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SF1670 inhibits apoptosis and inflammation *via* the PTEN/Akt pathway and thus protects intervertebral disc degeneration

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Abstract. – OBJECTIVE: Intervertebral disc degeneration (IVDD) is associated with the apoptosis of nucleus pulposus (NP) cells. Previous studies have shown that PTEN plays crucial roles in cell survival and apoptosis. The effect of PTEN inhibitors on cell survival following IVDD has been rarely examined. In this study, we investigated the protective effect of SF1670, as a specific PTEN inhibitor, on an *in vitro* NP cells degenerated model.

PATIENTS AND METHODS: We collected human disc samples from IVDD patients and detected PTEN expression in them with different degenerated degrees. NP cells were isolated from the samples and exposed to IL-1β with or without SF1670. Then, cells viability was determined by CCK-8 assay. We also measured the levels of collagen II, p16, p53, PTEN, Akt, aggrecan, caspase 3/9, Bax, Bcl-2, and several inflammatory factors in NP cells.

RESULTS: We found that the expression of PTEN markedly increased in severely degenerated disc tissues. The data showed that IL-1β upregulated the expressions of p16, p53, PTEN, caspase 3/9, and Bax, but decreased the expressions of collagen II, Akt, aggrecan, and Bcl-2. Surprisingly, the treatment with SF1670 could significantly reverse the regulatory effects of IL-1β. Moreover, relative levels of IL-6, IL-8, TNF-α, and MMP3/9/13 were significantly suppressed by SF1670 stimuli compared with IL-1β group.

CONCLUSIONS: Overall, these results demonstrated that SF1670 prevented NP degradation *via* suppressing apoptosis and inflammation through inhibition of PTEN and activation of Akt. SF1670 may become a novel target for the therapy of IVDD in the future.

Key Words:
Nucleus pulposus cells, Intervertebral disc degeneration, SF1670, Inflammation, Apoptosis.

Introduction

Degenerative disc diseases, manifesting as low back pain, affect over 70% of people in the same age, which lead to the reduction of life quality or even disability[1]. It is widely accepted that intervertebral disc degeneration (IVDD) is the leading cause of low back pain. However, the pathogenesis of IVDD has not yet been fully elucidated, whose inflammatory and apoptosis (programmed cell death) of nucleus pulposus (NP) cells play essential roles in this process[2-5]. The human intervertebral disc is composed of three interdependent parts including the gelatinous NP tissues containing a large amount of collagen II and other extracellular matrices (ECM), the outer annulus fibrosus, and the top and bottom cartilages endplates. Because of the complex structures, mechanism of IVDD involves lots of biomolecules and signaling pathways[6-8]. Therapy strategy for intervertebral disc disease nowadays mainly contains biological, genomics, and clinical treatment[9,10]. In this present study, we aim to seek an efficient biological cytokine to prevent IVDD and explore its potential mechanism.

Disablement of NP cells contributes to the onset of IVDD, whose apoptosis of NP cells plays a significant role in the pathogenesis pro-

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cess[11]. The loss of NP cells leads to a decrease in the synthesis of matrix protective genes to maintain the stability of IVD, such as type II collagen and aggrecan[12,13]. During the progress of IVDD, NP cells usually undergo multiple biologic changes, accompanying cell apoptosis and increased inflammation. Apoptosis in IVDD primarily involves three pathways: the mitochondrial pathway[14], death receptor pathway[15], and ER pathway[16]. By the forefront of biomarkers (Bax, Bcl-2, caspase-3/4/8/9) of these three apoptosis pathways, ER pathway is the most vital one in the mild stage of IVDD degeneration[14]. Undoubtedly, it is of great significance to intervene on IVDD at the beginning of the mild stage.

Activation of Akt, which is related to a classic intracellular survival pathway, leads to inhibition of several downstream substrates of cell apoptosis such as HIF-1α, Bcl-2, caspase 3, and caspase 9[17,18]. Phosphatase and tensin homolog protein (PTEN), a negative regulator of Akt phosphorylation, has an important significance in the cell apoptosis[19]. Therefore, dysregulated PTEN is of significance in IVDD. Current studies found that pretreatment with SF1670, a developed specific PTEN inhibitor, is capable of enhancing PIP3 signaling in transplanted neutrophils[20] and protecting PC12 cells against cell death[21]. However, the protective effect of SF1670 against IVDD has not yet been investigated. In the present study, for the first time, we explored the effects of pretreatment with SF1670 for protecting NP cells against apoptosis and inflammation.

Patients and Methods

Patient Tissue Samples Collection

This retrospective trial was approved by the Ethics Committee of the Affiliated Hospital of Weifang Medical University. All patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki. A total of 14 patients (8 males and 6 females; mean age: 49 years old, from 38 to 73 years) undergoing lumbar disc herniation operations in our hospital from July 2017 to August 2018 were enrolled. The magnitude of IVDD in each segment was determined based on the Pfirrmann classification score according to the Magnetic Resonance Imaging (MRI) before surgery. In particular, grades I and II indicated no significant IVDD; while grades III, IV, and V indicated intervertebral disc degeneration. We divided the disc tissues into two groups: 1) Mild degenerated group (grades I and II), 2) Severe degenerated group (grades III, IV, and V). The tissues were stored in liquid nitrogen immediately after resection.

NP Cells Isolation and Cell Culture

The central gelatinous NP tissues were removed from the intervertebral disc and washed three times with sterile phosphate-buffered saline solution (PBS). Then, the samples were cut into small pieces and sequentially digested with 0.25% trypsin (Gibco, Grand Island, NY, USA) for 10 min and 0.25% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 12 h. The NP cell pellets were re-suspended in DMEM/F12 containing 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). First-generation NP cells were used in the following experiment groups: 1) control group (free from intervention); 2) IL-1β group (treated with 10 ng/ml IL-1β); 3) IL-1β+SF1670 group (treated with 10 ng/ml IL-1β and 2 μM SF1670). The cells were cultured for 5 days for the next steps.

Western Blot Analysis

After NP cells were treated with different stimuli for 5 days, the total protein was isolated with the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Then, an equal protein sample of each group was added in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blockage in 5% milk, the membranes were incubated with desired primary antibodies: collagen II (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), AKT (1:1000; Cell Signaling Technology, Danvers, MA, USA), PTEN (1:1000; Cell Signaling Technology, Danvers, MA, USA), Caspase-3/9 (1:1000; Cell Signaling Technology, Danvers, MA, USA), Bax (1:1000; Cell Signaling Technology, Danvers, MA, USA), Bcl-2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), and β-Actin (as loading control, 1:1000; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed three times with Phosphate-Buffered Saline-Tween 20 (PBST) and incubated with secondary antibody for 1 h at room temperature. Finally, the membranes were washed again, incubated in enhanced chemiluminescence (ECL)

substrate (Beyotime, Shanghai, China), and exposed using developing film. Band intensities were measured using ImageJ software (NIH, Bethesda, MD, USA).

Immunofluorescence

The coverslips were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton-X for 15 min at room temperature. Then, the coverslips were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature to avoid non-specific binding. The coverslips were washed and incubated with primary antibodies: collagen II (1:100, Cell Applications, San Diego, CA, USA), p16 (1:200, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The coverslips were washed with PBS, and subsequently incubated with Alexa Fluor488 conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h in the dark at room temperature. The staining intensity was measured using the Image-Pro Plus software (Version 5.1, Media Cybernetics, Inc., Silver Spring, MD, USA).

Real Time-Polymerase Chain Reaction (RT-PCR) Analysis

Briefly, the total RNA of NP cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) with a reverse transcription kit (Roche, Basel, Switzerland). The real time-PCR analysis on collagen II, PTEN, aggrecan, p16, p53, Akt, IL-6/8, TNF-α, MMP3/9/13, Bax, and Bcl-2 were performed using SYBR Green Master (TOYOBO, Osaka, Japan). Relative gene expression was achieved by normalization to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated according to the method of $2^{-\Delta\Delta Ct}$. The primers used for real time-PCR were listed in Table I.

Enzyme-Linked Immunosorbent Assay (ELISA)

Contents of IL-6, IL-8, and TNF-α secreted by NP cells were measured using an ELISA kit (R & D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Cell Viability Assay

Cell viability was determined by the Cell Counting Kit (CCK-8) assay (Dojindo, Molecular Technologies, Kumamoto, Japan). NP cells were seeded at a density of 1×10^4 cells per well in 96-well plate and incubated for cell attachment. The cells were then treated with specific drugs for indicated time points. After treatments, the cells were incubated with CCK-8 reagent according to the manufacturer's instructions. Absorbance was measured at 570 nm using a microplate reader (Labsystems Multiskan, Vantaa, MS, Vantaa, Finland). Cell viability was shown as a percentage relative to non-treated value.

Statistical Analysis

Data were analyzed by the Statistical Product and Service Solutions (SPSS) Version 22.0 software package (IBM Corp., Armonk, NY, USA)

Table I. Primer sequences of the genes for RT-PCR.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
Aggrecan	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
PTEN	TGGATTCGACTTAGACTTGACCT	GGTGGGTTATGGTCTTCAAAAGG
Akt	AGCGACGTGGCTATTGTGAAG	GCCATCATTCTTGAGGAGGAAGT
P16	GATCCAGGTGGGTAGAAGGTC	CCCCTGCAAACTTCGTCCT
P53	GGTTCCTGCCCCAGGATGTTG	GGAACATCTCGAAGCGCTCA
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-8	TTTTGCCAAGGAGTGCTAAAGA	AACCCTCTGCACCCAGTTTTC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
MMP3	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP9	CTGGACAGCCAGACACTAAAC	CTCGCGGCAAGTCTTCAGAG
MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
Bax	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
Bcl-2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

and expressed as mean ± standard deviation (SD). The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using the One-way analysis of variance (ANOVA) test, followed by the post-hoc test (Least Significant Difference). p-value<0.05 indicated a statistical significance.

Results

Expression of PTEN In Human NP Tissues According to Pfirrmann Grades

Western blot and RT-PCR were used to measure the expressions of collagen II and PTEN in degenerated discs extracted from 4 patients in Mild group and Severe group, respectively. The protein level of collagen II significantly decreased in the Severe group compared with the Mild group (Figure 1A). Meanwhile, PTEN was markedly upregulated in the Severe group as well (Figure 1A). Their mRNA levels were similarly changed (Figures 1B, 1C). The primers sequences used for RT-PCR were list in Table I. Collagen II, the major matrix component of the NP, maintains the structural stability of NP tissue, and its content can indicate the healthy condition of NP cells. Thus, it is concluded that the PTEN level increased with the deterioration of disc degeneration.

SF1670 Treatment Increased NP Cells Viability In Vitro

After isolating the NP cells from samples, IL-1β was applied to establish the degenerated model in the first-generation NP cells according to previous method[22]. Firstly, we aimed to examine the optimized concentration of SF1670 applying in NP cells by CCK-8 assay. As shown in Figure 2A, the viability of NP cells achieved the highest level after 2 μM SF1670 treatment for 3 h. Therefore, the concentration of 2 μM was used for the following treatment of anti-IL-1β. As shown in Figure 2B, IL-1β treatment markedly decreased NP cells, which was protected by SF1670 treatment. It is suggested that SF1670 pretreatment provided a favorable environment to NP cells.

SF1670 Treatment Reversed NP Cells Degeneration In Vitro

To test the effect of SF1670 on protecting NP cell senescence, we detected the expressions of p16 and p53, the representative makers of cell senescence. The results showed that IL-1β en-

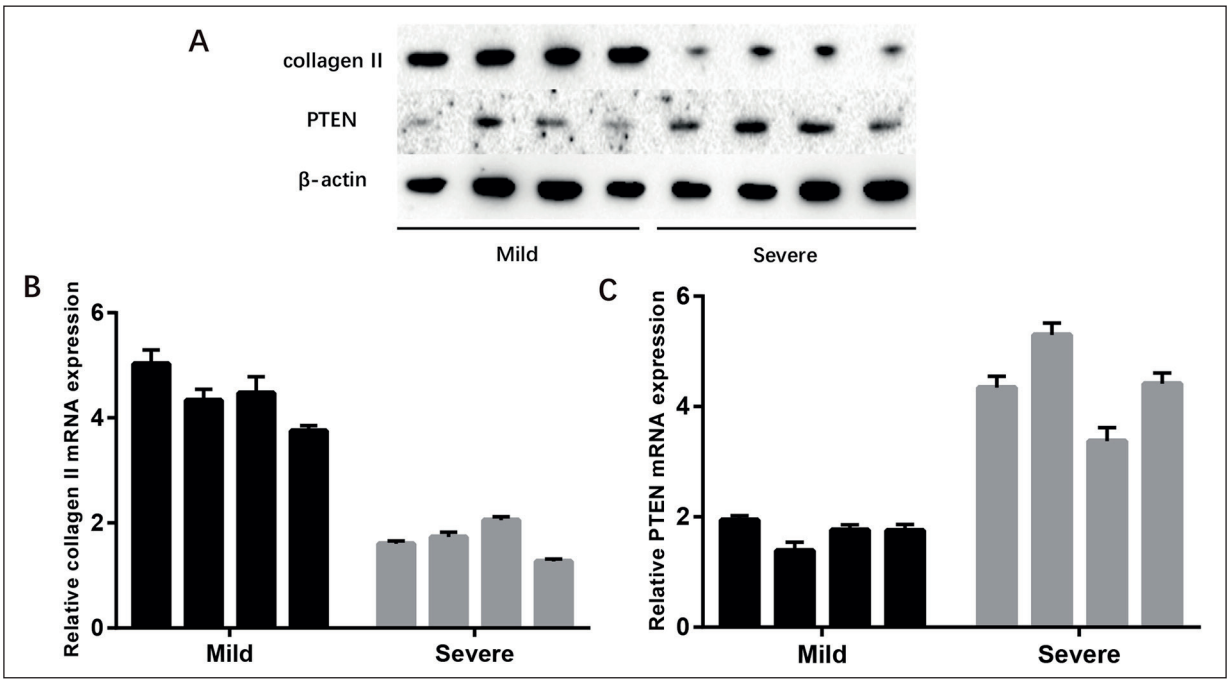

Figure 1. Expression of PTEN in human NP tissues according to Pfirrmann grades. Expressions of collagen II and PTEN in human disc nucleus pulposus tissue was determined by Western blot (**A**) and RT-PCR (**B, C**).

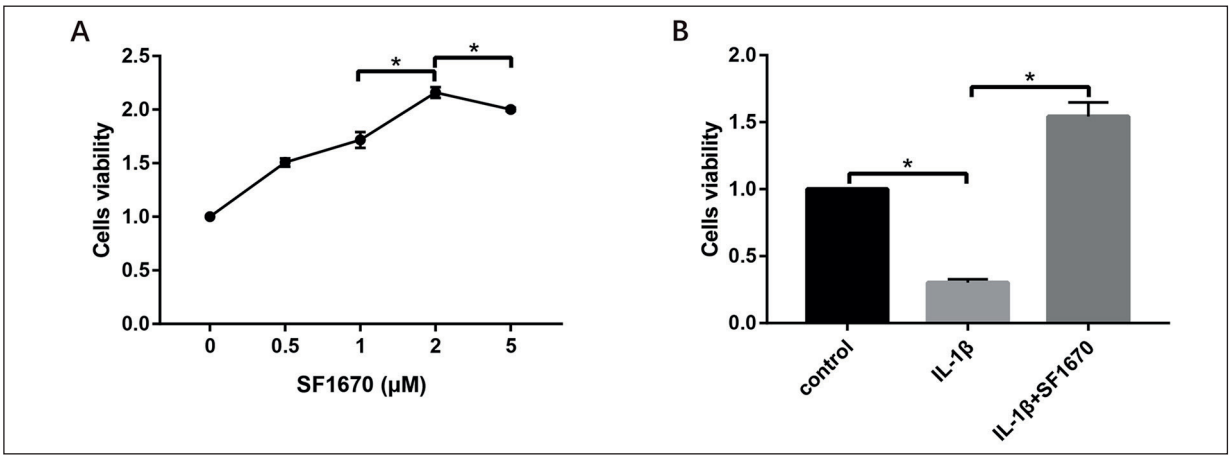

Figure 2. SF1670 treatment increased NP cells viability *in vitro*. **A,** CCK-8 assay for NP cells with treatment of 0, 0.5, 1, 2, and 5 μM SF1670. **B,** CCK-8 assay for NP cells in three groups. ("*" means that there is a statistical difference between two groups).

hanced the expressions of p16 and p53 in NP cells compared with the control group. However, pretreatment of SF1670 markedly downregulated p16 expression compared with IL-1β group (Figure 3A). Similarly, collagen II and aggrecan expressions were upregulated by SF1670 stimuli compared with IL-1β group (Figure 3B, 3C). As the inhibitor of PTEN, we detected the expressions

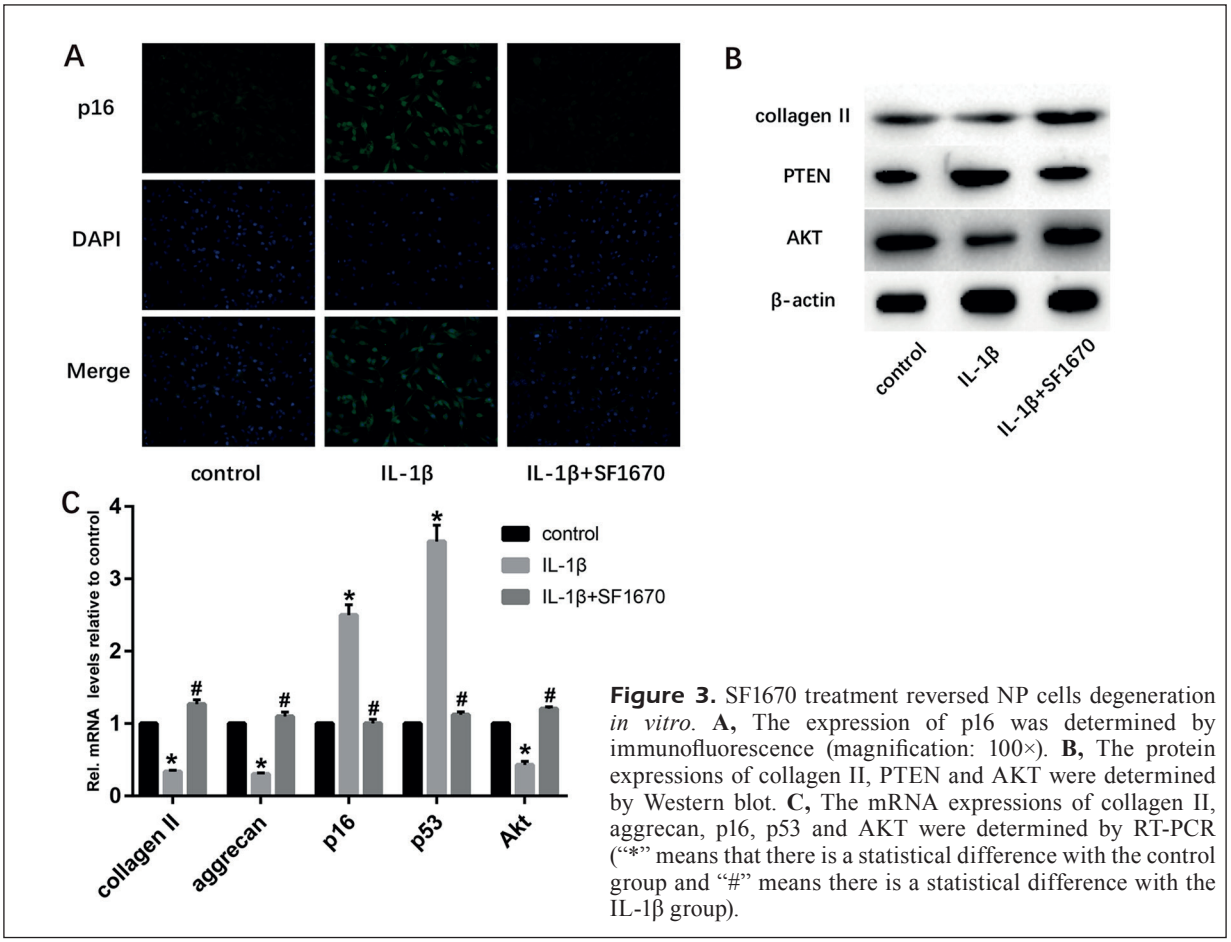

Figure 3. SF1670 treatment reversed NP cells degeneration *in vitro*. **A,** The expression of p16 was determined by immunofluorescence (magnification: 100×). **B,** The protein expressions of collagen II, PTEN and AKT were determined by Western blot. **C,** The mRNA expressions of collagen II, aggrecan, p16, p53 and AKT were determined by RT-PCR ("*" means that there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

of PTEN and its downstream protein Akt. As depicted in Figure 3B and 3C, there was a significant change in both mRNA and protein expressions of total PTEN between IL-1β and control group. Conversely, SF1670 markedly enhanced Akt activity through the suppression of PTEN function (Figures 3B, 3C). The results indicated that SF1670 could significantly improve the degeneration of NP cells by suppressing PTEN and activating Akt.

SF1670 Treatment Inhibited Inflammation of NP Cells In Vitro

To determine the function of SF1670 in influencing the inflammatory responses in NP cells, mRNA levels of TNF-α, IL-6, IL-8, MMP3, MMP9, and MMP13 were determined using RT-PCR. After stimulation of IL-1β, the relative levels of TNF-α, IL-6, IL-8, MMP3, MMP9, and MMP13 were significantly upregulated in NP cells (Figure 4A). Following incubation with SF1670, these inflammatory factors showed a downward trend (Figure 4A). ELISA determination on the contents of TNF-α, IL-6, and IL-8 yielded the same results (Figures 4B-4D). These finding suggested that SF1670 protected inflammation in human NP cells.

SF1670 Attenuated Apoptosis of NP Cells In Vitro

Subsequently, the regulatory effect of SF1670 on apoptosis of NP cells was investigated by detecting the expression levels of caspase-3, caspase-9, Bax, and Bcl-2. Caspase-3, caspase-9, and Bax were upregulated, while Bcl-2 was downregulated in IL-1β-induced NP cells degeneration (Figure 5A). The above trends were partially abolished by SF1670 pretreatment. The mRNA level changes of them were shown in Figure 5B.

Discussion

Apoptosis has been demonstrated to participate in IVD degeneration for many years. Recent studies suggested that anti-apoptosis of NP cells may be beneficial to IVDD. Clarify-

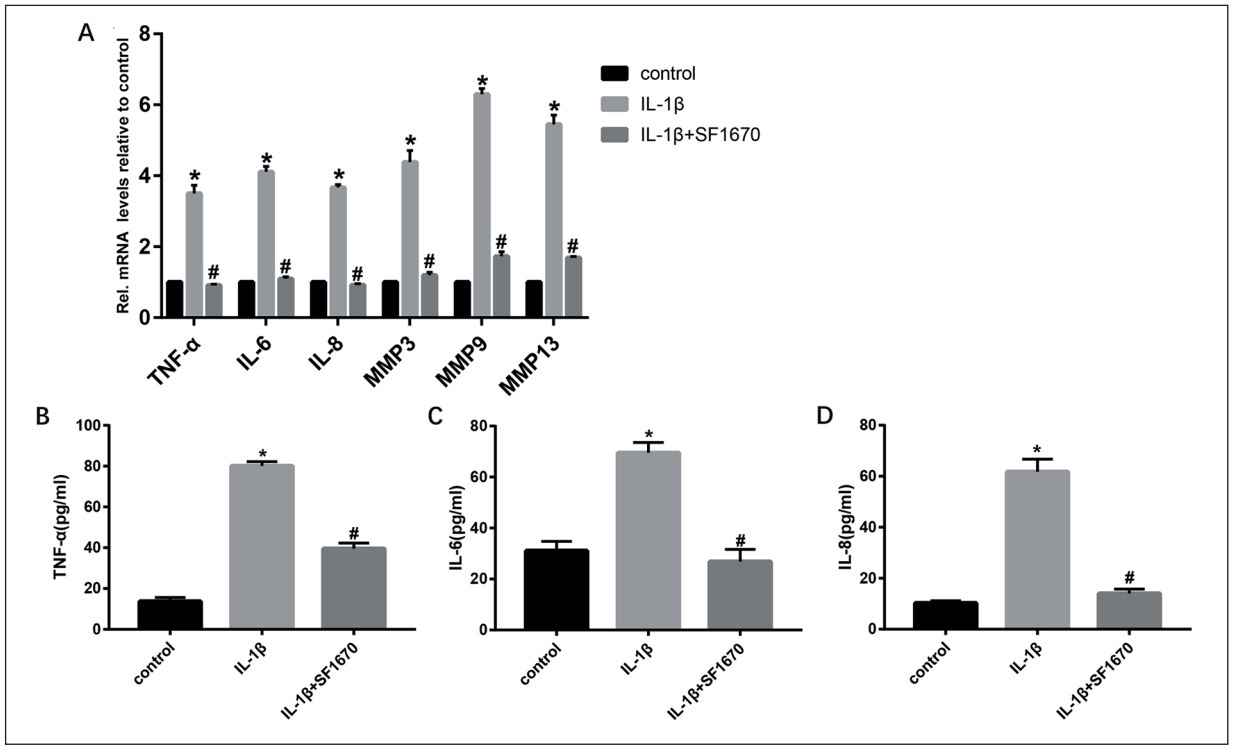

Figure 4. SF1670 treatment inhibited inflammation of NP cells *in vitro*. **A,** The mRNA expressions of TNF-α, IL-6, IL-8, MMP3, MMP9, and MMP13 were determined by RT-PCR. The expressions of TNF-α (**B**), IL-6 (**C**), and IL-8 (**D**) were determined by ELISA. ("*" means that there is a statistical difference with the control group and "#" means that there is a statistical difference with the IL-1β group).

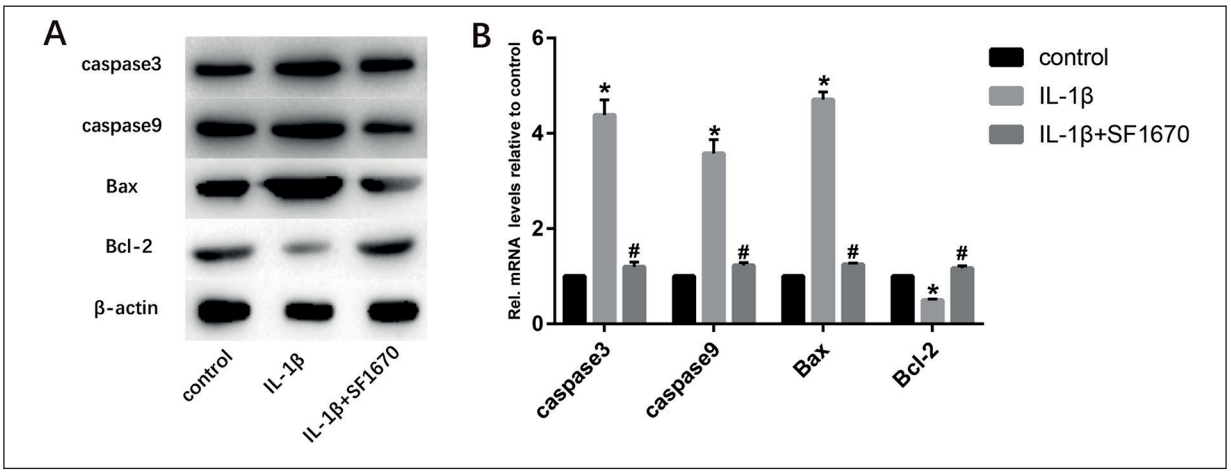

Figure 5. SF1670 attenuated apoptosis of NP cells *in vitro*. The expressions of caspase3, caspase9 Bax, and Bcl-2 were determined by Western blot (**A**) and RT-PCR (**B**). ("*" means that there is a statistical difference with the control group and "#" means that there is a statistical difference with the IL-1β group).

ing both intrinsic and extrinsic mechanisms of apoptosis pathways is of great significance for the therapeutic benefits[23,24]. PTEN is a well-known tumor-suppressor gene that maintains a balance between cell viability and cell death[25]. PTEN function is independent of the 3-kinase (PI3K)/Akt pathway[26,27], and the downregulation of PTEN results in Akt activation and apoptosis suppression. Akt might play its anti-apoptotic role by regulating caspase-3, Bax, and Bcl-2 in the antagonist of cell death[28]. It is reported that the activation of the phosphatidylinositol PI3K/Akt pathway protects IVDD by inhibiting apoptosis, promoting cell proliferation, and elevating ECM content[29]. SF1670 is a potent inhibitor of the phosphatase activity of PTEN. By enhancing the neutrophil activity, SF1670 exerts an anti-bacterial capacity of neutropenic recipient mice[20]. However, the protective effect ofSF1670 on IVDD is still unknown.

To the best of our knowledge, this present study is the first study demonstrating the protective effects of IL-1β-induced NP cell degenerated model through SF1670 treatment. The results demonstrated that IL-1β accelerated the degeneration speed of NP cells with downregulated collagen II and aggrecan, as well as upregulated p16 and p53. However, SF1670 pretreatment upregulated collagen II and aggrecan, and downregulated p16 and p53 compared with the IL-1β group. Moreover, CCK-8 assay indicated that SF1670 protected the proliferation of NP cells against IL-1β treatment. Since SF1670 is the specific inhibitor of PTEN, we have verified that SF1670 intervened PTEN and its downstream Akt.

We analyzed various cell death-related genes, including MMP3, MMP9, MMP13, Bax, and Bcl-2 in NP cells. IL-1β degenerated NP cell by activating cell apoptosis, which was partially reversed by SF1670. It has previously been reported that the suppression of the PTEN/Akt signaling pathway protects against pulmonary[30] and vascular inflammation[31]. Based on the above research, we also measured the relative levels of the inflammatory factors IL-6, IL-8, and TNF-α. The results elucidated that the inflammation was significantly inhibited after the treatment of SF1670. In future experiments, we will validate our findings in *in vivo* models. Taken together, our findings revealed that SF1670 could be a promising reagent for the treatment of intervertebral disc diseases.

Conclusions

Overall, these results demonstrated that SF1670 prevented NP degradation *via* suppressing apoptosis and inflammation through inhibition of PTEN and activation of Akt. SF1670 may become a novel target for the therapy of IVDD in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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