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Original Article

Effects of atorvastatin calcium on expression of iNOS and VEGF in articular chondrocytes of rats with knee osteoarthritis

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Abstract: Objective: This study aimed to investigate the effect of atorvastatin calcium on the degeneration of articular cartilage by regulating expression of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) in rats with knee osteoarthritis (KOA). Methods: Sprague-Dawley rats were randomly divided into normal control, model, and atorvastatin calcium treatment groups. The rat KOA model was established by the Hulth method. After 8 weeks, expression of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β in the serum of rats were detected by enzyme-linked immunosorbent assay, knee was examined by x-ray, pathological observation was conducted by hematoxylin and eosin staining of articular cartilage, and the expression levels of iNOS and VEGF in cartilage tissue were detected. Results: The serum levels of TNF-α and IL-1β were significantly higher in the model group than in the other two groups. X-ray findings showed an improvement in knee joint space, articular cartilage destruction, and osteophyte formation in the atorvastatin calcium group compared with the model group. Atorvastatin calcium could significantly improve the pathological changes in the knee joint. Mankin's score of articular cartilage decreased significantly in the atorvastatin calcium treatment group compared with the model group. The expression levels of iNOS and VEGF in articular cartilage were significantly higher in the model group than in the other two groups. Conclusion: Atorvastatin calcium inhibited expression of iNOS and VEGF in articular cartilage and inflammation in the knee joint, and improved articular cartilage degeneration in rats with KOA.

Keywords: Atorvastatin calcium, iNOS, knee osteoarthritis, KOA, VEGF

Introduction

Osteoarthritis (OA) is a disease characterized by the regression of articular cartilage, with knee osteoarthritis (KOA) being the most common. KOA is characterized by the destruction of articular cartilage and synovial inflammation. The incidence of KOA gradually increases with the progression of aging [1]. The latest epidemiological survey shows that the total prevalence of OA is about 18%, with the prevalence in males and female being 11% and 19%, respectively. The morbidity of females was higher than that of male [2]. Currently, many treatment methods are available for KOA, but they are not specific because of their unclear pathogenesis. Atorvastatin calcium is a lipid-lowering drug popular in recent years, and its protective effect on OA has been shown in *in vitro* experiments [3], but little research has been done on its mechanism in treating OA. In this study, atorvastatin calcium was used to cure KOA in Sprague-Dawley (SD) rats. X-ray imaging of the knee, cartilage histomorphology observation, and enzyme-linked immunosorbent assay (ELISA) was used to determine expression levels of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β in the serum, and immunohistochemical methods were used to determine the expression levels of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) in cartilage. This study aimed to investigate whether atorvastatin calcium could inhibit expression of TNF-α and IL-1β, thereby inhibiting expression of iNOS and VEGF and improving articular cartilage degeneration in rats with KOA.

Materials and methods

Laboratory animals, feeding conditions, and feed

A total of 30 healthy specific-pathogen-free male SD rats weighing 220 ± 20 grams were purchased from the Animal Experimental Center of

Zhejiang Chinese Medical University. The breeding conditions were as follows: clean-level experimental breeding room, temperature 23°C ± 2°C, humidity 50%-70%, caged feeding, natural day-and-night lighting, and free access to water and diet. The feed was purchased from the Animal Experimental Center of Zhejiang Chinese Medical University.

Drugs, reagents, and instruments

Atorvastatin calcium was purchased from Pfizer, ELISA kit from Cusabio, anti-iNOS antibody and anti-VEGF antibody from Cell Signaling Technology, Varioskan Flash microplate reader from Thermo Fisher Scientific, and YTX-3000 high-resolution x-ray inspection equipment from Kodak, USA.

Animal grouping, model preparation, and drug delivery

A total of 30 male SD rats were randomly divided into normal control, atorvastatin calcium treatment, and model groups, with 10 rats in each group. The KOA model was prepared by the Hulth method as follows [4]: The rats in the treatment and model groups were anesthetized by an intraperitoneal injection of 0.3 mL/100 gram chloral hydrate. After successful anesthesia, a small surgical incision was made inside the right knee joint, the medial collateral ligament and anterior cruciate ligament of the right knee joint were cut, the medial meniscus was removed, and the posterior cruciate ligament was cut. The incision was sutured after the drawer test confirmed the successful establishment of the model. Within 3 days after surgery, 1 mL of penicillin sodium (40,000 U/mL) was injected into the muscle of the rats to prevent infection.

After successful establishment of the model, the atorvastatin calcium group was intra-gastrically administered at 10 mg/(kg × d). The model and normal control groups were injected with an equal volume of normal saline (NS) once per day. Each group was fed with a normal diet. The total duration of treatment was 8 weeks.

Drawing materials

Eight weeks following the experiment, after abdominal anesthesia, six rats in each group were randomly selected for x-ray examination of the right knee joint. Then, the rats in each group were subjected to cardiac blood sampling. The samples were centrifuged at 3000 rpm for 20 minutes, and serum was separated and stored at -20°C. The right knee joints of each group were quickly excised, and six knee joints of each group were randomly selected and stored in 10% formaldehyde solution.

Observation indicators and methods

Oral x-ray was used to examine changes in the knee joint cartilage and joint space. Pathological observation and Mankin's score of the knee joint were performed by formaldehyde fixation, decalcification, paraffin embedding, sectioning, and hematoxylin and eosin (HE) staining. The ELISA method was used to determine the expression levels of TNF-α and IL-1β in the serum. Immunohistochemistry was used to determine the expression levels of iNOS and VEGF in the cartilage.

Statistical analysis

All data were presented as mean ± SD ($\bar{x} \pm s$). SPSS 19.0 statistical analysis and GraphPad Prism 7.00 were used. One-way ANOVA was used to compare the levels of TNF-α and IL-1β in serum, Mankin's score of articular cartilage of rats in each group, and the effect of atorvastatin calcium on the protein levels of iNOS and VEGF in cartilage. The pairwise comparison was performed by q test. $P < 0.05$ was considered statistically significant.

Results

Effect of atorvastatin calcium on the TNF-α and IL-1β levels of rats with KOA

The levels of TNF-α and IL-1β in the serum were significantly higher in the model group than in the control group ($P < 0.01$), while the levels of TNF-α and IL-1β were significantly lower in the atorvastatin calcium group than in the model group ($P < 0.01$) (**Table 1**).

X-ray examination of rats in each group after 8 weeks

The X-ray examination showed normal knee joint space, normal articular cartilage, smooth articular surface, and no osteophyte formation in the control group (**Figure 1A**). In the model

Table 1. Comparison of levels of TNF-α and IL-1β in the serum of each group after 8 weeks ($\bar{x}$ ± s, n=10)

	TNF-α (pg/mL)	IL-1β (pg/mL)
Control group (A)	94.81 ± 6.79	11.28 ± 1.87
Model group (B)	214.73 ± 16.53	26.88 ± 1.91
Atorvastatin calcium group (C)	152.42 ± 9.61	18.22 ± 1.45
F value	261.99	198.43
Mean Diff.	A vs. B -119.90	A vs. B -15.59
	B vs. C 62.31	B vs. C 8.66
95.00% CI of diff.	A vs. B -132.90 to -106.90	A vs. B -17.54 to -13.64
	B vs. C 49.32 to 75.30	B vs. C 6.72 to 10.60
P value	A vs. B < 0.0001	A vs. B < 0.0001
	B vs. C < 0.0001	B vs. C < 0.0001

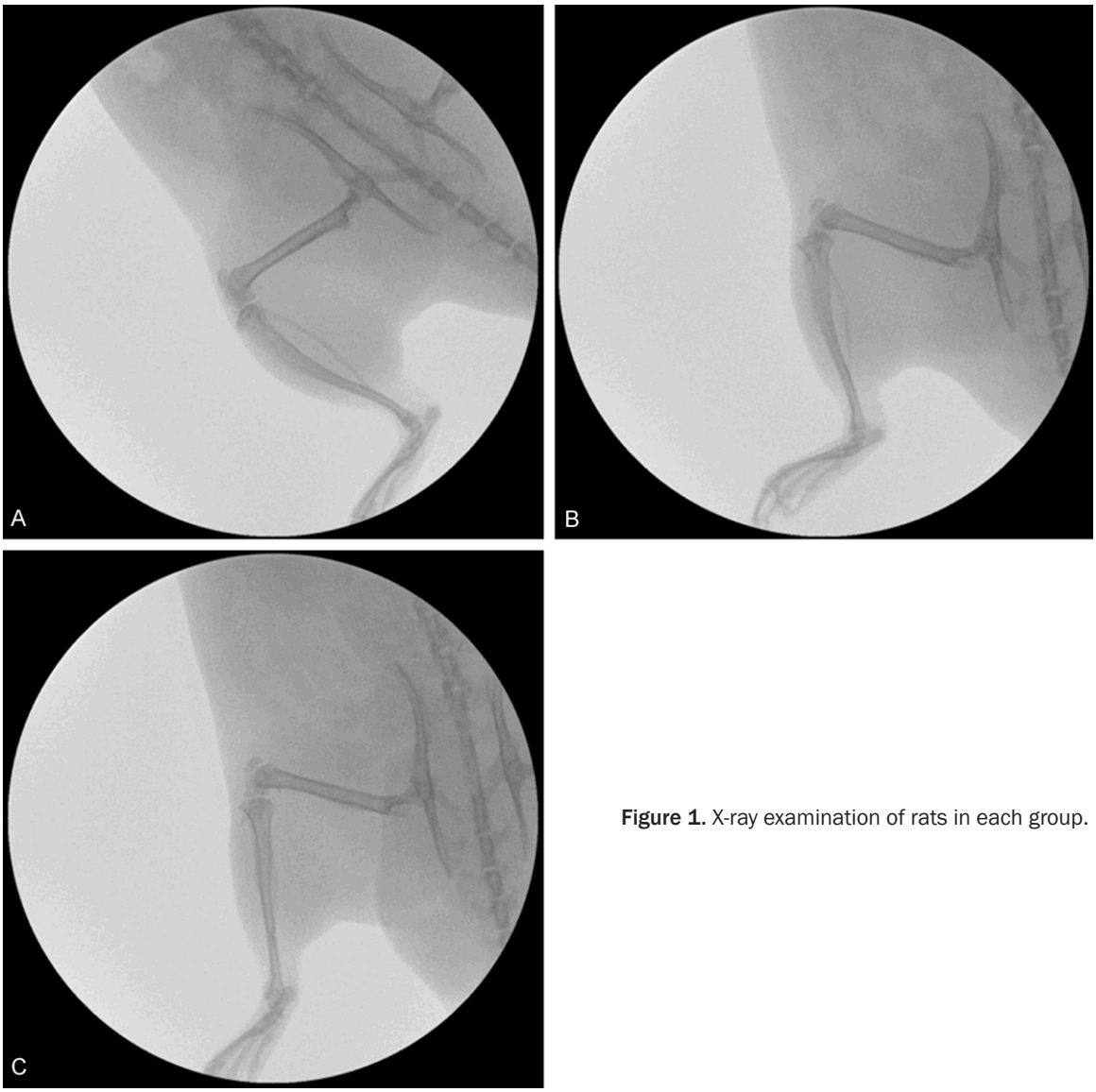

Figure 1. X-ray examination of rats in each group.

group (**Figure 1B**), the joint space was significantly narrow, the articular cartilage became thinner, sclerosis was seen in the margin of the articular surface, obvious osteophyte forma-

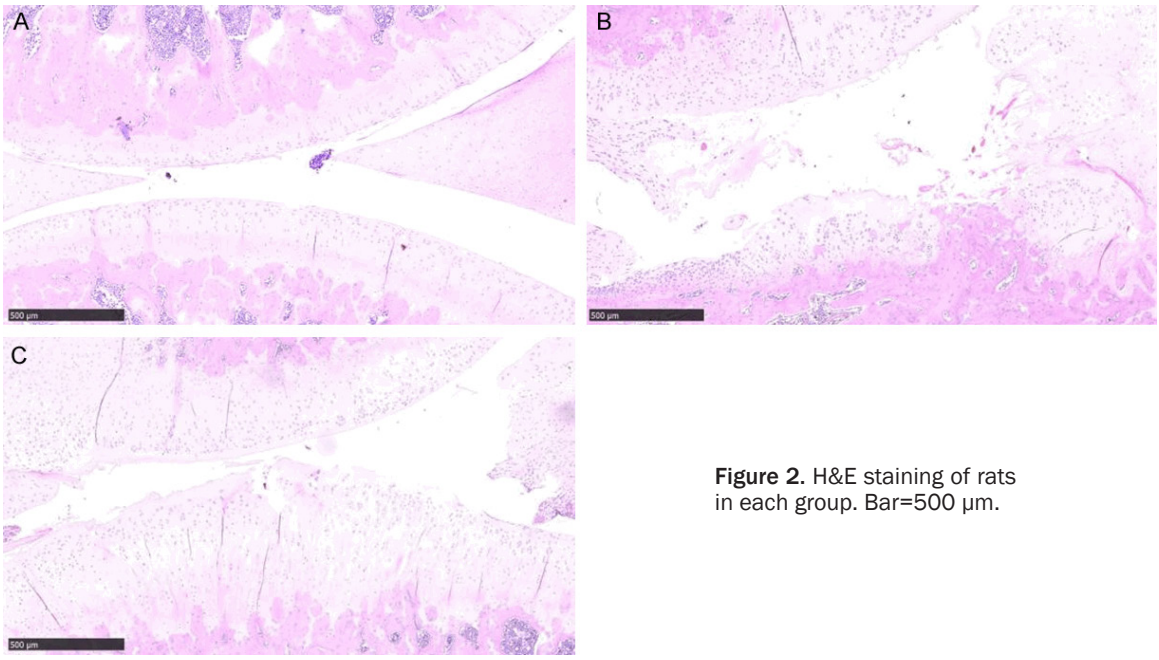

Figure 2. H&E staining of rats in each group. Bar=500 μm.

Table 2. Mankin's score of articular cartilage of rats in each group ($\bar{x} \pm s$, n=6)

Group	Mankin's score
Control group (A)	0.50 ± 0.55
Model group (B)	7.83 ± 0.98
Atorvastatin calcium group (C)	5.00 ± 0.89
F value	119.11
Mean Diff.	A vs. B -7.33
	B vs. C 2.83
95.00% CI of diff.	A vs. B -8.58 to -6.08
	B vs. C 1.59 to 4.07
P value	A vs. B < 0.0001
	B vs. C < 0.0001

tion occurred, and the joint was subluxated. In the atorvastatin calcium treatment group, the joint space was slightly narrower than that in the control group. Mild thinning of the articular cartilage with few osteophytes was observed. The lesion was reduced compared with the model group (**Figure 1C**).

Effect of atorvastatin calcium on the histopathological changes in articular cartilage in rats with KOA

After 8 weeks, H&E staining showed that the articular cartilage in the control group was smooth and intact, the cartilage layers and tidal lines were clear, the chondrocytes were well arranged and evenly distributed, and the structural integrity of trabecular bone was intact (**Figure 2A**). In the model group, the articular cartilage was thinner, the superficial and middle chondrocytes were absent, the tidal lines were disordered with partial fracture, the bone trabeculae were partially broken, and some were accompanied by cartilage necrosis (**Figure 2B**). The structure and morphology of articular cartilage and bone trabeculae of rats treated with atorvastatin calcium improved to varying degrees compared with the model group (**Figure 2C**).

Mankin's score of articular cartilage was significantly higher in the model group than in the normal ($P < 0.01$) and treatment groups ($P < 0.01$) (**Table 2**).

The effect of atorvastatin calcium on the levels of iNOS and VEGF protein in the cartilage

The results of immunohistochemistry showed that the levels of iNOS and VEGF protein were significantly higher in the model group (**Figures 3B**, **4B** and **5**) than in the control ($P < 0.01$; **Figures 3A**, **4A** and **5**) and treatment groups ($P < 0.01$; **Figures 3C**, **4C** and **5**).

Discussion

TNF-α and IL-1β have a pivotal role in the pathological progression of arthritis [5] and are the

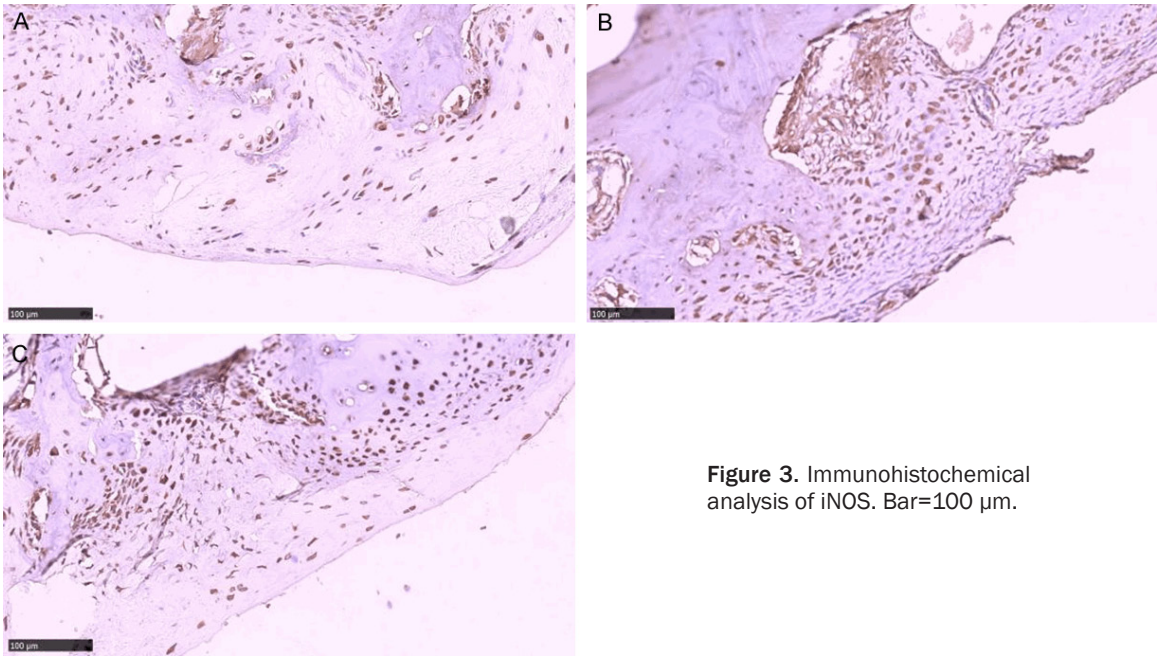

Figure 3. Immunohistochemical analysis of iNOS. Bar=100 μm.

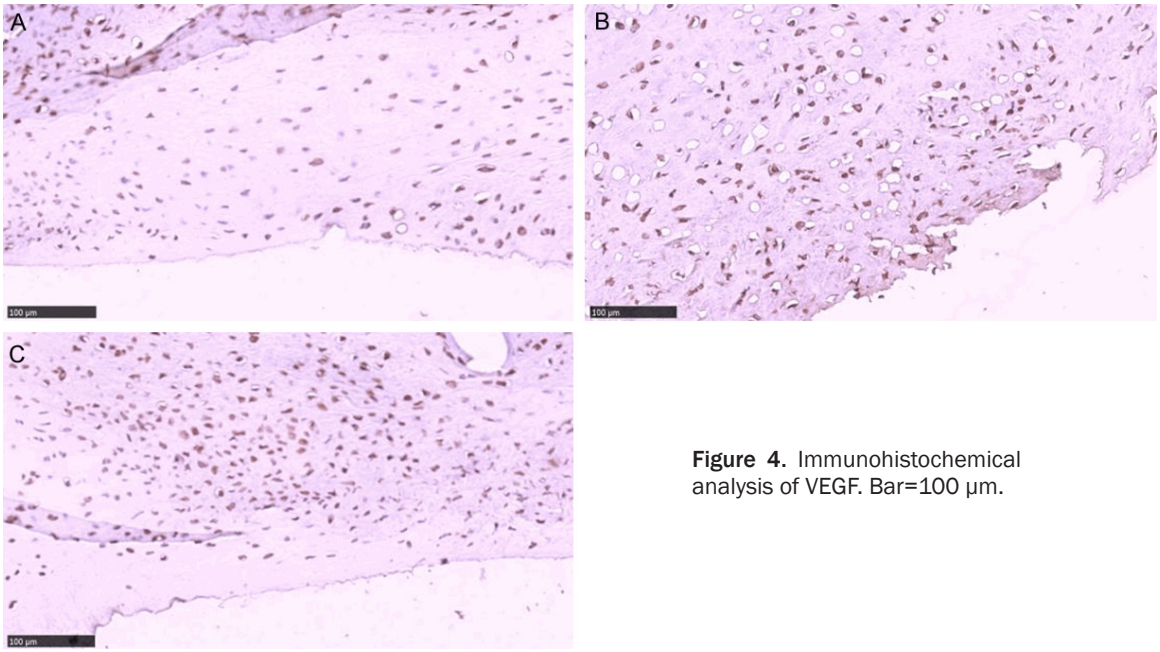

Figure 4. Immunohistochemical analysis of VEGF. Bar=100 μm.

most important cytokines in promoting the degradation and destruction of articular cartilage matrix [6]. Among them, IL-1β is essential for the apoptosis of chondrocytes. It can inhibit synthesis of proteoglycans and type II collagen in chondrocytes and promote high expression of metalloproteinases and synthesis and secretion of type I and III collagen, resulting in denaturation of chondrocytes [7, 8]. The latest research showed that IL-1β could also affect the catabolism of chondrocytes in OA by regulating specific miRNAs [9]. TNF-α plays a synergistic role with IL-1β in the course of OA and induces chondrocyte apoptosis [10]. Zheng et al. effectively reduced cartilage erosion and Osteoarthritis Research Society International (OARSI) scores by inhibiting the expression of IL-1β and TNF-α in the OA model [11].

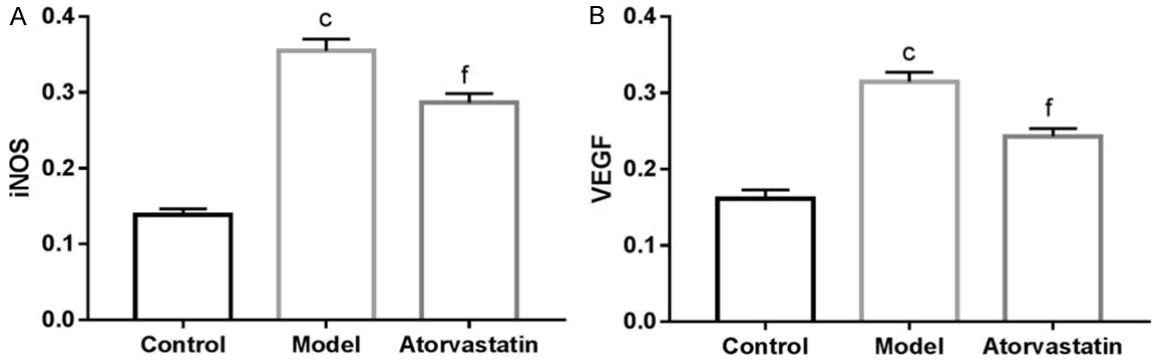

Figure 5. Effect of atorvastatin calcium on the protein levels of iNOS and VEGF in cartilage ($\bar{x} \pm s$, n=6). Compared with Control group, $^{b}P < 0.05$, $^{c}P < 0.01$; compared with Model group $^{e}P < 0.05$, $^{f}P < 0.01$).

IL-1β causes chondrocytes to produce iNOS [12]. iNOS is a potent, non-calcium-dependent synthase that continually induces the production of large amounts of nitric oxide (NO). NO is an important inflammatory mediator in the pathogenesis of OA. It promotes the release of pro-inflammatory cytokine cyclooxygenase (COX)-2, inhibits the synthesis of proteoglycan and type II collagen, and induces the apoptosis of chondrocytes. Rasheed et al. [13] found that the nuclear factor kappa-B (NF-κB) pathway was activated in the progression of KOA, and iNOS was induced by downregulating the expression of hsa-miR-26a-5p in this pathway.

VEGF is known to be involved in many pathological processes of KOA, including synovitis, cartilage degeneration, subchondral bone cyst and sclerosis, osteophyte formation, and pain [14]. Maria et al. [15] showed that inflammatory factors, such as IL-1β and TNF-α, promoted the secretion of VEGF in OA chondrocytes, thereby promoting angiogenesis through the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway by binding to its receptors, thus aggravating the progression of KOA [16].

Atorvastatin calcium is one of the liver 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, having a good inhibitory effect on cholesterol synthesis. It is widely used in cardiovascular diseases. Recent studies have found that, in addition to anti-cholesterol synthesis, it also has anti-inflammatory, anti-oxidative, immune regulatory, and other functions [17]. Its anti-inflammatory function is related to the inhibition of inflammatory factor synthesis, regulation of microRNA expression, blocking of the isopentenylation of small G protein, interference with toll-like receptor 2 (TLR2) and TLR4 signaling, and increased peroxisome proliferator-activated receptor (PPAR) expression [18-22].

In this study, rats with KOA were treated with atorvastatin calcium. After 8 weeks of the treatment, the levels of TNF-α and IL-1β in the serum of the model group increased significantly, and the articular cartilage was severely damaged. However, in the treatment group, the levels of TNF-α and IL-1β dramatically decreased and the destruction of articular cartilage also alleviated compared with the model group. The articular cartilage pathology and x-ray changes were analyzed according to Mankin's scoring criteria [23]. The rats in the model group were regarded as in the mid-stage KOA, and those in the treatment group rats in the early-stage KOA. Thus, it was suggested that TNF-α and IL-1β played an important role in the progression of KOA, and atorvastatin calcium was effective in reducing TNF-α and IL-1β, thereby improving the destruction of articular cartilage in rats with KOA.

Relevant *in vitro* studies showed that cartilage destruction could be relieved by inhibiting the production of iNOS induced by IL-1β in the chondrocytes of rats [12]. Jansen et al. [24] used the Mankin's scale to validate KOA histologically. VEGF was involved in early pathological changes in KOA. The results of this study showed that the expression of iNOS and VEGF was high in the model group but decreased significantly in the treatment group, indicating that atorvastatin calcium could effectively reduce the production of iNOS and VEGF in chondrocytes of rats with early-stage KOA.

This study concluded that atorvastatin calcium had a protective effect on early KOA, and its mechanism might be related to the inhibition of the expression of TNF-α and IL-1β, thereby restricting the expression of iNOS and VEGF, and finally alleviating the apoptosis of knee joint chondrocytes. The pathogenesis of KOA is very complicated. Whether only atorvastatin calcium interferes with the expression of related inflammatory factors in KOA or other mechanisms also participate in the development of KOA and whether atorvastatin calcium is effective for mid-term or advanced KOA need further investigation.

Disclosure of conflict of interest

None.

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