Ketorolac tromethamine alleviates IL-1β-induced chondrocyte injury by inhibiting COX-2 expression

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Abstract. Osteoarthritis (OA) is one of the most frequently diagnosed chronic diseases, and its prevalence is rising as life expectancy increases. The present study was designed to investigate the role of ketorolac tromethamine (KT) in OA by establishing an in vitro model in ATDC5 cells. The OA model was established through induction using 10 ng/ml IL-1β. KT was then used to treat the ATDC5 cells. An MTT assay was adopted to detect the viability of ATDC5 cells with or without IL-1β induction, and cyclo-oxygenase-2 (COX-2) expression in IL-1β-induced ATDC5 cells was measured via reverse transcription-quantitative (RT-q)PCR and western blotting. To explore the effects of KT on proliferation and apoptosis in IL-1β-induced ATDC5 cells, COX-2 was overexpressed and RT-qPCR was employed to detect the mRNA expression of COX-2. The viability of IL-1β-induced ATDC5 cells was detected by using a Cell Counting Kit-8 assay. In addition, levels of apoptosis and apoptosis-related proteins were determined using TUNEL staining and western blotting, respectively. Additionally, the effects of KT on oxidative stress in IL-1β-induced ATDC5 cells were also investigated. The expression levels of nitric oxide (NO) and inducible NO synthase (iNOS) were detected via NO kit assay and western blotting, respectively. In addition, the expression levels of oxidative stress-related proteins, including reactive oxygen species (ROS), superoxide dismutase (SOD) and prostaglandin E2 (PGE2), were determined using ELISA. To investigate the effects of KT on the inflammatory response and extracellular matrix (ECM) degradation, ELISA and western blotting were adopted to detect inflammatory-related proteins and ECM degradation-related proteins. Results from MTT assay indicated that KT decreased ATDC5 cell viability in a concentration-dependent manner. The expression of COX-2 was found to be downregulated in IL-1β-induced ATDC5 cells after treatment with KT, according to RT-qPCR and western blotting results. KT inhibited apoptosis and the expression levels of NO, iNOS and inflammatory-related proteins in IL-1β-induced ATDC5 cells, while COX-2 overexpression reversed these inhibitory effects. However, the increased proliferation of IL-1β-induced ATDC5 cells after the stimulation of KT was decreased by COX-2 overexpression. Additionally, KT upregulated Bcl-2, SOD, type II collagen and aggrecan expression levels in IL-1β-induced ATDC5 cells, whereas Bax, ROS, matrix metalloproteinase (MMP)1 and MMP13 expression levels were downregulated. KT promoted the proliferation of IL-1β-induced ATDC5 cells, whereas COX-2 overexpression reversed the promotive effects of KT, revealing that KT could alleviate IL-1β-induced chondrocyte injury by suppressing COX-2 expression.

Introduction

Osteoarthritis (OA), which is one of the most common joint diseases diagnosed globally, is characterized by the gradual degeneration of articular cartilage, secondary paroxysmal synovitis and bone reconstruction (1). Inflammatory molecules, specifically IL-1β, are some of the key mediators involved in OA pathophysiology (2). It is reported that ~10% of men and 18% of women aged >60 years are affected by OA (3). Furthermore, OA contributes to impaired mobility in the elderly, as well as pain, loss of function and decline in quality of life (4). To date, the pathophysiology of OA is poorly understood and the treatment for OA is under investigation (5). Thus, it is of great urgency to explore the underlying mechanism of OA and to seek effective therapeutic options for the treatment of OA.

Ketorolac tromethamine (KT), a novel injectable/oral non-steroidal anti-inflammatory analgesic that was first commercialized in the United States in March 1990, has no obvious opiate receptor activity and can be used alone or combined with other opiate analgesics to relieve postoperative pain (6,7). It has been noted that KT has anti-inflammatory effects (8). Shrestha et al (9) reported that the considerable pain and loss of function resulting from acute gouty arthritis could be alleviated...
through the use of KT. It was also revealed that the consistent administration of KT could relieve the pain of rheumatoid arthritis (RA) (10). The viability of cartilage cells was verified to be increased after exposure to KT, indicating that KT is an ideal therapeutic agent for the treatment of OA (11). In view of this, the present study was conducted to investigate our hypothesis that KT could relieve chondrocyte injury.

Cyclooxygenase-2 (COX-2) is an isoform of cyclooxygenase, which is an enzyme involved in the biosynthesis of prostaglandin, that plays an indispensable role in relieving pain, inflammatory response and improving joint function for patients suffering from RA (12,13). It has been reported that COX-2 is a target of OA and RA therapy due to its pain, inflammatory response and improving joint function (14). Anderson et al (15) reported that COX-2 acted as a prominent player in the inflammation involved in adjuvant arthritis, and that the inhibition of COX-2 could improve adjuvant arthritis (15). Additionally, KT was reported to relieve the inflammatory response in OA through the inhibition of COX-2 expression (11). Therefore, in the present study, the hypothesis that KT could alleviate chondrocyte injury by targeting COX-2 expression was assessed.

Materials and methods

Cell culture, treatment and transfection. The chondrogenic cell line, ATDC5, was purchased from RIKEN BioResource Center and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO2. The cells were stimulated with IL-1β (10 ng/ml) for 24 h and then 5, 10 and 20 mg/ml KT were used to treat these IL-1β-induced ATDC5 cells at 37˚C for 24 h; IL-1β-induced ATDC5 cells without KT as used as the control.

With the aim of overexpressing COX-2 expression in ATDC5 cells, the pcDNA3.1 vector containing full-length COX-2 (OV-COX-2) and empty vector (OV-NC) were all designed and synthesized by Thermo Fisher Scientific, Inc. In addition, the cells that were not transfected with the plasmid were used as the control group. The transfection of ATDC5 cells was performed with Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at a concentration of 50 ng/ml. Following transfection for 48 h at 37˚C, the transfection efficiency was detected by reverse transcription-quantitative PCR (RT-qPCR) 48 h post-transfection.

MTT assay. The proliferation of KT-treated ATDC5 cells was detected by performing an MTT assay (Beyotime Institute of Biotechnology). The cells were seeded into 96-well plates at a density of 2x10^3 cells/well and incubated at 37˚C for 24 h. Following this, 10 µl MTT solution was added into each well and then the cells were incubated with formazan lysis solution for another 4 h at 37˚C until all purple crystals were dissolved. Finally, the absorbance at 490 nm was detected using a spectrophotometer.

RT-qPCR. The total RNA from ATDC5 cells was extracted with TRIzol® reagent (Thermo Fisher Scientific, Inc.) and then reverse transcribed into cDNA using PrimeScript Reverse Transcriptase (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. qPCR was conducted via SYBR-Green PCR Master Mix kit (Takara Biotechnology Co., Ltd.) on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The following thermocycling conditions were used for qPCR: 95˚C for 10 min; followed by 40 cycles of denaturation at 95˚C for 10 sec and annealing/extension at 60˚C for 60 sec. The following primers (GenScript) were used for qPCR: COX-2 forward, 5′-AGG ACTCTGTGCTAGAAGGA-3′ and reverse, 5′-TGACATGGA TTGGAACAGCA-3′; and GAPDH forward, 5′-ACCTTTAAG AGGGATGCTGC-3′ and reverse, 5′-CCCCATACGGCC AAATCCGT-3′. Gene expression levels were quantified using the 2^−ΔΔCt method (16) and normalized to the internal reference gene GAPDH.

Western blot analysis. The total proteins from transfected ATDC5 cells treated by IL-1β and KT were extracted with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) and levels were determined using a BCA protein assay kit (Thermo Fisher Scientific Inc.). The proteins (30 µg/lane) were separated on a 10% gel using SDS-PAGE and then transferred onto PVDF membranes. Membranes were blocked with 5% skimmed milk for 2 h at room temperature. Subsequently, the membranes were incubated overnight at 4˚C with the following primary antibodies: Anti-COX-2 (1:5,000; cat. no. Ab179800), anti-Bcl2 (1:1,000; cat. no. Ab182858), anti-Bax (1:1,000; cat. no. Ab32503), anti-GAPDH (1:1,000; cat. no. Ab8245), anti-matrix metalloproteinase (MMP)1 (1:1,000; cat. no. Ab137332), anti-MMP13 (1:1,000; cat. no. Ab219620), anti-inducible NO synthase (iNOS) (1:1,000; cat. no. Ab178945), anti-Collagen II (1:1,000; cat. no. Ab34712), anti-Aggrecan (1:1,000; cat. no. Ab216965) and anti-β-actin (1:1,000; cat. no. Ab8227; all Abcam).

Following primary incubation, the membranes were incubated with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:5,000; cat. no. Ab6721; Abcam) at room temperature for 4 h. Finally, the images of protein bands were visualized using ECL reagent (MilliporeSigma). Protein expression levels were semi-quantified using ImageJ software (version 1.46; National Institutes of Health) with GAPDH as the loading control.

Cell Counting Kit-8 (CCK-8) assay. CCK-8 assay was employed to assess cell viability. The transfected cells treated by IL-1β and KT were inoculated into 96-well plates at 5x10^3 cells/well for 24 h and then incubated with 10 µl CCK-8 reagent (Beyotime Institute of Biotechnology) at 37˚C for another 2 h. The absorbance value at 450 nm was detected using a microplate reader (BioTek Instruments, Inc.).

TUNEL. To detect the apoptosis of transfected ATDC5 cells treated by IL-1β and KT, TUNEL assay kit (C1086; Beyotime Institute of Biotechnology) were employed according to the manufacturer's protocol. ATDC5 cells were fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized in 0.25% Triton X-100 for 20 min at room temperature. Then, the cells (1x10^5 cells/well) were incubated with TUNEL reaction solution at 37˚C for 1 h, followed by staining with
1 mg/ml DAPI at 37°C for 30 min and mounted in an anti-fade reagent (Beijing Solarbio Science & Technology Co., Ltd.). Finally, a fluorescence microscope was applied to observe and capture images of TUNEL-positive cells in five fields of view selected at random.

Measurement of nitric oxide (NO) production. The production of NO in transfected ATDC5 cells treated by IL-1β and KT was detected using a NO assay kit (S0021; Beyotime Institute of Biotechnology) according to the manufacturer's specifications. In brief, the cell supernatants were plated into 96-well plates and then incubated with Griess Reagent for 15 min at room temperature. Following which, the absorbance was measured at 540 nm (magnification, x200).

ELISA kits (Beyotime Institute of Biotechnology) were applied according to the manufacturer’s protocol to detect the levels of inflammatory cytokines, including IL-6 (cat. no. P1326) and TNF-α (cat. no. PT512). The expression level of prostaglandin E2 (PEG2) was detected using a PGE2 kit (Shanghai Tongwei Industrial Co., Ltd.; cat. no. TW8620). The reactive oxygen species (ROS) ELISA Kit (AMEKO, LianShuo Biological; cat. no. DRE901Mu) and superoxide dismutase (SOD) ELISA Kit (Excell Bio; cat. no. EMC56-96) were used to evaluate ROS and SOD levels in cells, respectively.

The optical density was recorded using a microplate reader (Bio-Rad Laboratories, Inc.) under the circumstance of λ=450 nm and the levels of inflammatory cytokines were calculated with the standard curve.

Statistical analysis. The experimental data are displayed as the mean ± SD and were assessed with SPSS software 22.0 (IBM Corp). Two-tailed Student's t-tests (unpaired) were used to compare the differences between two groups. Comparisons among multiple groups were performed with one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

KT increases viability and decreases COX-2 expression of IL-1β-induced ATDC5 cells. To ensure the findings in the following experiments were reliable, the viability of ATDC5 cells following the application of MTT was detected. Overall, KT increased ATDC5 cell viability in a dose-dependent manner (Fig. 1A). After induction with IL-1β, the viability of ATDC5 cells was detected with another MTT assay. As shown in Fig. 1B, the viability of IL-1β-induced ATDC5 cells treated with KT was increased compared with that of the IL-1β group. In addition, the relative protein and mRNA expression levels of COX-2
in IL-1β-induced ATDC5 cells were decreased after treatment with KT in a concentration-dependent manner (Fig. 1C and D). Therefore, the concentration of KT with the best inhibitory effect (20 mg/ml) was selected for the following experiment.

**KT promotes IL-1β-induced ATDC5 cell proliferation and suppresses apoptosis by inhibiting COX-2 expression.** As revealed in Fig. 2A, the expression of COX-2 in IL-1β-induced ATDC5 cells was significantly upregulated following transfection with COX-2 overexpression plasmids. Results from the CCK-8 assay revealed that the viability of IL-1β-induced ATDC5 cells was markedly increased after treatment with KT compared with that of the IL-1β group. However, the increased cell viability was then decreased by COX-2 overexpression (Fig. 2B). In addition, the decreased apoptosis of IL-1β-induced ATDC5 cells treated with KT was increased by
COX-2 overexpression, suggesting that COX-2 overexpression reversed the inhibitory effects of KT on IL-1β-induced ATDC5 cells (Fig. 2C and D). Compared with that in the IL-1β + KT + Ov-negative control (NC) group, the expression of Bcl-2 was downregulated by COX-2 overexpression, while the expression of Bax was upregulated (Fig. 2E).

**KT suppresses oxidative stress in IL-1β-induced ATDC5 cells by inhibiting COX-2 expression.** As presented in Fig. 3A, the decreased NO expression caused by co-treatment of IL-1β and KT was increased by COX-2 overexpression when compared with that in the IL-1β + KT + Ov-NC group. The relative levels of iNOS were found to be downregulated in IL-1β-induced ATDC5 cells after treatment with KT. Nevertheless, COX-2 overexpression reversed the inhibitory effects of KT, evidenced by the increased iNOS expression compared with those in the IL-1β + KT + Ov-NC group (Fig. 3B). Additionally, compared with the IL-1β + KT + Ov-NC group, COX-2 overexpression upregulated the expression levels of MMP1 and MMP13, but downregulated the expression levels of type II collagen (collagen II) and aggrecan (Fig. 4C).

Discussion

OA, which is regulated by multi-level and complex biological interactions, is the most common form of arthritis diagnosed in the musculoskeletal system (17,18). OA is characterized by pain, swelling and stiffness of the joints, and contributes to disability worldwide (19). Furthermore, OA has caused a large number of direct and indirect social and economic costs worldwide (20). At present, the exact underlying mechanism of OA remains unclear and currently no effective cure exists (5). Therefore, it is of great importance to identify a more effective therapy for the treatment of OA.

**KT suppresses the inflammatory response and extracellular matrix (ECM) degradation of IL-1β-induced ATDC5 cells by inhibiting COX-2 expression.** Results from Fig. 4A suggested that the increased levels of IL-6 and TNF-α in IL-1β-induced ATDC5 cells were decreased by KT treatment. However, COX-2 overexpression partially abolished the inhibitory effects of KT on IL-1β-induced ATDC5 cells, evidenced by the increased levels of IL-6 and TNF-α in comparison with those in the IL-1β + KT + Ov-NC group. In addition, KT treatment effectively reduced the level of PGE2 in IL-1β-induced ATDC5 cells, while COX-2 overexpression also reversed this positive effect (Fig. 4B). Additionally, compared with the IL-1β + KT + Ov-NC group, COX-2 overexpression upregulated the expression levels of MMP1 and MMP13, but downregulated the expression levels of type II collagen (collagen II) and aggrecan (Fig. 4C).
In the present study, it was found that the decreased viability of IL-1β-induced ATDC5 cells was increased following treatment with KT. It was also revealed that KT suppressed oxidative stress, inflammatory response and ECM degradation of IL-1β-induced ATDC5 cells by inhibiting COX-2 expression, revealing that KT exerted protective effects on IL-1β-induced ATDC5 cells by targeting COX-2.

The inflammatory response has an important role in OA. As cytokines regulate the biological function of cells and the degeneration process of cartilage, they act as important regulators in the pathogenesis of OA (23,24). As a critical inflammatory cytokine, TNF-α has been shown to promote the occurrence of initial events in the process of OA and has roles in the synthesis of other cytokines, such as IL-6 (25). Furthermore, IL-6 has been reported to activate the immune system and strengthen the inflammatory response (26). In the present study, it was discovered that the levels of IL-6 and TNF-α were markedly increased following use of IL-1β compared with those of the control group. However, the levels of IL-6 and TNF-α declined following treatment with KT. Yin et al (27) demonstrated that COX-2 has a critical role in the pathological process of inflammation due to its functions in promoting pro-inflammatory mediators and cytokines. In the present study, it was identified that COX-2 overexpression increased the levels of IL-6 and TNF-α in comparison with the IL-1β + KT + Ov-NC group.

Oxidative stress is another important factor in OA. It has been revealed that ROS can destroy chondrocytes and the ECM of chondrocytes. Excessive ROS production results in chondrocyte senescence and death, as well as the dysfunction of subchondral bone (28,29). Srivastava et al (30) reported that Curcuma longa extract alleviates OA of the knee by reducing oxidative stress biomarkers. Similarly, the
present study discovered that the increased ROS expression stimulated by IL-1β was significantly decreased after treatment with KT.

ECM degradation has also been revealed to be involved in OA. Daheshia and Yao (31) suggested that pro-inflammatory cytokines could induce cartilage degradation by activating MMPs. MMPs, which are a type of proteolytic enzyme, can degrade the ECM components in OA (32). Cartilage ECM structure, which is primarily composed of collagen II and aggrecan, serves as a vital player in supporting joint movements (33,34). In the present study, it was determined that the expression levels of collagen II and aggrecan were downregulated by IL-1β, while the expression levels of MMP1 and MMP13 were upregulated, indicating that IL-1β could induce ECM degradation. Nevertheless, the degradation of collagen II and aggrecan, as well as the upregulation of MMP1 and MMP13, were notably reversed by treatment with KT.

In conclusion, KT suppressed the oxidative stress, inflammatory response and ECM degradation of IL-1β-induced ATDC5 cells, while COX-2 overexpression reversed the inhibitory effects of KT on IL-1β-induced ATDC5 cells. These results revealed that KT could relieve chondrocyte injury by targeting COX-2 overexpression.

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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

CL conceptualized and designed the present study. YC acquired, analyzed and interpreted data. CL and YC drafted the manuscript and revised it critically for important intellectual content. CL and YC confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agreed to be held accountable for the current study in ensuring questions related to the integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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