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Kaempferol protects chondrogenic ATDC5 cells against inflammatory injury triggered by lipopolysaccharide through down-regulating miR-146a

Rui Jiang[a,1], Peng Hao[b,1], Guisheng Yu[b], Chuanan Liu[b], Chuandong Yu[b], Yan Huang[a], Yongkun Wang[a,*]

[a] Department of Orthopaedics, China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin, China
[b] Department of Orthopaedics, Heze Municipal Hospital, Heze 274031, Shandong, China

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ABSTRACT

Kaempferol is a kind of bioflavonoid exerts diverse pharmacological activities, including anti-apoptotic and anti-inflammatory activities. Kaempferol has been recognized as an effective agent for alleviating the clinical symptoms of osteoarthritis (OA). This study aimed to provide evidence that Kaempferol has potential in the management of OA. Lipopolysaccharide (LPS) stimulation induced a significant cell death and inflammatory injury in ATDC5 cells, as evidenced by the decreased cell viability, the induced apoptosis, the activated caspase-3, and the excessive production of IL-6, IL-8 and TNF-α. Precondition of cells with Kaempferol prevented apoptosis and the release of proinflammatory cytokines triggered by LPS. miR-146a was down-regulated by Kaempferol treatment, and Decorin was up-regulated by miR-146a overexpression. Consistently, both silence of miR-146a and Decorin exhibited Kaempferol-like effects towards ATDC5 cells stimulated by LPS. Moreover, Decorin silence activated PI3K/AKT/mTOR signaling pathway. In rat model of OA, the expression of miR-146a and Decorin in cartilage tissues was repressed by Kaempferol. Also, the activated PI3K/AKT/mTOR signaling pathway in OA animal model was enhanced by Kaempferol administration. These data suggested that Kaempferol exerted potential anti-OA effects through down-regulation of miR-146a, and thus repressing the expression of Decorin.

1. Introduction

Osteoarthritis (OA), also known as degenerative arthritis, is one of the most common degenerative joint diseases, which has a tremendous influence on the health of older people around the world. The main clinical manifestations of OA include recurrent joint pain and gradually increased joint movement disorder. Current treatments for OA are limited to pain management. In the late stage of disease process, joint-replacement surgery may be recommended [1], but there are problems getting the transferred cartilage to integrate well with the existing cartilage at the transfer site [2].

Kaempferol ($C_{15}H_{10}O_6$, molecular weight: 286.23), 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, is a kind of bioflavonoid isolated from a wide variety of plants, including the root of *Kaempferia galanga L.*, grapefruit, tea and broccoli [3]. Kaempferol is structurally similar to quercetin [4]. It has been reported to exert diverse pharmacological activities, such as inhibiting the oxidative stress, and attenuating morphological changes, cell apoptosis [5] and inflammation [6]. Besides, several studies suggested that Kaempferol also possess angiogenic [7] and anti-cancer [8] activities. Lee and his colleagues demonstrated that long-term daily consumption of a Kaempferol-rich diet can help to prevent the development of rheumatoid arthritis, a systemic inflammatory disease [9]. Another two investigations recognized Kaempferol as a novel therapeutic active agent for preventing, stopping and retarding the progression of OA [10,11]. Although sporadic literatures have reported the potential of Kaempferol in the management of OA, more effort are required to fully appreciating its complexity and to reveal the underlying mechanisms of which Kaempferol retarding the progression of OA.

microRNAs (miRNAs or miRs) are a kind of endogenous noncoding RNA, with 17–25 nucleotides. miRNAs are highly conserved and prevalent in eukaryotic cells. Recent decades, miRNAs have been identified as key regulators in a wide range of biological processes, as they can negatively regulate the expression of their target genes at post-transcriptional level. A number of miRNAs have been shown to be pivotal in the pathogenesis of OA in recent epigenetic studies [12], like miR-577

* Corresponding author at: Department of Orthopaedics, China-Japan Union Hospital of Jilin University, No.126, Xiantai Street, Changchun 130033, Jilin, China.
E-mail address: yongkunwang42@sohu.com (Y. Wang).
[1] Co-first authors.

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[13], miR-140-5p/miR-149 [14], miR-140-3p and miR-140-5p [15], etc. Among which, several literatures have focused on the importance of miR-146a in the development of OA, as it is a negative regulator of leukocyte immune response. miR-146a is intensely expressed in low-grade OA cartilage and its expression can be elevated by IL-1β stimulation [16]. Further studies suggested that miR-146a facilitated OA through targeting Camk2d, Ppp3r2 [17], CXCR4 [18] or TRAF6 [19]. All these demonstrated miR-146a as an OA-related miRNA.

This study aimed to investigate the effect of Kaempferol precondition on ATDC5 cells stimulated by lipopolysaccharide (LPS). And also, the regulatory role of Kaempferol in the expression of miR-146a was detected to decode a novel underlying mechanism of which Kaempferol affected ATDC5 cells.

2. Materials and methods

2.1. Cell culture

ATDC5 cells purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) were cultured in DMEM:Ham's F12 (1:1) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 2 mM Glutamine (Sigma-Aldrich) and 5% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). The cells were routinely cultured in a humidity incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO_2.

ATDC5 cells were treated by 5 μg/ml LPS (from *Escherichia coli* O111:B4, Sigma-Aldrich) for 5 h, as previously described [20].

Kaempferol with purity > 97.0% (HPLC) was purchased from Sigma-Aldrich, and was dissolved in DMSO (Sigma-Aldrich). The cells were treated by different concentrations (0–50 μM) of Kaempferol for 12 h before LPS stimulation.

2.2. Cell counting kit-8 (CCK-8) assay

Viability of ATDC5 cells after Kaempferol and/or LPS treatment was assessed using CCK-8 assay (Dojindo Molecular Technologies, Kyushu, Japan). Briefly, 5×10^3 ATDC5 cells in 96-well plate were washed twice with PBS and then 10 μl CCK-8 solution was added into each well. The plate was maintained at 37 °C for 1 h. After that, the absorbance of each well at 450 nm was recorded using Micro-plate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.3. Apoptosis assay

Apoptosis of ATDC5 cells after Kaempferol and/or LPS treatment was tested using Annexin V-FITC/PI apoptosis detection kit (Invitrogen, Carlsbad, CA, USA). Briefly, 1×10^5 ATDC5 cells in 6-well plate were collected and disposed as following step: washed with PBS for three times, stained using kit solution for 25 min at 37 °C in the dark, and subjected to flow cytometry analysis using Guava EasyCyte flow cytometer (Guava Technologies, Hayward, CA, USA). Data were quantified using FCS Express software (De Novo software, Los Angeles, CA, USA).

2.4. Caspase-3 activity assay

Caspase-3 activity was measured by using a Caspase-3 Assay Kit (Fluorometric) (ab39383) purchased from Abcam (Cambridge, MA). In brief, after the indicated treatment, the cells in 6-well plates were collected and resuspended in 50 μl chilled Cell Lysis Buffer. The cells were incubated on ice for 10 min, and then 50 μl 2 × Reaction Buffer containing 10 mM DTT was added into each sample. DEVD-AFC substrate with a final concentration of 50 μM was added and the sample was incubated at 37 °C for 1 h. Enzyme activity was calculated based on absorption values at 400 nm. Fold change of caspase-3 activity can be determined by comparing with the level of the un-treated control.

2.5. Transfection

The mimic, inhibitor and negative control (NC) specific for miR-146a were purchased from GenePharma (Shanghai, China). The sequences of miR-146a mimic, inhibitor and NC were listed as follows. Mimic sense, 5′-UGA GAA CUG AAU UCC AUG GGU U-3′, mimic antisense, 5'-CCC AUG GAA UUC AGU UCU CAU U-3′; inhibitor, 5′-AAC CCA UGG AAU UCA GUU CUC A-3′; NC, 5′-UCA CAA CCU CCU AGA AAG AGU AGA-3′. pc-Decorin for expression of Decorin was established by inserting full-length of Decorin into pc-DNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA). The empty pc-DNA3.1 plasmid was used as a blank control. sh-Decorin for silencing of Decorin was established by inserting the Decorin shRNA into pGPU6/Ne plasmid (GenePharma). pGPU6/Ne plasmid with non-targeting sequences was used as its blank control. Cell transfection was performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) in line with the manufacturer's instruction. Transfection efficiency was verified by RT-qPCR.

2.6. ELISA

After the indicated treatment, culture supernatant was collected from 24-well plates and the concentrations of IL-6, IL-8 and TNF-α were respectively measured by using mouse IL-6 ELISA Kit (Abcam, Cambridge, MA), mouse IL-8 ELISA Kit (R&D Systems, Minneapolis, MN), and mouse TNF-α ELISA Kit (Cusabio, Wuhan, China).

2.7. Northern blot

Total RNAs were extracted from cell by using TRIzolTM Plus RNA Purification kit (Invitrogen). RNA extracts were resolved over polyacrylamide gel and transferred onto nylon membrane (Beyotime, Shanghai, China), After UV-crosslinked, hybridization was performed by using DIG-labeled oligonucleotide probes which specific against miR-146a. DIG luminescent detection kit (Roche, Basel, Switzerland) was used for probe detection.

2.8. In vivo experiments

At total of 24 SPF grade of male Sprague-Dawley rats (250 ± 20 g) were purchased from Vital River Laboratories (Beijing, China). The animal experiments performed in this study were approved by the Animal Ethics Committee of China-Japan Union Hospital of Jilin University and performed according to the instruction of the institute. The rats were randomly divided into three groups: Control, OA and OA + KFL groups (8 rats per group). Rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (Tocris, Avonmouth, UK). Rat model of OA was made as previous described [21]. Right knee was subsequently exposed using an 8–10 mm medial parapatellar approach with the patella laterally dislocated before the anterior cruciate ligament was transected, and the medial meniscus was completely resected in a manner that did not injure the articular cartilage. The rats in OA + KFL group were intragastrically administrated with 5 mg/kg/day Kaempferol for 3 months before OA surgery. The rats in KFL untreated group received same volume of vehicle. After 4 weeks of surgery, rats were sacrificed and cartilage samples were collected for detecting the expression of miR-146a, Decorin and core proteins in PI3K/AKT/mTOR pathway.

2.9. RT-qPCR

Total RNAs in ATDC5 cells and cartilage tissues were isolated using TRIzolTM Plus RNA Purification kit (Invitrogen, Carlsbad, CA, USA). cDNA was transcribed using SuperScriptTM IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The mRNA levels of IL-6, IL-8, TNF-α, and Decorin were measured by using SYBRTM Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The level of

miR-146a was measured by mirVanaTM qRT-PCR miRNA Detection kit (Invitrogen, Carlsbad, CA, USA). β-actin acted as an endogenous control for IL-6, IL-8, TNF-α, and Decorin, and U6 for miR-146a. Data were quantified using $2^{-\triangle\triangle Ct}$ method [22]. The primary sequences used in this procedure were listed as follows. IL-6: 5′-AAG CCA GAG TCC TTC AGA GAG AT-3′ (forward), 5′-TTG GAT GGT CTT GGT CCT TAG C-3′ (reverse); IL-8: 5'-CGG CAA TGA AGC TTC TGT AT-3′ (forward), 5′-CCT TGA AAC TCT TTG CCT CA-3′ (reverse); TNF-α: 5′-CTA CTC CCA GGT TCT CTT CAA-3′ (forward), 5′-GCA GAG AGG AGG TTG ACT TTC-3′ (reverse); Decorin: 5′-TCA CTC AGC CAA CTG CTC GC-3′ (forward), 5′-AAG ATG GCA TTG ACA GCG GAA G-3′ (reverse); β-actin: 5'-CAT CCG TAA AGA CCT CTA TGC CAA C-3′ (forward), 5′-ATG GAG CCA CCG ATC CAC A-3′ (reverse); miR-146a: 5′-TGA GAA CTG AAT TCC ATG GGT T-3′ (forward), 5′-CGA TTA CTT GGT GGA TGT TGG-3′ (reverse); U6: 5′-GCT TCG GCA GCA CAT ATA CTA AAA T-3′ (forward), 5′-CGC TTC ACG AAT TTG CGT GTC AT-3′(reverse).

2.10. Western blot

Total proteins in ATDC5 cells and cartilage tissues after the relevant treatment were isolated by using RIPA Lysis buffer (Beyotime, Shanghai, China). The purity of the extracts was tested by BCA Protein Assay kit (Beyotime, Shanghai, China). Protein sample in equal concentration was electrophoresed in polyacrylamide gels and transferred onto polyvinylidene fluoride (PDVF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies at 4 °C overnight for detection of Bcl-2 (ab692), Bax (ab182733), caspase-3 (ab90437 and ab2302), PARP (ab32138), cleaved-PARP (ab32064), IL-6 (ab6672), IL-8 (ab154390), TNF-α (ab1793), Decorin (ab137508), PI3K (ab191606), p-PI3K (ab182651), AKT (ab8805), p-AKT (ab38449), mTOR (ab32028), p-mTOR (ab84400), and β-actin (ab8226, Abcam). The membranes were washed with Tris-Buffered Saline and Tween (TBST, Beyotime, Shanghai, China) and then incubated with Goat Anti-Mouse IgG H&L (FITC) (ab6785) and Goat Anti-Rabbit IgG H&L (HRP) (ab6721, Abcam) for 1 h at room temperature. Signals of proteins were captured using Bio-Rad ChemiDocTM XRS system (Bio-Rad Laboratories, Hercules, CA, USA). The intensities of bands were analyzed using Image LabTM software (Bio-Rad Laboratories, Hercules, CA, USA).

2.11. Statistics

All experiments were repeated three or eight times. The results of multiple experiments are presented as the mean ± SD. Graphpad 6.0 software (Graphpad, San Diego, CA, USA) was used for statistical analysis. Differences between groups were calculated by using one-way analysis of variance (ANOVA). Statistical significance was established at $P < 0.05$.

3. Results

3.1. Kaempferol protects ATDC5 cells against LPS-mediated apoptosis and inflammation

ATDC5 cells were treated by various concentrations of Kaempferol for 12 h, and the viability of cells was monitored to evaluate the cytotoxicity of Kaempferol. CCK-8 assay results shown in Fig. 1A indicated that Kaempferol with concentrations < 50 μM did not affect cell viability significantly, but 50 μM of Kaempferol significantly reduced cell viability ($P < 0.05$). Thus, 40 μM was selected as a Kaempferol-treating condition for use in the following experiments. Fig. 1B-1D showed that, LPS induced a significant cell death in ATDC5 cells, as cell viability was decreasd ($P < 0.01$), apoptosis rate was increased ($P < 0.001$), and caspase activity ($P < 0.001$) was enhanced by LPS stimulation. These observation were coupled with the down-regulated expression of Bcl-2, the up-regulated expression of Bax, as well as the cleavage of caspase-3 and PARP (Fig. 1E), suggesting LPS significantly damaged ATDC5 cells. However, Kaempferol remarkably prevented LPS-induced damage, as cell viability loss, apoptosis and caspase-3 activation made by LPS were all attenuted by pretreating with Kaempferol (Fig. 1B-1E). Same trends were observed in the release of proinflammtory cytokines. As results shown in Fig. 1F-1H, LPS induced significant releases of IL-6, IL-8, and TNF-α in ATDC5 cells, while the enhanced release of proinflammatory cytokines made by LPS was attenuated by Kaempferol. Collectively, these results suggested that pretreating ATDC5 cells with Kaempferol effectively prevented LPS-induced cell death and inflammatory injury.

3.2. Kaempferol down-regulates the expression of miR-146a

The expression changes of miR-146a in ATDC5 cells after Kaempferol and/or LPS treatment were measured. RT-qPCR data in Fig. 2A showed that LPS significantly increased miR-146a expression as compared to the control group ($P < 0.001$). However, when comapred to the LPS group, miR-146a expression was significantly decreased in KFL + LPS group ($P < 0.001$). The expression changes of miR-146a were further verified by Northern blot analysis. Results in Fig. 2B showed that, miR-146a expression was up-regulated by LPS while down-regulated by Kaempferol, indicating miR-146a as a downstream effector of Kaempferol.

3.3. Kaempferol protects ATDC5 cells against LPS-mediated apoptosis and inflammation through down-regulating miR-146a

To test the abovementioned hypothesis, the expression of miR-146a in ATDC5 cells was altered by miR-transfection. Results in Supplementary Fig. 1 showed that, miR-146a overexpression damaged ATDC5 cells in the absence of LPS. Besides, miR-146a overexpression is able to aggravate LPS-induced cell damage. Transfection of cells with miR-146a mimic significantly enhanced LPS-induced cell viability loss, apoptosis, caspase-3 activation, as well as the release of proinflammatory cytokines. As expected, silence of miR-146 exhibited contrary effects on these items. The result indiated that silence of miR-146a results in a similar protective effects on chondrocytes as Kaempferol. Next, LPS-injured ATDC5 cells were pre-treated with Kaempferol in combination with miR-146a mimic to see if Kaempferol conferred its protective function via down-regulatin of miR-146a. Results in Fig. 3A–C revealed that, Kaempferol failed to protect ATDC5 cells against LPS-induced cell viability loss and apoptosis when miR-146a was overexpressed. This result confirmed our hypothesis that Kaempferol protected ATDC5 cells against LPS-mediated apoptosis and inflammation through down-regulating miR-146a.

3.4. miR-146a positively regulates Decorin

Decorin is a matrix-organizing proteoglycan, playing an essential role in the pathology of degenerative joint disease. It has been reported that Decorin deficiency altered extracellular matrix (ECM) composition and articular cartilage stiffness [23]. Herein, we found that both the mRNA and protein levels of Decorin were down-regulated by miR-146a inhibitor transfection ($P < 0.05$), while were up-regulated by miR-146a mimic transfection ($P < 0.01$, Fig. 4A–B). It seems that Decorin is positively regulated by miR-146a, and Decorin may act as a downstream gene of miR-146a.

3.5. Kaempferol confers its protective functions via down-regulating miR-146a and thus modulating Decorin

Next, ATDC5 cells were transfected with pc-Decorin (a Decorin expressing vector), sh-Decorin (a shRNA specific for Decorin) or the negative controls (pcDNA3.1 and sh-NC). We found that, Decorin overexpression also could damage ATDC5 cells in the absence of LPS.

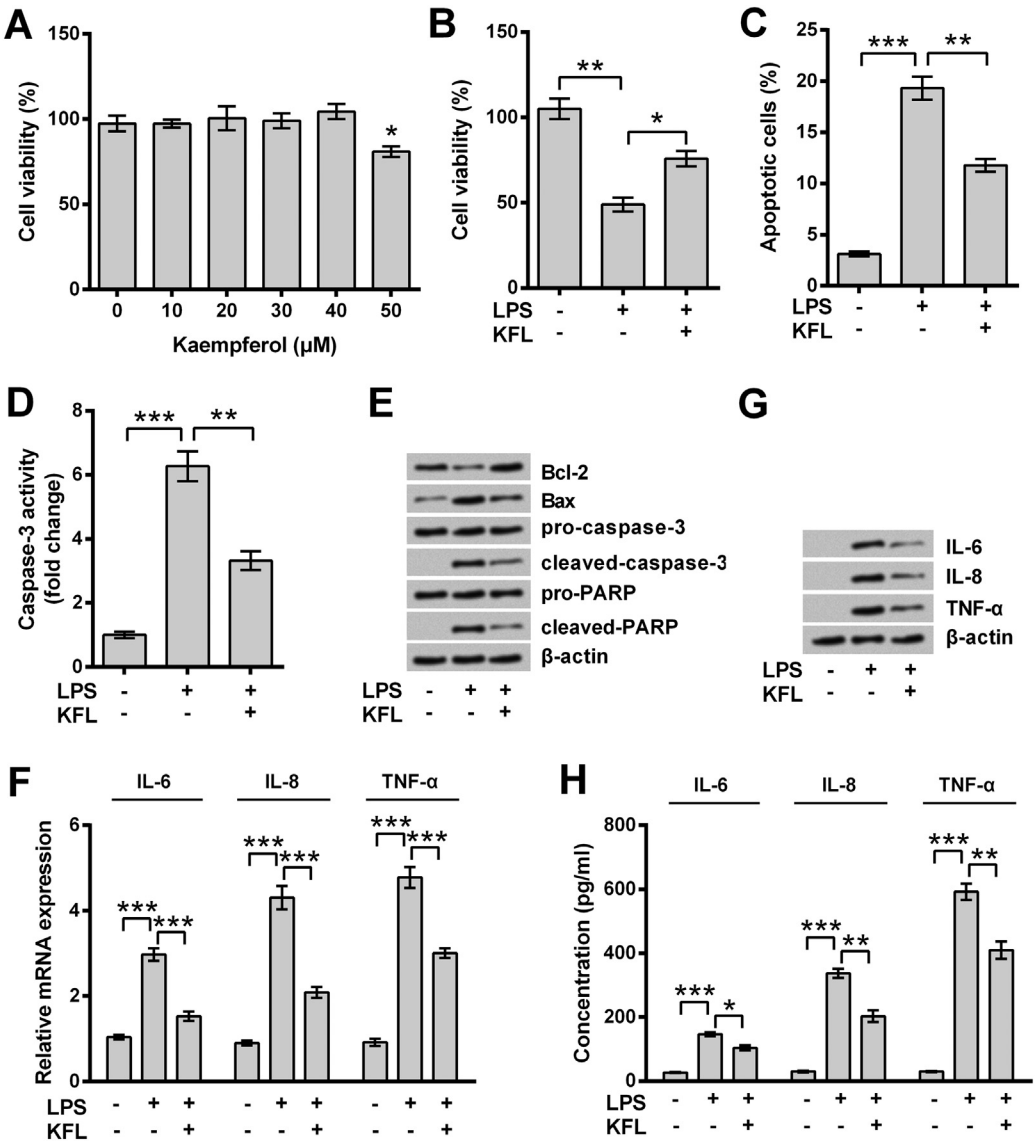

Fig. 1. Effect of Kaempferol on LPS-injured ATDC5 cells. (A) Viability of ATDC5 cells was monitored by CCK-8 assay following the treatment of different concentrations of Kaempferol. ATDC5 cells were treated by 40 μM Kaempferol for 12 h, and/or 5 μg/ml LPS for 5 h. (B) Viability, (C) apoptosis rate, (D) caspase-3 activity, and (E) protein levels of apoptosis-related factors were measured by CCK-8 assay, Annexin V-FITC/PI double staining, caspase-3 activity assay and Western blot. (F) mRNA levels, (G) protein levels, and (H) concentrations of proinflammatory cytokines were respectively measured by qRT-PCR, Western blot and ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Moreover, LPS-induced cell viability loss, apoptosis, caspase-3 activation, as well as the release of proinflammatory cytokines were all accelerated by pc-Decorin transfection (Supplementary Fig. 2). However, LPS-induced these alterations were all attenuated by sh-Decorin transfection (Supplementary Fig. 2). These finding suggested that silence of Decorin exerted protective functions on LPS-injured ATDC5 cells. Thus, we further studied the involvement of Decorin in Kaempferol's protective functions. Results in Fig. 5A–C indicated that, miR-146a overexpression did not impede Kaempferol's protective functions when Decorin was silenced. Altogether, these results indicated that Kaempferol conferred its protective functions possibly via down-regulating miR-146a and thus modulating Decorin.

3.6. Silence of Decorin activates PI3K/AKT/mTOR signaling pathway

PI3K/AKT/mTOR signaling pathway has been demonstrated to be involved in both the degradation of ECM and the death of chondrocytes [24]. More interestingly, several reports have revealed that Kaempferol exerted its beneficial effects via regulating PI3K/AKT/mTOR signaling pathway [4,25]. Thus, we are interested in investigating the regulatory role of Decorin in PI3K/AKT/mTOR signaling pathway to reveal the underlying mechanism of which Decorin silence protected ATDC5 cells. Western blotting results in Fig. 6A-6B showed that, LPS treatment significantly increased the ratios of p/t-PI3K ($P < 0.001$), p/t-AKT ($P < 0.001$) and p/t-mTOR ($P < 0.05$). pc-Decorin transfection significantly abolished LPS-induced these increases ($P < 0.05$, $P < 0.01$ or $P < 0.001$), while sh-Decorin transfection significantly accelerated them ($P < 0.01$ or $P < 0.001$).

3.7. In vivo effects of Kaempferol on miR-146a and Decorin expression as well as the activation of PI3K/AKT/mTOR signaling

Finally, the regulatory effects of Kaempferol on miR-146a and Decorin expression revealed above was further confirmed in an animal model of OA. As results shown in Fig. 7A, miR-146a expression in OA cartilage tissues was remarkably higher than that in normal controls ($P < 0.001$). Also, the mRNA ($P < 0.001$) and protein levels of Decorin were highly expressed in OA group as compared to normal control group (Fig. 7B–C). As expected, the expression of miR-146a and Decorin induced in OA model was repressed by Kaempferol, which further confirmed the inhibitory effects of Kaempferol on the expression of miR-146a and Decorin.

In addition, the regulatory effects of Kaempferol on PI3K/AKT/mTOR pathway were tested in vivo. As seen in Fig. 7D–E, phosphorylation of PI3K, AKT and mTOR was induced in animal model of OA, and was further enhanced by Kaempferol administration.

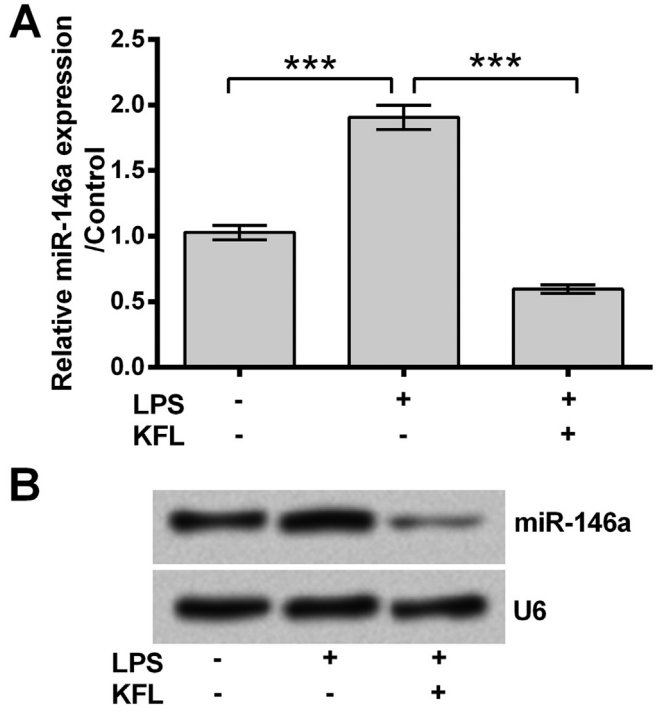

Fig. 2. Effect of Kaempferol on the expression of miR-146a. ATDC5 cells were treated by 40 μM Kaempferol for 12 h, and/or 5 μg/ml LPS for 5 h. The expression of miR-146a was detected by (A) qRT-PCR and (B) Northern blot analysis. $^{***}P < 0.001$.

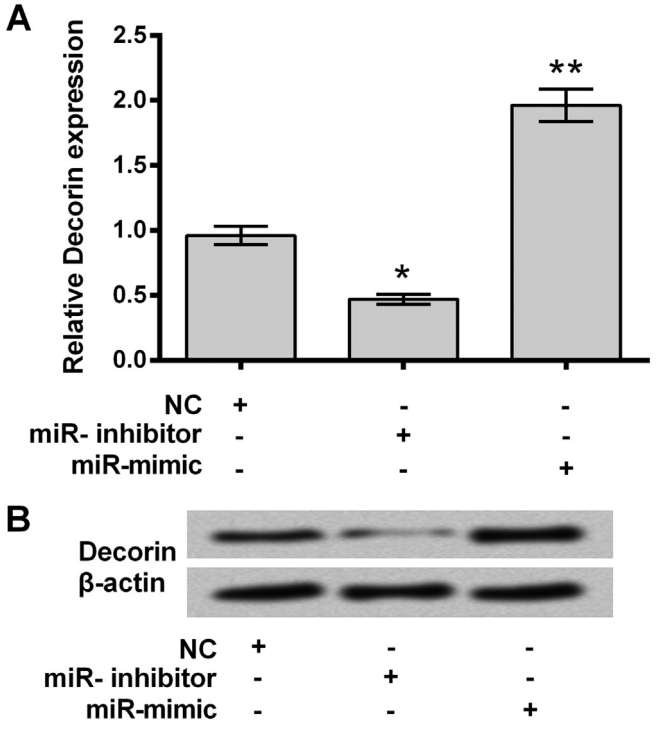

Fig. 4. Effect of miR-146a dysregulation on the expression of Decorin. ATDC5 cells were transfected with miR-146a mimic, inhibitor or NC. Thereafter, the (A) mRNA and (B) protein levels of Decorin were detected respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$.

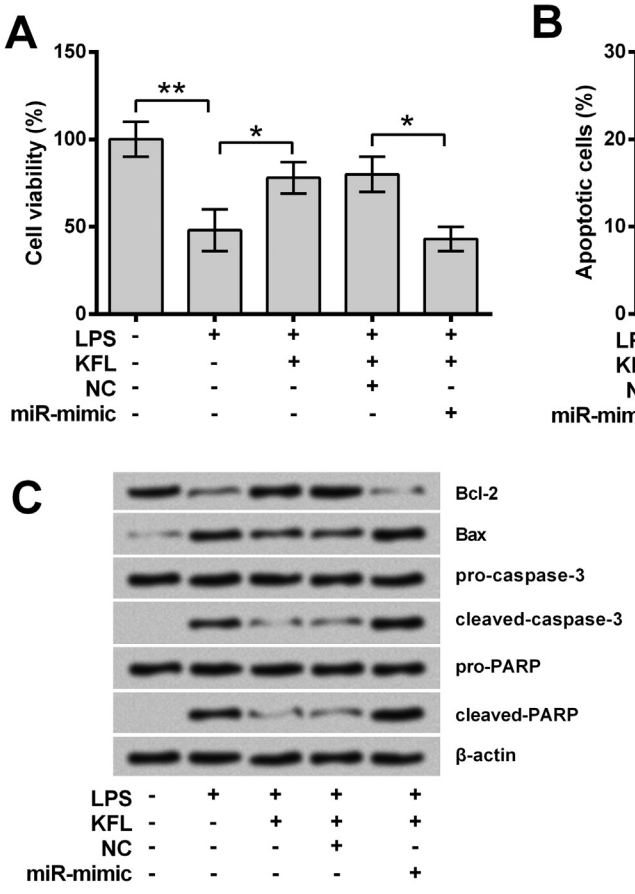

Fig. 3. Effects of miR-146a overexpression on ATDC5 cells treated by LPS plus Kaempferol. ATDC5 cells were transfected with miR-146a mimic or NC, and then treated by 40 μM Kaempferol for 12 h alone or in combination with 5 μg/ml LPS for 5 h. (A) Cell viability, (B) apoptosis rate, and (C) protein levels of apoptosis-related factors were measured by CCK-8 assay, Annexin V-FITC/PI double staining, and Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

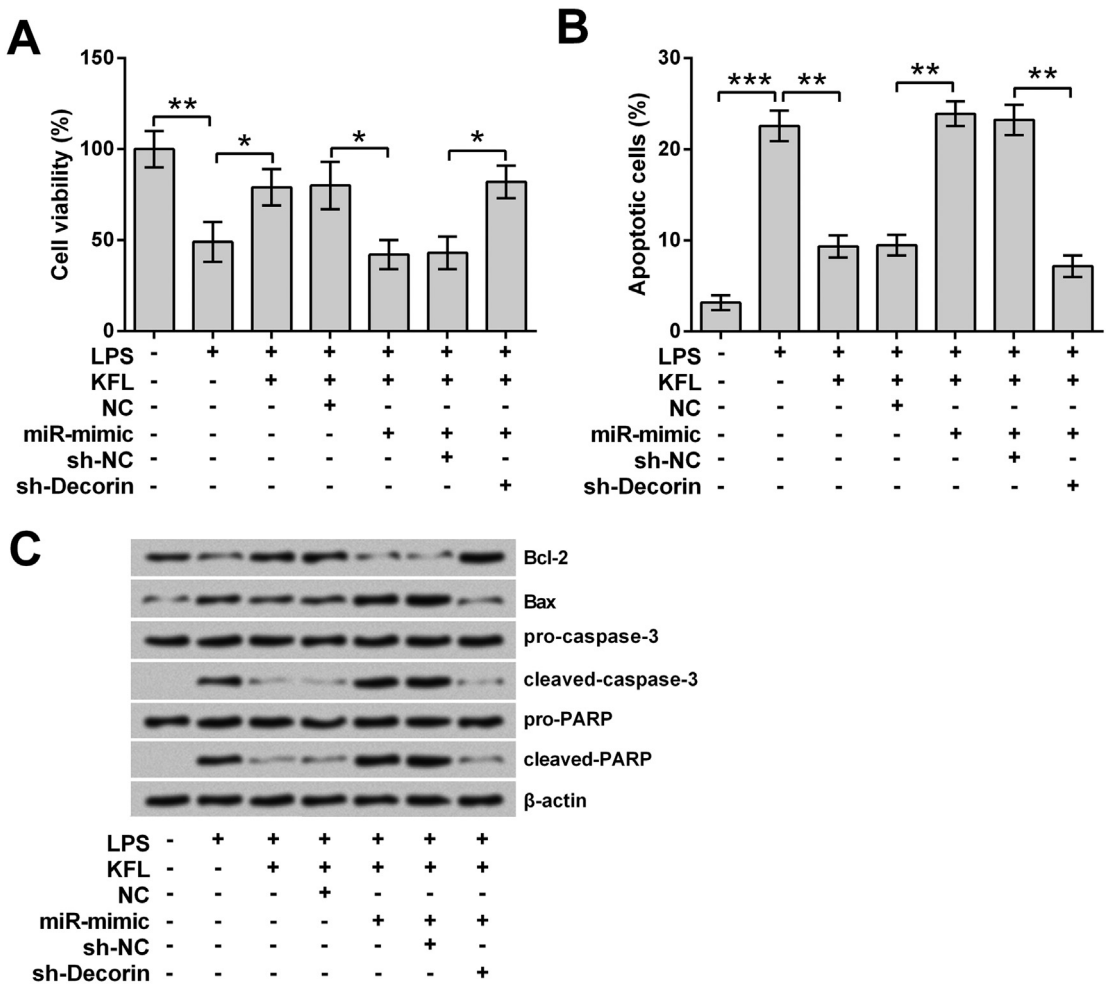

Fig. 5. Effects of Decorin silence on miR-146a-overxpressed ATDC5 cells treated by LPS plus Kaempferol. ATDC5 cells were co-transfected with miR-146a mimic and sh-Decorin, and then treated by 40 μM Kaempferol for 12 h alone or in combination with 5 μg/ml LPS for 5 h. (A) Cell viability, (B) apoptosis rate, and (C) protein levels of apoptosis-related factors were measured by CCK-8 assay, Annexin V-FITC/PI double staining, and Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

4. Discussion

It was generally thought that chondrocytes die by apoptosis is one of the major pathways for OA pathology [26]. Other than apoptosis, inflammation also contributes to the progression of OA. Affected by a variety of proinflammatory factors, including IL-6 and IL-8, the chondrocyte and cartilage microenvironment undergoes alterations in OA patients that subsequently contributes to chondrocyte dysfunction [27]. In vitro models of OA are well-established by using TNF-α [29], IL-1β [30] and LPS [31], and they serve as important adjuncts and surrogates

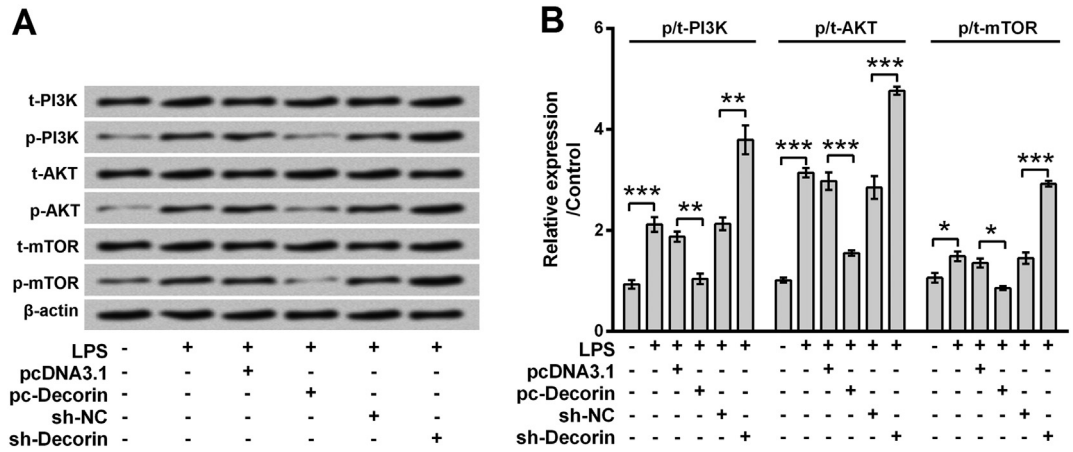

Fig. 6. Effect of Decorin dysregulation on the activation of PI3K/AKT/mTOR signaling pathway. ATDC5 cells were transfected with pc-Decorin, sh-Decorin, pcDNA3.1 or sh-NC, and then treated by 5 μg/ml LPS for 5 h. (A-B) The expression changes of core proteins in PI3K/AKT/mTOR signaling pathway were determined by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

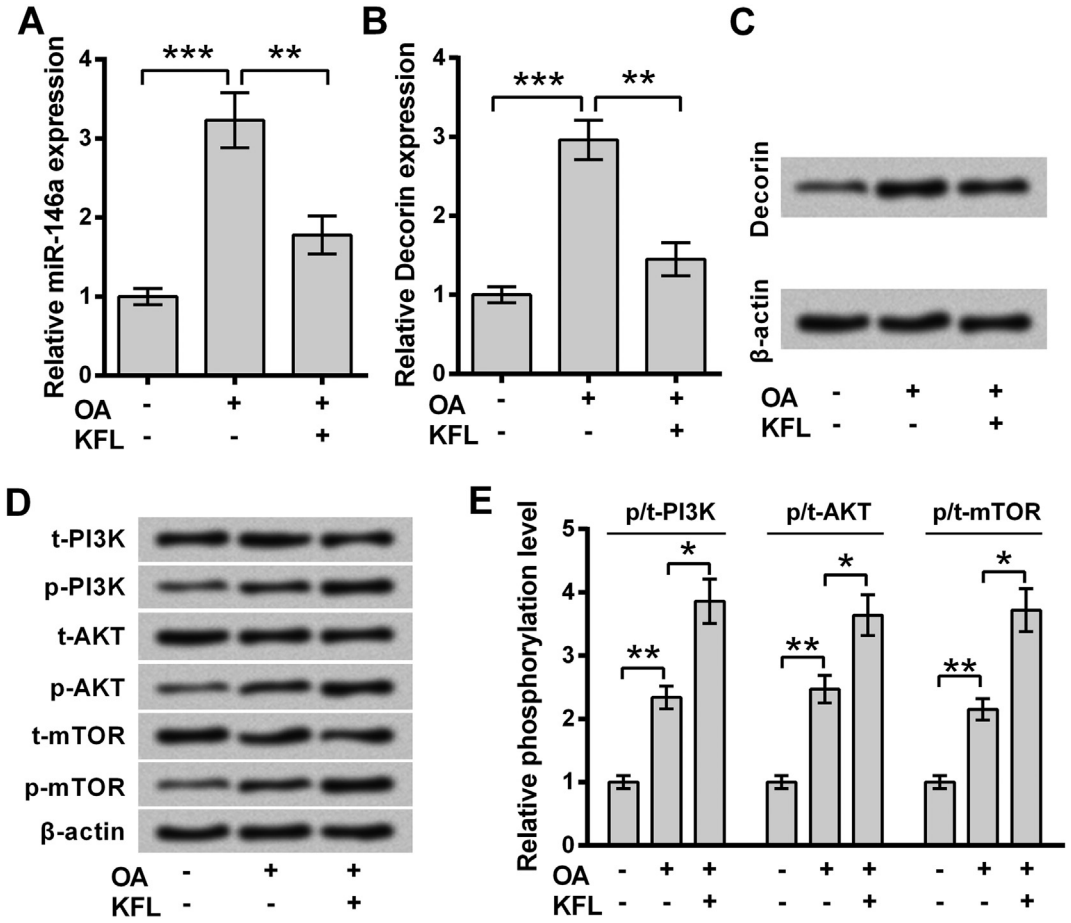

Fig. 7. In vivo effects of Kaempferol on miR-146a and Decorin expression as well as the activation of PI3K/AKT/mTOR signaling. Sprague-Dawley rats were used to establish an animal model of OA. The rats were intragastrically administrated with 5 mg/kg/day Kaempferol for 3 months before OA surgery. After 4 weeks of surgery, (A) miR-146a expression, (B) mRNA and (C) protein levels of Decorin expression were measured by qRT-PCR and Western blot. (D-E) The expression changes of core proteins in PI3K/AKT/mTOR signaling pathway were measured by Western blot analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

for studies of OA in human. In this study, LPS was used as a stimulus to establish an in vitro model of OA on murine chondrogenic ATDC5 cells. It is well-known that, LPS is able to activate Toll-like receptor 4 (TLR-4) signaling, which is involved in development of inflammation as well as catabolism and anabolism of osteoblasts and osteocytes [32]. Besides, the LPS infected cells may shut off protein synthesis and undergo programmed cell death via TLR-4-dependent signaling. In line with these findings, LPS stimulation induced a significant cell death and inflammatory injury in ATDC5 cells. Precondition of Kaempferol prevented apoptosis and the release of proinflammatory cytokines triggered by LPS. Kaempferol protected ATDC5 cells might be via down-regulation of miR-146a, and thus repressing the expression of Decorin as well as activating PI3K/AKT/mTOR signaling pathway. The revealed regulatory relationship was also confirmed in a rat model of OA. The expression of miR-146a and Decorin in cartilage tissues was repressed by Kaempferol. Also, the activated PI3K/AKT/mTOR signaling pathway in OA animal model was further enhanced by Kaempferol administration.

It is well-established that Kaempferol exhibits strong anti-apoptosis and anti-inflammatory activities in response to various kinds of stimulus including propacetamol [6], ethanol [34], hyperglycemia [35], and LPS [36]. The anti-apoptotic and anti-inflammatory effects of Kaempferol on rat OA chondrocytes stimulated with IL-1β has been reported [10]. Also, trials in patients with OA suggested that administration with Kaempferol alleviated the clinical symptoms of OA [11]. In this study, we demonstrated that Kaempferol appeared to protect ATDC5 cells against LPS induced apoptosis and inflammation. Supporting evidence from this study included findings that viability of ATDC5 cells was increased, apoptosis rate was decreased, anti-apoptotic protein Bcl-2 was up-regulated, pro-apoptotic protein Bax was down-regulated, and apoptotic executioner caspase-3 and DNA-repairing enzyme PARP were deactivated. Also, the excessive production of proinflammatory cytokines, IL-6, IL-8, and TNF-α induced by LPS was attenuated by Kaempferol precondition.

In the regard of OA, numbers of miRNAs have been identified to be involved in the pathogenesis of OA [12–15]. In this study, we focused on miR-146a to further reveal whether Kaempferol protected ATDC5 cells via regulation of miR-146a. We found that miR-146a expression was up-regulated in response to LPS, which is in line with previous studies [18,37]. We additionally found that Kaempferol treatment decreased miR-146a expression. And also, rescue assay results showed that miR-146a silence exhibited a Kaempferol-like effect totally, as apoptosis and the release of proinflammatory cytokines were both repressed in LPS-stimulated cells. Collectively, these data suggested that Kaempferol protected ATDC5 cells against LPS-mediated cell damage via down-regulation of miR-146a.

Decorin is a member of small leucine-rich repeat proteoglycans family and is a kind of matrix-organizing proteoglycans. It has been recognized as a functional component of ECM, and thus playing an essential role in pathology of degenerative joint disease [23]. At late stage of OA, Decorin is up-regulated at both transcriptional and translational levels [38], indicating Decorin up-regulation is associated with the development of OA. In this research, Decorin was found to be a downstream gene of miR-146a, and exerted aggravating effect on LPS-

injured ATDC5 cells. This phenomenon was consistence with a previous finding that disruption of Decorin led to higher stiffness of articular cartilage matrix, and conferred resistant to OA [23].

PI3K/AKT/mTOR signaling pathway is one of the most prominent signal cascades to regulate numerous cellular responses, including cell-cycle progression, apoptosis, migration and inflammation. It is well-accepted that PI3K/AKT/mTOR signaling determines the fate of chondrocyte under stimulating conditions of IL-1β [39] and TNF-α [40]. PI3K/AKT/mTOR signaling pathway is activated in response to LPS [41], as PI3K can be activated by TLR4 (a receptor of LPS) signaling. This was also confirmed in this study, that PI3K/AKT/mTOR signaling pathway was activated in ATDC5 cells following LPS treatment. More importantly, we observed that the activation of PI3K/AKT/mTOR signaling induced by LPS was repressed by Decorin overexpression, and was accelerated by Decorin silence.

In conclusion, the present study demonstrated that precondition of Kaempferol prevented apoptosis and inflammation triggered by LPS. The protective effect of Kaempferol might be attributed to its ability of down-regulating miR-146a, and thus repressing the expression of Decorin. This study suggested that Kaempferol could be a promisingly therapeutic agent for the management of OA, and highlighted a possible underlying mechanism of this action.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.02.014.

Abbreviations

OA	osteoarthritis
LPS	lipopolysaccharide
ELISA	enzyme linked immunosorbent assay
CCK-8	cell counting kit-8

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Competing interests

The authors declare that they have no competing interests.

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