European Review for Medical and Pharmacological Sciences 2018; 22: 786-795

Leptin inhibits apoptosis of nucleus pulposus cells via promoting autophagy

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Abstract. – OBJECTIVE: To investigate whether leptin can regulate the apoptosis of nucleus pulposus cells by adjusting the autophagy of human degenerative nucleus pulposus cells and its possible molecular mechanism.

PATIENTS AND METHODS: The human degenerative nucleus pulposus cells were extracted and cultured, then treated with leptin, leptin inhibitor and leptin neutralizing antibody; the expressions of light chain 3 (LC3) II/I and Beclin-1 were detected by Western blot, and the change of apoptosis rate was detected by flow cytometer. After the nucleus pulposus cells were treated with leptin, bafilomycin A, the autophagy inhibitor, was used to inhibit the autophagy. Western blot was used to detect the expressions of LC3II/I and cleaved caspase 3, and the apoptosis rate was detected by flow cytometer. Finally, Western blot was used to detect the expressions of Akt and extracellular regulated protein kinases 1/2 (Erk1/2) signal pathway associated proteins after nucleus pulposus cells were treated with LY294002, the phosphatidylinositol 3-kinase (PI3K) inhibitor, and PD98059, the extracellular regulated protein kinases (Erk1/2) inhibitor.

RESULTS: After the cells were treated with leptin, the expressions of LC3II/I and Beclin-1 could be increased, and the apoptosis rate of nucleus pulposus cells could be decreased ($p<0.05$). After the degenerative nucleus pulposus cells were treated with leptin inhibitor or neutralizing antibody, the expressions of LC3II/I and Beclin-1 were decreased and the apoptosis rate of nucleus pulposus cells was increased ($p<0.05$). Bafilomycin A could increase the expression of LC3II/I ($p<0.05$), increase the apoptosis rate ($p<0.05$), and the effect of leptin of decreasing the apoptosis rate of nucleus pulposus cells. The results of Western blot showed that LY294002 could inhibit the expression of LC3II/I by inhibiting the Akt phosphorylation ($p<0.05$), but leptin could partially offset the inhibiting effect of LY294002 on the expression of LC3II/I. PD9805 could partially decrease the expression of LC3II/I via inhibiting the Erk1/2 phosphorylation, but leptin could not reverse the inhibiting effect of PD98059 on the expression of LC3II/I.

CONCLUSIONS: Leptin inhibits the apoptosis of degenerative nucleus pulposus cells via promoting autophagy, and leptin regulates the autophagy of human degenerative nucleus pulposus cells through the phosphorylated Erk1/2 signal.

Key Words:
Leptin, Autophagy, Nucleus pulposus cell of intervertebral disc, Apoptosis.

Introduction

Intervertebral disc degeneration is the main cause of a series of diseases, such as discogenic back pain, herniation of intervertebral disc, degenerative spinal stenosis, and lumbar spondylolisthesis, etc. Therefore, degenerative intervertebral disc disease is a kind of common degenerative disease affecting people's health. At present, the main treatment method of such diseases includes the conservative anti-inflammatory and analgesic therapy, physical therapy and surgical operation of intervertebral discectomy, replacement and spinal fusion, etc.[2] However, these methods only play a role as symptomatic treatment without intervention on the fundamental causes of biological behavior of intervertebral disc degeneration, especially the early- and mid-stage intervertebral disc degeneration that does not need the surgical treatment yet[3]. Therefore, how to delay or even inhibit intervertebral disc degeneration from the pathogenesis of intervertebral disc degeneration has been a problem to be solved urgently in the field of spine surgery.

Intervertebral disc nucleus pulposus cell apoptosis is the main reason for intervertebral disc degeneration. Although the exact molecular mechanism of disc degeneration is not clear, the current study shows that the cell apoptosis in intervertebral disc degeneration is increased significantly, and the cell over-apoptosis caused by non-physiological causes is the main reason for the reduction of intervertebral disc cells[4]. In the nucleus pulposus tissues, the extracellular

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matrix is secreted by nucleus pulposus cells. The increased apoptosis of nucleus pulposus cells will lead to the loss of a series of microscopic organic structures and biochemical components: decreased cell number, degradation of extracellular matrix and reduced hydration of matrix. Thus, it can be seen that the inhibition of nucleus pulposus cell apoptosis is expected to become a potential target for the prevention of intervertebral disc degeneration.

Autophagy is a process that cells phagocytose and degrade their own cytoplasm and organelle through the lysosomal system, which plays an important role in the organism growth and development, self-stabilization, maturation, and differentiation of cells, etc[5]. Before cell death, autophagy can be observed in the cells, showing that autophagy may be associated with apoptosis. In the annulus fibrosus of the intervertebral disc in rabbits, autophagy can protect the degeneration of annulus fibrosus by inhibiting apoptosis[6,7]. Xu et al[8] studied and found that the promotion of autophagy can inhibit the calcification of endplate cartilage of intervertebral disc in rats. Recently, scholars[9-11] in China have found that with the rat-tail intervertebral disc degeneration, autophagy of nucleus pulposus cells is gradually enhanced. The above results show that autophagy may play an important role in the process of intervertebral disc degeneration.

Leptin is a small molecular protein mainly secreted by the white adipose tissue, which is why it is named "leptin". It can affect the hypothalamic nucleus by negative feedback, thereby adjusting appetite, promoting fat metabolism, and causing weight loss[12]. Recent studies[13-15] have shown that leptin is not only involved in energy metabolism, but also involved in a variety of physiological and pathological processes, such as cell proliferation, apoptosis, inflammatory response, angiogenesis, tumorigenesis, tumor metastasis, which plays an important negative effect. The role of leptin is realized through the combination of leptin receptor. It has been reported that leptin expression is detected in the synovial fluid, osteophytes and articular cartilage of osteoarthritis patients. Gruber et al[16] have proved that human intervertebral disc cells cultured *in vitro* can naturally secrete the leptin protein that reaches the detection level. Zhao et al[17] also detected the leptin and its specific receptors in nucleus pulposus cells of the lumbar intervertebral disc.

In summary, the apoptosis of nucleus pulposus cells is an important factor in disc degeneration, and autophagy is the key link to determine the occurrence of apoptosis; leptin is involved in the cell proliferation and apoptosis process, and human intervertebral disc cells can naturally secrete leptin and express specific receptors. Thus, we speculate that leptin can regulate the autophagy to regulate the apoptosis of nucleus pulposus cells, thus affecting the process of disc degeneration. The intervention study was conducted from the perspective of three key factors that are crucial for the survival of nucleus pulposus cells, namely leptin, autophagy, and apoptosis with the nucleus pulposus cells in patients with intervertebral disc degeneration as the subjects, to investigate the molecular mechanism of leptin that regulates the autophagy to adjust the nucleus pulposus cell apoptosis, so as to find a new breakthrough for the research and prevention of intervertebral disc degeneration.

Patients and Methods

Clinical Specimens

In this study, the acquisition of human body specimen had been approved by Ethics Committee in our hospital. The specimens of human intervertebral disc nucleus pulposus were from the intervertebral disc nucleus pulposus tissues resected from patients admitted to Spine Surgery Department of our hospital, and the informed consent of patients had been obtained. The normal nucleus pulposus tissues (Nor) were from a total of 4 young patients with lumbar vertebral fracture. Degenerative intervertebral disc nucleus pulposus tissues (DDD) were from a total of 25 middle-aged and elderly patients with degenerative disc disease.

Materials of Human Normal and Degenerative Intervertebral Disc Nucleus Pulposus Cells

The intervertebral disc specimens were placed in a sterile glass dish with DMEM/F12 complete culture solution containing 15% fetal bovine serum. The ice cubes were used to maintain the low temperature, and the specimen was quickly sent to the laboratory. Then, the tough annulus fibrosus, endplate cartilage tissues, a little muscle and bone structure around the nucleus pulposus tissues were removed using muscle forceps in the sterile operating table. Phosphate-buffered saline (PBS) was used to clean the bloodstain in nucleus pulposus tissues for 3-5 times. The separated and

clean nucleus pulposus tissues were cut using muscle forceps into comminuted meats with the size of 1×1×1 mm^3, and placed in 15 ml centrifuge tube. Then, 0.25% trypsin was added, and the tube was vibrated for digestion in water bath at 37°C for 20 min. After digestion, it was centrifuged at 800 r/min×5 min, and the supernatant was discarded. 0.2% type II collagenase was added for the secondary digestion and, then, the tube was vibrated for digestion in water bath at 37°C for 2-3 h. After that, 2 ml DMEM/F12 was added immediately to terminate the digestion. The digestive solution in centrifuge tube was filtered and centrifuged at 800 r/min×5 min, and the cell sediments at the bottom were retained. 10 ml DMEM/F12 complete culture solution containing 15% fetal bovine serum was added, and transferred into the culture flask for inoculation after the cells were blown and beat uniformly.

Culture and Passage

The culture flask was placed in the incubator containing 5% CO_2 at 37°C, and 3 ml DMEM/F12 complete culture solution containing 15% FBS was added at 3 d, in order to avoid losing the non-adherent nucleus pulposus cells due to too-early solution replacement. The solution was replaced for the first time at 5-7 d when the cells were completely adhered. After that, the solution was replaced every 2-3 d according to the cell growth situation. When the primary cell confusion rate reached about 90% (cultured for 2-3 weeks), the first subculture was performed. The culture medium was abandoned and PBS was used to wash once, and cells were digested with 1.5 ml 0.25% trypsin for 1 min. The digestion process was observed under microscope (Olympus, Tokyo, Japan) in real time. When most of the cells began to shrink, DMEM/F12 complete culture solution containing 15% fetal bovine serum was added immediately to terminate the digestion. The straw was used to gently beat and blow the cells repeatedly to fall off cells. The cell suspension was collected and centrifuged (800 r/min×10 min), and PBS was used to wash twice. Then, the suspension was centrifuged (800 r/min×10 min); the suspension cells were added, and the passage was performed as the appropriate density of 1:2 or 1:3 according to the cell quantity.

Nucleus Pulposus Tissue and Cells Observed by Electron Microscope

The nucleus pulposus tissue was washed with PBS and cut into the particle with the size of 1 mm^3. Five to six particles were placed in 2.5% glutaraldehyde fixative solution, fixed at room temperature for 24 h, and PBS was used to wash particles for 15 min for 3 times. 2% osmic acid stationary liquid was used to fix at room temperature for 2-3 hours and PBS was used to wash particles for 15 min for 3 times, followed by tissue dehydration (50% ethanol, 70% ethanol, 90% ethanol, 90% ethanol, 90% acetone (1:1), 90% acetone) at 4°C for 20 min, 100% acetone at room temperature for 20 min, 3 times, pure acetone + embedding solution (1:2) overnight, overnight at 37°C, 50-70 nm ultra-thin slice, 3% uranyl acetate-lead citrate double staining, observation under electron microscope and film.

Western Blot Detection

The extraction of total protein in nucleus pulposus cell was performed according to the instruction of tissue protein extraction kit (KeyGEN BIO, Beijing, China), and the entire process was completed on the ice. Protein concentration was determined according to the instruction of bicinchoninic acid (BCA) protein concentration kit (Beyotime, Shanghai, China). According to the protein molecular mass, the separation gel and concentrated gel in different concentrations were prepared; the protein samples received the electrophoresis under a constant voltage of 60V after sample loading. According to the protein molecular mass, the appropriate polyvinylidene difluoride (PVDF) membrane was selected. The appropriately diluted primary antibodies (LC3 1:1000, Beclin-11:1000, β-actin 1:500, Caspase-3 1:1000, Akt 1:1000, p-Akt 1:1000, extracellular regulated protein kinases 1/2 (Erk1/2) 1:1000, p-Erk1/2 1:1000, mammalian target of rapamycin (mTOR) 1:1000, p-mTOR 1:1000) were added and the samples were placed in refrigerator overnight at 4°C. ECL reaction liquid was prepared, vibrated and fully mixed. The membrane and ECL reaction liquid were co-cultured (at room temperature in a dark place for 5 min) and, then, the images in imager (Bio-Rad, Hercules, CA, USA) were collected.

Statistical Analysis

SPSS13.0 statistical software was used, and experimental data were presented as mean ± standard deviation (S.D). Independent sample *t*-test was used for the data significance test, and $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of Autophagosomes in Human Normal and Degenerative Nucleus Pulposus Cells

We observed the expression of autophagosomes in normal (Nor) and degenerative nucleus pulposus cells (DDD) by transmission electron microscope, the golden standard of autophagy detection. Under TEM, we found the double-layer or multi-layer autophagosomes in normal nucleus pulposus cells and degenerative nucleus pulposus cells. The number of normal nucleus pulposus specimens was limited, and the proportion of nucleus pulposus cells in the whole nucleus pulposus tissue was very small, so it was difficult to conduct the concrete quantitative analysis of the autophagosome observed. However, from the perspective of observation condition, the number of autophagosomes in normal nucleus pulposus cells was significantly more than that in degenerative nucleus pulposus cells. Western blot showed that the number of LC3II/I and Beclin-1 in normal nucleus pulposus cells was more than that in degenerative nucleus cells (Figure 1).

Leptin Promoted Autophagy of Degenerative Nucleus Pulposus Cells

After nucleus pulposus cells were treated with leptin (Lept), Western blot was used to detect the expressions of LC3II/I and Beclin-1 in each group, so as to study the effect of leptin on autophagy of degenerative nucleus pulposus cells. After the treatment with leptin, the expressions of LC3II/I and Beclin-1 were significantly increased. When leptin antagonist (Anta) and leptin neutralizing antibody (Anti), the expressions of LC3II/I and Beclin-1 were significantly decreased (Figure 2). The differences between above results and those of control group (Cont) were statistically significant (Figure 2ABC).

Leptin Inhibited the Apoptosis of Degenerative Nucleus Pulposus Cells

After nucleus pulposus cells were treated, the apoptotic cells in each group were counted by flow

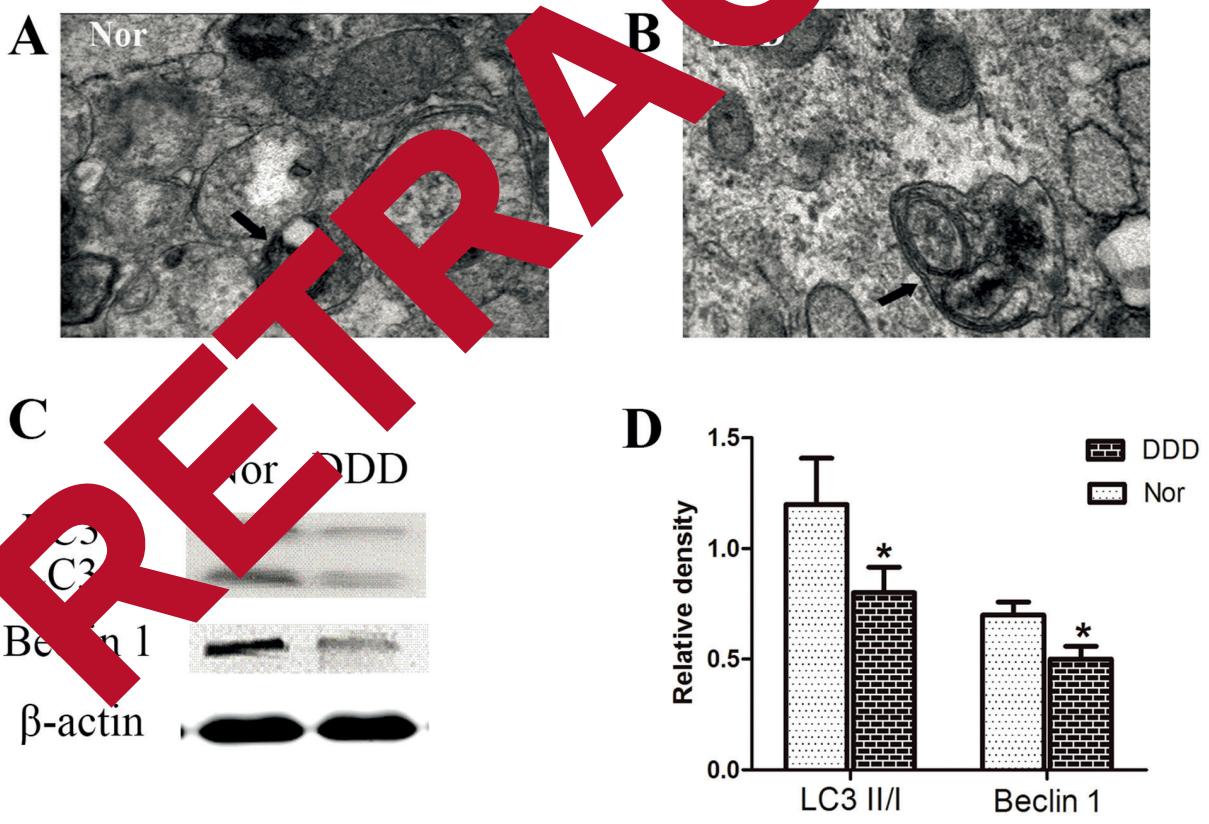

Figure 1. Expression of autophagosomes in human normal and degenerative nucleus pulposus cells. (A) The expression of autophagosomes in normal by TEM. (B) The expression of autophagosomes in degenerative nucleus pulposus cells by TEM. (C) Western blots analysis reveals the expression of LC3II/I and Beclin-1. (D) Semiquantitative analysis of LC3II/I and Beclin-1 (*p<0.05).

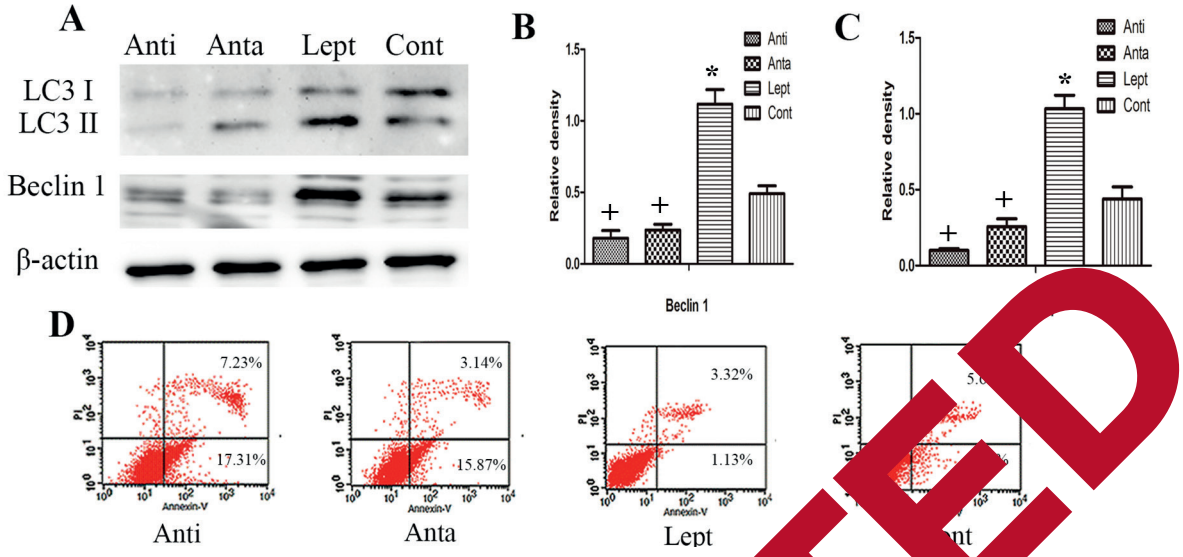

Figure 2. Leptin promoted autophagy and inhibited the apoptosis. ***(A)*** W[illegible]ts analysis[illegible]s the expression of LC3II/I and Beclin-1. ***(B)*** Semiquantitative analysis of LC3II/I. ***(C)*** Semiqua[illegible]tative [illegible]lysis of Bec[illegible]n-1. ***(D)*** The apoptotic cells in each group counted by flow cytometer (**p*<0.05 *versus* Cont, + *p*<0.05 *versus* L[illegible]

cytometer. The results showed that the apopt[illegible]is rate of Cont group was 9.77% (Figure 3), [illegible] that of leptin group was 4.45%, indicating tha[illegible]e apoptosis rate was decreased significantly a[illegible] treatment with leptin. The apoptosis [illegible]s of An[illegible] group and Anti group were 19.0[illegible] a[illegible]4.54%, respectively, indicating that th[illegible]optos[illegible]ates of degenerative nucleus pulpo[illegible]s [illegible]we[illegible] significantly when lep[illegible] was [illegible]bited; the differences between t[illegible]ove resu[illegible]nd those of control group [illegible]e [illegible]istically [illegible]nificant (Figure 2D).

Leptin In[illegible]ted [illegible] Apoptosis of Degener[illegible]leus Pulposus Cells via Prom[illegible]phag[illegible]

[illegible] st[illegible]ther leptin protects the [illegible]ptosis [illegible] degen[illegible]ve nucleus pulposus cells [illegible]pro[illegible]he autophagy, we treated the cel[illegible] bafilomycin A (Bafi A); the autophagy inhib[illegible] Western blot was used to detect the expressi[illegible] of key proteins in autophagy and apoptosis, and flow cytometer was used to detect the change in cell apoptosis rate. After treatment of nucleus pulposus cells, the protein expressions of LC3II/I and cleaved caspase 3 in each group were detected by Western blot. The results showed that the expression of LC3II/I in Bafi A group, Lept group and Lept + Bafi A group was significantly higher than that in Cont group, and the [illegible]s had the statistical significance. Compared with that in Lept group, the expression [illegible]C3II/I in Bafi A group and Lept + Bafi A gro[illegible] was increased significantly; but there was no significant difference in the expression of LC3II/I between Bafi A group and Lept + Bafi A group. Compared with that in Cont group, the expression of cleaved caspase 3 in Lept group was significantly decreased, and the expressions of cleaved caspase 3 in Bafi A group and Lept + Bafi A group were increased significantly. Compared with that in Lept group, the expressions of cleaved caspase 3 in Bafi A group and Lept + Bafi A group were increased significantly, and there was no significant difference in the expression of cleaved caspase 3 between Bafi A group and Lept + Bafi A group. After treatment of nucleus pulposus cells, the changes in apoptosis rate were detected by flow cytometer. The results showed that the apoptosis rate of nucleus pulposus cells in Cont group was 8.12%, that in Lept group was decreased to 3.55%, and that in Bafi A group and Lept + Bafi A group was increased to 35.47% and 30.76%, respectively. The above results showed that compared with that in Cont group, the apoptosis rate in Lept group was significantly decreased and that in Bafi A group and Lept + Bafi A group was significantly increased; compared with that in Lept group, the apoptosis rate in Lept + Bafi A group was significantly increased,

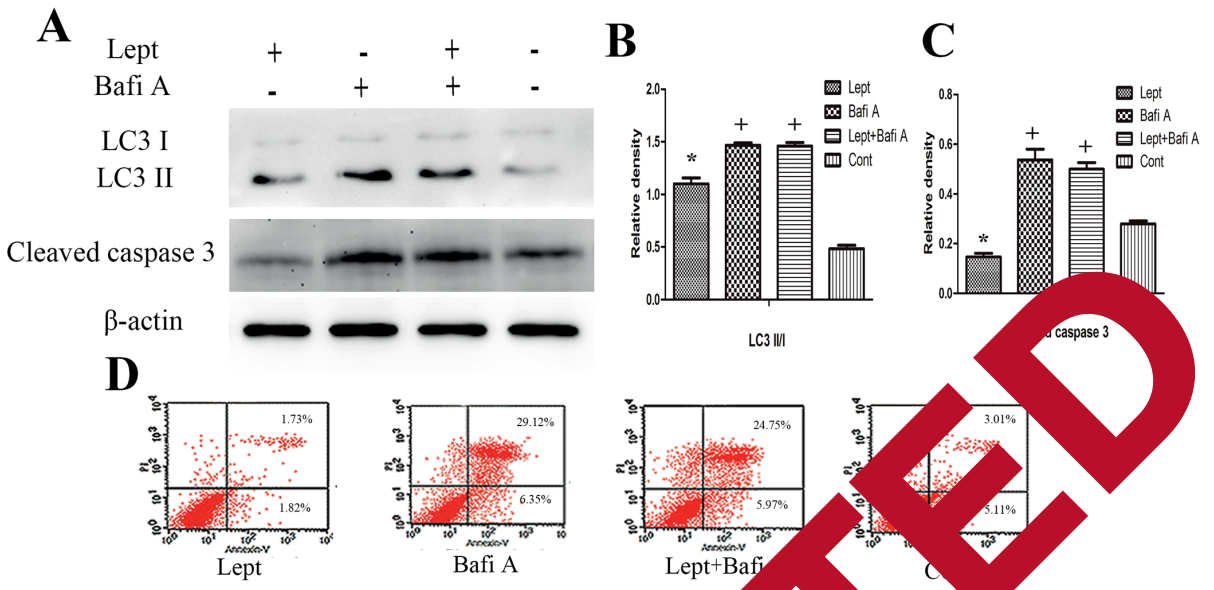

Figure 3. Leptin inhibited the apoptosis of degenerative nucleus pulposus cells via p[illegible]ting autophagy. ***(A)*** Western blots analysis reveals the expression of LC3II/I and Cleaved caspase 3. ***(B)*** Se[illegible]itative ana[illegible]f LC3II/I. ***(C)*** Semiquantitative analysis of Cleaved caspase 3. ***(D)*** The apoptotic cells in each group [illegible]ted by flow cytome[illegible] (*$p<0.05$ *versus* Cont, +$p<0.05$ *versus* Lept.).

indicating that when the autophagic activity w[illegible]as inhibited, leptin could not stop the apopto[illegible] nucleus pulposus cells, so leptin inhibited [illegible]e apoptosis of degenerative nucleus pulposus c[illegible] by promoting autophagy (Figure 3).

Leptin did not Promote A[illegible]phac[illegible] of Degenerative Nucleus Pu[illegible]us [illegible] Akt Pathway

To test whether l[illegible] promot[illegible]utophagy through Akt pathw[illegible]w[illegible]ed Weste[illegible]blot to detect the expre[illegible]n of LC[illegible]I, the key protein in autophagy[illegible]aved caspase[illegible]e key protein in apoptosi[illegible]kt a[illegible]mTOR, the key pathway proteins, a[illegible]hei[illegible]hosphorylated proteins after degene[illegible]ive [illegible]s pul[illegible]us cells were treated wit[illegible]002 [illegible]e results showed that [illegible]pared[illegible]ith tha[illegible] Con group, Lept could [illegible]ote[illegible]ssion of Akt phosphorylation pro[illegible]romote the expression of LC3II/I, inhibit the ex[illegible]ion of cleaved caspase 3, and inhibit the expressi[illegible]f mTOR phosphorylation protein, but the total expressions of Akt and mTOR were not affected. After cells were treated with LY294002, the phosphatidylinositol 3-kinase (PI3K) inhibitor, the expression of LC3II/I was inhibited, the expression of cleaved caspase 3 was increased and the phosphorylation level of mTOR was increased. When degenerative nucleus pulposus cells were treated with leptin and LY294002, the expression of L[illegible]/I [illegible]increased compared with treatment with LY294002, indicating that leptin can partially [illegible]t the inhibiting effect of LY294002 on [illegible]agy. The above results suggested that leptin does not promote the autophagy of degenerative nucleus pulposus through promoting Akt phosphorylation (Figure 4).

Leptin Promoted the Autophagy of Degenerative Nucleus Pulposus Cells Via Erk-mTOR Pathway

In order to test whether leptin promotes the autophagy via Erk pathway, Western blot method was used to detect the expressions of LC3II/I, the key protein in autophagy, cleaved caspase 3, the key protein in apoptosis, and Erk1/2 and mTOR, the key pathway proteins, and their phosphorylated proteins, after the treatment of degenerative nucleus pulposus cells with PD98059 (PD). The results showed that compared with Cont group, leptin could promote the expression of Erk1/2 phosphorylated protein, promote the expression of LC3II/I, the key protein in autophagy, inhibit the expression of cleaved caspase 3, the key protein in apoptosis and inhibit the expression of mTOR phosphorylated proteins, but the total expressions of Erk1/2 and mTOR were not affected. When cells were treated with PD98059, the MEK inhibitor, the expression of LC3II/I was inhibited, while the expression of cleaved caspase

3 was increased, so was the phosphorylation level of mTOR. When degenerative nucleus pulposus cells were treated with leptin and PD98059, the expression of LC3II/I did not change compared with that when cells were treated with PD98059 alone, indicating that leptin could not offset the inhibiting effect of PD98059 on autophagic activity. The above results suggested that leptin regulates the autophagy of degenerative nucleus pulposus cells via Erk-mTOR pathway (Figure 5).

Discussion

Autophagy involves a wide range of physiological and pathological processes of various diseases, such as the tumor, metabolic and neurodegenerative disorders, cardiovascular and pulmonary diseases, which is closely related to exercise and aging[18-20]. The main role of autophagy is to remove the senile organelles in cells, useless biological macromolecules and damaged cell structures, which is the "scavenger" of cells[21]. In our work, we firstly observed the autophagosome in human nucleus pulposus cells using autoradiometric gold standard, transmission electron microscopy, and proved the presence of human nucleus pulposus cells. Further observation revealed that the number of autophagosomes in nucleus pulposus cells of young patients was significantly lower than aged patients. However, because the num of cells in the nucleus pulposus is scarc cess of normal nucleus pulposus is difficu ly 4 cases of normal nucleus p us speci ns were obtained in the wh expe nt), a he autophagy process m be very s y as short as 8 min fr autop osome mation to degradation), it is ffic to dir tly quantify the number autop osom bserved by transmissi tron mi .

Leptin s lved in process of cell proliferation a apoptosis, and human int bral disc c can naturally secrete the l n and express specific receptors. Therefore, suggest th eptin may promote the autophagy

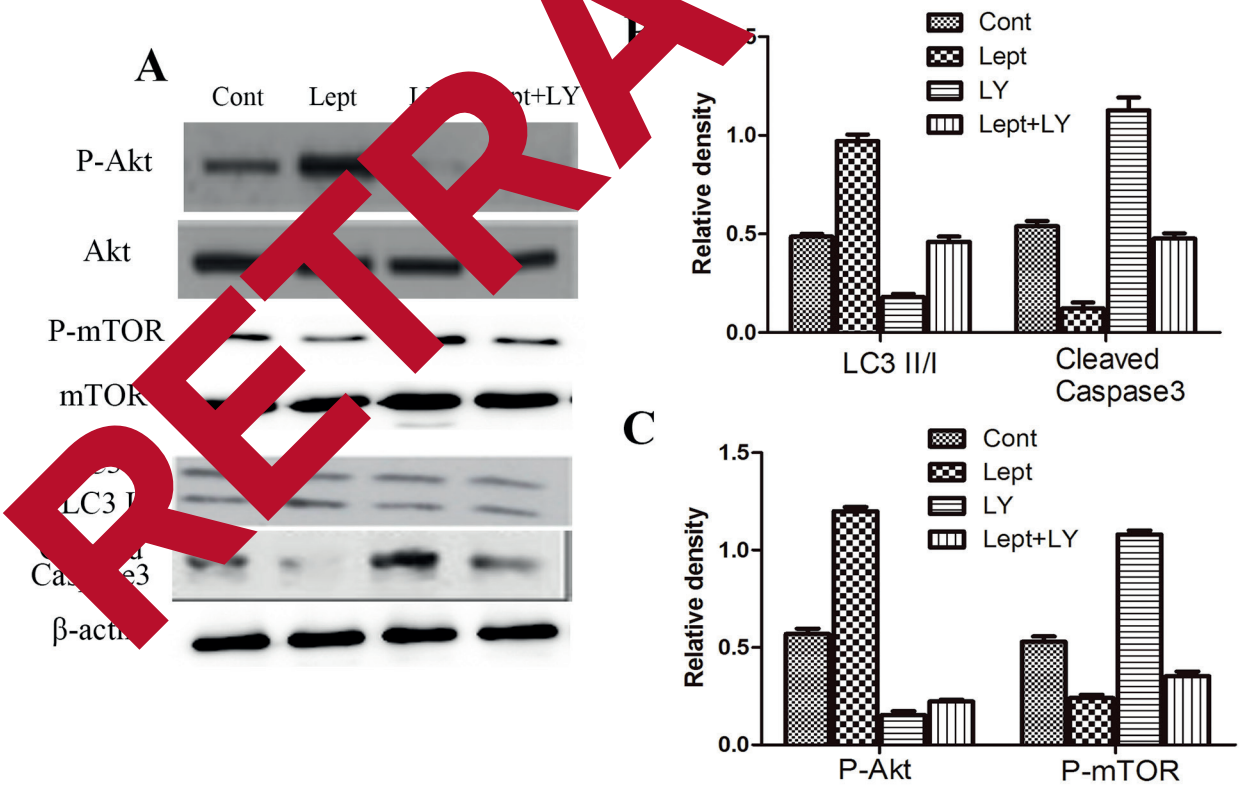

Figure 4. Leptin did not promote autophagy of degenerative nucleus pulposus cells via Akt pathway. ***(A)*** Western blots analysis reveals the expression of P-Akt, Akt, P-mTOR, mTOR, LC3II/I, Cleaved caspase 3. ***(B)*** Semiquantitative analysis of LC3II/I and Cleaved caspase 3. ***(C)*** Semiquantitative analysis of P-Akt and P-mTOR.

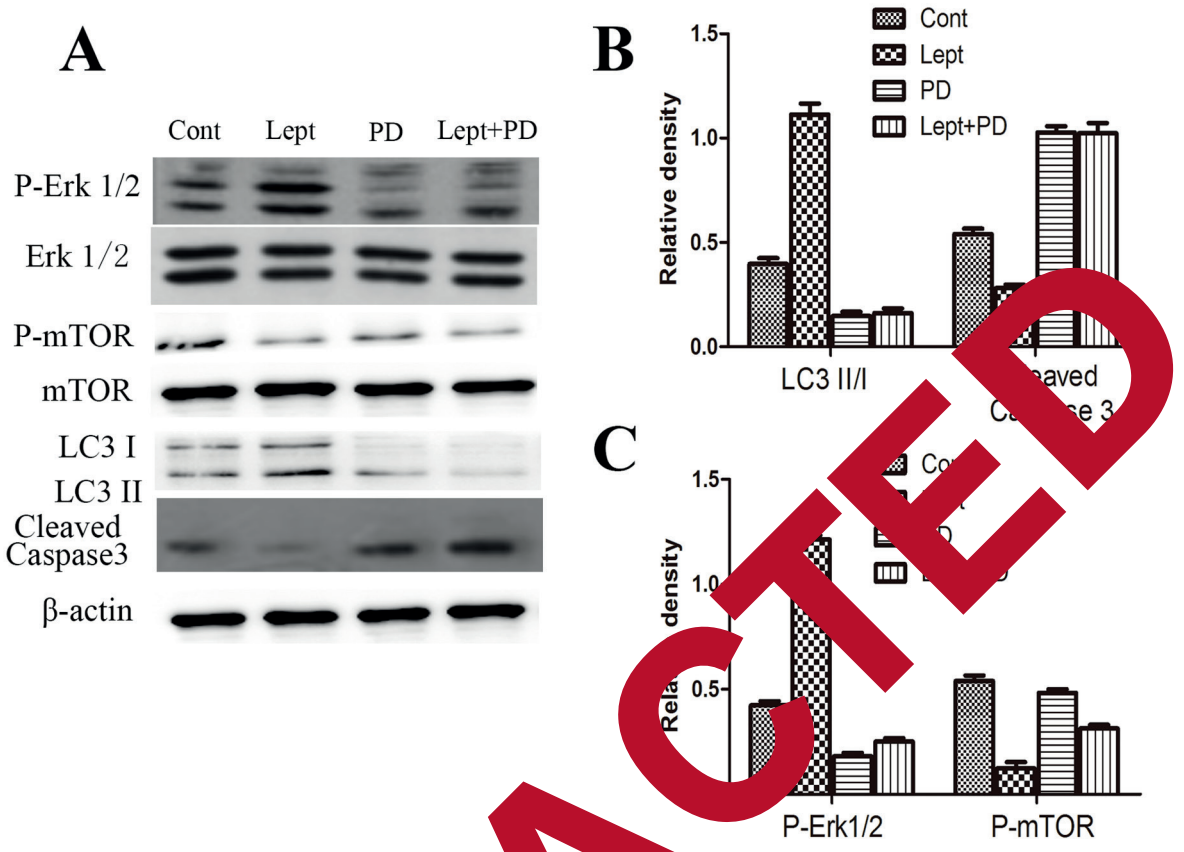

Figure 5. Leptin promoted the autophagy of degene[illegible]posus cells via Erk-mTOR pathway. ***(A)*** Western blots analysis reveals the expression of P-Erk1/2, Erk1/2, [illegible]R, mTOR, LC3II/I, Cleaved caspase 3. ***(B)*** Semiquantitative analysis of LC3II/I and Cleaved caspase [illegible]iquanti[illegible]e analysis of P-Erk1/2 and P-mTOR.

of degenerative nucleus p[illegible]osus [illegible]. The results in this study also confi[illegible]our spec[illegible]ion. After degenerative nucle[illegible]u[illegible]s cells w[illegible]treated with leptin, au[illegible]hagy wa[illegible]nhanced, which was manifest[illegible]n the enhanc[illegible]xpressions of LC3II/I and [illegible]clin-1[illegible]d increased apoptosis rate of nucleus p[illegible]os[illegible]ells; b[illegible]t, when degenerative nucle[illegible]ulpo[illegible]cells [illegible]e treated with leptin ant[illegible]neu[illegible]i[illegible]antibody, the autophagic [illegible]vity w[illegible]inhibi[illegible], which was manifested in [illegible]lecr[illegible]ession of LC3II/I and Beclin-1, and [illegible]increased apoptosis rate of nucleus pulpo[illegible]ells. The above results suggested that leptin ca[illegible]romote the autophagy of degenerative nucleus pulposus cells and inhibit the apoptosis of degenerative nucleus pulposus cells. To further test whether leptin inhibits nucleus pulposus cell apoptosis via promoting autophagy, we used bafilomycin A, the autophagy inhibitor, to inhibit autophagy of nucleus pulposus cells. Bafilomycin A is a kind of specific inhibitor of vacuolated H+-ATP enzyme that inhibits autophagy by blocking the binding between autophagosomes and lysosomes, which is manifested in the increased autophagosome and decreased autolysosomes[22]. We found that after the treatment of degenerative nucleus pulposus cells with leptin + bafilomycin A, the expression of LC3II/I was significantly increased compared with that when cells were treated with leptin alone. Interestingly, although the expression of LC3II/I also had an increasing trend after cells were treated with leptin + bafilomycin A compared with that when cells were treated with bafilomycin A alone, the differences had no statistical significance. Chen et al[15] also described a similar result in his paper, but did not explain such a phenomenon. We inferred that it is possibly because bafilomycin A blocks the autophagic flux, reducing the effect of leptin of inducing autophagosome formation. In other words, when bafilomycin A blocks the binding between autophagosome and lysosome, more autophagosomes will be accumulated in nucleus pulposus cells, and leptin cannot promote

the formation of more autophagosomes via the regulation similar to negative feedback. Of course, this is only an assumption, and further experiments are needed to prove it. We also found that when nucleus pulposus cells were treated with bafilomycin A, the expression of cleaved caspase 3 in degenerative nucleus pulposus cells was significantly increased and the apoptosis rate was also increased significantly, no matter whether leptin was added. All the above results confirmed that leptin inhibits the apoptosis of human degenerative nucleus pulposus cells by promoting autophagy. It was reported in previous studies that the activation of PI3K/Akt signaling pathway in some cells could induce autophagy via phosphorylated mTOR[23-25]. We found that leptin could promote the expression of Akt phosphorylated protein, promote the expression of LC3II/I, the key protein in autophagy, inhibit the expression of cleaved caspase 3, the key protein in apoptosis, and inhibit the expression of mTOR phosphorylated protein, but the total expressions of Akt and mTOR were not affected. When cells were treated with LY294002, the PI3K inhibitor, the expression of LC3II/I was inhibited, while the expression of cleaved caspase 3 was increas was the phosphorylation level of mTOR. W degenerative nucleus pulposus cells were trea with leptin and LY294002, the e ssion LC3II/I was increased compared wher cells were treated with LY2940 lone, i cating that leptin could partially o the effect of LY294002 on a hagi ity. These results proved that le an media utophagy via pathways other and our lts also supported this [22,23]. So previous studies also argued th itogen-activ protein kinase (MAPK)/E athw layed an important role in the regulati of c proliferation, differentiation, apopto etc he re s of this work showed tha an p e expression of Erk1/2 phor ed pro , promote the expression protein in autophagy, inhibit the ession of cleaved caspase 3, the key prote apoptosis, and inhibit the expression of mTO hosphorylated protein, but the total expressions of LC3II/I and mTOR are not affected. When cells were treated with PD98059, the extracellular regulated protein kinases (MEK) inhibitor, the expression of LC3II/I was inhibited, the expression of cleaved caspase 3 was increased and the phosphorylation level of mTOR was also increased. When degenerative nucleus pulposus cells were treated with leptin and PD98059, the expression of LC3II/I did not change compared with that when cells were treated with PD98059 alone, indicating that leptin could not offset the inhibiting effect of PD98059 on autophagic activity. The above results indicated that Erk-mTOR signal axis plays an important role in promoting the expression of LC3II/I and inhibiting the activation of cleaved caspase 3.

Conclusion

Our results showed th leptin inhi the apoptosis of human de erative nu posus cells via promoting ophag and fu r study found that lept la the tophagy of degenerative leus posus ls via Erk-mTOR path instead TOR signaling pathway esu ults pro d some ideas and basis for the fu r investigation of internal rel between le and intervertebral disc d eneration.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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