Baicalin Ameliorates Cartilage Injury in Rats With Osteoarthritis via Modulating miR-766-3p/AIFM1 Axis

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Summary

The study aims to elucidate the therapeutic mechanism of Baicalin (BAI) in alleviating cartilage injury in osteoarthritic (OA) rat models, concentrating on its regulation of the miR-766-3p/AIFM1 axis. An OA rat model was developed with unilateral anterior cruciate ligament transection (ACLT). Interventions comprised of BAI treatment and intra-articular administration of miR-766-3p inhibitor. For evaluation, histopathological staining was conducted to investigate the pathological severity of knee cartilage injury. The levels of oxidative stress (OS) indicators including MDA, SOD, and GSH-Px, were quantified using colorimetric assays. Inflammatory factors (IFs; TNF-α, IL-1β, and IL-6) in knee joint lavage fluids were assessed using ELISA, while RT-PCR was employed to quantify miR-766-3p expression. TUNEL apoptosis staining was utilized to detect chondrocyte apoptosis, and western blotting examined autophagy-related markers (LC3, Beclin, p62), extracellular matrix (ECM) synthesis-associated indices (COL2A, ACAN, MMP13), and apoptosis-inducing factor mitochondrion-associated 1 (AIFM1). Histological examination revealed a marked amelioration of cartilage injury in the BAI-treated OA rat models compared to controls. BAI treatment significantly reduced inflammation and OS of knee joint fluid, activated autophagy, and decreased chondrocyte apoptosis and ECM degradation. Interestingly, the inhibitory effects of BAI on these pathological markers were significantly decreased by the miR-766-3p inhibitor. Further assessment revealed that BAI efficiently promoted miR-766-3p expression while inhibiting AIFM1 protein expression. BAI potentially mitigates articular cartilage injury in OA rats, likely through modulation of miR-766-3p/AIFM1 axis.

Keywords: Baicalin • microRNA • AIFM1 • Osteoarthritisv • Rat

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Introduction

As a degenerative joint condition commonly seen in older adults, osteoarthritis (OA) primarily presents with progressive chondrodegeneration, synovitis and subchondral sclerosis [1], and is usually caused by aging, heredity, overweight, joint injury or deformation [2]. According to epidemiological surveys [3,4], OA afflicts 9.8 % of men and 18 % of women among the elderly aged 60 years or older, with a very high incidence and disability rate, seriously impacting patients' health and quality of life. The disease progression of OA, a condition with complex pathogenesis, is mainly attributed to the reduction of chondrocytes and the degradation of extracellular matrix (ECM) induced by internal and external factors such as inflammatory stimuli, metabolic disorder and mechanical stress, which leads to the destruction of the entire joint tissue structure [5]. The current treatment of OA mainly relies on non-specific medication such as antipyretics, non-steroidal anti-inflammatory drugs (NSAIDs), and analgesics to diminish inflammation and relieve pain, as well as supplementation of chondroitin sulfate, hyaluronic acid, etc. to protect cartilage to a certain extent.

But these therapies have shortcomings such as slow onset and poor effectiveness. Therefore, it is urgent to find novel therapeutic drug therapies for OA, so as to obviously improve the clinical therapeutic effect of OA.

Baicalin (BAI) is a flavonoid component isolated from the root of the Chinese herb Scutellaria baicalensis Georgi (Labiatae), which is considered to exert antioxidation, anti-inflammatory, anticancer, diuretic and antithrombotic and other pharmacological activities [6-9], allowing it to safely and effectively treat a series of diseases such as inflammation and cardio-cerebrovascular diseases. A number of studies have reported [10] the potent anti-inflammatory properties of BAI in *in vitro* cell research. Li *et al.* [11], for example, found that BAI can effectively inhibit interleukin (IL)-6, IL-8 and other inflammatory factors (IFs) in human OA chondrocytes by modulating iNOS, thus alleviating the inflammatory stimulation process. BAI also playsan anti-inflammatory role in human OA chondrocytes stimulated by IL-1β [12]. Meanwhile, BAI down-regulates caspase-3 protein, promotes Bcl-2 expression, and protects chondrocytes from IL-1β-induced chondrocyte apoptosis [13]. However, *in vivo* research on BAI in OA is still lacking, and further elucidation is needed regarding its mechanism of action. microRNAs (miRNAs), a small molecule non-coding RNA family, are critical in biological and pathological processes like inflammation and cancer, by regulating gene expression through the 3'end of targeted gene mRNAs. According to literature [14], various miRNAs are strongly linked to chondrocyte apoptosis and articular cartilage (AC) ECM degradation, and are essential in OA procession. Among them, miR-766-3p has been shown to be antineoplastic in the progression of liver, breast and other cancers by suppressing tumor growth, invasion and metastasis [15,16]. The previous *in vitro* study of our research team [17] showed that BAI played an anti-inflammatory part in human OA via IL-1β inducement of chondrocytes to significantly upregulate miR-766-3p. Therefore, this study aims at studying BAI's impact on AC injury in OA rats and evaluating the specific role played by miR-766-3p/AIFM1 axis in BAI's alleviation of AC injury, which can render a strong theoretical foundation for revealing the possible molecular mechanisms underlying BAI treatment of OA, with practical guiding significance for promoting the early clinical application of BAI.

Materials and methods

Experimental animals and grouping

Twelve-week-old male SD rats (weight: 250±20g: Animal Center of Sichuan University), kept in standard alternating light/dark (12h-12h) conditions, were fed a standard commercial diet and tap water. They were humanely cared for and all steps were performed sterile tactically. General anesthesia was realized through 3 % pentobarbital sodium (Tocris, UK) intraperitoneal injection at 40 mg/kg, with all efforts made to minimise animal suffering. Using an 8-10 mm medial parapatellar approach, the right knee of the narcotized rats was exposed, and the patella was displaced laterally prior to anterior cruciate ligament (ACL) transection (ACLT), followed by complete excision of the medial meniscus without damaging the AC. After skin suture, the post-surgical animals were allowed to move, eat and drink at their will. The animals were grouped as follows with 9 rats in each group: sham, OA (OA group), OA + 100 mg/kg BAI (BAI group), and OA + 100 mg/kg BAI + miR-766-3p inhibitor intra-articular injection (BAI + miR-766-3p inhibitor group). Sham rats received the same operation but skipped the ACLT procedure. For BAI and BAI + miR-766-3p inhibitor groups, rats were subjected to injection of different BAI volumes daily immediately after ACLT operation for 14 days. While the injected solution in control group was replaced by normal saline. In the third group, 5 nmol of miR-766-3p inhibitor (volume: 100 μL) was intra-articularly injected through the medial parapatellar approach (MPA) one week after surgery, and 5 nmol of miR-NC was used as the control.

Histopathological staining

Rats were killed 6 weeks later for AC specimen collection. After being paraformaldehyde-fixed for 24 hours and 10 % EDTA (pH 7.4)-decalcified for 21 days, the tissue samples were treated with paraffin-embedding and preparation into slices 5-μm in thickness. Medial and lateral compartments of the retrieved AC were then prepared as consecutive sections at 200-μm intervals. The selected slices were then subjected to xylene dawaxing, rehydration by graded ethanol rinses, and hematoxylin-eosin (H&E) and Safranin O/Fast Green dye (SO/FG; Servicebio, China) dyeing. Pathological damage was evaluated using the Mankin score system.

Enzyme-linked immunosorbent assay (ELISA)

After random selection and decapitation of 7 rats, 2 mL of blood was drawn for 2 h of room temperature standing. Meanwhile, the knee joint was quickly separated and the suprapatellar bursa was cut, after which the joint was washed with normal saline to collect the lavage solution. Measurements of IFs (tumor necrosis factor, TNF-α; interleukin-1β, IL-1β; and IL-6) in rat knee joint lavage fluid were made as instructed by the manufacturer's protocols of commercial ELISA kits from R&D (Minneapolis, USA). Protein concentrations in tissue homogenates were determined with protein determination dyes (Bio-Rad Laboratory, USA).

Oxidative stress (OS) detection

The level of reactive oxygen species (ROS), reflected in malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) contents, were measured as described above. Additionally, a CLA-2100 high performance chemiluminescent (CL) analyzer from Tohoku Electronic Industrial Co., Japan, was utilized for the determination of superoxides and peroxynitrous acid. To put it simply, a phosphate buffer (200 μL)-supplemented tissue homogenate (400 μL) was put into a stainless steel plate, and the background count of CL was read for 60 seconds. CL counting was performed after the injection of 100 μL of fluorescein Octyl-β-d-glucopyranosiduronic acid into the machine. The CL Analyzer data acquisition software ordered from Tohoku Electric Power, Japan, was employed for data analyses.

Western blotting

Total proteins were isolated from the cultured cells by radioimmunoprecipitation (RIPA) lysates and the concentrations were determined. Protein specimens of an equal amount were then isolated by SDS-PAGE and moved to a PVDF membrane for blocking in 5 % skim milk-supplemented 0.1 % Tris-buffered saline with Tween (TBST), overnight incubation (4 ℃) with I antibodies, and 1 hour of cultivation with HRP-bound II antibodies. After that, an enhanced chemiluminescence (ECL) kit was employed for the detection of protein signals, with GAPDH as an internal control. Bcl-2, BAX, Pro-caspase-3, and Cleave-caspase-3 from Abcam (Cambridge, Massachusetts, USA), ACAN, COL2A, MMP13, and GAPDH from Santa Cruz, USA, as well as LC3, Beclin-1 (Proteintech), and p-62 from Proteintech (Chicago, Illinois, USA) were the antibodies used.

TUNEL apoptosis assay

For apoptosis determination, cells were subjected to 1 h of room temperature staining with PI/FITC-annexin V (Sigma, St Louis, MO) after different treatments. Then came the assessment of early/late apoptotic and necrotic cells following the Annexin V-FITC Apoptosis-Hoechst Staining Kit (Abcam) protocol. In short, the cells harvested were centrifuged (626 g, 4 ℃) for 5 minutes, followed by 2 PBS rinses and another 5-minute centrifugation under the same conditions. Subsequently, cells (1×105) were collected from each group for resuspension in a binding buffer and room temperature staining with Annexin V-FITC and propidium iodide (5 μL each) in darkness. Within 1 hour, flow cytometry was performed with BD FACSAria Flow Cytometer (BD Biosciences) and CellQuest Pro software (*v*5.1; BD Biosciences) for apoptosis analysis.

qRT-PCR

Total RNA was separated from cartilage tissue with TRIzol reagent (Invitrogen, Carlsbad, CA) and its concentration was measured. A Retrovirus PrimerScriptTM kit was then utilized to reverse transcribe mRNA into cDNA. miRNA determination was carried out with a miRNA-specific TaqManTM MicroRNA kit. This was followed by the use of FastStart Universal SYBR Green master mix to perform quantitative PCR. The 2−Δct method was utilized to work out miRNA and mRNA expression normalized against U6 and GAPDH, respectively (see below for sequences of primers). Normal (Normal) and OA rat-derived cartilage tissues (normal and OA chondrocytes) were inoculated into cell culture bottles at 3,000 cells/cm2. The medium used was DMEM/F12 complete medium plus 10 % fetal bovine serum, 100 U/ml penicillin and 100 J.tg/mL streptomycin, and the culture was conducted in a cell incubator under the conditions of 37˚C and 50 % CO_2, with the solution changed once every 3 days. After the cells became 90 % confluent, 25 % trypsin-EDTA was used for timely digestion and passage at the ratio of 1:3. In subsequent cell experiments, the 2rd generation (P2) chondrocytes were uniformly used. Excess chondrocytes were stored in liquid nitrogen and resuscitated as needed.

Statistical analysis

The software used in this study for statistical data analysis and mapping was SPSS. Between-group and multi-group differences (significance threshold: $P<0.05$)

of continuous variables (denoted by $\bar{x}\pm s$) were identified by t-tests and one-way analysis of variance (ANOVA), respectively.

Results

BAI alleviates knee cartilage injury in OA rats by up-regulating miR-766-3p expression

According to histopathological staining results, Sham rats had intact knee cartilage surface and no chondrocyte injury; in OA model group, the knee cartilage tissue was seriously damaged, the number of chondrocytes was significantly reduced, the AC lamellar structure was blurred, and the chondrocytes was disorderedly arranged; BAI-treated OA rats showed markedly alleviated pathological injury of knee cartilage than OA model rats; while intra-articular miR-766-3p inhibitor injection obviously inhibited BAI amelioration of knee cartilage injury in OA rats (P<0.05; Fig. 1A). Meanwhile, higher Mankin scores in OA model rats versus Sham rats were determined (P<0.05), while OA rats in BAI group had markedly lower scores than model group (P<0.05); and significantly higher Mankin scores were determined in BAI + miR-766-3p inhibitor group versus BAI group (P<0.05; Fig. 1B). It is suggested that BAI can ameliorate knee cartilage injury in OA rats, and inhibiting miR-766-3p expression can obviously affect the anti-injury effect of BAI on OA rats.

In addition, as indicated by RT-PCR, knee cartilage miR-766-3p expression was obviously decreased in OA model rats versus Sham rats (P<0.05); OA rats in BAI group showed markedly higher knee cartilage miR-766-3p levels than OA model rats (P<0.05); however, the promotion of BAI on miR-766-3p expression was obviously blocked by intra-articular injection of miR-766-3p inhibitor (P<0.05; Fig. 1C). It is suggested that BAI can alleviate knee cartilage injury in OA rats, possibly via up-regulating miR-766-3p.

BAI affects IFs in knee lavage fluid of OA rats by up-regulating miR-766-3p

According to ELISA results, OA model rats had higher IFs (TNF-α, IL-1β and IL-6) in knee joint lavage fluid than Sham rats (P<0.05), and BAI treatment validly reduced these IFs (P<0.05); while miR-766-3p inhibitor intervention could effectively block the inhibitory effect of BAI on inflammatory responses in joint lavage fluid of OA rats (P<0.05; Fig. 2). Thus, BAI is effective in mitigating knee joint inflammation in OA rats, and inhibiting miR-766-3p can block the anti-inflammatory effect of BAI in OA rats.

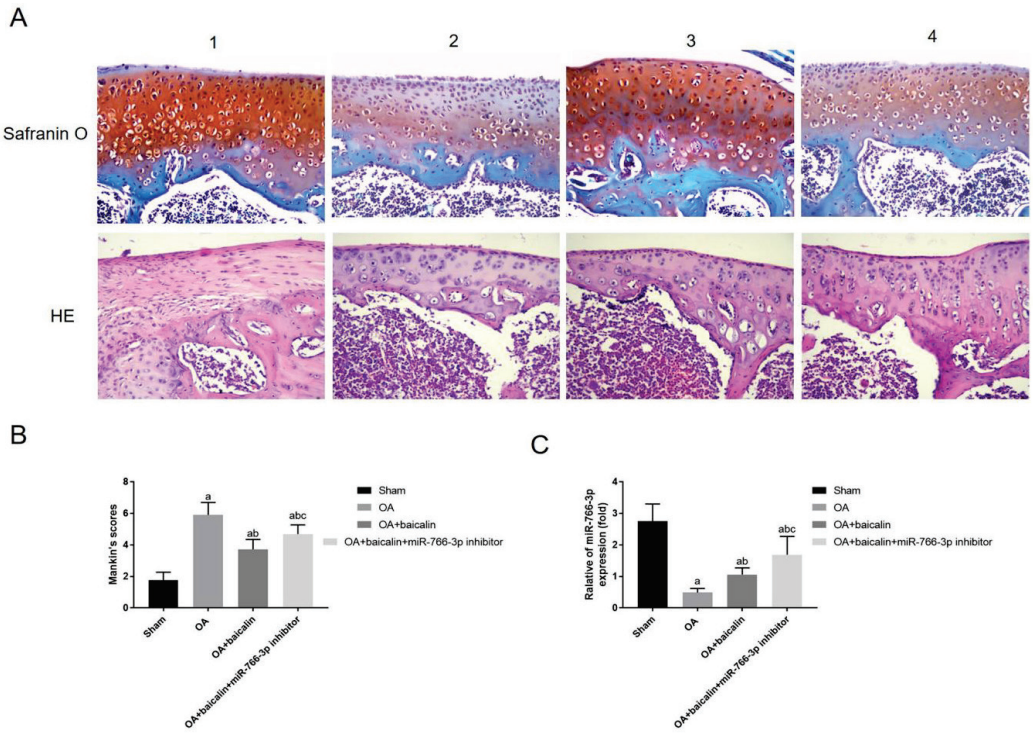

Fig. 1. Baicalin up-regulates miR-766-3p to alleviate knee cartilage injury in OA rats. **A**) Pathological injury of knee cartilage visualizd by Safranin O and HE stain. **B**) Mankin scores assessment. **C**) miR-766-3p expression assessment using RT-PCR. **1**) Sham Group; **2**) OA Group; **3**) OA + Baicalin Group; **4**) OA+ Baicalin + miR-766-3p inhibitor Group. aP<0.05, bP<0.05, and cP<0.05 vs. Sham group.

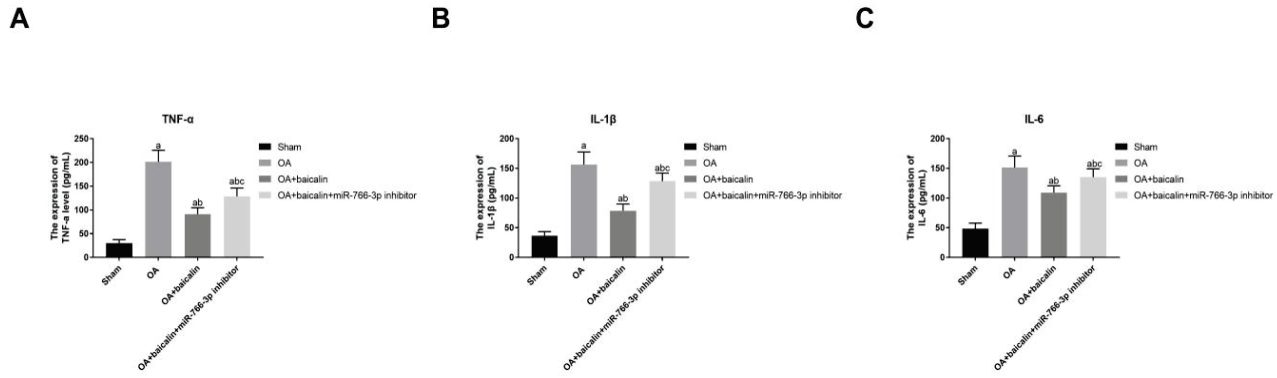

Fig. 2. Baicalin up-regulates miR-766-3p to inhibit the inflammatory factors TNF-α (**A**), IL-1 β (**B**), IL-6 (**C**) levels in OA rats. [a]*P*<0.05, [b]*P*<0.05, and [c]*P*<0.05 versus Sham group.

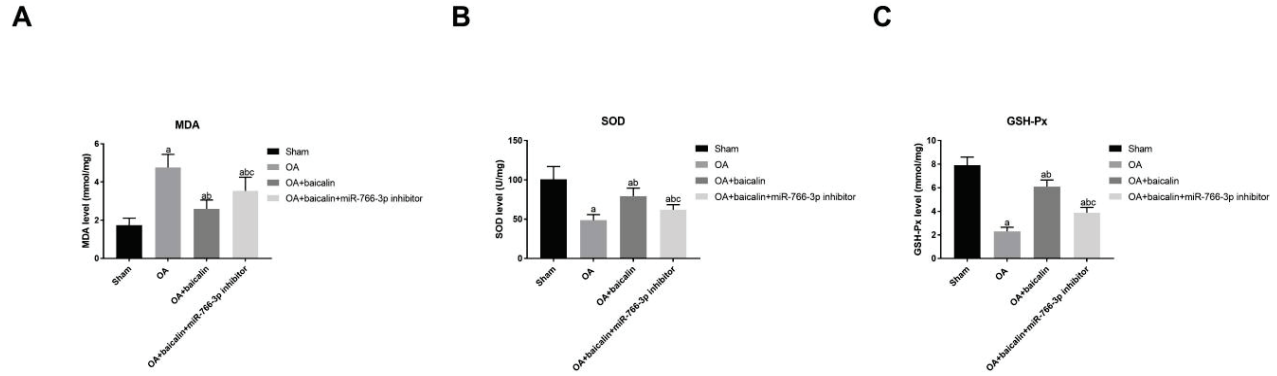

Fig. 3. Baicalin up-regulates miR-766-3p to influence MDA (**A**), SOD (**B**), GSH-Px (**C**) levels related to oxidative stress in knee joint lavage fluid of OA rats. [a]*P*<0.05, [b]*P*<0.05, and [c]*P*<0.05 versus Sham group.

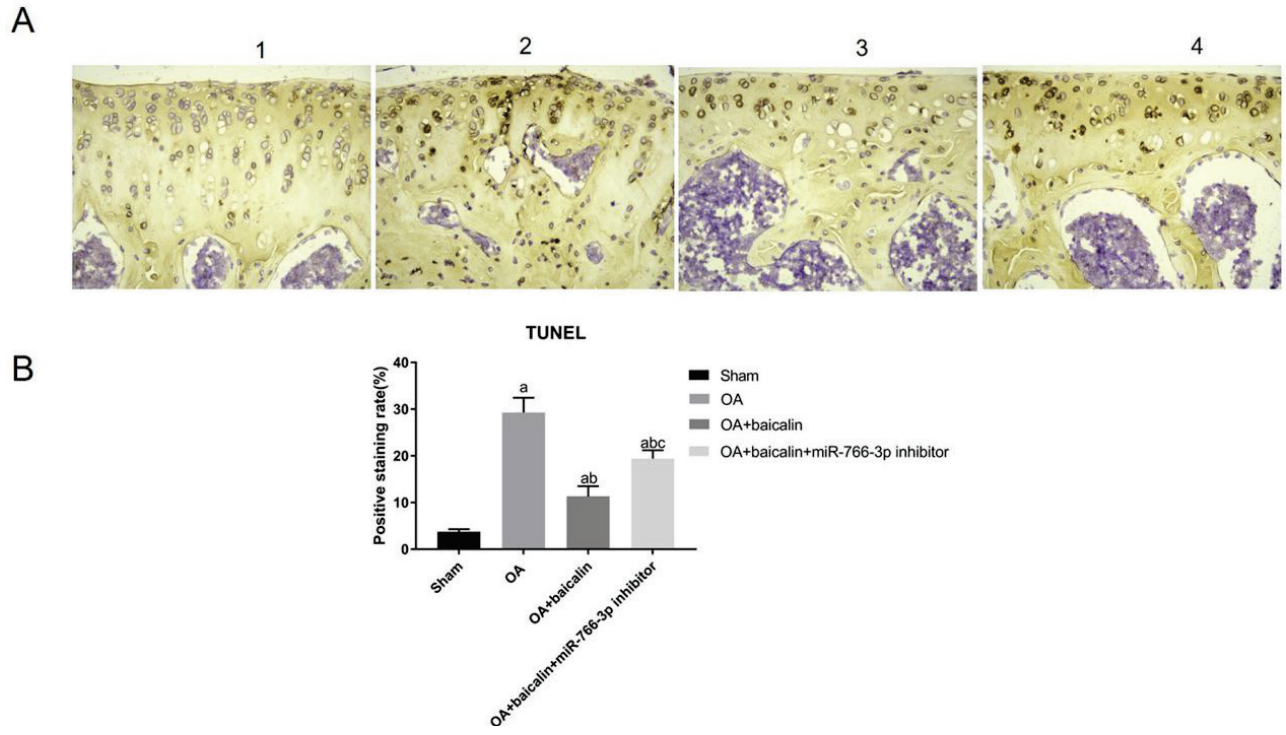

Fig. 4. Baicalin up-regulates miR-766-3p to suppress knee chondrocyte apoptosis in OA rats. **A**) TUNEL staining. **B**) quantitative analysis of TUNEL stain. **1**) Sham Group; **2**) OA Group; **3**) OA+ Baicalin Group; **4**) OA+ Baicalin+ miR-766-3p inhibitor Group. aP<0.05, bP<0.05, and cP<0.05 versus Sham group.

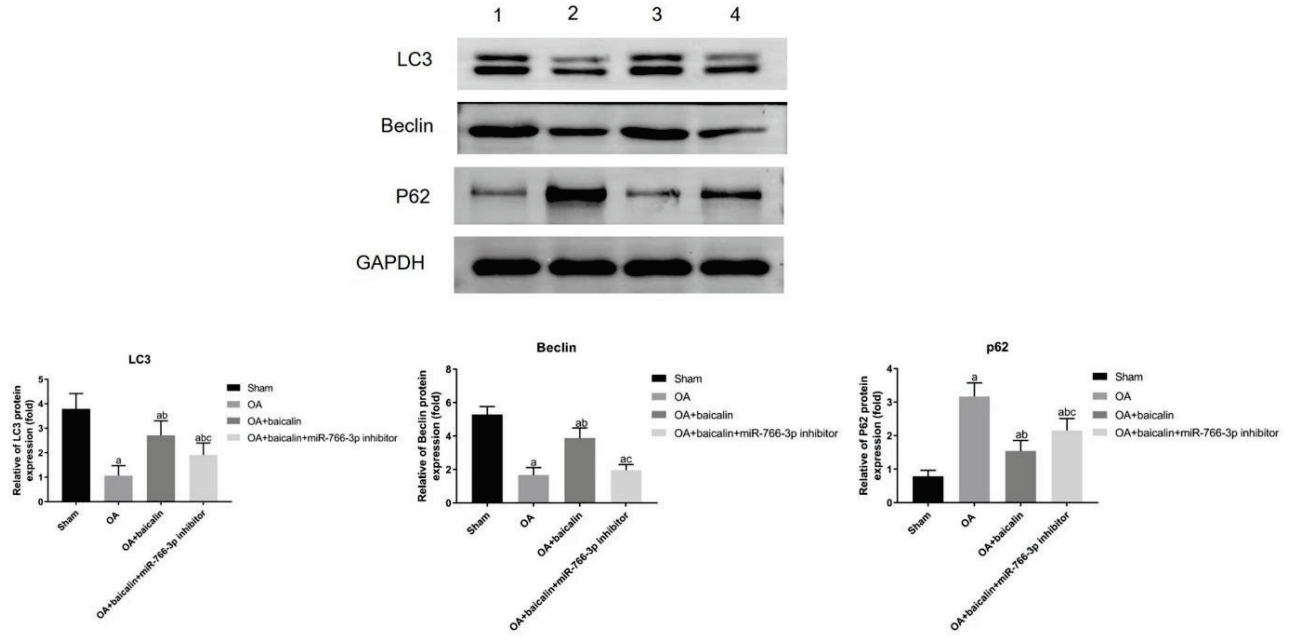

Fig. 5. Baicalin activates autophagy of knee cartilage tissue in OA rats by upregulating miR-766-3p. **A**) Western blot of Autophagy associated proteins. **B**) quantitative analysis of Western blot. **1**) Sham Group; **2**) OA Group; **3**) OA+ Baicalin Group; **4**) OA+ Baicalin+ miR-766-3p inhibitor Group. Compared with Sham group, aP<0.05; Compared with Sham group, bP<0.05; Compared with Sham group, cP<0.05.

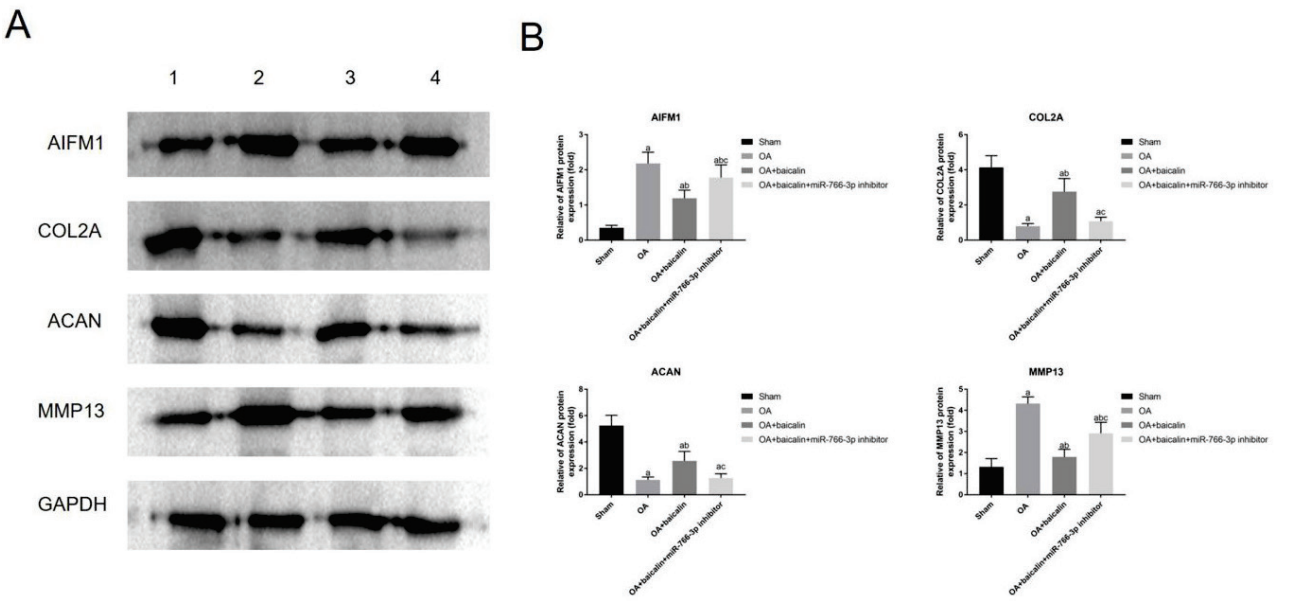

Fig. 6. Baicalin regulates miR-766-3p/AIFM1 axis to suppress ECM degradation of knee cartilage in OA rats. **A**) Western blot of ECM degradation related protein. **B**) quantitative analysis of Western blot. **1**) Sham Group; **2**) OA Group; **3**) OA+ Baicalin Group; **4**) OA+ Baicalin+ miR-766-3p inhibitor Group. aP<0.05, bP<0.05, and cP<0.05 versus Sham group.

BAI influences OS (MDA, SOD and GSH-Px) in knee lavage fluid of OA rats by up-regulating miR-766-3p

The OS-associated indexes (MDA, SOD and GSH-Px) in rat knee joint lavage fluid in each group were detected. Markedly higher MDA while statistically lower SOD and GSH-Px were determined in knee joint lavage fluid of OA model rats than in Sham rats (P<0.05); BAI treatment led to an evident reduction in MDA and an obvious increase in both SOD and GSH-Px contents in joint lavage fluid of OA rats (P<0.05); while MDA in the lavage fluid of OA rats in BAI + miR-766-3p inhibitor group were markedly elevated compared with BAI group,

and SOD and GSH-Px were reduced (P<0.05; Fig. 3). It shows that BAI may reduce the OS response of knee joint in OA rats by regulating miR-766-3p levels.

BAI inhibits knee chondrocyte apoptosis in OA rats via up-regulating miR-766-3p

The results of TUNEL staining showed that the knee chondrocyte apoptosis level was evidently elevated in OA model than in Sham group (P<0.05), and that in BAI group was lower versus model group (P<0.05); while the knee chondrocyte apoptosis level in OA rats treated with miR-766-3p inhibitor was higher compared to BAI-intervened rats (P<0.05; Fig. 4). It is suggested that BAI can inhibit knee chondrocyte apoptosis via up-regulating miR-766-3p in OA rats.

BAI activates knee cartilage autophagy inOA rats by up-regulating miR-766-3p

The measurements of LC3, Beclin and p62, autophagy-related markers, revealed that LC3 and Beclin protein levels in knee cartilage were markedly lower in OA model group compared to Sham group, while p62 protein expression was higher (P<0.05); BAI-treated OA rats had higher LC3 and Beclin protein levels and lower p62 protein expression in cartilage tissue than model group (P<0.05), suggesting that BAI can activate knee cartilage autophagy in OA rats. Moreover, LC3 and Beclin protein expression lowered remarkably after miR-766-3p inhibitor intervention compared with BAI group, while p62 protein expression levels were evidently elevated (P<0.05). Thus, by up-regulating miR-766-3p, BAI may activate knee cartilage autophagy in OA rats. See Fig. 5 for details.

BAI inhibits ECM degradation in knee cartilage of OA rats by modulating miR-766-3p/AIFM1 axis

AC is mainly composed of chondrocytes and cartilage ECM, and ECM synthesis-related markers type II collagen (COL2A) and aggrecan (ACAN), and ECM degradation-related indicator MMP-13 are closely related to cartilage ECM degradation. The results of this study showed lower COL2A and ACAN while higher MMP-13 protein expression in rat cartilage in OA model group versus Sham group (P<0.05). BAI-treated OA rats showed higher cartilage tissue COL2A and ACAN and lower MMP13 protein levels compared with OA model rats (P<0.05). While miR-766-3p inhibitor treatment resulted in markedly lower COL2A and ACAN levels and higher MMP13 protein expression than BAI group (P<0.05). Hence, BAI is effective in inhibiting ECM degradation in cartilage tissue, the effect of which can be effectively blocked by miR-766-3p inhibitor intervention. Moreover, AIFM1 protein showed markedly enhanced expression in knee cartilage of OA rats and was inhibited by BAI treatment, while miR-766-3p inhibitor intervention could block this inhibition. Based on the results of previous cell studies *in vitro*, we believe that BAI modulates the miR-766-3p/AIFM1 axis to achieve the suppression of knee cartilage ECM degradation in OA rats. See Fig. 6 for details.

Discussion

Over the years, NSAIDs have long been used in clinical practice to treat OA, but rather than hinder OA progression, these drugs can only relieve clinical symptoms such as joint pain and swelling, in addition to inducing a range of serious side effects. Thus, finding safe and effective drug therapies that can both relieve clinical symptoms and slow OA progression is critical. BAI is a major flavonoid isolated from the dried roots of Scutellaria baicalensis Georgi, which has anti-inflammatory effects [18]. This study demonstrated the ability of BAI to markedly suppress inflammatory and OS reactions in OA rats, which is mainly reflected in alterations in MDA, SOD, GSH-Px related to OS and IFs (TNF-α, IL-1β and IL-6). Additionally, BAI greatly inhibited the degradation of ACAN and COL2A, with excessive chondrocyte death and ECM degradation. The chemical and genetic regulation of ECM synthesis and apoptosis has been shown to play an active role in disease progression in cell or animal OA models [19, 20]. But the therapeutic potential of BAI in OA has been rarely reported [21, 22]. Herein, we discussed the ameliorative action of BAI against knee cartilage injury in OA rats and molecular mechanisms, and found that BAI administration alleviated apoptosis and ECM degradation of knee cartilage tissue in OA rats.

An increasing body of evidence shows that ECM degradation and chondrocyte apoptosis in OA rats are related to the pathogenesis and development of OA [23-25]. Herein, we utilized a simulated rat OA model of cartilage injury for investigation. As expected, knee chondrocytes apoptosis and matrix metalloproteinase expression were significantly enhanced in OA model rats, and cartilage autophagy and ECM synthesis were obviously reduced. BAI treatment reversed the harmful effects of OA in rats, which indicated that BAI might be

protective against knee cartilage injury in OA rats and inhibit inflammatory damage to knee cartilage. Moreover, the molecular mechanism of BAI is suggested to be linked to autophagy activation and autophagy flux recovery of chondrocytes in OA.

Autophagy, a cellular homeostatic mechanism, is involved in all kinds of pathological events [26], with evidence indicating that moderate autophagy can effectively inhibit apoptosis [27]. Decreased autophagy activity is thought to characterize AC in OA cases [28]. Further analysis revealed significant changes in the levels of Beclin-1 and p62, autophagy markers, after OA modeling in rats, which was manifested as reduced Beclin-1 and enhanced p62, suggesting that the OA rat model adversely modulates chondrocyte autophagy. While increased autophagosome protein levels and promoted autophagy flow were observed after BAI intervention. In addition, the beneficial effect of BAI was diminished after miR-766-3p inhibitor inhibition of autophagy flux, which suggests that BAI up-regulates miR-766-3p to promote autophagy flux, thus playing a protective role.

miRNAs, in virtue of their role in modulating chondrocytes' biological behavior in OA, have been considered treatment targets or diagnostic biomarkers for the disease [29, 30]. Recent studies have linked the abnormal expression of miRNAs to OA onset and development [31, 32]. miR-766-3p elevates with increasing patient age and exerts anti-inflammatory activity in fibroblast-like synovial cells in human rheumatoid arthritis (RA), according to a previous study [33]. Herein, we demonstrate for the first time that BAI may protect cartilage tissue from chondrocyte apoptosis and autophagy inhibition in OA rats by up-regulating miR766-3p. In addition, our previous literature confirmed that miR-766-3p may target the AIFM1 mRNA 3-UTR. Combining the results of qRT-PCR and Western blotting in this study, it can be seen that the level of AIFM1 protein increased significantly after miR-766-3p inhibitor injection. Thus, BAI can mitigate cartilage injury in rats with OA by regulating miR-766-3p/AIFM1 axis.

The key findings of this study support the potential of Baicalin in ameliorating articular cartilage injury in OA rats through the modulation of the miR-766-3p/AIFM1 axis. However, to strengthen the robustness of this statement, our future study would include additional data that provide a more comprehensive understanding of the molecular mechanisms and the therapeutic effects of Baicalin. Specifically, additional experiments or analyses focusing on the direct interaction between Baicalin and the miR-766-3p/AIFM1 axis, as well as the downstream effects of this modulation on chondrocyte function and extracellular matrix maintenance, would further illuminate the precise mechanisms underlying Baicalin's protective effects in osteoarthritis. These additional insights will help provide a more thorough and convincing demonstration of the proposed mechanism of action of Baicalin.

To sum up, BAI can alleviate cartilage injury in OA rats and protect OA rats from cartilage injury *via* inhibiting chondrocyte apoptosis and promoting autophagy, and that the protective effect may be related to the upregulation of miR-766-3p to reduce AIFM1 expression, suggesting the promising clinical value of BAI for OA treatment.

Authors' Contributions

Jiuxiang Liu conceived the study design and the content concept; Jiangqi Chen, Hao Zhou, performed the data collection, extraction and analyzed the data. Qiang Zuo, interpreted and reviewed the data and drafts. Feng Liu reviewed the final draft. All authors read and approved the final draft.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

OA, Osteoarthritis; BAI, Baicalin; miR-766-3p, microRNA 766-3p; AIFM1, Apoptosis-Inducing Factor Mitochondrion-Associated 1; AC, Articular Cartilage; ECM - Extracellular Matrix; IFs, Inflammatory Factors; OS, Oxidative Stress; H&E, Hematoxylin-Eosin; ELISA, Enzyme-Linked Immunosorbent Assay; RT-PCR, Reverse Transcription Polymerase Chain Reaction; MDA, Malondialdehyde SOD, Superoxide Dismutase; GSH-Px, Glutathione Peroxidase; CL, Chemiluminescent; SD, Standard Deviation; RA, Rheumatoid Arthritis; NSAIDs, Non-Steroidal Anti-Inflammatory Drugs; IL, Interleukin

References

1. Sharma L. Osteoarthritis of the knee. New England J Med 2021;384:51-59. https://doi.org/10.1056/NEJMcp1903768
2. Schram B, Orr R, Pope R, et al. Risk factors for development of lower limb osteoarthritis in physically demanding occupations: A narrative umbrella review. Journal of occupational health, 2020;62:e12103. https://doi.org/10.1002/1348-9585.12103
3. Vos T, Lim S S, Abbafati C, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet 2020;396:1204-1222. https://doi.org/10.1016/S0140-6736(20)30925-9
4. March L, Smith E U R, Hoy D G, et al. Burden of disability due to musculoskeletal (MSK) disorders. Best practice & research Clinical rheumatology 2014;28: 353-366. https://doi.org/10.1016/j.berh.2014.08.002
5. Vincent T L. Targeting mechanotransduction pathways in osteoarthritis: a focus on the pericellular matrix. Current opinion in pharmacology 2013;13:449-454. https://doi.org/10.1016/j.coph.2013.01.010
6. Lee W, Ku S K, Bae J S. Anti-inflammatory effects of Baicalin, Baicalein, and Wogonin in vitro and in vivo. Inflammation, 2015;38:110-125. https://doi.org/10.1007/s10753-014-0013-0
7. Duan X, Guo G, Pei X, et al. Baicalin inhibits cell viability, migration and invasion in breast cancer by regulating miR-338-3p and MORC4. OncoTargets Therap 2019;12:11183. https://doi.org/10.2147/OTT.S217101
8. Wang X, Yu J, Sun Y, et al. Baicalin protects LPS-induced blood-brain barrier damage and activates Nrf2-mediated antioxidant stress pathway. Int Immunopharmacol 2021;96:107725. https://doi.org/10.1016/j.intimp.2021.107725
9. Hu S, Jiang L, Yan Q, et al. Evidence construction of baicalin for treating myocardial ischemia diseases: A preclinical meta-analysis. Phytomedicine 2022;107:154476. https://doi.org/10.1016/j.phymed.2022.154476
10. Wang X, Xie L, Long J, et al. Therapeutic effect of baicalin on inflammatory bowel disease: A review. J Ethnopharmacol 2022;283:114749. https://doi.org/10.1016/j.jep.2021.114749
11. Li B, Chen K, Qian N, et al. Baicalein alleviates osteoarthritis by protecting subchondral bone, inhibiting angiogenesis and synovial proliferation. J Cell Mol Med 2021;25:5283-5294. https://doi.org/10.1111/jcmm.16538
12. Dinda B, Dinda S, DasSharma S, et al. Therapeutic potentials of baicalin and its aglycone, baicalein against inflammatory disorders. Eur J Med Chem 2017;131:68-80. https://doi.org/10.1016/j.ejmech.2017.03.004
13. Ding W, Du D, Chen S. LIPUS promotes synthesis and secretion of extracellular matrix and reduces cell apoptosis in human osteoarthritis through upregulation of SOX9 expression. Int J Clin Exp Pathol 2020;13:810.
14. Chen C, Zhang C, Cai L, et al. Baicalin suppresses IL-1β-induced expression of inflammatory cytokines via blocking NF-κB in human osteoarthritis chondrocytes and shows protective effect in mice osteoarthritis models. Int Immunopharmacol 2017;52:218-226. https://doi.org/10.1016/j.intimp.2017.09.017
15. You Y, Que K, Zhou Y, et al. MicroRNA-766-3p inhibits tumour progression by targeting Wnt3a in hepatocellular carcinoma. Mol Cells 2018;41:830.
16. Oh K, Lee D S. In vivo validation of metastasis-regulating microRNA-766 in human triple-negative breast cancer cells. Laboratory Animal Research 2017;33:256-263. https://doi.org/10.5625/lar.2017.33.3.256
17. Li Z, Cheng J, Liu J. Baicalin protects human OA chondrocytes against IL-1β-induced apoptosis and ECM degradation by activating autophagy via MiR-766-3p/AIFM1 axis. Drug Design, Development and Therapy 2020; 2645-2655. https://doi.org/10.2147/DDDT.S255823
18. L. Cui, L. Feng, Z.H. Zhang, X.B. Jia, The anti-inflammation effect of baicalin on experimental colitis through inhibiting TLR4/NF-kappaB pathway activation, Int Immunopharmacol 2014;23:294-303. https://doi.org/10.1016/j.intimp.2014.09.005
19. Khan NM, Ahmad I, Haqqi TM. Nrf2/ARE pathway attenuates oxidative and apoptotic response in human osteoarthritis chondrocytes by activating ERK1/2/ELK1-P70S6K-P90RSK signaling axis. Free Radic Biol Med 2018;116:159-171. https://doi.org/10.1016/j.freeradbiomed.2018.01.013
20. Li Y, Wu Y, Jiang K, et al. Mangiferin prevents TBHP-induced apoptosis and ECM degradation in mouse osteoarthritic chondrocytes via restoring autophagy and ameliorates murine osteoarthritis. Oxid Med Cell Longev 2019;2019:8783197. https://doi.org/10.1155/2019/8783197

21. Yang X, Zhang Q, Gao Z, Yu C, Zhang L. Baicalin alleviates IL-1β-induced inflammatory injury via down-regulating miR-126 in chondrocytes. Biomed Pharmacother 2018;99:184-190. https://doi.org/10.1016/j.biopha.2018.01.041
22. Chen D-S, Cao J-G, Zhu B, Wang Z-L, Wang T-F, Tang J-J. Baicalin attenuates joint pain and muscle dysfunction by inhibiting muscular oxidative stress in an experimental osteoarthritis rat model. Arch Immunol Ther Exp 2018;66:453-461. https://doi.org/10.1007/s00005-018-0518-6
23. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol. 2011;7:33-42. https://doi.org/10.1038/nrrheum.2010.196
24. Daheshia M, Yao JQ. The interleukin 1β pathway in the pathogenesis of osteoarthritis. J Rheumatol 2008;35:2306-2312. https://doi.org/10.3899/jrheum.080346
25. Hussein MR, Fathi NA, El-Din AME, et al. Alterations of the CD4+, CD8+ T cell subsets, Interleukins-1β, IL-10, IL-17, tumor necrosis Factor-α and soluble intercellular adhesion molecule-1 in rheumatoid arthritis and osteoarthritis: preliminary observations. Pathol Oncol Res 2008;14:321-328. https://doi.org/10.1007/s12253-008-9016-1
26. Mizushima N. Physiological functions of autophagy. Curr Top Microbiol Immunol 2009;335:71-84. https://doi.org/10.1007/978-3-642-00302-8_3
27. Caramés B, Taniguchi N, Otsuki S, Blanco FJ, Lotz M. Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. Arthritis Rheum 2010;62:791-801. https://doi.org/10.1002/art.27305
28. Saberi Hosnijeh F, Bierma-Zeinstra SM, Bay-Jensen AC. Osteoarthritis year in review 2018: biomarkers (biochemical markers). Osteoarthr Cartil. 2019;27:412-423. https://doi.org/10.1016/j.joca.2018.12.002
29. Wang X, Li M, Hu Y. miR-29c-3p Attenuates beta-Amyloid-Induced Neurotoxicity in Alzheimer's Disease Through Regulating beta-Site Amyloid Precursor Protein-Cleaving Enzyme 1. Physiol Res 2023;72:833-841. https://doi.org/10.33549/physiolres.935084
30. Zhu B, He J, Ye X, Pei X, Bai Y, Gao F, Guo L, Yong H, Zhao W. Role of Cisplatin in Inducing Acute Kidney Injury and Pyroptosis in Mice via the Exosome miR-122/ELAVL1 Regulatory Axis. Physiol Res 2023;31;72:753-765. https://doi.org/10.33549/physiolres.935129
31. Le LT, Swingler TE, Clark IM. Review: the role of microRNAs in osteoarthritis and chondrogenesis. Arthritis Rheum. 2013;65:1963-1974. https://doi.org/10.1002/art.37990
32. Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. PLoS One 2008;3:e3740. https://doi.org/10.1371/journal.pone.0003740
33. Sharma A, Diecke S, Zhang WY, et al. The role of SIRT6 protein in aging and reprogramming of human induced pluripotent stem cells. J Biol Chem 2013;288:18439-18447. https://doi.org/10.1074/jbc.M112.405928