Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.297

RESEARCH ARTICLE

Capsaicin Regulates Pyroptosis and Autophagy in Chondrocytes Associated with Osteoarthritis in Rat Models Via the AMPK/mTOR Signaling Pathway

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ARTICLE HISTORY (25-309)

Received: April 13, 2025
Revised: October 16, 2025
Accepted: October 24, 2025
Published online: December 27, 2025

Key words:
AMPK/mTOR pathway
Autophagy
Capsaicin
Osteoarthritis
Pyroptosis

ABSTRACT

Autophagy and pyroptosis in chondrocytes contribute to cartilage degeneration in osteoarthritis (OA). Capsaicin (Cap), a bioactive compound derived from chili peppers, exhibits anti-inflammatory and antioxidant properties. However, the underlying mechanisms by which Cap exerts its chondroprotective effects in OA remain poorly understood. This study aims to elucidate the molecular basis of Cap-mediated chondroprotection. OA models were established using SD rats and primary rat chondrocytes to evaluate the effects of Cap on extracellular matrix (ECM) degradation, inflammation, pyroptosis, autophagy, and the AMPK/mTOR signaling pathway. To investigate the mechanisms underlying Cap's chondroprotective effects, 3-Methyladenine and dorsomorphin were employed to inhibit autophagy and AMPK signaling, respectively. Cap mitigates cartilage damage and alleviates pain through activation of the AMPK/mTOR signaling pathway, while promoting chondrocyte autophagy and suppressing NLRP3 inflammasome activation and subsequent inflammatory responses. Notably, inhibition of autophagy or AMPK signaling in chondrocytes partially abrogates the protective effects of Cap on cartilage. Our findings demonstrate that Cap exerts its therapeutic effects in OA by activating the AMPK/mTOR pathway and enhancing autophagy in chondrocytes, thereby sequentially inhibiting pyroptosis and ECM degradation.

To Cite This Article: Zhang Z, Yang L, Du S, Han L, Liu K, Lu A, Ma T, Gao R and Bai H, 2025. Capsaicin regulates pyroptosis and autophagy in chondrocytes associated with osteoarthritis in rat models via the AMPK/mTOR signaling pathway. Pak Vet J, 45(4): 1766-1775. http://dx.doi.org/10.29261/pakvetj/2025.297

INTRODUCTION

Osteoarthritis (OA) is a degenerative entire joint disease, including articular cartilage, synovial membrane, subchondral bone, and periarticular tissues. It is a prevalent condition in humans, horses, dogs, and cats. In veterinary clinical practice, OA-associated chronic pain significantly impairs locomotor function and life quality, frequently progressing to premature retirement or euthanasia (Barbeau-Grégoire *et al.,* 2022; Seabaugh *et al.,* 2022; Mustonen *et al.,*2023; Anderson *et al.,* 2025). The primary pathogenic progression of OA involves the progressive degradation of the extracellular matrix (ECM), subchondral bone remodeling, osteophyte formation, and synovitis. These pathological changes collectively contribute to joint dysfunction and pain (Hodgkinson *et al.,* 2022; Young *et al.,* 2022). Current treatments for OA predominantly focus on conservative therapy. Approved pharmacological interventions include non-steroidal anti-inflammatory drugs (NSAIDs), hyaluronic acid (HA), and opioids, which primarily alleviate pain but do not address cartilage degeneration and may lead to significant adverse effects with long-term use (Hunter and Bierma-Zeinstra, 2019). Therefore, there is an urgent need to develop novel therapeutic strategies that effectively slow OA progression, relieve clinical symptoms, and minimize adverse effects.

Chondrocytes are the only cell type present in cartilage and are responsible for synthesizing extracellular matrix (ECM) components and degradative enzymes, thereby playing a key role in maintaining cartilage homeostasis (Fujii *et al.,* 2022). The NLRP3 inflammasome complexes comprising a sensor molecule,

the adaptor apoptosis--associated speck-like protein containing a CARD (ASC) and the effector pro-caspase-1(Chen *et al.,* 2021; Liu *et al.,* 2023). Studies have demonstrated that NLRP3 inflammasome activation-induced pyroptosis is closely associated with ECM degradation in rat OA models (Zhang *et al.,* 2024; Guo *et al.,* 2024). In OA cartilage, activation of autophagy can promote the degradation of NLRP3, inhibit chondrocyte pyroptosis, and prevent ECM degradation. Furthermore, the AMPK/mTOR signaling pathway is regarding to regulating cartilage autophagy and pyroptosis (Li *et al.,* 2021).

Capsaicin (CAP), the primary active substance extracted from chili peppers, has been widely studied in analgesic, anti-inflammatory, antioxidant, and thermogenic effects. Capsaicin showed potential benefits for obesity, diabetes, cardiovascular diseases, and arthritis (Srinivasan, 2016; Lv *et al.,* 2021; Thongin *et al.,* 2022; Huang *et al.,* 2023a). However, the precise mechanism underlying the effects of CAP on OA remains to be fully elucidated and requires further experimental validation. In this study, we investigated whether chondrocyte autophagy and pyroptosis, regulated by the AMPK/mTOR signaling pathway, serve as key mechanisms through which CAP attenuates the progression of OA.

MATERIALS AND METHODS

Animals: SD rats (24 weeks, male, 280g-300g) were obtained from the Liaoning Changsheng Experimental Animal Resources Center (Liaoning, China). All the animals were accommodated in well-ventilated, dry and clean animal housing facilities (room temperature 23±1°C, the humidity was 50±5%), with 12 hours of daily light exposure and unrestricted access to water and food. The shavings bedding material was changed on a weekly basis. All the experiments were carried out in accordance with the guidelines of the Chinese Animal Experiment Ethics Committee, and approved by Animal Ethics Committee of Inner Mongolia Agricultural University (NND2023052).

The OA model of rat induced by ACLT and Cap treatment: Forty male SD rats were averagely divided into four groups randomly: the control (sham operation, Con) group, the OA model group (ACLT), the capsaicin control group (Cap), and the capsaicin treatment group (Cap+ACLT). The OA model was established in the rats through anterior cruciate ligament transection (ACLT) of the right hind limb based on the method described in our previous studies (Bai *et al.,* 2022). In the Con and Cap group, only the joint capsule was revealed, after which the wound was closed following standard procedures.

After the surgery, the Con group and ACLT group rats were injected with normal saline (50μL), into the joint cavities, while the Cap group and the Cap+ACLT group was injected with capsaicin (50μM, 50μL, MCE). The dose and volume for intra-articular administration of Cap were determined according to previously studies(Kalff *et al.,* 2010; Lv *et al.,* 2021). The drug treatment was administered twice a week for six consecutive weeks. Following six weeks of treatment, blood was collected from the caudal vein, and the serum was frozen. Subsequently, all rats were euthanized through inhaling isoflurane, and the right hind limb knee joints were fully excised and preserved in 4% formaldehyde solution for subsequent pathological histological analysis.

Behavioral evaluation of pain: The Von Frey Filaments test was utilized to evaluate the paw withdrawal threshold (PWT) of rats through simplifying the up-down procedure (Bonin *et al.,* 2014). Prior to the test, the rats were placed in cages for acclimation for approximately 30 minutes. Once the animals were in a quiescent state and displayed no exploratory behavior, they were stimulated with von Frey filaments of diverse diameters perpendicularly in the central area of the right hind paw, thereby causing the bending of the nylon fiber. Each stimulation persisted for 6-7s, and the PWT was evaluated. The test was replicated thrice. The Von Frey test was executed at 1, 2, 3, 4, 5, and 6 weeks post-operatively.

Histopathological analysis: The knee joints (n=3 for each treatment group) that were fixed with 4% polyformaldehyde underwent decalcification, dehydration with ethanol, and embedding in paraffin. Then the joints were sliced up at 4μm-thick and dewaxed with xylene. The slices were stained using hematoxylin and eosin (H&E) and safranin O and fast green (SF). The histological scores were assessed by two experienced pathologists in double-blinded, and the extent of joint damage in each treatment group was scored in accordance with the OARSI scoring criteria (Pritzker *et al.,* 2006; Berg *et al.,* 2025).

Immunohistochemical analysis: The Immunohistochemical (IHC) sectioning approach is in accordance with histological examination. After the dehydration of the slides, antigen retrieval is carried out using citrate buffer, followed by blocking with rabbit serum. The sections are incubated with anti-Beclin-1 (1:100, Abclonal, A7353), LC3B (1:100, Immunoway, YN5524), p62 (1:100, Abclonal, A11250), p-AMPK(1:100, Abclonal, AP1102), ADAMTS-5 (1:100, Affinity, DF13288), MMP13 (1:100; Novus, OTI2D8), NLRP3 (1:100, Wanlei, WL02635), GSDMD (1:100, Affinity, AF4012), and caspase-1 (1:100, Wanlei, WL02996a) at 4°C overnight. Subsequently, incubation with HRP-conjugated secondary antibodies is conducted for 1h. DAB is employed for staining, hematoxylin is utilized to re-stain the nucleus, and neutral resin is used for mounting the slides. The sections are observed under a microscope, and the mean density is counted using Image-Pro Plus 6.0.

Primary chondrocyte culture of rat and drug administration: SD rats aged 2-3 weeks were euthanized following isoflurane anesthesia. Small pieces of cartilage from the femoral condyles, tibial plateaus, and caput femoris were aseptically dissected using sterile surgical blades and transferred to serum-free DMEM/F12 culture medium. The isolated cartilage tissues were then minced into fragments of approximately 1mm² in size. After trypsin incubation for 30 minutes, 0.2% type II collagenase was incubated for digestion at 37°C for 4h. Subsequently, the cells were centrifuged at 400g, re-suspended, and inoculated into culture flasks at 37°C in a 5% CO_2 environment. Further studies were implemented when the cell fusion rate reached 60-70%.

Cells were pre-incubated with Cap (50μM) for 24h, or were pre-treated with 3-Methyladenine (5mM, 3-MA, an autophagy inhibitor, MCE) or Dorsomorphin (10μM, Compound C, an AMPK inhibitor, MCE) for 30min prior to treatment with Cap. Subsequently, H_2O_2 (300μM) was added to the culture medium for 24h.

Lactate dehydrogenase release assay: Chondrocytes were plated (5000 cells/well), and a blank control group, a sample control group, as well as a maximum enzyme activity group were established. Subsequently, the test groups were exposed to drug treatment. One hour before detection, Lactate dehydrogenase release (LDH) release solution was added to the wells. At the treatment endpoint, LDH working solution was incubated in the dark. The microplate reader (BioTek) is employed to determine the OD value at 490nm and calculate the LDH release rate.

Western blot analysis: After determination of the concentration total protein, protein separation was carried out using gel electrophoresis, and subsequently transfer them onto a NC membrane. Room temperature blocking was executed, and the primary antibody Beclin-1 (1:1000, Abclonal, A7353), LC3B (1:1000, Immunoway, YN5524), p62 (1:1000, Abclonal, A11250), ADAMTS-5 (1:1000, Affinity, DF13288), MMP13 (1:1000; Novus, OTI2D8), NLRP3 (1:1000, Wanlei, WL02635), GSDMD (1:100, Affinity, AF4012), caspase-1 (1:1000, Wanlei, WL02996a), AMPK(1:1000, Abclonal, A1229), p-AMPK(1:1000, Abclonal, AP1102), mTOR(1:1000, Abclonal, A2445), and p-mTOR(1:1000, Wanlei, WL03694) were subjected to overnight incubation at 4°C. Following TBST washing, the HRP-labeled secondary antibody was incubated, and images were collected using a chemiluminescence imaging system. The gray value was analyzed using Image J software.

ELISA analysis: An ELISA kit (Jingmei, Co., Ltd. JiangSu, China) was utilized to determine the concentrations of IL-1β (JM-01454), IL-6 (JM-01597R), IL-10 (JM-01602R), IL-18 (JM-01601R), Cartilage oligomeric matrix protein (COMP, JM-10490R), COX-2 (JM-10333R), PGE_2 (JM-01475R), and C-telopeptides of type II collagen (CTX-II, JM-01488R) in the serum and cell supernatants of each group. The processes were performed following the guidelines provided in the manual. A microplate reader (Epoch, BioTek, USA) was used to measure the absorbance at 450nm. The concentrations were computed based on the standard curve.

Data analysis and statistics: All data were analyzed via GraphPad Prism 10, and the results were depicted as Mean±SEM. Differential analysis was conducted using one-way ANOVA, and multiple comparisons were performed with Tukey's post-hoc test.

RESULTS

Intra-articular administration of Cap alleviates cartilage degeneration and associated pain resulting from ACLT in SD rats: To assess the effects of Cap on OA cartilage, pathological histological staining was employed to examine cartilage lesions. HE staining showed that the Con group exhibited a smooth cartilage surface with chondrocytes organized in a clustered arrangement. In contrast, the ACLT group displayed defects in the superficial cartilage layer, along with disorganization of chondrocytes in both the superficial and middle layers. Compared to the ACLT group, the Cap+ACLT group showed reduced severity of structural damage, characterized by minor superficial defects and mild disarray of chondrocytes in the superficial layer (Fig. 1A). The SF staining indicated that in the ACLT group, the cartilage surface was eroded, with damage extending into the middle layer, chondrocyte hypertrophy and decreased proteoglycan content, as evidenced by diminished red staining. These findings were corroborated by OARSI scoring. In the Cap+ACLT group, the cartilage surface remained relatively intact, with microfractures and disordered surface layers. Additionally, proteoglycan content was increased, and the OARSI score was significantly lower compared to the ACLT group ($P<0.01$) (Fig. 1B–D). These results demonstrate that Cap therapy alleviates cartilage degeneration and proteoglycan loss in OA- affected rats.

To evaluate the analgesic effect, the PWT was measured using the von Frey method, and levels of PGE_2 and COX-2 were quantified using ELISA kits. The von Frey test results (Fig. 1G) showed a significant reduction in PWT in ACLT rats compared to Con group ($P<0.01$). No significant difference in PWT between the two groups was observed at two weeks post-surgery ($P>0.05$). However, starting from the third week, the Cap treatment group exhibited a progressive and significant increase in PWT ($P<0.05$), suggesting a marked attenuation of pain sensitivity in Cap-treated animals. Furthermore, the levels of PGE_2 and COX-2 (Fig. 1E and F) were significantly elevated in OA rats relative to the Con group ($P<0.01$), and Cap administration effectively reduced these inflammatory mediators ($P<0.01$). The results suggest that Cap therapy alleviates pain in OA rats, with pain sensitivity gradually diminishing over the course of treatment.

Cap triggers autophagy in OA chondrocytes: As shown in Fig. 2A, the mean density of Beclin-1, LC3-B, and p62 in the cartilage of rats in the ACLT group were markedly reduced compared to those in the Con group ($P<0.01$). In contrast, in the Cap+ACLT group, treatment with Cap markedly elevated the levels of these autophagy-related proteins in cartilage ($P<0.01$). To further investigate autophagy modulation, primary rat chondrocytes were pretreated with 3 3-MA, a specific autophagy inhibitor. As shown in Fig. 2B, Beclin-1, LC3-B, and p62 proteins expression in chondrocytes were significantly downregulated after H_2O_2 stimulation for 24h ($P<0.01$), an effect that was effectively reversed by Cap pretreatment ($P<0.01$). Additionally, 3-MA pretreatment reversed the regulatory effect of Cap on these autophagy markers. The results suggest that the chondroprotective effect of Cap is associated with the activation of autophagy.

Cap modulates pyroptosis and ECM degradation in rat chondrocytes via autophagy: To determine cartilage matrix degradation and pyroptosis, we analyzed the expression MMP-13 (a major protease that degrades type II collagen), ADAMTS-5 (a protease that degrades

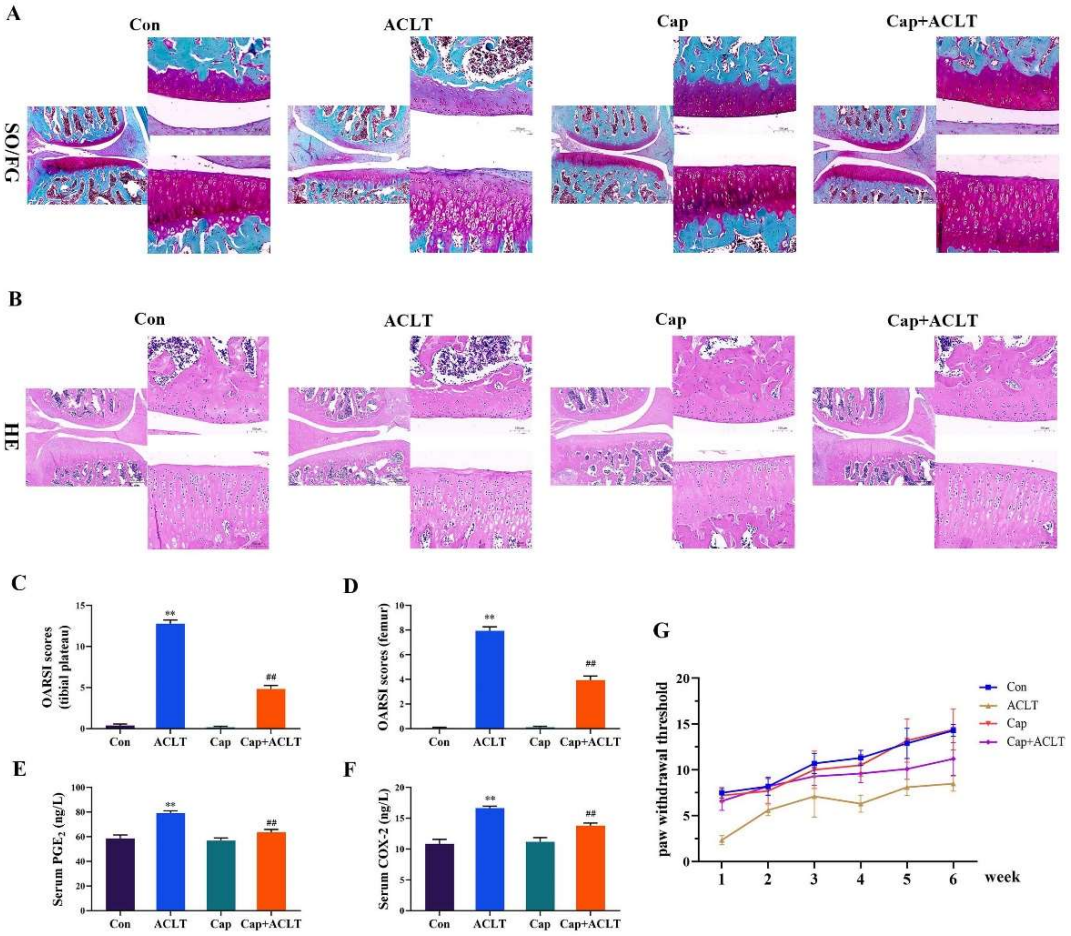

Fig. 1: Cap treatment alleviates cartilage damage and pain induced by ACLT in Rats OA. (A) HE staining (n=3). (B) SF staining (n=3). (C) and (D) OARSI scores in tibial plateau and femur. (E) and (F) Levels of PGE_2 and COX2 in rat serum (n=6). (G) PWT results obtained by von Frey (n=10). All results were described using the mean±SEM. * indicates P<0.05, ** indicates P<0.01 *vs* the Con group. # indicates P<0.05, ## indicates P<0.01 *vs* the ACLT group.

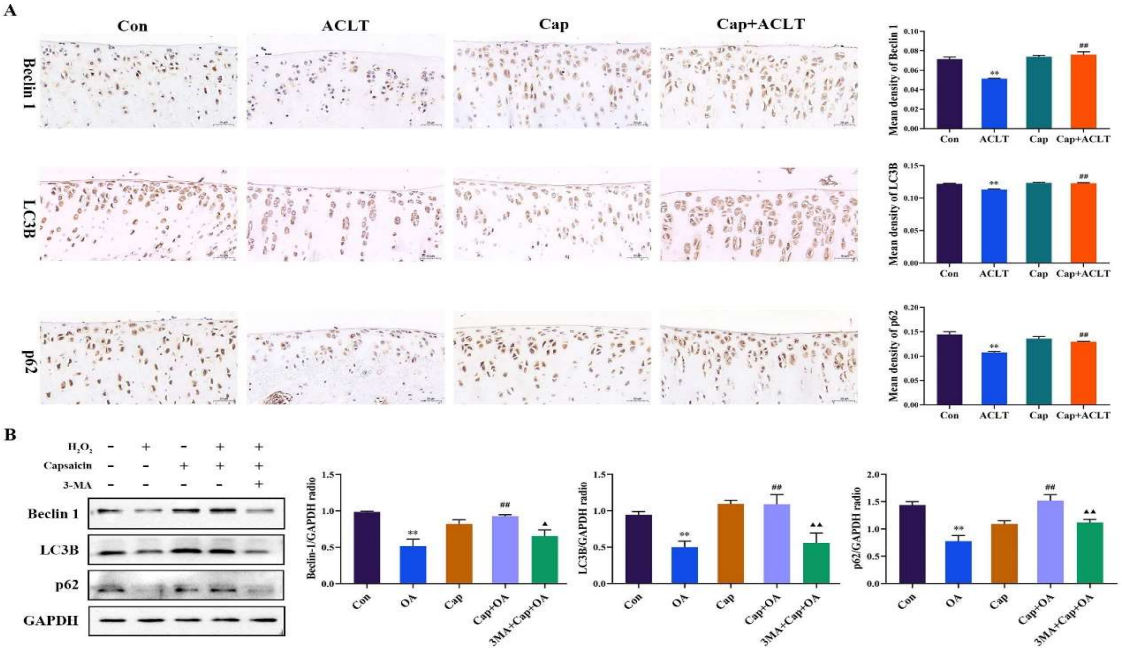

Fig. 2: Cap activates autophagy in rat cartilage and chondrocytes in OA. (A) Immunohistochemical staining and quantification of average optical density for Beclin-1, LC3-B, and p62 proteins (n=3) in cartilage. (B) Representative images and results analysis of Beclin-1, LC3-B, and p62 proteins (n=3). All results were described using the mean±SEM. *, #, and ▲ indicates P<0.05 compared with Con, ACLT or OA, and Cap+OA group, respectively. **, ##, and ▲▲ indicates P<0.01.

proteoglycans), and proteins involved in the classical pyroptosis pathway in the tibial plateau using IHC (Fig. 3A). Compared with the Con rats, the average density of MMP-13, ADAMTS-5, NLRP3, caspase-1, and GSDMD were significantly elevated in the tibial plateau of ACLT group ($P<0.01$). In contrast, in the Cap+ACLT treatment group, these protein levels were opposite to those in the ACLT group. No significant difference in the above markers were detected in the Cap group alone. Furthermore, we analyzed the effects of Cap on pyroptosis and matrix degradation in primary chondrocytes. As shown in Fig. 3B-H, the LDH release rate, the expression levels of NLRP3, caspase-1, GSDMD, MMP-13, and ADAMTS-5 proteins were significantly upregulated ($P<0.01$) after H_2O_2 stimulation. Notably, pretreatment with Cap significantly attenuated these H_2O_2-induced effects ($P<0.05$). Additionally, 3-MA pretreatment counteracted the regulatory effects of Cap on the classical pyroptosis pathway (LDH release and pyroptosis proteins) and matrix degradation proteins ($P<0.01$). The above results indicate that Cap regulates autophagy to inhibit chondrocyte pyroptosis and ECM degradation.

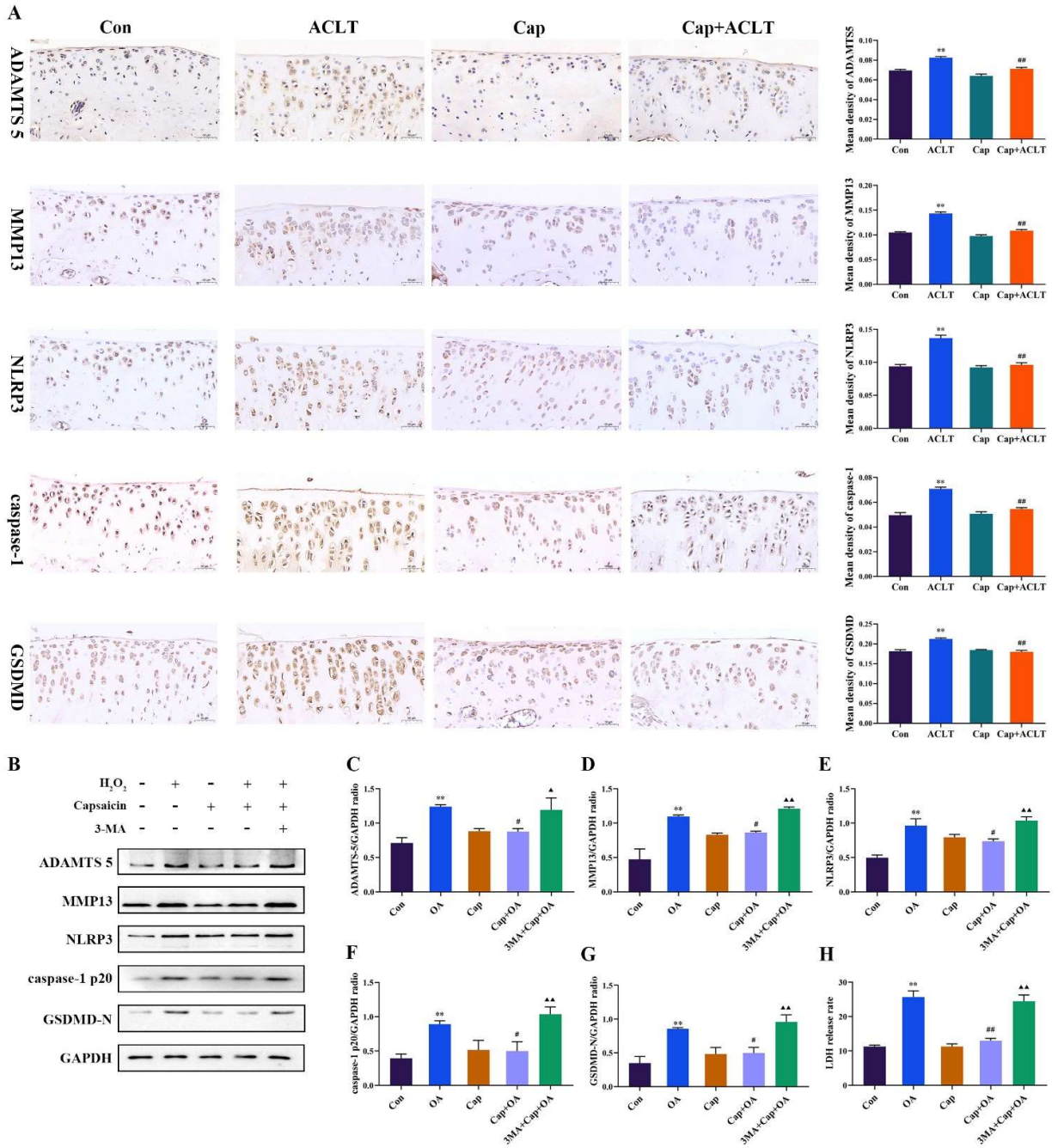

Fig. 3: Cap inhibits ECM degradation and pyroptosis in OA via autophagy-mediated mechanisms. (A) Immunohistochemical staining and quantification of average optical density for ADAMTS-5, MMP-13, NLRP3, caspase-1, and GSDMD proteins (n=3). (B-G) Representative Western blot images and analysis of ADAMTS-5, MMP-13, NLRP3, caspase-1p20, and GSDMD-N proteins in rat chondrocytes (n=3). (H) Analysis of LDH release rate in chondrocytes (n=6). All results were described using the mean ± SEM. *, #, and ▲ indicates $P<0.05$ compared with Con, ACLT or OA, and Cap+OA group, respectively. **, ##, and ▲▲ indicates $P<0.01$. Cap, capsaicin. 3-MA, 3-Methyladenine.

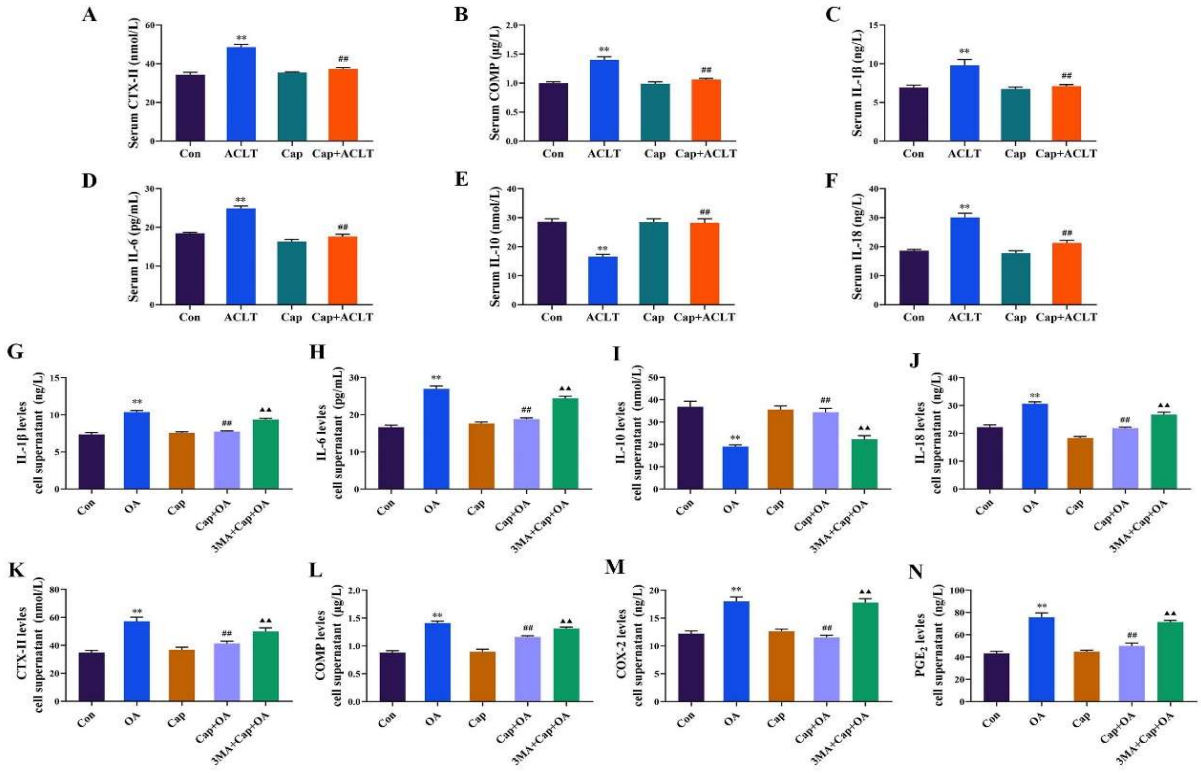

Fig. 4: Cap suppresses the ECM degradation and inflammatory responses. (A) and (B) The level of COMP and CTX-II in rat serum (n=6). (C-F) The concentrations of IL-1β, IL-6, IL-10, and IL-18 in rat serum (n=6). (G-N) The level of IL-1β, IL-6, IL-10, IL-18, CTX-II, COMP, COX-2, and PGE_2 in the cell supernatant of rat chondrocytes (n=6). All results were described using the mean±SEM. *, #, and ▲ indicates P<0.05 compared with Con, ACLT or OA, and Cap+OA group, respectively. **, ##, and ▲▲ indicates P<0.01. Cap, capsaicin. 3-MA, 3-Methyladenine.

Cap regulates collagen metabolism and inflammation through autophagy: The collagen metabolism and inflammatory cytokines in the serum of ACLT-induced OA rats were examined (Fig.4A-F). Compared with the Con rats, the serum levels of collagen II metabolism markers (COMP and CTX-II) and pro-inflammatory mediators (IL-1β, IL-6, and IL-18) were significantly increased (P<0.01) in the ACLT rats. In contrast, IL-10 levels were significantly reduced. Notably, Cap treatment effectively reversed these effects. In vitro experiments further revealed that H_2O_2 stimulation markedly increased the levels of collagen II metabolism markers and pro-inflammatory cytokines in the chondrocyte culture supernatant stimulated (P<0.01), whereas IL-10 showed an opposite trend (P<0.01). Cap pretreatment significantly reversed these H_2O_2-induced effects (P<0.05), while 3-MA pretreatment counteracted the protective effects of Cap (Fig.4G-N). Collectively, these results indicate that Cap inhibits collagen catabolism and inflammation through the modulation of autophagy.

Cap modulates chondrocytes autophagy through the AMPK/mTOR pathway: The IHC staining of tibial plateau results (Fig. 5A) showed that the average optical density of p-AMPK was significantly lower in OA cartilage induced by ACLT compared to the Con group, whereas this effect was reversed in the Cap+ACLT group. As shown in Fig. 5B, H_2O_2 stimulation significantly decreased p-AMPK expression and the p-AMPK/AMPK ratio, while increasing p-mTOR expression and p-mTOR/mTOR ratio in chondrocytes. Notably, Cap pretreatment significantly attenuated these changes (P<0.05). Furthermore, we used Compound C to inhibit AMPK activation. In the CC+Cap+OA group, Compound C significantly inhibited Cap-induced activation of p-AMPK and increased the p-mTOR/mTOR ratio. Additionally, Beclin-1, LC3-B, and p62 protein levels were significantly downregulated in the compared to the Cap+OA group (P<0.01). These findings indicate that Cap promotes chondrocyte autophagy via the AMPK/mTOR signaling pathway, thereby exerting a chondroprotective effect.

Cap regulates pyroptosis, inflammation, and ECM degradation via the AMPK/mTOR signaling pathway: To further determine the effects of the AMPK/mTOR signaling pathway in pyroptosis, matrix degradation, and inflammation in OA chondrocytes. LDH release (Fig. 6G), the expression of canonical pyroptosis-related proteins (NLRP3, caspase-1, and GSDMD; Fig. 6A, D–F), and levels of ECM degradation markers (ADAMTS-5 and MMP-13; Fig. 6A–C) were elevated in CC+Cap+OA group (P<0.05). Furthermore, COMP and CTX-II levels (Fig. 6L and M) were significantly increased in the cell supernatant (P<0.05), along with upregulation of inflammatory mediators including IL-1β, IL-6, IL-18, COX-2, and PGE_2 (Fig. 6H, I, K, N, O; P<0.05). In contrast, IL-10 levels were significantly decreased (P<0.01; Fig. 6J). These findings suggest that Cap inhibits pyroptosis, inflammation, and ECM degradation through modulating autophagy via the AMPK/mTOR signaling pathway.

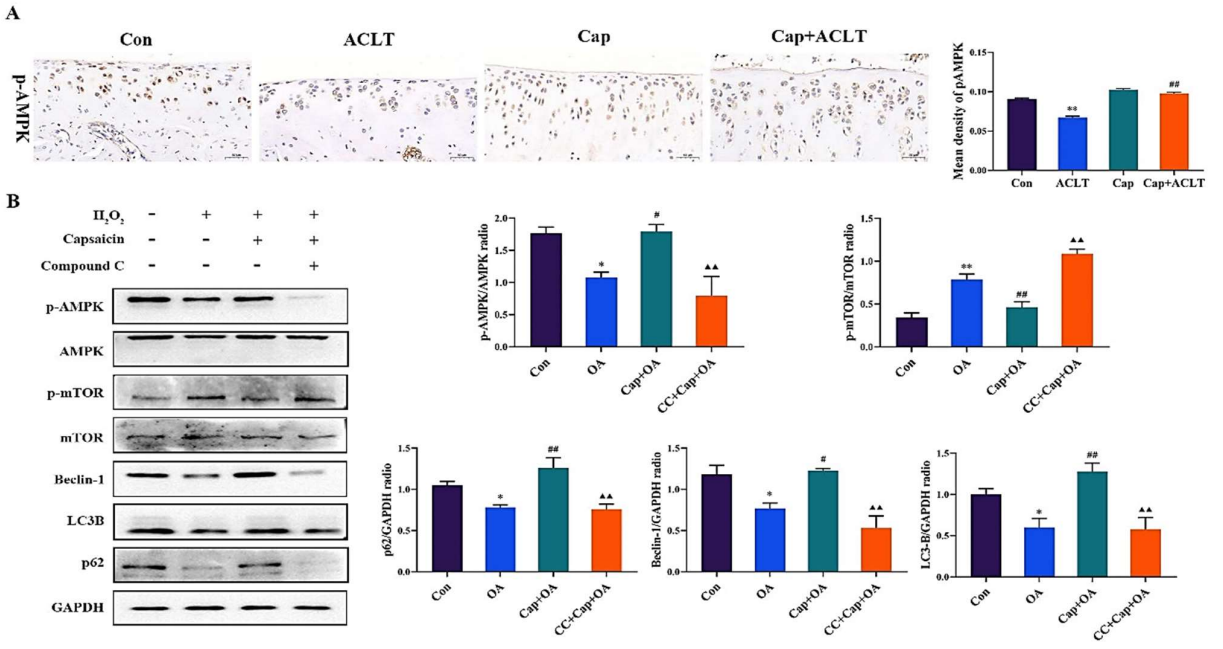

Fig. 5: Cap activates chondrocyte autophagy via the AMPK/mTOR pathway. (A) Immunohistochemical results and quantification of average optical density for p-AMPK proteins in cartilage. (B) Representative Western blot images and analysis of AMPK, p-AMPK, p-mTOR, mTOR, Beclin-1, LC3-B, and p62 proteins in chondrocytes. All results were described using the mean±SEM (n=3). *, #, and ▲ indicates P<0.05 compared with Con, ACLT or OA, and Cap + OA group, respectively. **, ##, and ▲▲ indicates P<0.01. Cap, capsaicin. CC, Compound C (Dorsomorphin).

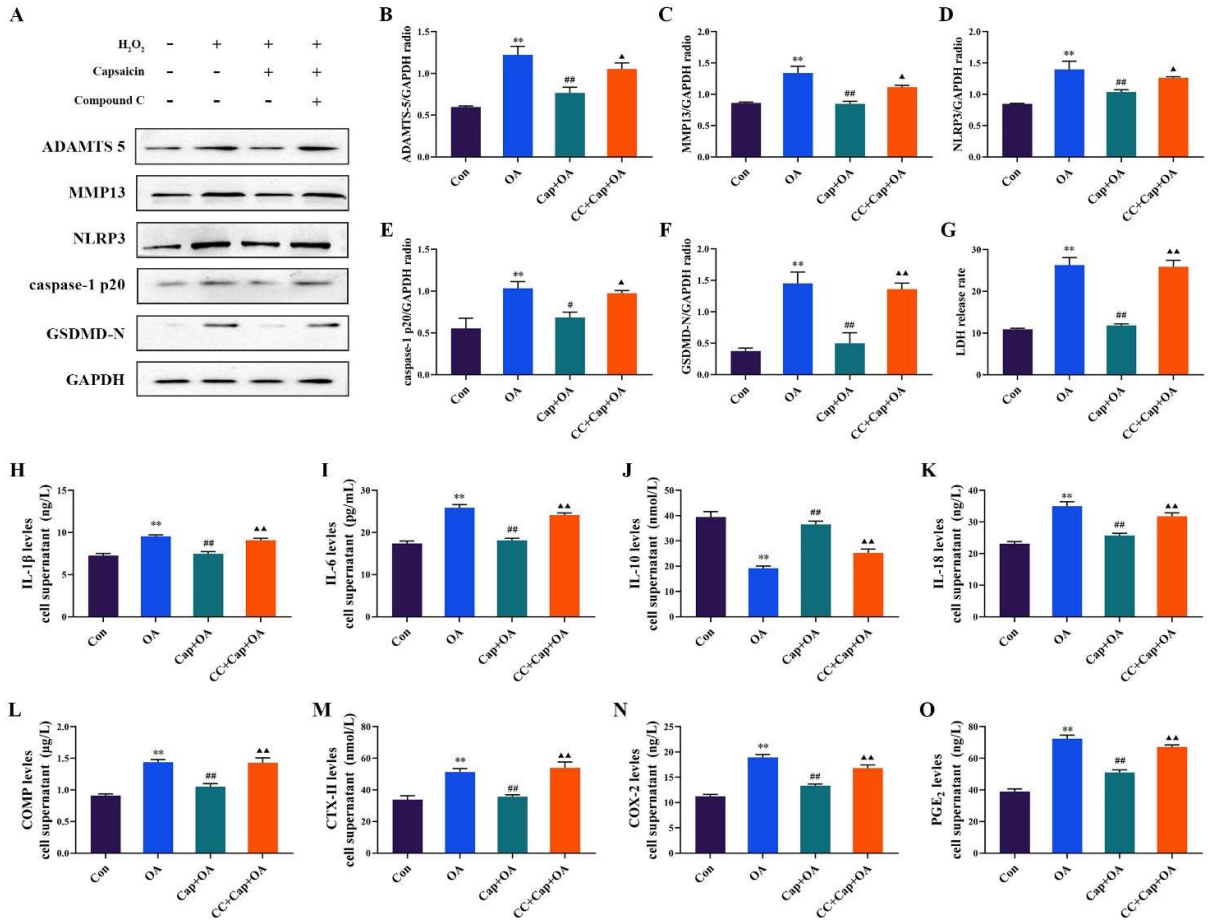

Fig. 6: Cap inhibits chondrocyte pyroptosis, inflammatory responses, and ECM degradation via the AMPK/mTOR signaling pathway. Representative Western blot images (A) and analysis of ADAMTS-5 (B), MMP-13 (C), NLRP3 (D), caspase-1 p20 (E), and GSDMD-N (F) proteins in rat chondrocytes (n=3). (G) Analysis of LDH release rate in chondrocytes (n=6). The concentrations of IL-1β (H), IL-6 (I), IL-10 (J), IL-18 (K), COMP (L), CTX-II (M), COX-2 (N), and PGE_2 (O) in the cell supernatant (n = 6). All results were described using the mean±SEM. *, #, and ▲ indicates P<0.05 compared with Con, ACLT or OA, and Cap+OA group, respectively. **, ##, and ▲▲ indicates P<0.01. Cap, capsaicin. CC, Compound C (Dorsomorphin).

DISCUSSION

Cap is a natural alkaloid derived from Capsicum species and functions as a selective agonist of TRPV1 channel. It is widely used for alleviate pain associated with diabetic neuropathy, rheumatoid arthritis, and osteoarthritis (Liu *et al.,* 2022b; He *et al.,* 2025). Cap exhibits significant first-pass metabolism and may induce gastric mucosal irritation, rendering it unsuitable for oral administration. Consequently, local application is considered as a more appropriate and effective alternative (Rollyson *et al.,* 2014). In veterinary clinical practice, Cap has been shown to exert anti-inflammatory and antiviral effects across various animal species (Yang *et al.,* 2024; Zhang *et al.,* 2025). Moreover, Cap is utilized as an analgesic agent for the management of pain associated with equine OA (Braucke *et al.,* 2020; Robinson *et al.,* 2020). Our study showed that intra-articular injections of Cap (50μM, 50μL, twice a week) effectively alleviated OA-related pain and cartilage degeneration induced by ACLT in rats. Specifically, this treatment significantly reduced pain-related behaviors, decreased serum levels of inflammatory cytokines, and attenuated pathological changes. Furthermore, previous research has shown intra-articular injection of Cap in a rat significantly mitigates OA-related pathological alterations, such as joint swelling, cartilage defects, and osteophyte formation (Lv *et al.,* 2021). Although Cap has been demonstrated to alleviate acute pain and mitigate cartilage damage in various rat OA models, studies investigating the precise mechanisms underlying its chondroprotective effects remain limited.

The NLRP3 inflammasome, composed of NOD-, LRR-, and pyrin domains, functions as a cytoplasmic pattern recognition receptor that facilitates caspase-1 activation， leading to GSDMD-mediated pyroptosis and the maturation of pro-inflammatory cytokines (Coll *et al.,* 2022). Dysregulation of inflammasomes in articular cartilage results in synovial inflammation and progressive cartilage destruction, while excessive activation of the NLRP3 inflammasome is implicated in the chronic low-grade inflammation in OA (Zhang *et al.,* 2024). NLRP3 expression is significantly upregulated in synovial tissues, and levels of IL-1β and IL-18 are markedly elevated in synovial fluid, promoting extracellular matrix ECM breakdown (Tang *et al.,* 2023; Fang *et al.,* 2024). During OA progression, alterations in cartilage ECM metabolism are characterized by reduced synthesis and enhanced degradation, accompanied by significant upregulation of MMP-13 and ADAMTS-5, which further accelerate cartilage destruction (Fang *et al.,* 2024). Moreover, cartilage metabolic biomarkers such as COMP and CTX-II are substantially increased during OA progression and have potential utility as biomarkers for monitoring therapeutic response (Bay-Jensen *et al.,* 2018). Our in vitro experiments show that Cap inhibits classical pyroptosis pathway protein, reduces the release of inflammatory cytokines in OA, downregulates the expression of MMP-13 and ADAMTS-5, and decreases levels of cartilage metabolism markers (COMP and CTX-II). These findings indicate that Cap mitigates ECM degradation in OA by suppressing NLRP3/caspase-1-mediated pyroptosis and associated inflammatory responses.

Autophagy is a highly conserved intracellular process characterized by degradation and reuse of impaired components, playing an essential function in regulating chondrocyte survival and maintaining intracellular homeostasis (Lv *et al.,* 2022). In chondrocytes, autophagy effectively clears intracellular toxic substances, and impairment of this process has been shown to accelerate OA progression (Liu *et al.,* 2022a , Jin *et al.,* 2022). Autophagy levels fluctuate across the stages of OA. In early stages, autophagy is upregulated in chondrocytes as a protective mechanism against oxidative stress and cell death. As OA advances, autophagy becomes inhibited, leading to a significant reduction in autophagy markers such as ULK1, Beclin1, and LC3 (Shapiro *et al.,* 2014). Our study demonstrated that the expression of essential autophagy-related proteins (Beclin-1, LC3-B, and p62) was substantially downregulated in cartilage and primary chondrocytes from an OA model, indicating impaired autophagic function. Conversely, Cap treatment significantly enhances autophagic activity.

Following autophagy impairment, chondrocytes exhibit increased catabolic activity, resulting in elevated production of matrix-degrading enzymes MMP13 and ADAMTS-5 in OA, thereby accelerating the loss of ECM (Shapiro *et al.,* 2014; Kraeutler *et al.,* 2020). In the DMM-induced rat OA model, activation of autophagy in chondrocytes inhibits NLRP3 inflammasome activation, thereby mitigating OA-associated cartilage degeneration (Li *et al.,* 2024). These findings suggest that enhancing autophagy in chondrocytes may represent a promising therapeutic strategy for OA treatment. In this study, we employed the classical autophagy inhibitor 3-MA to investigate the mechanism underlying Cap-mediated cartilage protection. Our results demonstrate that 3-MA effectively reverses Cap-induced autophagy activation and inhibition of pyroptosis, consequently abolishing Cap's anti-inflammatory effects and its capacity to suppress ECM degradation in OA.

It has been reported that signaling molecules such as mTOR and AMPK play critical roles in mediating autophagy in OA chondrocytes (Wang *et al.,* 2020). During OA progression, upregulation of mTOR inhibits autophagic signaling, thereby diminishing the protective effects of autophagy on cartilage and promoting cartilage degeneration. mTORC1 serves as a central inhibitor of autophagy and is regulated by upstream signaling pathways, including PI3K/AKT, MAPK, and AMPK (Chen and Long, 2018). AMPK promotes autophagy through phosphorylating TSC2 and RAPTOR, which leads to the inhibition of mTORC1 (Alao *et al.,* 2023). In this study, we examined alterations in the AMPK/mTOR pathway in both rat OA models and H_2O_2-stimulated chondrocytes. Consistent results from in vivo and in vitro experiments revealed that Cap treatment activated AMPK, as evidenced by increased p-AMPK expression, while simultaneously suppressing mTOR.

Certain natural compounds, such as bilobalide and geniposide, activated AMPK/mTOR pathway, which is associated with enhanced autophagy in chondrocytes, thereby enhancing cell proliferation and protecting chondrocytes from inflammation and ECM degradation (Ma *et al.,* 2022; Huang *et al.,* 2023b). Furthermore, studies have shown that moderate exercise induces

chondrocyte autophagy through the AMPK/mTOR signaling pathway, thus alleviating monosodium iodoacetate (MIA)-induced pyroptosis in OA rats (Li *et al.,* 2021). In this study, the AMPK inhibitor Compound C (Dorsomorphin) was used to block Cap-induced activation of the AMPK signaling pathway, thereby elucidating the mechanistic basis of Cap's chondroprotective effects. Our findings demonstrate that Dorsomorphin abolishes Cap-mediated regulation of the AMPK/mTOR pathway, resulting in suppressed chondrocyte autophagy and subsequently exacerbating pyroptosis, inflammatory responses, and ECM degradation.

Conclusions: Collectively, Cap regulates the AMPK/mTOR signaling pathway, thereby inducing autophagy, suppressing NLRP3/caspase-1-mediated pyroptosis, and attenuating cartilage inflammatory responses and ECM degradation

Authors contribution: ZZ, LY, KL, and LH: Methodology, Investigation, and Data curation; ZZ, HB and RG: Project administration, Supervision and Funding acquisition; ZZ, SD, AL. and TM, Formal analysis, Software, Validation, ZZ, and HB, Writing - original draft, review & editing. All authors have reviewed and endorsed the final version of the manuscript.

Funding: This study was supported by Inner Mongolia Natural Science Foundation (No. 2025QN03165 and 2025QN03104), the Initial Scientific Research Foundation of Inner Mongolia Agricultural University (NDYB2022-4 and NDYB2022-7), and the National Natural Science Foundation of China (No. 31960724).

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