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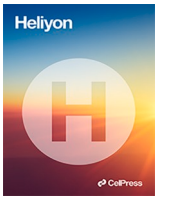

Research article

Luteolin regulating synthesis and catabolism of osteoarthritis chondrocytes via activating autophagy

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A R T I C L E I N F O

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A B S T R A C T

Osteoarthritis (OA) is a prevalent bone and joint disease characterized by degeneration. The dysregulation between chondrocyte synthesis and breakdown is a key factor in OA development. Targeting the degenerative changes in cartilage tissue degradation could be a potential treatment approach for OA. Previous research has established a strong link between autophagy and the regulation of chondrocyte functions. Activating autophagy has shown promise in mitigating cartilage tissue degeneration. Currently, osteoarthritis treatment primarily focuses on symptom management, as there is no definitive medication to stop disease progression. Previous studies have demonstrated that luteolin, a flavonoid present in Chinese herbal medicine, can activate autophagy and reduce the expression of MMP1 and ADAMTS-5. This study utilized an in vitro osteoarthritis model with chondrocytes stimulated by IL-1β, treated with varying concentrations of luteolin. Treatment with luteolin notably increased the levels of synthesis factors Aggrecan and Collagen II, while decreasing the levels of decomposition factors MMP-1 and ADAMTS-5. Moreover, inhibition of autophagy by Chloroquine reversed the imbalances in chondrocyte activities induced by IL-1β. In an in vivo model of knee osteoarthritis induced by medial meniscal instability (DMM), luteolin was administered as a therapeutic regimen. After 12 weeks, knee cartilage tissues from mice were analyzed. Immunofluorescence and immunohistochemical staining revealed a decrease in P62 expression and an increase in Beclin-1 in the cartilage tissues. Additionally, cartilage wear in the knee joints of mice was alleviated by safranin O and fast green staining. Our study findings underscore the significant role of luteolin in effectively rebalancing chondrocyte activities disrupted by IL-1β. Our results strongly indicate that luteolin has the potential to be developed as a novel therapeutic agent for the treatment of osteoarthritis, offering promising prospects for future drug development.

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1. Introduction

Osteoarthritis (OA) is a chronic joint disease that manifests through symptoms such as joint swelling, pain, and limited movement [1]. It is primarily caused by the narrowing of the joint space, the development of bone spurs, and the hardening of weight-bearing cartilage surfaces. These factors contribute to a gradual deterioration of joint function over time. The prevalence of OA has notably risen as a result of the aging populace, significantly influencing the well-being and productivity of individuals [2]. Clinical treatments for OA mainly focus on symptom control and improving function, without providing a permanent cure [3]. Given the large population affected by OA in China, it imposes a substantial economic burden on society and families. Therefore, it is crucial to research on the treatment for OA articular cartilage degeneration has been the current hot spots.

The pathological features of OA was widely believed that caused by mechanical wear and aseptic inflammation. Numerous studies have demonstrated that the breakdown of extracellular matrix (ECM) components is the underlying cause of cartilage degeneration in OA [4,5]. The ECM primarily consists of Collagen II and Aggrecan, which benefit articular cartilage adapt to mechanical stress during joint movement [6,7]. Aseptic inflammation of the joint leads to a significant increase in the concentration of interleukin-1β (IL-1β), which is considered a key pro-inflammatory factor [8]. In vitro experiments applying IL-1β to stimulate chondrocytes have shown an increase in Matrix Metalloproteinases (MMPs) and A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS) [9, 10]. Chondrocytes, as the sole cell type in cartilage tissue, play an significant role in synthesizing Collagen II and Aggrecan, as well as degrading MMPs and ADAMTS matrix proteolytic enzymes [11]. Consequently, the study focuses on understanding the specific pathway that regulates the synthesis and catabolism of chondrocytes, which could potentially uncover effective avenues for exploring the treatment of cartilage degeneration in OA.

Autophagy is a dynamic intracellular process that serves as a self-protection mechanism for eukaryotic cells [12]. Research has shown that damaged and denatured organelles and macromolecular substances in the cytoplasm are wrapped by the cell membrane structure to form autophagosomes. These autophagosomes then fuse with the lysosome membrane to form autophagolysosomes, which are ultimately degraded by the lysosomes. This degradation process benefits maintain the renewal of organelles and the metabolism of the cell itself. Previous studies have indicated a close relationship between decreased autophagy levels and the progression of OA [13, 14]. Activation of autophagy has been found to promote the upregulation of Collagen II and Aggrecan in chondrocytes, inhibit the expression of ADAMTS-5 and MMP-1 proteolytic enzymes, and maintain chondrocyte homeostasis. Huang et al. demonstrated that SGK1 phosphorylates FoxO1, leading to a significant reduction in autophagy levels in chondrocytes and a tendency towards spontaneous OA [15]. Similarly, Wang et al. found that injecting shikonin into the joint cavity of mice with knee OA improved the autophagy level in cartilage tissue, effectively inhibiting cartilage degeneration [16]. These findings collectively highlight the crucial role of autophagy in maintaining the synthesis and catabolic balance of cartilage tissue [17]. Therefore, further investigation into the regulation of chondrocyte autophagy and its underlying mechanisms holds promise for better understanding cartilage tissue degeneration and developing novel approaches for OA treatment.

The current treatment for OA is limited to symptomatic treatment and does not have the ability to completely block or reverse the progression of the disease. Therefore, it is vital to further investigate potential drugs for treating OA and understand the specific molecular mechanism involved. Luteolin, a weakly acidic tetrahydroxy flavonoid, is a representative component of natural flavonoids that is widely found in plants. Since its first isolation in 1955, luteolin has been extensively studied for its biological activities and pharmacological effects, including antioxidant, anti-inflammatory, and protection against ischemic vascular damage. Wu et al. discovered that luteolin can inhibit SGK1, promoting the entry of FOXO3a into the nucleus and increasing autophagy levels [18]. Luteolin not only demonstrates favorable activity on affected cells but also exhibits minimal toxicity and high selectivity towards cells. Previous studies have indicated that luteolin acts as a positive regulator of autophagy. However, the expression of luteolin undergoes changes during the degenerative process of articular cartilage in OA, and its regulatory effects on the autophagy level, synthesis, and catabolism of chondrocytes remain unclear. Therefore, we propose to treat chondrocytes and mice models with luteolin in order to observe its effects. This study aims to provide a theoretical basis for the future application of luteolin in OA treatment and explore its possible mechanism of action.

2. Materials and methods

2.1. Toluidine blue and safranin O staining

Chondrocytes were evenly distributed on slides in 12-well cell plates. When the cell confluence reached 90 %, subsequent cell experiments were performed. Paraformaldehyde solution (4 %) was applied to the samples and allowed to fix at room temperature for a period of 15 min. Subsequently, the wells were washed three times using sterile PBS to remove any residual fixative. Following the washing steps, the samples were stained with a 1 % solution of toluidine blue for a duration of 2 h at room temperature. After removing the excess staining solution from the culture plate, sterile PBS was added again and soaked for 3 min. The slide was carefully lifted using small tweezers, placed on a slide, sealed, and observed under a microscope for image capture.

2.2. Cell viability detection

The cell suspension was diluted according to the seed plate concentration, 100 μl (10 % fetal bovine serum cell medium, 20 mL fetal bovine serum (obtained from CLARK, United States) and 2 mL cyan-streptomycin (purchased from Beyotime, China) were combined to create a 10 % serum culture solution. This solution was then added to each well, and the cells were placed in a 5 % CO2 cell incubator at

37 °C for a period of 12 h. Once the cells had properly adhered to the walls, media containing varying concentrations of drugs were introduced to the control group, while the blank group received culture medium devoid of any drugs. Following a 24h incubation period in the 5 % CO2 cell incubator at 37 °C, 10 μl of CCK-8 solution was added to each well and further incubated in the 5 % CO2 incubator at 37 °C. Subsequently, the 96-well plate was removed, and the optical density (OD) value of each well at a wavelength of 450 nm was measured using an enzyme-labeled instrument.

2.3. RNA extraction and reverse transcription of chondrocytes

RNA was harvested from chondrocytes by utilizing the Trizol kit (Thermo Fisher Scientific, USA), as per the manufacturer's instructions. The concentration and quality of the RNA were evaluated using a spectrophotometer. Subsequently, the RNA was reverse-transcribed with the RT-PCR kit (Vazyme Biotech, China). The resultant cDNA was subjected to quantitative real-time PCR employing the SYBR qPCR SuperMix Plus kit (Vazyme Biotech, China). The anticipated outcomes were achieved through iterative denaturation, annealing, and extension cycles. Upon standardization, the information was assessed utilizing the $2^{-\Delta\Delta Ct}$ approach. All relative gene primers are listed as follows: MMP-1 (human):forward:5′-AAATAGTGGCCCAGTGGTTG-3′,reverse:5′-CACATCAGGCACTCCACATC-3. CollagenII(human):forward:5′-GGAGCAGCAAGAGCAAGGAGAAG-3′, reverse: 5′-TGGACAGCAGGCGTAGGAAGG-3'; Aggrecan (human): forward:5′-ACCCCTGAGGAACAGG-3′,reverse:5′-GTGCCAGATCATCACCACAC-3'. ADAMTS-5 (human): forward: 5′-CTTGACGTTCGGGCCTGA-3′, reverse: 5′-CACTGTTTCTGGGTGCAG3′,LC3(human):forward,5′-CATGAGCGAGTTGGTCAAGAT-3′, reverse,5′-TCGTCTTTCTCCTGCTCGTAG-30′; p62(human): forward, 5′-TGTGGAACATGGAGGGAAGAG-3′,reverse,5′-TGTGCCTGTGCTGGAACTTTC-3′.Beclin-1(human):forward,5′-AAGACAGAGCGATGGTAG-3′,reverse,5′-CTGGGCTGYGGYAAGTAA-3′.

2.4. Protein extraction and western-blotting

1 mL 0.25 % pancreatic enzyme (Beyotime, Shanghai, China) was added to each well of the cell culture plate, the cell suspension was transferred into 15 mL sterile EP tube, and centrifuged at 1200 rpm for 5 min. After blowing and mixing well, 1.5 mL of cell lysate was added to each tube (RIPA lysate: PMSF: phosphatase inhibitor: protease inhibitor = 1000 μL: 10 μL: 10 μL: 1 μL), and the lysate was cracked on ice for 30 min. The protein concentration was determined by BCA method and then add 5xSDS-PAGE protein loading buffer (Beyotime, Shanghai, China) into the protein supernatant, mix thoroughly, and boil for 15 min.

The sample volume was calculated according to the protein concentration and added into the upper sample hole. Markers were added at both ends of the sample for marking. Protein tailoring of PVDF membrane was carried out according to Marker. The PVDF membrane was placed in a sealing solution (5 % TBST milk) and slowly shaken at room temperature for 2 h. The PVDF membrane was incubated overnight with primary antibody at 4 °C. Western blot analysis used the following antibodies: Collagen II (1:200; Cell Signaling Technology, USA, not for immunofluorescent staining), Aggrecan (1:500; Cell Signaling Technology, USA), ADAMTS-5 (1:500; WANLEIBIO, China), MMP-13 (1:1000; WANLEIBIO, China), Beclin-1 (1:800; WANLEIBIO, China), LC3 (1:500; Cell Signaling Technology, USA), P62 (1:1000; Cell Signaling Technology, USA), GAPDH (1:1500; WANLEIBIO, China). PVDF membrane was incubated with secondary antibody at room temperature for 2 h. The target protein was prepared according to the instructions of ECL luminescent solution, and suitable imaging conditions were selected according to different molecular weights, so that the target protein could be stored after imaging.

2.5. Immunofluorescence staining

Immunofluorescence is the reaction of fluorescently labeled specific antibodies with antigens. The fluorescent pigment is labeled on the antibody (or antigen) but does not affect the antigen-antibody activity. After binding with its corresponding antigen (or antibody), the localization is observed under the immunofluorescence microscope. The sterile slide was put into the cell culture plate to complete the cell climbing, and the slide was immersed with paraformaldehyde (4 %), and the time was fixed at room temperature for 15 min. Add the prepared TritonX-100 (diluted to 0.5 % with PBS) into each hole and let it stand at room temperature for 20 min. Add goat serum (5 %) to cover the slide and close for 30 min. A sufficient amount of primary antibody was added to the cell pad and placed at 4 °C overnight. Second antibody was added in dark drops and placed for 1 h, DAPI was added to stain cell nuclei and incubated for 5 min, and the sealing solution (including anti-fluorescence quench agent) was added to the slide, observed and photographed under a fluorescence microscope.

2.6. Immunchemistry staining

Immunchemistry staining is the specific reaction of antigen and antibody to carry out in situ color reaction in tissue cells. The knee tissue of mice was taken and fixed in formalin solution (4 %) for 24 h. After decalcification (EDTA decalcification solution,PH7.3), wax blocks were embedded. The tissue sections (thickness 5 μm) were placed in 50 °C water to adhere to the slides, and the slices were baked for 25 min (temperature set at 90 °C). Cool naturally, add the prepared antigen repair solution (2.5min), and slowly cool again to room temperature. 100 μL of endogenous peroxidase blocker was added and allowed to incubate for 10 min at room temperature. Next, the primary antibody was added and allowed to incubate for 30 min at room temperature or overnight at 4 °C. Next, 100 μL of the enzyme-labeled antibody complex was added and incubated at room temperature for 20 min. The slide was then placed in a chamber with freshly prepared DAB dyeing solution for 5 min until color development, followed by 5 min of hematoxylin redyeing. The slide was further treated in double steaming water for 15 min return it to blue. Finally, after sealing the disc, the images were observed

under an optical microscope.

2.7. Mice OA model and experiment design

C57BL/6 (7 weeks old) male mice were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. in Shandong, China. These mice were housed at the Animal Center of Anhui Medical University under constant temperature conditions of 21 °C. The animal research protocols were approved by the Animal Protection and Use Committee of Anhui Medical University with the ethics number LLSC20190547. The mice, weighing between 22 and 26 g, were anesthetized using intraperitoneal injections of 10 % chloral hydrate. Surgical procedures involved exposing the medial condyle of the femoral knee and the medial tibial plateau by making a median anterior knee incision. The medial collateral ligament was severed, and the connection between the medial meniscus and tibial plateau was cut to induce instability. Following the operation, luteolin at a dose of 20 mg/kg or normal saline was injected into the mice's knee joint every other week using microsyringes starting from 2 weeks post-surgery.

2.8. Safranin O-fast green staining

The paraffin sections were deparaffinized, followed by xylene I for 20 min, xylene II for 20 min, anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, and then immersed in 75 % alcohol for another 5 min. The sections were then immersed in a solid green dye solution for 5–10 min until the cartilage appeared colorless and the differentiation solution was slightly absorbed. Subsequently, the sections were immersed in saffranine dye for 15–30 s and dehydrated quickly using anhydrous ethanol. They were then cleaned with xylene for 5 min, followed by sealing with neutral gum. The samples were then analyzed using microscopy for image acquisition and analysis.

2.9. Statistical analysis

Statistical analysis was conducted using SPSS 18.0 (Chicago, Illinois, USA) and image processing was performed using Prism 9.0 (San Diego, California, USA). To evaluate significant differences between the groups, the two-sample independent T-test was employed. In addition, a diverse multivariate analysis of variance (ANOVA) was utilized to compare multiple groups. Mean values with corresponding standard deviations are reported for all data.

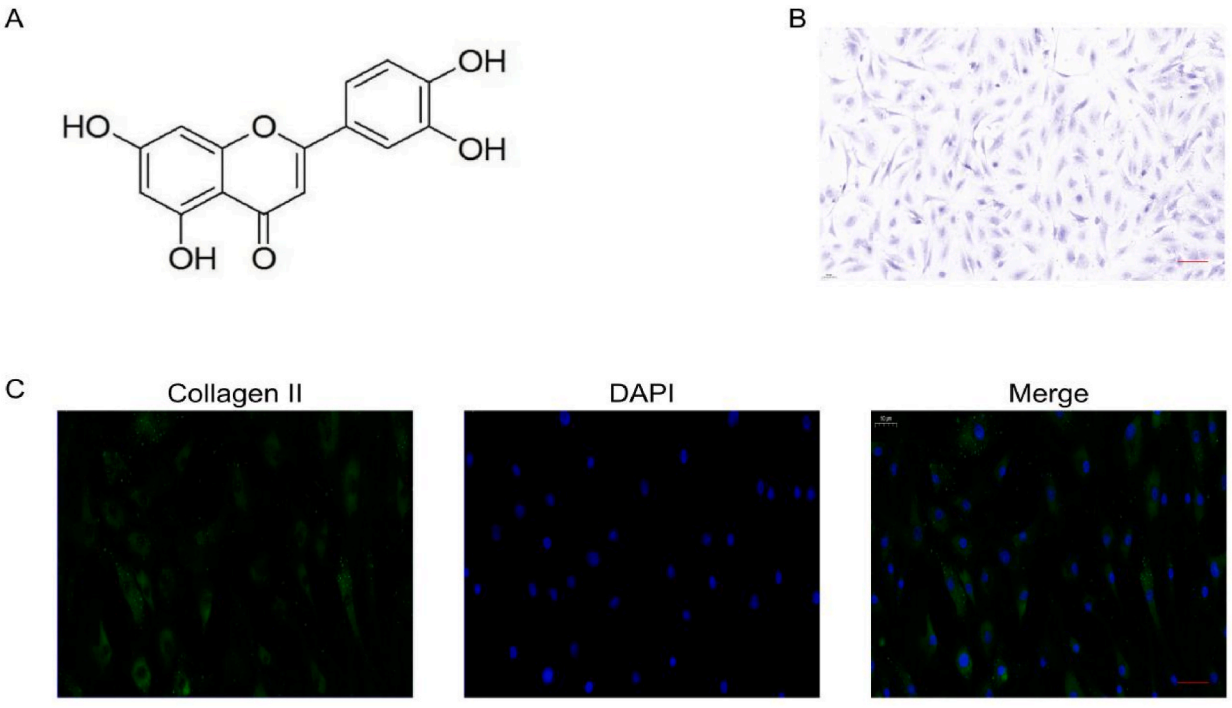

Fig. 1. (A) The composition of luteolin. (B) Staining with toluidine blue reveals the presence of purple-colored proteoglycan in chondrocytes (200X, scale bar 50 μm). (C) Immunofluorescence analysis of chondrocytes was conducted to detect type II collagen. This was done in combination with DAPI fluorescent staining (200X, scale bar 50 μm). The nuclei of chondrocytes were stained blue with DAPI, while type II collagen staining appeared green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Structure of luteolin and chondrocytes identification

Luteolin, which is an inherent compound of flavonoid nature (Fig. 1A), was utilized in this study. Immunofluorescence staining and toluidine blue staining techniques were employed to detect and characterize chondrocytes. Immunofluorescence staining specifically targeted collagen II in the cytoplasm, resulting in green staining, while no positive staining was observed in the nucleus. Moreover, toluidine blue staining revealed the spindle-shaped morphology of chondrocytes, with purple staining indicating the presence of proteoglycans in the cytoplasm. Both staining methods conclusively confirmed the cellular identity as chondrocytes (Fig. 1B and C).

3.2. The effects of luteolin on the activity of chondrocytes and Glycosaminoglycan (GAGs) levels in IL-1β treated chondrocytes

Chondrocyte viability was evaluated by the CCK-8 test kits. Results demonstrated that different concentrations of luteolin (5, 10, 20 μmol/L) did not exhibit any harmful impact on chondrocytes. Nevertheless, with an escalation in luteolin concentration to 40 and 80 μmol/L, there was a marked decline in cell viability. Consequently, luteolin doses (5, 10, 20 μmol/L) were chosen for subsequent trials. Additionally, various concentrations of luteolin (5, 10, 20 μmol/L) were co-incubated with the chondrocytes in the presence or absence of IL-1β. The findings illustrated that there were no significant alterations in cell viability (Fig. 2A). Furthermore, safranin O staining was utilized to assess the GAGs content in the chondrocytes. The IL-1β group displayed decreased levels of GAGs, indicating extracellular matrix content, in contrast to the IL-1β+luteolin group. This indicates that luteolin might aid in preserving the anabolic and catabolic equilibrium in chondrocytes (Fig. 2B).

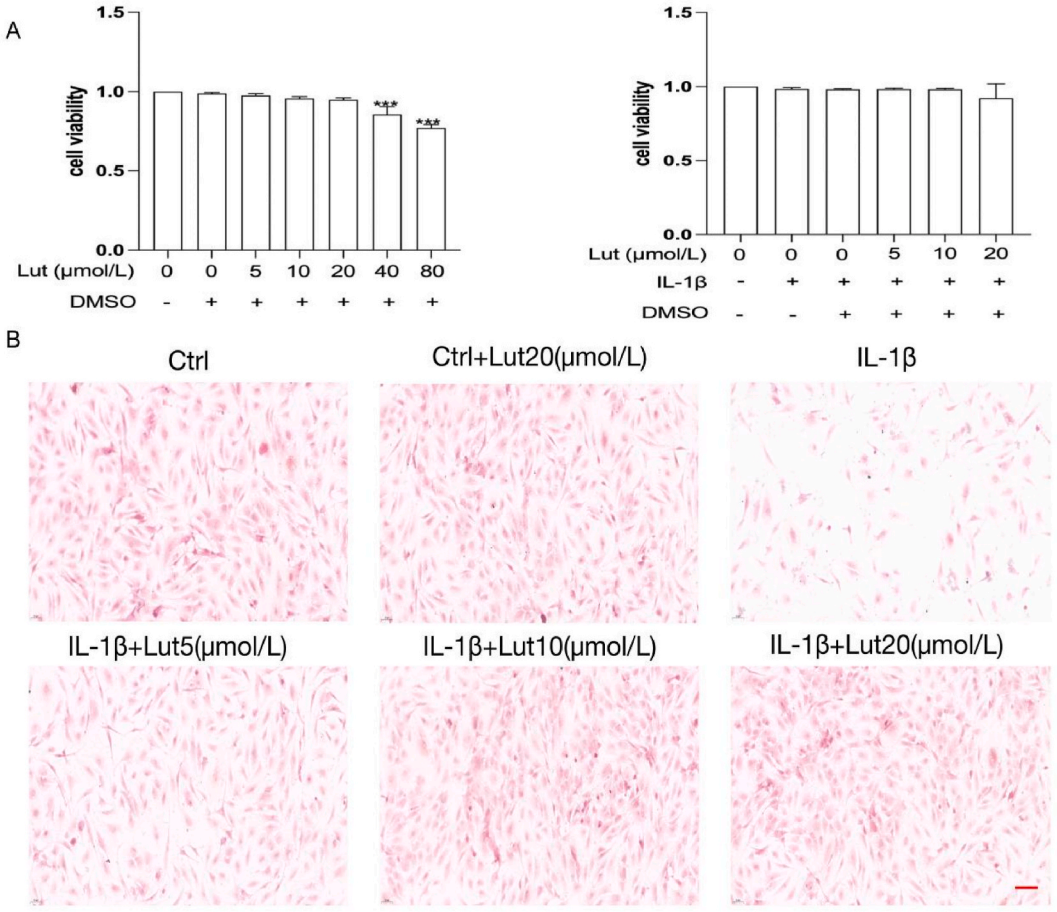

Fig. 2. Preservation of chondrocytes viability was observed upon administration of lut. (A) Chondrocytes were subjected to varying concentrations of lut (0, 5, 10, 20, 40, 80 μmol/L) for a duration of 24 h. Our findings indicated no considerable cytotoxicity after 24 h of lut treatment at 5, 10, and 20 μmol/L, irrespective of the presence of IL-1β. (B) Following pre-treatment with lut, chondrocytes were exposed to 10 ng/mL IL-1β for an additional 24 h. The utilization of Safranin O staining facilitated cell visualization (200x, Scale bar, 50 μm). The obtained results were expressed as mean ± SD. Statistical analysis revealed a significant disparity compared to the control. ***P < 0.001vs. Control group.

3.3. Luteolin regulates the expression of MMP-1, ADAMTS-5, collagen II, aggrecan, P62, Beclin-1 and LC3 in chondrocytes induced by IL-1β

An analysis using qRT-PCR was carried out to explore the effects of luteolin on the imbalance between anabolism and catabolism in chondrocytes triggered by IL-1β. Specifically, the concentrations of MMP-1, ADAMTS-5, Collagen II, and Aggrecan were investigated in an in vitro setting. The findings demonstrated that following stimulation with IL-1β, the mRNA levels of the inflammatory markers MMP-1 and ADAMTS-5 in chondrocytes markedly increased, whereas the levels of Collagen II and Aggrecan decreased ($P < 0.05$) compared to the control group. However, after treatment with luteolin, these indexes exhibited a reversal trend. The expressions of Collagen II and Aggrecan increased, while MMP-1 and ADAMTS-5 significantly decreased ($P < 0.05$). This demonstrates that luteolin treatment effectively inhibits IL-1β-induced inflammatory effects. Additionally, considering previous literature reports emphasizing autophagy as a critical protective mechanism for maintaining chondrocyte metabolic balance, we also assessed the expression levels of autophagy markers (Beclin-1, LC3, and P62) using qRT-PCR. The results demonstrated a significant increase in the expression of Beclin-1 and LC3 in chondrocytes treated with luteolin and IL-1β, while the expression of P62 was significantly decreased (Fig. 3A–G).

3.4. Western blot analysis was performed to confirm the efficacy of luteolin as a potential protective treatment for osteoarthritis

Western blot analysis was performed to assess the expression levels of MMP-1, ADAMTS-5, Collagen II, Aggrecan, P62, Beclin-1, and LC3II/I. As expected, the results demonstrated that luteolin significantly decreased the expression of MMP-1 and ADAMTS-5, while increasing the expression of Collagen II and Aggrecan (Fig. 4 A, B). Furthermore, the results revealed that treatment with luteolin + IL-1β significantly enhanced the expression of Beclin-1, the ratio of LC3 II/LC3 I, and reduced the expression of P62 in chondrocytes compared to the IL-1β treatment group. These findings indicate a notable activation of the autophagy signaling pathway. Based on these findings, we reconfirmed that luteolin has the potential to serve as a protective agent for chondrocytes in the treatment of osteoarthritis by regulating autophagy (Fig. 4 C).

3.5. Luteolin promotes synthetic catabolic balance via regulating autophagy

Combined with the previous findings, autophagy plays a crucial role in maintaining the balance between chondrocyte synthesis and

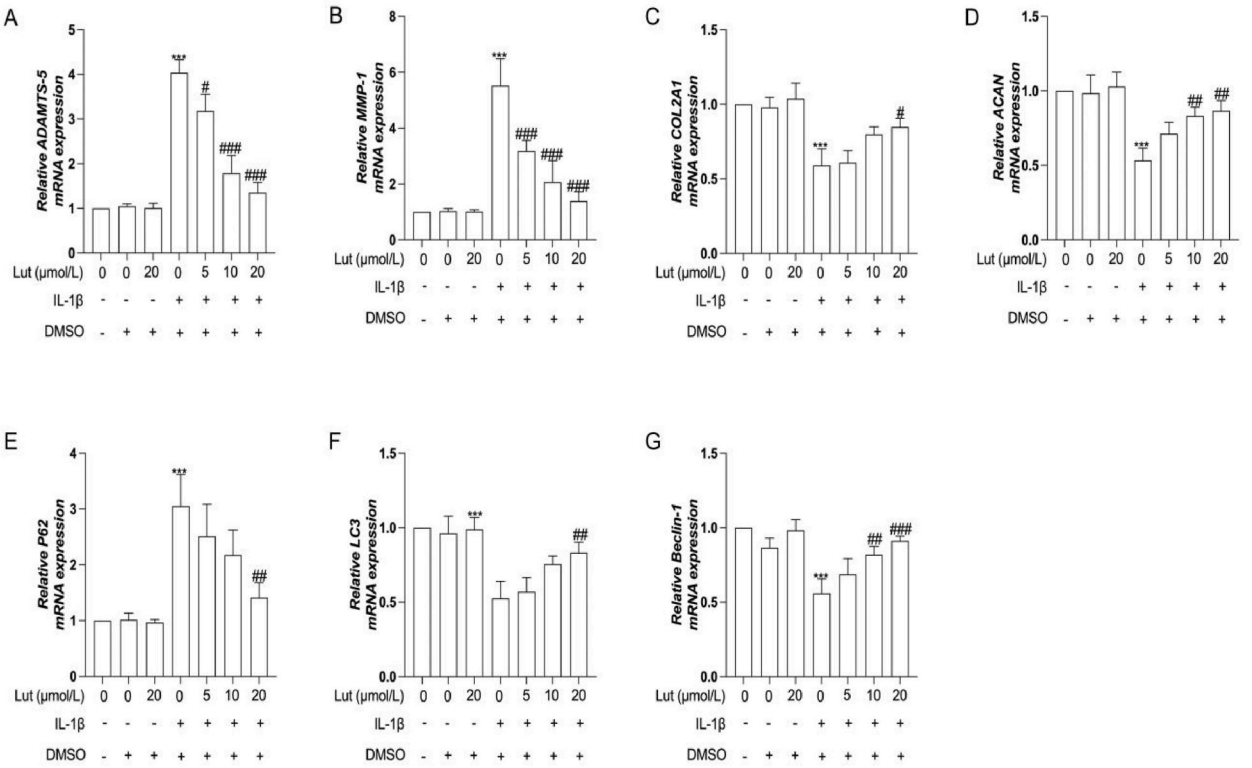

Fig. 3. Figure A–G. Chondrocytes were treated with lut and IL-1β for 24 h. The expression levels of MMP-1, ADAMTS-5, Collagen II, Aggrecan, P62, Beclin-1, and LC3 were detected using qRT-PCR. Statistical analysis showed that the expression levels were significantly different compared to the control group. Additionally, the expression levels were also compared to the IL-1β group, where significant differences were observed. ***$P < 0.001$ vs. Control group; ($P < 0.05$, $P < 0.01$, $P < 0.001$ for #, ##, and ###, respectively).

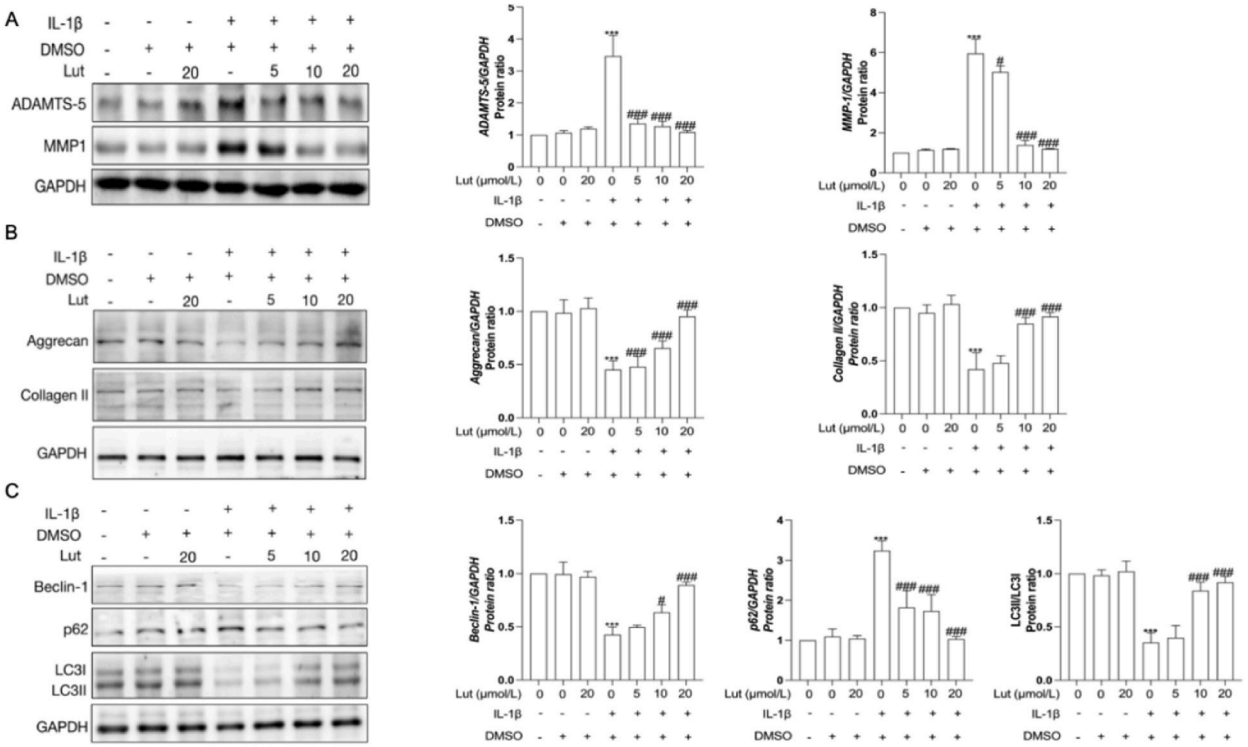

Fig. 4. A–C: Western blot assay was conducted to detect the expression of related phenotypes and inflammatory factors in chondrocytes. The results showed that treatment with IL-1β led to a decrease in the expressions of Collagen II and Aggrecan, while the expressions of inflammatory factors ADAMTS-5 and MMP-1 were increased. However, when chondrocytes were induced by IL-1β and treated with different concentrations of luteolin, the expression levels of these related proteins were reversed. In addition, the expression levels of autophagy markers Beclin-1, LC3, and P62 were measured in chondrocytes treated with luteolin for 24 h. The data were presented as mean ± standard deviation. ***P < 0.001 vs. control; #P < 0.05, ###P < 0.001 vs. IL-1β group.

catabolism. To investigate this further, we conducted recovery experiments using an autophagy inhibitor called chloroquine (CQ). Western blot analysis was performed to measure the protein levels of chondrocytes synthesis and catabolism indicators. The results revealed that, compared to the IL-1β+ luteolin group, the IL-1β+ luteolin + CQ group showed up-regulation of decomposition factors MMP-1 and ADAMTS-5, as well as down-regulation of synthesis factors Collagen II and Aggrecan (Fig. 5 A, B). Additionally, Safranin O staining was used to assess the level of chondrocyte GAGs, providing further evidence that IL-1β-induced metabolic imbalance in chondrocytes is mediated through autophagy inhibition (Fig. 5 C).

3.6. Immunofluorescence showed that luteolin activated the autophagy signaling pathway in chondrocytes

Autophagy is crucial for maintaining the balance between synthesis and breakdown in chondrocytes. To investigate this, we analyzed the expression of autophagy pathway marker proteins, specifically p62 and Beclin-1. In normal chondrocytes, there was a low proportion of p62-positive cells, while a high proportion of cells were positive for Beclin-1. Immunofluorescence assay results revealed that the IL-1β + luteolin group exhibited a significant decrease in the number of p62-positive chondrocytes compared to the IL-1β group. Conversely, the number of beclin-1 positive chondrocytes increased, indicating activation of the autophagy pathway (Fig. 6A and B).

3.7. Luteolin effectively alleviated OA induced by DMM in mice

The staining technique using safranin O and fast green was utilized to identify cartilage destruction in mice, with the OARSI score serving as a measure of injury severity. Our findings indicated that cartilage degradation in the DMM + luteolin group was slightly less pronounced than in the DMM group. Additionally, the OARSI score was higher in the DMM + luteolin group compared to the DMM group. This suggests that luteolin may have a protective effect against cartilage damage in this model of osteoarthritis. These findings indicate that the activation of autophagy by Luteolin may potentially alleviate DMM-induced osteoarthritis in the mice model (Fig. 7).

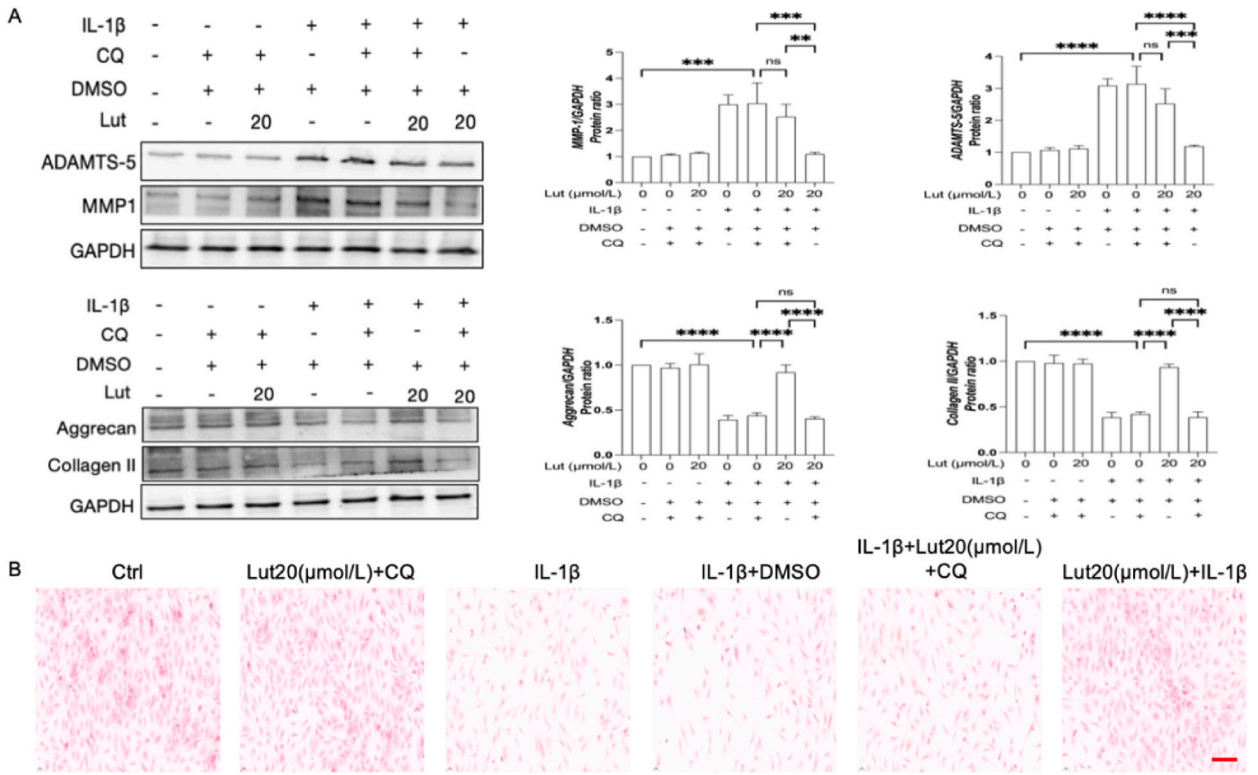

Fig. 5. Figure A–B illustrates the changes in the synthesis of catabolism-related proteins in the chondrocytes model of osteoarthritis after luteolin treatment with CQ in IL-1β-induced chondrocytes. (A) The protein expression levels of Collagen II, Aggrecan, ADAMTS-5, and MMP-13 were detected using Western blot. (B) Safranin O staining demonstrated that GAGs deposition was reduced once again in the IL-1β+ luteolin + CQ group (200x magnification, scale bar; 50 μm). The data is presented as mean ± standard deviation, with statistical significance denoted as **P < 0.01, ***P < 0.001, and ****P < 0.0001.

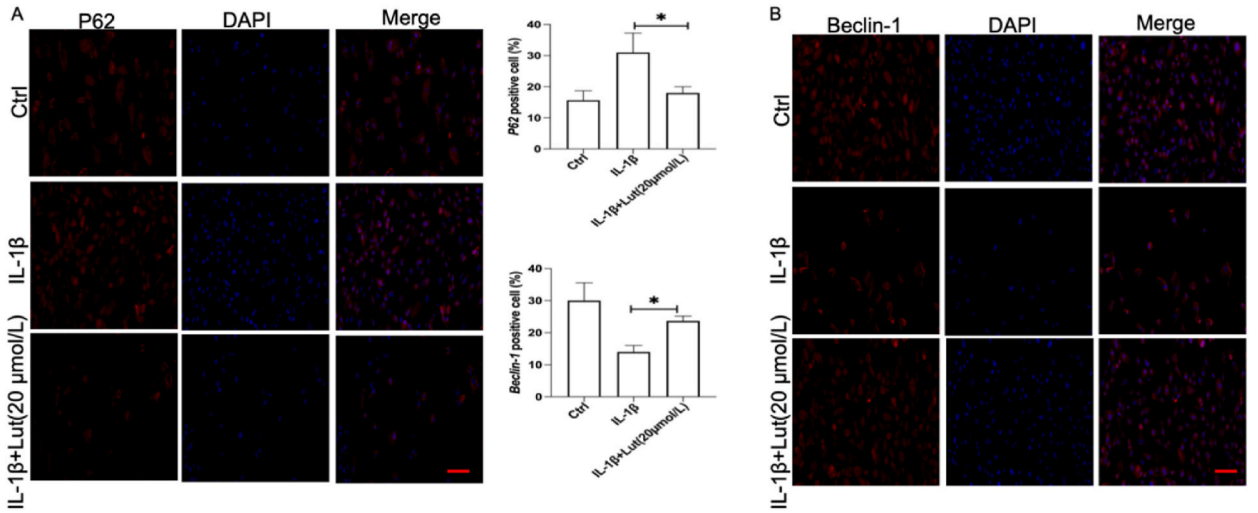

Fig. 6. (A–B) Immunofluorescence staining was performed to assess the expression intensity of autophagy markers in chondrocytes treated with IL-1β and IL-1β+ luteolin. Positive cells were indicated by red fluorescence, while the nucleus was stained blue using DAPI (200x, scale bar; 50 μm). The data is presented as mean ± standard deviation, with statistical significance denoted as *P < 0.05 IL-1β group vs. IL-1β + luteolin (20 μmol/L) group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

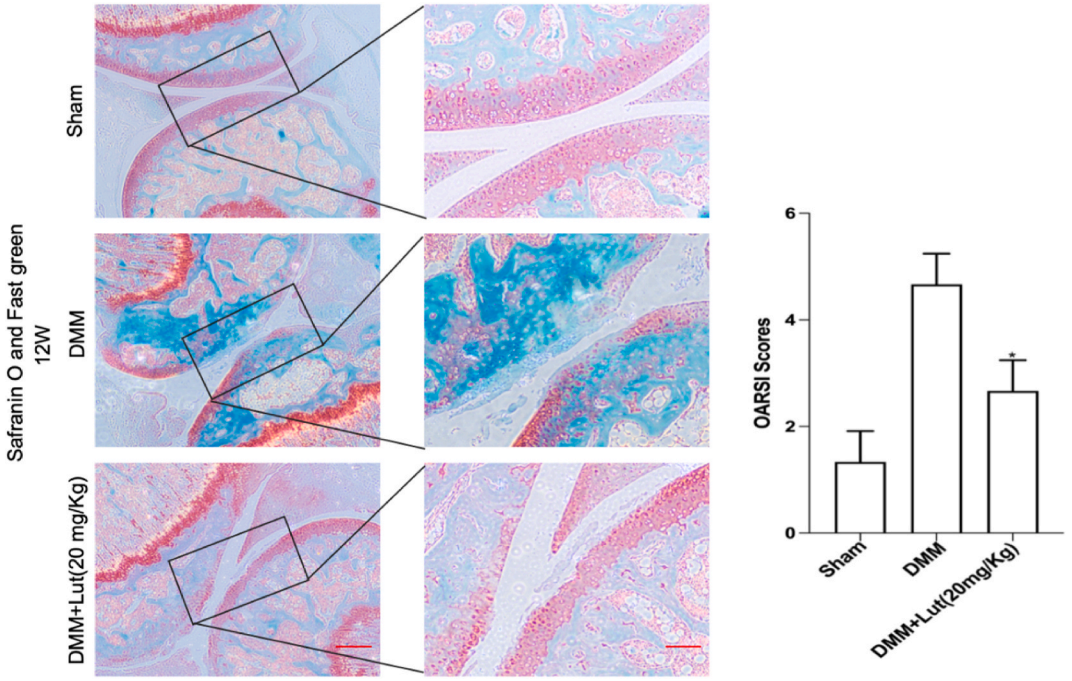

Fig. 7. Safranin O and Fast green staining results and OARSI score of mice knee joint.The data is presented as mean ± standard deviation, *P < 0.05 DMM + Lut group vs. DMM group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.8. Luteolin improves the progression of osteoarthritis by activating autophagy

To investigate the mechanism of action of luteolin, we utilized immunohistochemical analysis to examine the expression of autophagy pathway marker proteins, namely p62 and Beclin-1. Our findings revealed that compared to the sham group, the DMM group exhibited a significant increase in p62 expression, along with a notable decrease in Beclin-1 expression. These results indicate that autophagy levels were suppressed in the chondrocytes of the DMM group. Notably, the luteolin treatment group exhibited significantly higher p62 expression than the DMM group, while Beclin-1 expression was significantly improved compared to the DMM group, resulting in a reduction in positive p62 expression. Overall, these findings suggest that luteolin can activate the autophagy pathway and alleviate cartilage matrix degradation in OA mice (Fig. 8A and B).

3.9. The model of luteolin improves the imbalance of protein metabolism induced by IL-1β in chondrocytes via regulating autophagy

The study revealed that luteolin effectively increased the expression of Collagen II and Aggrecan in chondrocytes stimulated by IL-1β, while also reducing the levels of ADAMTS-5 and MMP-1. Previous studies on autophagy and osteoarthritis-related articular cartilage degeneration have established a link between autophagy and the expression of Collagen II and Aggrecan in OA articular cartilage, as well as the degradation of cartilage matrix proteolytic enzymes like ADAMTS-5 and MMP-1. Chloroquine was used to inhibit the autophagy pathway. The ability of luteolin to counteract the metabolic imbalance caused by IL-1β in human primary chondrocytes is attributed to its ability to enhance autophagy levels (Fig. 9).

4. Discussion

Osteoarthritis (OA) is a joint disease characterized by cartilage degeneration, joint space narrowing, and joint swelling, significantly impacting the patient's daily life and work [19,20]. In current clinical drug treatment, the primary options are non-steroidal anti-inflammatory analgesics and nutritional cartilage drugs, which aim to alleviate symptoms. However, prolonged use of these drugs can result in severe side effects. Additionally, due to the chronic nature of OA, lifelong or long-term treatment may be necessary to halt or slow its progression. Consequently, there is an urgent need for safe and effective drugs to address this issue.

Luteolin, a natural pigment found widely in nature, has garnered significant attention in recent years due to its anti-inflammatory biological effects [21]. It acts as a potent mast cell release inhibitor, effectively suppressing mast cell degranulation and the release of inflammatory mediators, such as cytokines, thus exhibiting anti-inflammatory properties [22,23]. Additionally, luteolin has demonstrated extensive immunomodulatory effects on various inflammatory cells, including neutrophils, monocytes, fibroblasts, and lymphocytes [24]. It achieves this mainly by inhibiting cell activation and the release of inflammatory mediators (such as histamine and

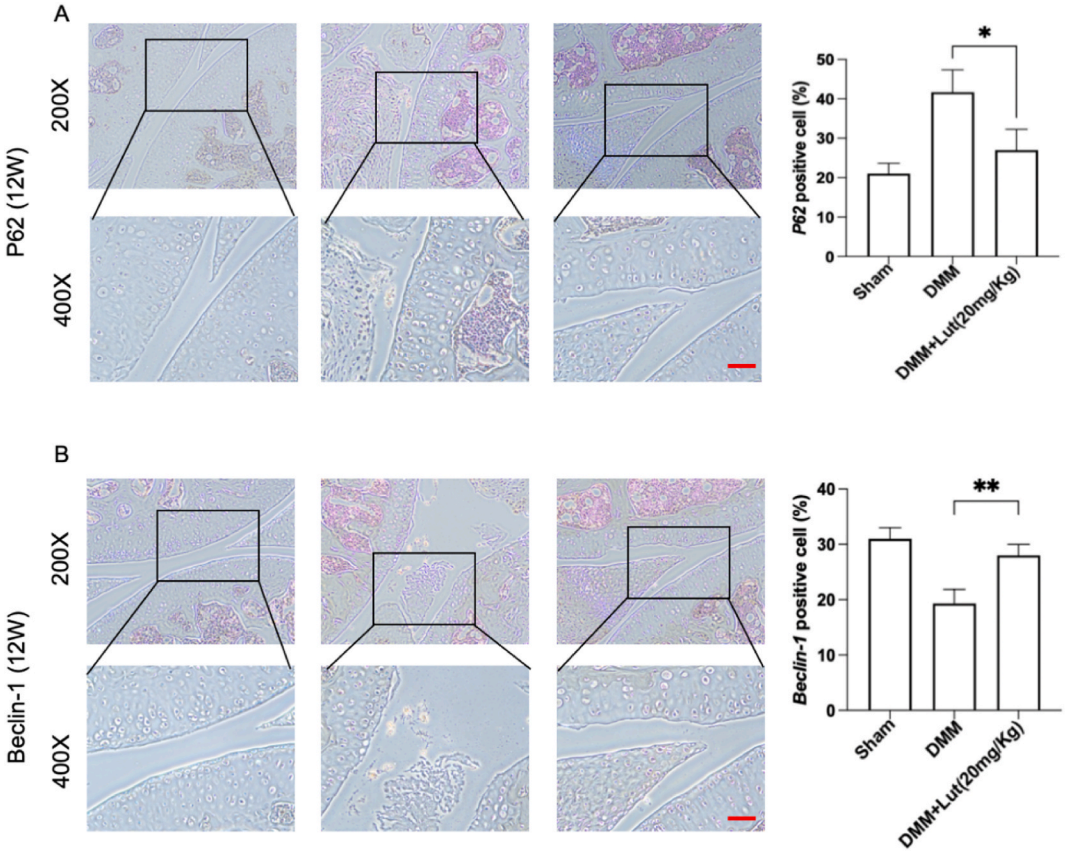

Fig. 8. The expression of autophagy pathway protein p62 and Beclin-1 was detected by immunohistochemical staining in mice knee sections (200x, 50 μm). The data is presented as mean ± standard deviation, *P < 0.05 DMM + Lut(20)vs. DMM group, **P < 0.01 vs. DMM group,.

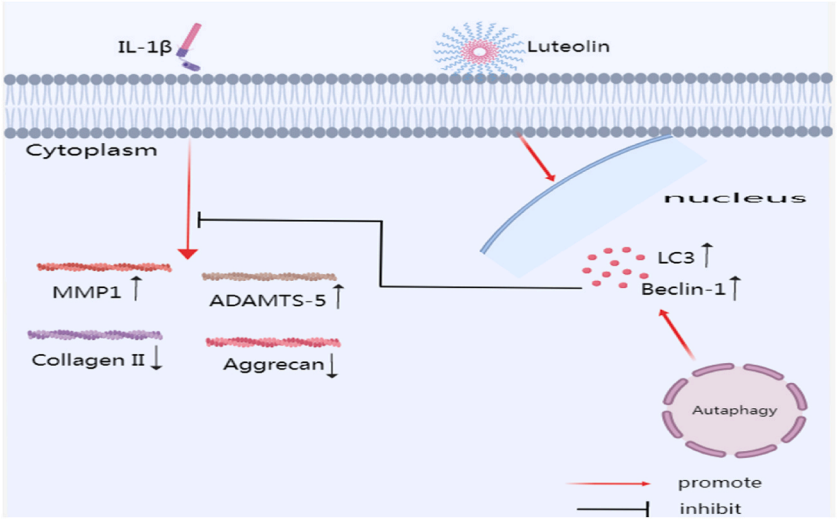

Fig. 9. Luteolin regulates autophagy to alleviate IL-1β mediated catabolic and anabolic imbalances.

prostasin-like substances) when subjected to inflammatory stimulation. Earlier studies have indicated that the pro-inflammatory cytokine IL-1β can down-regulate the expression of cartilage matrix proteins like Collagen II and Aggrecan in chondrocytes, while up-regulating the expression of cartilage matrix proteolytic enzymes like ADAMTS-5 and MMP-1 [25,26]. This imbalance in metabolism promotes the degeneration of OA articular cartilage. The extracellular matrix (ECM) of cartilage tissue primarily consists of

type II collagen (90%–95 %), aggrecan, water, and electrolytes. In the degradation of ECM in OA cartilage, MMP-1 is recognized as the primary protease responsible for ECM degradation, while chondrocytes secrete ADAMTS-5, which is involved in the breakdown of extracellular matrix aggrecan [27,28]. Considering the aforementioned structural characteristics of cartilage tissue, collagen II and aggrecan were chosen as the anabolic markers for chondrocytes, while ADAMTS-5 and MMP-1 were utilized as the catabolic markers for cartilage tissue. In this study, we observed that luteolin effectively inhibits the decrease of Collagen II and Aggrecan in chondrocytes caused by IL-1β. Additionally, luteolin down-regulates the expression of ADAMTS-5 and MMP-1, thereby promoting their synthesis and maintaining a catabolic balance. However, the specific mechanism by which luteolin regulates chondrocyte inflammation remains unclear. Therefore, investigating how luteolin alleviates chondroinflammatory changes is of great clinical significance for the treatment of osteoarthritis.

Autophagy is a crucial self-protective mechanism in the body, playing a vital role in regulating cellular metabolism and energy balance [29,30]. It involves the formation of autophagosomes, which encapsulate damaged organelles and misfolded proteins in the cytoplasm. These autophagosomes then fuse with lysosome membranes to form autophagolysosomes, where degradation occurs to preserve the structural integrity of cells and tissues [31,32]. Previous studies have established a close association between autophagy and the degeneration of cartilage in OA. Normal cartilage tissues exhibit a rich presence of important autophagy-related factors such as Beclin1 and LC3II. Wang et al. conducted a study on a knee OA surgery model in DMM mice and found that intra-articular injection of shikonin significantly enhanced cartilage autophagy levels [16,33]. This activation of autophagy effectively inhibited articular cartilage degeneration, further highlighting the involvement of autophagy in maintaining the normal physiological function of cartilage tissue. In this experiment, the qRT-PCR and western blotting analysis revealed that the expression levels of MMP1 and ADAMTS5 were decreased in the IL-1β + luteolin group compared to the IL-1β group. Additionally, the expression levels of Collagen II and Aggrecan were increased. Moreover, treatment with luteolin significantly increased the levels of Beclin-1 and LC3-II/LC3-I in chondrocytes, while decreasing the levels of the autophagy receptor protein p62 after IL-1β-induced decrease. These findings are consistent with previous studies and suggest that luteolin promotes autophagy to maintain the homeostasis of anabolism and catabolism in chondrocytes. Immunofluorescence analysis revealed that treatment with luteolin increased the expression of Beclin-1 and inhibited the expression of p62 in chondrocytes, providing further evidence of luteolin's role in activating autophagy. To investigate this further, we conducted a response experiment using the autophagy inhibitor CQ [34]. Western blotting results demonstrated that CQ effectively suppressed the expression levels of Collagen II and Aggrecan, while ADAMTS-5 and MMP-1 were significantly increased. In the knee OA model of DMM mice, luteolin promotes the anabolism of OA chondrocytes by up-regulating autophagy, thus delaying the progression of OA. These findings further support the notion that blocking autophagy with CQ disrupts the balance between anabolism and catabolism in chondrocytes.

Subsequently, the DMM-induced OA model was established in C57BL/6 mice, followed by immunohistochemistry to detect key genes p62 and Beclin-1 [35]. The results confirmed the significant role of luteolin in activating autophagy in vivo. Additionally, safranin O-fast green staining demonstrated that luteolin can effectively reduce the degeneration of articular cartilage induced by DMM. The degree of improvement in cartilage degeneration was objectively described using the OARSI score [36]. These findings suggest that luteolin exhibits a protective effect on OA through the activation of autophagy.

To summarize, our research suggests that luteolin possesses the ability to boost the production of extracellular matrix in chondrocytes when tested outside a living organism. Furthermore, it offers protection against the deterioration of cartilage when evaluated within a living organism. Moreover, the role of autophagy in sustaining the equilibrium between anabolic and catabolic processes provides additional evidence for the potential use of luteolin as a therapeutic intervention for osteoarthritis.

5. Limitations

At the cellular level, it was confirmed that luteolin exhibits fewer indicators of osteoarthritis regression and a limited number of experimental approaches. The dosage of luteolin for osteoarthritis in mice was relatively straightforward at the animal level. In future, our focus will be on enhancing the in vivo research content in this area and strengthening the evidence chain.

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Data availability statement

The data that support the findings of this study are available on request from the author, Yetian Li , upon reasonable request.

CRediT authorship contribution statement

Yetian Li: Funding acquisition. **Zhenfei Ding:** Writing – review & editing. **Fuen Liu:** Writing – original draft. **Shuang Li:** Data curation. **Wei Huang:** Formal analysis. **Shusheng Zhou:** Visualization. **Yongsheng Han:** Visualization. **Ling Liu:** Visualization. **Yan Li:** Visualization. **Zongsheng Yin:** Investigation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yetian Li reports financial support was provided by The First Affiliated Hospital of Anhui Medical University. Fuen Liu reports financial support was provided by The First Affiliated Hospital of Anhui Medical University. Yetian Li reports a relationship with The First Affiliated Hospital of Anhui Medical University that includes: funding grants. None If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31028.

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