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RESEARCH ARTICLE

Baicalein Suppresses NLRP3-Mediated Chondrocyte Pyroptosis and Extracellular Matrix Degradation in Osteoarthritis Via P2X7 Receptor

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ABSTRACT

Osteoarthritis (OA) is a degenerative joint disorder marked by the breakdown of cartilage and inflammatory responses. P2X7R is a switch of inflammation and also an activator of NLRP3 inflammasome and IL-1β. Recent studies have focused on plant extracts to prevent or delay OA development. Baicalein has been linked to positive effects for inflammation, and might be a potential therapeutic agent. In this study, we performed baicalein in an anterior cruciate ligament transection (ACLT) - induced OA rat model to evaluate its effects on cartilage histological changes, cartilage catabolism, pro-inflammatory cytokines, and NLRP3-indcued signaling pathway. Moreover, to further clarify the target of baicalein towards the upstream molecular of NLRP3, we used BzATP (a selective agonist of P2X7R) before baicalein administration both in OA rats and primary chondrocytes of rats. Our results show that baicalein relieves OA cartilage torn, including increased safranin O-fast green staining for proteoglycan and decreased OARSI score, as well as inhibiting MMP-13 and ADAMTS-5, which contribute to cartilage degradation. Additionally, baicalein downregulated the expression of P2X7 and NLRP3-induced axis both *in vivo* and *in vitro*; moreover, it reduces the contents of IL-1β, IL-6, IL-18, COX-2 and PGE-2, but increases IL-10. However, these results were reversed by BzATP partly. In conclusion, baicalein inhibits OA inflammation and cartilage catabolism through the P2X7-NLRP3 axis as an OA protective agent.

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INTRODUCTION

Osteoarthritis (OA) is a comprehensive joint disorder that involves multiple tissues, including cartilage, subchondral bone, synovium, and infrapatellar fat pad, and the surrounding ligaments (Motta *et al.*, 2023 ;Tang *et al.*, 2025). OA is characterized as a joint disorder caused by cell stress and extracellular matrix degradation, leading to cartilage degradation, synovium inflammation and fibrosis, and subchondral bone remodelling (Kraus *et al.*, 2015). The imbalance between anabolic and catabolic processes of cartilage primes degradation and decrease of type II and proteoglycan, which are mainly driven by matrix metalloproteinase 13 and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) (Zheng *et al.*, 2021). During OA, chondrocytes respond to change in the surrounding environment by activating multiple downstream signaling pathway networks to regulate key cellular processes in OA, in particular inflammation. The P2X7 purinergic receptor (P2X7R) belongs to the ATP-activated ion channel family of receptors (Sainz *et al.*, 2023). The activation of P2X7R has been implicated in various processes, of concern cytokine production and inflammasome activation. Extracellular ATP, the unique physiological activator of P2X7R, was regarded as a signal to induce the release of IL-1β. It is considered that P2X7R stimulation activates the NLRP3 (Adinolfi *et al.*, 2018). Our previous study has shown that NLRP3 inflammasome and the activation of caspase-1 were involved in OA inflammation, specifically reflected in massive release of IL-1β and IL-18, which were key pro-inflammatory cytokines promoting cartilage degradation (Bai *et al.*, 2022). Thus, targeting P2X7R or NLRP3 will be a promising therapeutic agent.

To this day, non-steroidal anti-inflammatory drugs (NSAIDs) are used to relieve pain for symptomatic osteoarthritis patients. Joint replacement was suitable for patients with severe joint wear and tear. It is noteworthy that the incidence rate of peptic ulcer disease in OA patients is twice as high as that in non-OA patients, likely reflecting the adverse effects of NSAIDs for OA management (Dell'Isola *et al.*, 2024). The development of new OA treatment drugs is necessary and urgent. Recently, some plant-derived extracts have demonstrated strong potential in alleviating OA symptoms. Baicalein is one of the active components in *S. baicalensis*, which exhibits multiple activities, such as antiviral, anti-inflammation, antioxidant, and antitumor (Bai *et al.*, 2020a; Park *et al.*, 2024). Although multiple mechanisms are involved, in drug development, it is still necessary to identify the target points of these mechanisms. Therefore, the objective of the present study is to clarify the involvement of P2X7R and NLRP3 in the mechanism of action of baicalein.

MATERIALS AND METHODS

Animals and study groups: Eight-week-old Sprague Dawley (SD) rats were procured from the Changsheng Laboratory Animal Center in Liaoning Province. All rats were housed under standardized conditions, including a temperature range of 21–23°C, relative humidity of 45-50%, a 12-hour light/dark cycle, adequate ventilation, and a clean environment. Food and water were provided without restriction, and the bedding material was renewed on weekly basis.

ACLT-induced OA model and drug intervention: A total of forty rats were randomly assigned to four groups (n = 10): sham group, anterior cruciate ligament transection (ACLT) - induced osteoarthritis group (OA group), baicalein treatment group (BAI + OA group), and BzATP inhibitor group (BzATP + BAI + OA group). Under isoflurane anesthesia, the sham group underwent only a right hind limb joint capsule incision followed by routine suture closure. The other groups underwent ACLT surgery to induce rat OA. After incising the joint capsule, the anterior cruciate ligament was transected, and the surgical site was closed using a 5-0 absorbable suture. Post-surgery, the BAI + OA group received oral administration of baicalein at a daily dose of 35mg/kg. The BzATP + BAI + OA group received both oral baicalein (35mg/kg) and intra-articular injections of BzATP (a P2X7 receptor agonist, 0.3mg per rat, 50μL) into the knee joint capsule of the right hind limb twice weekly. The duration of all treatments was maintained for a period of six weeks.

Sample collection: All rats were subjected to weekly pain behavioral assessments. After 6 weeks, the rats were euthanized under isoflurane anesthesia, and blood samples as well as whole-joint specimens were collected for molecular biological and histological analyses, respectively.

Rat chondrocyte culture and drug treatment: Under sterile conditions, cartilage from the knee and hip joints of rats aged 14-21 days postnatally was harvested (cut into small cubes with 1 mm^3), digested with trypsin, and further digested with type II collagenase. The digestion process was terminated using Dulbecco's Modification of Eagle's Medium/Ham's F-12 (DMEM/F12) culture medium (Dalian Meilun Biotechnology Company, China) supplemented with 10% fetal bovine serum (FBS, Bioind Company, Israel). After centrifugation, the cells were resuspended in complete culture medium (DMEM/F12 containing 10% FBS and antibiotic solution) and seeded into cell culture flasks for subculture(Bai *et al.*, 2022).

The *In vitro* experiments were divided into four groups: the Sham group, the H_2O_2-treated group (OA group), the BAI + OA group, and the Bz + BAI + OA group. Chondrocytes in the Con group received no treatment. In the OA group, cells were exposed to 300μM H_2O_2 for 24hours. For the BAI + OA group, 50μM BAI was added 24hours prior to H_2O_2 stimulation. In the Bz + BAI + OA group, 40μM BzATP (MedChemExpress, USA) was administered 30 minutes before BAI treatment, followed by H_2O_2 stimulation.

Lactate dehydrogenase levels detection: The lactate dehydrogenase (LDH) assay kit (Beyotime Company, China) was employed for the experiment. Blank control wells and wells with maximal enzyme activity were established. Following the addition of the LDH working solution, the absorbance values were detected using a microplate reader, and the LDH levels were subsequently calculated.

Pathological histological analysis: Full joint specimens were harvested and fixed in 4% paraformaldehyde, followed by decalcification in EDTA decalcification solution. Tissues were then dehydrated through a graded ethanol series, embedded in paraffin to form tissue blocks, and sectioned at 4 μm. Paraffin-embedded sections were stained with safranin O-fast green (SO-FG) or hematoxylin-eosin (HE), prior to dehydration in absolute ethanol and clearing in xylene. Finally, sections were mounted with neutral resin. The degree of articular cartilage damage (in the tibial plateau and femur) in each group was quantitatively evaluated using the Osteoarthritis Research Society International (OARSI) scoring system (Pritzker *et al*., 2006).

Immunohistochemical analysis: After dewaxing cartilage tissue sections, the following procedures were performed sequentially: antigen retrieval, blocking with bovine serum albumin (BSA), incubation with primary antibodies and subsequent HRP-conjugated secondary antibodies, DAB staining, hematoxylin counterstaining, and mounting with neutral gum to complete IHC staining. Sections were then observed under different magnifications, and images were captured. Average optical density values were calculated for quantitative assessment. The primary antibodies employed included Collagen II (1:100, AF0135, Affinity), MMP-13 (1:150, OTI2D8), P2X7 (1:100, A10511, Abclonal), NLRP3 (1:150, WL02635, WanLei), caspase-1 (1:150, WL02996a, WanLei), and GSDMD (1:100, AF4012, Affinity).

Western blot analysis: Chondrocyte protein samples were separated by SDS-PAGE and transferred to nitrocellulose (NC) membranes. Membranes were then incubated with primary antibodies against ADAMTS5 (DF13288, Affinity), MMP13 (OTI2D8, Novus), P2X7 (A10511, Abclonal), NLRP3 (WL02635, WanLei), caspase-1

(WL02996a, WanLei), GSDMD (ab219800, ABCAM), and GAPDH, each diluted at 1:1000. Subsequently, membranes were incubated with HRP-linked secondary antibodies for binding, followed by chemiluminescent detection. Protein expression levels were quantified by capturing images with a chemiluminescence imaging system and analyzing band intensities.

Enzyme-linked immunosorbent assay (ELISA): Levels of Prostaglandin E_2 (PGE_2), cyclooxygenase 2 (COX-2), inflammatory cytokines interleukin (IL-1β, IL-6, IL-10, and IL-18), type II collagen C-terminal peptide (CTX-II), and cartilage oligomeric matrix protein (COMP) in serum and cell culture supernatant were quantified using ELISA kits (Jingmei Biotechnology, Jiangsu, China). All experiments were performed according to the manufacturer's protocols, with standard curves generated for each analyte. Absorbance was measured at 450 nm to determine the concentrations of target markers.

Statistical analysis: All experimental data were showed as mean±SD (Standard Deviation). Statistical analyses were performed using GraphPad Prism software (Version 10.0). Multiple group comparisons were conducted via one-way analysis of variance (ANOVA) with Tukey's post hoc test. Statistical significance was defined as a p-value less than 0.05.

RESULTS

BAI alleviates cartilage degradation in ACLT induced rat OA via the P2X7 receptor: SO/FG and HE staining were used to assess the cartilage damage (Fig. 1). In the OA group (ACLT surgery group), articular cartilage exhibited significant structural impairment, characterized by surface defects, disordered chondrocyte arrangement with decreased cellular density, and notable proteoglycans loss (reduced red staining). Additionally, the OARSI score was significantly increased in OA group ($P<0.01$, Fig. 1C). However, the pathological injury of cartilage was alleviated and the red staining of proteoglycans has increased after BAI administration, as well as reduced OARSI score. These results demonstrate that BAI treatment effectively mitigated cartilage damage; however, this therapeutic effect was markedly abrogated by intra-articular injection of BzATP.

BAI modulates chondrocyte inflammation by regulating P2X7 receptor: To explore whether the chondroprotective effect of BAI is mediated by P2X7 receptor, immunohistochemistry and Western blotting were employed to evaluate P2X7 protein expression both in vivo and in vitro. As illustrated in Fig. 2A and 2B, P2X7 protein expression in cartilage was significantly higher in the OA group than in the Sham group ($P<0.01$), and this upregulation was attenuated by BAI treatment. Nevertheless, the regulatory effect of BAI on P2X7 was reversed following intra-articular injection of the P2X7 agonist BzATP. Consistent with these in vivo findings, similar results were observed in in vitro experiments (Fig. 2C). Collectively, these data indicate that the chondroprotective effect of BAI may be mediated through the P2X7 receptor.

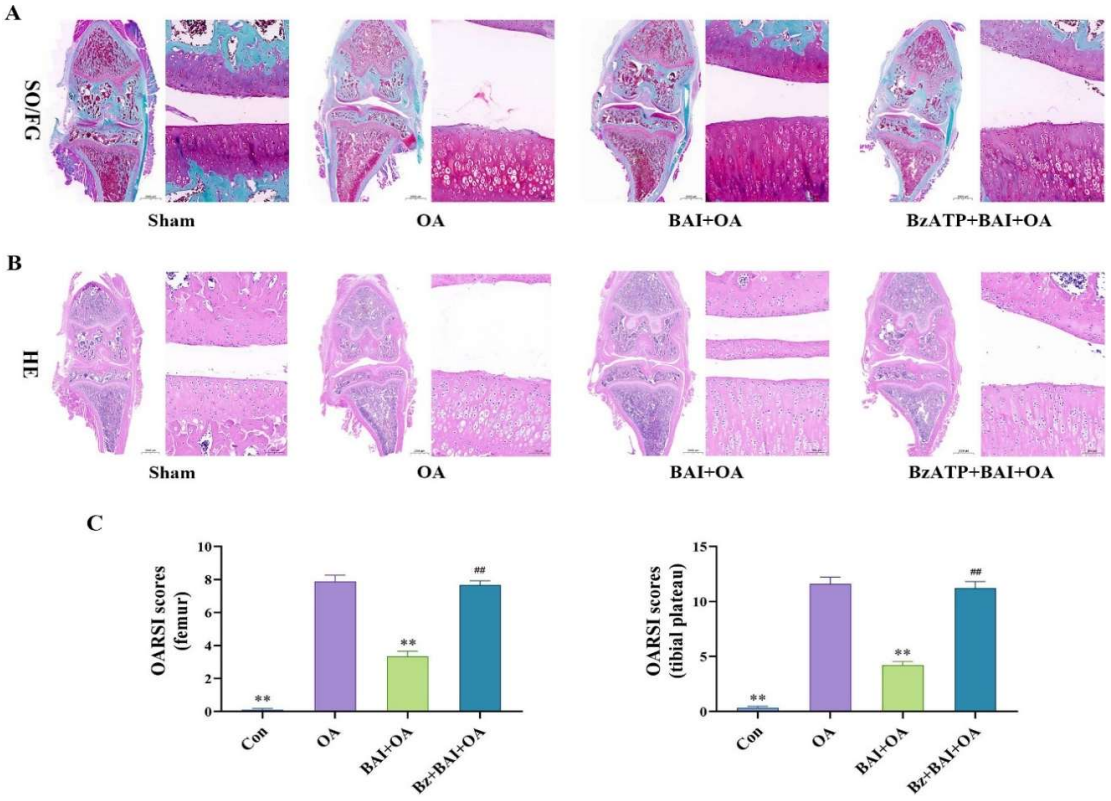

Fig. 1: BAI attenuates cartilage degeneration through P2X7 receptor in an ACLT-induced rat OA model. (A) Safranin O-fast green (SO/FG) staining and (B) hematoxylin-eosin (HE) staining in cartilage. (C) The OARSI score in the tibial plateau and femur. * indicates significant difference compared to the OA group, # indicates significant difference compared to the BAI + OA group (where * or # represents $P<0.05$, ** or ## represents $P<0.01$).

BAI modulates collagen metabolism in chondrocytes of OA rats via the P2X7 receptor: As shown in Fig. 3, immunohistochemical staining and ELISA were used to determine changes in type II collagen and metabolic markers in cartilage. BAI treatment markedly enhanced the expression of Col-II in cartilage (Fig.3A and B) and substantially decreased the contents of CTX-II and COMP in serum (Fig. 3C) and cell supernatant (Fig. 3D), whereas BzATP significantly abrogated the effects of BAI. Our findings indicate that BAI may modulate cartilage metabolism via inhibition of the P2X7 receptor.

BAI regulates inflammatory in chondrocytes via the P2X7 receptor: As presented in Fig. 4, the contents of inflammatory cytokines detected in rat serum and chondrocyte culture supernatant are summarized. Compared with the OA group, following BAI treatment, the contents of PGE_2, COX-2, IL-1β, IL-6, and IL-18 in serum and cell supernatant were markedly reduced (P<0.01), whereas the contents of IL-10 were significantly elevated (P<0.01). However, BzATP substantially abrogated the anti-inflammatory effects of BAI on cartilage. Our findings indicate that BAI may modulate cartilage inflammation through inhibition of the P2X7 receptor.

BAI suppresses pyroptosis and matrix degradation in rat OA cartilage via the P2X7 receptor: Immunohistochemical analysis of cartilage is presented in Fig. 5. Relative to the OA group, BAI treatment significantly downregulated the levels of MMP13, NLRP3, caspase-1, and GSDMD-N in cartilage (P<0.01). Notably, intra-articular injection of BzATP substantially diminished the therapeutic effects of BAI on inhibiting cartilage pyroptosis and matrix degradation (Fig. 5A). In the chondrocyte OA model, BAI significantly reduced the protein levels of NLRP3, caspase-1 p20, GSDMD-N, ADAMTS-5, and MMP13 proteins (P<0.01), whereas BzATP effectively reversed these BAI-induced regulatory effects (Fig. 5B). Collectively, these results demonstrate that BAI play a critical role in suppressing chondrocyte pyroptosis and cartilage matrix degradation by modulating the P2X7 receptor.

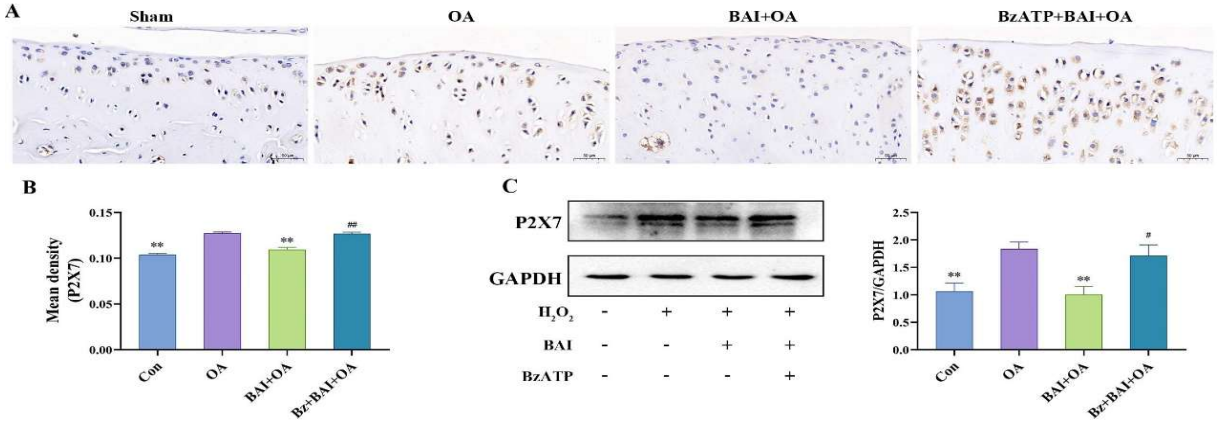

Fig. 2. BAI suppresses P2X7 expression in OA development both in vivo and in vitro. (A) and (B) Immunohistochemical staining and average optical density for P2X7 protein in articular cartilage. (C) Relative expression levels of P2X7 protein in chondrocytes (n=3). * indicates significant difference compared to the OA group, # indicates significant difference compared to the BAI + OA group (where * or # represents P<0.05, ** or ## represents P<0.01).

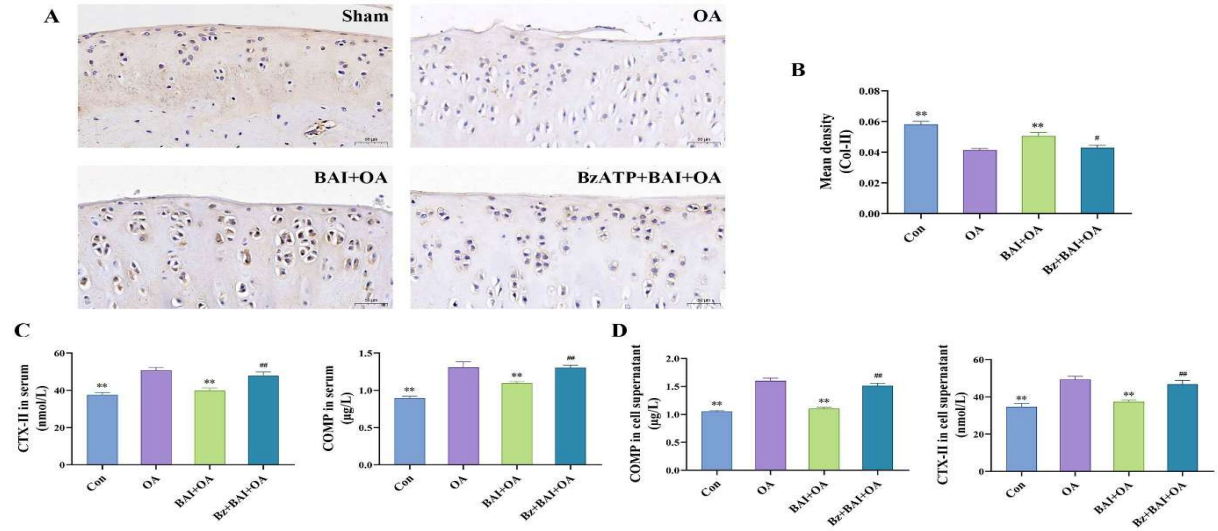

Fig.3: BAI regulates collagen II metabolism in rat OA both in vivo and in vitro. (A) and (B) Immunohistochemical staining and average optical density for collagen II (Col-II) in articular cartilage. The levels of CTX-II (C) and COMP (D) in serum and cell supernatant (n=6). * indicates significant difference compared to the OA group, # indicates significant difference compared to the BAI + OA group (where * or # represents P<0.05, ** or ## represents P<0.01).

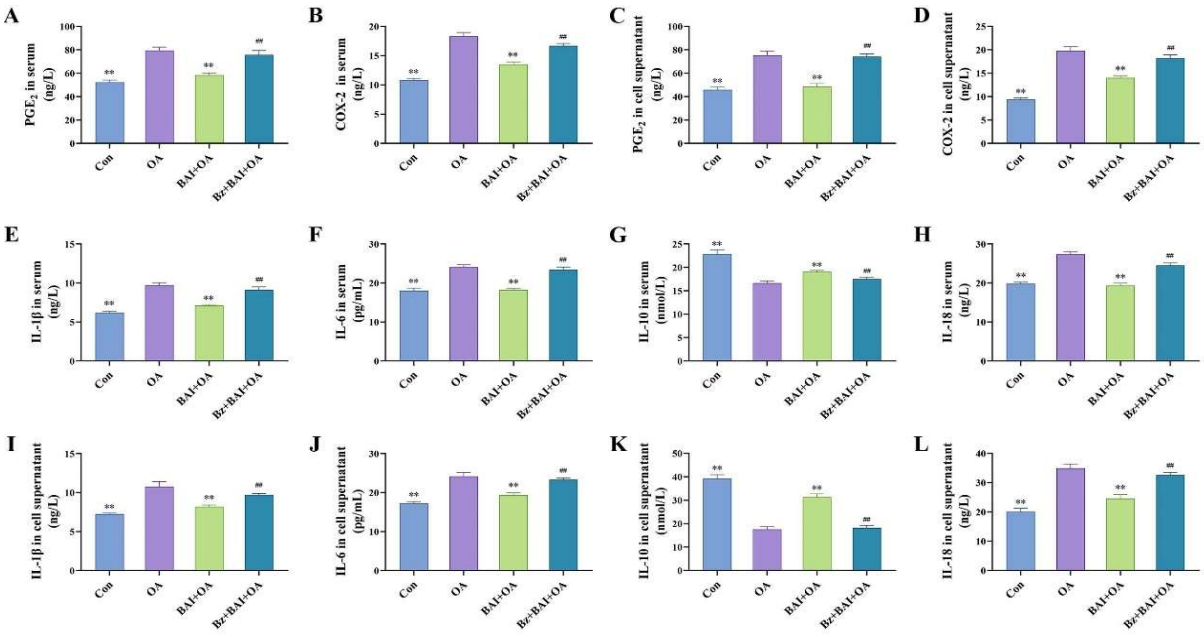

Fig.4: BAI inhibits the inflammatory response by P2X7 receptor in rat OA. (A) PGE_2, (B) COX-2, and (E-H) IL-1β, IL-6, IL-10, and IL-18 in serum (n =6). (C) PGE_2, (D) COX-2, and (I-L) IL-1β, IL-6, IL-10, and IL-18 in cell supernatant (n =6). * indicates significant difference compared to the OA group, # indicates significant difference compared to the BAI + OA group (where ** or ## represents P<0.01).

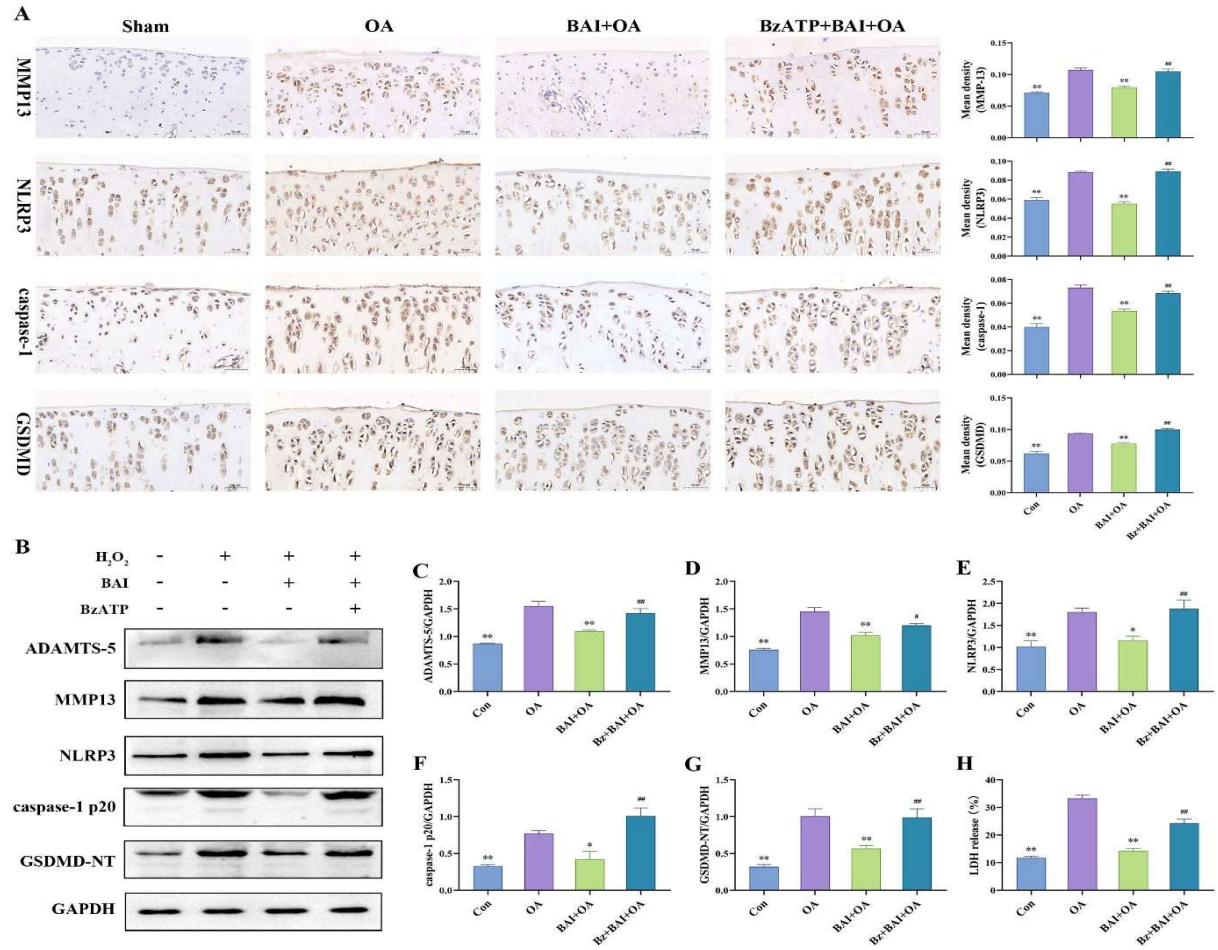

Fig. 5: BAI inhibits pyroptosis and matrix degradation in OA both in vivo and in vitro. (A) Immunohistochemical staining and average density for MMP13, NLRP3, caspase-1, and GSDMD (n =3). (B-G) Relative protein expression levels of ADAMTS-5, MMP13, NLRP3, caspase-1, and GSDMD in chondrocytes OA (n =3). (H) LDH levels in chondrocytes (n =6). * indicates significant difference compared to the OA group, # indicates significant difference compared to the BAI + OA group (where * or # represents P<0.05, ** or ## represents P<0.01).

DISCUSSION

The initial pathological features of OA are still unclear, but the degeneration of articular cartilage is a key event involved. Chondrocytes, the only cell type in cartilage, are driven into a pro-inflammatory state during OA which promotes joint inflammation. IL-1β and TNFα contribute to OA inflammation via activating NF-κB pathway and other signaling pathways, leading to ECM degradation and pain (Bai *et al.*, 2020b; Liu *et al.*, 2023; Sun *et al.*, 2024). In our present study, our results showed activation of the NLRP3 inflammasome, a key mediator in the production of mature IL-1β, along with increased levels of cleaved caspase-1 and Gasdermin-D (GSDMD) in both *In vivo* and *In vitro* models in cartilage. This is consistent with our previous research findings that NLRP3 is involved in inflammation in OA cartilage (Bai *et al.*, 2022). In addition, the administration of baicalein inhibited classic pyroptosis pathway proteins and cytokines. As is reported, NLRP3-induced pyroptosis caused cell death and, moreover, triggered inflammation. Our results indicated the anti-inflammatory effects of baicalein. Multiple studies have focused on the anti-inflammatory activity of baicalein, such as endometriosis (Park *et al.*, 2024), OVA-induced airway inflammation (Peng *et al.*, 2024), and neurodegenerative disease (Yan *et al.*, 2020). Thereby, the effect of baicalein targeting OA inflammation needs more attention.

Although NLRP3/caspase-1 axis is involved in the anti-inflammatory mechanism of baicalein, the upstream targets are still unknown. P2X7R is the switch of inflammation, and also an activator of NLRP3 inflammasome, displayed as inducing the upstream event of NLRP3, such as the intracellular K^+ decrease, intracellular Ca^{2+} increase, and ROS production (Yaron *et al.*, 2015). Our findings demonstrate increased expression of the P2X7 receptor in osteoarthritic cartilage, while administration of baicalein decreased the P2X7 expression. In order to verify whether P2X7 activated NLRP3-induced axis, we performed BzATP (a selective P2X7R agonist) before baicalein administration in OA rats, and the results showed BzATP partly eliminated the effects of baicalein in NLRP3-induced inflammation, such as downregulating the expression of NLRP3, caspase-1, GSDMD, and the decreased levels of IL-1β and IL-18. These results suggested that NLRP3-induced inflammation, activated by P2X7, is involved in the effects of baicalein in OA. The previous study has indicated the upregulated expression of P2X7 mRNA in OA cartilage; furthermore, blocking of P2X7 with a selective antagonist inhibited cartilage degradation and NF-κB pathway(Hu *et al.*, 2016). Interestingly, the early activation of P2X7 delayed OA development in the OA rat model; further excessive activation of P2X7 plays a negative role in cartilage, such as increasing apoptosis(Li *et al.*, 2021c). Our present study detected the P2X7 expression six weeks after ACLT operation; however, the upregulation of P2X7 was not accompanied by OA protective effects, but rather by the loss of the articular cartilage layer. This indicated the overexpression of P2X7 during this stage.

The regulatory effect of baicalein in OA is still in the exploratory stage, in particular anti-inflammatory effect is of great concern. Relevant evidence has been demonstrated that baicalein limits OA progression through various processes in animal models, involving inhibiting chondrocyte ferroptosis (Wan *et al.*, 2023), ameliorating subchondral bone remodelling and inhibiting synovial inflammation (Li *et al.*, 2021a). Nevertheless, our research does provide a new insight, that baicalein suppress P2X7-mediated NLRP3 activation, leading to downregulation of MMP-13, ADAMTS-5 in chondrocytes, and ultimately reducing cartilage catabolism, leading to reduced expression of MMP-13 and ADAMTS-5 in chondrocytes, and ultimately decreasing cartilage catabolism. This finding is consistent with another study indicating that the ATP/P2X7 signal is a potential target for the anti-inflammatory effect of baicalein (Nuka , 2018).

Notably, P2X7-mediated regulation of autophagy through the AMPK/mTOR signaling pathway exhibited an inhibitory effect on pyroptosis (Li *et al.*, 2021b). Whether this mechanism applies to the regulation of the P2X7-NLRP3 axis by baicalein remains to be determined.

We observed that baicalin can not only inhibit pro-inflammatory cytokines, but also suppress the contents of COX-2 and PGE-2, indicating that it may have a certain relieving effect on OA pain. PGE2 is a major COX-2 product; both of these factors are the target of NSAIDs and COX-2 inhibitors, regarding the pain symptoms of OA (Amin *et al.*, 1997). Of course, further experiments will be needed in the future to confirm our hypothesis, for instance, through behavioral experiments. The pain mechanism of OA is complex, involving multiple central and peripheral sensitization pathways. However, there is no doubt that inflammation plays an indispensable role in OA pain. Therefore, in the future, the development of drugs for relieving OA symptoms should focus on different OA phenotypes and determine the contribution of inflammation in each case.

The effect of IL-6 in OA is still uncertain. The current available evidence suggests the dual effect of IL-6. IL-6-deficient mice show a reduced presence of inflammatory cells in cartilage and exhibit decreased susceptibility to collagen-induced OA (Alonzi T, 1998). However, administration of IL-6 into the joint cavity of IL-6-deficient mice attenuates OA damage (van de Loo FA, 1997). Our results showed that IL-6 levels are slightly elevated, while baicalein reduces its expression, indicating a potential target of baicalein of IL-6 and associated molecular crosstalk. Additionally, baicalein increased the levels of IL-10, a major anti-inflammatory cytokine in OA serum. In a word, the effect of baicalein in OA rats mainly targeting inflammation in cartilage. However, our research has certain limitations. More detailed research is needed in the future to determine the anti-inflammatory mechanism of baicalein, such as its effect on the upstream activators of NLRP3 and its impact on other inflammatory-related signaling pathways.

Conclusions: The findings in our study demonstrated that baicalein inhibits OA inflammation and cartilage degradation through suppressing the P2X7-NLRP3 axis, as well as decreases pain-related cytokines, such as COX-2 and PGE-2. The present evidence suggests the protective effects of baicalein in OA as a symptom relief medication.

Authors contribution: ZZ, HB, AL, LH, and KL were responsible for methodology, investigation, and data curation. ZZ and YH contributed to conceptualization, formal analysis, and software validation. HB and PH contributed to project administration, supervision and funding acquisition. HB and ZZ prepared the initial draft of the manuscript, while YH, SD, and PH were responsible for reviewing and editing the content. All authors have read and approved the final version of the manuscript.

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