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Effect of PI3K/AKT/HIF-1α signaling pathway on oxidative stress and apoptosis in nucleus pulposus cells

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Abstract. – OBJECTIVE: The aim of the study was to explore the expression of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/AKT)/hypoxia-induced factor-1 alpha (HIF-1α) signaling pathway and the apoptosis of nucleus pulposus cells (NPCs) under different oxygen concentrations to clarify the biological characteristics of NPCs and the molecular mechanism of intervertebral disc degeneration (IVDD).

MATERIALS AND METHODS: Normal and degenerated human NPCs were collected. Leibovitz's medium with 100 μmol/L CoCl2 was used to establish a hypoxic culture environment, and 100 μmol/L H_2O_2 was used to establish an oxidative stress culture environment. Third-generation NPCs were divided into 6 groups: normal NPCs + hypoxia, normal NPCs + normoxia, normal NPCs + oxidative stress, degenerated NPCs + hypoxia, degenerated NPCs + normoxia, and degenerated NPCs + oxidative stress. Normal NPCs + hypoxia was used as the experimental control group. Cell viability and proliferation were detected by using the Cell Counting Kit-8 (CCK-8) method. Cell apoptosis rate was assessed by flow cytometry, and expression levels of PI3K, AKT, and HIF-1α were determined by Real-Time-Polymerase Chain Reaction (RT-PCR) and Western blotting.

RESULTS: The cell proliferation rate of both normal and degenerated NPCs decreased with increasing oxygen concentration. Conversely, the apoptosis rate increased as the oxygen concentration increased ($p<0.05$). Compared with the control group, whether the cells degenerated had a very significant impact on the apoptosis rate ($p<0.001$), and oxygen concentration also had a highly significant impact on both the cell proliferation rate and apoptosis rate (both $p<0.001$). The interaction between cell degeneration and oxygen concentration significantly affected both cell proliferation and apoptosis rates ($p<0.05$). Considering the expression levels of PI3K, AKT, and HIF-1α, normal NPCs had the highest levels under low oxygen concentrations, followed by oxidative stress and normoxia. In degenerated NPCs, the expression levels also decrease as the oxygen concentration increases.

CONCLUSIONS: The PI3K/AKT/HIF-1α signaling pathway plays a significant role in inhibiting oxidative stress, antagonizing NPC apoptosis, and consequently delaying IVDD.

Key Words:
Oxygen concentration, Nucleus pulposus cells, Cell apoptosis, Oxidative stress, Biological characteristics, PI3K/AKT/HIF-1α.

Introduction

Intervertebral disc degeneration (IVDD) is one of the main causes of shoulder, neck, and back pain and mainly occurs in middle-aged and elderly people, with an over 90% prevalence rate. IVDD is becoming increasingly common, and its incidence shows a younger trend, resulting in a serious socioeconomic burden[1]. At present, the pathological mechanism of IVDD is still unclear, but it is generally believed that apoptosis, oxidative stress, and the inflammatory response, play an important role[2]. Healthy intervertebral disc tissue is in a microenvironment of low oxygen (1% to 2%) and low nutrients[3,4]. The generation and clearance of reactive oxygen species (ROS) is in a dynamic balance in nucleus pulposus cells (NPCs). Once the dynamic balance is disrupted and causes oxidative stress[5], the oxygen concentration in the intervertebral disc will change,

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inducing NPC apoptosis and leading to IVDD[6]. Therefore, exploring the mechanism of NPC apoptosis under different oxygen concentrations is important in understanding the pathological mechanism of IVDD.

The phosphatidylinositol-3 kinase (PI-3K)/protein kinase B (AKT) signaling pathway plays an important role in cell proliferation and apoptosis[7]. The pathway can be activated in an oxidative stress environment to inhibit apoptosis and promote extracellular matrix (ECM) generation, thereby delaying the IVDD process[8]. Hypoxia-inducible factor 1-alpha (HIF-1α) is a downstream gene of the PI3K/AKT signaling pathway that plays an important role in maintaining oxygen balance, ECM metabolic balance, and the homeostasis of the intervertebral disc microenvironment[7-9]. At present, studies have shown that the PI3K/AKT signaling pathway is involved in the IVDD process[10] and that HIF-1α participates in the apoptosis of NPCs[11]. This work will further explore the expression of the PI3K/AKT/HIF-1α signaling pathway and apoptosis of NPCs under different oxygen concentrations to clarify the biological characteristics of NPCs and the molecular mechanism of IVDD and then to provide targets and strategies for clinical therapy.

Materials and Methods

Study Design

The study was designed by an *in vitro* cell experiment, and the differences among groups were compared by two-way analysis of variance (ANOVA).

Time and Location

The experiment was conducted at the Second Affiliated Hospital of Hunan University of Traditional Chinese Medicine and Wuhan Genecreate Bioengineering Co., Ltd. from January, 2021 to January, 2023.

Main Reagents and Instruments

Normal and degenerated NPCs were purchased from Pricella Biological Company (CM-H170). Dulbecco's Modified Eagle Medium (DMEM, HyClone Co., GE Healthcare, Chicago, IL, USA), Fetal Bovine Serum (FBS, Gibco Co., Grand Island, NY, USA), Cell Counting Kit-8 (CCK-8, Solarbio Co., Beijing, China), TRIzol (Invitrogen Co., Waltham, MA, USA), ReverTra Ace qPCR RT Kit (Toyobo Co., Osaka, Japan), SYBR Green Realtime PCR Master Mix (Toyobo Co., Osaka, Japan), TritonX-100 (Sinopharm Co., Beijing, China), CHAPS (Sigma Co., St. Louis, MO, USA), CAPSO (Sigma Co., St. Louis, MO, USA), Phosphatase Inhibitor Cocktail (CWBIO Co., Beijing, China), Protease Inhibitor Cocktail (Beyotime Co., Shanghai, China), PMSF (Sigma Co., St. Louis, MO, USA), DL-Dithiothreitol (DTT, Solarbio Co., Beijing, China), Plant Protease Inhibitor Cocktail (Beyotime Co., Shanghai, China), BCA Protein Assay Kit (CWBIO Co., Beijing, China), Skimmed milk powder (Solarbio Co., Beijing, China), Prestained Dual Color Protein Marker (UElandy Co., Suzhou, China), Super ECL Prime (UElandy Co., Suzhou, China), Ultrasensitive ECL Kit (UElandy Co., Suzhou, China), HRP-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (Proteintech Co., Chicago, IL, USA), HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (Proteintech Co., Chicago, IL, USA), Pipettes, Centrifuges, Cell incubators, Microplate readers, Electrophoresis instruments, Quantitative PCR amplification instruments, Ultraviolet spectrophotometers, High-speed homogenizers, Ultrasonic cell disruptors, and Water baths cauldron.

Cell Culture

Normal and degenerated human NPCs purchased from Pricella Biotechnology Co. (Thermo Scientific, Grand Island, NY, USA) were used in the experiment, and the cells were passaged when the cell fusion rate reached 85%. The second-generation cells were used for identification, and the third-generation cells were used for subsequent experimental research.

Identification of Normal and Degenerated NPCs

The identification of normal and degenerated NPCs was performed as previously described[12]. The second-generation cells were seeded in 24-well plates and washed thoroughly with PBS after adhering. Then, 4% paraformaldehyde was used to fix the cells for 15 minutes, and the cells were thoroughly washed with PBS. Triton X-100 (0.5%) was used for permeabilization at room temperature for 20 minutes. After removing the blocking solution, the cells were placed in a wet box and incubated overnight at 4°C. The next day, after fully washing with PBS, diluted fluorescent (Cy3)-labeled goat anti-rabbit IgG was added at a dilution ratio of 1:100 and incubated in a wet box at 20-37°C for 1 hour. After washing with PBS,

DAPI was added for nuclear staining in the dark for 5 minutes and then washed. After removing the liquid from the slides, the slides were sealed with a mounting solution containing an Anti-Fluorescence Quenching Agent and then observed and photographed under a fluorescence microscope to observe the staining and morphological characteristics of the cells.

Grouping

Leibovitz's medium with 100 μmol/L $CoCl_2$ was used to establish a hypoxic culture environment, DMEM with 10% FBS and 100 μmol/L H_2O_2 was used to construct an oxidative stress culture environment, and the normoxic environment was 37°C, with an air oxygen concentration and saturated humidity. Then, the third-generation NPCs were divided into 6 groups: normal NPCs + hypoxia, normal NPCs + normoxia, normal NPCs + oxidative stress, degenerated NPCs + hypoxia, degenerated NPCs + normoxia, and degenerated NPCs + oxidative stress. Normal NPCs + hypoxia was used as the experimental control group.

CCK-8 Assay for Cell Viability

CCK-8 assay was performed as previously described[13]. The cells were harvested with trypsin, counted, and inoculated in a 96-well plate with 1,000 cells and 100 μl medium per well. The plate was placed at 37°C in a 5% CO_2 incubator for 0 h, 12 h, 24 h, and 48 h. CCK-8 solution (10 μL) was added to each well and mixed. The cells were cultured for 2 h, and the absorbance value at 450 nm was measured with a microplate reader. The measurement was repeated 3 times for each well.

Detection of Apoptosis by Flow Cytometry

Cell apoptosis was detected by Annexin V-FITC/PI double staining as previously described[14]. After the cells in each group were cultured for 48 hours, the cells were collected and centrifuged to discard the supernatant. Annexin V-FITC and PI staining agents were added and incubated with the cells. Cell apoptosis was detected by flow cytometry. The experiment was repeated 3 times.

Protein Expression Detection of PI3K, AKT, and HIF-1α

Western bolt was performed as previously described[15]. A total of 10^7 cells (approximately 50 μL) were added to 0.5 mL RIPA lysate containing phosphatase inhibitor cocktail (1 mM), protease inhibitor cocktail (1 mM), phenylmethanesulfonyl fluoride (PMSF, 1 mM), and DTT (1 mM) and gently pipetted to mix. After standing in an ice bath, the mixture was centrifuged in a high-speed refrigerated centrifuge. The supernatant was collected, and the concentration of the total protein was determined by the Bicinchoninic acid (BCA) method. A concentrated gel was prepared according to the molecular weight and expression level of the tested protein and the number of samples. An appropriate amount of total protein was placed in a 0.6 mL Eppendorf (EP) tube, buffer was added, and the tube was mixed well before electrophoresis. After removing the gel and removing the stacking gel, the polyvinylidene fluoride (PVDF) membrane and transfer filter paper were cut to the same size as the separation gel to perform the transfer. After the transfer was completed, the membrane was removed, and the excess liquid was absorbed and placed in an incubation bag. Diluted primary and secondary antibodies were added to the bag. After incubation, the membrane was washed with Tris Buffered Saline Tween (TBST), and 500 μL of enhanced chemiluminescence (ECL) reaction solution was added. After reaction for 30 s and exposure for 10 min, the protein expression results were observed.

mRNA Expression Detection of PI3K, AKT, and HIF-1α

Total ribonucleic acid (RNA) in cells was extracted by the TRIzol method as previously described[16]. The concentration of RNA was detected with a Nanodrop2000c ultramicro spectrophotometer (Thermo Scientific, Grand Island, NY, USA). One microgram of total RNA was taken and reverse transcribed into c-deoxyribo nucleic acid (cDNA), and the operation steps were carried out according to the manufacturer's instructions. Real-Time Fluorescent Quantitative PCR was carried out using cDNA as a template and actin as an internal reference. The reaction conditions were as follows: 95°C for 1 minute and a total of 40 amplification cycles of 95°C for 15 s and 60°C for 30 s. Finally, the relative expression level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The experimental data were preliminarily tabulated with Excel 2010 and analyzed by analysis of variance with SPSS 19.0 (SPSS Corp., Armonk, NY, USA) statistical analysis software.

Multivariate analysis of variance was used to evaluate the main effect of NPC degeneration and the oxygen environment and their interaction. The least significant difference (LSD) test was used for post-hoc pairwise comparisons (significance level α=0.05). GraphPad Prism 8.0.1 (La Jolla, CA, USA) was used for plotting. $p < 0.05$ indicated a significant difference.

Results

Identification of Normal and Degenerated NPCs

As shown in Figures 1 and 2, the second-generation normal NPCs showed a polygonal shape, while the degenerated NPCs mainly showed a spindle shape. Both types of NPCs had large nuclei and abundant cytoplasm. COLII is a specific protein of NPCs that is often used to identify NPCs. Red fluorescence represented the expression of COLII protein with a positive rate greater than 90%; that is, the cell purity was more than 90%. The results indicated that the cultured cells were NPCs.

Effects of Different Oxygen Concentrations on the Cell Viability of Normal and Degenerated NPCs

The cell proliferation curves of the 6 groups are shown in Figure 3. After culture for 12, 24, and 48 h, the proliferation rates of normal and degenerated NPCs decreased with increasing oxygen concentration, and the difference was statistically significant ($p < 0.05$). The proliferation rate of degenerated NPCs in hypoxic and oxidative stress environments was slightly higher than that of normal NPCs in hypoxic and oxidative stress environments after culture for 48 h, and the differences were statistically

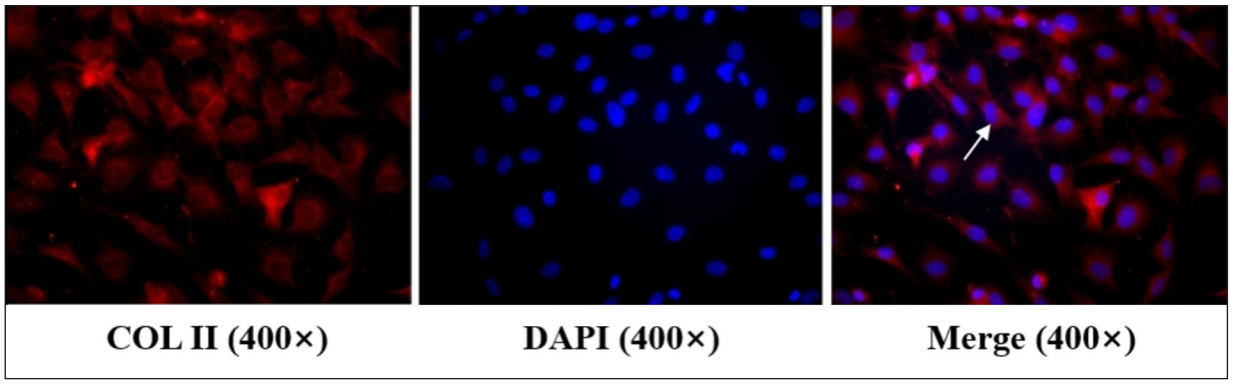

Figure 1. Immunofluorescence results of normal NPCs**.** COL II, collagen II; DAPI, 4',6-diamidino-2-phenylindole; NPCs, nucleus pulposus cells.

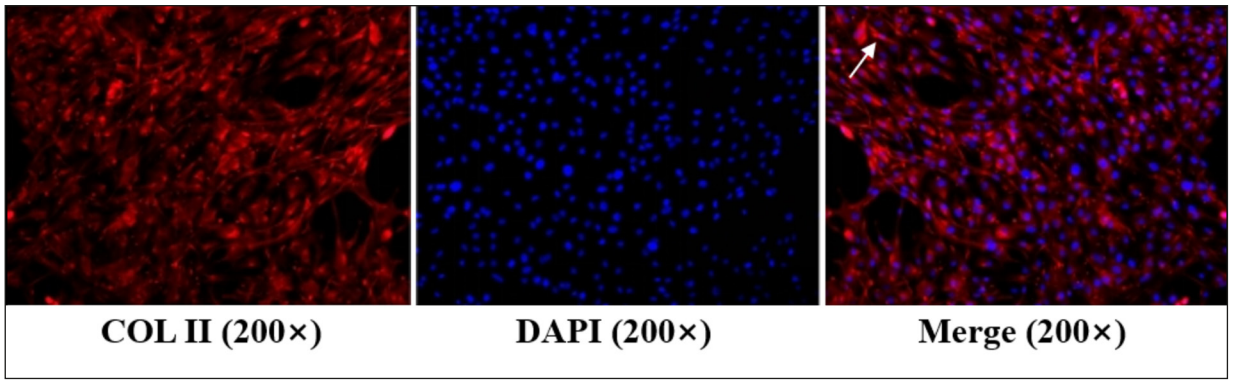

Figure 2. Immunofluorescence results of degenerated NPCs. COL II, collagen II; DAPI, 4',6-diamidino-2-phenylindole; NPCs, nucleus pulposus cells.

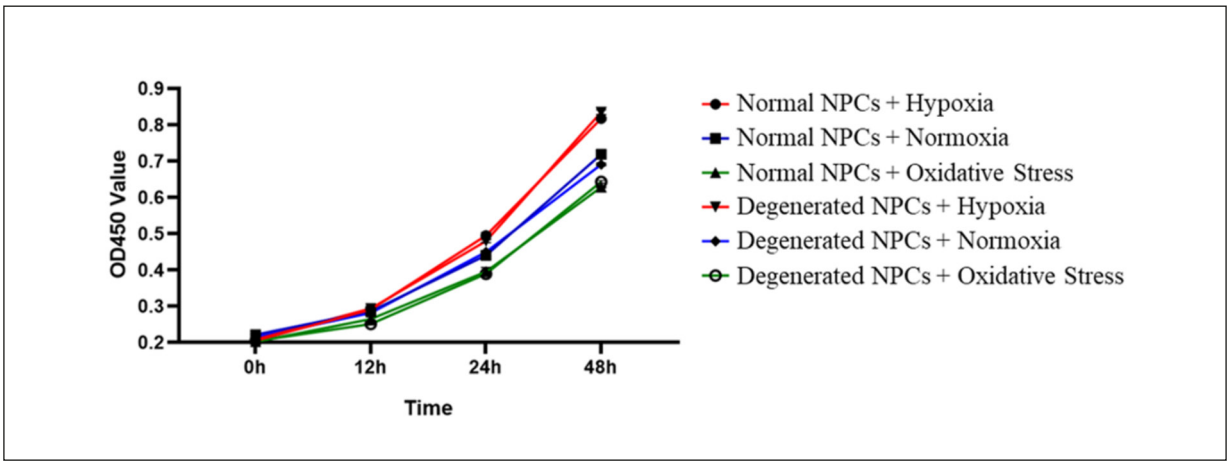

Figure 3. Comparison of cell viability of normal and degenerated NPCs under different oxygen concentrations at different time points. NPCs, nucleus pulposus cells.

significant ($p < 0.05$). The cell proliferation rate of normal NPCs was slightly higher than that of degenerative NPCs under normoxic conditions, and the difference was statistically significant ($p < 0.05$).

When considering normal NPCs + hypoxia as the control group, whether NPCs degenerated had no significant effect on the proliferation rate ($p > 0.05$), but the oxygen concentration had an extremely significant impact on the proliferation rate ($p < 0.001$). The interaction had a significant impact on the proliferation rate ($p < 0.05$), and the impact was extremely significant after 48 hours of culture ($p < 0.001$).

Effects of Different Oxygen Concentrations on the Apoptosis Rate of Normal and Degenerated NPCs

The apoptosis rate of the 6 groups is shown in Figure 4. The apoptosis rates of normal and degenerated NPCs increased with increasing oxygen concentration, and the differences were statistically significant ($p < 0.05$). Under hypoxia and normoxia, there was no significant difference between normal and degenerated NPCs ($p > 0.05$). Under oxidative stress, the apoptosis rate of degenerated NPCs was significantly higher than that of normal NPCs ($p < 0.05$). When considering normal NPCs + hypoxia as the control group,

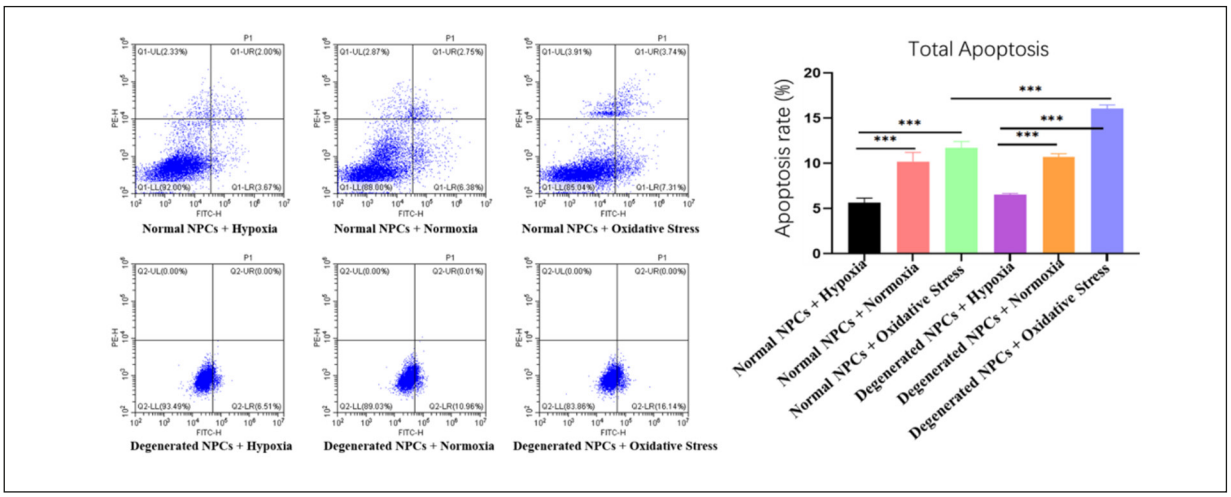

Figure 4. Comparisons of the apoptosis rates of normal and degenerated NPCs under different oxygen concentrations. NPCs, nucleus pulposus cells. ***$p < 0.001$.

whether NPCs degenerated, oxygen concentration, and their interaction had extremely significant impacts on the apoptosis rate ($p < 0.001$).

Protein expression results of PI3K, AKT, and HIF-1α

As shown in Figure 5, the protein expression of PI3K, AKT, and HIF-1α in normal and degenerated NPCs under different oxygen concentrations showed similar trends. In normal NPCs, the protein expression of these three proteins was the highest under hypoxia, followed by oxidative stress, and the lowest under normoxia. In degenerated NPCs, the expression levels of these three proteins decreased with increasing oxygen concentration, and the difference was statistically significant ($p < 0.05$).

Under different oxygen concentrations, the protein expression levels of PI3K, AKT, and HIF-1α between normal and degenerated NPCs were different. Under normoxia, there was no significant difference in the protein expression of HIF-1α between normal and degenerated NPCs ($p > 0.05$), but under hypoxic and oxidative stress conditions, the protein expression of HIF-1α in normal NPCs was significantly higher than that in degenerated NPCs ($p < 0.05$). Under different oxygen concentrations, the protein expression levels of PI3K and AKT in normal NPCs were significantly higher than those in degenerated NPCs ($p < 0.05$). When considering the Normal NPC + hypoxia group as a reference, whether NPCs degenerated, oxygen concentration and their interaction had extremely significant impacts on the expression of PI3K, AKT, and HIF-1α ($p < 0.001$).

mRNA expression Results of PI3K, AKT, and HIF-1α in Each Group

As shown in Figure 6, the mRNA levels of PI3K, AKT, and HIF-1α in normal and degenerated NPCs under different oxygen concentrations showed similar trends. In normal NPCs, the mRNA expression levels of PI3K, AKT, and HIF-1α under oxidative stress conditions were not significantly different from those under hypoxic conditions ($p > 0.05$), but the expression levels of these mRNAs under normoxia were significantly lower than those under hypoxic conditions ($p < 0.05$). In degenerated NPCs, the mRNA expression levels of PI3K, AKT, and HIF-1α under normoxia and oxidative stress were significantly different from those under hypoxia ($p < 0.05$), and the levels of those mRNAs decreased with increasing oxygen concentration. When considering the Normal NPC + hypoxia group as a reference, whether NPCs degenerated, oxygen concentration and their interaction had extremely significant impacts on the mRNA expression of PI3K, AKT, and HIF-1α ($p < 0.001$).

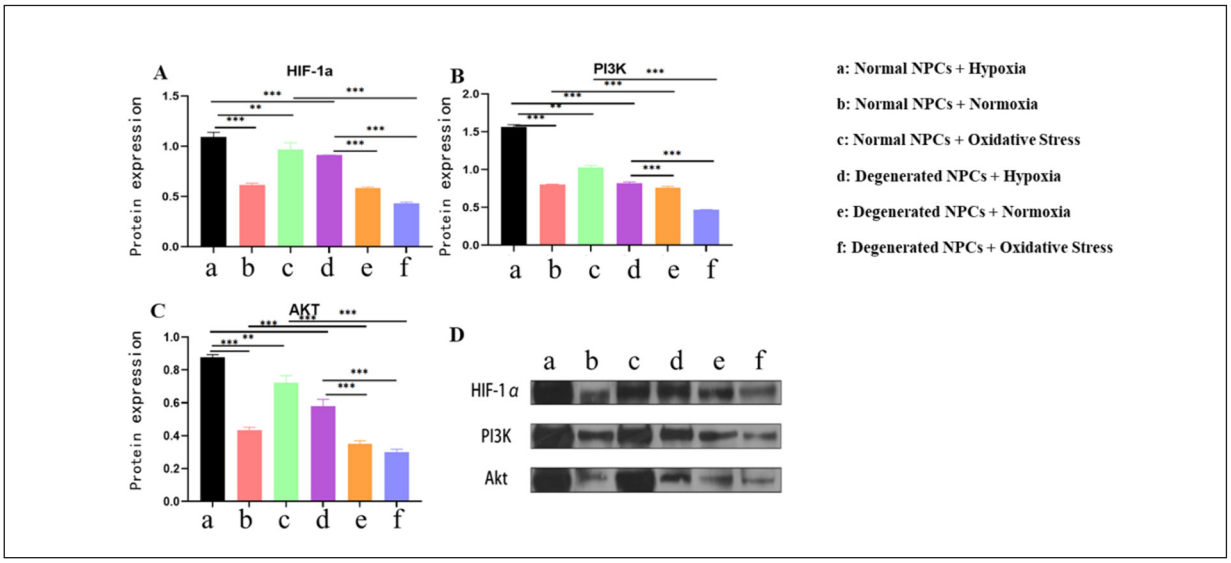

Figure 5. Protein expression of PI3K, AKT, and HIF-1α in normal and degenerated NPCs under different oxygen concentrations (**A**, **B**, **C**); (**D**), Western blot results. NPCs, nucleus pulposus cells; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; HIF-1α, hypoxia-induced factor-1 alpha. $^{**}p < 0.01$. $^{***}p < 0.001$.

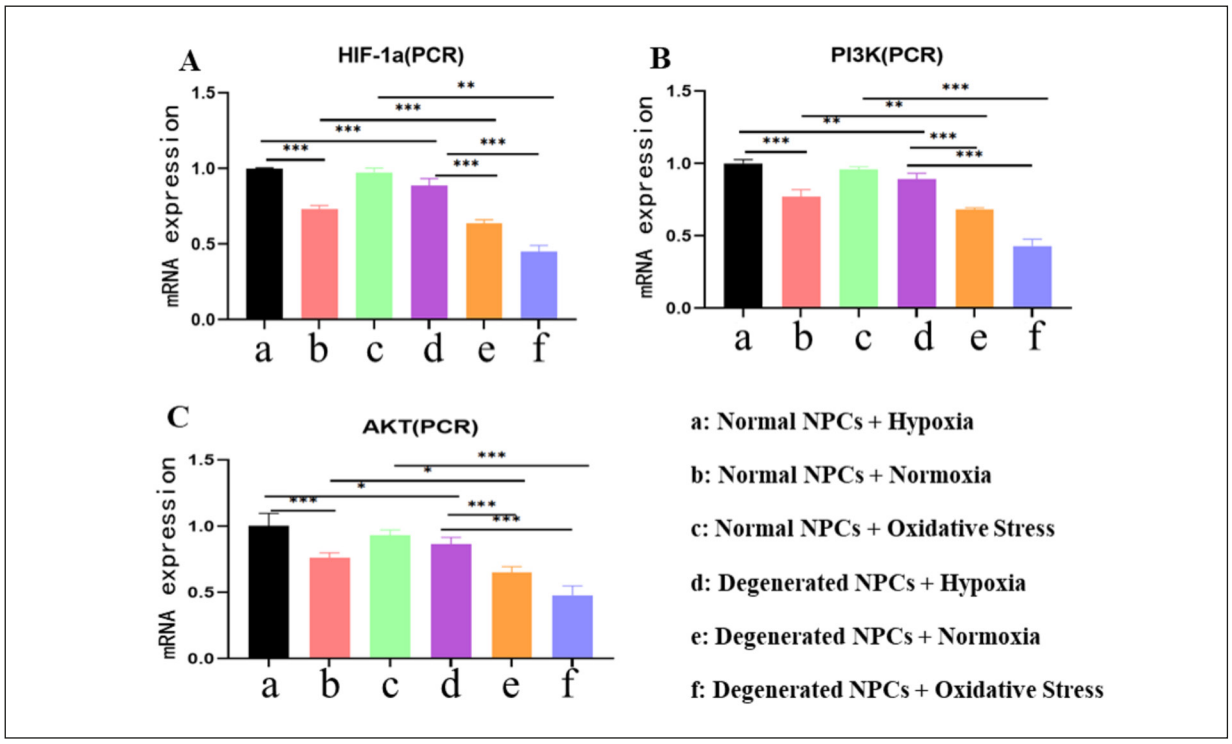

Figure 6. The mRNA expression of HIF-1α (**A**), PI3K (**B**), and AKT (**C**) in normal and degenerated NPCs under different oxygen concentrations. NPCs, nucleus pulposus cells; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; HIF-1α, hypoxia-induced factor-1 alpha; PCR, polymerase chain reaction. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

Discussion

In this study, we found that the proliferation and apoptosis of NPCs are closely related to the oxygen concentration and cells degeneration. The PI3K/AKT/HIF-1α signaling pathway has antagonistic regulation in cell degeneration and different oxygen concentration environments to maintain reactive oxygen species stability. This pathway plays a significant role in inhibiting oxidative stress, antagonizing NPC apoptosis, and consequently delaying IVDD.

IVDD is a complex disease caused by multiple factors, and its pathogenic mechanism is also relatively complicated, but the core lies in the degeneration of the nucleus pulposus[17]. The normal quantity and function of NPCs are crucial for maintaining the metabolic balance of the ECM and maintaining the stability of the microenvironment and normal mechanical function of the intervertebral disc[18]. In general, the intervertebral disc is in an environment with relatively low nutrient and oxygen contents and relatively high concentrations of metabolites[19]. In a high oxygen concentration and oxidative stress environment, H_2O_2 induces NPC apoptosis by downregulating mitochondrial membrane potential, releasing cytochrome c and other endogenous pathways[20,21], decreasing the synthesis and metabolic capacity of ECM[6], changing the collagen composition of the annulus fibrosus, and decreasing moisture. The above changes lead to weakened intervertebral disc elasticity and decreased longitudinal load-resistance capacity, further accelerating the IVDD process. Therefore, exploring the proliferation and apoptosis of normal and degenerated NPCs under different oxygen concentrations will help clarify the mechanism of IVDD and provide evidence for finding therapeutic targets.

PI3K-activated AKT can directly inhibit the apoptosis of NPCs by regulating apoptosis-related factors, including reducing GSK-3 activation and the phosphorylation of caspase-9, or indirectly inhibit the apoptosis of NPCs by inhibiting NF-κB[22,23]. In a hypoxic environment, the PI3K/AKT signaling pathway is upregulated and activated[24], which is of great significance for protecting NPCs and delaying their apoptosis[25]. As a downstream factor of the PI3K/AKT signaling pathway, HIF-1α can be activated by the PI3K/AKT signaling

pathway under hypoxic conditions, which accelerates intervertebral disc recovery, and alleviate the degenerative process[26,27]. Hosford et al[28] found that hyperoxia can reduce the expression of HIF in rat lung tissue, thereby inhibiting the growth of transitional cell carcinoma and the expression of VEGF protein[29]. However, hyperoxia can treat acute cerebral infarction by promoting the expression of HIF-1α in the cerebral cortex and hippocampus[30]. Therefore, we detected the levels of the PI3K/AKT/HIF-1α signaling pathway and the apoptosis of NPCs under different oxygen concentrations to explore the mechanism of delayed intervertebral disc degeneration.

In the experiment, CCK-8 and flow cytometry results found that the proliferation activity of normal and degenerated NPCs was inversely proportional to the oxygen concentration, and the apoptosis rate was directly proportional to the oxygen concentration. With the increase in oxygen concentration, the cell proliferation activity of normal and degenerated NPCs gradually decreased, and the cell apoptosis rate gradually increased. The results confirmed that the hypoxic environment is more conducive to maintaining the activity and cell proliferation of NPCs, which is consistent with the hypoxic internal environment of normal intervertebral discs. As the oxygen concentration increased, the activity of NPCs decreased, and the apoptosis rate increased. By analyzing the factors of NPC degeneration, oxygen concentration, and the interaction between them, the results indicated that normal or degenerated NPCs had no significant effect on the cell proliferation rate, and the cell proliferation rate was mainly affected by the oxygen concentration – the higher the oxygen concentration was, the lower the proliferation rate. The interaction factor also had a significant effect on the cell proliferation rate, and as time increased, the effect gradually increased. The state of cell degeneration, oxygen concentration, and the interaction between them all had a significant impact on the apoptosis rate of NPCs, indicating that the apoptosis rate of NPCs increases with increasing oxygen concentration and that the proliferation rate decreases with increasing oxygen concentration. In the microenvironment of the intervertebral disc, when the oxygen concentration increases, the enzymatic antioxidant mechanism cannot effectively remove ROS, which causes an imbalance in ROS and oxidative stress reactions. At the same time, ROS inhibits the synthesis of proteoglycans and destroys the protein structure of the extracellular matrix, causing damage to ECM synthesis[31-35], combined with NPC degeneration caused by physiology or pathology, ultimately accelerating the IVDD process.

The protein and mRNA expression levels of PI3K, AKT, and HIF-1α in normal and degenerated NPCs under different oxygen concentrations were the same. In normal NPCs, the protein expression levels of the three genes under hypoxic conditions were slightly higher than those in oxidative stress environments ($p < 0.05$), while the mRNA expression levels were not significantly different ($p < 0.05$), and the expression levels of those genes were the lowest under normoxia. In degenerated NPCs, the protein and mRNA expression levels of the three genes decreased with increasing oxygen concentration ($p < 0.05$). These results may be related to the basic state of cells. Under hypoxic conditions, the function of PHD2 is inhibited, and HIF-1α cannot be hydroxylated, which is conducive to the stability and massive accumulation of HIF-1α[36]. As the oxygen concentration increased, HIF-1α was hydroxylated, and the expression level gradually decreased. In normal NPCs, when the oxygen concentration increases and reaches the oxidative stress status, the PI3K/AKT signaling pathway is activated to antagonize the apoptosis caused by oxidative stress and promote the translation of the downstream factor HIF-1α[37]. Therefore, in normal NPCs, the mRNA expression levels of PI3K, AKT, and HIF-1α under hypoxia are close to those under oxidative stress. In degenerated NPCs, there are a large number of apoptotic cells. In response to rising oxygen concentrations, the antagonistic ability of the PI3K/AKT signaling pathway in oxidative stress environments decreased[38]. Therefore, the protein and mRNA levels of PI3K/AKT/HIF-1α in degenerated NPCs are the lowest under oxidative stress. On the other hand, by analyzing NPC degeneration, oxygen concentration, and their interaction, whether NPCs degenerated, oxygen concentration change, and their interaction all had extremely significant effects on the protein and mRNA expression of PI3K, AKT, and HIF-1α ($p < 0.001$). The results indicate that the PI3K/AKT/HIF-1α signaling pathway plays an important role in the process of NPC degeneration and apoptosis caused by increasing oxygen concentrations and oxidative stress and that NPC degeneration also affects the regulatory ability of the PI3K/AKT/HIF-1α signaling pathway[38]. HIF-1α can effectively maintain the metabolic balance

of ROS, and the PI3K/AKT/HIF-1α signaling pathway can also effectively alleviate oxidative stress reactions to prevent NPC apoptosis and delay the IVDD process[39].

Limitations

In the experiment, the CCK8 results showed that after 48 h of culture, the proliferation rate of degenerated NPCs in hypoxic and oxidative stress environments was slightly higher than that of normal NPCs in hypoxic and oxidative stress environments ($p<0.05$), which is inconsistent with the trends observed in the rest of the experiment. The reason may be due to insufficient experimental time and intervention conditions. Therefore, in future research, the culture time should be extended, and the oxygen concentration scale should be refined to explore the proliferation rate of NPCs further. There was no drug intervention in this research to verify the theoretical conjecture of this study further. The expression of other signaling pathways and therapeutic targets under different oxygen concentrations must be further explored.

Conclusions

The proliferation and apoptosis of NPCs are closely related to the oxygen concentration and the degenerated status of the cells themselves. The PI3K/AKT/HIF-1α signaling pathway has an antagonistic regulatory effect in different NPC degeneration statuses and different oxygen concentration environments, which may be related to activation of the PI3K/AKT signaling pathway to promote the transcription and translation of HIF-1α, thereby maintaining the metabolic balance of ROS. This pathway is of great significance for inhibiting oxidative stress, antagonizing NPC apoptosis, and delaying the IVDD process. In-depth research on the regulation of this pathway can comprehensively elucidate the pathogenic mechanism of IVDD and provide more treatment ideas and therapeutic targets.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Ethics Approval

The ethics approval was obtained from the Hunan University of Chinese Medicine (Approval No.: LL2022111703, dated 28/10/2022).

Authors' Contribution

Wuji Xu and Xianbo Zheng conceived the structure of the manuscript. Minghan Zhou and Hui Zhang did the experiments and made the figures. Minghan Zhou reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Availability of Data and Materials

The authors give their consent for the article publication.

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References

1) Pulickal T, Boos J, Konieczny M, Sawicki LM, Müller-Lutz A, Bittersohl B, Gerß J, Eichner M, Wittsack HJ, Antoch G, Schleich C. MRI identifies biochemical alterations of intervertebral discs in patients with low back pain and radiculopathy. Eur Radiol 2019; 29: 6443-6446.
2) Xin Z, Wu X, Ji T, Xu B, Han Y, Sun M, Jiang S, Li T, Hu W, Deng C, Yang Y. Bakuchiol: A newly discovered warrior against organ damage. Pharmacol Res 2019; 141: 208-213.
3) Feng G, Jin X, Hu J, Ma H, Gupte MJ, Liu H, Ma PX. Effects of hypoxias and scaffold architecture on rabbit mesenchymal stem cell differentiation towards a nucleus pulposus-like phenotype. Biomaterials 2011; 32: 8182-8189.
4) Zhong WX, Zhang GS, Tang J. The effect of Nrf2 activators tBHQ and 4-octyl itaconate on the nucleus pulposus cell degeneration. Eur Rev Med Pharmacol Sci 2021; 25: 5189-5198.
5) Wang Y, Cheng H, Wang T, Zhang K, Zhang Y, Kang X. Oxidative stress in intervertebral disc de-

generation: Molecular mechanisms, pathogenesis and treatment. Cell Prolif 2023; 56: e13448.

6) Zhan D, Lin M, Chen J, Cai W, Liu J, Fang Y, Li Y, Wu B, Wang G. Hypoxia-inducible factor-1α regulates PI3K/AKT signaling through microRNA-32-5p/PTEN and affects nucleus pulposus cell proliferation and apoptosis. Exp Ther Med 2021; 21: 646.
7) Xiang Q, Zhao Y, Lin J, Jiang S, Li W. The Nrf2 antioxidant defense system in intervertebral disc degeneration: Molecular insights. Exp Mol Med 2022; 54: 1067-1075.
8) Chen J, Bai M, Ning C, Xie B, Zhang J, Liao H, Xiong J, Tao X, Yan D, Xi X, Chen X, Yu Y, Bast RC, Zhang Z, Feng Y, Zheng W. Gankyrin facilitates follicle-stimulating hormone-driven ovarian cancer cell proliferation through the PI3K/AKT/HIF-1α/cyclin D1 pathway. Oncogene 2016; 35: 2506-2517.
9) Ouyang ZH, Wang WJ, Yan YG, Wang B, Lv GH. The PI3K/Akt pathway: a critical player in intervertebral disc degeneration. Oncotarget 2017; 8: 57870-57881.
10) Wu J, Yu L, Liu Y, Xiao B, Ye X, Zhao H, Xi Y, Shi Z, Wang W. Hypoxia regulates adipose mesenchymal stem cells proliferation, migration, and nucleus pulposus-like differentiation by regulating endoplasmic reticulum stress via the HIF-1α pathway. J Orthop Surg Res 2023; 18: 339.
11) Luo L, Jian X, Sun H, Qin J, Wang Y, Zhang J, Shen Z, Yang D, Li C, Zhao P, Liu M, Tian Z, Zhou Y. Cartilage endplate stem cells inhibit intervertebral disc degeneration by releasing exosomes to nucleus pulposus cells to activate Akt/autophagy. Stem Cells 2021; 39: 467-481.
12) Li Y, Zhuang Q, Tao L, Zheng K, Chen S, Yang Y, Feng C, Wang Z, Shi H, Shi J, Fang Y, Xiao L, Geng D, Wang Z. Urolithin B suppressed osteoclast activation and reduced bone loss of osteoporosis via inhibiting ERK/NF-κB pathway. Cell Prolif 2022; 55: e13291.
13) McKinnon KM. Flow Cytometry: An Overview. Curr Protoc Immunol 2018; 120: 5.1.1-5.1.11.
14) Pillai-Kastoori L, Schutz-Geschwender AR, Harford JA. A systematic approach to quantitative Western blot analysis. Anal Biochem 2020; 593: 113608.
15) Jozefczuk J, Adjaye J. Quantitative real-time PCR-based analysis of gene expression. Methods Enzymol 2011; 500: 99-109.
16) Francisco V, Pino J, González-Gay MÁ, Lago F, Karppinen J, Tervonen O, Mobasheri A, Gualillo O. A new immunometabolic perspective of intervertebral disc degeneration. Nat Rev Rheumatol 2022; 18: 47-60.
17) Zou YP, Zhang QC, Zhang QY, Jiang LB, Li XL. Procyanidin B2 alleviates oxidative stress-induced nucleus pulposus cells apoptosis through upregulating Nrf2 via PI3K-Akt pathway. J Orthop Res 2023; 41: 1555-1564.
18) Huang YC, Leung VY, Lu WW, Luk KD. The effects of microenvironment in mesenchymal stem cell-based regeneration of intervertebral disc. Spine J 2013; 13: 352-362.
19) Lotz JC, Chin JR. Intervertebral disc cell death is dependent on the magnitude and duration of spinal loading. Spine (Phila Pa 1976) 2000; 25: 1477-1483.
20) Rannou F, Lee TS, Zhou RH, Chin J, Lotz JC, Mayoux-Benhamou MA, Barbet JP, Chevrot A, Shyy JY. Intervertebral disc degeneration: the role of the mitochondrial pathway in annulus fibrosus cell apoptosis induced by overload. Am J Pathol 2004; 164: 915-924.
21) Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT. The PI3K Pathway in Human Disease. Cell 2017; 170: 605-635.
22) Xiao Q, Teng Y, Xu C, Pan W, Yang H, Zhao J, Zhou Q. Role of PI3K/AKT Signaling Pathway in Nucleus Pulposus Cells. Biomed Res Int 2021; 2021: 9941253.
23) Beitner-Johnson D, Rust RT, Hsieh TC, Millhorn DE. Hypoxia activates Akt and induces phosphorylation of GSK-3 in PC12 cells. Cell Signal 2001; 13: 23-27.
24) Cheng X, Zhang G, Zhang L, Hu Y, Zhang K, Sun X, Zhao C, Li H, Li YM, Zhao J. Mesenchymal stem cells deliver exogenous miR-21 via exosomes to inhibit nucleus pulposus cell apoptosis and reduce intervertebral disc degeneration. J Cell Mol Med 2018; 22: 261-276.
25) Zheng J, Chang L, Bao X, Zhang X, Li C, Deng L. TRIM21 drives intervertebral disc degeneration induced by oxidative stress via mediating HIF-1α degradation. Biochem Biophys Res Commun 2021; 555: 46-53.
26) Liu Z, Li C, Meng X, Bai Y, Qi J, Wang J, Zhou Q, Zhang W, Zhang X. Hypoxia-inducible factor-lα mediates aggrecan and collagen Π expression via NOTCH1 signaling in nucleus pulposus cells during intervertebral disc degeneration. Biochem Biophys Res Commun 2017; 488: 554-561.
27) Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci 2011; 4: 51.
28) Hosford GE, Olson DM. Effects of hyperoxia on VEGF, its receptors, and HIF-2alpha in the newborn rat lung. Am J Physiol Lung Cell Mol Physiol 2003; 285: L161-168.
29) Duan S, Shao G, Yu L, Ren C. Angiogenesis contributes to the neuroprotection induced by hyperbaric oxygen preconditioning against focal cerebral ischemia in rats. Int J Neurosci 2015; 125: 625-634.
30) Kang L, Xiang Q, Zhan S, Song Y, Wang K, Zhao K, Li S, Shao Z, Yang C, Zhang Y. Restoration of Autophagic Flux Rescues Oxidative Damage and Mitochondrial Dysfunction to Protect against Intervertebral Disc Degeneration. Oxid Med Cell Longev 2019; 2019: 7810320.
31) Liao Z, Luo R, Li G, Song Y, Zhan S, Zhao K, Hua W, Zhang Y, Wu X, Yang C. Exosomes from mes-

enchymal stem cells modulate endoplasmic reticulum stress to protect against nucleus pulposus cell death and ameliorate intervertebral disc degeneration in vivo. Theranostics 2019; 9: 4084-4100.

32) Zhang H, He F, Li P, Hardwidge PR, Li N, Peng Y. The Role of Innate Immunity in Pulmonary Infections. Biomed Res Int 2021; 2021: 6646071.

33) Song P, Jo HS, Shim WS, Kwon YW, Bae S, Kwon Y, Azamov B, Hur J, Lee D, Ryu SH, Yoon JH. Emodin induces collagen type I synthesis in Hs27 human dermal fibroblasts. Exp Ther Med 2021; 21: 420.

34) Chen MM, Li Y, Deng SL, Zhao Y, Lian ZX, Yu K. Mitochondrial Function and Reactive Oxygen/Nitrogen Species in Skeletal Muscle. 2022; 10: 826981.

35) Kaufman B, Scharf O, Arbeit J, Ashcroft M, Brown JM, Bruick RK, Chapman JD, Evans SM, Giaccia AJ, Harris AL, Huang E, Johnson R, Kaelin W Jr, Koch CJ, Maxwell P, Mitchell J, Neckers L, Powis G, Rajendran J, Semenza GL, Simons J, Storkebaum E, Welch MJ, Whitelaw M, Melillo G, Ivy SP. Proceedings of the Oxygen Homeostasis/Hypoxia Meeting. Cancer Res 2004; 64: 3350-3356.

36) Itsuji T, Tonomura H, Ishibashi H, Mikami Y, Nagae M, Takatori R, Tanida T, Matsuda KI, Tanaka M, Kubo T. Hepatocyte growth factor regulates HIF-1α-induced nucleus pulposus cell proliferation through MAPK-, PI3K/Akt-, and STAT3-mediated signaling. J Orthop Res 2021; 39: 1184-1191.

37) Zhan D, Lin M, Chen J, Cai W, Liu J, Fang Y, Li Y, Wu B, Wang G. Hypoxia-inducible factor-1α regulates PI3K/AKT signaling through microRNA-32-5p/PTEN and affects nucleus pulposus cell proliferation and apoptosis. Exp Ther Med 2021; 21: 646.

38) Wang J, Zhang Y, Cao J, Wang Y, Anwar N, Zhang Z, Zhang D, Ma Y, Xiao Y, Xiao L, Wang X. The role of autophagy in bone metabolism and clinical significance. Autophagy 2023; 19: 2409-2427.