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Dexmedetomidine suppresses oxidative stress and inflammation of nucleus pulposus cells by activating the PI3K/Akt signaling pathway

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Objective: Intervertebral disc degeneration (IVDD) is very common in the elderly, so it is particularly important to find appropriate prevention or treatment. The aim of this study was to explore the effect of dexmedetomidine (DEX) on the degeneration of nucleus pulposus (NP) cells and its mechanism. *Methods*: We established a mouse model of IVDD and cultured mouse NP cells and treated them with IL-1β and DEX. The effect of DEX on NP cells was determined by detecting the extracellular matrix of NP cells, changes in ROS levels and inflammatory mediators. LY294002, a PI3K inhibitor, is used to inhibit the activity of the PI3K/Akt signaling pathway. The effect of DEX on the PI3K/Akt signaling pathway was determined by studying the effects of DEX on PI3K/Akt signaling pathway-related molecules and the effect of LY294002 on NP cells degeneration. DEX significantly increased the disc height index and attenuated IVDD in mice. *Results*: DEX significantly inhibited the expression of MMP3/9 in NP cells, effectively inhibiting the degradation of extracellular matrix. In addition, oxidative stress levels and inflammatory levels in NP cells are also attenuated by DEX. The expression of PI3K, Akt and p-Akt was significantly increased in DEX-stimulated NP cells, indicating that DEX increased the activity of the PI3K/Akt signaling pathway. DEX promotes PI3K/Akt signaling pathway, inhibits oxidative stress and inflammatory of NP cells, thereby slowing the degeneration of NP cells. *Conclusion*: DEX promotes PI3K/Akt signaling pathway, inhibits oxidative stress and inflammatory of NP cells, thereby slowing the degeneration of NP cells.

1. Introduction

Low back pain (LBP) is a common disease that plagues the general population (Wilke et al. 2017). Studies have shown that about 80% of people experience chronic LBP for more than four weeks in their lifetime, and the disability rate caused by chronic LBP is as high as 10% or more (Patrick et al. 2014). Intervertebral disc degeneration (IVDD) and a series of secondary diseases (such as lumbar disc herniation, lumbar spondylolisthesis, lumbar spinal stenosis, degenerative scoliosis, etc.) are the main causes of chronic LBP (Hoy et al. 2010). A number of factors can directly or indirectly cause IVDD, however most of the factors are changes in the nucleus pulposus (NP) cells and their extracellular matrix (Navone et al. 2017). It is mainly characterized by a decrease in some protective components of intervertebral discs, such as a decrease in proteoglycans (mainly aggrecan) and collagen II, and an increase in extracellular spinal destructive factors (such as matrix metalloproteinases, MMP) (Kadow et al. 2015). In addition, the increase of oxidative stress level and inflammation level in the NP of intervertebral disc caused by various factors is also an important cause of IVDD (Zhang et al. 2016).

The PI3K/Akt signaling pathway mainly promotes matrix anabolism, inhibits apoptosis and promotes cell proliferation, which may play an important role in delaying the IVDD process (Murata et al. 2013). Dexmedetomidine is a powerful and highly selective alpha 2 adrenergic receptor agonist that reduces sympathetic tone and indirectly increases vagal tone (Fung et al. 1993). Studies on DEX in recent years have found that DEX plays a protective role in the organ through anti-inflammatory and antioxidant effects in ischemia reperfusion (Kang et al. 2008). In addition, studies have shown that DEX can effectively reduce the occurrence of apoptosis of renal tubular cells *in vitro* (Kang et al. 2008). It was found (Shen et al. 2017) that dexmedetomidine can play an anti-inflammatory and antioxidant role by activating the PI3K/Akt signaling pathway, thereby reducing lung injury. However, the effect and mechanism of DEX on intervertebral disc degeneration have not been studied. Therefore, we used DEX to study its anti-oxidation and anti-inflammatory effects on IVDD.

2. Investigations and results

2.1. DEX delays degeneration of mouse intervertebral disc and reduces degradation of extracellular matrix

We used the tail suspension method to construct a model of IVDD and gave mice subcutaneous injection of DEX (2 mg/kg). The results of DHI showed that the DHI of the mice in the degenerative group was significantly lower than that of the control group, while the DHI of the mice in the treatment group was significantly increased relative to the degenerative group. Western blot and RT-PCR results showed that DEX can significantly reduce the expression of MMP3 and MMP9 in the intervertebral disc, and increase the expression of extracellular matrix collagen II and aggrecan. The results of immunohistochemistry also confirmed this. These results indicated that DEX effectively delayed IVDD in mice.

2.2. DEX inhibits the degradation of extracellular matrix of NP cells

During the degeneration of NP cells, increased expression of MMP leads to degradation of the extracellular matrix. To determine the effect of DEX on degeneration of NP cells, we examined the effects of DEX on MMP3, MMP9 and extracellular matrices in NP cells.

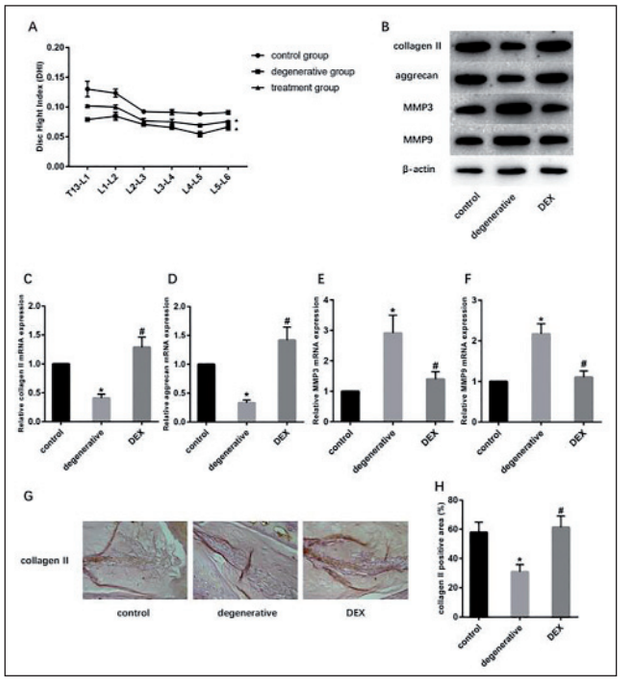

Fig. 1: DEX delays degeneration of mouse intervertebral disc and reduces degradation of extracellular matrix. DHI was determined by x-ray (A). Results of the expression of collagen II, aggrecan, MMP3 and MMP9 were determined by western blot (B) and RT-PCR (C~F). The expression of collagen II was determined by immunohistochemical staining (G, H). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the degenerative group).

First, we tested the cell viability of 0, 1, 2, 5, 10, 20 μM DEX-stimulated nucleus cells for 24 h by CCK8. The results showed that 5 μM DEX had the best effect (Fig. 1A). Then we used 5 μM DEX to stimulate the NP cells for 0, 1, 6, 12, 24, 48 h, and then tested their effects on the viability of NP cells. The results showed that the cell viability was best at 24 h (Fig. 1B). Different concentrations of DEX were used to stimulate NP cells. After 24 h, we extracted total RNA and supernatant from cells, and detected different concentrations of DEX on NP collagen II, aggrecan, MMP3 and MMP9 by RT-PCR (Fig. 1C-F) and ELISA (Fig. 1G, H). The effect of expression showed that DEX significantly increased the expression of collagen II and aggrecan, decreased the expression of MMP3 and MMP9 and showed a dose-dependent manner.

2.3. DEX inhibits NP cells oxidative stress

We studied the molecular mechanism of DEX's role in anti-oxidative stress in NP cells to determine the effect of DEX on degeneration of NP cells. Recombinant mouse IL-1β (10 ng/ml) was used to stimulate degeneration of NP cells. Western blot results showed that IL-1β-stimulated NP cells expressed low levels of SOD1 and SOD2, while DEX increased SOD1 and SOD2 expression (Fig. 2A). RT-PCR results showed that DEX significantly attenuated the effect of IL-1β on the low expression of SOD1, SOD2, GPX1 and GPX3 (Fig. 2B-D). The results of the ELISA were similar to those of western blot and RT-PCR (Fig. 2E-G). The results of immunofluorescence also indicate that DEX can increase the expression of SOD1 (Fig. 2H). In addition, flow cytometry results showed that the level of ROS in the NP cells of the IL-1β+DEX group was significantly lower than that of the IL-1β group (Fig. 2I). These results indicate that DEX can significantly reduce the level of oxidative stress in NP cells.

2.4. DEX inhibits inflammation levels in NP cells

Inflammation is an important part of the process of IVDD. The results of Western blot (Fig. 3A) and RT-PCR (Fig. 3B-D) showed that the expression of IL-6, IL-8 and TNF-α increased significantly

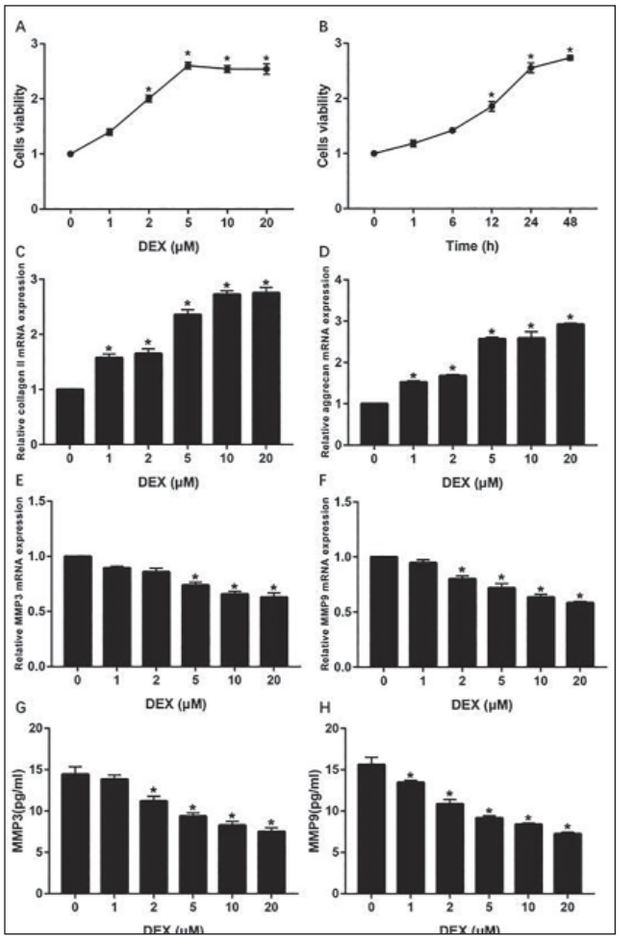

Fig. 2: DEX inhibits the degradation of extracellular matrix of NP cells. Optimum concentration (A) and time (B) of DEX was determined by CCK8. Results of the mRNA expression of collagen II (C), aggrecan (D), MMP3 (E) and MMP9 (F) were determined by RT-PCR. Results of the expression of MMP3 (G) and MMP9 (H) were determined by ELISA. ("*" means there is a statistical difference with the DEX 0 μM group or DEX 0 h group).

after IL-1β stimulation. DEX can alleviate the effects of IL-1β and reduce the expression of IL-6, IL-8 and TNF-α. The results of the ELISA were similar to those of the western blot (Fig. 3E-G). This suggests that DEX can significantly reduce the level of inflammation in NP cells.

2.5. DEX promotes the activity of the PI3K/Akt signaling pathway in NP cells

The PI3K/Akt signaling pathway is an important signaling pathway in the process of IVDD. The PI3K/Akt signaling pathway promotes the expression of SOX9 in NP cells, thereby promoting the expression of collagen II and aggrecan, and alleviating the degeneration of NP cells. We used the PI3K inhibitor LY294002 to inhibit the activity of the PI3K/Akt signaling pathway. Western blot (Fig. 4A) and RT-PCR (Fig. 4B-E) results showed that the expression of PI3K, Akt and p-Akt was significantly decreased after IL-1β stimulated NP cells, while DEX increased the activity of PI3K/Akt signaling pathway. After inhibition of the PI3K/Akt signaling pathway by LY294002, the expression of collagen II and aggrecan was significantly reduced. The results of cellular immunofluorescence (Fig. 4F) were similar to those of western blot. This indicates that DEX can increase the activity of the PI3K/Akt signaling pathway, and the degree of degeneration of NP cells is aggravated after the PI3K/Akt signaling pathway is inhibited. Therefore, DEX may delay the degeneration of intervertebral disc nucleus cells by activating the PI3K/Akt signaling pathway.

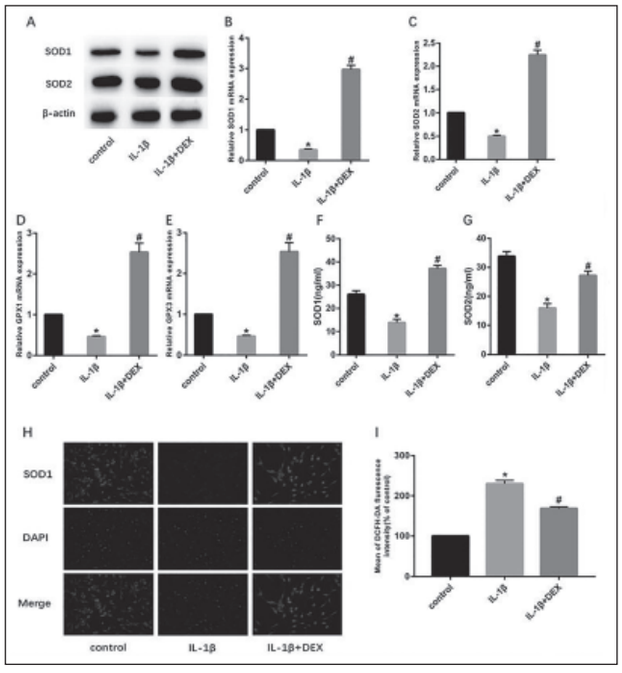

Fig. 3: DEX inhibits NP cells oxidative stress. The expression of SOD1, SOD2, GPX1 and GPX3 in the intervertebral disc of mice was detected by western blot (A) and RT-PCR (B~E). ELISA was performed to detect the expression of SOD1 (F) and SOD2 (G). The expression of SOD1 was determined by immunofluorescence (H). The level of ROS was detected by flow cytometry (I). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

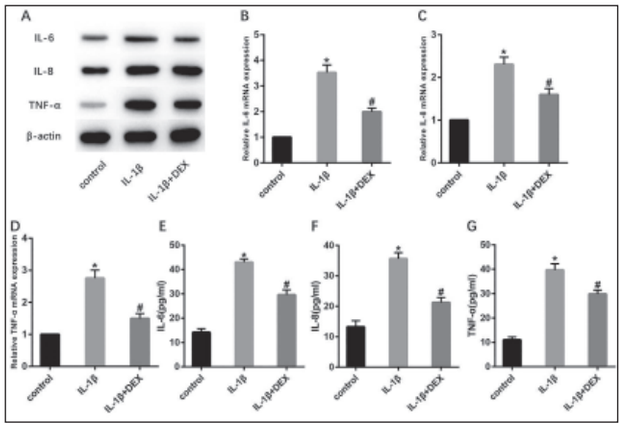

Fig. 4: DEX inhibits inflammation levels in NP cells. The expression of IL-6, IL-8 and TNF-α in three groups were detected. A, western blot. B~D, RT-PCR. E~G, ELISA. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

3. Discussion

There is increasing evidence that the mechanism of IVDD may be related to oxidative stress. In the body, ROS mainly include hydrogen peroxide, superoxide anion and hydroxyl radicals, which can regulate and control multiple functional activities of cells, and the presence of low concentrations of ROS is an important condition for signal transduction in cells (Zhang et al. 2018). Under normal conditions, ROS production and ROS clearance in the body are in a dynamic balance. Due to various reasons, when the production of ROS increases or the ability of the body to clear ROS decreases, the body will have oxidative stress, which can cause lipid peroxidation, DNA breakage, protein damage and cell damage (Sinha and Dabla 2015). Studies have shown that many factors such as age, compression, neovascularization and trauma can increase the production of ROS in the body, trigger apoptosis

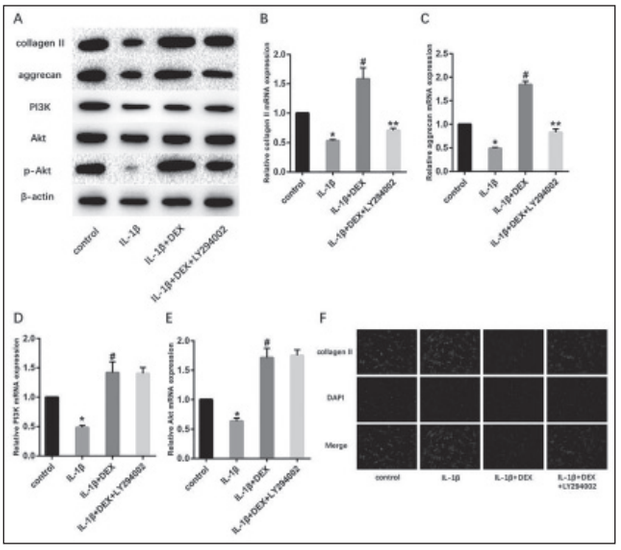

Fig. 5: DEX promotes the activity of the PI3K/Akt signaling pathway in NP cells. The expression of collagen, aggrecan, PI3K, Akt and p-Akt in four groups was determined by western blot(A). Results of mRNA expression of collagen II, aggrecan, PI3K and AKT in four groups were determined by RT-PCR (B~E). Immunofluorescence was performed to detect the expression of collagen II in four groups (F). ("*" means there is a statistical difference with the control group, "#" means there is a statistical difference with the IL-1β group and "**" means there is a statistical difference with the IL-1β+DEX group).

of NP cells and accelerate cell senescence, eventually leading to IVDD (Feng et al. 2017). By studying Ercc1 mice, a spontaneous age-dependent disc degeneration mouse, Nasto et al. (2013) found that mitochondria-derived ROS promote age-related IVDD. Mitochondria are a major endogenous source of ROS and are also most sensitive to ROS. Excessive ROS will cause mitochondrial oxidative stress, reduce mitochondrial membrane potential, and induce apoptosis. Our study found that after stimulation of NP cells with DEX, the cytokines SOD1 and SOD2, which play an important role in anti-oxidative stress, are significantly increased, and ROS levels are also significantly reduced. This suggests that the anti-oxidative stress of DEX can significantly reduce the level of oxidative stress in NP cells.

Inflammatory factors are involved in and promote the progression of IVDD. Studies by Phillips et al. (2013) have shown that the expression level of inflammatory factors in degenerated intervertebral disc tissue is significantly higher than that in normal intervertebral disc tissue, and the expression level of inflammatory factors is positively correlated with the degree of IVDD. Inflammatory factors such as interleukin, tumor necrosis factor, nitric oxide, and prostaglandin E2 can be detected in degenerated intervertebral disc tissue. These inflammatory factors are the main factors leading to the inflammatory reaction of intervertebral disc tissue, and play an important role in the process of IVDD. In this study, IL-1β-induced NP cells showed a significant inflammatory response. After treatment of NP cells with DEX, the expression of IL-6, IL-8 and TNF-α in NP cells was significantly reduced, and the level of inflammation was significantly reduced. This suggests that DEX has an anti-inflammatory effect in NP cells, which helps to delay the IVDD.

The PI3K/Akt pathway plays an important role in delaying IVDD by promoting matrix anabolism, inhibiting apoptosis, and promoting cell proliferation (Xie et al. 2019). In this study, we used the PI3K inhibitor LY294002 to inhibit the activity of the PI3K/Akt signaling pathway. The expression of PI3K, Akt and p-Akt in DEX-treated NP cells was significantly increased, and LY294002 effectively weakened the promotion effect of DEX on the synthesis of extracellular matrix of nucleus pulpoedullary. This suggests that this DEX has a positive effect on the PI3K/Akt signaling pathway. In addition, in *in vivo* experiments, DEX effectively increased the DHI of the mouse spine and reduced the degradation of the extra-

cellular matrix. This also validated the protective effect of DEX on the intervertebral disc.

This is the first report to uncover the anti-oxidant and anti-inflammatory effect of DEX on IVDD via thePI3K/Akt signaling pathway. DEX could be a useful drug to slow down the progression of IVDD.

4. Experimental

4.1. Animal and grouping

A total of 30 C57/BL6 mice were used in this study. Mice were divided into three groups: control group, degeneration group and treatment group. We constructed the IVDD model by a tail suspension method. The mouse's tail is suspended on a pulley at the top of the cage. The height of the suspension was controlled so that the mice's hind legs were just off the ground. The suspension time is one month. Mice in the treatment group were given daily intraperitoneal injection of DEX (2 mg/kg, Hengrui Medicine, China) on a suspended basis. The experiment was approved by the Ethics committee of our hospital.

4.2. X-ray

The extent of disc degeneration in mice was determined by radiography of lateral spinal surfaces and calculation of disc height index (DHI). DHI is the ratio of the height of the disc to the height of the adjacent upper and lower vertebrae.

Table 1: Primer sequences of RT-PCR

Name	sense/anti-sense	Sequence(5'-3')
collagen II	sense	GGGAATGTCCTCTGCGATGAC
	anti-sense	GAAGGGGATCTCGGGGTTG
aggrecan	sense	GGTGAACCAGTTGTGTTGTC
	anti-sense	CCGTCCTTTCCAGCAGTC
MMP3	sense	ACATGGAGACTTTGTCCCTTTTG
	anti-sense	TTGGCTGAGTGGTAGAGTCCC
MMP9	sense	CTGGACAGCCAGACACTAAAC
	anti-sense	CTCGCGGCAAGTCTTCAGAG
SOD1	sense	GGTGAACCAGTTGTGTTGTC
	anti-sense	CCGTCCTTTCCAGCAGTC
SOD2	sense	CAGACCTGCCTTACGACTATGG
	anti-sense	CTCGGTGGCGTTGAGATTGTT
GPX1	sense	ATCATATGTGTGCTGCTCGGCTAGC
	anti-sense	TACTCGAGGGCACAGCTGGGCCCTTGAG
GPX3	sense	AGAGCCGGGGACAAGAGAA
	anti-sense	ATTTGCCAGCATACTGCTTGA
IL-6	sense	ACTCACCTCTTCAGAACGAATTG
	anti-sense	CCATCTTTGGAAGGTTCAGGTTG
IL-8	sense	AGCCCAGAACACTGGTCTC
	anti-sense	ACTCAGGATTTCAATGGTGCC
TNF-α	sense	CTACCATCACCGCACTGAGAT
	anti-sense	GGTCACTTCACCATAGTGGACA
PI3K	sense	GGTGACTGTGTGGGACTTATTGA
	anti-sense	CTGATGTAGTGTGTGGCTGTTGA
AKT	sense	CAGGTTCACCCAGTGACAACTCA
	anti-sense	CACGAGACAGGTGGAAGAAGAGC
GAPDH	sense	ACAACTTTGGTATCGTGGAAGG
	anti-sense	GCCATCACGCCACAGTTTC

MMP3: matrix metalloproteinase 3; MMP9: matrix metalloproteinase 9; SOD1: Superoxide Dismutase 1; SOD2: Superoxide Dismutase 2; GPX1: Glutathione Peroxidase 1; GPX3: Glutathione Peroxidase 3; IL-6: Interleukin 6; IL-8: Interleukin 8; TNF-α: Tumor Necrosis Factor-α; PI3K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; AKT: Serine/Threonine Kinase; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

4.3. Immunohistochemical staining

The spinal tissue of the mice was isolated and decalcified. After dehydration entrapment of the spine tissue, we made paraffin sections of the mouse spine. We then stained the paraffin sections using the immunohistochemistry kit (Keygen, China) according to the manufacturer's instructions and observed the expression of collagen II.

4.4. NP cells isolation and cell treatment

Mouse primary NP cells were purchased from Shanghai Saibaikang Biological Company. NP cells were maintained in DEME/F12 medium (Hyclone, USA) containing 10% fetal bovine serum (Invitrogen, USA) and 1% antibiotics (Invitrogen, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Recombinant mouse IL-1β (Biochrom, UK) is used to stimulate the degeneration of NP cells. LY294002, a PI3K inhibitor, was used to inhibit the PI3K/Akt signaling pathway. DEX was dissolved in ddH_2O for the next experiment.

4.5. Western blot analysis

The NP cells are cultured in a six-well plate. After different treatments, the NP cells were washed twice with PBS. We used RIPA lysate to solubilize NP cells and extract total protein. The BSA kit (Beyotime, China) was used to detect protein concentration. Total cellular proteins (20 μg per well) were resolved via 10% SDS-PAGE and then transferred to PVDF membranes. The primary antibody (Collagen II, Abcam, United Kingdom, Rabbit, 1:5000; aggrecan, Abcam, United Kingdom, Rabbit, 1:3000; SOD1, Abcam, United Kingdom, Rabbit, 1:3000; SOD2, Abcam, United Kingdom, Rabbit, 1:3000; IL-6, Abcam, United Kingdom, Rabbit, 1:1000; IL-8, Abcam, United Kingdom, Rabbit, 1:1000; TNF-α, Abcam, United Kingdom, Rabbit, 1:1000; PI3K, Abcam, United Kingdom, Rabbit, 1:1000; Akt, Abcam, United Kingdom, Rabbit, 1:1000; p-Akt Abcam, United Kingdom, Rabbit, 1:1000; β-actin, Proteintech, USA, 1:5000) was added to the PVDF membrane and incubated overnight at 4 °C. The next day, after washing the PVDF membrane with TPST, we added the secondary antibody (Abcam, United Kingdom, 1:3000) to the PVDF membrane and incubated for 2 hours at room temperature. Finally, we analyzed the results using Image DEX software.

4.6. Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from NP cells using Trizol reagent (Invitrogen, USA). Then, we use a ReverTraAce qPCR RT Kit (TOYOBO, Japan) to convert RNA to cDNA. The SYBR Green PCR Master Mix (TIANGEN, China) was used to set up PCR reactions. GAPDH expression levels were used to normalize relative gene expression levels, and the data were presented as fold changes using the formula $2^{-\Delta\Delta CT}$, as recommended by the manufacturer. The primer sequences of each gene were listed in the Table.

4.7. Immunofluorescence

NP cells were transferred to 24-well plates. After different treatments on the cells, we took the 96-well plates and discarded medium. After washed by phosphate-buffered saline (PBS), the NP cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X 100 in PBS for 10 min. NP cells were blocked with 5% BSA-PBS for 30 min and incubated with primary antibody SOD1 (Abcam, United Kingdom, Rabbit, 1:500) and PI3K (Abcam, United Kingdom, Rabbit, 1:500) overnight at 4 °C. Next day, after washed by PBS, the NP cells were incubated with a fluorescent DEXeled secondary antibody (Abcam, United Kingdom, 1:500) for 1 h at room temperature and incubated with DAPI for 10 min. The cells were then imaged with fluorescence microscope.

4.8. Cell viability assay

NP cells were transferred to 96-well plates. After different treatments on the cells, we added 10 μl of CCK8 (GLPBIO, USA) to each well. The 96-well plate was then placed in an incubator for 2 h. A microplate reader was used to measure the absorbance of each well.

4.9. Intracellular ROS levels

Intracellular ROS level was detected by flow cytometry. After different treatments on the cells, we collected NP cells and washed them three times with cold PBS. The cells were intubated with DCFH-DA (10 μM Kaiji, Nanjing, China) for 20 min in 37 °C followed immediately by flow cytometry analysis in a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) to detect the total ROS level.

4.10. Enzyme linked immunosorbent assay (ELISA)

NP cells were transferred to six-well plates. Each well contained 5×10^4 cells. After different treatments on the cells, the concentration of SOD1/2 and MMP3/9 in the cell supernatant was detected using ELISA Kits (EDEXscience, Wuhan, China) according to the manufacturer's instructions.

4.11. Statistical analysis

Each set of experiments was repeated three times. We use SPSS20.0 for statistical analysis. For measurement data, we use the mean±standard deviation for analysis. For comparison between groups, we used one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

Conflicts of interest: None declared.

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