

# $\beta$ -ecdysterone protects against apoptosis by promoting autophagy in nucleus pulposus cells and ameliorates disc degeneration

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**Abstract.** Increasing cell apoptosis is one of the major causes of intervertebral disc degeneration (IDD).  $\beta$ -ecdysterone has been demonstrated to protect PC12 cells against neurotoxicity. A previous study revealed that  $\beta$ -ecdysterone may be involved in the regulation of autophagy in osteoblasts. Therefore, we hypothesized that  $\beta$ -ecdysterone may possess therapeutic effects on IDD via autophagy stimulation. The effect of  $\beta$ -ecdysterone on IDD was explored by *in vitro* experiments. The results demonstrated that  $\beta$ -ecdysterone attenuated the apoptosis induced by tert-butyl hydroperoxide via promoting autophagy in nucleus pulposus cells. Beclin-1, an indispensable protein for the stimulation of autophagy, is upregulated and stabilized by  $\beta$ -ecdysterone in a dose- and time-dependent manner in nucleus pulposus cells. Inhibition of autophagy with 3-methyladenine partially abrogated the protective function of  $\beta$ -ecdysterone against apoptosis of nucleus pulposus cells, indicating that autophagy participated in the protective effect of  $\beta$ -ecdysterone on IDD. Additionally,  $\beta$ -ecdysterone promoted the expression of anabolic genes while inhibiting the expression of catabolic genes in nucleus pulposus cells. Collectively, the present study demonstrated that  $\beta$ -ecdysterone may protect nucleus pulposus cells against apoptosis by autophagy stimulation and ameliorate disc degeneration, which indicates that  $\beta$ -ecdysterone may be a potential therapeutic agent for IDD.

## Introduction

Lower back pain represents a significant musculoskeletal disorder that is one of the primary causes of poor quality of life and healthcare-associated expenditure worldwide (1). The number of people suffering from lower back pain has increased in previous years (2). The cause of lower back pain is complex and multifactorial. At present, the molecular mechanism regulating lower back pain remains largely unknown. Emerging evidence indicates that intervertebral disc degeneration (IDD) is a major potential factor leading to lower back pain (3,4). Nevertheless, no effective drug for IDD treatment has been developed until recently.

Intervertebral discs are avascular and consist of a gelatinous inner core, nucleus pulposus, the annulus fibrosus and tough outer rings, which cooperatively endows specific mechanical function of the disc and allows multi-axial flexibility of the spine (5,6). It has been demonstrated that the nucleus pulposus cells reside in the gelatinous nucleus pulposus, are vital for the physiological functions of the disc and produce a high level of extracellular matrix (ECM) proteins including aggrecan, collagen II and other factors (7-9). Adequate ECM is a prerequisite to ensure the internal pressure of the intervertebral disc and the performance of normal physiological disc functions (10,11). Abnormal apoptosis in nucleus pulposus cells has been demonstrated to contribute to the process of IDD (12-15).

Autophagy is a catabolic process by which dysfunctional proteins or organelles are degraded to relieve the cellular stresses (16). An increasing number of studies have suggested that there is a close association between autophagy and apoptosis in the pathological processes of certain degenerative diseases, including IDD and Alzheimer's disease (17,18). A previous study also revealed that increasing autophagy levels in nucleus pulposus cells may decrease apoptosis and alleviate IDD (19).

$\beta$ -ecdysterone is a major component of Chinese herbal medicines.  $\beta$ -ecdysterone has been demonstrated to exhibit a number of functions, including anabolic and hepatoprotective effects (20). Certain studies have indicated that  $\beta$ -ecdysterone may increase the synthesis of collagen protein and inhibit cell apoptosis by regulation of autophagy (20-22). However,

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the roles of  $\beta$ -ecdysterone on nucleus pulposus cells and IDD remain incompletely characterized. We hypothesized that  $\beta$ -ecdysterone may have protective functions on IDD via autophagy stimulation.

Increased oxidative stress is a pathological cause of apoptosis in nucleus pulposus cells (23). Therefore, the present study used tert-butyl hydroperoxide (TBHP) to induce oxidative stress and explore the effects of  $\beta$ -ecdysterone on apoptosis of nucleus pulposus cells under oxidative stress and IDD.

## Patients and methods

**Patient samples.** All the subjects were patients (three males and two females; Age,  $59.3 \pm 7.2$  years) undergoing lumbar spine surgery admitted to Hubei Provincial Hospital of Traditional Chinese Medicine (Wuhan, China) between May 2014 and December 2015. The IDD tissues were harvested under sterile conditions and immediately sent to the laboratory (within 30 min of harvesting). Written informed consent was obtained from each patient. The study was approved by the Ethics Committee of Hubei Provincial Hospital of Traditional Chinese Medicine. Patients with ankylosing spondylitis or diffuse idiopathic skeletal hyperostosis were excluded. Complete Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium with serum at 4°C was used as transport medium. The nucleus pulposus tissues were carefully isolated from IDD tissues by a scalpel microscopically under sterile conditions.

**Reagents and antibodies.** 3-methyladenine (3-MA), TBHP and the type II collagenases were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The primary antibodies against sequestosome-1 (p62; 1:2,000; cat. no. ab207305), horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab7090) and GAPDH (1:2,000; cat. no. ab9485; all Abcam, Cambridge, UK). The microtubule-associated proteins 1A/1B light chain 3A (LC-3; 1:2,000; cat. no. 12741), Beclin-1 (1:2,000; cat. no. 3495), Bax (1:2,000; cat. no. 5023) and cleaved caspase 3 (1:2,000; cat. no. 9661) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). DAPI was obtained from Beyotime Institute of Biotechnology (Haimen, China). The cell culture reagents were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

**Western blotting.** Total protein was extracted from nucleus pulposus cells by radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). The protein lysates (40  $\mu$ g per lane) were separated using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc.). The membrane was blocked using 5% non-fat milk in PBS (Thermo Fisher Scientific, Inc.) containing 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA) at room temperature for 2 h. Subsequently, the membrane was incubated for 2 h at 25°C with specific primary anti-human antibodies, followed by incubation for 1 h at 25°C with a goat horseradish peroxidase-conjugated secondary antibody.

Membranes were then washed with PBS for 10 min at room temperature, and the protein bands were visualized using an Enhanced Chemiluminescence Western Blotting kit (Pierce; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocol. Protein densitometry was performed using ImageJ software (version 1.41; National Institutes of Health, Bethesda, MD, USA). GAPDH was used as a control. The experiment was repeated 3 times.

**Protein degradation detection.** Chx (Cycloheximide, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; 1  $\mu$ g/ml) was added into cultured cell medium. Nucleus pulposus cells were cultured for 0, 12, and 24 h. Then cells were collected and Beclin-1 expression was measured using western blotting.

**Isolation and culture of human nucleus pulposus cells.** The human nucleus pulposus tissues were carefully isolated from IDD tissues by a scalpel microscopically under sterile condition as previously described (19). Then, they were washed with PBS twice and cut into 1 mm<sup>3</sup> fragments. The fragments of nucleus pulposus tissues were digested in 0.25% trypsin solution for 30 min at 37°C, following 3–4 h in 0.2% type II collagenase at 37°C. Tissue debris was removed by passing through a 200  $\mu$ m filter and then the nucleus pulposus cells were resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. When the cells grew to 80–90% confluence, they were digested by 0.25% trypsin solution and sub-cultured in culture flasks. The third generation of nucleus pulposus cells was used for all experiments.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** The total RNA was extracted from the cells in a 6-well plate using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.). The RNA quality and concentration was determined using a Thermo Scientific NanoDrop ND-100 (Wilmington, DE, USA). A total of 1  $\mu$ g of total RNA was used to synthesize cDNA using a Reverse Transcription System kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The thermocycling conditions were: 37°C for 25 min, incubated at 85°C for 5 sec in 20  $\mu$ l of reaction volume. For the PCR amplification, a 20  $\mu$ l reaction volume was used, including 10  $\mu$ l 2X SYBR Premix Ex Taq mixture (Takara Bio, Inc., Otsu, Japan), 0.2 mmol/l primer, 2  $\mu$ l 2-fold diluted cDNA and sterile distilled water. The reaction and detection were conducted in a light cycler (Roche Diagnostics GmbH, Mannheim, Germany). The thermocycling conditions were as follows: Denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and elongation at 60°C for 1 min. The cycle threshold (Cq) values were collected and normalized to the level of the housekeeping gene GAPDH according to the 2<sup>- $\Delta\Delta$ Cq</sup> method (24). The primer sequences were as follows: Collagen type II alpha 1 (Col2a1) forward (F), 5'-ACG CTCAAGTCGCTGAACAA-3' and reverse (R), 5'-TCA ATCCAGTAGTCTCCGCTCT-3'; Aggrecan F, 5'-TCCAAA CCAACCCGACAAT-3' and R, 5'-TCTCATAGCGATCTT TCTTCTGC-3'; a disintegrin and metalloproteinase with

thrombospondin motifs 5 (Adamts-5) F, 5'-CGACAAGAGTCTGGAGGTGAG-3' and R, 5'-CGTGAGCCACAGTGA AAGC-3'; matrix metalloproteinase-3 (MMP-3) F, 5'-ATGATGAACGATGGACAGATGA-3' and R, 5'-CATTGGCTGAGTGAAGAGACC-3'; Beclin-1 F, 5'-TCCGGGCTCCCGAGG-3' and R, 5'-TTCTCTCTGGGTCTCTCCTG-3'; Bax F, 5'-TCATGGGCTGGACATTGGAC-3' and R, 5'-GAGACAGGACATCAGTCGC-3'; GAPDH F, 5'-ATGTTGCAACCGGGAAGGAA-3' and R, 5'-AGGAAAAGCATCACC CGGAG-3'.

**Cell culture treatment protocols.** To establish the apoptosis model of nucleus pulposus cells, different concentrations of TBHP (50, 100, 200, 300 and 500  $\mu$ M) were added into the culture medium of nucleus pulposus cells for 24 h. Cells were pretreated with different concentrations of  $\beta$ -ecdysterone (10, 50, 100 and 200  $\mu$ M) for 12 h prior to the addition of TBHP (100  $\mu$ M) to investigate its effect on cell apoptosis. To examine the role of autophagy in  $\beta$ -ecdysterone-mediated cell protection, nucleus pulposus cells were pretreated with 10  $\mu$ M 3-MA, an autophagy inhibitor, for 1 h prior to  $\beta$ -ecdysterone administration. All experiments were performed in triplicate. Small interfering (si)-Beclin-1 transfection (5'-CGGAGAGGAGCCATTTATTGAA-3'; 50 nM) was performed using Lipofectamine 2000 siRNA transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The efficiency was confirmed 48 h after transfection, using RT-qPCR.

**Cell viability assay.** Cell viability was assayed with the Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol. In brief, the nucleus pulposus cells were plated in 96-well plates (5,000 cells/cm<sup>2</sup>) and incubated in DMEM with 10% FBS at 37°C for 24 h. Then, the cells were treated with TBHP,  $\beta$ -ecdysterone and 3-MA, as aforementioned. Following treatment, the cells were washed with PBS, and then 100  $\mu$ l DMEM containing 10  $\mu$ l CCK-8 solution was added to each well, and the plate was incubated for an additional 1 h. The absorbance of the wells was then measured at 450 nm using a microplate reader.

**ELISA assay.** Nucleus pulposus cells were pretreated with  $\beta$ -ecdysterone for 12 h prior to TBHP stimulation for 24 h. Culture supernatants were collected and stored at -20°C until analysis. The concentration of Col2a1 (cat. no. DY7589-05), Aggrecan (cat. no. DY1220), Adamts-5 (DY2198-05) and MMP-3 (cat. no. DMP300) in cell culture supernatants was measured using ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

**Statistical analysis.** Experiments were performed at least three times. The results are presented as mean  $\pm$  standard deviation. Statistical analyses were performed using SPSS statistical software program v.18.0 (SPSS, Inc., Chicago, IL, USA). Data were analyzed by Student's t-test or one-way analysis of variance followed by Tukey's post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**$\beta$ -ecdysterone treatment decreases apoptosis levels in nucleus pulposus cells.** To investigate the effect of  $\beta$ -ecdysterone on nucleus pulposus cells, the cytotoxic effect of  $\beta$ -ecdysterone was first evaluated. Nucleus pulposus cells from patients with lumbar vertebral fracture were treated with  $\beta$ -ecdysterone at different concentrations (0, 10, 50, 100, 200 and 500  $\mu$ M) or for different times (0, 6, 12, 18, 24 and 48 h). As indicated in Fig. 1A and B,  $\beta$ -ecdysterone was not cytotoxic to nucleus pulposus cells for 24 h or at concentrations of  $\leq 100$   $\mu$ M. However, the cell viability was significantly downregulated following TBHP treatment in a dose-dependent manner (Fig. 1C). Then, nucleus pulposus cells were treated with  $\beta$ -ecdysterone and TBHP, and it was identified that  $\beta$ -ecdysterone markedly protected nucleus pulposus cells from TBHP-induced cell apoptosis (Fig. 1D and E). The expression of B cell lymphoma 2-associated X protein (Bax) and cleaved caspase 3 were also measured by RT-qPCR and western blot analysis. The results indicated that pretreatment with  $\beta$ -ecdysterone decreased the mRNA level of Bax and the protein level of cleaved caspase 3 induced by TBHP in nucleus pulposus cells (Fig. 1F and G). Taken together,  $\beta$ -ecdysterone exhibited a marked protective effect against TBHP-induced apoptosis in nucleus pulposus cells.

**$\beta$ -ecdysterone treatment induces autophagy in nucleus pulposus cells.** A previous study indicated that  $\beta$ -ecdysterone inhibited cell apoptosis by induction of autophagy in osteoblasts (20). To determine whether  $\beta$ -ecdysterone has a role in autophagy in nucleus pulposus cells, cells were treated with different concentrations of  $\beta$ -ecdysterone or for different time intervals in the present study. LC3-II/LC3-I, beclin-1 and p62 were recognized as indicators of autophagy formation. Their levels in nucleus pulposus cells treated with  $\beta$ -ecdysterone were assessed by western blot analysis, and it was identified that the protein levels of LC3-II and beclin-1 were significantly increased in a dose-dependent manner, while the expression level of p62 was decreased following treatment (Fig. 2A-C). Their expression levels were then determined at indicative time points following  $\beta$ -ecdysterone treatment (100  $\mu$ M). As demonstrated, the expression levels of LC3-II and Beclin-1 were increased, while the expression of p62 was significantly downregulated (Fig. 2D-F). Collectively,  $\beta$ -ecdysterone is an effective stimulus for autophagy in nucleus pulposus cells.

**Autophagy induced by  $\beta$ -ecdysterone is essential for protection against TBHP-induced apoptosis in nucleus pulposus cells.** Previous evidence indicates that autophagy protects cells from apoptosis, including in nucleus pulposus cells (25). To determine whether  $\beta$ -ecdysterone-induced autophagy is responsible for the protection against TBHP-mediated apoptosis, autophagy was inhibited with 3-MA. The administration of 3-MA abolished the autophagy induced by  $\beta$ -ecdysterone in nucleus pulposus cells (Fig. 3A). As demonstrated, the protein levels of LC3-II and beclin-1 were significantly decreased while the expression of p62 was increased following 3-MA treatment (Fig. 3A-C). These experiments indicated that  $\beta$ -ecdysterone may effectively

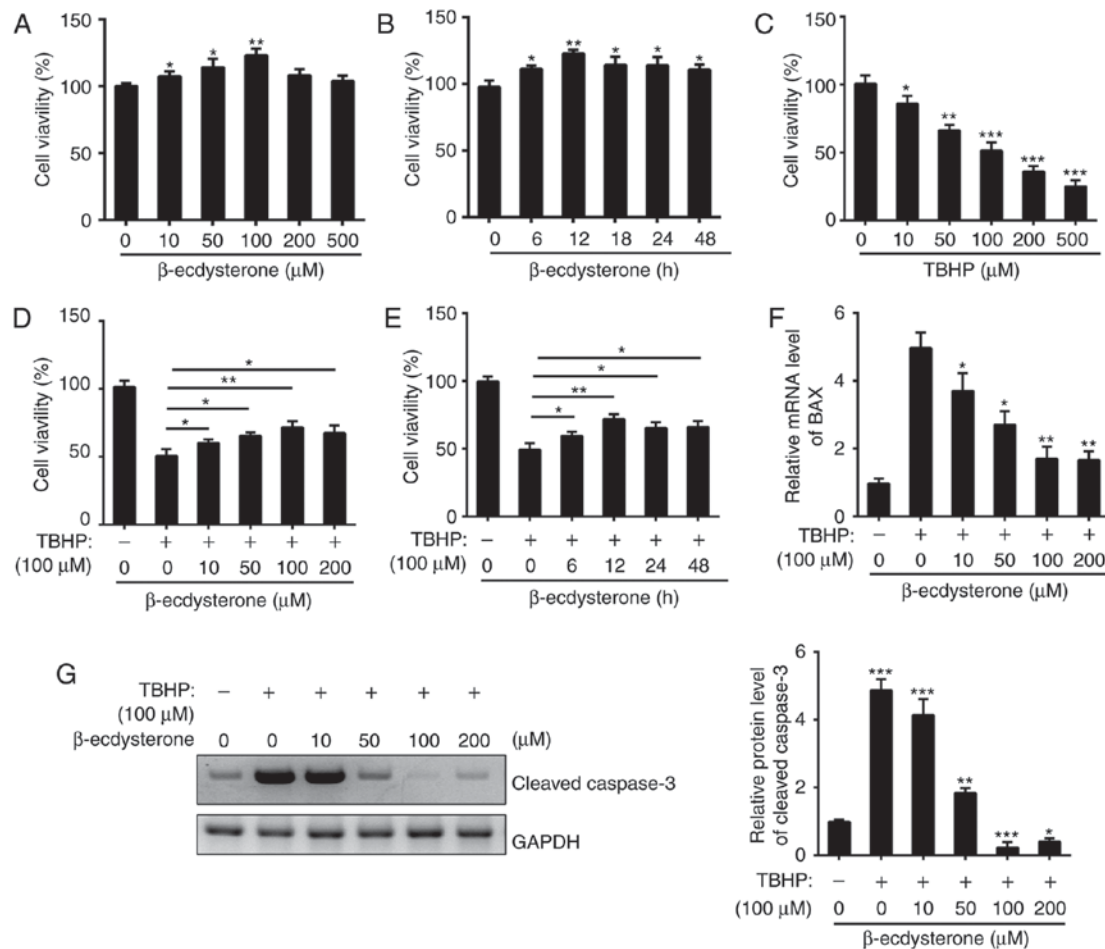


Figure 1.  $\beta$ -ecdysterone treatment decreases apoptosis in nucleus pulposus cells. (A) Nucleus pulposus cells were treated with different concentrations of  $\beta$ -ecdysterone for 24 h and then cell viability was determined by CCK-8 assay. (B) Nucleus pulposus cells were treated for different time intervals with 100  $\mu$ M  $\beta$ -ecdysterone and then cell viability was determined by CCK-8 assay. (C) Nucleus pulposus cells were treated with different concentrations of  $\beta$ -ecdysterone for 4 h and then cell viability was determined by CCK-8 assay. CCK-8 results of nucleus pulposus cells treated with TBHP and (D) increasing  $\beta$ -ecdysterone concentrations and (E) increasing treatment durations. (F) The mRNA expression of Bax in nucleus pulposus cells treated with TBHP or TBHP plus  $\beta$ -ecdysterone was measured by reverse transcription quantitative polymerase chain reaction. (G) Protein content of cleaved caspase 3 in nucleus pulposus cells treated with TBHP and or TBHP plus  $\beta$ -ecdysterone was measured by western blot analysis. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. control. CCK-8, Cell Counting kit-8; TBHP, tert-butyl hydroperoxide; Bax, B-cell lymphoma 2-associated X protein.

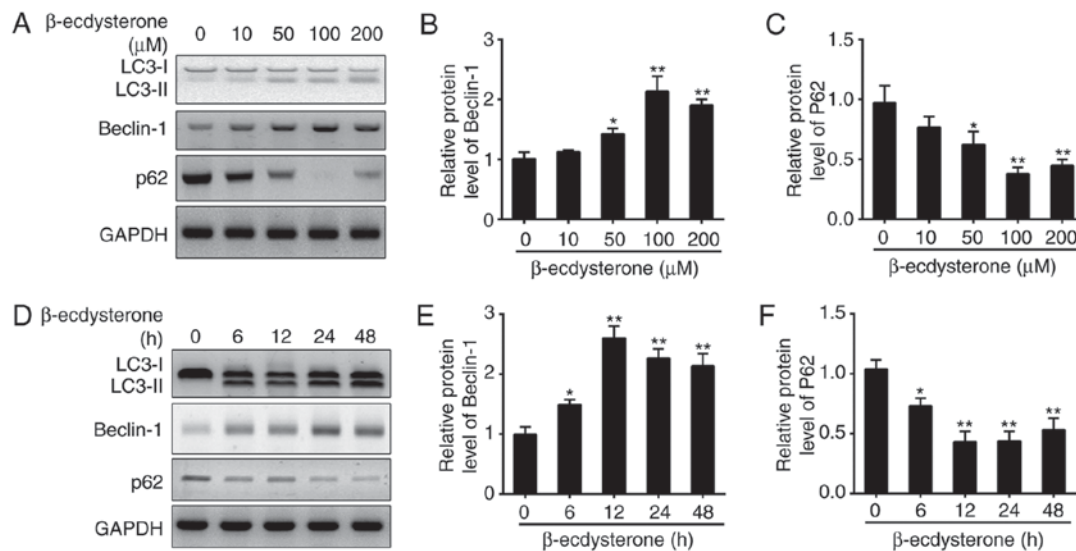


Figure 2.  $\beta$ -ecdysterone treatment induces autophagy in nucleus pulposus cells. Protein levels of (A) LC3, (B) Beclin-1 and (C) p62 in nucleus pulposus cells treated with different concentrations of  $\beta$ -ecdysterone were measured by western blot analysis. Protein levels of (D) LC3, (E) Beclin-1 and (F) p62 in nucleus pulposus cells treated with  $\beta$ -ecdysterone for different time intervals were measured by western blot analysis. \* $P$ <0.05 and \*\* $P$ <0.01 vs. control. LC3, Microtubule-associated proteins 1A/1B light chain 3A; p62, sequestosome-1.



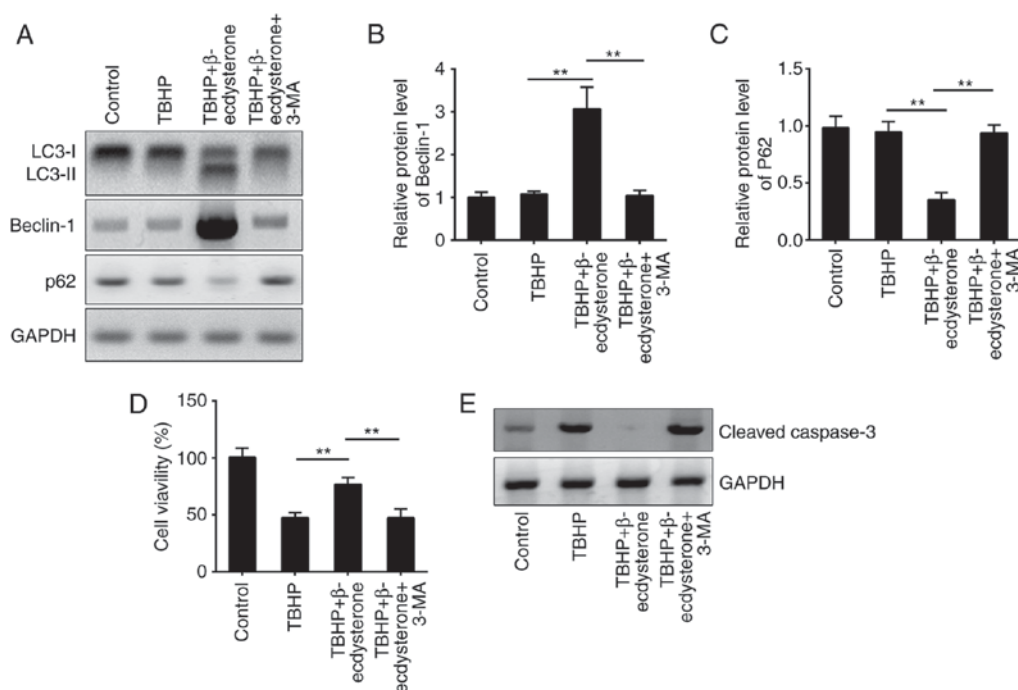


Figure 3. Autophagy induced by  $\beta$ -ecdysterone is essential for the protection against TBHP-induced apoptosis in nucleus pulposus cells. Protein levels of (A) LC3, (B) beclin-1 and (C) p62 in nucleus pulposus cells treated with TBHP,  $\beta$ -ecdysterone and/or 3-MA. (D) Viability of cells treated with TBHP,  $\beta$ -ecdysterone and/or 3-MA was measured by Cell Counting kit-8 assay. (E) Protein level of cleaved caspase 3 in nucleus pulposus cells treated with TBHP,  $\beta$ -ecdysterone and/or 3-MA was determined by western blot analysis. \*\* $P < 0.01$  vs. control. LC3, Microtubule-associated proteins 1A/1B light chain 3A; p62, sequestosome-1; TBHP, tert-butyl hydroperoxide; 3-MA, 3-methyladenine.

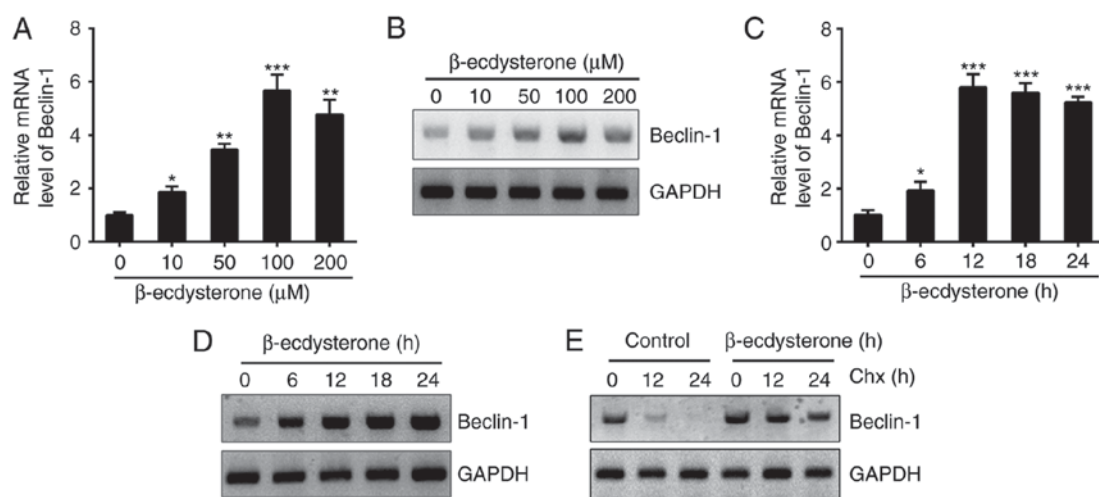


Figure 4.  $\beta$ -ecdysterone promotes the mRNA and protein levels of beclin-1 in nucleus pulposus cells. mRNA and protein levels of beclin-1 in nucleus pulposus cells treated with different concentrations of  $\beta$ -ecdysterone were determined by (A) RT-qPCR and (B) western blot analysis. mRNA and protein levels of beclin-1 in nucleus pulposus cells treated with  $\beta$ -ecdysterone for different time intervals were determined by (C) RT-qPCR and (D) western blot analysis. (E) Nucleus pulposus cells were treated with 100  $\mu$ M  $\beta$ -ecdysterone for 12 h and then Chx was added. The protein levels of Beclin-1 were determined at the indicated time points. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control. RT-qPCR, reverse transcription quantitative polymerase chain reaction.

induce autophagy flux in TBHP-treated nucleus pulposus cells, which may be completely reversed by 3-MA treatment. To additionally determine whether autophagy was involved in  $\beta$ -ecdysterone-mediated protection against apoptosis in nucleus pulposus cells, the cell viability and the expression level of cleaved caspase 3 were examined. As indicated,  $\beta$ -ecdysterone increased the proportion of cell viability and inhibited the protein level of cleaved caspase 3 in TBHP-treated cells, while addition of 3-MA completely reversed this trend (Fig. 3D and E).

*$\beta$ -ecdysterone promotes the mRNA and protein levels of Beclin-1 in nucleus pulposus cells.* Subsequently, the present study aimed to determine the molecular mechanism by which  $\beta$ -ecdysterone regulated autophagy in nucleus pulposus cells. To examine whether  $\beta$ -ecdysterone may promote the expression of Beclin-1; cells were treated with  $\beta$ -ecdysterone at different concentrations and it was identified that the Beclin-1 mRNA protein levels were markedly upregulated (Fig. 4A and B). Similarly, administration with  $\beta$ -ecdysterone for

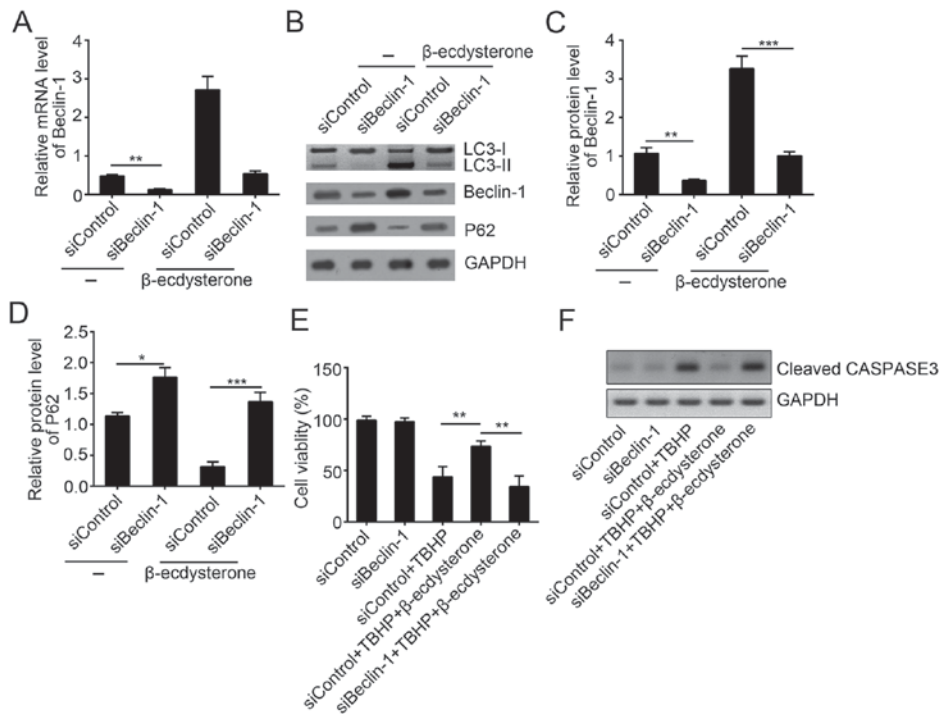


Figure 5.  $\beta$ -ecdysterone induces autophagy in a Beclin-1 dependent manner. (A) The mRNA level of beclin-1 was measured by reverse transcription quantitative polymerase chain reaction in nucleus pulposus cells transfected with siControl or siBeclin-1, and then treated with 100  $\mu$ M  $\beta$ -ecdysterone. (B-D) The protein expression of (B) LC3, (C) beclin-1 and (D) p62 in the nucleus pulposus cells treated as aforementioned, with 100  $\mu$ M  $\beta$ -ecdysterone, was assessed by western blot analysis. (E and F) Cell viability and apoptosis were measured by (E) Cell Counting kit-8 and (F) western blot analysis assays in the nucleus pulposus cells as aforementioned, with 100  $\mu$ M  $\beta$ -ecdysterone. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. control. si, small interfering; LC3, Microtubule-associated proteins 1A/1B light chain 3A; p62, sequestosome-1.

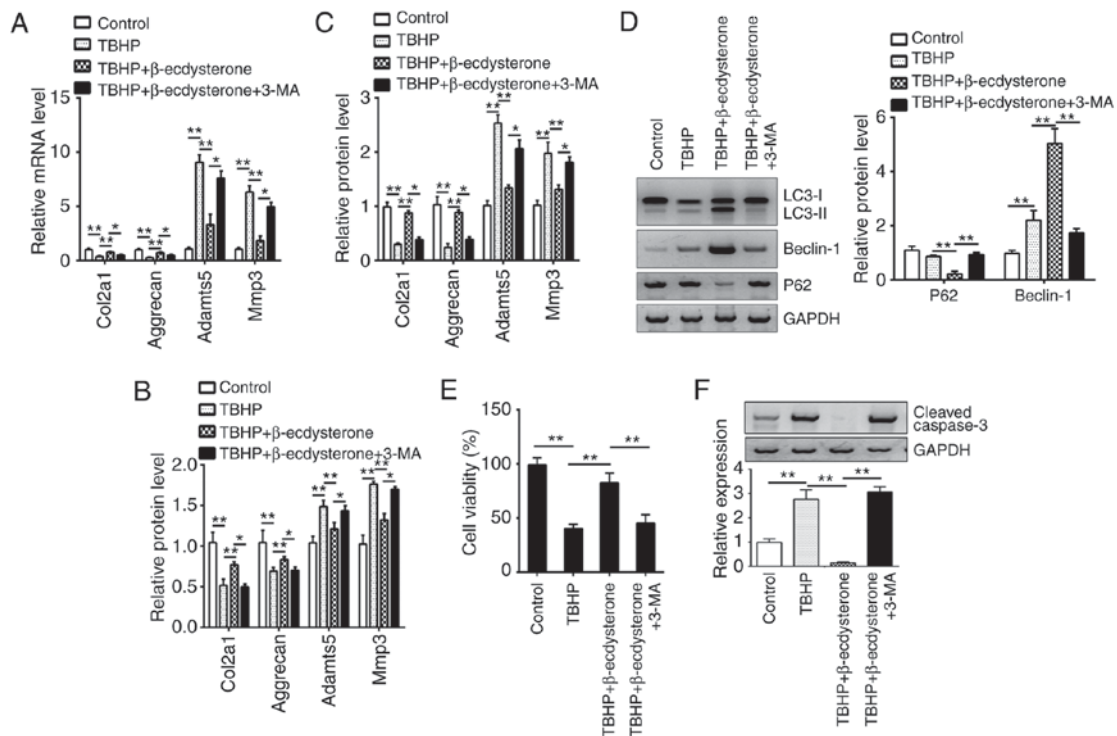


Figure 6.  $\beta$ -ecdysterone regulates the expression of degeneration-associated genes via autophagy. (A) The mRNA expression of Col2a1, Aggrecan, Adamts-5 and MMP-3 were measured by reverse transcription quantitative polymerase chain reaction in the nucleus pulposus cells from normal human intervertebral discs. (B) The protein expression levels of Col2a1, Aggrecan, Adamts-5 and MMP-3 were measured by ELISA in the nucleus pulposus cells from normal human intervertebral discs. (C) The protein expression levels of Col2a1, Aggrecan, Adamts-5 and MMP-3 were measured by ELISA in the nucleus pulposus cells from patients with IDD. (D) The protein levels of LC3, beclin-1 and p62 were determined by western blot analysis in nucleus pulposus cells from patients with IDD. Cell viability and apoptosis were determined by (E) CCK8 and (F) western blot analysis assays in nucleus pulposus cells from patients with IDD. \* $P$ <0.05 and \*\* $P$ <0.01 vs. control. Col2a1, collagen type II alpha 1; Adamts-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; MMP-3, matrix metalloproteinase 3; IDD, intervertebral disc degeneration; LC3, Microtubule-associated proteins 1A/1B light chain 3A; p62, sequestosome-1.

different time intervals also promoted the mRNA and protein levels of Beclin-1 (Fig. 4C and D). In addition, it was identified that  $\beta$ -ecdysterone significantly improved the stability of Beclin-1 in nucleus pulposus cells. As demonstrated in Fig. 4E, following the addition of Chx, the degradation of Beclin-1 in  $\beta$ -ecdysterone-treated cells was slower.

*$\beta$ -ecdysterone induces autophagy in a Beclin-1-dependent manner.* To additionally investigate the role of Beclin-1 in the process of  $\beta$ -ecdysterone-induced autophagy, nucleus pulposus cells were transfected with Beclin-1-siRNA (Fig. 5A). By western blot analysis, it was demonstrated that depletion of Beclin-1 protein levels by siRNA abolished the increased autophagy in nucleus pulposus cells (Fig. 5B-D). Then, the effect of Beclin-1 knockdown on the protective effect of  $\beta$ -ecdysterone was also evaluated; it was identified that  $\beta$ -ecdysterone improved the cell activity and inhibited the expression of cleaved caspase 3 in nucleus pulposus cells while Beclin-1 knockdown abolished this effects (Fig. 5E and F), which indicated that  $\beta$ -ecdysterone-mediated autophagy and protective function against apoptosis relied on upregulation of Beclin-1.

*$\beta$ -ecdysterone regulates the expression of degeneration-associated genes via autophagy.* Decrease in ECM proteins including aggrecan, collagen II and other factors often results in IDD (26,27). To additionally explore the degeneration of nucleus pulposus cells, the expression levels of major ECM synthesis genes (Col2a1 and aggrecan) and ECM degrading genes (MMP-3 and Adamts5) in nucleus pulposus cells were examined. As demonstrated, the mRNA levels of Col2a1 and aggrecan were downregulated following TBHP treatment, while TBHP upregulated the mRNA levels of MMP-3 and Adamts5 in nucleus pulposus cells (Fig. 6A). Notably,  $\beta$ -ecdysterone reversed the effects of TBHP on the mRNA levels of Col2a1, aggrecan, MMP-3 and Adamts5 via activation of autophagy (Fig. 6A), which was validated by the addition of 3-MA. Furthermore, the ELISA results also exhibited a similar trend.  $\beta$ -ecdysterone promoted the protein levels of collagen-II and aggrecan but inhibited that of MMP-3 and Adamts-5 (Fig. 6B). Finally, nucleus pulposus cells were isolated from patients with IDD, and it was identified that  $\beta$ -ecdysterone upregulated the protein levels of collagen-II and aggrecan but inhibited that of MMP-3 and adamts-5 by ELISA (Fig. 6C). In addition,  $\beta$ -ecdysterone also increased the autophagy of nucleus pulposus cells from patients with IDD and promoted their survival by inhibiting apoptosis (Fig. 6D-F).

## Discussion

Autophagy is a catabolic process by which dysfunctional proteins or organelles are degraded to relieve the cellular stresses (28). Emerging evidence has indicated that there is a closely association between autophagy and various diseases including neurodegeneration, infection and aging (29-31). An increasing number of studies have demonstrated that autophagy serves an important role in the pathology of disc degeneration (17,32,33). For example, NAD-dependent protein deacetylase sirtuin-1 protects against apoptosis in degenerative human disc nucleus

pulposus cells via promoting autophagy (28). Zhao *et al* (32) revealed that microRNA (miRNA)-129-5P modulates nucleus pulposus cell autophagy by targeting Beclin-1 in IDD. In addition, glucosamine exhibited a protective effect on nucleus pulposus cells via activation of autophagy in a mechanistic target of rapamycin-dependent manner (34). Diverse factors have been demonstrated to regulate autophagy, including miRNAs (35). A previous study also revealed the association between autophagy and oxidative stress-induced apoptosis in nucleus pulposus cells (36). Diverse factors may contribute to IDD, including genetic predisposition, infection, excessive biomechanical loading and aging (37,38). At present, no effective drug for IDD treatment has been developed. The present study identified that  $\beta$ -ecdysterone may regulate autophagy in nucleus pulposus cells and inhibit cell apoptosis. In addition, it was demonstrated that  $\beta$ -ecdysterone may be a potential therapy drug for IDD treatment.

A previous study has demonstrated that oxidative stress may lead to degenerated intervertebral discs (38). Emerging evidence has suggested that increased concentration of oxidation products may induce cell apoptosis by the mitochondrial pathway (36). Other studies have also revealed that various pathogenic factors may induce cell apoptosis via reactive oxygen, which leads to dysfunction of the mitochondria (39,40). A previous study demonstrated that TBHP induces the production of reactive oxygen species and then leads to apoptosis in nucleus pulposus cells (19). In the present study, a TBHP-induced apoptosis model was used to investigate the role of  $\beta$ -ecdysterone in the process of IDD. Autophagy is a process used to degrade useless proteins or organelles to maintain cellular functions (41). Increasing evidence has indicated that moderate autophagy exhibits a protective function against cellular pathologies, including IDD (42). In addition, a specific study revealed that autophagy-associated genes were downregulated in IDD tissues compared with healthy tissues, including Beclin-1 (28). Therefore, modulating autophagy in disc cells may be an effective therapeutic method for the treatment of patients with IDD. In addition, there is great clinical importance and an urgent requirement to develop novel drugs targeting autophagy for the therapy of IDD. To the best of our knowledge, the present study demonstrated for the first time that  $\beta$ -ecdysterone regulated autophagy in a Beclin-1 dependent manner in nucleus pulposus cells.

A large number of studies have demonstrated that apoptosis promotes the development of IDD (12). In the present study, it was identified that pretreatment with  $\beta$ -ecdysterone markedly inhibited the expression of Bax and the activation of caspase 3 in nucleus pulposus cells under oxidative stress, which indicated that  $\beta$ -ecdysterone protects nucleus pulposus cells by inhibiting apoptosis, at least in part. In addition, it was demonstrated that  $\beta$ -ecdysterone administration promoted the protein expression of ECM components including Col2A and aggrecan, whilst inhibiting the expression of catabolism of ECM components including MMP-3 and Adamts-5, which implied that  $\beta$ -ecdysterone may be of use in preventing IDD. Finally, it was also identified that  $\beta$ -ecdysterone may inhibit apoptosis by activating autophagy in nucleus pulposus cells isolated from patients with IDD, which suggests that  $\beta$ -ecdysterone may be a potential drug to ameliorate disc degeneration.

The present study demonstrated that  $\beta$ -ecdysterone may upregulate the mRNA and protein levels of Beclin-1, and stabilize Beclin-1. However, how  $\beta$ -ecdysterone regulates beclin-1 expression remains unknown. In addition, the molecular mechanism by which  $\beta$ -ecdysterone stabilizes beclin-1 remains to be investigated.

In conclusion, the present study provides evidence that treatment with  $\beta$ -ecdysterone induces autophagy in a Beclin-1-dependent manner in the nucleus pulposus cells, which confers an anti-apoptosis role against oxidative stress. These data revealed the therapeutic potential of  $\beta$ -ecdysterone in the prevention of the disc degeneration.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

FW and A-FY initiated and designed the present study, performed the experiments and wrote the manuscript. JY, C-JH and Z-WZ performed the western blot analysis. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The protocol for the present study was approved by the Institutional Ethics Committee of Hubei Provincial Hospital of Traditional Chinese Medicine and all enrolled patients signed a written informed consent document approving the use of their samples.

## Patient consent for publication

Patients have provided written informed consent for publication of their data.

## Competing interests

The authors declare that they have no competing interests.

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