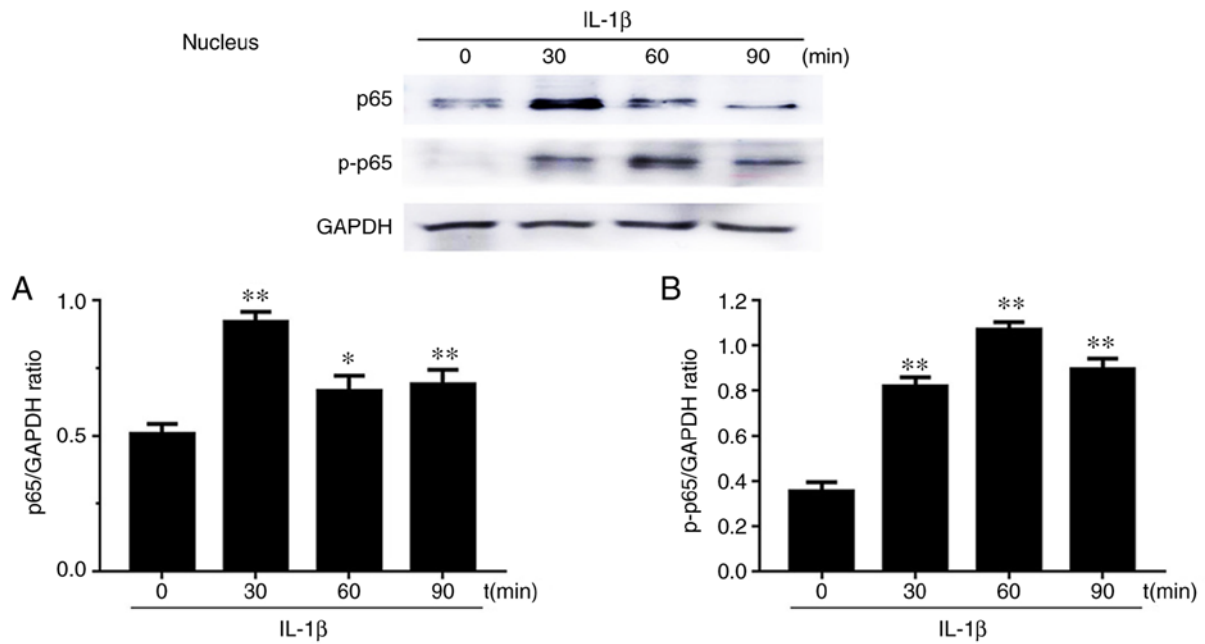


Figure 6. Effect of p65 and phosphorylated p65 expression levels in the nucleus. Western blotting was applied to assess the protein levels of p65 and phosphorylated p65 in the nucleus. Densitometry data of (A) p65 and (B) phosphorylated p65 was analysed. * $P < 0.05$ and ** $P < 0.01$.

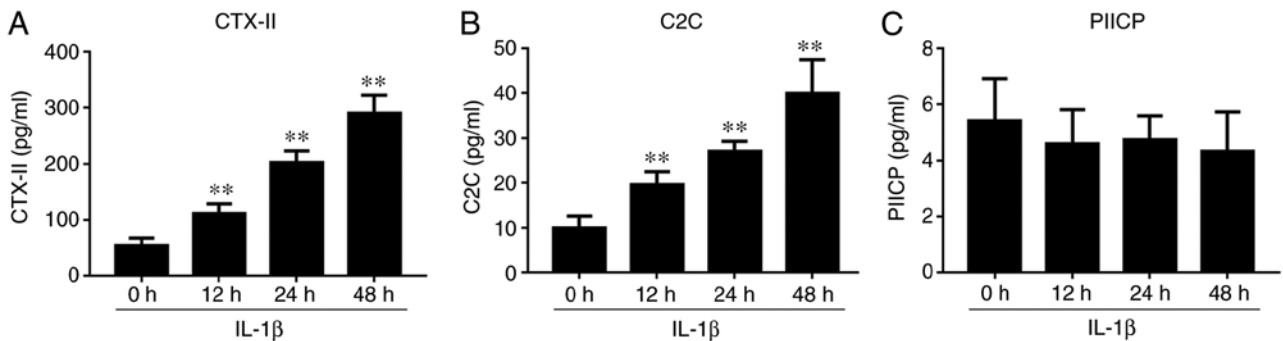


Figure 7. Effects of the levels of CTX-II, C2C, and PIICP in IL-1 β -induced chondrocytes. (A) CTX-II, (B) C2C and (C) PIICP expression changes in chondrocytes. ** $P < 0.01$. CTX-II, collagen type-II C-telopeptide fragments; C2C, type II collagen cleavage neoepitope; PIICP, procollagen type II carboxy-terminal propeptide; IL, interleukin

disappears (29). Part of the cartilage forms osteophytes (30). IL-1 β (10 ng/ml) (14) has been revealed as an important pro-inflammatory compound that plays key roles in numerous inflammatory responses (31). The development of OA is accompanied by an inflammatory response (32). Therefore, IL-1 β , as a pro-inflammatory agent, initiates the activation of inflammation and induces OA. It can be confirmed that articular cartilage is destroyed in OA development (12). In a study by De Visser *et al* articular cartilage damage was induced on femoral condyles and it was revealed that Fib3-3 was upregulated and may be a biomarker and related to joint degenerative changes (33). In addition, it was revealed by observing different biomarkers in an *in vitro* OA rat model induced with IL-1 α (1 ng/ml) that membrane-free stem cell components (MFSCC) had an effect on cartilage regeneration (34). In these experiments, biomarkers were assessed at specific time-points and did not exhibit trends. In the present study model, rat-specific chondrocytes were used, and different time-points were treated with IL-1 β , which could reflect the

human OA pathological process to some extent. OA progression is more intuitively reflected by observing biomarker changes at different time-points.

The conducted present experiment only diagnosed OA biomarkers at the cellular level. In follow-up research, we will verify this effect *in vivo*, which will provide a theoretical basis for the early diagnosis and treatment of OA. The present study revealed that the expression levels of MMP-3, MMP-13, and iNOS and the production of NO were upregulated in IL-1 β -induced chondrocytes (35,36). In the progression of OA, MMPs (including MMP-3 and MMP-13) are important regulators. MMP-3, as a matrix lysin, can decrease the expression of collagen and proteoglycans and activate other collagenases (37). MMP-13 is a collagenase that regulates matrix degradation and binds to type II collagen in the ECM (7,38). In addition, proteolytic enzymes and MMPs could be stimulated by IL-1 β , which are important factors for cartilage destruction (13). The results of a previous study on type II collagen degradation in chondrocytes were consistent

with those of the present study (4). Furthermore, the NF- κ B signaling pathway activated by IL-1 β , and western blot results revealed that p65 and p-p65 levels increased, which also confirmed the inflammatory response of chondrocytes (39). NF- κ B is a transcription factor that plays a vital role in the response to cellular stress as a transcriptional regulator (40). Inflammatory cytokines, such as IL-1 β , activate NF- κ B to regulate the facilitation of OA. Thus, NF- κ B is an important molecule that controls both the normal development and the pathological destruction of cartilage (41). NF- κ B is a heterodimeric, and p65 is one of its subunits (42). In the present study, IL-1 β stimulated the NF- κ B signaling pathway; then, p65 was phosphorylated in the cytoplasm and transferred to the nucleus. Therefore, the level of p65 significantly decreased in cytoplasmic extracts. However, the level of p-p65 exhibited the opposite result. In the chondrocyte nucleus, the level of p65 increased first and then decreased, while the level of p-p65 levels increased.

Western blot analyses revealed that the expression of iNOS increased in chondrocytes, and also, the concentration of NO increased in the chondrocyte culture supernatant (43,44). This suggests that activation of the NF- κ B pathway leads to the activation of related transcription factors, further leading to an increase in the synthesis of iNOS and NO. However, the increase of NO concentration stimulated the degradation of the cartilage matrix. In summary, in OA, the NF- κ B pathway is activated, which induces a cascade response downstream of the pathway, which promotes cartilage damage. In the present study, the *in vitro* OA model was induced by treating chondrocytes with IL-1 β for different periods of time to observe biomarker changes. According to the results, not only was the pathogenesis and early diagnosis of OA clarified, but also the screening of drugs used for the treatment of OA. The goal of the present study was to provide a summary and guide for the application of *in vitro* biomarkers for the development of drugs for OA. The present study provided insight into the treatment and relief of OA pathological processes.

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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

XM and LG designed the study. XM, ZZ and RL performed the cell experiments. XM, MS and XJ collected and analyzed data. XM, ZZ, YM, LG and ZW interpreted the data. XM

wrote and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of rats in the present study was approved by the Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University (Harbin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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