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The role of quinazoline in ameliorating intervertebral disc degeneration by inhibiting oxidative stress and anti-inflammation *via* NF-κB/MAPKs signaling pathway

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Abstract. – OBJECTIVE: Previous studies have shown that Quinazoline (QNZ) plays extremely important roles in the cellular physiological activity, but it has been rarely examined on cell behavior following intervertebral disc degeneration (IVDD). The aim of this study was to investigate whether QNZ mediates oxidative stress and inflammation contributed to IL-1β-induced nucleus pulposus (NP) cells degeneration *in vitro*.

PATIENTS AND METHODS: NP were isolated cells from human disc samples collected from patients and the IL-1β-induced NP cells degenerated model was constructed. The cells were randomly divided into 3 groups, namely, Control group, IL-1β group (10 μM), QNZ + IL-1β group (containing 10 nM QNZ and 10 μM IL-1β). Then, the cell viability was determined by CCK-8 assay, and the levels of collagen I, collagen II, aggrecan, p16, p53, β-galactosidase (β-gal), antioxidant enzymes, 8-hydroxy-2-deoxyguanosine (8-OHdG), NF-κB/MAPKs signaling-related proteins and inflammatory factors were examined using Western blot and reverse transcription quantitative polymerase chain reaction (RT-qPCR) in NP cells. Finally, the expressions of IL-1β, IL-6, and TNF-α in the cell supernatants were also determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS: This study showed that IL-1β promoted the progress of IDD, with markedly increased expressions of collagen I, p16, p53, and β-gal, as well as decreased expressions of collagen II and aggrecan. However, QNZ treatment could reverse the effects of IL-1β. It was found that cell proliferation was increased, ROS level was decreased, antioxidant enzymes were upregulated, and inflammatory factors were reduced after QNZ stimulation. Moreover, NF-κB/MAPKs signaling proteins IKKβ, IκBα, p65, ERK, JNK, and p38 were significantly dephosphorylated by QNZ.

CONCLUSIONS: These results indicated that QNZ prevented NP degradation via restraining oxidative stress and inflammation through inhibition of the NF-κB/MAPKs signaling pathway. QNZ may become a novel insight into the therapy of IVDD in the future.

Key Words:
Nucleus pulposus cells, Intervertebral disc degeneration, QNZ, Oxidative stress, NF-κB/MAPKs.

Introduction

Low back pain has brought a serious impact to both somatic problems of patients and socio-economic burden[1]. Intervertebral disc degeneration (IVDD) is known as the main cause of low back pain[2]. Since then, medical treatment to reverse or even regenerate disc degeneration is still limited[3]. Structural failure in the degenerative disc is characterized by disc height loss, annulus fibrosus fissures, cartilage endplate calcification, loss of extracellular matrix (ECM) in nucleus pulposus (NP). The pathogenesis of IVDD involves various molecules and complex signaling networks[4,5]. Our understanding of the pathogenesis of IVDD is limited, but a consensus has been reached that IVDD is a process that involves oxidative stress and inflammation response[6,7]. In the degenerative disc tissue samples, reactive oxygen species

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(ROS) levels and various inflammatory cytokines such as IL-1β, IL-6, and TNF-α are found to be increased. Of note, several studies[8,9] have demonstrated that ROS and inflammation can injury disc matrix metabolism so as to promote disc cell death. Therefore, inhibition of ROS and inflammation-induced NP cells degeneration may be a potential way to ameliorate IVDD.

The nuclear factor κB (NF-κB) signaling pathway has been shown previously to modulate inflammatory cytokine response and catabolic matrix enzymes in intervertebral[10], which leads to the NF-κB pathway to an attractive target involved in the IVDD protection[11]. QNZ, 6-amino4-(4-phenoxyphenylethylamino) quinazoline, is a novel NF-κB inhibit reagent[12] known for its in anti-inflammatory, antioxidant and anticancer activity. The mechanism of action of QNZ is multifactorial, of which interferes with the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways[13,14]. Besides, QNZ can protect the endogenous oxidants or radicals induced nuclear DNA damages[15]. No safety issues have been observed with QNZ, and side effects are very rarely reported.

However, the protective effect of QNZ against IVDD still remains unknown. The main purpose of this study was to investigate whether pretreatment with QNZ can attenuate human NP cells degeneration against ROS and inflammation induced by IL-1β *via* the suppression of NF-κB signaling pathway *in vitro*, which might contribute to better clarify prevention and treatment of QNZ for the future treatment of IDD in clinical settings.

Patients and Methods

Nucleus Pulposus Cells Isolation and Cell Treatment

Human disc tissues were collected from patients undergoing disc herniation surgery in our hospital. The project was approved by the Ethics Committee of West China Hospital Sichuan University, and written informed consent was obtained from all patients or relatives before the surgeries. 12 degenerative disc samples were donated in the last two years. Nucleus pulposus without the endplates were divided into two groups according to the Pfirrmann score of disc degeneration. Only Grade II the Mild degenerated NP tissues were used to cells isolation. NP tissues were washed three times with a sterile phosphate-buffered saline solution (PBS) before isolation. After that, the tissues were minced into small pieces and digested with 0.25% trypsin solution for 30 min at 37°C. Then, the samples were incubated in 0.15% type II collagenase at 37°C overnight, and the solution was pipetted onto a cell strainer with 100 μm pore sizes and resuspended in cell culture medium (Dulbecco's Modified Eagle's Medium and Ham's F-12 medium, 1:1, Thermo Fisher Scientific, Waltham, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, USA), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 μg/mL). Finally, the cells were seeded on six-well plates (Sigma-Aldrich, St. Louis, MO, USA) at 1×10^5 cells per well, pre-incubated with IL-1β (Beyotime, Shanghai, China), and stimulated by QNZ (dissolved in Dimethyl Sulfoxide, Tongtian, Shanghai, China) as indicated in the Results and Discussion section.

Western Blot (WB)

NP cells were harvested with radioimmunoprecipitation assay (RIPA) lysis buffer and proteins were isolated using a Nuclear/Cytosol Fractionation Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Then, the concentrations of protein were measured using the enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China), blocked with 5% skim milk for 1 h at room temperature to avoid non-specific binding, and incubated overnight with anti-aggrecan (1:1000, Abcam, Cambridge, MA, USA), anti-type I collagen (Cell Signaling Technology, Danvers, MA, USA), anti-SOD1/2 (1:2000, Millipore, Billerica, MA, USA), anti-pERK (1:1000, Millipore, Billerica, MA, USA), anti-pJNK (1:1000, Abcam, Cambridge, MA, USA), anti-p38 (1:3000, Abcam, Cambridge, MA, USA), anti-pIKKβ (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-pIκBα (1:1000, Abcam, Cambridge, MA, USA), anti-p65 (1:3000, Abcam, Cambridge, MA, USA) and anti-β-actin (1:3000, Cell Signaling Technology, Danvers, MA, USA) used as controls. After incubating with secondary antibody (1:2000; Abcam, Cambridge, MA, USA) at room temperature for 2 h, membranes were washed again, incubated in enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific, Waltham, MA, USA), and exposed using developing film.

Immunofluorescence

The cells were fixed in 4% paraformaldehyde (PFA) for 15 min, quenched with PBS, permeabilized with 0.25% Triton X-100 for 15 min and

blocked with 5% bovine serum albumin/PBS (fatty acid-free; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Cells were then incubated with the first antibody of type II collagen (1:200, Cell Signaling Technology, Danvers, MA, USA), anti-p16 (1:250, Abcam, Cambridge, MA, USA), anti-β-gal (1:800, Cell Signaling Technology, Danvers, MA, USA), and anti-8-OH (1:500, Abcam, Cambridge, MA, USA) overnight at 4°C. Next, these cells were incubated with a secondary antibody (1:200, Sigma-Aldrich, St. Louis, MO, USA) for 1 h, washed extensively, and treated with 300 nM 4,6-diamidino-2-phenylindole (DAPI, 1:500, Beyotime, Shanghai, China) for nuclear counterstaining. Finally, the cells were visualized using a fluorescence microscope (Zeiss, Oberkochen, Germany)

Quantitative Real Time-Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from NP cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The absorbance of RNA at 260/280 nm was measured to authenticate the quality of RNA, respectively. RNA was then reverse-transcribed into single-stranded complementary DNA (cDNA) using PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). Later, P16, type I collagen, type II collagen, p53, aggrecan, SOD1/2, GSH, CAT, POD, GPX3, IL-1β, IL-6, TNF-α, and MMP3/9/10/13 mRNA levels were determined by RT-qPCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Thereafter, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as normalization. Primers depending on the nucleotide sequence of the gene were obtained from the Gene Bank (Table I). Finally, melting curve analysis was performed to confirm the identity and specificity of the PCR products, and relative mRNA expressions were determined using the $2^{-\Delta\Delta Ct}$ method.

Cell Viability

Human NP cells viability was measured using a Cell Counting Kit (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Cells were seeded in 96-well culture plates at a density of 5×10^3 cells per well. 10 μL CCK-8 solution was applied at specific time points, and then they were incubated in the dark for 2 h at 37°C. After that, cell viability was assessed through absorbance detection at 450 nm using a spectrophotometer (ELx808 Absorbance Microplate Reader, Bio-Tek, Biotek Winooski, VT, USA).

Intracellular ROS Levels

Intracellular ROS level was detected by flow cytometry. NP cells were collected (1×10^6/group) after treatment and washed three times with cold PBS. Then, the cells were intubated with DCFH-DA (10 μM Kaiji, Nanjing, China) for 20 min in 37°C followed immediately by flow cytometry

Table I. RT-PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
aggrecan	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
Collagen I	TGGATTCGACTTAGACTTGACCT	GGTGGGTTATGGTCTTCAAAAGG
P16	GATCCAGGTGGGTAGAAGGTC	CCCCTGCAAACTTCGTCCT
P53	GGTTCCTGCCCCAGGATGTTG	GGAACATCTCGAAGCGCTCA
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
CAT	TGGAGCTGGTAACCCAGTAGG	CCTTTGCCTTGGAGTATTTGGTA
GSH	GGGAGCCTCTTGCAGGATAAA	GAATGGGGCATAGCTCACCAC
POD	TCCTGGCTAACGACAAATACGA	TTTCCCGGCCACCATAAAGG
GPX3	AGAGCCGGGGACAAGAGAA	ATTTGCCAGCATACTGCTTGA
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
MMP3	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP9	CTGGACAGCCAGACACTAAAC	CTCGCGGCAAGTCTTCAGAG
MMP10	TGCTCTGCCTATCCTCTGAGT	TCACATCCTTTTCGAGGTTGTAG
MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction

analysis in a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) to detect the total ROS level.

ELISA Assay

The anti-HE4 antibody was detected by indirect ELISA. Microtiter plates were coated overnight with 1 μg/mL of recombinant HE4 in 10 mM of sodium carbonate buffer (pH 9.6) at 4°C or 37°C for 2 h and then blocked with PBS containing 5% skim milk at 37°C after three washes. After washing the wells three times with Phosphate-Buffered Saline Tween-20 (PBST), 100 μL per well of mouse antiserum, culture supernatants, or mAbs diluted by PBS were added to the plates and incubated for 1.5 h at 37°C. After the plates were washed thoroughly with PBST, 100 μL of a secondary antibody (biotinylated rat anti-mouse antibody) at 1:10000 diluted in PBS was added into each well. After a second incubation for 1 h, the plates were washed six times, and then 50,000-fold diluted streptavidin-HRP in PBS was added and incubated at 37°C for 1 h. Tetramethylbenzidine substrate system (100 μL/well) was then added and incubated away from light for 3-5 min at 37°C. After the color developed, the reaction was halted by 50 μL/well of 2 mol/L sulfuric acid (H_2SO_4), and then the absorbance at 450 nm was determined with an ELISA reader (Biotek, Winooski, VT, USA).

Statistical Analysis

Data were represented as mean ± SD (Standard Deviation). The *t*-test was used for analyzing measurement data. The differences between the two groups were analyzed by using the Student's *t*-test. Comparison among multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Besides, qualitative data were described as percentages and analyzed using a Chi-square test. Graph Pad Prism 6.0 software (San Diego, CA, USA) was used to plot, and *p*-values were two-sided. $p<0.05$ represented statistical significance.

Results

QNZ Promotes NP Cells Proliferation In Vitro

Human NP cells isolated from the patients were used as the object in this study. To examine the cytotoxicity of QNZ on NP cells and decrease the direct effect of cytotoxicity on stimulation, CCK-8 assay was used to detect the optimize concentration of QNZ on cell viability in NP cells. Figure 1A shows cell viabilities following administration with QNZ at 0 nM, 5 nM, 10 nM, 20 μM, 30 μM for 48 h, and the results showed that NP cells showed the highest viability at the 10 nM. Thus, the concentrations of 10 nM were chosen for the following experiments.

To establish the NP cells degeneration model, IL-1β was used as the stimulation for cell culture (10 ng/mL) according to the previous method[16]. To evaluate the potential anti-senescent effects of QNZ on NP cells, human NP cells were cultured with both IL-1β and QNZ. After cells 48 h drugs

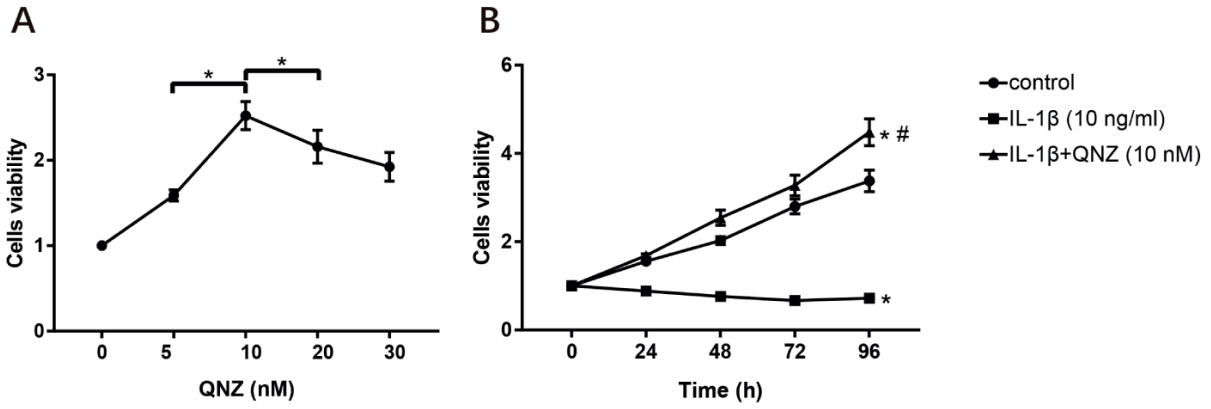

Figure 1. QNZ promotes NP cells proliferation *in vitro*. **A,** CCK8 assay for NP cells with treatment of 0, 5, 10, 20, and 30 nM QNZ. ("*" means there is a statistical difference between two groups). **B,** CCK8 assay for NP cells in three groups. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

treatment, the medium was replaced with normal culture medium, and the cell viability was analyzed by CCK8 method on indicated time points shown in Figure 1B. The results demonstrated that IL-1β inhibited the cell proliferation and QNZ seemed to be the antagonist of this effect. In brief, QNZ might have a role in promoting NP cells proliferation.

QNZ Alleviates NP Cells Senescence In Vitro

Expressions of collagen I, collagen II, and aggrecan were explored to measure the physiological status of NP cells, and it was found that IL-1β made NP cells to express much collagen I and less collagen II, but QNZ successfully reverse the effect of IL-1β, which meant QNZ decreased the degenerated phenotypes of NP cells (Figure 2A). Then, the NP cell senescence was tested by the aging maker β-gal (Figure 2B). The results showed that IL-1β significantly increased the β-gal expression compared with the control group. On the contrary, QNZ+IL-1β group showed significantly less β-gal expression compared with IL-1β group. After that, these differences were also observed in gene expression using RT-qPCR and achieved the same results (Figure 2C). Taken together, these findings indicated that QNZ exerted its an-

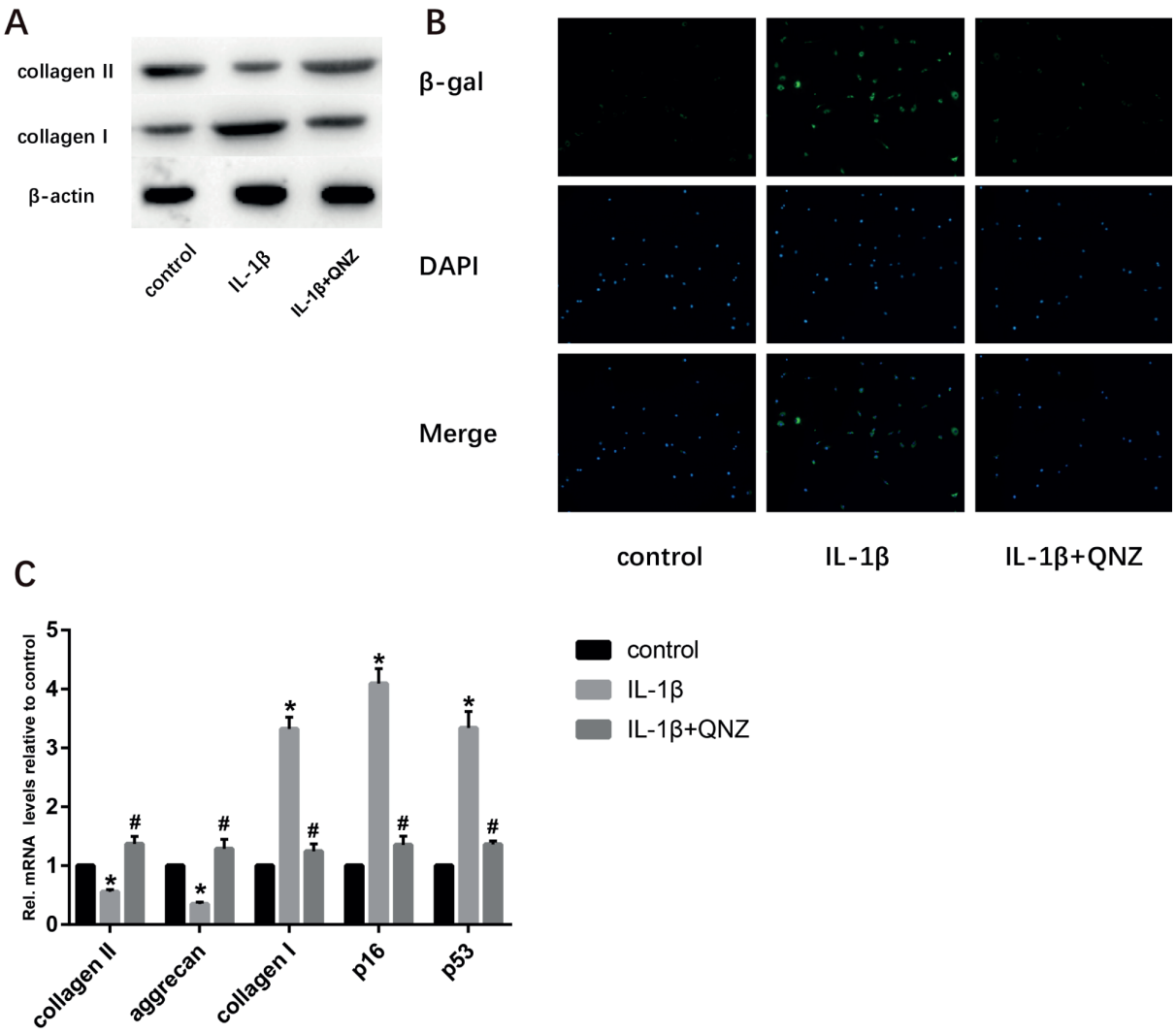

Figure 2. QNZ alleviates NP cells senescence in vitro. **A,** Expressions of collagen II and collagen I are determined by Western blot. **B,** Expression of β-gal is determined by immunofluorescence (magnification: 100×). **C,** MRNA expressions of collagen II, aggrecan, collagen I, p16 and p53 are determined by RT-PCR. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

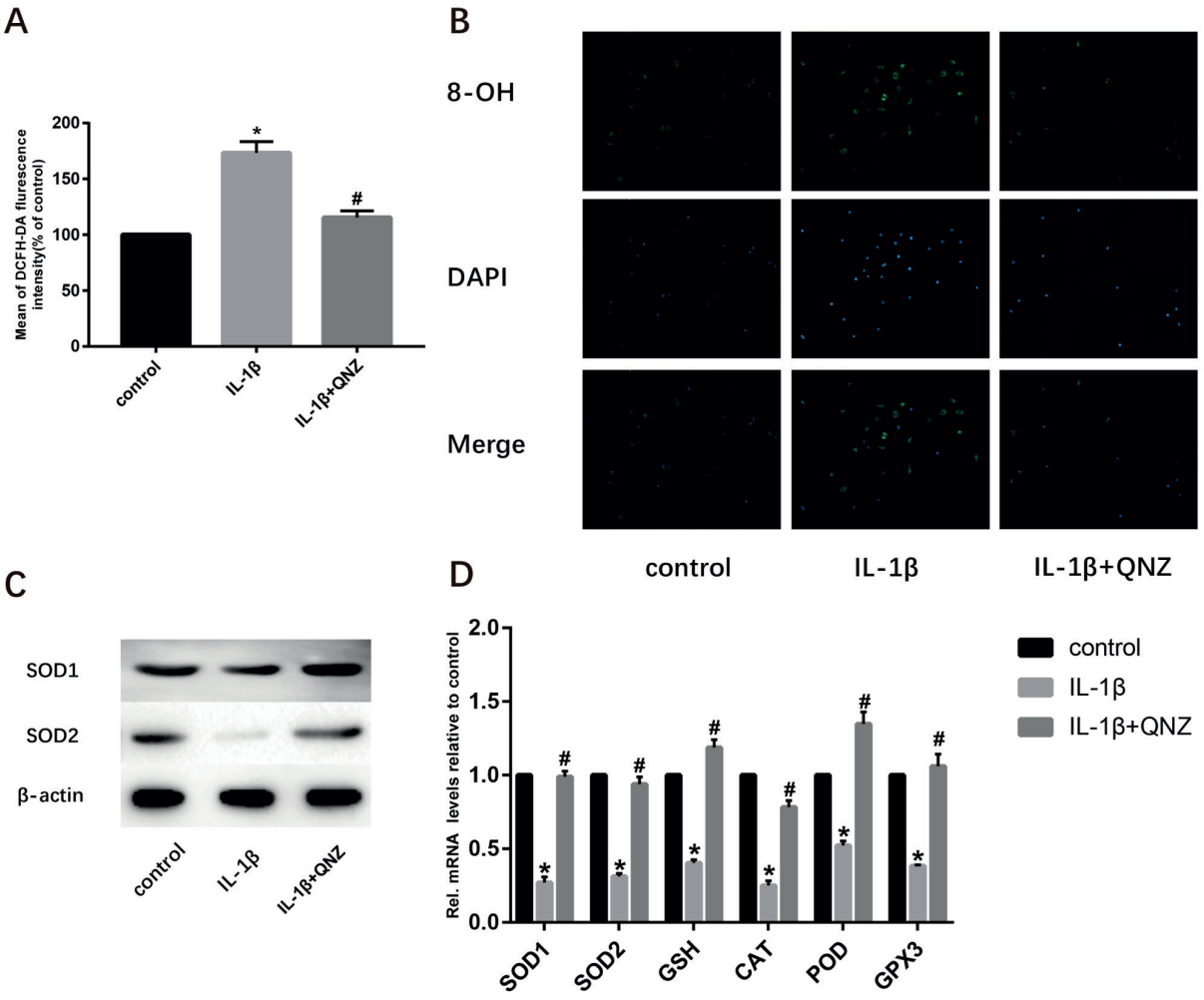

Figure 3. Effect of QNZ in anti-oxidation in NP cells. **A,** Intracellular ROS level is detected by flow cytometry. **B,** Expression of 8-OH is determined by immunofluorescence (magnification: 100×). **C,** Protein expressions of SOD1 and SOD2 are determined by Western blot. D, MRNA expressions of SOD1, SOD2, GSH, CAT, POD and GPX3 are determined by RT-PCR. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

ti-senescent effects and the protection of the extracellular matrix of NP cells.

Effect of QNZ in Anti-Oxidation in NP Cells

Oxidative stress has been reported to be involved in plenty of field of IVDD progress, which contains cell senescence, apoptosis, inflammation, and so on. However, the interaction between QNZ and oxidative stress during the IVDD is not well understood. To analyze the oxidative stress level, flow cytometry was used to measure the intracellular ROS level. The results showed that IL-1β increased the total ROS level of NP cells compared with the control group, hopefully, QNZ reduced it compared with the IL-1β group (Figure 3A). 8-hydroxy 2'-deoxyguanosine (8-OHdG), a base for the construction of DNA, is seen as oxidative DNA damage-maker. 8-OHdG positive NP cells were found to be quite much in degenerated NP cells and significantly decreased in the condition of QNZ treatment (Figure 3B). Apart from this, several anti-oxidative enzymes were also analyzed. The results showed that SOD1 and SOD2 protein expressed higher undergoing the QNZ treatment compared with IL-1β group (Figure 3C). Meantime, the RNA levels of SOD1, SOD2, GSH, CAT, POD, and GPX3 were all upregulated after QNZ treatment compared with only IL-1β stimulation (Figure 3D). For this part, it was found that QNZ enhanced the expression of anti-oxidative enzymes, so as to decrease the intra-

cellular ROS level of NP cells, which might be a novel direction for the study of ROS reduction.

QNZ Suppresses Inflammation by Inhibiting NF-κB/MAPKs Pathway

The activation of NF-κB/MAPKs is known to participate in the production of pro-inflammatory cytokines in various cells[11,17]. Meanwhile, QNZ is reported to exert potent anti-inflammatory function in smoke extract-induced COPD[18]. Therefore, the interventional effects of QNZ on NF-κB/MAPKs signaling pathway of human NP cells were explored. It was found that IL-1β markedly activated the NF-κB/MAPKs signaling pathway by upregulating the phosphorylation of ERK, p38, IKKβ, IκBα, and p65. However, the pretreatment with QNZ significantly decreased the phosphorylation levels of these kinases (Figure 4A). Besides, several relative inflammatory factors were also analyzed by RT-qPCR, and the results detected IL-1β, IL-6, TNF-α, MMP3, MMP9, MMP10, and MMP13 were completely downregulated by the stimulation of QNZ compared with the IL-1β group (Figure 4B). It came to the same consequence with the results of ELISA of IL-6 and TNF-α expression (Figure 4C, 4D). These results suggested that NF-κB/MAPKs signaling pathway might be the targets for the anti-inflammatory effect of QNZ.

Discussion

IVDD is characterized as the loss of water content and ECM, which represents an imbalance between anabolic and catabolic processes[19,20]. Disc

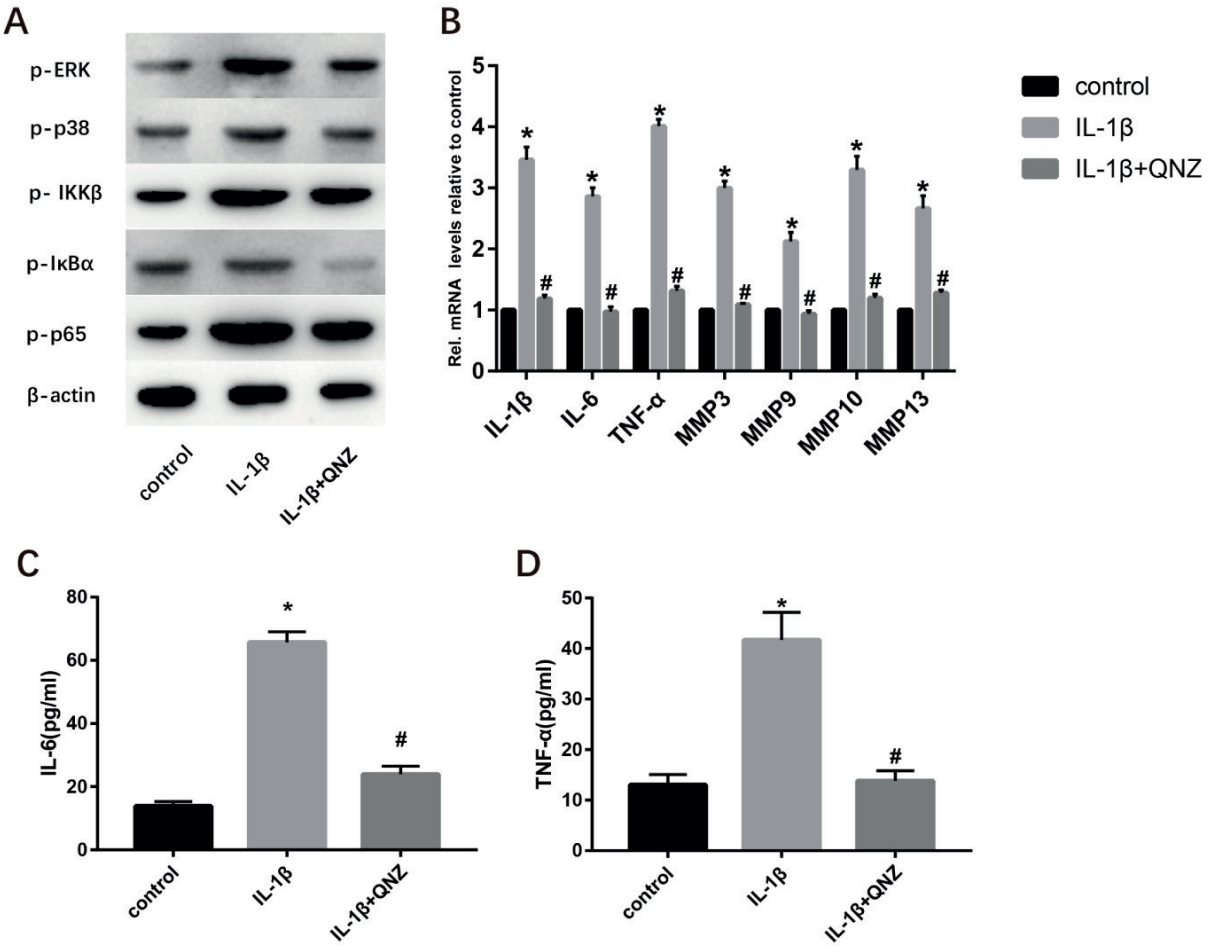

Figure 4. QNZ suppresses inflammation by inhibiting NF-κB/MAPKs pathway. ***A,*** Protein expressions of phosphorylation of ERK, p38, IKKβ, IκBα, and p65 are determined by Western blot. B, MRNA expressions of IL-1β, IL-6, TNF-α, MMP3, MMP9, MMP10, and MMP13 are determined by RT-PCR. ***C,*** and ***D,*** ELISA assay. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

degeneration is the main cause of spine diseases, and the pathophysiological process of IVDD is complicated. Aging coordinating with age-related stresses promotes NP senescence and accelerates IVDD[21]. Both acute and accumulated mechanical loading can induce disc degradation[22]. Infection and calcification in the endplates or sometimes in the disc are normal to be observed in the intervertebral disc diseases[23,24]. In addition to this, ROS and inflammatory reaction are also common in the NP cell during degenerative disc[25,26]. Current drug therapy of IVDD is hard to be achieved, therefore, new strategies are required to get involved in disease progression. The signaling pathways that regulate inflammation or other parameters are numerous, among which the activation of NF-κB/MAPKs pathway is involved in many key aspects of the inflammatory response triggering the production of many pro-inflammatory cytokines and inflammation-related genes[27,28]. In the present study, the protective effect of QNZ of human NP cells was explored and its underlying mechanisms of action were investigated. It was found that the fibrosis maker collagen I, senescent makers p16, p53, and β-gal were increased, but benefit content was decreased in the NP cells degeneration model caused by IL-1β. After treatment of QNZ, these degenerated makers associated with NP cells were significantly reduced compared with IL-β group. Besides, QNZ contributes to the promotion of NP cells proliferation. It is true that QNZ plays a positive effect on the degenerated NP cells, which indicates that it is might also good for the progress of IVDD.

Based on the previous findings in the literature, oxidative stress and inflammation are the key links in the progression of IVDD. QNZ has potent radicals scavenging properties which may mediate its beneficial physiological effects[29]. The excessive exist of ROS can induce oxidative stress and loss of cell vitality. This study revealed that QNZ significantly decreased ROS levels in the right dose. 8-OHdG is an oxidant of deoxyguanosine, a base for the construction of DNA. The interaction of oxygen radical with the nucleobases of the DNA strand, such as guanine, leads to its formation[30]. So, 8-OHdG was selected as an indicator of oxidative damage. The immunofluorescence of 8-OHdG positive cells was markedly decreased after QNZ treatment compared with degenerated group. These effects would be related to the upregulation of antioxidant enzymes, including SOD1, SOD2, GSH, CAT, POD, and GPX3. Reduction of collagen II and aggrecan, the major components of ECM in IVD, have been observed in the progress of disc degeneration. These changes are associated with the increased expression of matrix-degrading enzymes such as MMP3, MMP9, MMP13, and proinflammatory cytokines, such as IL-1β, IL-6, and TNFα[31,32]. The results of this study showed that QNZ stimulation suppressed the MMPs, IL-1β, IL-6, and TNFα expressions, as well as NF-κB activation in degenerated NP cells. It has been proved that p-ERK, p-p38MAPK, and p-JNK participate in NF-κB activation which regulates the gene expression of proinflammatory cytokines[33]. Besides, this work also showed that QNZ significantly inhibited MAPKs pathway activation which might be a part of its protection for NP cells.

QNZ is an organic compound whose derivatives, including drugs, also have protective effects on IVDD, e.g., Pan et al[34] found Gefitinib protected IVDD in rats. To our knowledge, this is the first report to uncover the antioxidant and anti-inflammatory effect of QNZ on IVDD *via* the NF-κB/MAPKs signaling pathway. These data suggest that QNZ could be a useful drug to slow down the progression of IVDD.

Conclusions

These results indicated that QNZ prevented NP degradation *via* restraining oxidative stress and inflammation through the inhibition of the NF-κB/MAPKs signaling pathway. QNZ may become a novel insight into the therapy of IVDD in the future.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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