

## Research Article

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# Zoledronate promotes ECM degradation and apoptosis via Wnt/ $\beta$ -catenin

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**Abstract:** This study examined the potential mechanism of zoledronate on interleukin (IL)-1 $\beta$ -induced temporomandibular joint osteoarthritis (TMJOA) chondrocytes, using IL-1 $\beta$ -induced rabbit immortalized mandibular condylar chondrocytes cultured with zoledronate. Cell viability, apoptosis, mRNA, and protein expression of relevant genes involved in extracellular matrix (ECM) degradation, apoptosis, and Wnt/ $\beta$ -catenin signaling were examined. The involvement of the Wnt/ $\beta$ -catenin signaling was examined using Wnt/ $\beta$ -catenin inhibitor (2-(4-(trifluoromethyl)phenyl)-7,8-dihydro-5H-thiopyrano[4,3-d]pyrimidin-4-ol (XAV-939)) and activator lithium chloride (LiCl). Aggrecan and type II collagen were downregulated by zoledronate, especially with 100 nM for 48 h ( $p < 0.01$ ), consistently with the upregulation of A disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4) ( $p < 0.001$ ), matrix metalloproteinase-9 (MMP-9) ( $p < 0.01$ ), caspase-3 ( $p < 0.001$ ) and downregulation of proliferating cell nuclear antigen (PCNA) ( $p < 0.01$ ). The apoptotic rate increased from 34.1% to 45.7% with 100 nM zoledronate for 48 h ( $p < 0.01$ ). The effects of zoledronate on ADAMTS4 ( $p < 0.001$ ), MMP-9 ( $p < 0.001$ ), caspase-3 ( $p < 0.001$ ), and PCNA ( $p < 0.01$ ) were reversed by XAV-939,

while LiCl increased caspase-3 expression ( $p < 0.01$ ). In conclusion, zoledronate enhances IL-1 $\beta$ -induced ECM degradation and cell apoptosis in TMJOA chondrocytes. Wnt/ $\beta$ -catenin signaling might be involved in this process, but additional studies are necessary to determine the exact involvement of Wnt/ $\beta$ -catenin signaling in chondrocytes after zoledronate treatment.

**Keywords:** temporomandibular joint, osteoarthritis, zoledronate, extracellular matrix, apoptosis, Wnt signaling pathway

## 1 Introduction

Osteoarthritis (OA) is a degenerative disease characterized by progressive cartilage damage, bone sclerosis, osteophyte formation, and chronic pain [1]. Temporomandibular joint (TMJ) osteoarthritis (TMJOA) is an important subtype of temporomandibular disorders (TMDs). The etiology of TMJOA is complex and multifactorial; it is often idiopathic since the TMJ performs the most complicated movement in the human body [2,3]. Inflammatory cytokines are increased in the synovial fluid of patients with TMJOA or animal models, suggesting the importance of inflammation in the progression of TMJOA [4,5]. The catabolic enzymes in the cartilage matrix that are involved in the degradation of the cartilage and destruction of subchondral bone are upregulated during the progression of TMJOA [6].

Interleukin (IL)-1 $\beta$  plays a pivotal role in cartilage destruction during the pathophysiological process of OA by promoting the release of degenerative matrix enzymes and inhibiting the synthesis of the extracellular matrix (ECM) proteins by chondrocytes [7]. IL-1 $\beta$  can induce the secretion of matrix metalloproteinases (MMPs) by chondrocytes, leading to ECM degradation and increased chondrocyte apoptosis [8,9]. Members of the A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family are more efficient than MMPs to cleave the aggrecan core protein [11]. Aggrecan and type II collagen are important components of the ECM in TMJ condyle cartilage [10,11].

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Therefore, IL-1 $\beta$  is usually used to induce chondrocyte degradation and apoptosis to study OA. Several lines of evidence derived from animal models support the involvement of the Wnt/ $\beta$ -catenin signaling pathway in the molecular mechanisms underlying cartilage degradation [12–15], as well as a potential treatment target against OA [16].

Bisphosphonates (BPs) are nonhydrolyzable analogs of inorganic pyrophosphate and are widely used for the treatment of disorders related to calcium metabolism, including osteoporosis, Paget's disease, multiple myeloma, and bone metastases [17]. They inhibit bone resorption by inducing osteoclast apoptosis. Nitrogen-containing BPs (pamidronate and risedronate) can protect the chondrocytes from dexamethasone-induced growth retardation and apoptosis [18]. Zoledronate (ZOL) can decrease the expression of vascular endothelial growth factor (VEGF) A in synovial cells, suppressing angiogenesis, reducing inflammatory changes, and alleviating pain [19]. Intra-articular injected ZOL could suppress synovial inflammation but not reduce cartilage degeneration in early OA models [20]. High-dose ZOL by intravenous injection may be chondroprotective in OA animal models [21,22]. On the other hand, BPs neither provide symptomatic relief nor slow down radiographic progression in knee OA [23]. ZOL has antiproliferative and proapoptotic effects in dental pulp stem cells and colorectal cancer cells [24,25]. Nonetheless, few studies examined the effects of ZOL on chondrocytes (especially on TMJOA chondrocytes), ECM degradation, apoptosis, and the mechanisms involved.

Several cases of TMJ disorders such as joint dislocation, ankylosis, destruction, and suppurative arthritis were reported after BP therapy, and BP-related osteonecrosis of the jaw attracted researchers' attention to the effect of ZOL on maxillofacial bone and cartilage diseases [26–28]. To the best of our knowledge, there is no evidence about the relationship between TMJOA and BP. TMJOA is a kind of OA characterized by the loss of articular cartilage and degradation of the cartilage matrix [1,2]. ZOL is widely used to treat bone-related disorders and could promote growth inhibition and apoptosis [19–22,24,25,27].

Therefore, this study aimed to examine the role and potential mechanisms of ZOL on IL-1 $\beta$ -induced TMJOA chondrocytes. We examined the expression levels of several pivotal genes and corresponding proteins involved in ECM degradation, cell apoptosis, and the Wnt/ $\beta$ -catenin signaling pathway.

## 2 Materials and methods

### 2.1 Cell culture

The rabbit immortalized mandibular condylar chondrocyte (IMCC) cell line used in this study was purchased from the Department of Oral Biology of the School of Stomatology of the Fourth Military Medical University (China). The IMCCs were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) and penicillin/streptomycin (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) at 37°C in a 5% CO<sub>2</sub> atmosphere.

A total of  $1 \times 10^5$  cell/mL IMCCs were plated on 6-well plates and treated with 10 ng/mL IL-1 $\beta$  (Novoprotein, Shanghai, China) to induce chondrocyte degradation. Samples were collected at different time points (1, 2, 3, 6, 12, and 24 h), and the expression levels of ECM-related genes were analyzed by reverse transcription-quantitative polymerase chain reaction (qRT-PCR), including aggrecan, type II collagen, ADAMTS-4, ADAMTS-5, and MMP-9.

### 2.2 Cell treatments

The chondrocytes were examined under a Leica DM4000B microscope (Leica, IL, USA) for any cellular morphological changes in the presence of the control (DMEM), 10 ng/mL IL-1 $\beta$  alone, or in combination with different concentrations (1 nM, 10 nM, 100 nM, and 1  $\mu$ M) and times (0, 24, 48, and 96 h) of ZOL (Aladin, Shanghai, China). IL-1 $\beta$ -induced IMCCs were treated with 10 or 100 nM ZOL for 24 or 48 h. Caspase-9 (apoptosis initiator) and caspase-3 (apoptosis effector) were examined by qRT-PCR and western blotting. The percentage of apoptotic cells was determined by flow cytometry. The expression of the proliferating cell nuclear antigen (PCNA) was determined [29]. XAV-939 (Selleck Chemicals, TX, USA) and LiCl (Sigma-Aldrich Co., MO, USA) were used as the inhibitor and activator of the Wnt/ $\beta$ -catenin signaling pathway, respectively, and were added to the ZOL-treated TMJOA chondrocytes for 24 and 48 h. Chondrocytes without ZOL were used as controls. The changes in chondrocyte morphology were examined using an inverted phase microscope (Nikon, Tokyo, Japan).

## 2.3 qRT-PCR

The total RNA was extracted from chondrocytes using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The first-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed on a Roche LightCycler 480 System (Roche, Basel, CH) with the SYBR Green Master Mix (Starbiolab, Hangzhou, China) and primers (Table 1). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene.

## 2.4 Western blot

The chondrocytes were lysed in ristocetin-induced platelet aggregation buffer (Beyotime Biotechnology, Shanghai, China) containing a protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA, USA). The protein concentration was determined using a bicinchoninic acid assay kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of proteins were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked with 5% nonfat milk for 1 h and incubated with primary antibody overnight at 4°C. The following primary antibodies were used to detect the proteins: rabbit anti-

aggrecan polyclonal antibody (1:500, Proteintech, Wuhan, China), rabbit anti-collagen II polyclonal antibody (1:500, Proteintech, Wuhan, China), rabbit anti-MMP-9 polyclonal antibody (1:500, Proteintech, Wuhan, China), rabbit anti-ADAMTS-4 polyclonal antibody (1:500, RuiyingBio, Suzhou, China), rabbit anti-Caspase-3 polyclonal antibody (1:500, Proteintech, Wuhan, China), and mouse anti-PCNA monoclonal antibody (1:500, RuiyingBio, Suzhou, China). The membranes were incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1,000, Beyotime, Shanghai, China) or a goat anti-mouse IgG conjugated to horseradish peroxidase (1:1,000, Beyotime, Shanghai, China) for 1 h. Incubation with a mouse anti-GAPDH monoclonal antibody (1:1,000, Abcam, Cambridge, UK) was performed as the loading sample control. The blots were revealed using the Clarity MAX™ Western enterochromaffin-like Substrate (BioRad, CA, USA) by ChemiDoc™ XRS + System (BioRad, CA, USA).

## 2.5 Flow cytometry

The chondrocytes were harvested and washed three times with phosphate-buffered saline (PBS) and resuspended in loading buffer (Sangon Biotech, Shanghai, China) to  $2-5 \times 10^5$  cells/mL. For the apoptosis analysis, the Annexin V-fluorescein isothiocyanate/propidium iodide kit (Sangon Biotech, Shanghai, China) was used strictly according to the manufacturer's protocol. The Becton, Dickinson (BD) FACSCanto™ II system (BD Biosciences, CO, USA) was used to detect the surface markers of the chondrocytes. The results were analyzed using the FlowJo software.

**Table 1:** Primer sequences used for qPCR

Gene	Primer sequences
Aggrecan	F: TTGGAGGTCGTGGTGAAGG R: TCCCGGATGCCGTAGGTT
Type II Collagen	F: ACGACATAATCTGTGAAGACCAAGG R: TGGCAGTGGCGAGGTCAGTAG
MMP-9	F: GTGAAGACGCAGACGGTGATT R: GGTACTCACACGCCAGAAGAAGC
ADAMTS-4	F: CTGACCACTTCGACACAGCCATC R: GTCATCATCTCCACGATAGCACAG
ADAMTS-5	F: AGTGTGGAGTATGCGGAGGAGAC R: TTCTTCTCAAGGCTAAGTAGGCAAGT
PCNA	F: TGAAGATAATGCGGACACCTTGCC R: TGGCTGAGGTCTCGGCATATACG
Caspase-3	F: GATTCTAAGCCACGGTGATGAAGGAG R: CACTGTCTGTCTCGATGCCACTG
Caspase-9	F: AGCCTGACCTGACCCCAAG R: CAGCCGTGAGACAGGATGACAAC
GAPDH	F: ATGGGGAAAGGTGAAGGTCG R: TAAAAGCAGCCCTGGTGACC

## 2.6 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT viability assay was performed to determine the appropriate concentrations of XAV-939 and LiCl. Chondrocyte viability was investigated using MTT (Sigma St Louis, MO, USA). Briefly, the chondrocytes (5,000 cells/well, 100  $\mu$ L medium) were cultured in a 96-well plate (Corning, NY, USA) and 20  $\mu$ L of MTT solution (5 mg/mL in PBS) was added and incubated for 4 h at 37°C. The medium was removed, 100  $\mu$ L of dimethyl sulfoxide was added (DMSO, Sigma St Louis, MO, USA), and the optical density was measured at 490 nm using a plate reader (Bio-Tek, Winooski, VT, USA).

## 2.7 Statistical analysis

All data were presented as mean  $\pm$  SEM from three independent experiments and were analyzed using analysis of variance and Student's *t*-test using SPSS 21 (IBM, Armonk, NY, USA). Plots were drawn using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). The *p*-values of  $< 0.05$  were considered statistically significant.

## 3 Results

### 3.1 Induction of IMCC degradation by IL-1 $\beta$ treatment for 6 h

After rabbit IMCCs were treated with 10 ng/mL of IL-1 $\beta$ , the expression of aggrecan, type II collagen, ADAMTS-4, and ADAMTS-5 did not significantly change after 1 and 3 h of culture (all  $p > 0.05$ ) but were all higher compared with controls at 6 h (all  $p < 0.05$ ) (Figure 1a–d). Compared with controls, the expression of MMP-9 was not significantly different at 1 h, was lower at 3 h ( $p < 0.05$ ), but was higher at 6 h ( $p < 0.01$ ) (Figure 1e). Therefore, the TMJOA cells were treated with 10 ng/mL of IL-1 $\beta$  for 6 h for the subsequent experiments, which could induce a decrease in chondrocytes ECM and upregulation of ADAMTS and MMPs.

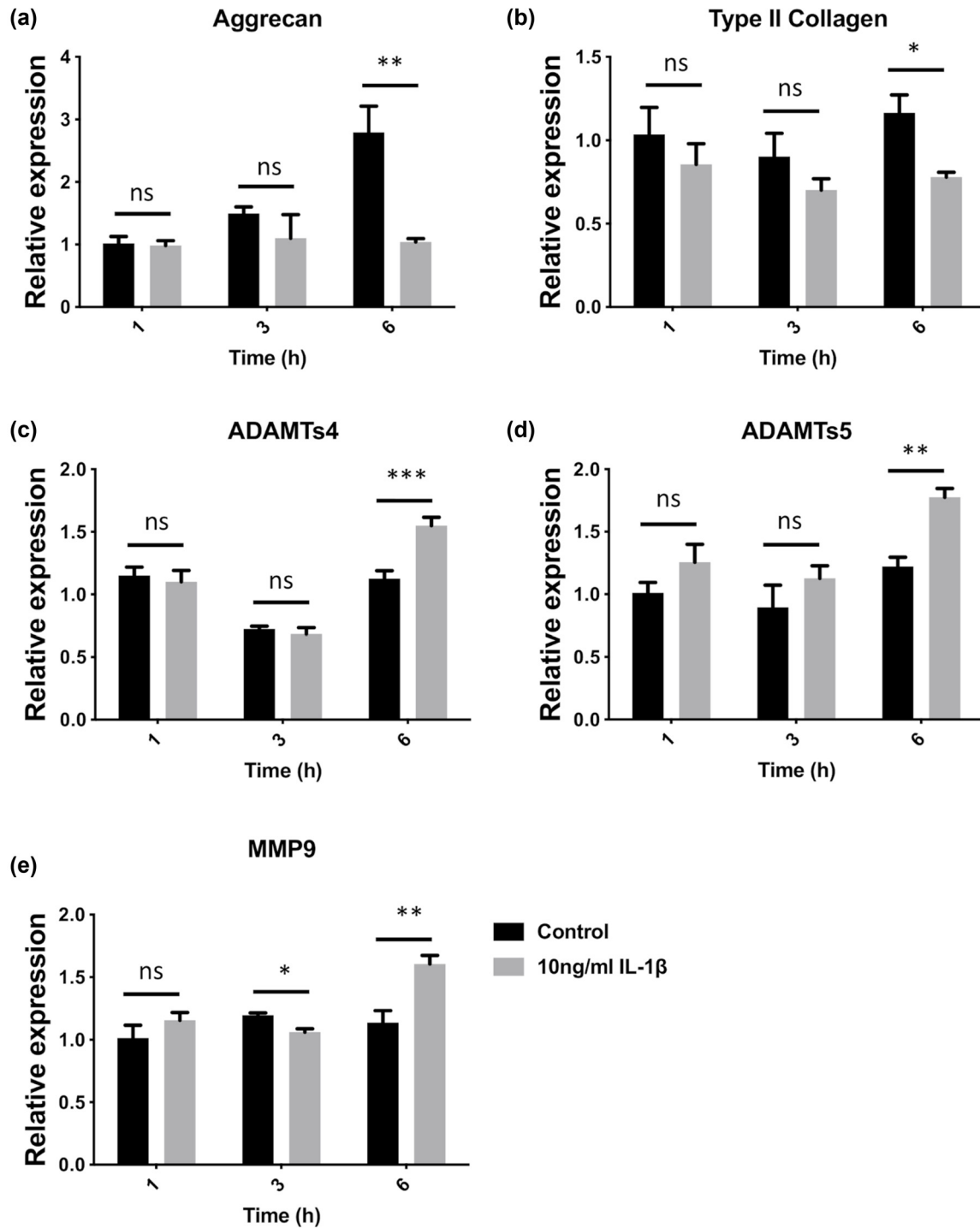
### 3.2 Changes in chondrocytes morphology and ECM degradation after ZOL treatment

The cellular morphology of the chondrocytes was polygonal with clear-cut boundaries and compact arrangement when being untreated or early during 10 ng/mL IL-1 $\beta$  treatment. With the increase of ZOL concentrations and the prolongation of treatment time, the chondrocytes became sparsely arranged, with irregular and elongated shapes. The typical proapoptotic and antiproliferation morphological changes appeared in the presence of 10 or 100 nM ZOL for 24 or 48 h (Figure 2). Figure 3a shows that ZOL at 10 nM for 24 h resulted in an increase in messenger RNA (mRNA) aggrecan expression at 24 h compared with IL-1 $\beta$  controls ( $p < 0.05$ ), while 100 nM ZOL led to a decrease in aggrecan expression at 48 h ( $p < 0.001$ ).

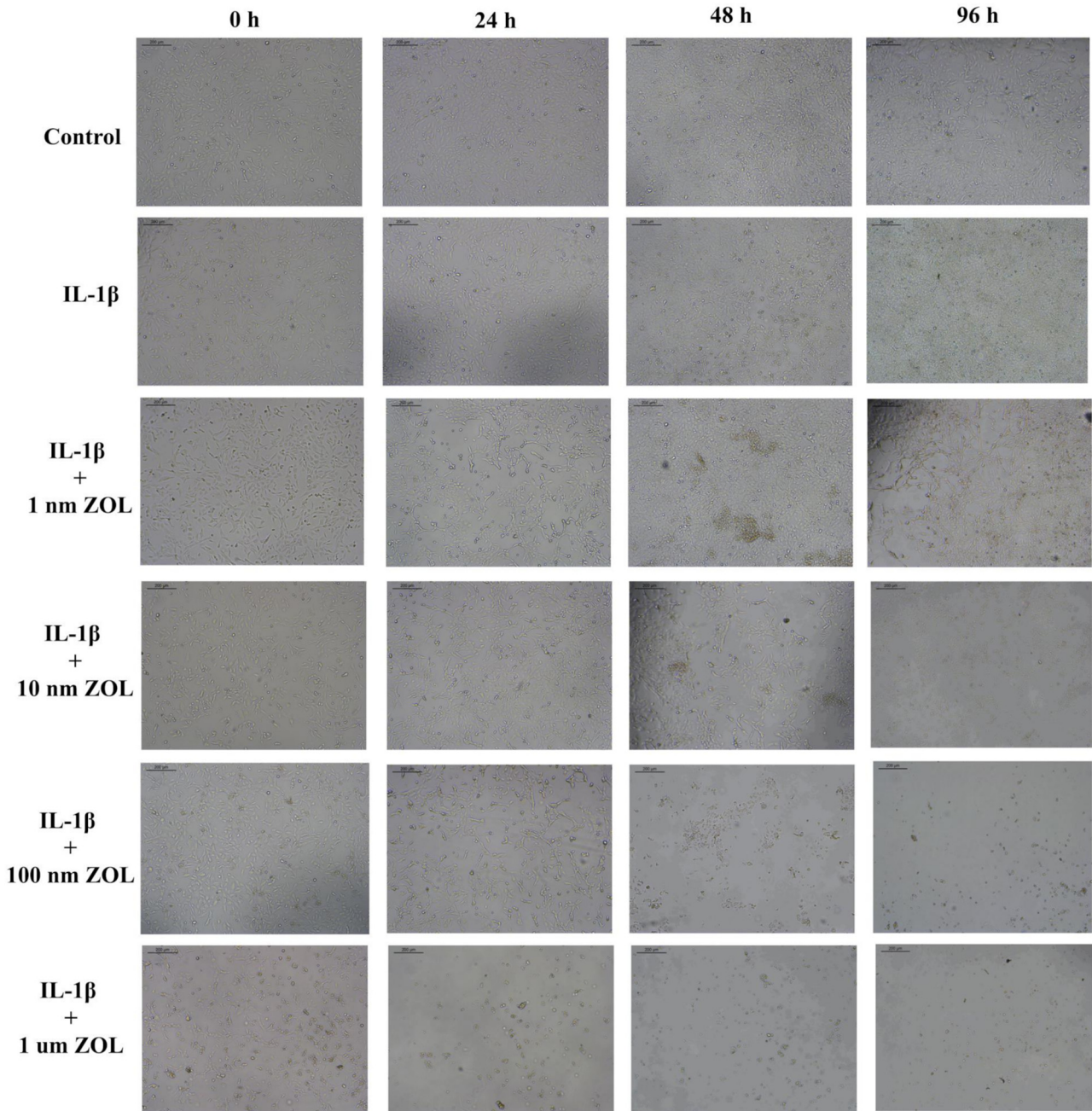
Figure 3b shows that ZOL 10 nM led to a decreased mRNA expression of type II collagen at 48 h ( $p < 0.05$ ), while 100 nM ZOL led to a decreased type II collagen expression at 24 ( $p < 0.001$ ) and 48 h ( $p < 0.01$ ). The mRNA expression of ADAMTS-4 first decreased at 24 h with 10 nM ZOL ( $p < 0.001$ ) but increased at 48 h ( $p < 0.01$ ) (Figure 3c), while it was increased with 100 nM ZOL at 24 ( $p < 0.05$ ) and 48 h ( $p < 0.001$ ). The mRNA expression of ADAMTS-5 was increased at 48 h with 10 nM ZOL ( $p < 0.01$ ) and decreased at 24 h with 100 nM ZOL ( $p < 0.05$ ) (Figure 3d). Figure 3e shows that the mRNA expression of MMP-9 was increased with 10 nM ZOL at 48 h ( $p < 0.01$ ), as well as with 100 nM ZOL at 24 ( $p < 0.01$ ) and 48 h ( $p < 0.01$ ). Figure 3f shows that the protein expression of those five proteins generally followed the mRNA trends. The results indicated that ZOL would cause the alterations of TMJOA ECM-related gene and protein expression and induce chondrocyte degradation.

### 3.3 Proapoptotic and anti-proliferation effects of ZOL on IL-1 $\beta$ -induced TMJOA chondrocytes

To assess the effect of ZOL on the apoptosis and proliferation of IL-1 $\beta$ -induced TMJOA chondrocytes, the mRNA and protein expression levels of caspase-3, caspase-9, and PCNA were determined. It was observed that the mRNA expression levels of caspase-3 and Caspase-9 were upregulated when treated with 10 nM for 24 ( $p < 0.05$ ) and 48 h ( $p < 0.001$ ), respectively (Figure 4a and b). The mRNA expression of PCNA was significantly downregulated when the cells were treated with 10 and 100 nM (both  $p < 0.001$ ) ZOL for 48 h (Figure 4c). Caspase-3 was upregulated, and PCNA was downregulated with ZOL treatment, independently of time and concentration (Figure 4d). Flow cytometry was performed to verify whether ZOL would affect cell apoptosis in TMJOA cells. The results showed no obvious differences after treatment for 24 h no matter which concentration, but after 48 h, the apoptotic rate increased from 34.1 to 45.7% in response to 100 nM ZOL (Figure 4e). It indicates that ZOL could induce TMJOA cell apoptosis in a dose- and time-dependent manner. When combining those results, ZOL could induce TMJOA cell apoptosis by upregulating caspase genes and suppressing the proliferation gene, especially with 100 nM for 48 h.



**Figure 1:** Expression levels of ECM-related genes in IL-1 $\beta$ -treated rabbit IMCCs. Expression levels of (a) aggrecan, (b) type II collagen, (c) ADAMTS-4, (d) ADAMTS-5, and (e) MMP-9 were analyzed by qRT-PCR. All data are presented as mean  $\pm$  SEM. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, ns: not significant.



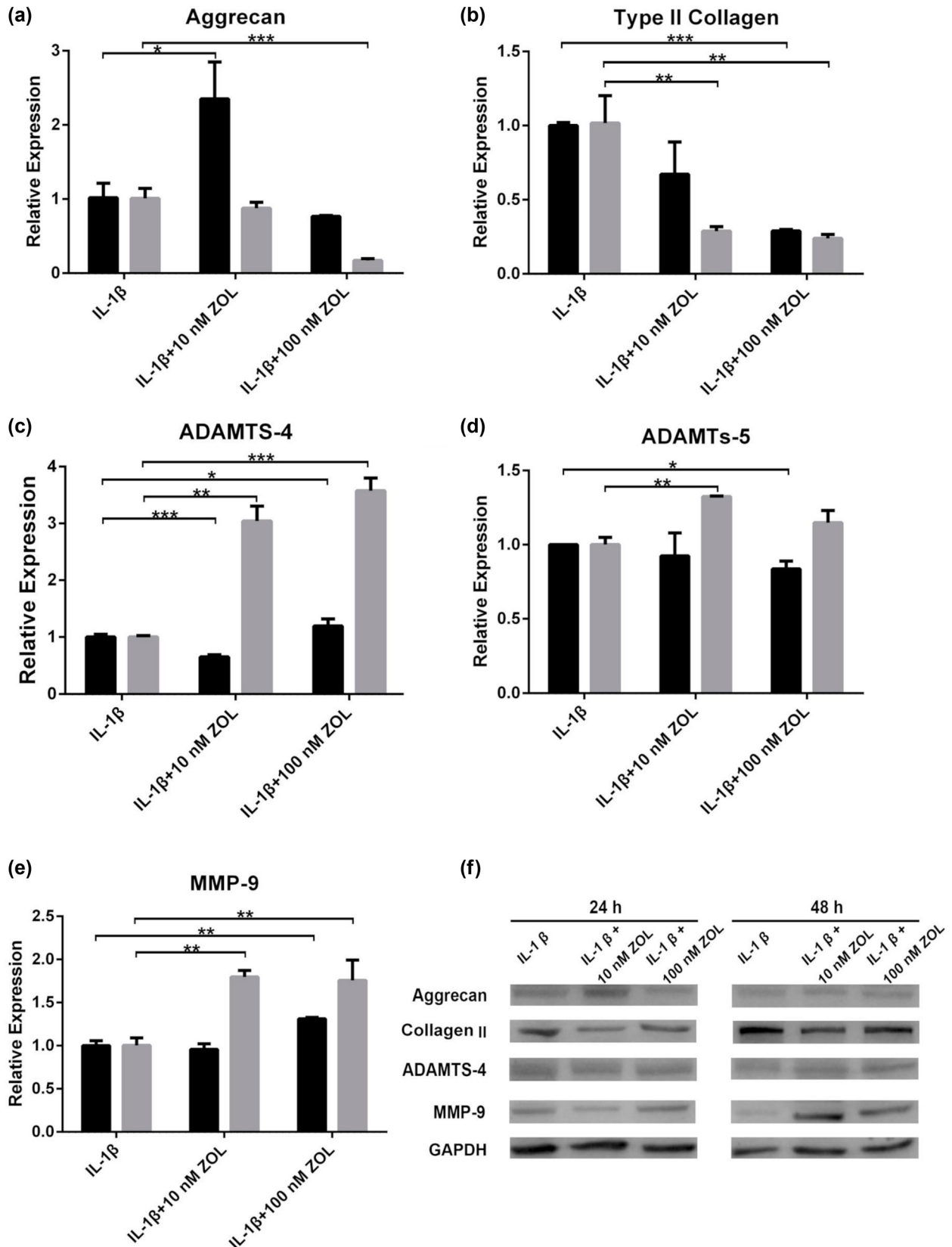
**Figure 2:** The morphological and abundance changes of TMJOA chondrocytes treated with different concentrations and times of ZOL (scale bar = 200  $\mu\text{m}$ ).

### 3.4 ZOL affects chondrocyte degradation, involving the Wnt/ $\beta$ -catenin signaling pathway

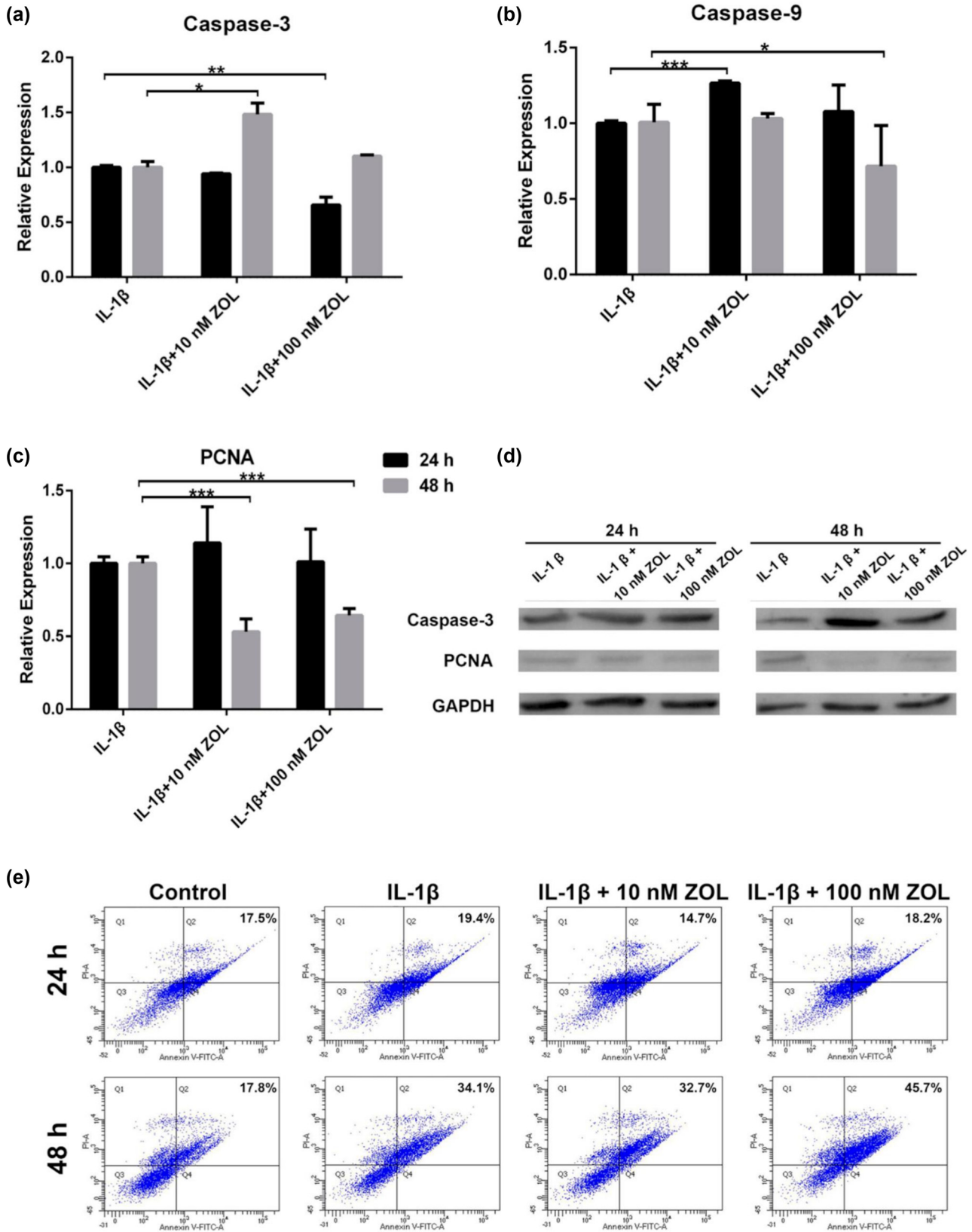
We performed the MTT viability assay to select appropriate concentrations of XAV-939 and LiCl. According to the trend in cell viability (Figure 5a and b) and previous

studies [30,31], we selected 1  $\mu\text{M}$  XAV-939 and 45 mM LiCl for the subsequent experiments.

After treating TMJOA chondrocytes with 100 nM ZOL for 48 h, LiCl was added to activate the Wnt/ $\beta$ -catenin signaling. Aggrecan, ADAMTS-4, and MMP-9 showed no changes compared with ZOL alone. On the other hand, the Wnt/ $\beta$ -catenin signaling inhibitor (XAV-939) upregulated

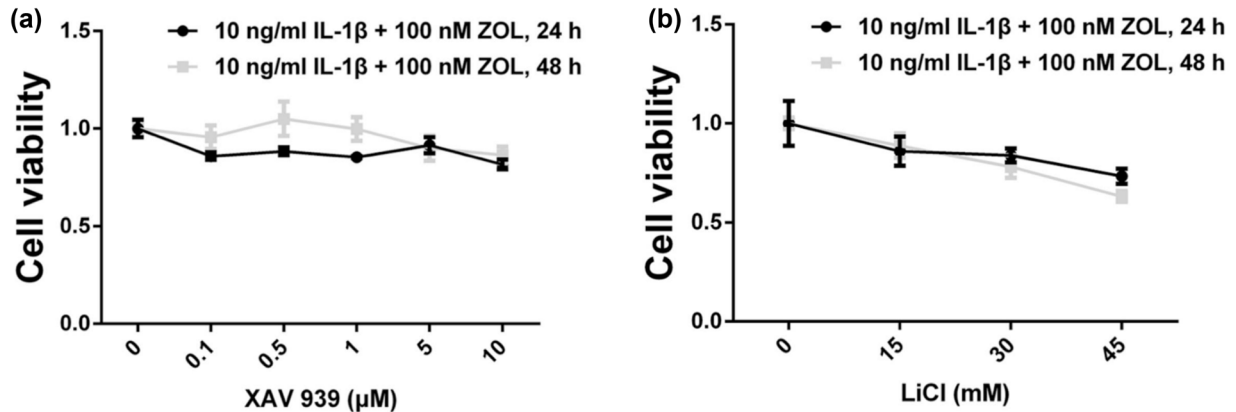


**Figure 3:** Expression levels of ECM-related genes and proteins after treatment with 10 or 100 nM ZOL in TMJOA chondrocytes. (a) Aggrecan, (b) type II collagen, (c) ADAMTS-4, (d) ADAMTS-5, and (e) MMP-9 were analyzed by qRT-PCR. (f) ECM-related proteins were detected by western blotting. Black bars represent the control group, and gray bars represent the 10 ng/mL IL-1 $\beta$  group. All data are presented as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  between the two groups linked by the line; all other comparisons are not significant.



**Figure 4:** Expression levels of apoptosis- and proliferation-related genes, proteins, and percentage of apoptotic cells after treatment with 10 or 100 nM ZOL in IMCCs. (a) Caspase-3, (b) Caspase-9, and (c) PCNA were analyzed by qRT-PCR. (d) Caspase-3 and PCNA proteins were detected by western blotting. (e) Flow cytometry analysis for the apoptotic cells. All data are presented as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  between the two groups linked by the line; all other comparisons are not significant.





**Figure 5:** Determination of the concentrations of XAV-939 and LiCl. Cell viability was measured by the MTT assay after XAV-939 (a) or LiCl (b) and 100 nM ZOL treatment of TMJOA cells for 48 h.

aggrecan ( $p < 0.001$ ) and downregulated ADAMTS-4 ( $p < 0.001$ ) and MMP-9 ( $p < 0.001$ ) (Figure 6a–c). Caspase-3 was upregulated by LiCl and downregulated by XAV-939 ( $p < 0.001$ ) (Figure 6d). PCNA was upregulated with XAV-939 treatment ( $p < 0.01$ ) (Figure 6e). Western blotting was conducted to examine the possible involvement of the Wnt/ $\beta$ -catenin signaling in the ZOL-induced regulation of chondrocytes degradation, and the results were consistent with the mRNA changes (Figure 6f). The results indicate that the effects of ZOL on TMJOA could involve the Wnt/ $\beta$ -catenin signaling pathway.

## 4 Discussion

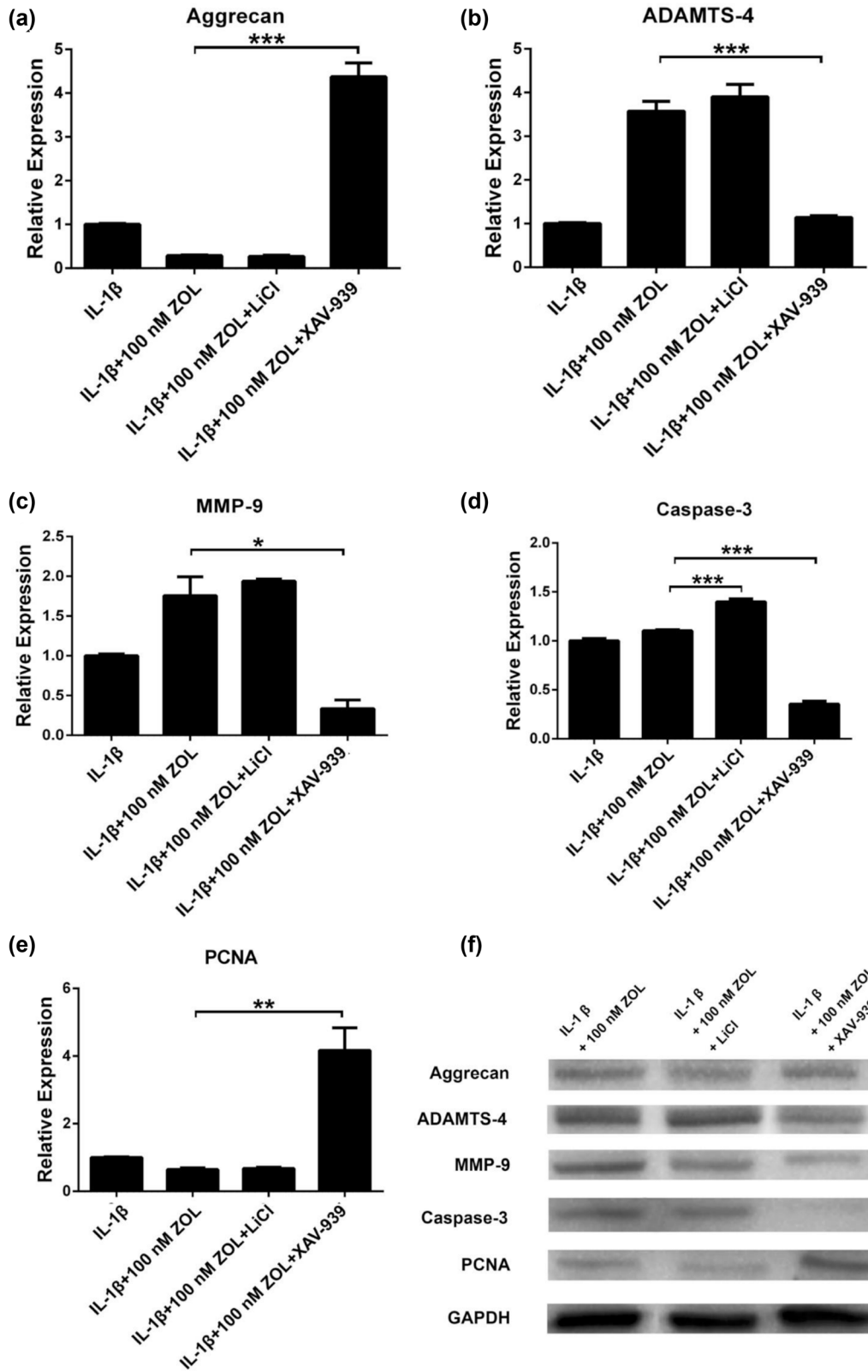
Few studies focused on the effects of ZOL on chondrocytes, especially on TMJOA chondrocytes, ECM degradation, apoptosis, and related mechanisms. This study examined the role and potential mechanism of ZOL on IL-1 $\beta$ -induced TMJOA chondrocytes. The results suggest that ZOL enhances the IL-1 $\beta$ -induced ECM degradation and cell apoptosis in TMJOA chondrocyte models. The results also suggest that Wnt/ $\beta$ -catenin signaling might be involved in this process, but additional studies are necessary to determine the exact involvement.

This study showed that IL-1 $\beta$ , as an inflammatory activator, increased the production of ECM by chondrocytes, which would be conducive to the development of OA while ZOL decreased ECM production, increased ECM degradation, and induced the apoptosis of chondrocytes, especially at a high concentration and over a long-term treatment in IL-1 $\beta$ -induced TMJOA chondrocyte model. On the other hand, Cinar *et al.* [20] showed that ZOL had chondroprotective effects in rats, which was also

observed in dogs [22]. Nevertheless, direct comparisons among these studies are difficult since the type and activity of the BPs used in those studies are different, the models are different, and the treatment time and doses are different. Therefore, considering those conflicting results, studies are still necessary to determine the effect of ZOL on TMJOA. It is possible that different joints react differently to ZOL because of different conditions like weight-bearing. Studies specifically aiming at TMJOA are necessary.

Studies showed that high-dose ZOL by intravenous injection might be chondroprotective in an OA animal model [21,22]. A recent meta-analysis draws a contradictory conclusion to prior reviews that BPs could not provide symptomatic relief or defer radiographic progression in knee OA [23], as supported by a more recent trial [32]. On other aspects, ZOL could inhibit the growth of hematopoietic cell transplant 116 colorectal cancer cells *in vitro* and *in vivo* and induce apoptosis through the mitochondria pathway [24]. ZOL also has antiproliferative and proapoptotic effects in dental pulp stem cells [25]. Yi *et al.* [19] showed that ZOL modulated the expression of VEGF-a and could affect the progression of OA. Bagi *et al.* [33] showed that BPs could not slow the progression of OA in rat models with traumatic OA. A recent study showed that ZOL had protective effects in rabbit OA models [34].

Wnt signaling is a conserved pathway associated with the response to cell differentiation and fate determination during embryogenesis and the late stages of development [35]. Evidence suggests that the activation of the Wnt/ $\beta$ -catenin signaling, the canonical pathway, could be involved in cartilage destruction in arthritis [14,36]. Notably, in this study, when Wnt/ $\beta$ -catenin signaling was activated, the expression levels of apoptosis genes were increased in ZOL-treated TMJOA chondrocytes, and



**Figure 6:** Involvement of Wnt/ $\beta$ -catenin signaling in the effects of ZOL to TMJOA degradation. Relative mRNA expression levels of (a) aggrecan, (b) ADAMTS-4, (c) MMP-9, (d) Caspase-3, and (e) PCNA were analyzed by qRT-PCR. (f) Western blotting analysis of those proteins. All data are presented as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  between the two groups linked by the line; all other comparisons are not significant.

when it was inhibited, ECM-related genes and proteins were increased and apoptosis caused by ZOL was reversed. Those results suggest the involvement of the Wnt/ $\beta$ -catenin signaling in TMJOA and the response of TMJOA chondrocytes to ZOL, but the determination of the exact regulation of Wnt/ $\beta$ -catenin signaling will require additional studies, including gene knockout, overexpression, and silencing.

It is interesting to note that caspase-3 decreased in response to IL-1 $\beta$  + 100 nM ZOL compared with IL-1 $\beta$  + 10 nM ZOL (Figure 4d), but apoptosis increased in response to IL-1 $\beta$  + 100 nM ZOL compared with IL-1 $\beta$  + 10 nM ZOL (Figure 4e) for 48 h. These results indicated that the apoptosis of chondrocytes was not only regulated by caspase-3 but also by other apoptosis factors that were not examined in this study. This study indicated that 48 h and a high concentration of ZOL can promote the apoptosis of chondrocytes, which may be affected by caspase-3, but future studies will have to examine multiple proteins involved in apoptosis.

This study has limitations. It was performed in cells, and the translation of the results to an *in vivo* setting and humans is unknown. In addition, no blank group was set up in the study design. Only a small panel of genes and proteins was investigated, and the exact mechanisms are not elucidated. Examining those pathways will have to be carried out in future studies. Finally, animal studies will have to be performed to verify the results and determine the effects of ZOL on joint development and joint diseases.

In conclusion, this is the first study that investigated the changes in aggrecan, collagen II, matrix metalloproteinase, and chondrocyte apoptosis in IL-1 $\beta$ -treated TMJ chondrocytes after ZOL treatment, and found that ZOL would enhance ECM degradation, promote cell apoptosis, and suppress cell proliferation in rabbit TMJOA cells and that these effects might involve the Wnt/ $\beta$ -catenin signaling, but additional studies are necessary for confirmation of the exact involvement of Wnt/ $\beta$ -catenin signaling in chondrocytes after ZOL treatment.

## Abbreviations

TMJ	temporomandibular joint
TMJOA	temporomandibular joint osteoarthritis
ECM	extracellular matrix
IMCCs	immortalized mandibular condylar chondrocytes
PCNA	proliferating cell nuclear antigen

OA	osteoarthritis
TMD	temporomandibular disorders
IL	interleukin
MMPs	matrix metalloproteinases
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
BPs	bisphosphonates
BRONJ	BP-related osteonecrosis of the jaw
IMCC	immortalized mandibular condylar chondrocyte
qRT-PCR	reverse transcription-quantitative polymerase chain reaction
ZOL	zoledronate

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**Author contributions:** JX, GC, and GX conceived the idea, designed the experiments, and interpreted the data. JX and YL performed the experiments, analyzed the data, and wrote the manuscript. All authors reviewed and approved the manuscript.

**Conflict of interest:** The authors of this work have nothing to disclose.

**Data availability statement:** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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