Taraxasterol inhibits inflammation in osteoarthritis rat model by regulating miRNAs and NF-κB signaling pathway

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Osteoarthritis (OA) has a high incidence rate in the elderly population and is a cause of chronic degenerative joint disease. Current therapeutic approaches to OA are effective but come with some side effects. Therefore, it is urgent to find new and effective OA treatments. This study aimed to clarify the function of taraxasterol (TAX) isolated from Taraxacum officinale in the papain-induced rat OA model. We observed that TAX alleviated the typical OA-caused phenomena in the joint. The expression of serum inflammatory mediators such as TNF-α, IL-6, and IL-1β was also repressed by TAX. In addition, NF-κB signaling pathway was repressed by TAX. Furthermore, two microRNAs: miR-140 and miR-146a were elevated after TAX treatment in OA rat model. Interestingly, several common targets of miR-140 and miR-146a, including HSPA4L, ST5, and ERBB4, were confirmed to be regulated by TAX. Inflammatory response related genes including S100A8, CCL3, A2M, LBP, and CCR1 were repressed by TAX in OA rat model. In summary, TAX inhibits inflammation in osteoarthritis rat model. Inflammatory mediators, NF-κB pathway and miR-140/miR-146a targets mediate the function of TAX.

Keywords: Taraxasterol, osteoarthritis, NF-κB pathway, miR-140, miR-146a

INTRODUCTION

Osteoarthritis (OA) has a high incidence rate in the elderly population and is a cause of chronic degenerative joint disease caused by OA results in a reduced quality of life and an increased economic burden (Litwic et al., 2013). Among the types of OA, osteoarthritis of the knee (KO/A) is the most common (Sliepen et al., 2018). Current therapeutic approaches including non-steroidal anti-inflammatory drugs (NSAIDs), arthroplasty, and hyaluronic acid injection are effective but the treatments have side effects (Michael et al., 2010). Therefore, it is urgent to find new and effective therapies for OA. Previous studies have shown that traditional Chinese medicine (TCM) alleviates OA symptoms, improving the joint function in patients with OA (Wang et al., 2020). These studies have shown that TCM can repress inflammatory mediators in OA, and promote the transformation of bone marrow derived mesenchymal stem cells into chondrocytes (Chen et al., 2007). However, a precise molecular mechanism of TCM in alleviating OA remains elusive.

Taraxasterol (TAX) is the main active compound isolated from Taraxacum officinale (Martinez et al., 2015). In traditional Chinese medicine, Taraxacum officinale is frequently used for infectious and inflammatory diseases such as pneumonia (Liu et al., 2010), hepatitis (Park et al., 2010), and mastopathy (Sweeney et al., 2005). The pharmacological activities of Taraxacum officinale include anti-inflammatory and anti-angiogenesis (Leon et al., 2008; Park et al., 2014). Previous studies have shown that TAX, an active component of Taraxacum officinale, represses inflammation via regulating proinflammatory cytokines and mediators. Both NF-κB and MAPK signaling pathways are targeted by TAX (Zhang et al., 2012; Xiong et al., 2014). In addition, TAX shows protective effect in endotoxic shock and allergic asthma (Zhang et al., 2014). In OA, TAX suppresses IL-1β – induced inflammation (Piao et al., 2015) and represses matrix metalloproteinases (MMPs), NO and PGE2, resulting in a protective effect on human chondrocytes (Piao et al., 2015). Here, one of our aims is to demonstrate the effect of TAX in the rat OA model and clarify mechanism of NF-κB signaling pathway regulation by TAX.

Interestingly, previous studies have shown that deregulation of miRNAs contributes to the progression of OA. Therefore, it is interesting to investigate whether miRNAs could mediate the function of TAX in OA treatment. Previous research has shown that miR-140 and miR-146a have therapeutic potential in OA treatment. MiR-140 alleviates OA progression by repressing NFκB, several ILS, and NF-κB signaling pathway (Guan et al., 2018; Hou et al., 2021). Here, we test whether miR-140 and miR-146a mediate the function of TAX in OA alleviation. We hope that this study will contribute to finding new drug candidates for OA therapy.

MATERIAL AND METHODS

Chemicals, antibodies, and kits

Taraxasterol (TAX) was purchased from Merck (PHL.84272, 10 mg, ≥98.0% by HPLC). The chemical structure of TAX is shown in Fig. 1A. TAX was dis-
solved in distilled water to a stock concentration of 1 mg/ml and stored in −20°C. Primary antibodies of IKK2 (15649-1-AP), NF-κB (66535-1-Ig), TAK1 (12330-2-AP), MMP-1 (10371-2-AP), MMP-2 (10373-2-AP), caspase-1 (22915-1-AP), and NLRP3 (19771-1-AP) were purchased from Proteintech (USA). Phosphorylated IκB (Ser32, #2859) was purchased from Cell Signaling Technology (USA). Rat TNF alpha ELISA Kit (ab236712), Rat IL-6 ELISA Kit (ab234570), and Rat IL-1 beta ELISA Kit (ab255730) were purchased from Abcam (USA).

Osteoarthritis rat model

Wistar rats (180–200 g) were purchased from CAVENS experimental animal company (Changzhou, China), and housed at room temperature (25±2°C) and humidity (75±5%) with 12:12 h light-dark cycle. Animals were randomly divided into 5 groups. 6 rats were included in each group. In control group, rats received vehicle injection only while OA model group was given 4% papain and 0.03 M cysteine solution into the knee joint. Both papain and cysteine were administrated intraarticularly three times (1, 4 and 7 day) to induce OA. For OA models, after the last papain and cysteine injection, TAX was administrated once a day orally to a rat at different doses, including 0 (untreated group) 2.5, 5 and 10 mg/kg. Healthy rats (n=6), not treated with the injections of 4% papain and 0.03 M cysteine solution, served as a control group. All rats in each group were sacrificed for further study at the 28th day of the experiment. Animal experiments were approved by the Ethics Committee of Changzhou Traditional Chinese Medicine Hospital.

ELISA

At the last day of establishing OA rat model, rats were anesthetized by diethyl ether and blood samples were collected from the retro-orbital vein puncture. Serum was obtained by centrifugation of a blood sample at 3500 rpm for 10 min. The concentration of TNF-α, IL-6, and IL-1β were tested using dedicated ELISA kits.
Table 1. Primers used in q-PCR analysis (5'-3', rat sequences).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>HSPA4L</td>
<td>TTCTCAGCTGTAACATCGTGCT</td>
<td>CCTGTCGCTGTACTCGTGTTG</td>
</tr>
<tr>
<td>ST5</td>
<td>ACTGAGATGACGCAACAAAATTTGG</td>
<td>CATCTCAGGAGGATCTCCTTGT</td>
</tr>
<tr>
<td>ERBB4</td>
<td>GTGCATGGACCTCCAGCTGTGACTAG</td>
<td>TCATTGAACTGCACAGGGCAAA</td>
</tr>
<tr>
<td>S100A8</td>
<td>AAATCGACATGGCTTCCTACAGG</td>
<td>CCCCACCTTTAC1CAACAGGCA</td>
</tr>
<tr>
<td>CCL3</td>
<td>TTCTGCTACCATGACACCTTCGTT</td>
<td>CGTGAATCTCAGGGGCTGTA</td>
</tr>
<tr>
<td>A2M</td>
<td>AGATGGTGAGATCTGTCGTTGTC</td>
<td>ACGGTCTGCTGGATCTGTTG</td>
</tr>
<tr>
<td>LBP</td>
<td>GATAGGCGACAGGAGGGCTT</td>
<td>GGCTATGAAACTCGTACTGCC</td>
</tr>
<tr>
<td>CCR1</td>
<td>CTCTGAGCTATGAGGAAGCTT</td>
<td>ACATGGAATACCAAGAAATCCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGATTCTACCCACGCGGATGT</td>
<td>TGATGGGCTTCCCTAGTTAG</td>
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Histopathological examination of the joints

The knee joints of rats were subject to histological analysis. 10% phosphate buffered formalin was used to fix the joints and then 10% EDTA was used for decalcification. The joints were embedded in paraffin and cut into thick sections which served for hematoxylin-eosin (H&E) staining as described elsewhere (Tsuchiya et al., 2005). The stained sections were assigned scores by at least two independent pathologists from the Changzhou Traditional Chinese Medicine Hospital.

Quantitative PCR (q-PCR) analysis

RNA from the collected blood samples was purified with QIAamp RNA Blood Mini Kit (QIAGEN, Germany) according to the instructions provided. RNA of joint tissue was collected and purified with RNeasy Kit (QIAGEN, Germany). 100 ng of total RNA was used for reverse transcription and generating cDNA library by using Verso cDNA Synthesis Kit (Thermo Fisher, USA). For total miRNA isolation, miRNeasy Micro Kit (QIAGEN, Germany) was used according to the provided protocol. For q-PCR, SYBR™ Green Master Mix (Thermo Fisher, USA) and miRCURY LNA SYBR Green PCR Kit (QIAGEN, Germany) were used for miRNA and mRNA, respectively. The reaction was performed at LightCycler® 480 System (Roche, Switzerland). The relative gene expression was calculated by using 2^ΔΔCt method, in which, GAPDH and U6 served as internal reference genes for the expression of mRNA and miRNA, respectively. For all the genes, expression was normalized to the internal reference genes. Q-PCR primers are listed in Table 1.

Western blot

At the last day of treatment on animals, rat joint was collected and frozen into liquid nitrogen until homogenization. After homogenization, protein was extracted by using T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher, USA). Protein concentration was determined by bicinchoninic acid assay. 40 μg protein of each group was loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. Then, membranes were blocked by 5% milk in Tris-buffered saline and Tween 20 (TBST) for 30 min at room temperature. Next, primary antibodies diluted in TBST at 1:1000 ratio were used for membrane incubation at 4°C overnight. After washing 3 times with TBST, membranes were incubated with secondary antibody at room temperature for 1 h. LI-COR Odyssey Imaging System was used for imaging with chemiluminescence reagent. Quantification was performed by the software of LI-COR Odyssey Imaging System and protein expression was normalized to β-actin. The western blot was performed in samples from 6 rats in each group. The representative image of Western blot was shown and the quantification represented statistics of 6 rats from each group. The expression of protein in untreated group (OA model group with 0 mg/kg TAX) was compared to control group (healthy). The protein expression in OA models treated with increased concentrations of TAX was compared to untreated group (OA model group with 0 mg/kg TAX).

Bioinformatic analysis

Gene expression data from two independent patient cohorts (GSE36700 and GSE98918) were obtained and downloaded from Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo). The raw data were downloaded as MINiML files. The extracted data were normalized and processed by log2 transformation. The microarray data were normalized using the pre-processCore package in R software (version 3.4.1). Probes were converted to gene symbols according to the platform annotation information of the normalized data. Probes with more than one gene were eliminated and the average value was calculated for genes corresponding to more than one probe. As an initial quality control step of the variance stabilized counts, batch effect was removed using the removeBatchEffect function of limma R package. MiRNA targets prediction was performed through TargetScan 7.2 (http://www.targetscan.org/vert_72/).

Statistics

SPSS 21.0 was used to calculate all the values (means ± standard error of the mean). Statistical analyses were performed with Student’s t-test in two groups comparison. One-way analysis of variance (ANOVA) test was used to verify the significance among multiple experimental groups. The statistical significance was p<0.05.

RESULTS

TAX represses inflammation in osteoarthritis rat model

To validate the effect of TAX, we generated an OA rat model by injecting 4% papain and 0.03 M cysteine purchased from Abcam (USA). Procedures were performed according to the manufacturer’s protocol.
solution into the rat knee joint. In H&E staining of the model joint, we observed irregularly stained matrix, loss of tide lines, and reduction in cartilage thickness. In addition, articular cartilage lesions were also present in the medial tibial plateaus in the OA model group (0 mg/kg TAX) (Fig. 1B). TAX treatments (5 mg/kg and 10 mg/kg) were able to reverse the described symptoms in the rat joint (Fig. 1B), in a concentration dependent manner, suggesting protective role of TAX in OA. Furthermore, we measured the levels of inflammatory mediators including TNF-α, IL-6 and IL-1β in rat serum. Compared to the OA model group, TAX treatments repressed TNF-α, IL-6, and IL-1β expression in serum in a concentration dependent manner (Fig. 1C). However, the levels of TNF-α, IL-6, and IL-1β were still higher in OA model after TAX treatments than in healthy control group. In summary, we propose that TAX alleviated OA symptoms via repressing inflammatory mediators: TNF-α, IL-6, and IL-1β.

TAX represses NF-κB signaling pathway

Previous studies have shown that activated NF-κB signaling pathway promotes OA, thereby we asked whether TAX alleviates OA by regulating NF-κB signaling pathway. The expression of key factors of NF-κB signaling pathway was analyzed with Western blot (Fig. 2). In OA model group (0 mg/kg TAX), the expression of IKK2 and NF-κB was increased compared to healthy control group, indicating activation of NF-κB signaling pathway in OA model. TAX treatment resulted in repression of IKK2 and NF-κB expression. The highest dose of TAX (10 mg/kg) showed the strongest inhibitory effect on the expression of the components of NF-κB signaling pathway. The expression of TAK1, MMP-1, MMP-2, caspase-1, and NLRP3 was also increased in OA model group (0 mg/kg TAX) compared to the healthy control group. TAX treatment suppressed the expression of these factors in the cartilage of OA model in a concentration-dependent manner. Therefore, we conclude that TAX influenced NF-κB signaling pathway by mediating both upstream (TAK1) and downstream factors (MMPs, caspase-1 and NLRP3) which coexisted with OA alleviation.

TAX induces miR-140 and miR-146a

miRNAs were shown to play an important role in OA, in particular, miR-140 and miR-146a are postulated as promising OA biomarkers. Both miR-140 and miR-146a attenuate inflammation in OA. Therefore, we quantified miR-140 and miR-146a expression in rat OA model. Q-PCR analysis revealed that the expression of miR-140 and miR-146a was decreased in OA model compared to the healthy control group (Fig. 3A and 3B), which was reversed by TAX treatment (2.5–10 mg/kg) (Fig. 3A and 3B) also in a concentration-dependent manner. Above results suggest that miR-140 and miR-146a mediate TAX function in OA. Since miRNAs exert their role via targeting downstream genes, we analyzed the potential targets of miR-140 and miR-146a in context of their possible regulation by TAX in OA. Through the analysis with TargetScan 7.2 online tool, we obtained a total of 434 miR-140 targets and 283 miR-146a targets. Based on GO analysis, the targets of miR-140 were mainly enriched in the biological processes of endocytosis, TGF-β pathway and focal adhesion. The targets of miR-146a were mainly enriched in trit morphogenesis (Fig. 3C and 3D). Interestingly, 20 genes were common targets for both miR-140 and miR-146a (Table 2). Therefore, miR-140 and miR-146a may have a synergetic effect in OA by regulating common targets. We analyzed these 20 common target genes in an independent OA patient cohort obtained from GSE36700. Out of these, three genes: HSPA4L, ST5, and ERBB4 were upregulated in OA patients (Fig. 3E). At the same time, q-PCR analysis showed that the expression of HSPA4L, ST5, and ERBB4 was increased in rat OA model (0 mg/kg TAX) compared to the healthy control group, and this increase was repressed by 10 mg/kg TAX treatment (Fig. 3F). Therefore, we conclude that HSPA4L, ST5, and ERBB4 are involved in the function of TAX in OA. In summary, both miR-140 and miR-146a mediate the function of TAX in OA and their gene targets are also involved in the regulation.

TAX represses genes related to inflammation in OA

Having shown that TAX alleviates inflammation in OA through repressing the NF-κB signaling pathway, we asked whether any other genes related to inflammation are regulated by TAX in OA. An independent OA patient cohort obtained from GSE36700. Out of these, three genes: HSPA4L, ST5, and ERBB4 were upregulated in OA patients (Fig. 3E). At the same time, q-PCR analysis showed that the expression of HSPA4L, ST5, and ERBB4 was increased in rat OA model (0 mg/kg TAX) compared to the healthy control group, and this increase was repressed by 10 mg/kg TAX treatment (Fig. 3F). Therefore, we conclude that HSPA4L, ST5, and ERBB4 are involved in the function of TAX in OA. In summary, both miR-140 and miR-146a mediate the function of TAX in OA and their gene targets are also involved in the regulation.

Table 2. Common gene targets for miR-140 and miR-146a.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene target</th>
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<tr>
<td>NUCK51, KCND3, CEF2, LCOR, HSPA4L, MARK1, CELF1, ERLEC1, EFIF4G2, TAF9B, ZNF652, ST5, PDE7A, RFX7, MFHAS1, ERBB4, IKZF3, STRN, POLR3H, TMEM120B</td>
<td>CAST1A, COL3A1, COL5A2, COL6A3, FAP, ITGB1, LOX, LOXL1, LOXL2, PLOD1, ADAM12, POSTN, CRISPLD2, ADAMTS14</td>
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Table 3. Up-regulated genes involved in inflammation responses and down-regulated genes involved in in extracellular structure organization processes.

<table>
<thead>
<tr>
<th>Biological processes</th>
<th>Gene symbol</th>
</tr>
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<tr>
<td>inflammation responses</td>
<td>A2M, APOE, C1QA, C1QB, C3, C4D, C5L, DNASL13, CFH, CFI, IGF1, LBP, LPL, MGST2, SERPINF1, PLA2G2A, S100A8, S100A9, S100A12, CCL3, CCL14, CCL18, TEC, KLF4, CALCR, GPRCSB</td>
</tr>
<tr>
<td>extracellular structure organization</td>
<td>COL1A1, COL3A1, COL5A2, COL6A3, FAP, ITGB1, LOX, LOXL1, LOXL2, PLOD1, ADAM12, POSTN, CRISPLD2, ADAMTS14</td>
</tr>
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</table>
patient cohort GSE98918 was investigated. Heat map shows the top 20 genes differentially expressed in 12 OA and 12 non-OA patients (Fig. 4A). The remaining differentially expressed genes were shown in the volcano diagram (Fig. 4B). In detail, a total of 106 genes were upregulated (log2[Fold change (FC)] over 1.0) in OA patients, and 114 genes were downregulated (log2[FC] less than −1.0) (Fig. 4B). Gene ontology (GO) enrichment analysis indicated that the upregulated genes (with FC over 1.5) were enriched in biological processes of inflammatory response, external stimulus response and cell chemotaxis (Fig. 4C). 25 of the upregulated genes were enriched in inflammatory response process and are listed in Table 3. The downregulated genes (with FC less than 0.5) were enriched mainly in extracellular structure and matrix organization processes (Fig. 4D) and are listed in Table 3. We measured expression of all 25 upregulated genes related to inflammation in blood samples collected from rat OA model and healthy controls. We identified 5 genes (S100A8, CCL3, A2M, LBP and CCR1) that showed significantly higher expression (FC over 1.5-fold) in rat OA model (0 mg/kg TAX) compared to healthy controls (Fig. 4E). TAX treatment (10 mg/kg) repressed the expression of these 5 genes compared to the untreated OA model group (0 mg/kg TAX) (Fig. 4E). Next, we evaluated the expression of S100A8, CCL3, A2M, LBP and CCR1 in the joints. In OA model group (0 mg/kg TAX), the expression of these 5 genes was significantly elevated compared to healthy controls, whereas TAX treatment (10 mg/kg) largely reversed this.
increase (Fig. 4F). In summary, TAX protected the joint from inflammation through repression of genes related to inflammation responses.

DISCUSSION

Taraxasterol (TAX) is a type of pentacyclic-triterpene isolated from *Taraxacum officinale*. Previous studies have shown that TAX represses inflammation in rheumatoid arthritis and other diseases (Xueshibojie et al., 2016; Chen et al., 2019; Yang et al., 2021). In this study, we explored the effect of TAX in rat OA model generated by injection with 4% papain and 0.03 M cysteine solution into the knee joint. In line with previous studies showing TAX represses inflammatory mediators, we confirmed that TAX represses the expression of TNF-α, IL-6, and IL-1β in rat OA model. The clinical data from the OA cohorts strongly suggests that joint tissues are destructed by a catabolic process in which TNF-α and IL-1β play important roles. In vitro studies indicated that blocking of IL-1β activity prevents cartilage destruction in OA (van Vulpen et al., 2015). In addition, increased expression of TNF-α and IL-1β was observed in OA synovial membrane, synovial fluid, and cartilage (Liu et al., 2017). TAX could protect the joints of rats in OA model by repressing TNF-α and IL-1β and preventing cartilage destruction. Interestingly, the effect of IL-1 can be amplified by IL-6 (Studer et al., 2011), therefore the regulation of ILs is critical for the function of TAX in OA treatment.

Since deregulated NF-κB activity is implicated in inflammation and rheumatic diseases, including osteoarthritis (Choi et al., 2019), in this study we investigated the effect of TAX on NF-κB signaling pathway. Indeed, IKK2 expression was elevated in rat OA joint samples, which provides an explanation for the increased activity of NF-κB signaling pathway in the OA model. TAX treatment remarkably repressed the expression of IKK2 and the activity of NF-κB signaling pathway compared to the untreated OA. Higher concentration of TAX showed stronger repression on NF-κB signaling pathway compared to the untreated OA. Therefore, we conclude that the function of TAX in alleviating OA depends on NF-κB signaling pathway repression via inhibiting IKK2 expression. Furthermore,
several MMPs, including MMP-1 and MMP-2, were also repressed by TAX in OA model. NF-κB promotes the expression of MMPs through inducing TNF-α and IL-1β (Csaki et al., 2009). Consequently, TAX regulates the entire NF-κB pathway in which TNF-α, IL-1β and MMPs are involved. Since activated MMPs degrade articular cartilage in arthritis (Mort & Billington, 2001), TAX could protect the joint from damage by preventing articular cartilage degradation.

Previous studies demonstrated that both miR-140 and miR-146a mediate OA progression via regulating different target genes (Nugent, 2016). In our study, in line with other studies, both miR-140 and miR-146a expression was decreased in rat OA model, which was reversed with TAX. Interestingly, previous studies have shown that miR-140 and miR-146a synergistically inhibit TLR4 stimulation, leading to the activation of NF-κB signaling pathway and in consequence, triggering downstream inflammation mediators (Papathanasiou et al., 2020). MiR-140 and miR-146a are upstream regulators of TAX in OA. In addition, we analyzed the common targets of miR-140 and miR-146a. The expression of HSPA4L1, ST5, and ERBB4 was changed in OA and reversed by TAX in the OA model. HSPA4L1 and ERBB4 promote inflammation in diseases (Wang et al., 2015; Schumacher et al., 2017), which corroborates the hypothesis that the targets of miR-140 and miR-146a mediate the function of TAX in repressing inflammation. Nevertheless, those potential target genes still need to be validated in further experiments.

Arthritis comprises of three categories: OA, rheumatoid arthritis (RA) and psoriatic arthritis (Hauge & Geurts, 2017). In general, OA is the type of arthritis in which the abnormalities of joints of knees and hips are significant. Loss of matrix proteoglycans, fibrillation of cartilage surface and eventual loss of collagenous matrix are all common in OA. D Systemic autoimmune disorder is the main cause of RA, and chronic inflammation is its main feature (MacDonald et al., 2021). Interestingly, previous studies have shown that the genes with changed expression in OA and RA rarely overlapped (Li et al., 2016). Therefore, the strategy of OA or RA therapies should vary between individual cases. Here, an independent OA patient cohort was analyzed to reveal the effect of TAX on other genes of inflammation. The genes differentially expressed in the OA patients compared to healthy controls were enriched the biological pathways of inflammation responses. By q-PCR validation, S100A8, CCL3, A2M, LBP and CCR1 were confirmed to be regulated by TAX in rat OA model. Notably, both CCL3 and CCR1 are related to MMPs expression (Kato et al., 2013; Wang et al., 2019), which contributes to the articular cartilage degradation. Thus, TAX repression of inflammation mainly depends on suppressing the NF-κB pathway, involvement of miRNA and inflammatory mediators’ regulation. Nevertheless, rescue experiments are still needed to show impact of TAX treatment in S100A8, CCL3, A2M, LBP and CCR1 expression in OA model.

In conclusion, TAX inhibits inflammation in osteoarthritis rat model. Inflammatory mediators, NF-κB pathway and miR-140/miR-146a targets are involved in TAX function in OA.

Declarations

**Data Availability.** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Acknowledgement.** None.

**Conflict of interests.** The authors declare that there is no conflict of interest regarding the publication of this article.

**REFERENCES**


