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VO-OHpic attenuates intervertebral disc degeneration *via* PTEN/Akt pathway

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Abstract. – OBJECTIVE: Intervertebral disc degeneration (IVDD) is mainly associated with a chronic process of the nucleus pulposus (NP) cells disabled. Also, it is accepted to be the basic result of low back pain. The role of phosphatase and tensin homolog (PTEN) in negatively regulating the Akt/PKB signaling, which is the major cell survival pathway, has been documented in previous studies. The present work aimed to investigate the role of PTEN inhibitor VO-OHpic (VO) in the protection of IVDD and to explore its potential mechanisms.

PATIENTS AND METHODS: NP cells isolated from patients' lumbar discs were subjected to different concentrations of IL-1β or H2O2 to establish NP cells degenerated model. Cell proliferation was analyzed by the Cell Counting Kit-8 (CCK-8) assay. The expression levels of collagen II, aggrecan, PTEN, PI3K, Akt, SOD1, SOD2, p16, and β-galactosidase (β-gal) were detected by Western blotting, immunofluorescence staining or RT-PCR. Flow cytometry was used to measure the ROS level and cell cycle distribution.

RESULTS: Our research showed that collagen II, aggrecan, PI3K, and Akt markedly decreased in IL-1β- or H_2O_2-induced degenerated NP cells. VO could reverse the effects of IL-1β and H2O2 by the PTEN inhibition. Also, we found that VO increased the antioxidant enzymes SOD1, SOD2, CAT, GSH, POD production, and suppressed the ROS in the disc. Besides, data showed VO promoted NP cells proliferation by cell cycle mediation.

CONCLUSIONS: These results suggest that VO treatment prevents NP degradation *via* restraining oxidative stress and increasing cell proliferation through the PTEN/Akt pathway *in vitro*. VO may become a novel cytokine for the therapy of IVDD in the future.

Key Words:
Nucleus pulposus cells, Intervertebral disc degeneration, VO-OHpic, Oxidative stress, PTEN.

Introduction

Millions of people around the world have suffered from low back pain for decades, which is the most prevalent cause of pain and disability for the elderly[1,2]. However, the complex and multifactorial pathological mechanism of low back pain is still not well understood. It has been widely recognized that intervertebral disc degeneration (IVDD) is the primary factor resulting in low back pain in recent years[3,4]. Of these, it is acknowledged that increased reactive oxygen species (ROS) and decreased proliferation of nucleus pulposus (NP) cells play essential roles in this process[5,6]. The balance between the rates of production and breakdown of ROS is responsible for the low proliferation rate under ambient oxygen tension[7]. Overproduction of ROS could directly degenerate the NP cells and perturb the homeostasis of disc matrix, including reduced collagen II and aggrecan synthesis[8,9].

Phosphatase and tensin homolog (PTEN), a lipid phosphatase and tumor suppressor, has been proved to be associated with the regulation of cell proliferation, differentiation, apoptosis, and oxidative stress[10-12]. PTEN is an upstream negative regulator of Akt signaling pathway which is involved in cell survival, while the inhibition of PTEN is capable of activating Akt by upregulating PI3K[13]. Once activated, the PI3K/Akt pathway has been elucidating to have the ability to enhance endogenous antioxidant activity in intestinal epithelial cells, macrophages, and human umbilical vascular endothelial cells[14,15]. VO-OHpic (VO), a specific vanadium-based PTEN inhibitor[16], has been suggested to have a protective role in some fields. VO inhibits pro-inflammatory cytokines IL-1β and TNF-α expression and upregulates anti-inflammatory IL-10 expression in a sudden cardiac arrest model[17]. Additionally, VO treatment

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can reduce apoptosis and promote cardiomyocyte survival *in vitro*[18].

Current therapeutic strategies for IVDD are only restricted to symptomatic treatments, but without addressing the novel methods to promote intervertebral disc reparation and regeneration[19]. However, whether direct inhibition of PTEN via VO inhibits ROS and adverse IVDD remains unknown. In this study, we applied two methods to induce degeneration in human nucleus pulposus cells, evaluated the senescent degree and ROS level in the cells, and tried to uncover the function of VO involved in the progress of IVDD. We believe that understanding the VO attenuates human cells under oxidative stress is important to provide better therapeutic options for degenerative discogenic diseases.

Patients and Methods

NP Cell Isolation and Cell Culture

This research was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University. The present study contained 10 samples of intervertebral discs (Pfirrmann classification score, Grade 1-2, classified on the basis of the MRI status of the disc degeneration) obtained from patients who underwent discectomy for thoracolumbar fractures. These samples were collected from April 2018 to July 2018. Written informed consent was obtained from all patients before operations.

Tissues were collected during surgery and immediately preserved in cold cell culture medium. Under sterile conditions, the NP was cut into small fragments and digested with 0.2% Type II collagenase and 0.25% trypsin for 6 h. Then, cell pellets were filtered and washed in phosphate-buffered saline (PBS). Finally, the collected NP cells were seeded in culture medium [DMEM/F-12 contains 10% fetal bovine serum (FBS), 1% penicillin/streptomycin]. NP cells were treated with IL-1β (10 μM; AmyJet Scientific, China) with or without VO (30 nM; Selleck, Houston, TX, USA), and the culture medium was changed at the indicated times. Each treatment was conducted and replicated in three different wells.

Western Blotting

NP cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China). Total proteins were isolated by a Nuclear/Cytosol Fractionation Kit (BioVision Inc, Milpitas, CA, USA) according to the manufacturer's instructions. Protein concentrations were measured using bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Next, equal protein sample of each group was applied in the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies against the following proteins: PTEN (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PI3K (1:1000; Abcam, Cambridge, MA, USA), Akt (1:3000; Cell Signaling Technology, Danvers, MA, USA), SOD1/2 (1:1000; Abcam, USA), p16 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-gal (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (used as loading controls, 1 :3000; Cell Signaling Technology, Danvers, MA, USA). After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; Abcam, Cambridge, MA, USA) for 2 h at room temperature. Protein bands were detected using the enhanced chemiluminescence (ECL) system.

Immunofluorescence

Human NP cells were washed three times with PBS and 4% paraformaldehyde (PFA) was used to fix the samples at room temperature. After 15 min of fixation, cells were permeabilized with 0.1% Triton-X subsequent pretreatment with 5% bovine serum albumin (BSA) for 1 h at room temperature to avoid non-specific binding. Cells were washed and incubated with primary antibodies: collagen II (1:200, Cell Signaling Technology, Danvers, MA, USA), aggrecan (1:800, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The next day, cells were re-probed with Alexa Fluor® 488 (Beyotime, China) and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, China). The staining intensity was measured using a laser scanning confocal microscope (Olympus Fluoview, Tokyo, Japan).

Flow Cytometry

To determine the intracellular ROS generation level, NP cells were stained with 20, 7'-dichlorofluorescein diacetate (DCFH-DA, Kaiji, China). Briefly, cells were incubated with 10 mM

DCFH-DA for 30 min in the dark. Finally, the cells were analyzed for DCF fluorescence by flow cytometry. For cell cycle analysis, cells in different cycles were counted and represented as a percentage of the total cell count by propidium iodide (PI) staining (0.2 mg/mL, Kaiji, China) measured by flow cytometry according to the manufacturer's instructions.

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the NP cells by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity was measured using an A260/A280 ratio by spectrophotometer. RNA was reverse-transcribed into cDNA with a reverse transcription kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The real-time PCR analysis was set up for measuring the mRNA expression of collagen II, aggrecan, PTEN, PI3K, Akt, SOD1/2, CAT, GSH, POD, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used for the target genes were shown in Table I. GAPDH was used as internal control. Each sample was assessed in triplicate. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method following normalization to the controls.

Cell Viability Assay

The viability of NP cells was determined by Cell Counting Kit-8 (CCK-8; Beyotime, China) assays according to the manufacturer's instructions. 1×10^4 cells/well were seeded in 96-well plates and incubated in 100 ul of complete culture medium. Each treatment was repeated in five wells. 10 ul CCK-8 was added to 100 μl culture media each well. Then, these cells were incubated for 2 h. Finally, the absorbance of the sample from each well was measured using an auto microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm. Cell viability was shown as the percentage of viable cells relative to untreated cells.

Statistical Analysis

Data are presented as the mean ± standard using SPSS version 22.0 software (IBM, Armonk, NY, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using the One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). *p*-values < 0.05 were considered statistically significant.

Results

VO Alleviates IL-1β Induced Human NP Cells Degeneration Via the PTEN/Akt Pathway

IL-1β has been well documented as an inducer of NP cells degeneration via promoting inflammation. Therefore, we used 10 ng/ml IL-1β induced NP cells degeneration according to the previous method[20]. First, we used CCK-8 assay to test the cytotoxicity of VO on NP cells and to apply the optimized concentration for cell culture. We measured cell viabilities following the concentration of VO at 0 μM, 10 μM, 30 μM, 50 μM, 100 μM for 48 h. Results showed that 30 μM contributed to the best viability to NP cells (Figure 1A). 30 μM of VO was used in the following investigation.

To evaluate whether VO treatment protected human NP cells degeneration, RT-PCR was performed for collagen II and aggrecan. In the

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
PI3K	TATTTGGACTTTGCGACAAGACT	TCGAACGTACTGGTCTGGATAG
Akt	AGCGACGTGGCTATTGTGAAG	GCCATCATTCTTGAGGAGGAAGT
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
CAT	TGGAGCTGGTAACCCAGTAGG	CCTTTGCCTTGGAGTATTTGGTA
GSH	GGGAGCCTCTTGCAGGATAAA	GAATGGGGCATAGCTCACCAC
POD	TCCTGGCTAACGACAAATACGA	TTTCCCGGCCACCATAAAGG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

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

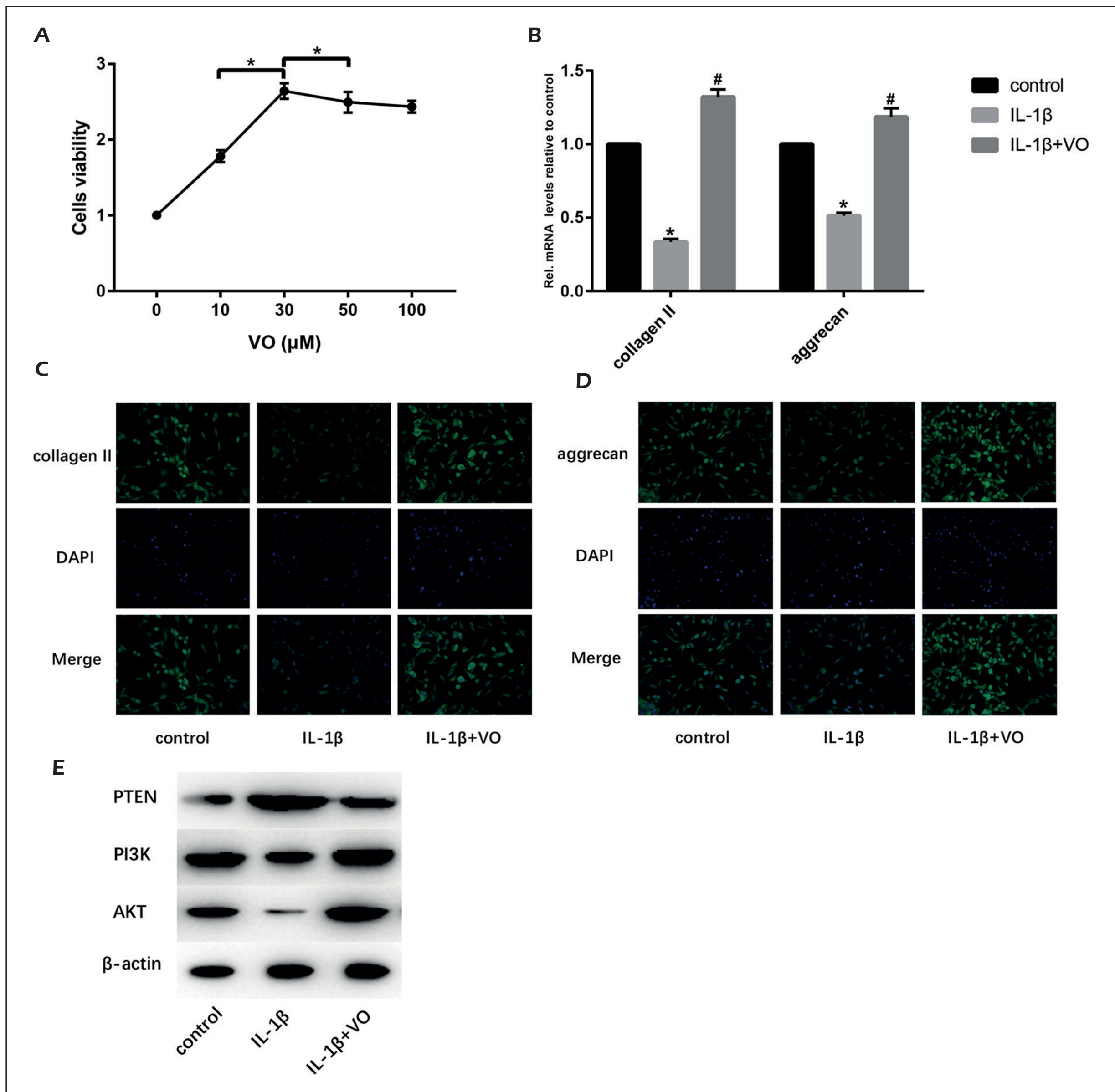

Figure 1. VO alleviates IL-1β induced human NP cells degeneration via the PTEN/Akt pathway. **A,** CCK8 assay for NP cells with treatment of 0, 10, 30, 50 and 100 μM VO ("*" means there is a statistical difference between two groups). The expression of collagen II and aggrecan was determined by RT-PCR (**B**) and immunofluorescence (**C-D**) ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group) (100×). **E,** Protein expression of PTEN, PI3K, and AKT was determined by Western blot.

treatment of IL-1β, mRNA expression of collagen II or aggrecan significantly decreased compared with control group. Moreover, collagen II and aggrecan levels were significantly upregulated after the stimulation of VO compared with IL-1β group (Figure 1B). Also, we carried out immunofluorescence staining to analyze the expression of collagen II and aggrecan and found the same result with RT-PCR (Figure 1C, 1D). As a function of VO in the inhibitor of PTEN, we further tested PTEN and its downstream targets PI3K and Akt in both protein and mRNA level in human NP cells. Results suggested that IL-1β successfully promoted the expression of PTEN to downregulate the PI3k and Akt expression. With the inclusion of VO treatment, PTEN expressed significantly lower levels compared to IL-1β group, reversely, PI3K, and Akt were up-

regulated (Figure 1E). Collectively, this data suggests that VO administration reduces degenerated actions, evidenced by the rise of collagen II and aggrecan expression, which might be associated with the inhibition of PTEN and activation of PI3K and Akt.

VO Promotes Human NP Cells Proliferation by Cell Cycle Mediation

To explore the effect of VO on proliferation to NP cells, we used CCK-8 assay to compare within different time points. As shown in Figure 2A, IL-1β made a decreased proliferation to NP cells compared to control group, while VO contributed to a positive influence on NP cells compared to IL-1β culture. It is well known that cell cycle stagnation leads to a decline in cell proliferation. Therefore, flow cytometry analysis was used to teste NP cell cycles. The result indicated that, compared to control group, a higher percentage of NP cells bogged down in the G0-G1 phase, and fewer cells remained in the S phase in IL-1β group. However, VO could ensure that more cells pass through the G1 to the S phase compared to IL-1β group (Figure 2B). In short, what we found indicated that VO could promote human NP cells proliferation via mediating cell to pass better through the G1 to the S phase.

VO Alleviates H_2O_2 Induced Human NP Cells Degeneration In Vitro

To determine the association of the VO with the oxidative stress level during IVDD, we used H_2O_2-induced NP cell degeneration model according to Chen et al[21]. First, NP cells were pre-treated with 400 μM H_2O_2 for 1 h before the following investigations. As shown in Figure 3A and B, collagen II, and aggrecan positive cells significantly decreased in NP cells in H_2O_2 group compared with control group. After the stimulation of VO, the expression of collagen II and aggrecan increased compared with H_2O_2 group. Also, we analyzed the p16 and β-gal protein levels of each group. The result indicated that these two senescent markers reduced at the condition of co-culture with VO compared with H_2O_2 group (Figure 3C). Also, VO proved to promote proliferation at H_2O_2-induced NP cell degenerated condition according to the CCK-8 assay (Figure 3D). Based on the above data, we concluded that VO could alleviate the H_2O_2-induced human NP cells degeneration *in vitro*.

VO Reduces ROS Level and Increases Anti-Oxidative Enzymes Activity in Human NP Cells

To verify whether VO alleviates human NP cells degeneration by anti-oxidative function, we used flow cytometry to test the total ROS level of each group. As it is shown in Figure 4A, H_2O_2 upregulated human NP cells reactive oxygen species significantly, and VO treatment decreased the ROS compared with H_2O_2 group. Several anti-oxidative enzymes SOD1, SOD2, CAT, GSH, and POD were measured by Western Blot or RT-PCR. Results suggested that these enzymes were all upregulated after VO stimulation compared with H_2O_2 group (Figure 4B, 4C). Collectively, our data suggested that VO treatment attenuated ROS and resulted in improved anti-oxidative enzymes activity.

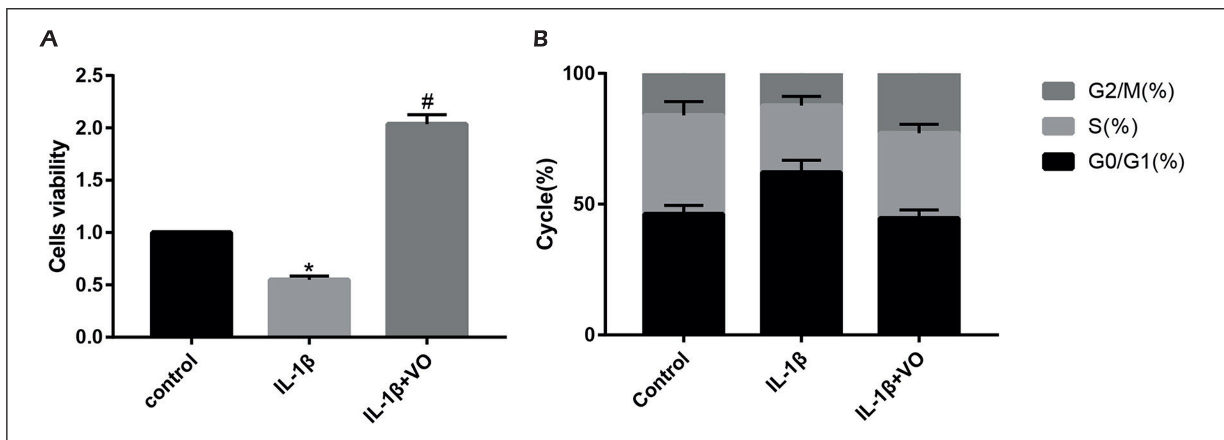

Figure 2. VO promotes human NP cells proliferation by cell cycle mediation. (**A**) CCK-8 assay for NP cells in three groups. (**B**) cell cycles in three groups were determined by flow cytometry. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

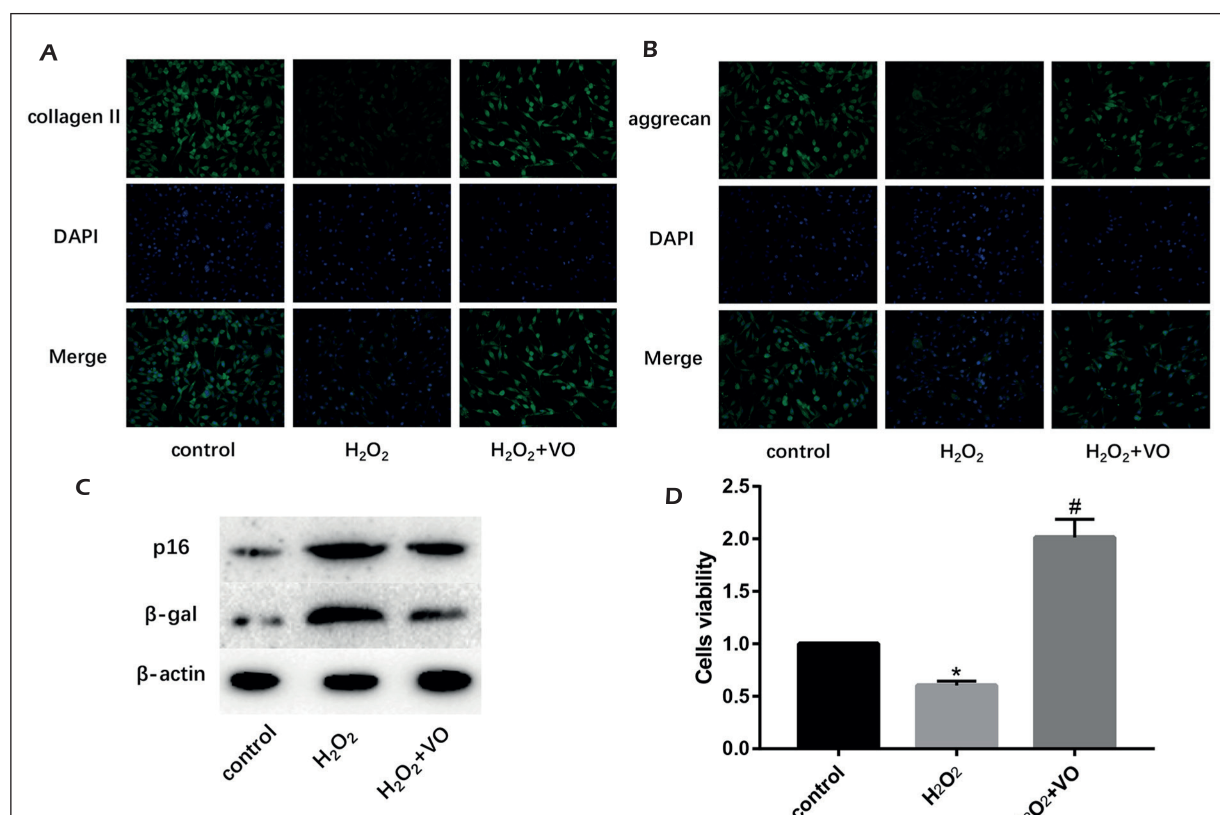

Figure 3. VO alleviates H_2O_2 induced human NP cells degeneration *in vitro*. The expression of collagen II (**A**) and aggrecan (**B**) was determined by immunofluorescence (100×). **C,** Protein expression of p16 and β-gal was determined by Western blot. **D,** CCK-8 assay in three groups. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the H_2O_2 group).

Discussion

ROS family is a chemically reactive chemical species containing oxygen such as hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), and hypochlorite ion (OCl^-). Due to the similar effects to ROS, nitric oxide (NO) and other reactive nitrogen species are also regarded as the ROS superfamily. ROS production is a result of aerobic metabolism. Due to poor vascularization, the microenvironment of the intervertebral disc is characterized by hypoxia[22,23]. H_2O_2 has been elucidated to exist in human NP tissues, indicating that disc cells are the ROS generator in the microenvironment of discs[24]. However, the excessive ROS production promoted disc degeneration has been widely reported, which means that the ROS level increases with the IVDD progression[25,26]. Various factors have been proved to increase ROS production in discs, such as mechanical stress, high glucose excitement, and pro-inflammatory cytokines. Apart from these, ROS themselves can also enhance ROS production in disc cells[26,27]. ROS serve as signaling messengers in many signaling pathways, such as the PTEN/Akt pathway, the NF-κB pathway, and the MAPKs pathway. In this study, we aim to explore the impact of VO in the IVDD through the PTEN/Akt pathway[28,29].

PTEN is a negative regulator of the PI3K/AKT cell survival pathway, and inhibition of PTEN has been associated with several biological advantages in various cancers[30], metabolic disorders[31], and intervertebral disc diseases[32]. However, research on specific inhibition of PTEN and its subsequent downstream effects in IVDD has not been sufficiently involved. In that regard, we used VO to inhibit PTEN in IL-1β-induced human NP cells degenerated model and evaluated its effects on PTEN/Akt pathway, cell proliferation, degeneration, and especially ROS levels.

The findings of the present work have shown that VO promotes NP cells proliferation within

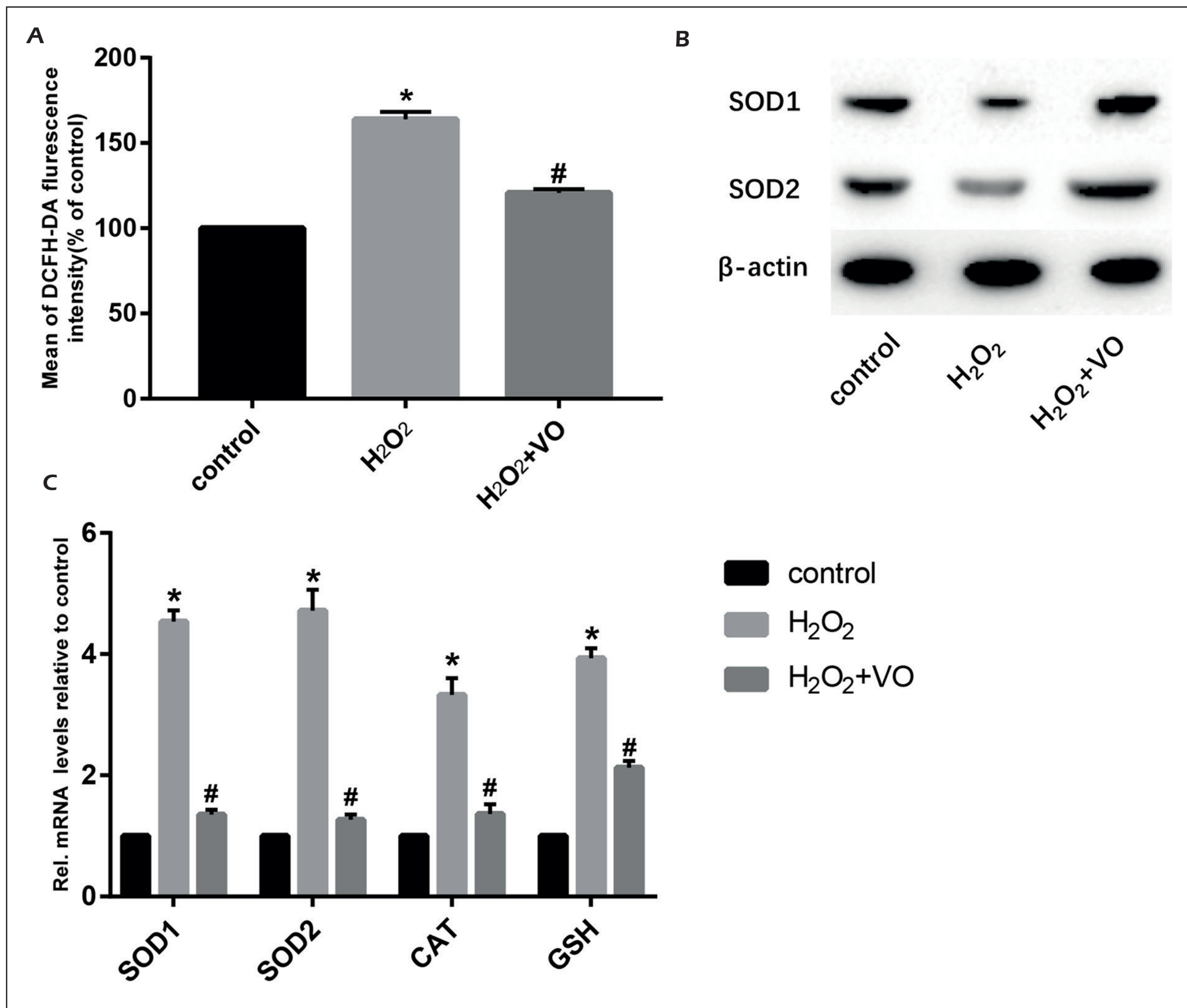

Figure 4. VO reduces ROS level and increases anti-oxidative enzymes activity in human NP cells. **A,** ROS levels in three groups were determined by flow cytometry. **B,** Protein expression of SOD1 and SOD2 was determined by Western blot. **C,** mRNA expression of SOD1, SOD2, CAT, and GSH was determined by RT-PCR ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the H_2O_2 group).

the proper concentration range, and this might be associated with the cell cycle mediation. Accordingly, it has been demonstrated that cells pass through the G1/S checkpoint smoothly contributing to the advanced proliferative ability[33]. Apart from this, collagen II and aggrecan, known as the crucial contents of ECM, were upregulated by the VO treatment compared with IL-1β group, meaning that VO showed a protecting effect in the NP cells degenerated progress. In our report, IL-1β activated the PTEN expression of NP cells to suppress its downstream PI3K and Akt levels. As expected, the expression of PTEN was significantly decreased by VO and resulted in the activation of PI3K and Akt. To explore the anti-oxidant function of VO, H_2O_2 was used to make the NP cells a peroxidation state. Specific increases of ROS level have been granted detected in the high H_2O_2 environment. Nonetheless, VO also showed an excellent antioxidant capacity, which was shown in suppressing total ROS level and upregulating various antioxidant enzymes expression. Therefore, the degenerated phenotype of NP cells significantly reduced. Notably, through the inhibition of PTEN, NP cells displayed a healthier state with advanced proliferation and lowered ROS level. However, there are some limitations in the present research. How the PTEN/Akt pathway interacts with the cell cycle needs to be further

explored. The present work provides valuable information regarding the protective role of VO *in vitro*. Additional studies are needed to clarify and confirm this unique mechanism *in vivo*.

Conclusions

These results suggest that VO treatment prevents NP degradation via restraining oxidative stress and increasing cell proliferation through the PTEN/Akt pathway *in vitro*. VO may become a novel cytokine 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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