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Original Paper

Aquaporin-3 Attenuates Oxidative Stress-Induced Nucleus Pulposus Cell Apoptosis Through Regulating the P38 MAPK Pathway

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Key Words
Nucleus pulposus • Apoptosis • Aquaporin-3 • Oxidative damage

Abstract
Background/Aims: Previous studies have shown that oxidative damage is a main contributor to disc nucleus pulposus (NP) cell apoptosis. Aquaporin-3 (AQP-3) facilitates reactive oxygen species (ROS) scavenging and thus alleviates oxidative injury in other cells. This study aims to investigate the role and mechanism of AQP-3 in regulating NP cell apoptosis under oxidative damage. ***Methods:*** Rat NP cells were treated with H_2O_2 for 48 hours, while control NP cells were free of H_2O_2. Recombinant AQP-3 lentiviral vectors were used to investigate the effect of enhanced AQP-3 expression levels in NP cells. NP cell apoptosis was assessed by flow cytometry, caspase-3 activity, gene expression of apoptosis-related molecules (Bax, Bcl-2 and caspase-3), and protein expression of cellular apoptosis markers (cleaved PARP and cleaved caspase-3). Additionally, intracellular ROS content and activity of the p38 MAPK pathway were evaluated. ***Results:*** Compared with the control NP cells, oxidative damage in the treatment cells significantly increased cell apoptosis ratios and caspase-3 activity, upregulated gene expression of Bax and caspase-3, downregulated gene expression of Bcl-2, and increased protein expression of cleaved PARP and cleaved caspase-3, as well as increased intracellular ROS content and activity of the p38 MAPK pathway. However, AQP-3 overexpression partly alleviated cell apoptosis, decreased intracellular ROS content, and inhibited the p38 MAPK pathway in NP cells under oxidative damage. ***Conclusion:*** Oxidative damage can significantly downregulate AQP-3 expression. Enhancing AQP-3 expression in NP cells partly attenuates cellular apoptosis through regulating the p38 MAPK pathway under oxidative damage.

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Introduction

The intervertebral disc (IVD) is a specialized organ that contributes to normal spinal biomechanical function. Disc degeneration is one of the major causes of lower back and leg pain, which can seriously affect life quality and contribute considerably to socioeconomic burden [1, 2]. It has been reported that approximately 80% of adults experience disc degeneration at least once in their lifetime [3]. Currently, the exact pathogenesis of disc degeneration remains unclear.

The central nucleus pulposus (NP) of adult discs contains many chondrocyte-like NP cells, which synthesize NP matrix proteins and thus maintain the normal mechanical properties of an individual disc [4]. During disc degeneration, NP cell apoptosis is a common cellular feature, and apoptotic NP cells increase in number as disc degeneration advances [5]. Since NP cells are the sole cells to generate and repair intervertebral disc extracellular matrix after maturation, a decrease in disc NP cells is theoretically correlated with a decrease in extracellular matrix (ECM) production within the NP region [6, 7]. Hence, inhibition or attenuation of NP cell apoptosis is a feasible strategy to retard and/or reverse disc degeneration.

According to previous research, the significant structural failure of degenerative discs partly results from oxidative stress, which is initiated by the excessive accumulation of reactive oxygen species (ROS) [8]. Several studies have identified some oxidative stress markers in degenerative human discs, such as carboxymethyl-lysine, advanced glycation end products (AGEs), and peroxynitrite [9-11]. ROS have been regarded as potential pro-apoptotic factors for NP cells *in vitro* [38, 40, 41, 44]. ROS accumulation has been shown to induce NP cell apoptosis through the mitochondrial apoptosis pathway and the death receptor pathway [12-14]. Additionally, many external stimuli can cause intracellular ROS accumulation, such as mechanical overloading, nutrition deprivation, and inflammatory cytokines [15, 16]. The findings above indicate that oxidative stress-induced NP cell apoptosis may play an important role in disc degeneration.

Aquaporins (AQPs) belong to a kind of small, integral membrane protein family which controls cell permeability to water and other small molecules [17]. AQP-3 is one important member of the AQP family. A previous study has identified the presence of AQP-3 in disc tissue and reported that AQP-3 expression in disc NP cells and annulus fibrosus (AF) cells of aged rats is significantly decreased compared with that of young rats [18]. Similarly, it has also been reported that AQP-3 expression in degenerative human disc tissue is reduced compared with normal human disc tissue [19]. Importantly, AQP-3 has been shown to participate in facilitating H_2O_2 diffusion [20, 21]. Recently, the diffusion of H_2O_2 across the plasma membrane to the extracellular fluid has also been regarded as a potential ROS elimination pathway [22, 23]. Hence, it can be deduced that AQP-3 may be helpful in the attenuation of oxidative damage-induced cellular injury.

In the present study, our main aim was to investigate AQP-3 expression under oxidative damage *in vitro* and further investigate the role of AQP-3 in regulating NP cell apoptosis under oxidative damage. NP cell apoptosis was evaluated by an Annexin V-FITC staining method, caspase-3 activity, and expression of apoptosis-related proteins.

Materials and Methods

NP cell isolation and culture

Thirty-seven healthy Sprague Dawley rats were used in this study. These rats were reared at standard conditions in a temperature-controlled room (23-25° C). All experimental protocols were in accordance with the relevant guidelines [SYCT (YUE) 2011-0217] of the Ethics Committee at the Third Affiliated Hospital of Sun Yat-sen University. Briefly, the rats' lumbar spinal column (L2-L6) was separated, and individual lumbar discs were obtained. NP cells from the inner most NP region were collected using a method previously described [24]. The collected NP cells and partially digested NP tissues were cultured in DMEM/F12

medium containing 10% fetal bovine serum (FBS, Gibco, USA) under standard conditions (37°C, 20% O_2 and 5% CO_2). After NP cells reached 70%-80% confluence, they were digested using 0.25% trypsin solution (Gibco, USA) and subcultured in appropriate culture bottles. Passage-two NP cells were used throughout all experiments in this study. H_2O_2 (100 μg/mL) was used to induce oxidative damage in accordance with previous studies [25]. After all NP cells were treated with H_2O_2 for 48 hours, they were used in the following assays. NP cells untreated with H_2O_2 were used as controls.

Cell transfection

NP cells were seeded at 4×10^3 cells/well in a 24-well plate. After cell adherence, 20 μL of recombinant lentiviral vectors LV5-AQP-3 (Genepharma, Shanghai, China) for 36 hours to allow overexpression of AQP-3 (NP-AQP-3). NP cells transfected with empty lentiviral vectors were used as controls (NP-AQP-3-NC). Once successful transfection was observed through fluorescence induced by uptake of the vector, the cell medium was refreshed for 2 days with 1 mL of complete culture medium containing puromycin (1 μg/mL) to eliminate nontransfected NP cells. Finally, the transfected NP cells were subcultured once for amplification and then used for subsequent experiments. The transfection efficacy was verified by real-time PCR and fluorescence observation.

Cell apoptosis ratio detection

NP cell apoptosis was evaluated using an Annexin V-FITC staining method according to the manufacturer's instructions (Beyotime, China). After NP cells were cultured, the culture medium was collected, and NP cells were washed with phosphate buffered solution (PBS) 3 times. Then, the adhered NP cells were collected by digestion with 0.25% trypsin without ethylene diamine tetraacetic acid (EDTA). The collected culture medium and the NP cells were then subjected to centrifugation (1000 g, 5 min). Subsequently, 1×10^5 cells in each group were incubated with 195 μL Annexin V-FITC binding buffer, 5 μL Annexin V-FITC solution, and 10 μL propidium iodide (PI) with agitation. Then, the cells were stained for 20 minutes at room temperature in a dark room. Finally, they were subjected to a flow cytometry assay (FACS Aria; BD Company). Apoptotic NP cells were identified as Annexin V positive-stained and PI-negative-stained cells, as well as double positive-stained cells.

Caspase-3 activity analysis

After NP cells were cultured for 48 hours, caspase-3 activity was analyzed using a caspase-3 activity detection kit (Beyotime, China). Specifically, the culture medium and the adhered NP cells were collected as described above. After NP cells were pelleted by centrifugation (1000 g, 5 min), 1×10^5 NP cells from each experimental group were lysed using RIPA lysis solution (Beyotime, China). Then, 40 μL reaction buffer, 50 μL lysate, and 10 μL Ac-DEVD-pNA were mixed and incubated for 8 hours at 37°C. Finally, the optical density (OD) of the lysate at a wavelength of 405 nm was measured using a multimode reader. The caspase-3 activity, normalized to the total protein concentration, was calculated from a standard curve using pNA as the standard.

Intracellular ROS measurement

Briefly, once NP cells were incubated for 48 hours with conditioned medium, they were then incubated with DCFH-DA (10 μM) for 30 minutes. The uncombined DCFH-DA was removed by washing with FBS. Finally, NP cells (1×10^5 cells in each group) were used to measure the ROS content using a reactive oxygen species assay kit (Nanjing Jiancheng Bioengineering Institute, China). ROS content is expressed as relative fluorescence units (RFU) detected at an excitation/emission wavelength of 490/585 nm.

Real-time polymerase chain reaction (PCR) analysis

At the end of culturing, total RNA was extracted from NP cells using TRIzol reagent (Invitrogen Life Technologies, USA). Then, 1 μg of total RNA was reverse transcribed to make cDNA using a TIANScript II RT kit (TIANGE BIOTECH CO., LTD, China). cDNA, specific gene primers, and SYBR Green Mix (TOYOBO, Japan) were added together and real-time PCR (Thermo, USA) was performed. The cycling parameters were: 3 min at 95°C, followed by 30 amplification cycles of 10 seconds at 95°C, 10 seconds at 57°C and 15 seconds at 72°C. The specific primers for target genes are shown in the Table 1. Gene expression was normalized to β-actin and was calculated with the $2^{-\triangle\triangle Ct}$ method.

Table 1. Primers of target genes

Gene	Forward (5'-3')	Reverse (5'-3')	Amplicon size
β-actin	CCGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC	116bp
AQP-3	TGGGCCTTGTGGTCCTGGTCA	GGGGTGGATACAGGGAGCGT	105bp
Bcl-2	GGGGCTACGAGTGGGATACT	GACGGTAGCGACGAGAGAAG	123bp
Bax	GGCGAATTGGCGATGAACTG	CCCAGTTGAAGTTGCCGTCT	98bp
Caspase-3	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCAGGGT	134bp

Western blot analysis

Once cultured, NP cells were washed with ice-cold PBS for 3 times, and cell lysate was collected using RIPA lysis buffer (Beyotime, China). Total protein was collected by centrifugation at 15, 000 g for 20 minutes at 4°C, and protein concentration was determined using a BCA Protein Assay Kit (Beyotime, China). Then, equal concentrations of cell proteins were resolved on 8%-12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad, CA). After PVDF membranes were blocked with 5% bovine serum albumin (BSA), they were incubated with primary antibodies (β-actin: Abcam, ab8226; cleaved PARP: Cell Signaling Technology, #94885; cleaved caspase-3: Cell Signaling Technology, #9661; AQP-3:Abcam, ab125219; p38 MAPK: Cell Signaling Technology, #8690; p-p38 MAPK: Cell Signaling Technology, #4511. All diluted 1:1000) for 18-24 hours at 4°C, followed by incubation with HRP-conjugated secondary antibodies (Diluted at 1:4000, Beyotime, China) for 2 hours at room temperature. Immunolabeling was developed using a SuperSignal West Pico Trial Kit (Thermo, USA). The intensity of protein bands was analyzed using ImageJ software (National Institutes of Health, USA).

Statistical analysis

All assays were performed in triplicate. The numerical data are presented as the mean ± SD and analyzed using SPSS 17.0 software. After the homogeneity test for variance, differences between two groups were analyzed by Student's T-test. However, differences between three groups were assessed by one-way analysis of variance (ANOVA), and an LSD post hoc test was performed. p-value < 0.05 indicated statistical significance.

Results

Oxidative damage increased intracellular ROS and downregulated AQP-3 expression of NP cells

Because intracellular ROS can be scavenged by diffusion through the integral membrane protein AQP-3 in other cells [10, 23], we evaluated intracellular ROS content and AQP-3 expression under oxidative damage in NP cells. The results showed that intracellular ROS content was significantly increased compared with control NP cells (Fig. 1A) and that AQP-3 expression in the H_2O_2-treated NP cells was significantly decreased compared with the control NP cells at both the gene (Fig. 1B) and protein levels.

Oxidative damage promoted NP cell apoptosis

NP cell apoptosis ratios, caspase-3 activity, and expression of apoptosis-related molecules (Bax, caspase-3 and Bcl-2) were used to evaluate NP cell apoptosis. The results showed that oxidative damage significantly increased the number of apoptotic NP cells (Fig. 2A), enhanced caspase-3 activity (Fig. 2B), upregulated gene expression of pro-apoptotic molecules (Bax and caspase-3) and downregulated gene expression of an anti-apoptotic molecule (Bcl-2) (Fig. 2C). Similarly, protein expression of cellular apoptosis-related markers (cleaved caspase-3 and cleaved PARP) was also upregulated under oxidative damage (Fig. 2D).

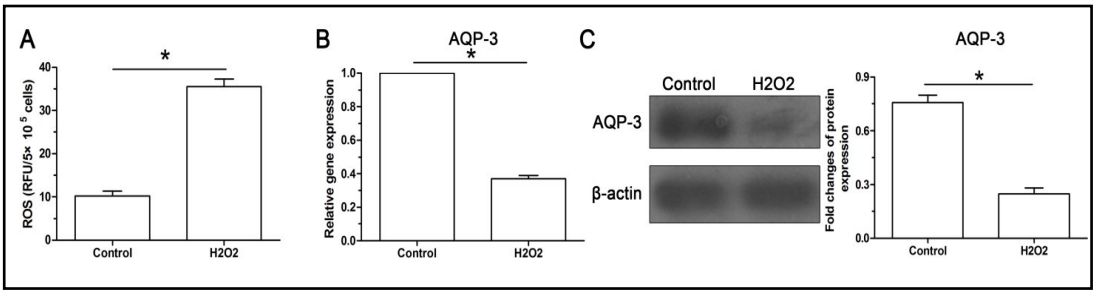

Fig. 1. Oxidative damage increased intracellular ROS and downregulated AQP-3 expression in nucleus pulposus (NP) cells. A: Reactive oxygen species (ROS) content measurement, which was indicated as relative fluorescence units (RFU). B: Gene expression of AQP-3. C: Protein expression of AQP-3. Data are shown as the mean ± SD (n=3). *: Indicates a significant difference ($p<0.05$) between two groups.

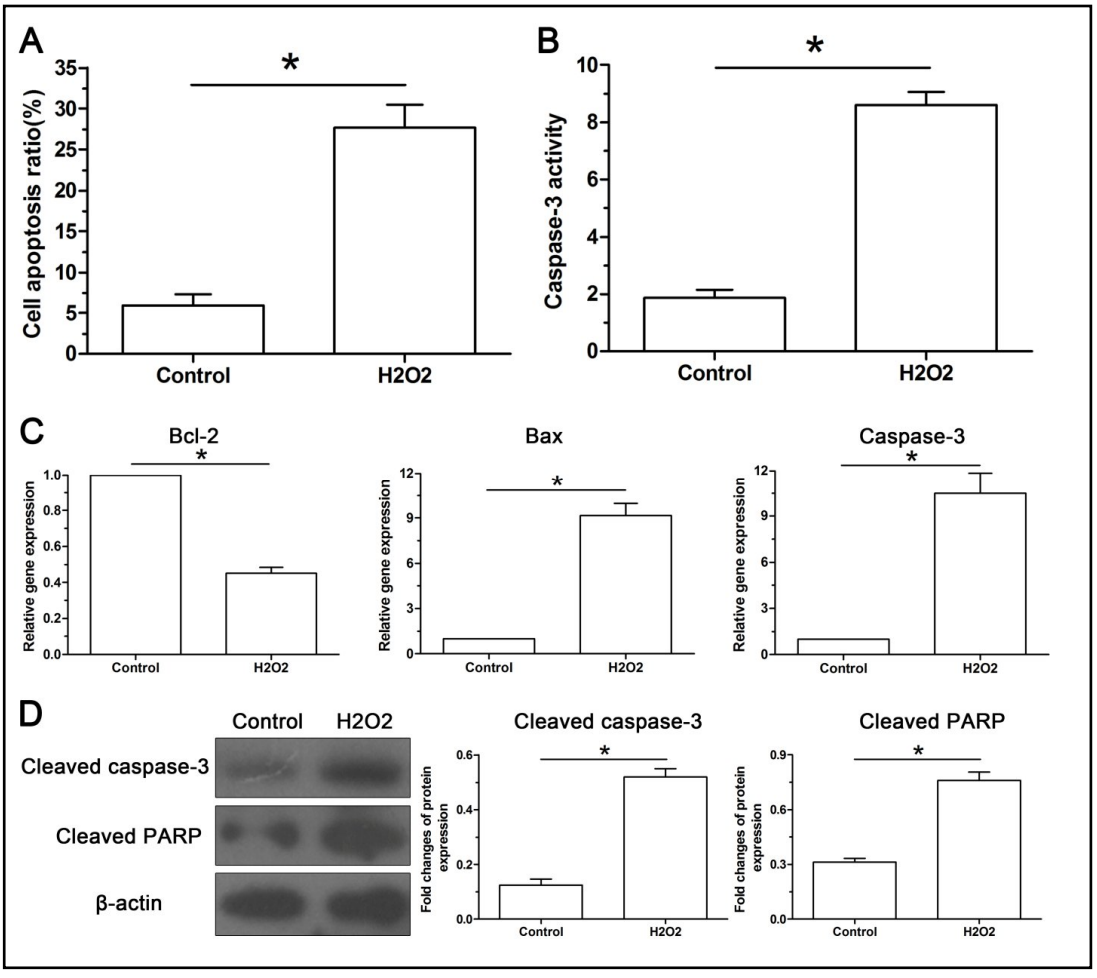

Fig. 2. Oxidative damage promoted nucleus pulposus (NP) cell apoptosis. A: NP cell apoptosis ratio evaluated by flow cytometry assay. B: Caspase-3 activity measurement. C: Gene expression of apoptosis-related molecules (Bcl-2, Bax and caspase-3). D: Protein expression of apoptosis-associated markers (cleaved caspase-3 and cleaved PARP). Data are shown as the mean ± SD (n=3). *: Indicates a significant difference ($p<0.05$) between two groups.

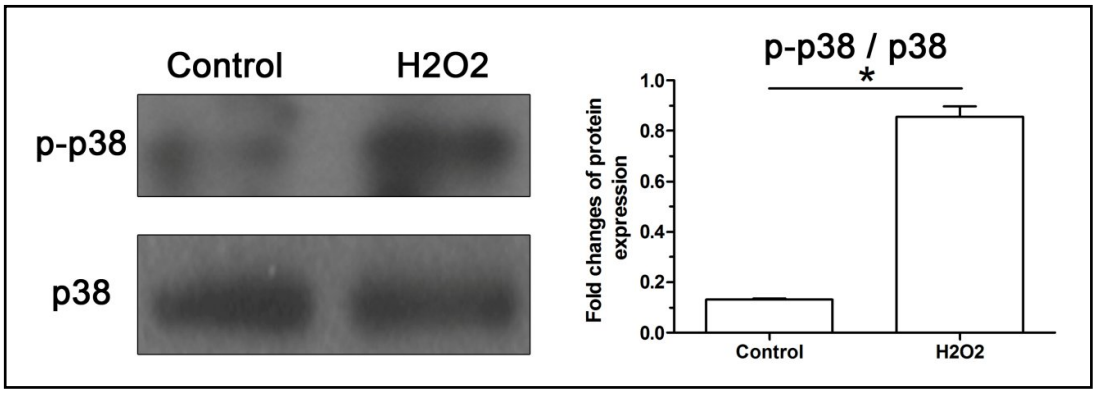

Fig. 3. Oxidative damage increased activity of the p38 MAPK pathway in nucleus pulposus (NP) cells. The activity of the p38 MAPK pathway was reflected by the protein expression of p-p38 MAPK. Data are shown as the mean ± SD (n=3). *: Indicates a significant difference (p<0.05) between two groups.

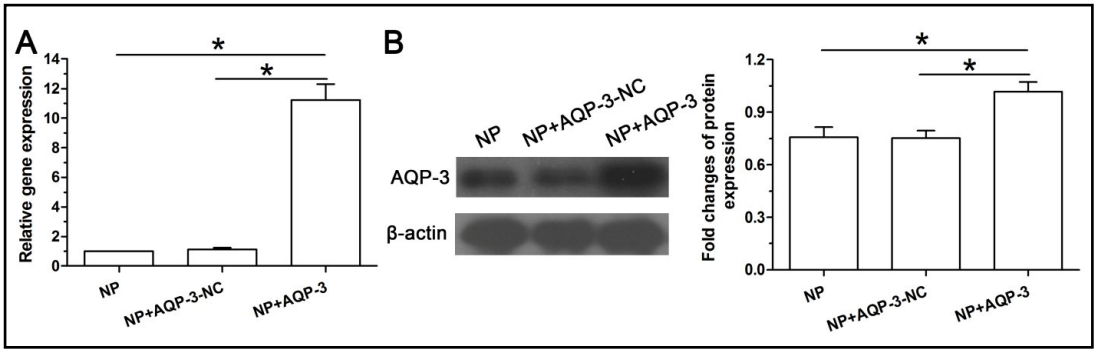

Fig. 4. Verification of AQP-3 overexpression in nucleus pulposus (NP) cells. A: Verification of the gene expression of AQP-3. B: Verification of the protein expression of AQP-3. Data are shown as the mean ± SD (n=3). *: Indicates a significant difference (p<0.05) between two groups.

Oxidative damage increased activity of the p38 MAPK pathway

Mitogen-activated protein kinases (MAPKs) are important in regulating cellular biology [26]. Previous studies have demonstrated that the p38 MAPK pathway can be activated under oxidative damage [27] and inhibition of the p38 MAPK pathway has the potential to protect against disc degeneration [28]. Here, our results confirmed that activity of the p38 MAPK pathway was significantly increased by oxidative damage (Fig. 3).

AQP-3 overexpression decreased intracellular ROS content of NP cells under oxidative damage

From the above results, we found a negative relationship between AQP-3 expression and NP cell apoptosis under oxidative damage. To fully verify the effects of AQP-3 on NP cell apoptosis under oxidative damage, we enhanced AQP-3 expression

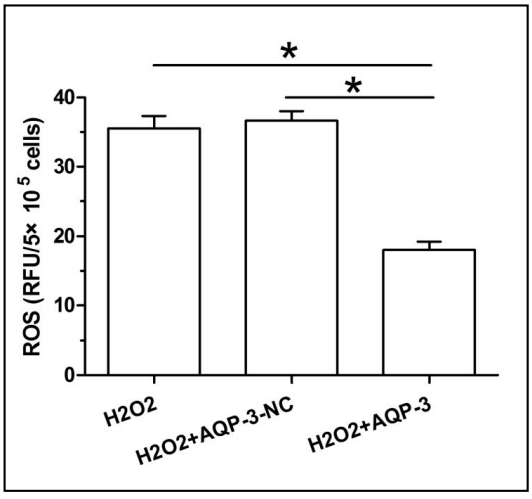

Fig. 5. AQP-3 overexpression decreased intracellular reactive oxygen species (ROS) content of nucleus pulposus (NP) cells under oxidative damage. Data are shown as the mean ± SD (n=3). *: Indicates a significant difference (p<0.05) between two groups.

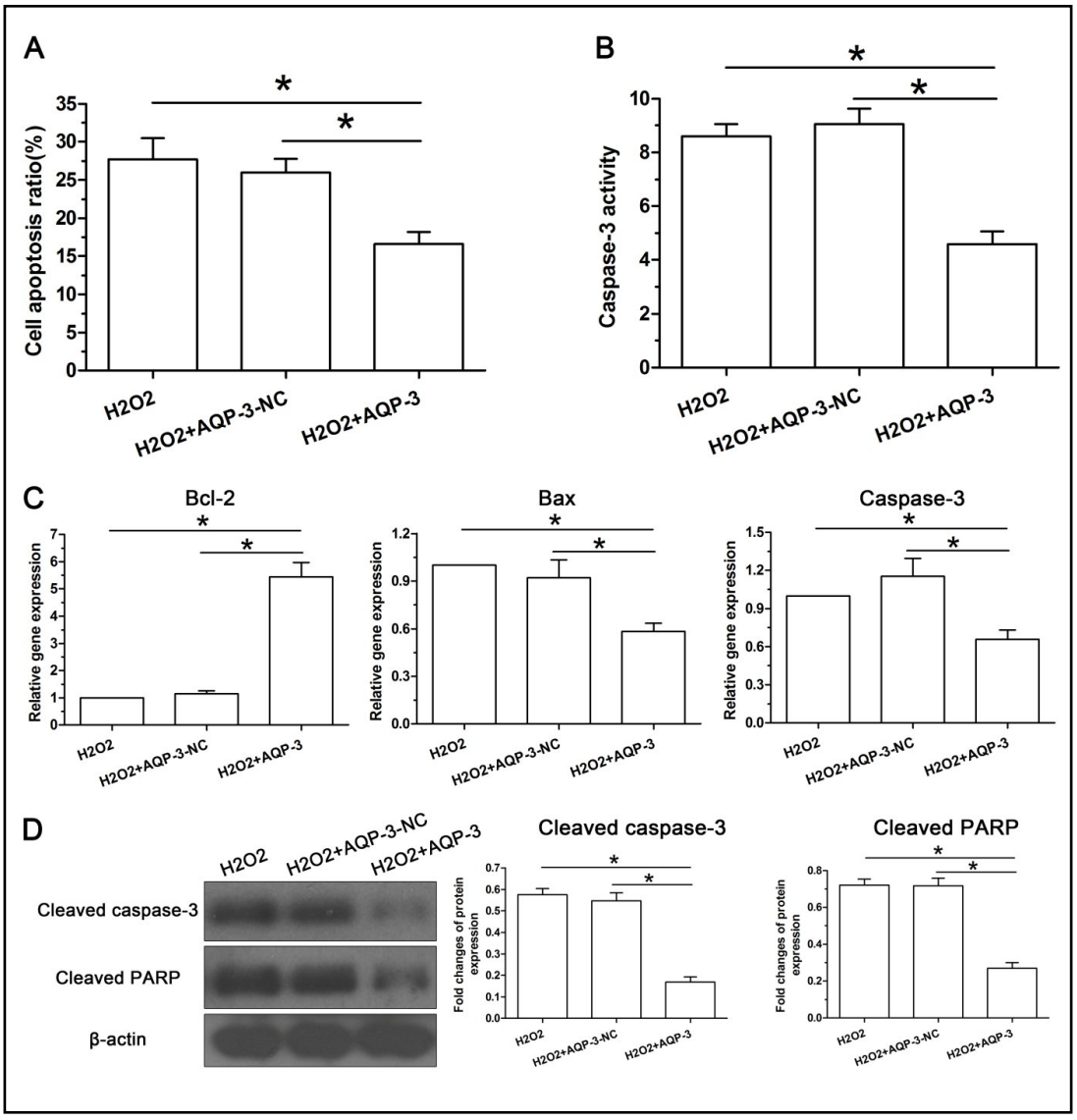

Fig. 6. AQP-3 overexpression attenuated nucleus pulposus (NP) cell apoptosis under oxidative damage. A: NP cell apoptosis ratios evaluated by flow cytometry assay. B: Caspase-3 activity measurements. C: Gene expression of apoptosis-related molecules (Bcl-2, Bax and caspase-3). D: Protein expression of apoptosis-associated markers (cleaved caspase-3 and cleaved PARP). Data are shown as the mean ± SD (n=3). *: Indicates a significant difference ($p<0.05$) between two groups.

using recombinant lentiviral vectors (Fig. 4). The results showed that the intracellular ROS content was partly decreased after enhancing AQP-3 expression in NP cells under oxidative damage (Fig. 5).

AQP-3 overexpression attenuated NP cell apoptosis under oxidative damage

After enhancing AQP-3 expression in NP cells under oxidative damage, we found that the apoptotic NP cells (Fig. 6A) and caspase-3 activity (Fig. 6B) were decreased. Moreover, PCR results showed that gene expression of pro-apoptotic molecules (Bax and caspase-3) was downregulated, whereas an anti-apoptotic molecule (Bcl-2) was upregulated (Fig. 6C) in the AQP-3 overexpressed NP cells. In addition, protein expression of the apoptosis-related markers (cleaved caspase-3 and cleaved PARP) was decreased after enhancing AQP-3 expression (Fig. 6D).

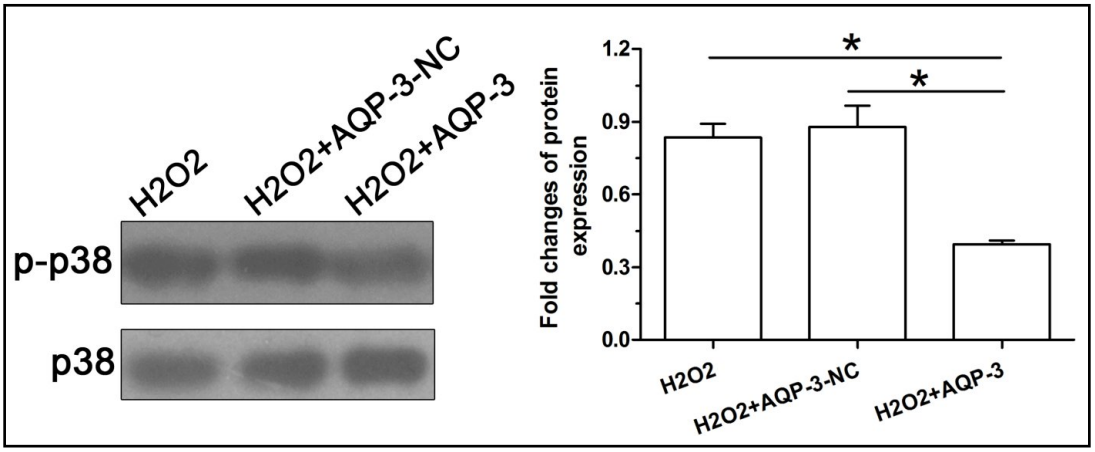

Fig. 7. AQP-3 overexpression decreased the activity of the p38 MAPK pathway in nucleus pulposus (NP) cells under oxidative damage. The activity of the p38 MAPK pathway was reflected by the protein expression of p-p38 MAPK. Data are shown as the mean ± SD (n=3). *: Indicates a significant difference ($p<0.05$) between two groups.

AQP-3 overexpression decreased activity of the p38 MAPK pathway under oxidative damage

After AQP-3 overexpression, we found that activity of the p38 MAPK pathway in the NP-AQP-3 cells was significantly decreased compared with the control NP cells or NP-AQP-3-NC cells under oxidative damage (Fig. 7).

Discussion

Disc degeneration is an important cause of disability [1]. Currently, no therapies have been developed to reverse and/or attenuate disc degeneration. Oxidative stress has been shown to be involved in disc degeneration and is responsible for disc NP cell apoptosis during degeneration. However, the exact mechanism driving degeneration remains unclear. In this study, we report for the first time that oxidative damage decreases AQP-3 expression and that enhancing AQP-3 expression attenuates NP cell apoptosis under oxidative damage through the regulation of the p38 MAPK pathway.

Disc degeneration is also an aging-related disease. According to the free-radical theory of aging, oxidative stress initiated by ROS accumulation contributes to the functional decline that occurs during aging [8]. Generally, ROSs include the superoxide anion (O^{2-}), nitric oxide (NO), and hydroxyl radicals (OH), which are byproducts of cell oxidative metabolism [29]. Once the intracellular ROS accumulation has exceeded the cell's capacity to scavenge, it can lead to oxidative damage and concomitant cellular damage. Several musculoskeletal diseases, including osteoarthritis and osteoporosis, are related to oxidative stress [30, 31]. Oxidative stress also plays an important role in disc degenerative diseases as oxidative stress markers (i.e., carboxymethyl-lysine, AGEs and peroxynitrite) are significantly elevated in degenerative human discs [9-11]. Previously, several studies have demonstrated that oxidative damage induced by external stimuli promotes NP cell apoptosis [32-34]. In line with this, our present results confirm that oxidative damage significantly increases the NP cell apoptosis ratio.

AQPs are a type of channel protein that can transport water and other small substrates across the cell membrane. Currently, researchers have identified a total of thirteen AQP isoforms (AQP 0-12) with different permeability properties and cellular localizations [35]. Among these AQP isoforms, AQP-3 expression is decreased in degenerative disc cells [18]. Because AQP-3 also plays a vital role in establishing an H_2O_2 transport system, previous studies have demonstrated that AQP-3 is able to alleviate oxidative damage through

elimination of intracellular ROS-like substrates [36, 37]. In this study, we used H_2O_2 to induce an oxidative damage. We found that oxidative damage significantly downregulated AQP-3 expression. In light of the protective effects of AQP-3 in alleviating oxidative damage and the stimulatory effects of oxidative damage on NP cell apoptosis, we enhanced AQP-3 expression in NP cells under oxidative damage. Our results showed that AQP-3 overexpression partly decreased apoptotic NP cells, caused caspase-3 activity to decline, facilitated gene expression of pro-apoptotic molecules (Bax and caspase-3), and promoted protein expression of cellular apoptosis markers (cleaved PARP and cleaved caspase-3), indicating that AQP-3 overexpression attenuates NP cell apoptosis under oxidative damage.

In cultured disc cells, it is known that oxidative damage caused by H_2O_2 treatment activates some important signaling pathways to initiate cellular self-protection and/or the opposite, cellular destruction, such as the p38 MAPK, ERK1/2, c-Jun N-terminal kinase (JNK), PI3K/Akt, and nuclear translocation of nuclear factor (NF)-κB pathway [38]. Because the p38 MAPK pathway is commonly regarded as a disadvantageous signal for the normal, healthy bioactivities of disc cells [28], we focused our study on the activity of the p38 MAPK pathway. The results showed a negative relationship between AQP-3 expression and activity of the p38 MAPK pathway in NP cells under oxidative damage and showed that AQP-3 overexpression partly decreases the pathway's activity in NP cells of the same condition. Combined with the above findings, these results indicate that AQP-3 may attenuate oxidative damage-induced NP cell apoptosis through regulating activity of the p38 MAPK pathway.

Conclusion

In summary, this study investigated the role of AQP-3 in regulating NP cell apoptosis and its potential signaling transduction under oxidative damage. Our results showed that oxidative damage downregulates AQP-3 expression and that enhancing AQP-3 expression in NP cells attenuates cellular apoptosis under oxidative damage. This study sheds light on a new mechanism through which oxidative damage induces NP cell apoptosis and indicates AQP-3 as a potential target to attenuate oxidative stress-induced disc NP cell apoptosis.

Disclosure Statement

The authors report no conflicts of interest related to this work.

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