β-Ecdysone attenuates cartilage damage in a mouse model of collagenase-induced osteoarthritis via mediating FOXO1/ADAMTS-4/5 signaling axis

Authors: Junzhu Han, Jianzhong Guan and Xunbing Zhu

DOI: 10.14670/HH-18-341
Article type: ORIGINAL ARTICLE
Accepted: 2021-04-20
Epub ahead of print: 2021-04-20
β-Ecdysone attenuates cartilage damage in a mouse model of collagenase-induced osteoarthritis via mediating FOXO1/ADAMTS-4/5 signaling axis

Running title: β-Ecdysone mitigates osteoarthritis.

Junzhu Han 1,2, Jianzhong Guan 1,3, Xunbing Zhu 2,4

1 Department of Orthopedics, Jinan University, Guangzhou, Guangdong Province, China; 2 Department of Orthopedics, the Second Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui Province, China; 3 Department of Orthopedics, the First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui Province, China; 4 Anhui Key Laboratory of Tissue Transplantation, Bengbu, Anhui Province, China.

* Corresponding author: Dr. Jianzhong Guan, Department of Orthopedics, the First Affiliated Hospital of Bengbu Medical College, No. 287 Changhuai Road, Bengbu 233000, Anhui Province, China. E-mail: jzguan2002@163.com; Tel: +86-05523086162.
Abstract

Background: β-Ecdysone has been reported to perform a protective effect to prevent interleukin 1β (IL-1β)-induced apoptosis and inflammatory response in chondrocytes. In our study, the chondroprotective effects of β-Ecdysone were explored in a mouse model of collagenase-induced osteoarthritis (OA).

Methods: Injection of collagenase in the left knee was implemented to establish a mouse model of OA. The histomorphological analysis was detected using safranine O staining. Serum pro-inflammatory cytokines were measured by ELISA assays. Protein expression in the femur and chondrocytes was analyzed using western blot. Chondrocyte apoptosis was evaluated by terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) staining.

Results: Treatment of OA mice with β-Ecdysone supplementation significantly inhibited the production of pro-inflammatory cytokines. Histologic examination exhibited that the degradation of proteoglycans and the loss of trabecular bone were observed in collagenase-injected mice. However, OA-like changes were attenuated by β-Ecdysone administration in collagenase-injected mice. Both in vivo and in vitro models, nuclear forkhead box O1 (FOXO1) protein expression was significantly reduced in the femur of collagenase-treated mice and IL-1β-stimulated chondrocytes. However, β-Ecdysone treatment was able to rescue FOXO1 protein expression in the nucleus to inhibit the transcription and translation of a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4 (ADAMTS-4) and ADAMTS-5.

Conclusion: The findings suggested that β-Ecdysone functioned as a FOXO1 activator to protect collagenase-induced cartilage damage. FOXO1 might be a potential molecular target of β-Ecdysone for the effective prevention and treatment of OA.
**Keywords:** β-Ecdysone; osteoarthritis; FOXO1; chondrocyte
Introduction

OA is a degenerative joint disease, which is characterized by the degradation of articular cartilage, thickening of subchondral bone and formation of osteophytes that evoke severe joint pain and disability in elderly populations worldwide (Cordaro et al., 2019; Fusco et al., 2020; Gugliandolo and Peritore, 2020). Recently, naturally-derived products contain a variety of bioactive molecules that have been shown to prevent the progression of OA (Bannuru et al., 2018; Yu et al., 2020).

β-Ecdysone is recognized as a natural steroid-like compound that can be extracted from arthropods and is implicated in the initiation of metamorphosis, also known as the molting process (Dai et al., 2015a). β-Ecdysone is also identified and purified from multifarious medicinal plants, such as achyranthes root and Tinospora cordifolia (Boo et al., 2010; Seidlova-Wuttke et al., 2010). β-Ecdysone, as a multi-potent agent which performs a variety of biological functions to alleviate neurological disorders (Chakraborty and Basu, 2017), glucocorticoid-induced osteoblast apoptosis, and ovariectomy-evoked bone loss and chondrocyte dysfunction (Kapur et al., 2010; Dai et al., 2015b, 2017). A previous study corroborates that β-Ecdysone increased the thickness of joint cartilage, growth plate and the proliferative and hypertrophic zones of the epiphyseal growth plate in estrogen deficiency-induced osteoporotic rats (Kapur et al., 2010). However, the chondroprotective activities and underlying molecular mechanism of β-Ecdysone are unclear in a mouse model of collagenase-induced OA.

FOXO1 is a ubiquitously expressed transcriptional regulator that modulates cellular differentiation, angiogenesis, tumor progression, autophagy, apoptosis and lifespan and can
be mobilized in response to oxidative stress, DNA damage and inflammatory response (van der Horst and Burgering, 2007; Luo et al., 2015; Kim et al., 2018; Xing et al., 2018). In addition, FOXO1 plays a crucial role in the pathogenesis of age-related diseases, such as primary osteoporosis, Alzheimer's syndrome, rheumatoid arthritis and degenerative joint disease (Lowe et al., 2007; Iyer et al., 2013; Grabiec et al., 2015; Lee et al., 2020). Mechanically, the physiological function of FOXO1 is dragged by its phosphorylation and nucleocytoplasmic shuttling that lead to nuclear export and silencing of FOXO1-dependent transcriptional function (Yuan et al., 2009; Peng et al., 2020). In the human aged joint, the downward trend of FOXO1 expression is quite explicit in the superficial zone of articular cartilage in response to persistent weight loading (Akasaki et al., 2014a). In vivo and in vitro models of OA, phosphorylation or cytoplasmic accumulation of FOXO1 contributes to the progression of articular cartilage degeneration, chondrocyte hypertrophy and apoptosis (Akasaki et al., 2014a). A previous study authenticates that knockdown of FOXO1 triggers cell death, as well as up-regulation of the production of ADAMTS4 (Akasaki et al., 2014b). ADAMTS4 and ADAMTS5 are identified as the two major matrix-associated zinc metalloendopeptidases that predispose to the destruction of aggrecans in arthritic diseases and have been validated as the therapeutic targets for the treatment of OA (Zhang et al., 2018).

Herein, we investigated the function of β-Ecdysone on pathomorphological changes of the knee joint in a mouse model of collagenase-induced OA. Moreover, we hypothesized that FOXO1/ADAMTS-4/5 signaling axis might be a potential target of β-Ecdysone to prevent OA.
Materials and methods

Animal model. Collagenase-induced mouse model of OA was performed as described previously (van der Kraan et al., 1990). In brief, male C57BL/6J mice (8-week old; 25 ± 2 g; Beijing HFK Bio-Technology co., LTD., China) were intra-articularly injected with collagenase (6 µl solution containing 10 U; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in the left knee. A total of 24 mice were averagely divided into four groups: (1) mice in Vehicle group received normal saline (0.9%) injection (n = 6); mice in OA group received collagenase injection (n = 6); mice in β-Ecdysone low concentration (Ecd-L) group received collagenase injection combined with β-Ecdysone (1 mg/kg; Sigma-Aldrich) administration by intra-articular injection (5 times/week; n = 6); mice in β-Ecdysone high concentration (Ecd-H) group received collagenase injection combined with β-Ecdysone (10 mg/kg) administration by intra-articular injection (5 times/week; n = 6). OA mice with or without β-Ecdysone treatment for 6 weeks were sacrificed for specimen collection. The animal experiment was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University.

Chondrocyte isolation and culture. Chondrocytes were isolated from normal mouse cartilage as described previously (Jonason et al., 2015). In brief, articular cartilage was dissected under sterile conditions and digested with 0.2% collagenase. Chondrocytes were cultured in DMEM/F12 medium (Invitrogen; Thermo Fisher Scientific, Inc.). Chondrocytes (1x10^5) were exposed to tumor necrosis factor-α (TNF-α; 50 ng/mL) to simulate OA model in vitro. After TNF-α-stimulated chondrocytes treatment with β-Ecdysone (10 nM or 100 nM), chondrocytes were collected for further experiments, including TUNEL, RT-PCR and western blot assays. Technical duplications (n = 3) and biological duplications (n = 3) were performed.
in each cell experiment. The concentration of β-Ecdysone (10 nM or 100 nM) was applied in our study according to our preliminary experiment and previous reference (Sheu et al., 2015).

**Cell transfection.** FOXO1 expression plasmids (vector-FOXP3) and a negative control empty plasmids (vector-con) were purchased from GeneCopoeia, Inc. (Rockville, MD, USA). Plasmids were transfected into chondrocytes using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocols. Briefly, Lipofectamine 2000 and vector-FOXP3 were mixed at room temperature for 20 min. Next, the mixture was added into a 6-well cell culture plate to culture the cells (1 × 10^5 cells/well) at 37 °C with 5% CO2 for 8 h.

**ELISA assay.** Cardiac blood samples were collected from all mice after β-Ecdysone administration for 6 weeks. Serum samples were obtained by centrifugation (x1500g; 15 min at 4°C). Serum proinflammatory cytokines, TNF-α, IL-1β and IL-6, were measured using ELISA assays from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA), according to the manufacturer’s protocol.

**Safranin O staining.** Left knees were collected and fixed with 4% formalin at room temperature for 7 days and decalcified in 0.5 M EDTA (pH=8.0) for 2 weeks, and then embedded in paraffin. Left knees were cut into 5 µm sections that were stained with Safranin O (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China). Stained slides were visualized under an optical microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany), and bone area/total area (BA/TA) was analyzed using an OsteoMeasure system (OsteoMetrics Inc., Decatur, GA, USA). Osteoarthritis Research Society International (OARSI) score was performed to assess cartilage damage in mice as described previously.
Reverse transcription-quantitative PCR (RT-qPCR). Total RNA in femur or chondrocyte was extracted using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA (2 µg) was used to synthesize cDNA with moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed by Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.) with the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.), following initial denaturation at 95°C for 3 min, 40 cycles were performed of denaturation at 95°C for 15 sec, annealing at 56°C for 20 sec and extension at 72°C for 20 sec. The relative expression levels of mRNA were calculated using the $2^{\Delta\Delta Ct}$ method. The PCR primers were listed as follows: forward 5’-AGTACCCGCATCTGCACAAC-3’ and reverse 5’-ACGAAGGGTCTCTTCTCGCT-3’ for SRY-box containing gene 9 (Sox9); forward 5’-GGTTCACAGAGGTTACCCAG-3’ and reverse 5’-ACCAGGGGAACCACCTCTCAC-3’ for collagen type II alpha 1 chain (Col2a1); forward 5’-GGCCTGGAACAGTCTCTGGC-3’ and reverse 5’-TGTCATCGTCTCATCGTCA-3’ for matrix metallopeptidase 3 (MMP3); forward 5’-CCCAGGCCGAGTTAACC-3’ and reverse 5’-GTTGCTCATAAAGTGGTGC-3’ for FOXO1; forward 5’-AGGTCCGGTGTAACGGATTTG-3’ and reverse 5’-GGGGTCTGGATGGCAACA-3’ for glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene.

Western blot analysis. Western blot analysis was performed as described previously (Liu et al., 2018). The following primary antibodies were used: FOXO1 rabbit mAb (CST; #2880); Histone H2A.X (D17A3) XP® rabbit mAb (CST; #7631); Rabbit Anti-ADAMTS4 antibody (Abcam; ab185722), Rabbit Anti-ADAMTS5 antibody (Abcam; ab182795), β-actin rabbit
mAb (Abcam; ab179467). The secondary antibody was used as follows: goat anti-rabbit IgG H and L (HRP) (Abcam; ab205718). Protein bands were obtained using an ECL chemiluminescence kit (Santa Cruz Biotech, Santa Cruz, CA, USA) with Bio-Rad Gel Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantitative data were analyzed using Quantity One® software version 4.5 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Chondrocyte apoptosis.** After chondrocyte exposure to TNF-α (50 ng/mL) with or without β-Ecdysone (10 nM or 100 nM) treatment or transfection of FOXO1 overexpressed plasmids, TUNEL assay (Roche) was utilized to analyze apoptotic cell proportion. In brief, chondrocytes were attached to slides, and then 50 µL TUNEL was added into slides that were incubated at 37 ℃ for 60 min. TUNEL positive staining cells were counted under the fluorescence microscope (Olympus Corporation, Japan).

**Statistical analysis.** The data are expressed as mean and standard deviation. Statistical analysis was performed with the GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Inter-group comparisons were calculated using one-way analysis of variance. A P value less than 0.05 was used to indicate significant differences.
Results

**β-Ecdysone inhibits serum pro-inflammatory cytokines in OA mice.** With regard to the anti-inflammatory effect of β-Ecdysone, IL-1β-induced inflammatory response in rat chondrocytes is attenuated by β-Ecdysone treatment (Zhang et al., 2014). In our study, serum pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, were distinctly elevated in collagenase-treated mice compared with those in the control group (**Figure 1A-1C**). As shown in **Figure 1A and 1C**, Ecd-L or Ecd-H administration caused a significant suppression of TNF-α and IL-6 production in serum of OA mice. Moreover, Ecd-H treatment significantly reduced serum IL-1β of OA mice (**Figure 1B**).

**β-Ecdysone prevents collagenase-induced histopathological changes in OA mice.** Compared with normal mice, safranin O positive staining of proteoglycans and chondrocytes was dramatically reduced in articular cartilage of OA mice. However, Ecd-L and Ecd-H groups exhibited a more positive staining of proteoglycans in the deep layer of cartilage that that of in OA mice (**Figure 2A**). Compared with OA mice, administration of Ecd-L or Ecd-H markedly improved OARSI score in collagenase-treated mice (**Figure 2B**). In addition, cartilage metabolism-related markers, Sox9, Col2a1 and MMP3, were analyzed in the femur using RT-qPCR. The findings suggested that Sox9 and Col2a1 mRNA were significantly decreased, while MMP3 was markedly increased in the femur of OA mice compared with the control group. However, Ecd-H administration increased the expression of Sox9 and Col2a1 and inhibited the expression of MMP3 compared with OA group (**Figure 2C**). In collagenase-treated mice, the degeneration and breakage of trabecular bone were observed in the epiphysiolsis of the distal femur. Compared with normal mice, BA/TA was significantly decreased in the distal femur of collagenase-treated mice compared with the control group.
However, Ecd-H administration had no obvious protective effect to prevent trabecular bone loss in collagenase-treated mice (Figure 2D and 2E). These findings indicate that β-Ecdysone has a beneficial effect to impede collagenase-induced articular cartilage injury.

β-Ecdysone mediates the FOXO1/ADAMTS-4/5 pathway in OA mice. In collagenase-treated mice, FOXO1 mRNA and protein levels in the nucleus were dramatically decreased in the femur. However, administration of β-Ecdysone was able to enhance FOXO1 production in OA mice (Figure 3A and 3B). Western blot analysis also found that ADAMTS-4 and ADAMTS-5 protein levels were significantly higher in the femur of OA mice than those in normal mice. However, β-Ecdysone treatment was able to restrain ADAMTS-4 and ADAMTS-5 activity (Figure 3C).

β-Ecdysone represses TNF-α-induced chondrocyte apoptosis. In an vitro experiment, TUNEL staining exhibited that TNF-α (50 ng/mL) treatment led to a significant increase in the apoptotic proportion of chondrocytes, while β-Ecdysone (100 nM) treatment reversed TNF-α-induced chondrocyte apoptosis (Figure 4A and 4B).

β-Ecdysone accelerates FOXO1 nuclear translocation. As shown in Figure 6A and 6B, TNF-α-stimulated caused a decrease in FOXO1 mRNA and nuclear protein expression. However, β-Ecdysone treatment reversed the inhibition of FOXO1 mRNA and nuclear protein expression caused by TNF-α. ADAMTS-4 and ADAMTS-5 protein expression levels significantly elevated in chondrocytes following TNF-α stimulation. TNF-α-induced the up-regulation of ADAMTS-4 and ADAMTS-5 expression was repressed by β-Ecdysone in chondrocytes (Figure 5C and 5D). To further explore the function of FOXO1 in
TNF-α-induced chondrocyte injury, TUNEL staining was performed to evaluate the protective effect of FOXO1 on TNF-α-induced chondrocyte apoptosis. As shown in Figure 6A and 6B, TNF-α-induced chondrocyte apoptosis was impeded by the over-expression of FOXO1 in vitro.
Discussion

Collagenase-induced OA is characterized by activation of inflammatory response and degeneration of articular cartilage by releasing extracellular matrix (ECM) catalytic enzymes (Nirmal et al., 2017). Consistent with previous studies (Nirmal et al., 2017; Jeong et al., 2018), ELISA assays revealed that injection of collagenase stimulated the release of circular pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6. Pharmacological investigations indicated that β-Ecdysone supplementation significantly inhibited the inflammatory response and cartilage degeneration in OA mice.

MMP3 functions as matrix metalloproteinase to degrade multifarious ECM proteins, such as fibronectin, collagens and proteoglycans (Nirmal et al., 2017; Wang and He, 2018). A growing body of evidence suggests that MMP3 also functions as an OA biomarker and a catabolic regulator to accelerate cartilage destruction (Yang et al., 2010; Pengas et al., 2018). In the present study, we examined MMP3 expression in the proximal femur of OA mice. In our study, collagenase-induced an increase in MMP-3 mRNA in OA mice was restrained by β-Ecdysone administration. Sox9 is necessary for chondrogenesis and enhances the transcription of cartilage synthesis-related factors, including Col2a1 (Bi et al., 1999; Lefebvre and Dvir-Ginzberg, 2017). In vivo and in vitro experimental measurements, administration of β-Ecdysone fulfilled chondroprotective functions via maintaining gene expression of chondrocyte anabolic marker, Sox9 and Col2a1.

FOXO1 serves as transcription factor to modulate chondrocyte homeostasis (Kurakazu et al., 2019). Oxidative stress-induced chondrocyte death may be associated with the reduction of FOXO1 (Akasaki et al., 2014b). FOXO1 knockdown facilitated apoptosis is accompanied
with caspase activation and up-regulates ADAMTS-4 production (Akasaki et al., 2014b). ADAMTS-4 and ADAMTS-5 are the key enzymes to corrode the cartilage extracellular matrix and play an important role in the progression of aggrecanase-mediated aggrecan degradation (Verma and Dalal, 2011). FOXO1 underlies the etiology of OA via a route of FOXO1 transferring from the nucleus to cytoplasm (Akasaki et al., 2014a). Decreased FOXO1 in the nucleus and increased its phosphorylation have been reported in IL-1β-induced metabolic disturbance of chondrocytes (Huang et al., 2020). Both in collagenase-treated mice and IL-1β-stimulated chondrocytes, nuclear FOXO1 protein levels were significantly reduced. However, β-Ecdysone treatment was able to rescue FOXO1 protein expression in the nucleus to inhibit the transcription and translation of ADAMTS-4 and ADAMTS-5. FOXO1 knockdown restrains chondrogenic differentiation and blocks Sox9 and Col2a1 expression (Kurakazu et al., 2019). In our study, overexpression of FOXO1 elevated Sox9 and Col2a1 mRNA expression in IL-1β-stimulated chondrocytes. Moreover, overexpression of FOXO1 protected against IL-1β-induced apoptosis of chondrocytes. These findings suggest that FOXO1 is a pivotal transcription factor in maintaining chondrocyte homeostasis and functions as a potential treatment target for preventing OA progression.

In conclusion, the current study suggests that β-Ecdysone supplementation effectively exhibited anti-inflammatory activity in OA mice. β-Ecdysone was able to improve collagenase-induced cartilage damage and repress IL-1β-induced chondrocyte apoptosis. In addition, β-Ecdysone served as FOXO1 activator to mediate its transcriptional targets, including Sox9, Col2a1, ADAMTS-4 and ADAMTS-5. These findings reveal that FOXO1 may be a potential molecular target of β-Ecdysone for the treatment of OA.
Declarations

Competing interests: The authors declare that they have no competing interests.

Funding: This research was supported by Scientific Research Project of Health Commission of Anhui Province, Grant. No: AHWJ2021b126, and Natural Science Foundation of Bengbu Medical College (Grant. No: 2020byzd186).

Consent for publication: Not applicable.

Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions: Study design: J-H and J-G; Literature research, Data acquisition and Data analysis: J-H, J-G and X-Z; Manuscript preparation and Manuscript editing: J-H, J-G and X-Z; Manuscript review: J-H, J-G and X-Z; Cell experiments: J-H, J-G and X-Z; Animal experiments: J-H, J-G and X-Z; All authors read and approved the final manuscript.
References


Yu G., Xiang W., Zhang T., Zeng L., Yang K. and Li J. (2020). Effectiveness of boswellia and
boswellia extract for osteoarthritis patients: A systematic review and meta-analysis.

BMC. Complement. Med. Ther. 20, 225.


Figure legends

Figure 1. β-Ecdysone inhibits serum pro-inflammatory cytokines in OA mice. Collagenase-induced OA mice treatment with β-Ecdysone (low concentration: 1 mg/kg or high concentration: 10 mg/kg) for 6 weeks, serum pro-inflammatory cytokines, TNF-α (A), IL-1β (B) and IL-6 (C), were measured using ELISA assays. ***P < 0.001 compared with vehicle group; $P < 0.05$, $$P < 0.01$ and $$$P < 0.001$ compared with OA group. Ecd-L, β-Ecdysone low concentration; Ecd-H, β-Ecdysone high concentration.

Figure 2. β-Ecdysone prevents collagenase-induced histopathological changes in OA mice. Collagenase-induced OA mice treatment with β-Ecdysone (low concentration: 1 mg/kg or high concentration: 10 mg/kg) for 6 weeks, left femurs were collected for safranin O staining to evaluate proteoglycan and chondrocyte less (A; amplification 40X; scale bar = 100 μm). OARSI score was analyzed in collagenase-treated mice with or without β-Ecdysone supplementation (B). Cartilage metabolism-related markers, Sox9, Col2a1 and MMP3, were measured using RT-qPCR in the femur (C). Histological change in the epiphysiolsis of the distal femur was observed by safranin O staining (D; amplification 100X; scale bar = 40 μm) and quantified with the ratio of BA/TA (E). *P < 0.05 compared with vehicle group; $P < 0.05$, $$P < 0.01$ and $$$P < 0.001$ compared with OA group. Ecd-L, β-Ecdysone low concentration; Ecd-H, β-Ecdysone high concentration; BA, bone area; TA, total area; ns, no significance.

Figure 3. β-Ecdysone mediates the FOXO1/ADAMTS-4/5 signaling axis in OA mice. Collagenase-induced OA mice treatment with β-Ecdysone (low concentration: 1 mg/kg or high concentration: 10 mg/kg) for 6 weeks, FOXO1 mRNA (A) and protein levels in the
nucleus (B) were measured using RT-qPCR and western blot analysis in the femur. ADAMTS-4 and ADAMTS-5 protein levels were measured using western blot in the femur of OA mice (C). * \( P < 0.05 \) and *** \( P < 0.001 \) compared with vehicle group; $ \( P < 0.05 \) and $$$$ \( P < 0.001 \) compared with OA group. Ecd-L, \( \beta \)-Ecdysone low concentration; Ecd-H, \( \beta \)-Ecdysone high concentration.

**Figure 4.** \( \beta \)-Ecdysone represses TNF-\( \alpha \)-induced chondrocyte apoptosis. TNF-\( \alpha \) (50 ng/mL) stimulated chondrocytes treatment with \( \beta \)-Ecdysone (low concentration: 10 nM or high concentration: 100 nM) for 48 h, TUNEL staining was performed to analyze apoptotic cell proportion (A and B; amplification 200X; scale bar = 10 \( \mu \)m). * \( P < 0.05 \) compared with control group; $ \( P < 0.05 \) compared with TNF-\( \alpha \) treated group.

**Figure 5.** TNF-\( \alpha \)-induced the reduction of nuclear FOXO1 in chondrocyte is activated by \( \beta \)-Ecdysone. TNF-\( \alpha \) (50 ng/mL) stimulated chondrocytes treatment with \( \beta \)-Ecdysone (low concentration: 10 nM or high concentration: 100 nM) for 48 h, FOXO1 mRNA (A) and protein levels in the nucleus (B) were measured using RT-qPCR and western blot. ADAMTS-4 and ADAMTS-5 protein levels were measured using western blot (C and D). * \( P < 0.05 \) and *** \( P < 0.001 \) compared with control group; $ \( P < 0.05 \) compared with TNF-\( \alpha \) treated group.

**Figure 6.** Overexpression of FOXO1 protects against TNF-\( \alpha \)-induced chondrocyte apoptosis. After transfection with vector-FOXO1 into TNF-\( \alpha \)-stimulated chondrocytes, TUNEL staining was implemented to analyze apoptotic cell proportion (A and B; amplification 200X; scale bar = 10 \( \mu \)m). * \( P < 0.05 \) compared with control group; $ \( P < 0.05 \) compared with TNF-\( \alpha \)
treated group.
A

![Graph showing FOXO1 mRNA expression](image)

B

![Graph showing FOXO1 protein expression](image)

C

![Graph showing relative protein expression of ADAMTS-4 and ADAMTS-5](image)
**A**

![Graph showing FOXO1 mRNA expression levels across Con, TNF-α, Ecd-10, and Ecd-100 conditions.

**B**

![Graph showing FOXO1 protein expression levels across Con, TNF-α, Ecd-10, and Ecd-100 conditions.

**C**

![Western blots for FOXO1/Nuc and Histone proteins under different conditions.

**D**

![Bar charts for ADAMTS-4 and ADAMTS-5 protein expression levels across Con, TNF-α, Ecd-10, and Ecd-100 conditions.](image)