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Original Article

Febuxostat mitigates IL-18-induced inflammatory response and reduction of extracellular matrix gene

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Abstract: Background: Osteoarthritis (OA) is a disease commonly diagnosed in the elderly population. It is reported that the reduction of extracellular matrix and infiltrated inflammation are two main factors responsible for the pathogenesis of OA. This investigation aims to explore the potential protective effects of Febuxostat against IL-18-induced insults in chondrocytes, as well as the possible mechanism. Materials and methods: The viability of chondrocytes was evaluated using the MTT assay. QRT-PCR and ELISA were used to measure the expressions and concentrations of IL-6, TNF-α, and CCL5, respectively. The accumulation of glycosaminoglycans (GAGs) was measured using Alcian blue assay. The chondrocytes were transfected with siRNA against Sox-9 in order to establish the Sox-9 knock-down chondrocytes. The expressions of *iNOS, Col2a1, Acan, and Sox-9* were measured using qRT-PCR. The production of NO was measured using Diaminofluorescein-FM diacetate (DAF-FM DA) staining. Results: The up-regulated expressions of IL-6, TNF-α, CCL5, iNOS, and NO stimulated by IL-18 were down-regulated by the introduction of Febuxostat. The expressions of *Col2a1, Acan, and Sox-9* were significantly reduced by IL-18 but greatly promoted by Febuxostat. The increased gene expressions of *Col2a1* and *Acan* induced by Febuxostat were abolished by knocking down Sox-9 in the chondrocytes. Conclusion: Febuxostat might mitigate IL-18-induced inflammatory response and reduction of the extracellular matrix gene mediated by Sox-9.

Keywords: Osteoarthritis, IL-18, chondrocytes, Sox-9, extracellular matrix

Introduction

Osteoarthritis (OA), the most common type of arthritis, is affecting millions of people worldwide [1]. Several physiological symptoms of OA have been found, including the degradation of articular cartilage [2]. Various pathological mechanisms have been explored attempting to achieve effective drugs for the treatment of OA [4-8]. Among them, blockage of the inflammatory response, activity of catabolic enzymes, and extracellular matrix degradation within chondrocytes have been considered as the most promising strategies [9-14]. Chondrocytes, the main type of cells in cartilage, play a key role in the metabolic balance and integrity of cartilage tissue. The dysfunction of chondrocytes has been associated with the development and progression of OA [15]. The total amount of chondrocytes in OA patients is less than that in healthy people and their apoptosis rate is higher. During the process of cell senescence, the level of reactive oxygen species (ROS) is promoted in the chondrocytes, which contributes to the imbalance between intracellular oxidation and anti-oxidation, finally resulting in apoptosis of the chondrocytes [16]. The inflammatory cells, such as macrophages, are recruited to the apoptotic sites, whereby the inflammatory factors will be released to further aggravate the inflammation in the cartilage tissues [17]. IL-18 is one of the important inflammatory cytokines, which could be expressed and released by a diversity of cells, including monocytes/macrophages and chondrocytes. IL-18 plays an important role in promoting inflammation by increasing the production of TNF-α, IL-6, and CCL5 [18]. Notably, IL-18 has been reported to induce the degeneration of extracellular matrix in chondrocytes by promoting the expression of MMP-13. Inhibition of IL-18-induced cartilage injury has been considered as a potential target for the therapeutic intervention of OA [19].

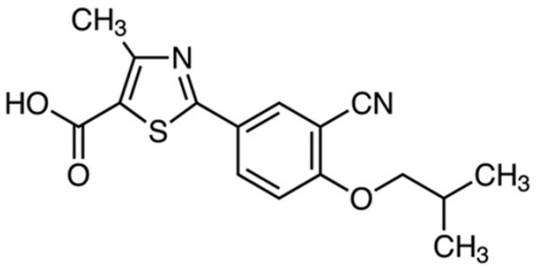

Figure 1. Molecular structure of Febuxostat.

Febuxostat is a novel inhibitor of xanthine oxidase and is used to treat hyperuricemia owing to its effects on suppressing the production of uric acid. The molecular structure of Febuxostat is shown in **Figure 1**. Febuxostat has already been proven in animal experiments to be effective in treating renal ischemia-reperfusion injury [20] and diabetic nephropathy [21] by regulating the process of oxidative stress. In the field of cardiovascular diseases, it is also reported that the process of atherosclerosis could be alleviated in the high-fat fed ApoE knock-down mice by Febuxostat [22]. Recently, Febuxostat was found to exert anti-inflammatory effects in multiple types of diseases [23, 24]. Here, the anti-inflammatory effects of Febuxostat on the injured chondrocytes induced by IL-18 will be investigated to explore the possible therapeutic effects of Febuxostat on OA.

Materials and methods

Cell culture and treatments

A retroviral vector pZipNeoSV (X) containing the SV40 large T antigen was transfected into human juvenile costal cartilage to isolate human T/C-28a2 tissue [25]. Briefly, the cartilage tissues were extracted from each donor followed by digestion with trypsin (Sigma-Aldrich, Missouri, USA) for 15 minutes at 37°C, and were subsequently incubated with type IV collagenase. The chondrocytes were isolated and placed into the cultural plates to be incubated at 37°C until 80% fusion. DMEM medium (Gibco, California, USA) containing 10% FBS, as well as 200 U/mL penicillin and 200 μg/mL streptomycin (P/S) (Gibco, California, USA), was used to culture the chondrocytes.

MTT assay

Chondrocytes were treated with Febuxostat (0.3, 3, 15, 30, and 300 μM) for 24 hours. After 3 washes, MTT in the serum-free medium at a final concentration of 1.0 mg/mL was added and incubated at 37°C for 4 hours. The reaction product was dissolved by adding 150 μL dimethylsulfoxide (DMSO) to each well. Absorbance at 570 nm was recorded.

Real-time PCR analysis

RNA was isolated using Trizol. Purified RNA was then used to reverse-transcribe cDNA using a one-step RT-PCR kit (Qiagen, Germany). Real-time PCR was then performed with SYBR Master Mix on a StepOne-Plus system. The expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the reference gene.

ELISA assay

After treatment, the secreted levels of TNF-α, IL-6, and CCL-5 were measured using ELISA kits. The cell culture supernatant was collected and stored at 80°C until use. Briefly, plates were coated and blocked. 50 μL samples and standards were then added into the designated wells and incubated for 1 hour. 100 μL of the detection antibody solution was then added and incubated for 2 hours, followed by with the addition of 100 μL of working streptavidin-HRP solution. After that, 100 μL of TMB substrate solution was then added. The reaction was then stopped with Stop solution. Absorbance at 450 nm was measured.

DAF-FM DA staining

Intracellular NO was quantified using DAF-FM DA staining [26]. After stimulation, cells were loaded with 5 μM DAF-FM DA and cultured for 20 minutes at 37°C. After washing 3 times, fluorescent signals were visualized using a fluorescent microscope.

Alcian blue staining

After indicated stimulation, chondrocytes were washed and fixed with 70% ethanol for 30 minutes at room temperature, followed by incubation with 1% Alcian blue 8GX (in 1% acetic acid) for another 30 minutes. The reaction was then stopped with 70% ethanol. After 3 washes with PBS, the slides were mounted, air-dried, and photographed.

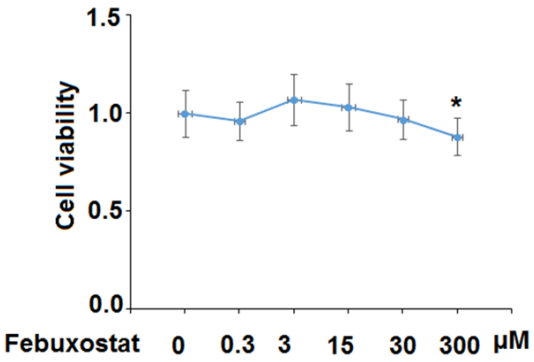

Figure 2. The effects of Febuxostat on cell viability of human T/C-28a2 chondrocytes. Cells were treated with Febuxostat at concentrations of 0.3, 3, 15, 30, 300 μM for 24 hours. Cell viability was assayed (*, $P < 0.05$ vs. vehicle group).

Transfection of small-interfering RNA (siRNA) molecules

The chondrocytes were transfected with siRNA against the mRNA of Sox-9 (Genscript, Nanjing, China). Lipofectamine® 2000 (Life Technologies, Carlsbad, USA) was used to transfect cells according to the manufacturer's instructions.

Western blot assay

Chondrocytes were lysed in RIPA Lysis Buffer (Beyotime, Shanghai, China). 20 μg of samples were loaded and separated using 10% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes and then blotted with antibodies against Sox-9 (1:1000, Cell Signaling Technologies, USA). β-actin (1:10000, Cell Signaling Technologies, USA) was used as the loading control. Images were analyzed using Image J.

Statistical analysis

GraphPad Prism 7.0 (GraphPad Software, USA) was used for statistical analysis. Data are expressed as Mean ± S.E.M. Results were statistically analyzed using ANOVA with Tukey's post hoc test for multigroup comparisons. Data with *P* values < 0.05 were considered significant.

Results

The concentration of Febuxostat was determined using MTT assay

To obtain the optimized concentration of Febuxostat with the highest efficacy and no toxicity, the chondrocytes were treated with 0.3, 3, 15, 30, and 300 μM Febuxostat for 24 hours, respectively. As shown in **Figure 2**, no significant decreased cell viability was observed on chondrocytes incubated with Febuxostat as the dosage increased from 0.3 to 30 μM. However, the cell viability decreased greatly as the concentration of Febuxostat increased from 30 to 300 μM. Therefore, 15 and 30 μM Febuxostat were used for the incubation concentrations in the subsequent experiments.

Inflammation induced by IL-18 was suppressed by Febuxostat

To evaluate the effects of Febuxostat on the inflammation, chondrocytes were stimulated with 100 ng/mL IL-18 in the presence or absence of 15 and 30 μM Febuxostat, respectively. The mRNA levels of TNF-α, IL-6, and CCL-5 were significantly promoted by stimulation with IL-18 and greatly suppressed by the introduction of Febuxostat (**Figure 3A**). **Figure 3B** shows the protein secretions of TNF-α, IL-6, and CCL-5 detected in the chondrocytes. The concentrations of IL-6 detected in the chondrocytes treated with blank medium, IL-18, IL-18+15 μM Febuxostat, and IL-18+30 μM Febuxostat were 300.8, 11892.5, 8335.9, and 6725.4 pg/mL, respectively. The concentrations of TNF-α in the chondrocytes treated with blank medium, IL-18, IL-18+15 μM Febuxostat, and IL-18+30 μM Febuxostat were 65.2, 356.9, 234.3, and 176.7 pg/mL, respectively (**Figure 3C**). The concentrations of CCL-5 in the chondrocytes treated with blank medium, IL-18, IL-18+15 μM Febuxostat, and IL-18+30 μM Febuxostat were 823.1, 6799.4, 4714.5, and 3658.6 pg/mL, respectively (**Figure 3D**).

Febuxostat reduced the expression of iNOS and the production of NO in the human T/C-28a2 cells

The gene expression level of iNOS was significantly increased by stimulation with IL-18 but greatly suppressed by the introduction of Febuxostat (**Figure 4A**). The increased release of NO induced by stimulation with IL-18 was significantly inhibited in the presence of Febuxostat (**Figure 4B**).

Febuxostat ameliorated IL-18-induced loss of glycosaminoglycans (GAGs)

Alcian blue assay indicates that IL-18 led to an obvious reduction in the accumulation of GAGs, which was rescued by treatment with

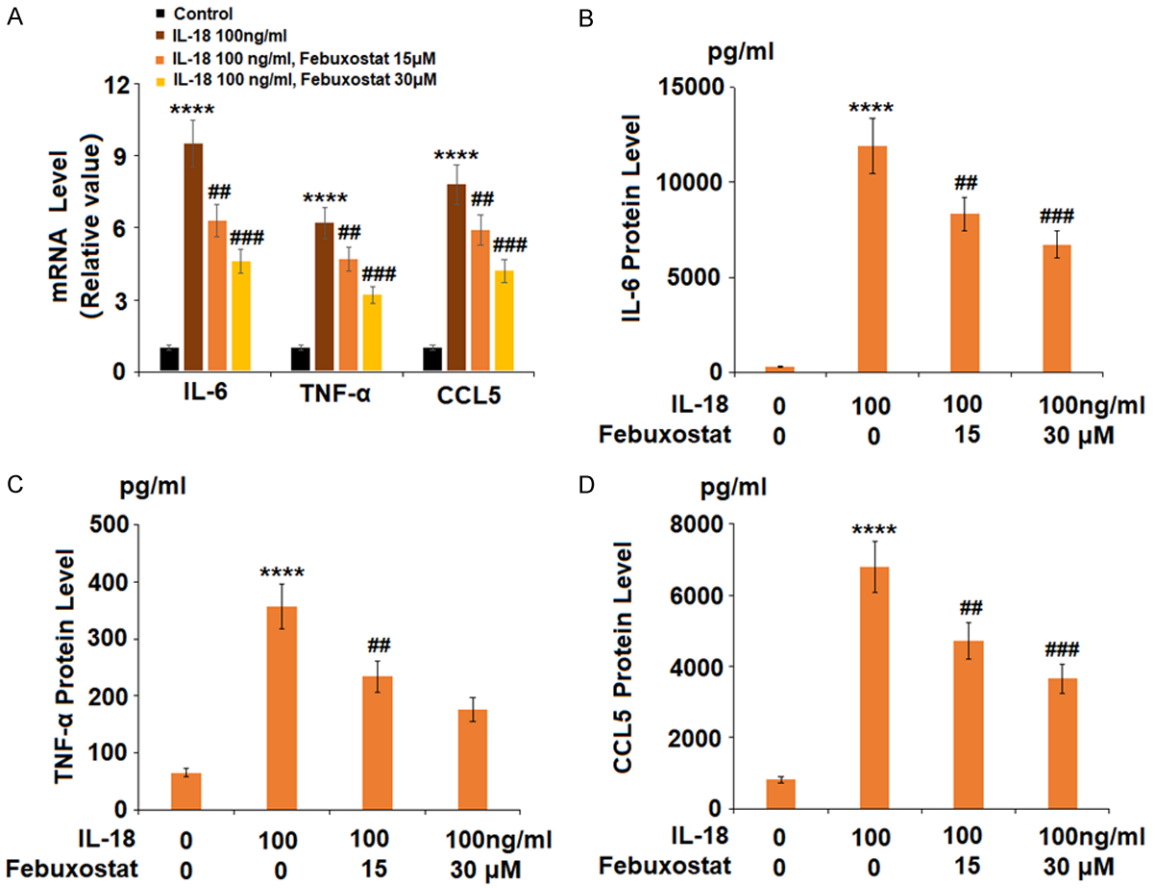

Figure 3. Febuxostat ameliorated IL-18-induced production of pro-inflammatory cytokines. Cells were cultured with IL-18 (100 ng/ml) or Febuxostat (15, 30 μM) for 12 hours. A. mRNA of *IL-6, TNF-α, and CCL5*; B. Secretions of IL-6; C. Secretions of TNF-α; D. Secretions of CCL5 (****, P < 0.0001 vs. vehicle group; ##, ###, P < 0.01, 0.001 vs. IL-18 group).

Febuxostat in a dose-dependent manner (**Figure 5A** and **5B**).

The down-regulation of Col2a1 and Acan by IL-18 was reversed by Febuxostat

To evaluate the effects of Febuxostat on the biological function of chondrocytes, the expressions of *Col2a1 and Acan* in human T/C-28a2 cells were evaluated using qRT-PCR following stimulation with IL-18 in the presence or absence of Febuxostat. As shown in **Figure 6**, we found that the gene expressions of *Col2a1 and Acan* were significantly reduced by IL-18, but rescued by Febuxostat in a dose-dependent manner.

Sox-9 was involved in the mechanism underlying the effects of Febuxostat against IL-18-induced injury on chondrocytes

To explore the possible mechanism underlying the repairing effects of Febuxostat against IL-18-induced injury on chondrocytes, the expression level of Sox-9 was evaluated following stimulation with IL-18. As shown in **Figure 7**, Sox-9 was significantly reduced by treatment with IL-18, and greatly up-regulated by the administration of Febuxostat. We further knocked down Sox-9 in chondrocytes by siRNA technology. As shown in **Figure 8A**, the expression of Sox-9 was extremely suppressed by the interference of siRNA against Sox-9, which indicates that Sox-9 was successfully knocked down in chondrocytes. The down-regulated gene expression levels of *Col2a1* and *Acan* were significantly up-regulated by treatment with Febuxostat, an effect greatly reversed by the knocking down of Sox-9 (**Figure 8B** and **8C**).

Discussion

Osteoarthritis (OA) is a disease with chronic degradation lesions on articular synovial tis-

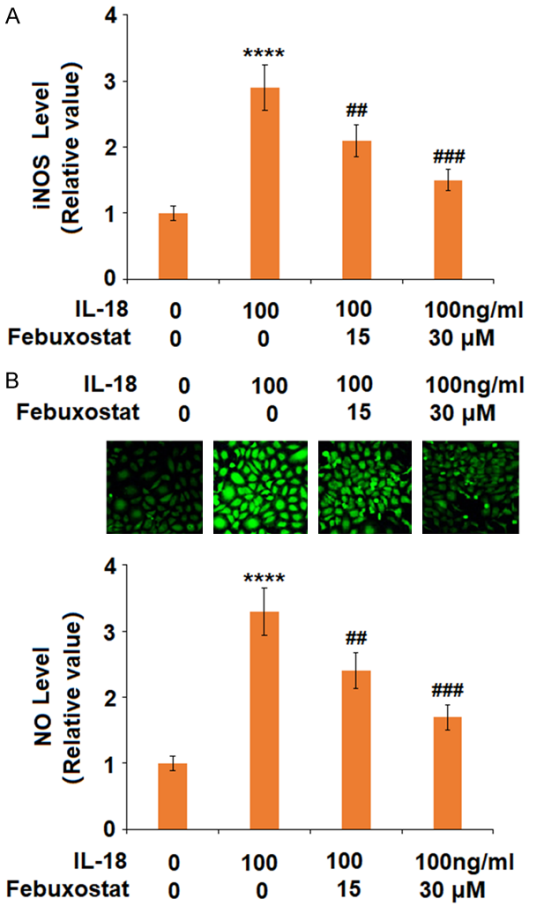

Figure 4. Febuxostat reduced the expression of iNOS and the production of NO. A. mRNA of *iNOS*; B. Production of nitric oxide (NO) as measured by DAF-FM DA (****, P < 0.0001 vs. vehicle group; ##, ###, P < 0.01, 0.001 vs. IL-18 group).

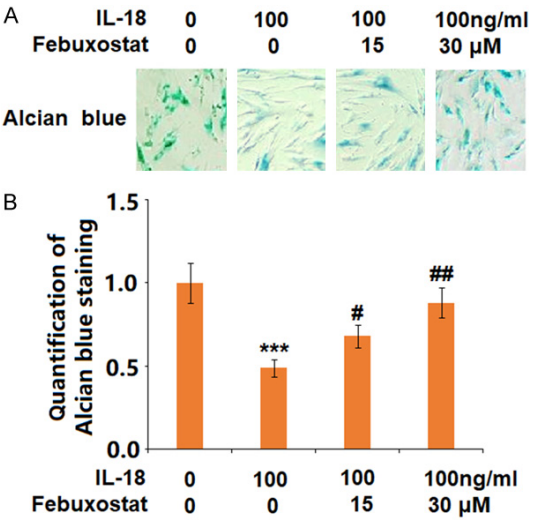

Figure 5. Febuxostat ameliorated IL-18-induced loss of glycosaminoglycans (GAGs). A. Representative results of Alcian blue staining; B. Quantification of Alcian blue staining (***, P < 0.001 vs. vehicle group; #, ##, P < 0.05, 01 vs. IL-18 treatment group, n=4).

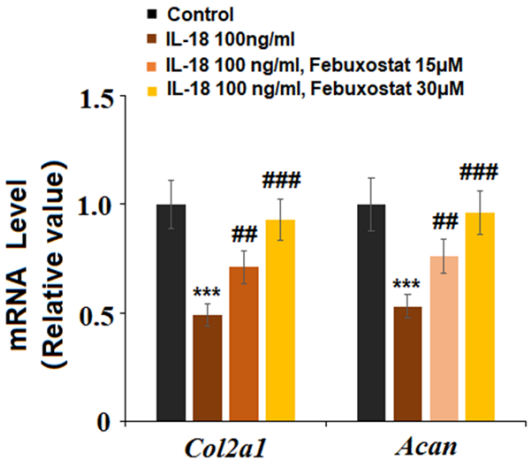

Figure 6. Febuxostat restored IL-18 (100 ng/ml)-induced reduction of *Col2a1* and *Acan*. mRNA of *Col2a1 and Acan* was measured using real-time PCR (****, P < 0.0001 vs. vehicle group; ##, ###, P < 0.01, 0.001 vs. IL-18 group).

sues, cartilage, subchondral bone, and peripheral tissues. It is characterized by knee joint pain, deformity, and dysfunction [27]. The pathological mechanism of OA is quite complicated and currently, no single theory can be widely accepted. The pathological changes involved in OA include hyperplasia of synovial cells, chronic infiltration of inflammatory cells, and excessive production of inflammatory factors [28]. There are three types of nitric oxide synthases, which are nNOS, eNOS, and iNOS. iNOS is closely related to the pathology of OA [29]. The secretion of prostaglandin E_2 (PEG_2) and cyclooxygenase-2 (COX-2) is induced by iNOS in the presence of inflammatory factors such as IL-18, which accelerates the destruction of articular cartilage. The proliferation of synovial fibroblasts is thereby triggered, and fibrosis of articular tissues induced [30]. The production of NO is dependent on the catalytic action of iNOS, accompanied by the excessive release of free radicals. Subsequently, the degradation of multiple types of mediums, such as type II collagen, type III collagen, matrix proteoglycan, and laminin is initiated and the repair of cartilage matrix is suppressed [31]. In the present study, we established an *in-vitro* OA model by stimulating the chondrocytes with IL-18, which was verified by increased production of inflammatory factors and chemokines, up-regulated iNOS, and promoted the release of NO. To avoid causing injury to the chondrocytes, the maximal concentrations that did not affect the proliferation of chondrocytes were chosen as the

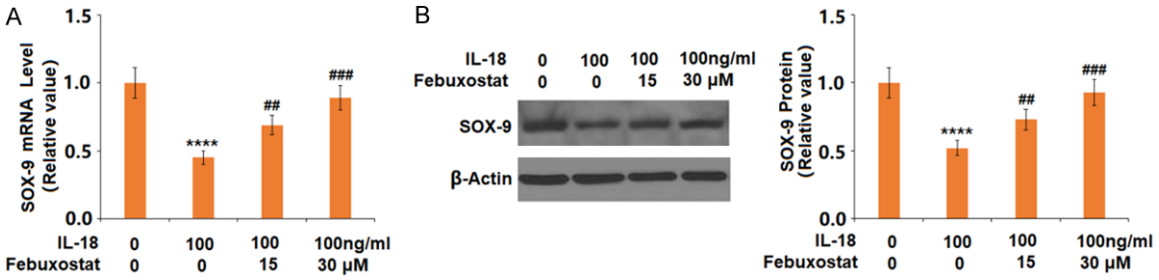

Figure 7. Febuxostat restored IL-18 (100 ng/ml)-induced reduction of human SOX-9. A. mRNA of *SOX-9*; B. Protein of SOX-9 (****, P < 0.0001 vs. vehicle group; ##, ###, P < 0.01, 0.001 vs. IL-18 group).

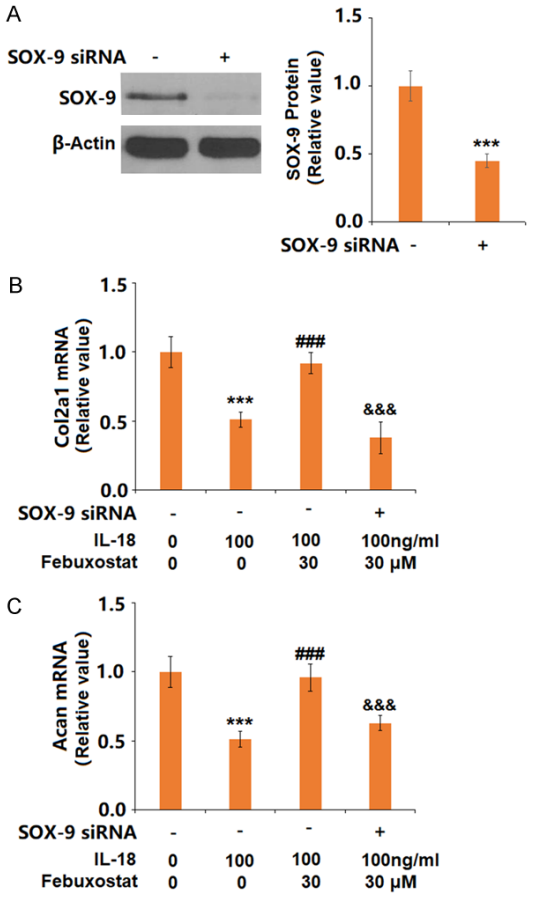

Figure 8. Knockdown of SOX-9 abolished the beneficial effects of Febuxostat against IL-18-induced reduction of *Col2a1* and *Acan*. Cells were transfected with SOX-9 siRNA, followed by incubation with IL-18 (100 ng/ml) or Febuxostat (30 μM) for 24 hours. A. Successful knockdown of SOX-9; B. mRNA of *Col2a1*; C. mRNA of *Acan* (***, P < 0.001 vs. vehicle group; ###, P < 0.001 vs. IL-18 group; &&&, P < 0.001 vs. IL-18+ Febuxostat group).

incubation concentrations of Febuxostat. We found that following incubation with Febuxostat, the production of inflammatory factors, expression of iNOS, and the release of NO were significantly suppressed, indicating a protective and repair effect of Febuxostat on the chondrocytes stimulated with IL-18. The function of Type II collagen and aggrecan is to maintain the integrity of the structure and function of cartilage [32]. The microenvironment of chondrocytes is changed by the reduction of cartilage matrix and the infiltration of inflammatory factors induced by the down-regulation of type II collagen, which finally contributes to the apoptosis of chondrocytes [33]. Here, the expression level of *Col2a1* was significantly inhibited by stimulation with IL-18 and was rescued by the introduction of Febuxostat, indicating that the protective and repair effect of Febuxostat on injured chondrocytes might be related to the up-regulation of *Col2a1*. In our future research, the therapeutic effects of Febuxostat on OA will be further investigated using an OA animal model.

As a gene associated with early embryonic development, Sox-9 is found to be involved in multiple processes of early embryonic development, such as the determination of gender and the development of chondrocytes. Along with other signaling pathways, Sox-9 is involved in regulating chondrocyte proliferation and differentiation as an activator of transcription [34]. Sox-9 and *Col2α1*, a specific proliferation gene for type II collagen in the chondrocytes, are found to be expressed in parallel in the chondrocytes [35]. *Col2α1* has been proven to be a Sox-9-dependent gene that regulates the expression of type II collagen in subsequent researches [36]. In addition, Sox-9 is reported to mediate the regeneration of chondrocytes, the formation of arthrosis, and the repair function on cartilage by regulating the Wnt/β catenin [37] and TGF-β/Smad signaling path-

Figure 9. A representative schematic of the molecular mechanisms.

ways [38]. Here, we found that the expression level of Sox-9 in the chondrocytes was significantly reduced by stimulation with IL-18, but was greatly promoted by the administration of Febuxostat, indicating an upregulation effect of Febuxostat on Sox-9. Furthermore, we found that the effects of Febuxostat on *Col2α1* and *Acan* were reversed in the Sox-9 knocked-down chondrocytes, indicating that the repair effects of Febuxostat on the injury of chondrocytes induced by IL-18 might be related to the upregulation of Sox-9. A graphic representation of the underlying molecular mechanism is shown in **Figure 9**. However, further investigation is to be explored in our future work to better explain the effects of Febuxostat on Sox-9, as well as the downstream signaling pathways.

Taken together, our data indicate that Febuxostat might mitigate IL-18-induced inflammatory response and reduction of extracellular matrix mediated by Sox-9.

Disclosure of conflict of interest

None.

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