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Original Article

Hypo-osmolality activates Wnt/β-catenin mediated by AQP1 in nucleus pulposus cells degeneration

Xiao Zhang[1], Zheng Lv[2], Chengjian Wei[1,*]

[1] Jiangsu Province Hospital of Chinese Medicine, The Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, PR China
[2] Wuhan Hospital of Traditional Chinese Medicine, Wuhan, China

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Abstract

The goals of this study were to investigate whether Wnt/β-catenin signaling plays a role in hypo-osmolality-related degeneration of nucleus pulposus (NP) cells, and if so, to define the mechanism underlying AQP1 in this effect. Human NP cells were cultured under hypo-osmotic (300/350/400 mOsm) and iso-osmotic (450 mOsm) conditions. The cell viability, AQP1, the expression of Wnt/β-catenin signaling, collagen II/I, and MMP3/9 were evaluated. To determine the effects of the Wnt/β-catenin signaling, we used the inhibitor and the activator of Wnt during the hypo-osmotic culture of NP cells. We also examined whether the silencing and overexpressing of the AQP1 gene would affect the Wnt/β-catenin expression in NP cells. Hypo-osmolality caused NP cell degeneration and activated the Wnt/β-catenin signaling but suppressed the AQP1 level. Inhibiting the Wnt/β-catenin signaling alleviated the hypo-osmolality-induced NP cell degeneration. On the contrary, activating Wnt/β-catenin aggravated the NP cell degeneration under hypo-osmotic conditions, which did not affect AQP1 expression. AQP1-overexpressed NP cells exhibited decreased Wnt/β-catenin signaling and alleviated cell degeneration under the hypo-osmotic condition. Besides, AQP1 silencing accelerated NP cell degeneration and activated Wnt/β-catenin expression compared with untreated control. Hypo-osmolality promotes NP cell degeneration via activating Wnt/β-catenin signaling, which is suppressed by AQP1 expression. The upregulation of AQP1 suppressed the Wnt/β-catenin signaling and alleviated the hypo-osmolality induced by the NP cell degeneration.

Keywords: Nucleus pulposus cells degeneration, AQP1, Wnt, β-catenin, Hypo-osmolality

1. Introduction

Intervertebral disc degeneration (IDD) is the primary pathological basis leading to spinal degeneration-related diseases [1]. The pathological characteristics of IDD mainly include reducing the number of functional nucleus pulposus (NP) cells, changes in extracellular matrix (ECM) components, decrease in osmotic pressure, and invasion of inflammation [2]. Usually, the intervertebral disc tissue is subjected to substantial compressive stress by increasing the NP tissue's osmotic pressure and adjusting the hydration state of the ECM [3]. There are many negatively charged glycosaminoglycan side chains on the aggrecan molecules in the ECM of the NP tissue. These side chains can be combined with sodium ions, potassium ions, etc., to form a high level of osmotic pressure, enabling the NP tissue to maintain its adsorption force for water molecules under high stress [4]. Van Dijk et al. have shown that the intervertebral disc's osmotic pressure value ranges from 430 to 496 mOsm/kg · H_2O (1 mOsm/kg · $H_2O \approx 25.6667$ kPa) [5].

In the process of IDD, the decrease in osmotic pressure in the NP leads to a reduction in the ability to absorb water, which in turn causes a reduction in the power of the intervertebral disc to regulate biological stress and gradually leads to the loss of intervertebral height. Wuertz et al. cultured human and bovine NP cells under hypo-osmotic (300 mOsm), iso-osmotic (400 mOsm), and hyper-osmotic (500 mOsm) conditions. As the medium's osmotic pressure increased, the type II collagen expression of NP cells gradually increased, while the expression of type I collagen was inhibited. They pointed out that high osmotic pressure could promote the synthesis of ECM, and the decrease of osmotic pressure may be one of the critical factors to accelerate the process of IDD [6].

Aquaporin (AQP) is a specific membrane channel protein, which is the primary molecular basis for water transport across the membrane, and it is widely present in different cells [7,8]. When the extracellular osmotic pressure drops, the cells swell and deform, or even swell. Cell inhibits volume increase through other mechanisms to maintain a steady state, among which volume increase limitation is a possible mechanism [9,10]. Fischbarg et al. found that AQP1 knockout mice have reduced rapid cell volume changes [11]. Thus, when the hypo-osmolality oc-

* Corresponding author.
E-mail address: drwcjtcm@sina.com (C. Wei).
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curs, a cell can adjust its volume through AQP1 transport water. Studies have shown that intervertebral discs mainly express AQP1 and AQP3 [12,13]. AQP1 only transports water molecules, and AQP3 is a dual channel of water and glycerol. The reduction of AQP1 and AQP3 in NP cells will reduce the water content and the nutrient permeability, which in turn affects cell function leading to IDD [14,15]. Recently, AQP1 was mentioned to crosstalk with the wnt/β-catenin pathway [16]. Xie et al. reported AQP3 protected IDD via the inhibition of Wnt/β-catenin signaling [17]. However, the mechanism underlying hypo-osmolality-induced IDD and whether it is related to Wnt/β-catenin signaling and AQP1 are not fully elucidated.

Here, we used the hypo-osmotic environment to culture human NP cells to investigate: (i) the role of Wnt/β-catenin in the hypo-osmolality caused NP cells degeneration; (ii) how AQP1 and influences Wnt/β-catenin signaling and the resulting NP cells degeneration.

2. Materials and methods

2.1. NP cells isolation and cultured under controlled osmolality

NP cells were isolated from human intervertebral disc tissues donated by the patients who underwent spinal surgery. Human NP tissue collection was approved by the Ethics Committee of The Affiliated Hospital of Nanjing University of Chinese Medicine and followed the Helsinki declaration. Briefly, the fragments of NP tissues in the disc were treated with 0.1% collagenase II for 8 h. After digestion, the cells were cultured in complete DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) at 37°C in a 5% CO_2 environment. After 1 week, the primary NP cells were split and replanted for the follow-up experiments.

Human NP cells were cultured under different osmotic conditions for an additional time by extra supplements of NaCl as previously method [18]. In the standard culture medium used as a control, the medium's osmotic stress was prepared as 450 mOsm (5). In addition to that, the ranged hypo-osmotic culture medium was adjusted to 300, 350, and 400 mOsm. Meanwhile, Wnt agonist 1 (agonist, 1 μM) and Adavivint (20 nM, Selleck, Shanghai, China), respectively, were the activators and inhibitors of Wnt signaling used during the cell culture.

2.2. Immunofluorescence

NP cells were seeded in 12-well plates (1.5×10^4 cells/well) and treated as designed. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 2 % FBS, and incubated overnight at 4°C with antibodies against AQP1 (1:200 dilutions; Abcam, Cambridge, MA, USA) and β-catenin (1:400 dilutions; Abcam, Cambridge, MA, USA). The next day, the cells were washed and continuously incubated with the secondary antibodies Alexa Fluor 488 and 647 (1:200 dilutions; Invitrogen, Carlsbad, CA, USA). For nuclear staining, the cells were incubated with 10 μM DAPI (Beyotime, Shanghai, China) at 25°C for 1 h. The fluorescence intensity was observed under a fluorescence microscope and measured using the Image-Pro Plus software.

2.3. Real-time PCR analysis

We determined the relative gene expression via mRNA level on the iCycler analysis system (Biorad, Munich, Germany). Briefly, the total RNA of each sample was prepared and extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, the RNA was measured and transcribed into cDNA using a reverse transcription kit (Roche, Basel, Switzerland). We used the sequences of the published primers (NCBI Entrez search system) for real-time PCR (Table 1) using SYBR Green Master (TOYOBO, Osaka, Japan). GAPDH was chosen as the housekeeping gene for normalization according to the $2^{-\Delta\Delta Ct}$ method.

2.4. Cell viability assay

The cell viability under different osmotic conditions was determined by the CCK8 assay. NP cells were seeded into 96-well plates (5000 cells/well) and treated as designed. After treatments, the cells were incubated with CCK8 reagent (Beyotime, Shanghai, China) according to the manufacturer's instructions. The intensity of the CCK8 product was measured at 570 nm using a microplate reader (Labsystems Multiskan, Finland).

2.5. siRNA and plasmid transfection

We silenced the AQP1 gene expression in NP cells by siRNA transfection and overexpressed the AQP1 gene expression by plasmid transfection. NP cells were seeded into 12-well plates (1.5×10^4 cells/well) one day before transfection. The next day, cells were treated with siRNA or plasmid targeting AQP1 (Thermo Scientific, Waltham, MA, USA) with a concentration of 300 ng using Lipofectamine 2000 (Beyotime, Shanghai, China). We changed the medium with a fresh growth medium six hours later.

Table 1. Primer sequences for PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen II	CCTGGCAAAGATGGTGAGACAG	CCTGGTTTTCCACCTTCACCTG
Collagen I	CCTGGTGCTAAAGGAGAAAGAGG	ATCACCACGACTTCCAGCAGGA
Wnt1	TCCTCATGAACCTTCACAATAACG	TTGCACTCTTGGCGCATCTC
Wnt3a	TGTGAGGTGAAGACCTGCTG	AAAGTTGGGGGAGTTCTCGT
Wnt4	GAAACGTGCGAGAAGCTCAAAG	AAAGGACTGTGAGAAGGCTACG
Wnt5a	TGCCACTTGTATCAGGACCA	TGTCTCTCGGCTGCCTATTT
Wnt6	GTCGACTTTGGGGATGAGAA	AAAGCCCATGGCACTTACAC
Wnt10a	GTTCCTAGCTCAGGCAGGTG	AAGTCTGTGGAGGGGGAGAT
MMP3	CACTCACAGACCTGACTCGGTT	AAGCAGGATCACAGTTGGCTGG
MMP9	GCCACTACTGTGCCTTTGAGTC	CCCTCAGAGAATCGCCAGTACT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

The cells were allowed to recover overnight and used for the follow-up experiments.

2.6. Statistical Analysis

Data were analyzed by the Statistic Package for Social Science (SPSS) 20.0 software package (IBM Corp, Armonk, NY, USA) with at least three independent triplicate experiments. The result was expressed as the mean ± standard deviation (SD). One-way ANOVA was used to compare the differences between more than two groups. Student's t-tests were also performed to compare the differences between the two groups. A P-value of less than 0.05 was considered statistical significance.

3. Results

3.1. Hypo-osmolality induces NP cells degeneration

Due to the normal osmolality in the intervertebral disc ranges from 430 to 496 mOsm [5], we prepared four different osmolality from 450 to 300 mOsm to explore how hypo-osmolality affects NP cells' fate. During 48 hours' culture, 400 and 450 mOsm conditions did not significantly affect the cell viability of NP cells. In contrast, the 350 mOsm environment reduced the cell viability 24 hours after culture, which further aggravated the injury 48 hours later. The most hypo-osmotic medium affected the cell viability most obviously, which began 12 hours after culture and gradually became severe until 48 hours later. Additionally, the cell viability was much injured in the condition of 300 mOsm compared to the same point-in-time of 350 mOsm (Figure 1A). As the unique marker of NP cells, the content of the type II collagen (collagen II) indicates the cell status, which decreases along with degeneration and is replaced by collagen I (collagen I) [19]. Moreover, the 350 mOsm conditions significantly reduced the collagen II gene expression after 48 hours' culture, and the collagen I level gradually increased after 12 hours. The medium of 300 mOsm disordered the collagen distribution acutely and fiercely. The reduction of collagen II started from 24 hours' culture, and the increase of collagen I was much higher than 350 mOsm at the same time point (Figure 1B, C). Thus, the cell viability was affected when the osmolality goes Hypo-osmotic with a disruption of the collagen synthesis. Since the 24 hours' 300 mOsm culture was sufficient to cause the degeneration of NP cells, we used this method to establish the cell degeneration model in the follow-up experiments.

3.2. Hypo-osmolality induces Wnt/β-catenin signaling activation

To determine whether Wnt/β-catenin signaling involves hypo-osmolality-induced NP cells degeneration, we suppled the activator (agonist [20]) or the inhibitor (Adavivint [21]) of Wnt/β-catenin signaling in the medium of 300 mOsm. The NP cells were divided into four groups: control (cultured in 450 mOsm for 24 hours with no intervention); 300 mOsm (cultured in 300 mOsm for 24 hours); 300 mOsm + Adavivint (cultured in 300 mOsm for 24 hours supplied with Adavivint); 300 mOsm + agonist (cultured in 300 mOsm for 24 hours supplied with agonist). As mentioned above, 24 hours' hypo-osmolality culture injured the cell viability of NP cells. However, the suppression of the Wnt/β-catenin signaling improved the cell viability under 300 mOsm conditions. In contrast, activating Wnt/β-catenin signaling aggravated the damage to cell viability (Figure 2A). As shown in Figures 2B and C, the AQP1 protein expression was significantly decreased in the hypo-osmotic condition, while the β-catenin expression was highly increased. Compared to the 300 mOsm group, the Adavivint suppressed the β-catenin expression, and the agonist further upregulated the β-catenin expression. Besides, the Wnt-related genes wnt1/3a/4/5a/6/10a mRNA expressions were also activated by hypo-osmolality, which can be inhibited by Adavivint and aggravated by the agonist (Figure 2D). Apart from this, when the Wnt signaling was suppressed, collagen II content was improved, and the collagen I and MMP3/9 levels were also resisted. On the contrary, the collagen I and MMP3/9 levels were further increased, resulting from supplying an agonist of Wnt signaling (Figure 2E, F).

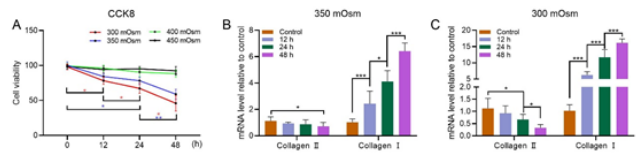

Fig. 1. Hypo-osmolality induces NP cell degeneration. NP cells were cultured in 300/350/400/450 mOsm medium for 12/24/48 hours. (A) Cell viability was determined by CCK8 assay. (*P < 0.05, **P < 0.01, red* indicated the 300 mOsm; blue* indicated the 350 mOsm). The RT-PCR analysis for collagen II and collagen I in (B) 350 mOsm, and (C) 300 mOsm by normalization to GAPDH. Results are expressed as mean ± SD. (*P < 0.05, ***P < 0.001).

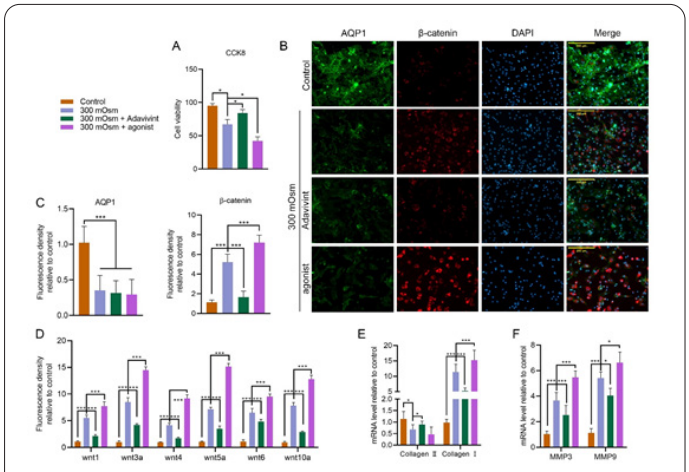

Fig. 2. Hypo-osmolality induces Wnt/β-catenin signaling activation. The NP cells were divided into four groups: control (cultured in 450 mOsm for 24 hours with no intervention); 300 mOsm (cultured in 300 mOsm for 24 hours); 300 mOsm + Adavivint (cultured in 300 mOsm for 24 hours supplied with Adavivint); 300 mOsm + agonist (cultured in 300 mOsm for 24 hours supplied with agonist). (A) Cell viability was determined by CCK8 assay. (B) Immunofluorescence staining of the AQP1 and β-catenin and (C) quantitative analysis of the fluorescence intensity. (D) The RT-PCR analysis for wnt1/3a/4/5a/6/10a, (E) collagen II, collagen I, and (F) MMP3/9 by normalization to GAPDH. Results are expressed as mean ± SD. (*P < 0.05, ***P < 0.001).

3.3. AQP1 overexpression alleviates the hypo-osmolality induced Wnt/β-catenin signaling activation

To determine whether the AQP1 overexpression would resist the hypo-osmolality caused by Wnt/β-catenin activation, we transfected the NP cells with the AQP1 plasmid. The NP cells were divided into three groups: control (normal NP cells cultured in 450 mOsm for 24 hours);

300 mOsm (normal NP cells cultured in 300 mOsm for 24 hours); 300 mOsm + AQP1-plasmid (AQP1-overexpressed NP cells cultured in 300 mOsm for 24 hours). Compared to the normal NP cells, AQP1 overexpressed NP cells were more capable of resisting the effects of hypo-osmolality, and the cell viability was more maintained (Figure 3A). The upregulated AQP1 under hypo-osmotic conditions inhibited the increase of β-catenin protein expression (Figure 3B, C) and wnt1/3a/4/5a/6/10a mRNA expressions (Figure 3D). Moreover, AQP1 overexpression also protected the collagen II content and suppressed the collagen I and MMP3/9 upregulation in hypo-osmotic conditions (Figure 3E, F).

3.4. AQP1 deficiency leads to Wnt/β-catenin signaling activation

Due to the AQP1 upregulation suppressed the Wnt/β-catenin signaling activation, we wondered whether the deficiency of AQP1 would lead to the activation of Wnt/β-catenin signaling. We silenced the AQP1 gene expression in NP cells via transfecting the siRNA. Since the AQP1 expression was already suppressed in the hypo-osmolality, we cultured the NP cells in the 450 mOsm medium to explore the relation between AQP1 deficiency and Wnt/β-catenin signaling. The NP cells were divided into four groups: control-3 d (normal NP cells cultured in 450 mOsm for 3 days); siAQP1-3 d (AQP1-silenced NP cells cultured in 450 mOsm for 3 days); control-7 d (normal NP cells cultured in 450 mOsm for 7 days); siAQP1-7 d (AQP1-silenced NP cells cultured in 450 mOsm for 7 days). As previously reported, the NP cells spontaneously degenerated in the monolayer culture in vitro [22]. During the three days' culture, the cell viability was not significantly affected when the AQP1 was silenced. However, silencing of the AQP1 gene injured the cell viability after seven days' culture compared to the normal NP cells (Figure 4A). As shown in Figures 4B and 4C, after silencing of AQP1, the β-catenin protein level was increased more than

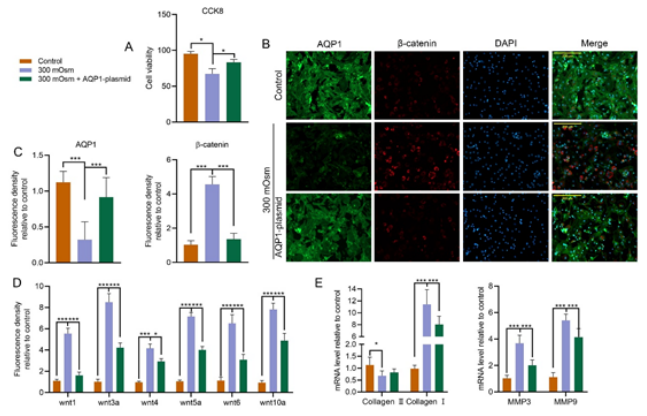

Fig. 3. AQP1 overexpression alleviates the hypo-osmolality induced Wnt/β-catenin signaling activation. The NP cells were divided into three groups: control (normal NP cells cultured in 450 mOsm for 24 hours); 300 mOsm (normal NP cells cultured in 300 mOsm for 24 hours); 300 mOsm + AQP1-plasmid (AQP1-overexpressed NP cells cultured in 300 mOsm for 24 hours). (A) Cell viability was determined by CCK8 assay. (B) Immunofluorescence staining of the AQP1 and β-catenin and (C) quantitative analysis of the fluorescence intensity. (D) The RT-PCR analysis for wnt1/3a/4/5a/6/10a, (E) collagen II, collagen I, and (F) MMP3/9 by normalization to GAPDH. Results are expressed as mean ± SD. (*P < 0.05, ***P < 0.001).

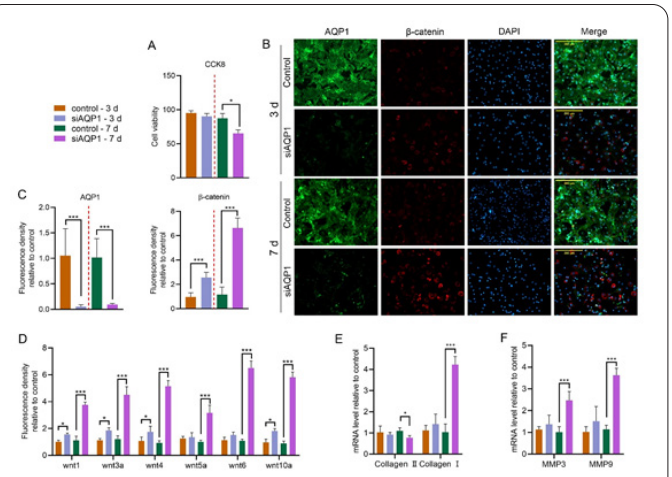

Fig. 4. AQP1 deficiency leads to Wnt/β-catenin signaling activation. The NP cells were divided into four groups: control-3 d (normal NP cells cultured in 450 mOsm for 3 days); siAQP1-3 d (AQP1-silenced NP cells cultured in 450 mOsm for 3 days); control-7 d (normal NP cells cultured in 450 mOsm for 7 days); siAQP1-7 d (AQP1-silenced NP cells cultured in 450 mOsm for 7 days). (A) Cell viability was determined by CCK8 assay. (B) Immunofluorescence staining of the AQP1 and β-catenin and (C) quantitative analysis of the fluorescence intensity. (D) The RT-PCR analysis for wnt1/3a/4/5a/6/10a, (E) collagen II, collagen I, and (F) MMP3/9 by normalization to GAPDH. Results are expressed as mean ± SD. (*P < 0.05, ***P < 0.001; data of siAQP1-3 d were normalized to control-3 d, data of siAQP1-7 d were normalized to control-7 d).

two times on day three compared to the control, which was continuously increased six-fold on day seven. Besides, AQP1 deficiency also led to an increase of wnt1/3a/4/10a expression on day three and immeasurably increased all the wnt1/3a/4/5a/6/10a genes on day seven (Figure 4D). Moreover, the collagen II/II and MMP3/9 mRNA expression were not changed on day three after AQP1 silencing, but they were obviously affected on day seven.

4. Discussion

In this study, we elucidated the role of AQP1-regulated Wnt/β-catenin signaling underlying the NP cell degeneration under hypo-osmolality. The hyper-osmotic environment in normal NP tissue is mainly maintained by negatively charged glycosaminoglycan side chains. A certain degree of hyper-osmolality is conducive to dispersing nutrients into the intervertebral disc and resisting pressure load [23]. We tested the status of NP cells under hypo-osmotic conditions and the changes of APQ1 and Wnt/β-catenin expressions. The data indicated that NP cells gradually underwent a degenerative phenotype when subjected to the hypo-osmotic environment with a decreased cell viability and collagen II expression, and an increase of collagen I and MMP3/9. Meanwhile, the AQP1 was suppressed, and the Wnt/β-catenin was activated. The NP cells in standard intervertebral discs are in a particular external environment under the action of low oxygen, high osmotic pressure, and continuous high stress. When the extracellular environment changes from hyper-osmolality to hypo-osmolality, NP cells' homeostasis is destroyed, and IDD occurs [24]. Homeostasis is crucial for NP cells to maintain their typical structure and physiological functions. Changes in osmotic pressure can lead to a disorder in the structure and function of intracellular proteins and

nucleic acids, which in turn induces autophagy, senescence, and even apoptosis [25].

When cells are in a hypo-osmolality state, the volume adjustment can be achieved by inhibiting water transport into the cells. Therefore, when the osmolality of the NP cells decreases, it is possible to regulate the distribution of water inside and outside the cell by reducing the expression of AQP1, thereby maintaining the primary cellular function and activity. Therefore, suppression of AQP1 is a regular regulation of cells to maintain the osmotic pressure balance [26,27]. The previous studies have verified the AQP1 downregulation is identified in the IDD [14], which is an adaptive role for these water channels during the hypo-osmotic condition. However, it is challenging to conclude that AQP1 reduction would be good for delaying the NP cell degeneration. On the contrary, the degenerative phenotype was improved after we upregulated the AQP1 gene expression in the hypo-osmotic condition. While inhibiting water transportation by reducing AQP1, the water content of the intervertebral disc and nutrient permeability are also reduced, thereby affecting the nutrient supply and then affecting cell function until apoptosis. Furthermore, the suppression of AQP1 would lead to the excessive activation of Wnt/β-catenin signaling [28]. Wnt/β-catenin is a classic Wnt signaling pathway closely related to various diseases and participates in regulating various life processes [29]. Excessive activation of Wnt/β-catenin signaling has been shown to cause severe intervertebral disc tissue structure changes, such as reduction of cell proliferation, cell cycle arrest, and MMPs activation [30].

We hypothesized that Wnt signaling activation might involve the hypo-osmolality-caused NP cell degeneration. Based on the Wnt/β-catenin activation in hypo-osmotic conditions, the excessive activation of Wnt using the agonist aggravated the reduction of cell viability and collagen II expression and increased the collagen I and MMP3/9 levels. Moreover, the suppression of Wnt via adding its inhibitor was sufficient to alleviate the injury caused by hypo-osmolality. Thus, Wnt/β-catenin is a key to understanding the hypo-osmolality-induced NP cell degeneration, which might be related to the decreased AQP1 expression. Shu et al. reported that AQP1 regulated the Wnt signaling in endometriosis mouse models and that AQP1 gene silencing promotes the Wnt signaling activation [31]. Zhang et al. found that AQP1 silencing impairs Alzheimer's disease in a mouse model by activating the Wnt signaling pathway [32]. Similarly, we also demonstrated as previously that AQP1 silencing contributed to the upregulation of Wnt signaling, which accelerated the NP cells degeneration, and the overexpression of AQP1 prevented the NP cells degeneration via suppressing the Wnt/β-catenin signaling.

In conclusion, we demonstrated that (1) hypo-osmolality suppresses AQP1 but activates Wnt/β-catenin signaling in NP cells; (2) the downregulation of AQP1 and upregulation of Wnt/β-catenin signaling is associated with NP cells degeneration; (3) AQP1 negative regulates the Wnt/β-catenin signaling in NP cells. Thus, our study elucidates that the NP cell degeneration in hypo-osmolality is related to the AQP1 reduction and the resulting Wnt/β-catenin signaling activation, which helps to understand the mechanism of osmolality changes in IDD.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the ethics committee of The Affiliated Hospital of Nanjing University of Chinese Medicine.

Informed Consent

Signed written informed consents were obtained from the patients and/or guardians.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

XZ and CW designed the study and performed the experiments, XZ and ZL collected the data, CW and ZL analyzed the data, XZ and CW prepared the manuscript. All authors read and approved the final manuscript.

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