Achyranthoside D (AD) improve intervertebral disc degeneration through affect the autophagy and the activation of PI3K/Akt/mTOR pathway

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Abstract

Purpose: This study aims to explore the potential mechanism of Achyranthoside D (AD) in improving intervertebral disc (IVD) degeneration (IDD).

Methods: The IDD model of SD rats and nucleus pulposus cells (NPCs) was established by lumbar cone annulus puncture and tert-butyl peroxide, respectively. Cell proliferation was detected by CCK8 assay. Apoptosis was detected by flow cytometry and TUNEL staining. IVD tissue injury was observed by HE staining. Alcian blue staining observed the glycoprotein secretion in IVD. Monodansylcadaverin (MDC) staining was used to detect the formation of autophagosomes. The LC3 expression was tested by immunofluorescence. The type II collagen, aggrecan and MMP3 expression were detected by ELISA. RT-qPCR was used to detect the Casp 3, Bax, Bcl2, Acan, Col2a1 and Mmp3 expression. The LC3, P62, type II collagen, aggrecan, Beclin1, Akt, MMP3, p-mTOR, PI3K, mTOR, p-PI3K and p-Akt expression were analyzed by western blot.

Results: The IVD tissue damage and apoptosis occurred in the Model group, and the glycoprotein secretion decreased. Compared with Model group, AD-H group alleviated the injury of IVD tissue, inhibited the apoptosis of cells, and increased the secretion of glycoprotein. 40 μg/mL AD restored the proliferation activity of NPCs. Compared to the Normal group, the NPCs apoptosis increased, the Collagen II, aggrecan and Bcl2 expressions were significantly decreased, the MMP3, Bax and Casp 3 expression were significantly increased, and the LC-3 II/I expression in IVD tissues were increased significantly in Model group, all of which was reversed in AD group. AD promoted the p-Akt, p-PI3K, p-mTOR, LC-3 II/I and Beclin1 expression, inhibited the P62 expression to alleviate the damage of nucleus pulposus cells and the degeneration of IVD.

Conclusion: AD improved IDD by affecting the PI3K/Akt/mTOR pathway and autophagy.
Introduction

Intervertebral disc (IVD) degeneration (IDD) is often accompanied by low back pain, which seriously causes huge economic losses and reduces the quality of human life. Many mechanical factors, such as mechanical, traumatic, genetic, and nutritional factors, may affect disc integrity. The degenerative process includes structural damage to the IVD and changes in the number and composition of cells, most commonly affecting the biomechanics of the nucleus pulposum and ultimately the entire spine. Moreover, there is evidence that environmental as well as genetic factors can change the IDD development, and the abnormal function of nucleus pulposus cells (NPCs) is association with the pathogenesis of IDD. At present, there is no long-term effective cures for IDD. Therefore, exploring the potential mechanism of NPCs in IDD disease may help to develop new therapies.

Autophagy has always been controversial in the study of IDD. In a way, autophagy make protective in diseases such as disc degeneration. On the other hand, excessive autophagy can aggravate degenerative diseases, and appropriate inhibition of autophagy has been proved to be effective in diseases treatment. Chen et al. proved metformin improved lumbar disc degeneration through activation of autophagy. Kang et al. also proved autophagy protected lumbar disc degeneration. However, the results of Chen et al. showed that H₂O₂ treatment induced autophagy, while autophagy inhibition could reduce the apoptosis of NPCs under oxidative stress. The above studies have proved autophagy was important in IDD, inhibiting or activating autophagy in NPCs or a new way to treat IDD.

In addition, some studies on the pharmacological mechanisms of resveratrol have demonstrated that the up-regulation of PI3K/Akt pathway could inhibit NPCs apoptosis. Yang et al. showed that bone protein one activated PI3K/Akt/mTOR pathway under hypertonic culture to protect the NPCs. Tan et al. showed that bone morphogenetic protein two inhibited apoptosis and alleviated IDD by upregulating the phosphorylation level of PI3K/Akt pathway. In addition, Lin et al. also demonstrated the activating PI3K/Akt pathway in the treatment of IDD. The above studies showed that the PI3K/Akt/mTOR pathway was bound up with the status and function of NPCs, or a potential pathway to inhibit or activate autophagy of NPCs.

Total saponins were one of the main active components in radix achyranthis bidentatae. Achyranthes aspera leaves have significant anticancer effects on Epstein-Barr virus activation and two-stage mouse skin cancer. Flavonoids, saponins and tannins in the ethanol extract of Achyranthes bidentata leaf have anti-ulcer activity. Ethanol extract of Achyrantha bidentata may delay intestinal absorption of dietary fat by inhibiting the activities of pancreatic amylase and lipase and play an anti-obesity role in high-fat diet treated mice. The saponins in achyranthes bidentata seeds have anti-hyperlipidemia and anti-oxidation properties, which may improve the lipid profile and blood anti-oxidation status of albino rats fed a high cholesterol diet. Animal experiments have shown that the saponin active ingredient in radix achyranthis bidentatae can treat synovial rheumatoid arthritis by inhibiting the proliferation of inflammatory cells and bone degradation, but its effect on IDD disease is still unknown. It is known that AD is one of the saponins isolated from the root of Achyranthes bidentata. Therefore, this study aims to explore how the Achyranthes D (AD) affect IDD in vivo and in vitro, to provide a new idea for the IDD treatment.

Material and methods

Animal experiment and grouping

Forty SD rats were divided into five groups randomly to explore the concentration of AD (CAS: 168,009–91-4, Wuhan Bid Winning Technology Co., Ltd) to alleviate IDD. The groups (8 rats/group) were as follow: control group (Normal), model group (IDD), low dose of AD group (60 μg/g/d, AD-L), medium dose of AD group (120 μg/g/d, AD-M) and high dose of AD group (240 μg/g/d, AD-H). The administration was continued for 7 days after modeling surgery, once a day.

Next, in order to elucidate the effect of AD on IDD disease and autophagy, rapamycin alone intervention and AD + rapamycin combined intervention were used. 40 SD rats were separated into five groups randomly. The groups (8 rats/group) included control group (Normal), model group (IDD), model + AD group (AD), model + Rapamycin group (Rapamycin, a potent inducer of autophagy) and model + AD + Rapamycin group (AD + Rapamycin). Rapamycin was purchased from MedchemExpress (Art-number: HY-10,219) and injected intraperitoneally at 5 mg/kg/d for 21 days after modeling surgery. AD (240 μg/g/d) was also administrated for 21 days after modeling surgery.
Animal model construction of IDD

SD rats were modelled by lumbar cone annulus puncture, and medication was given 2 weeks after surgery. In brief, rats were intrathecally anesthetized with pentobarbital sodium (30 mg/kg). The operator wears a medical surgical magnifying glass (EVC300, SurgiTel, USA) to perform the operation. The 18 G syringe needle was inserted vertically into the L6-7 and L8-9 lumbar discs in the vertical direction with 5 mm depth. After full penetration, rotate the needle 360° and hold it for 20s. In the control group, the lumbar discs were only exposed. For the model group, PBS was injected into the puncture site 2 weeks after puncture. After the experiment, all rats were sacrificed, blood and IVD tissue were collected. The semi-quantitatively of the in vivo degeneration cascade was also confirmed.

Cell experiment and grouping

Rat IVD NPCs (CP-R145, ProCell, China) were purchased from ProCell, and cultured normally in a special complete medium (CM-R145, ProCell, China) according to the instructions of the medium. To research the toxic of AD on rat NPCs, the NPCs were treated with 0, 10, 20, 40, 60 and 80 μg/mL AD, and the cell proliferation was detected by CCK8 assay. To investigate the most suitable concentration of tert-butyl peroxide to construct the microenvironment model of nucleus pulposus degeneration, the NPCs were treated with 0, 50, 100, 200, 300 and 500 μM tert-butyl peroxide (168,521, Sigma-Aldrich), and the cell proliferation was detected by CCK8 assay. To explore the optimal concentration of AD for IDD in vitro, 10, 20, 40 and 60 μg/mL AD were added under the treatment of tert-butyl peroxide, respectively. The NPCs proliferation was tested by CCK8 assay, and cell morphology was observed.

To investigate the mechanism of AD in the treatment of degeneration of IVD NPCs in rats, the IVD NPCs were separated into control group (Normal), model group (Model), model + AD group (AD), model + Rapamycin group (Rapamycin) and model + AD + Rapamycin group (AD+Rapamycin), randomly. Model group was modeled with tert-butyl peroxide to simulate the degenerative microenvironment of NPCs. The Rapamycin group was pretreated with 100 nM Rapamycin (HY-10,219, MedChemExpress) for 2 h, followed by retention treatment.

Hematoxylin-eosin (HE) staining

Rat IVD specimens were fixed by formaldehyde decalcification and then dehydrated and embedded in paraffin. The tissue was cut into 5 μm slices. The slices were dewaxed to water using gradient alcohol (75–100%). The slices were stained with hematoxylin for 1 min, washed with distilled water, and blue back with PBS. The slices were stained with eosin for 0.5 min, then washed with distilled water. The slices were dehydrated with alcohol gradient (95–100%), 5 min for each grade. Sections were placed in xylene, then sealed with neutral gelatin and observed by the microscope (BA410 T, Motic).

TUNEL staining

The TUNEL test kit (40306ES50, YEASEN, China) was used and proteinase K working fluid and incubation buffer were configured. The specific operation process is as follows: After the rat IVD tissue sections were dewaxed to water, 100 μL proteinase K working solution was added to the sample area of each section and incubated at 37°C for 20 min. The slices were immersed in 1 × PBS and rinsed for 5 min × 3 times. Each sample section was dropped for 100 μL 1 × Equilibration Buffer to cover the sample area and incubated. Then 50 μL TDT incubation buffer was added to the sample area of each section and incubated. DAPI working solution (Wellbio) was dyed at 37°C for 10 min. The slices were sealed with buffered glycerin (Wellbio) and observed by the fluorescence microscope (BA210 T, Motic). The number of apoptosis-positive cells (green) and the number of total nuclei (blue) were counted in three randomly selected regions of each section. The ratio of the number of apoptotic positive cells (green) to the number of total nuclei (blue) was calculated by cell counting method. Each selected region was observed 3 times.

Alcian blue staining

The AB-PAS staining kit (Wellbio) was used and the procedure was as follows. In short, after the rat IVD tissue sections were dewaxed to water, alcine blue staining solution was added and incubated. The sections were successively washed by running water and distilled water, then re-dyed with nuclear solid red and re-washed with distilled water. Then, the slices were baked at 60°C for 30–60 min, then taken out and placed in xylene for 10 min × 2 times. The slices were sealed with neutral glue (Sigma) and observed by the microscope (BA410 T, Motic).

Cell counting kit-8 (CCK8)

NPCs were seeded into 96-well plates at 100 μL per well with 5×10³ cells/well. CCK8 working fluid is prepared with complete medium. After cell culture adherent, each well was added 10 μL CCK8 working solution. The absorbance (OD) at 450 nm was analyzed by Bio-Tek enzyme plate (MB-530, Heales) after further incubation at 37°C, 5% CO₂, 20% O₂ and 95% air for 4 h.
Flow cytometry

The NPCs were digested by trypsin (C0201, Beyotime Biotechnology) without EDTA and collected. The cells were washed with PBS and centrifuged to collect NPCs. 500 μL binding buffer was used to suspend cells. NPCs were added with 5 μL annexin V-FITC (KGA108, Keygen Bio Tech) and mixed, then 5 μL propidium iodide was added. The cells reacted in dark and were observed by flow cytometry.

Autophagy Staining Assay with Monodansylcadaverin (MDC) staining

NPCs were cultured in 6-well plates (0.3 × 10⁶/well) for 24 h, and then treated with cardamristin (5, 20 μM), rapamycin (0.1 μM) and 2-DG (10 mM) for 48 h. The cells were washed with PBS and incubated with MDC (0.05 mM, CAS:10,121–91-2, D4008, Sigma-Aldrich) in the dark at room temperature. The cells were washed to observe under a fluorescence microscope (BA210 T, Motic).

Real-time quantitative reverse transcription PCR (qRT-PCR)

After the experiment, IVD tissues of rats and NPCs in different treatment groups were collected. The intervertebral disc tissue was digested with 0.2% collagenase type II for several hours by cell culture celosia. PBS containing calf serum was added to stop the process for total RNA extraction. Total RNA was extracted by Trizol reagent (Thermo, USA). The mRNA reverse transcription kit (CW2569, Beijing Comwin Biotech, China) was used to synthesize cDNA. For PCR amplification and quantitative analysis (QuantStudio1, Thermo, USA), a reaction volume of 30 mL was used including 15 mL 2× Sybgreen PCR Master Mix (CW2601, Beijing Comwin Biotech, China), 1 μL for each primer, 2 mL of double diluted cDNA and 11 μL of sterile distilled water. The relative mRNA levels of each gene were analyzed by 2^−ΔΔCT method. Table 1 lists the sequence of primer pairs.

Western blot

After the experiment, IVD tissues of rats and NPCs in different treatment groups were collected. Radioimmunoprecipitation analysis (RIPA), lysis buffer and Bicinchoninic acid (BCA) method were used to determine the protein concentration. A total of 200 μg protein samples were separated by 12% SDS-PAGE. The isolated proteins were transferred to a polyvinylidene fluoride membrane that had been activated by methanol and blocked by 5% skim milk. It is then incubated overnight with the primary antibody. The main primary antibodies included anti-LC3B (14,600-1-AP, 1: 1000, Proteintech, USA), anti-p62 (ab109012, 1: 10,000, abcam, UK), anti-type II collagen (28,459-1-AP, 1: 1000, Proteintech, USA), anti-Aggrcan (13,880-1-AP, 1: 1000, Proteintech, USA), anti-Beclin1 (ab210498, 1: 1000, abcam, UK), anti-MMP3 (ab179461, 1: 1000, abcam, UK), anti-p-mTOR (67,778-1-Ig, 1: 1000, Proteintech, USA), anti-mTOR (66,888-1-Ig, 1: 2500, Proteintech, USA), anti-PI3K (ab140307, 1: 2000, abcam, UK), anti-p-PI3K (ab182651, 1: 800, abcam, UK), anti-AKT (10,176-2-AP, 1: 1000, Proteintech, USA), anti-p-AKT (66,444-1-Ig, 1: 5000, Proteintech, USA) and anti-β-actin (66,009-1-Ig, 1:5000, Proteintech, USA). It was then incubated with secondary anti-IgG (1:6000, ProteinTech, USA) at 37°C for 90 min. Visualization was performed using chemiluminescence (Millipore, USA) and imaging analysis by software (GE Healthcare, Life Sciences, USA).

Enzyme linked immunosorbent assay (ELISA)

The 80 mg IVD tissues of rats were collected and washed with 1×PBS. The interdisc tissue was put into a tissue grinder with 800 μL 1×PBS to make homogenate, and then placed at −20°C overnight. After the cell membrane was damaged by repeated freeze-thaw treatment for two times, the tissue homogenate was centrifuged at 5000 g to get the supernatant. The supernatant of NPCs was centrifuged at 1000 g at 2–8°C for 15 min. The type II collagen (CSB-E08092r, CUSABIO BIOTECH CO,Ltd, China), aggrecan (RF10753, Shanghai Longcheng Biology, China) and MMP3 (CSB-E07410r, CUSABIO BIOTECH CO,Ltd, China) test kits and a multifunctional enzyme conjugate analyzer (MB-530, HEALES) were used to detect.

Immunofluorescence (IF)

Rat IVD sections were dewaxed with gradient alcohol (75–100%) to water. The slices were immersed in 0.01 M citrate solution and treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After blocking, the sections were incubated overnight with primary antibodies. The secondary antibodies were goat anti-rabbit IgG (1:100, Jackson ImmunoResearch, USA) and Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen, USA). The nuclei were stained with DAPI (1:5000, Invitrogen, USA). The slides were mounted with fluorescent mounting media and observed by a fluorescent microscope (BA210 T, Motic).

Table 1. The primer sequences.

| Gene   | Primer sequence (5’ → 3’) |
|--------|---------------------------|
| Casp 3 | FOR: ATACAGCCTAATTTCAGACC  REV: TCTCTCTTCCCTTACGCTCT |
| Bax    | FOR: TTGTACAGGGTTTCTACGAGG  REV: GCTCCAAGGTCAGCTCAGGT |
| Bcl2   | FOR: CTGGTGGACACATCGCTCT    REV: ATAGTCCAAAAAGGATCCCA |
| Acan   | FOR: ACAGACACCCCTACCTGGCC   REV: CCTCACATTGCTCCTGGTCGAT |
| Mmp3   | FOR: CCTCTGAGTCTTTCTATGGAGGG  REV: TGTCTGTAGCCAGGAGGTGT |
| Coll21  | FOR: GCCAGAGTGGCCGAAAATTAG  REV: CTGTACCACGGTGACCTCT |
| Actb   | FOR: ACATCCGTAAAGACCTCTAGGCC  REV: TACTCTGTCTTGGTCACCTC |

USA, anti-P62 (ab109012, 1: 10,000, abcam, UK), anti-type II collagen (28,459-1-AP, 1: 1000, Proteintech, USA), anti-Aggrcan (13,880-1-AP, 1: 1000, Proteintech, USA), anti-Beclin1 (ab210498, 1: 1000, abcam, UK), anti-MMP3 (ab179461, 1: 1000, abcam, UK), anti-p-mTOR (67,778-1-Ig, 1: 1000, Proteintech, USA), anti-mTOR (66,888-1-Ig, 1: 2500, Proteintech, USA), anti-PI3K (ab140307, 1: 2000, abcam, UK), anti-p-PI3K (ab182651, 1: 800, abcam, UK), anti-AKT (10,176-2-AP, 1: 1000, Proteintech, USA), anti-p-AKT (66,444-1-Ig, 1: 5000, Proteintech, USA). It was then incubated with secondary anti-IgG (1:6000, ProteinTech, USA) at 37°C for 90 min. Visualization was performed using chemiluminescence (Millipore, USA) and imaging analysis by software (GE Healthcare, Life Sciences, USA).
buffer (pH6.0), heated and boiled to heat repair antigen. The slices were immersed in 3% H2O2 to inactivate endogenous enzymes and rinsed with PBS. NPCs were treated with section, fixed with 4% paraformaldehyde for 30 min, and washed with PBS. The slices were added with 0.3% crotch laton, permeable at 37°C for 30 min, and rinsed with PBS. The sections were sealed with 5% BSA for 60 min at 37°C and washed with PBS. Primary antibody (LC3, 14,600-1-AP, 1:50, Proteintech, USA) was added to tissue sections and cell sections, respectively, at 4°C overnight. The slices were then dropped with 50–100 μL anti-rabbit-IgG-labeled fluorescent antibody and incubated. The slices were stained with DAPI solution at 37°C for 10 min, rinsed with PBS, sealed with buffered glycerin, and observed under a fluorescence microscope (BA210 T, Motic).

**Data statistics and analysis**

Statistical software SPSS 21.0 (IBM, USA) was used for analyze data in this study. All the data were presented as means ± SD. Normality and homogeneity of variance tests were performed first, which were consistent with normal distribution and homogeneity of variance. Un-paired t-test was used for comparison between groups, one-way analysis of variance or analysis of variance for repeated measures was used for comparison between groups, and Tukey’s post hoc test was used for comparison. p-value < 0.05 was considered to be statistically significant.

**Results**

**AD improved IVD tissue damage and glycoprotein secretion in rats with IDD**

To research the affect of AD on IDD, low, medium and high doses of AD were administered intragastrically to IDD rats. Apoptosis analysis of IVD tissue showed that apoptosis occurred in the IDD group, while AD alleviated apoptosis in a dose-dependent manner, indicating that high dose AD had a better therapeutic effect, which could be used for subsequent studies (Figures 1(a) and (b)). We did not found surgical complications, infection, premature deaths or abnormal behavior of rats in different groups. HE staining showed clear and complete IVD with intact annulus fibrosus and clear boundaries in the Normal group, while severe lamellar disorder and fiber fracture appeared in the IDD group, and the IVD tissue injury was reduced in AD group (Figure 1(c)). The semi-quantitatively of the in vivo degeneration cascade was significantly increased in the IDD group, and decreased after AD treatment (Figure 1(c)). However, there was no significantly changes in the body weight of rats in different groups (Figure 1(d)). Alcine blue staining showed that the glycoprotein secretion was decreased in IDD group, while the secretion of glycoprotein was more increased in AD group than IDD group (Figure 1(e)). All results indicated that 240 μg/g/d AD can improve the IVD tissue damage and glycoprotein secretion in rats with IDD.

**AD restored the proliferation of NPCs in vitro**

To analyze the toxic of AD on NPCs, different concentrations of AD were used to treat NPCs. We found AD had no significantly toxic on NPCs (Figure 2(a)). The cell injury model of nucleus pulposus was established by tert-butyl peroxide, and the cell activity was significantly inhibited with the increase of concentration (Figure 2(b)). Compared with the Normal group, the effect of 100 μM tert-butyl peroxide was more obvious, so 100 μM tert-butyl peroxide was used to construct in vitro NPCs injury model (Figure 2(b)). In vitro evaluation of the effect of AD treatment showed that the proliferation of NPCs was significantly inhibited in Model group compared to the Normal group (Figure 2(c)). Compared with the Model group, 40 μg/mL AD treatment significantly restored the proliferation activity of NPCs (Figure 2(c)). The observation of cell morphology found that compared with the Normal group, the cell density of the Model group decreased, the basic structure of the cells was incomplete, and the cells were damaged (Figure 2(d)). After different concentrations of AD treatment, it was found that the cell density of 40 μg/mL AD treatment group was higher and the cell structure was basically complete (Figure 2(d)). The results showed that 40 μg/mL AD restored the proliferation activity of NPCs in IDD.

**AD alleviated the damage of NPCs by affecting autophagy**

We further study the effect of AD on NPCs injury found that compared with Normal group, the apoptosis rate of NPCs in Model group was significantly increased (Figure 3(a)). Compared with the Model group, the apoptosis rate of NPCs after AD treatment decreased significantly (Figure 3(a)). Compared with Normal group, the Bcl2 expression was significantly decreased, and the Bax and Casp 3 genes expression were significantly increased in NPCs in the Model group (Figure 3(b)). AD group reversed the result of apoptosis-related gene expression in Model group (Figure 3(b)). MDC staining showed that the formation of autophagosomes in NPCs was increased in the Model group, and decreased in the AD group compared to the Model group (Figure 3(c)). Compared with Normal group, the type II collagen and aggrecan expression was decreased, the MMP3 expression was increased in Model group (Figures 3(d) and (e)). Compared with the Model group, the
type II collagen and aggrecan expressions were increased, while the MMP3 expression was decreased in the AD group (Figures 3(d) and (e)). The analysis of rat IVD tissue showed that compared with the Normal group, the aggrecan and type II collagen expression were significantly decreased, while the expressions of MMP3 and LC-3 II/I were significantly increased in Model group (Figure 3(f)). Compared with Model group, the expressions of type II collagen and aggrecan were significantly increased, while the expressions of MMP3 and LC-3 II/I were decreased in the IVD.
of rats in the AD group (Figure 3(f)). These results suggested that AD could alleviate the injury of NPCs by affecting autophagy.

**Activation of autophagy reversed the effect of AD on NPCs injury**

In order to investigate the role of autophagy in AD treatment of NPCs injury, Rapamycin was used to treat NPCs to investigate the role of autophagy in AD treatment of NPCs injury. Compared with Model group, LC3 expression was decreased in the AD group and increased in the Rapamycin group (Figures 4(a) and (b)). Compared with AD group, the expression of extracellular matrix related factors in nucleus pulposus was detected by ELISA. Compared with Model group, the expression of II collagen and aggrecan was decreased, while the expression of MMP3 was increased in the Rapamycin group, which was contrary to the results in AD group (Figure 4(c)). Compared with Rapamycin group, the expression of II collagen and aggrecan was decreased, while the expression of MMP3 was increased in the AD+Rapamycin group (Figures 4(a) and (b)). The expression of extracellular matrix related factors in nucleus pulposus was detected by ELISA. Compared with Model group, the Bax and Casp 3 expression was increased, and the Bcl2 expression was decreased in the AD+Rapamycin group (Figures 4(a) and (b)). Compared with Rapamycin group, the expression of Bax and Casp 3 was decreased, and the Bcl2 expression increased significantly in the AD+Rapamycin group.
AD+Rapamycin group (Figure 4(d)). The autophagy-related genes in NPCs showed that Beclin1 and LC-3 II/I were increased and P62 was decreased in Rapamycin group compared with Model group (Figure 4(e)). Compared with Rapamycin group, the expression of Beclin1 and LC-3 II/I were decreased and P62 was increased in the AD+Rapamycin group (Figure 4(e)). These results suggested that AD improved NPCs injury by regulating autophagy.

**Activation of autophagy reversed the effect of AD on IVD injury in rats**

In addition, we conducted in vivo experiments to reveal whether AD can improve IVD tissue injury in rats by affecting autophagy. We did not found surgical complications, infection, premature deaths or abnormal behavior of rats in different groups. HE staining Alcine blue staining showed

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**Figure 3.** AD alleviated the damage of NPCs by inhibiting autophagy. (a). The apoptosis of NPCs was detected by flow cytometry. (b). qRT-PCR was used to analyzed the Casp 3, Bax and Bcl2 expressions. (c). MDC staining was used to detect autophagosome formation in NPCs. (d, e). The Acan (aggrecan), Col2a1 (type II collagen) and Mmp3 (MMP3) expression in NPCs were analyzed by qRT-PCR and western blot. (f) Western blot was used to detected the type II collagen, aggrecan, MMP3 and LC-3 II/I expression in IVD of rats. Scale bar = 100 μm; The magnification is 100 times; #p < 0.05 vs Normal group; &p < 0.05 vs Model group.
AD treatment was reversed by Rapamycin, as well as the semi-quantitatively of the in vivo degeneration cascade (Figures 5(a) and (b)). However, there was no significantly changes in the body weight of rats in different groups (Figure 5(c)). Apoptosis analysis of IVD tissue showed that the apoptosis ratio was significantly increased in the AD+Rapamycin group (Figure 5(d)). IF analysis of LC3 expression in IVD tissue showed that compared with the IDD group, LC3 expression was significantly decreased in the AD group and increased in the Rapamycin group (Figure 5(e)). Compared with Rapamycin group, LC3 expression was significantly decreased in the AD+Rapamycin group (Figure 5(e)). The expression of extracellular matrix related factors in IVD tissue showed that compared with the IDD group, the type II collagen and aggrecan expressions were decreased, while the expression of MMP3 was increased in the Rapamycin group (Figure 5(f)). The results were contrary to those in the AD group.
Figure 5. Activation of autophagy reversed the therapeutic effect of AD on IVD injury in rats. (a). The IVD injury of rats was analyzed by HE staining and confirmed by the semi-quantitatively of the in vivo degeneration cascade. (b). The alcian blue staining analysis of rat IVD tissue. Scale bar = 250 μm and 100 μm; The magnification is 40 and 100 times. (c) The body weight of rats in different groups. (d). The apoptosis of IVD tissue was detected by TUNEL staining. Scale bar = 25 μm; The magnification is 400 times. (e). The expression of LC-3 protein in rat IVD tissue was detected by IF. Scale bar = 25 μm; The magnification is 400 times. (f). The expressions of type II collagen, aggrecan and MMP3 were detected by ELISA. (g). The Casp 3, Bax and Bcl2 expression was analyzed by qRT-PCR. (h). The expression of Beclin1, LC-3 II/I and P62 proteins were analyzed by western blot. *p < 0.05 vs normal group; #p < 0.05 vs Model group; †p < 0.05 vs Rapamycin group.
group (Figure 5(f)). Compared with Rapamycin group, the type II collagen and aggrecan expression was increased, while the expression of MMP3 was decreased in the AD+Rapamycin group (Figure 5(f)). The apoptosis proteins expression in IVD tissues showed that the Bax and Casp 3 expression was increased in the Rapamycin group, and the Bcl2 expression was decreased compared with IDD group (Figure 5(g)). Compared with Rapamycin group, the Bax and Casp 3 expression decreased, and the Bcl2 expression increased significantly in the AD+Rapamycin group (Figure 5(g)). The expression of autophagy-related genes in IVD tissues showed that Beclin1 and LC-3 II/I were increased and P62 was decreased in Rapamycin group compared with IDD group (Figure 5(h)). Compared with Rapamycin group, Beclin1 and LC-3 II/I expression were decreased and P62 expression was increased in the AD+Rapamycin group (Figure 5(h)). AD improved IVD tissue injury by regulating autophagy in IDD rats.

**Autophagy activators reversed the effect of AD on PI3K/Akt/mTOR pathway**

To explore the AD inhibition of autophagy in the treatment of rat IVD tissue injury, western blot was used to test the PI3K/Akt/mTOR pathway proteins expression. We found there was no significant change in the PI3K, Akt and mTOR expression in NPCs and rat IVD tissues (Figures 6(a) and (b)). The protein phosphorylation level analysis showed that the p-PI3K, p-Akt and p-mTOR expression in NPCs and rat IVD tissues were decreased in IDD group compared with Normal group (Figures 6(a) and (b)). Compared with the IDD group, the p-PI3K, p-Akt and p-mTOR expression were significantly increased in NPCs and rat IVD tissues in the AD group, and the expressions of p-PI3K, p-Akt and p-mTOR in the Rapamycin group were significantly decreased in NPCs and rat IVD tissues (Figures 6(a) and (b)). Compared with Rapamycin group, the expression of p-PI3K, p-Akt and p-mTOR in the AD+Rapamycin group was significantly increased, but lower than that in the AD group (Figures 6(a) and (b)). All indicated that AD could regulate autophagy by affecting PI3K/Akt/mTOR pathway to treat the injury of rat IVD tissue.

**Discussion**

Our study confirmed that AD alleviated the injury of IVD tissue in IDD rats. And AD alleviated the damage of NPCs and IVD tissue by affecting autophagy and PI3K/Akt/mTOR pathway. It was known that IDD was a chronic and progressive process characterized by dehydration of the IVD, reduction of the height of the IVD, and changes in the load distribution of the spine, leading to the destruction of the IVD structure and the occurrence of low back pain.36
Ligustrazine could reduce the expression of inflammatory factors and TGF-β1 in hypertrophic cartilage endplates to prevent IDD.37 Total saponins of Panax notoginseng can alleviate IDD by inhibiting osteoclast differentiation induced by nuclear factor-κB ligand and inhibiting abnormal osteoclast activation in porous cartilage endplates.38 Our study showed that the NPCs apoptosis increased, the expressions of Collagen II, aggrecan and Bcl2 were decreased, the expressions of MMP3, Casp 3 and Bax genes were increased, and the expressions of LC-3 II/I in IVD tissues were increased in IDD group, while AD treatment reversed this outcome. All the studies proved that AD improved IDD by down-regulating the degradation of ECM and apoptosis of IVD tissue.

The miR-21 promoted Collagen II and aggregated proteoglycan catabolism by up-regulating the Akt/mTOR pathway and inhibiting autophagy in NPCs, thereby alleviating disc degeneration.39 In addition, studies have shown that corticostatin (CST) can reduce the catabolism and apoptosis of NPCs during TNF-α-induced IVD degeneration, inhibit mitochondrial dysfunction of NPCs and alleviate IDD.40 Guo et al. showed that Moracin M might inhibit LPS-induced phosphorylation of PI3K and Akt, thereby promoting autophagy and inhibiting the production of inflammatory mediators in NPCs.41 Our study found AD promoted glycoprotein secretion and inhibited autophagosome formation, thus effectively alleviated the injury of IVD tissue in IDD rats, which were consistented with the above studies. All the research confirmed that AD alleviated the injury of IVD tissue in IDD rats by regulating abnormal NPCs metabolism and autophagosome formation.

Many studies have shown that many signal transduction pathways are involved in the regulation of autophagy.42 Among these pathways, some converge on targets of rapamycin (TOR), a highly conserved kinase important for autophagy regulation, such as PI3K-AKT, MAPK cascades and p53 signal.43 In some studies, the up-regulation of miR-19 increased the viability and proliferation of NPCs and reduced the autophagy of NPCs regulated by XIST, while overexpression of XIST inactivated PI3K/Akt signaling pathway.44 Estrogen could reduce intervertebral disc cell apoptosis in a variety of ways, including inhibiting TNF-α and IL-1β, inhibiting matrix metalloproteinases and reducing anabolism, activating PI3K/Akt pathway, reducing oxidative and promoting autophagy.45,46 Our study found AD could alleviate the injury of IVD tissue in IDD rats and NPCs damage by regulating the PI3K/Akt/mTOR pathway and autophagy, which were reversed by Rapamycin and inconsistented with the above studies. As the pivot point between anabolic and catabolic processes, mTOR complex 1 (mTORC1) signaling has been shown to play a role in regulating metabolism, translation and autophagy.47 We hypothesized that the regulation of autophagy by AD through mTOR includes the regulation between growth and metabolism, but there may be differences in nutritional factors or energy factors that cause inconsistency in studies.

We first explored the pharmacological effects of AD in IDD rats, and then analyzed the mechanism of AD in NPCs injury and IDD rats. However, we just used the rat NPC cells for the in vitro examination, and did not consider the differences in cell phenotypes between the human chondrogenic nucleus pulposus cells and the rat notochordal cells, this was a limitation in our study. At present, the derivatives of Achyranthiside have been used in clinical drug development,48 and our research proves that AD may be the basic compound for drug development. Saponins are sugar-bound natural compounds with a variety of biological activities, such as medicinal properties, antibacterial activity, antiviral activity, etc., which can be developed into natural drugs without side effects through biosynthesis and extraction, such as ginseng.49 Chemical synthesis has emerged as a practical alternative to natural saponins and their modified analogues to facilitate structure-activity relationships studies and the discovery of optimal structures for clinical applications, such as steroid/triterpene chemistry and carbohydrate chemistry.50 Therefore, we suspect that further development of the extraction and derivation process of AD may contribute to its application and treatment in orthopedic diseases.

Moreover, TGF-β1 has been found to down-regulate autophagy and delay IDD by up-regulating PI3K/Akt/mTOR pathway.51 In addition, Liu et al.52 found that the apelin-13/APJ system activated the PI3K/Akt pathway, reduced the degradation of ECM in the nucleus pulpodes, promoted proliferation, and reduced the levels of apoptosis and inflammation, thus delaying the degeneration of intervertebral discs. Curcumin can reduce inflammation and delay the progression of IDD through PI3K/Akt/NF-κB signaling pathway.53 In our study, AD promoted the PI3K, p-Akt, mTOR, p-PI3K, Akt, p-mTOR, Beclin1 and LC-3 II/I expression, and inhibited the P62 expression to alleviate the injury of IVD tissue in IDD rats and the damage of NPCs. AD could regulate autophagy through PI3K/Akt/mTOR pathway in the injury of IVD tissue in IDD rats and NPCs injury in vitro.

Conclusions

In conclusion, AD could alleviate the injury of IVD tissue in IDD rats and NPCs damage in vitro by regulating the PI3K/Akt/mTOR pathway and autophagy, or provide a new way for the treatment of IDD.

Declaration of Conflicting Interests

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Ethical statement
This study was approved by the Animal Ethics Committee, The Ethical statement issued by the Ministry of Science and Technology in 2006.

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