Original Paper

N-Cadherin-Mediated Activation of PI3K/Akt-GSK-3 β Signaling Attenuates Nucleus Pulposus Cell Apoptosis Under High-Magnitude Compression

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Key Words

Compression • Apoptosis • Nucleus pulposus cell • PI3K/Akt • N-cadherin

Abstract

Background/Aims: Mechanical overloading-induced nucleus pulposus (NP) apoptosis plays an important role in the pathogenesis of intervertebral disc degeneration. N-cadherin (N-CDH)-mediated signaling preserves normal NP cell phenotype. This study aims to investigate the effects of N-CDH on NP cell apoptosis under high-magnitude compression and the underlying mechanism behind this process. **Methods:** Rat NP cells seeded on scaffold were perfusion-cultured using a self-developed perfusion bioreactor for 5 days and experienced different magnitudes (2% and 20% compressive deformation, respectively) of compression at a frequency of 1.0 Hz for 4 hours once per day. The un-loaded NP cells were used as controls. Lentivirus-mediated N-CDH overexpression and inhibitor LY294002 were used to further investigate the role of N-CDH and PI3K/Akt pathway under high-magnitude compression, respectively. NP cell apoptosis was evaluated by caspase-3 activity measured using a commercial kit, flow cytometry, and expression of apoptosis-related molecules analyzed by real-time PCR and western blotting assays. **Results:** High-magnitude compression significantly increased apoptotic NP cells, caspase-3 activity and expression of pro-apoptotic molecules (Bax and caspase-3/cleaved caspase-3), but decreased expression of anti-apoptotic molecule (Bcl-2). High-magnitude compression decreased expression of N-CDH, p-Akt and p-GSK-3 β . However, N-CDH overexpression attenuated NP cell apoptosis and increased expression of p-Akt and p-GSK-3 β under high-magnitude compression. Further analysis showed that inhibition of the PI3K/Akt pathway suppressed NP cell apoptosis and

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decreased expression of p-GSK-3 β , but had no significant effects on N-CDH expression under high-magnitude compression. **Conclusion:** N-CDH can attenuate NP cell apoptosis through activating the PI3K/Akt-GSK-3 β signaling under high-magnitude compression.

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Introduction

Intervertebral disc degeneration (IDD) is a disease with high incidence of crowd worldwide [1, 2]. Currently, biological regeneration of IDD remains a difficult challenge for basic researchers and clinicians [3]. Further understanding of the pathogenesis of IDD contributes to the development of biologically effective treatments for IDD.

Maintenance of matrix homeostasis is important for disc function [4, 5]. Cell death-caused cellular loss leads to decrease in matrix biosynthesis and plays an important role in promoting disc degeneration [6, 7]. As a common type of disc cell death, cell apoptosis is reported to be associated with disc degeneration [8, 9]. It is well established that mechanical load affects disc cell biology in multiple aspects, including apoptosis, senescence and matrix degradation [10-16]. However, the mechanism behind this pathological process is unclear.

N-cadherin (N-CDH) belongs to the classical cadherin family of transmembrane glycoproteins [17]. It is first identified in the neural system and reported to participate in the development of neural crest and formation of neuronal connections [17, 18]. Recently, N-CDH is regarded as a molecular marker of normal nucleus pulposus (NP) cell and is down-regulated in NP cells with disc degeneration [19, 20]. Moreover, N-CDH-mediated signal has a biological function of maintaining NP cell phenotype and NP matrix biosynthesis [21, 22]. Additionally, N-CDH positively regulated expression of cartilage matrix-related molecules (aggrecan and collagen II) which are also main macromolecules of NP matrix [23]. These findings indicate that N-CDH is closely related with IDD. Though previous studies demonstrated that N-CDH down-regulation promoted cellular apoptosis in several types of cells [24, 25], it is unknown that whether N-CDH down-regulation is associated with NP cell apoptosis under the stimulation of external pathological factors.

In the present study, we mainly aimed to investigate the role and mechanism of N-CDH expression in NP cell apoptosis under high-magnitude compression. To achieve this, NP cells seeded in scaffold were perfusion-cultured and experienced different magnitudes of mechanical compression. The recombinant lentiviral vectors were used to enhance N-CDH expression in NP cells to further verify the role of N-CDH under high-magnitude compression. The inhibitor LY294002 was used to study the role of PI3K/Akt pathway in this regulatory process. NP cell apoptosis was evaluated by flow cytometry, caspase-3 activity and expression of apoptosis-related molecules.

Materials and Methods

Ethical statement

In this study, all experiment animals were used in accordance with the relevant guidelines [SYXK (YU) 2012-0012] of the Ethics Committee at Southwest Hospital affiliated to the Third Military Medical University.

Disc harvest and NP cell isolation

Healthy New Zealand rats (250 g, 6-8 weeks old) were sacrificed by excessive carbon dioxide. Then, the thoracic and lumbar discs were harvested as previously described [26, 27], and the NP tissues were separated under a dissecting microscope. After NP tissues were washed with phosphate buffer solution (PBS), NP tissues were sequentially digested with trypsin (0.25%, Gibco, USA) for 3-5 minutes and type I collagenase (0.25%, Sigma, USA) for 10 minutes. Subsequently, NP cell pellets were suspended in culture medium (DMEM/F12 with 10% fetal bovine serum (FBS, Gibco, USA) and 1% (v/v) penicillin-streptomycin (Gibco, USA)), and cultured under standard conditions (37°C, 20% O₂ and 5% CO₂).

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Fig. 1. The substance exchanger-based perfusion bioreactor system. A: General view of the machine. B: Photograph of this substance exchanger-based perfusion bioreactor. C: The scheme of this novel bioreactor system. D: Photograph of each unit of this perfusion bioreactor (1: medium reservoir; 2: substance exchanger; 3: peristaltic pump; 4-1: the first generation of tissue culture chamber; 4-2: the second generation of tissue culture chamber; 5: PO₂, PCO₂ and pH sensors).

NP cell transfection

Briefly, after NP cells were seeded in the 24-well plate and grown to approximately 50% confluence, NP cells were incubated with the recombinant lentiviral vectors LV5-N-CDH (Genepharma, Shanghai, China) for 48 hours to enhance N-CDH expression (NP-N-CDH). NP cells transfected with negative vectors were used as controls (NP-N-CDH-NC). To further obtain the transfected cell lines (NP-N-CDH and NP-N-CDH-NC), the transfected NP cells were incubated with culture medium containing puromycin (1 µg/mL) for 4-5 days. Finally, the real-time PCR and western blotting assays were used to verify the transfection efficacy.

Mechanical compression application

As described previously [28], after all transfected or un-transfected NP cell pellets were suspended in the collagen solution (1 mg/mL, Shengyou, China), NP cells were seeded in the prepared bovine decalcified bone matrix (DBM, 10 mm × 10 mm × 5 mm, 1 × 10⁷ cells per DBM) scaffold which were freely provided by Tissue Engineering Center of Third Military Medical University. Then, NP cells seeded in the scaffold were pre-cultured under standard conditions (37°C, 20% O₂ and 5% CO₂) for 2 days. Finally, the scaffolds seeded with NP cells were placed in the tissue culture chambers of the self-developed bioreactor (Fig. 1) [28] and perfusion-cultured for 5 days. Simultaneously, NP cells were dynamically compressed at different compressive magnitudes (low magnitude and high magnitude: 2% and 20% compressive deformation, respectively) at a frequency of 1.0 Hz for 4 hours once per day. The non-compressed NP cells were designed as the controls.

NP cell apoptosis detection by flow cytometry

After dynamic compression, NP cells seeded in the scaffolds were harvested by sequential digestion with 0.05% trypsin and 0.1% collagenase I. After NP cells were washed with PBS, NP cell apoptosis was evaluated by flow cytometry using the Annexin V/PI staining according to the manufacturer's instructions (Beyotime, China). These Annexin V positive-stained and PI-negative-stained cells and double positive-stained cells were regarded as apoptotic cells in this assay.

Caspase-3 activity measurement

As a key enzyme in the apoptosis process, caspase-3 activity of NP cells was also detected using a caspase-3 activity detection kit (Beyotime, China). Briefly, after dynamic compression, the protein supernatant was collected using lysis solution. Then, 80 µL reaction buffer, 10 µL lysate and 10 µL Ac-DEVD-pNA were mixed together and incubated for 8 hours at 37°C. Finally, caspase-3 activity normalized to the total protein was calculated by measuring the optical density (OD) at a wavelength of 405 nm.

Real-time polymerase chain reaction (PCR) analysis

Briefly, after DBM scaffolds were washed with PBS, they are homogenized on the ice in the Trizol reagent (Invitrogen, USA). Then, total RNA was extracted according to the manufacturer's instructions. Subsequently, extracted RNA samples were synthesized into cDNA with a reverse transcription kit (Roche, Switzerland). Finally, a reaction system containing cDNA, primers (Table 1) and SYBR Green Mix (DONGSHENG BIOTECH,

Table 1. Primers of target genes

Gene	Accession number	Forward (5'-3')	Reverse (5'-3')
β-actin	NM_031144.3	CCGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC
N-CDH	NM_031333.1	AAGAGGCCGAGACTTGTGAA	CACTGGGGATAAGGGAAGGT
Bcl-2	NM_016993.1	GGGGCTACGAGTGGGATACT	GACGGTAGCGACGAGAGAAG
Bax	NM_017059.2	GGCGAATTGGCGATGAACTG	CCCAGTTGAAGTTGCCGTCT
Caspase 3	NM_012922.2	GGAGCTTGGAAACGCGAAGAA	ACACAAGCCCATTTTCAGGGT

China) were used to perform the real-time PCR assay. β-actin was used as the reference gene and the relative gene expression of target genes was expressed as $2^{-\Delta\Delta Ct}$.

Western blotting analysis

Briefly, after total protein was extracted with RIPA lysate (Beyotime, China) and protein concentration was determined a protein BCA detection kit (Beyotime, China), protein samples in each group were subjected to SDS-PAGE, followed by transferring to the PVDF membrane. Then, the PVDF membranes were incubated with primary antibodies (β-actin: Proteintech, 60008-1-Ig; N-CDH, Abcam, ab18203; cleaved-caspase 3, Cell Signaling Technology, #9661; Bcl-2: Proteintech, 12789-1-AP; Bax: Proteintech, 50599-2-Ig. All diluted at 1:1000) at 4°C overnight, and corresponding HRP-conjugated secondary antibodies (ZSGB-BIO, China, diluted 1:1000) at room temperature for 1 hour. Finally, protein bands on the PVDF membrane were developed using the SuperSignal West Pico Trial Kit (Thermo). After the grey value of protein bands were measured using the Image J software (National Institutes of Health, USA), expression of target proteins were normalized to that of β-actin.

Statistical analysis

The data are expressed as means±SD and each experiment was performed in triplicate in this study. After the homogeneity test for variance, comparisons between groups were performed by one-way analysis of variance (ANOVA) using SPSS 13.0 software, and then post hoc test was determined by LSD test. A significant difference was indicated when the p-value < 0.05.

Results

High-magnitude compression promoted NP cell apoptosis

NP cell apoptosis rate was analyzed by flow cytometry, which showed that the rate of apoptotic NP cells in the high-magnitude compression group was higher than in the low-magnitude compression group and the control group (Fig. 2A). Because caspase-3 is a key enzyme in the apoptosis process, caspase-3 activity was analyzed to reflect NP cell apoptosis. Results showed that caspase-3 activity in the high-magnitude compression group was higher than in the low-magnitude compression group and the control group (Fig. 2B). To further investigate NP cell apoptosis under mechanical compression, expression of pro-apoptotic (Bax and caspase-3/cleaved caspase-3) and anti-apoptotic (Bcl-2) molecules was analyzed. Compared with the low-magnitude compression group and the control group, high-magnitude compression group significantly increased expression of pro-apoptotic molecules (Bax and caspase-3/cleaved caspase-3) whereas decreased expression of anti-apoptotic molecule (Bcl-2) both at gene (Fig. 2C) and protein (Fig. 2D) levels.

High-magnitude compression decreased N-CDH expression and inhibited activation of PI3K/Akt-GSK-3β signaling in NP cells

N-CDH-mediated signaling is important for maintaining normal biology of NP cells. Results showed that high-magnitude compression significantly decreased N-CDH expression compared with the low-magnitude compression and the control group (Fig. 3A). Additionally, expression of p-Akt and p-GSK-3β in the high-magnitude compression group was lower

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Fig. 2. High-magnitude compression promoted NP cell apoptosis. A and B: Flow cytometry analysis of cell apoptosis rate and caspase-3 activity of NP cells under different magnitudes (2% and 20% compressive deformation) of compression, respectively. C and D: Real-time PCR and western blotting analysis of pro-apoptosis (Bax and caspase-3/cleaved caspase-3) and anti-apoptosis (Bcl-2) molecules under different magnitudes (2% and 20% compressive deformation) of compression, respectively. Data are expressed as mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups. #: Indicates a significant difference when compared with the control group.

Fig. 3. High-magnitude compression decreased N-CDH expression and inhibited activation of PI3K/Akt-GSK-3 β signaling in NP cells. A: Real-time PCR and western blotting analysis of N-CDH expression of NP cells under different magnitudes (2% and 20% compressive deformation) of compression, respectively. B: Western blotting analysis of p-Akt and p-GSK-3 β expression of NP cells under different magnitudes (2% and 20% compressive deformation) of compression, respectively. Data are expressed as mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups. #: Indicates a significant difference when compared with the control group.

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Fig. 4. N-CDH overexpression promoted activation of PI3K/Akt-GSK-3 β signaling in NP cells under high-magnitude compression. A: Verification of the efficacy of N-CDH overexpression in NP cells using real-time PCR and western blotting assays. B: Western blotting analysis of N-CDH, p-Akt and p-GSK-3 β expression in N-CDH overexpressed NP cells under high-magnitude (20% compressive deformation) compression. Data are expressed as mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups. #: Indicates a significant difference when compared with the control group.

than in the low-magnitude compression group and the control group (Fig. 3B), indicating that activation of the PI3K/Akt-GSK-3 β signaling was inhibited under the high-magnitude compression.

N-CDH overexpression promoted activation of PI3K/Akt-GSK-3 β signaling in NP cells under high-magnitude compression

To further investigate the role of N-CDH under high-magnitude compression, N-CDH expression in the NP cells were overexpressed (Fig. 4A) using the recombinant lentiviral vectors. Then, we found that expression of p-Akt and p-GSK-3 β in the N-CDH overexpressed NP cells was up-regulated under high-magnitude compression (Fig. 4B), indicating that N-CDH expression is positively related with the activity of the PI3K/Akt-GSK-3 β signaling.

N-CDH overexpression attenuated NP cell apoptosis under high-magnitude compression

Results showed that N-CDH overexpression decreased the rate cell apoptosis (Fig. 5A) and caspase-3 activity (Fig. 5B) of NP cells under the high-magnitude compression. Furthermore, N-CDH overexpression significantly decreased expression of pro-apoptotic molecules (Bax and caspase-3/cleaved caspase-3) whereas increased expression of anti-apoptotic molecule (Bcl-2) both at gene (Fig. 5C) and protein (Fig. 5D) levels.

Inhibition of PI3K/Akt pathway in N-CDH overexpressed NP cells suppressed GSK-3 β phosphorylation but had no effects on N-CDH expression under high-magnitude compression

To investigate the role of PI3K/Akt pathway, inhibitor LY294002 was used to suppress activation of PI3K/Akt pathway in the N-CDH overexpressed NP cells (Fig. 6). Subsequently, we found that LY294002 significantly decreased expression of p-GSK-3 β but had no effects on N-CDH expression in N-CDH overexpressed NP cells under high-magnitude compression (Fig. 6), indicating that N-CDH is a upstream regulator of the PI3K/Akt-GSK-3 β signaling under high-magnitude compression.

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Fig. 5. N-CDH overexpression attenuated NP cell apoptosis under high-magnitude compression. A and B: Flow cytometry analysis of cell apoptosis rate and caspase-3 activity of N-CDH overexpressed NP cells under high-magnitude (20% compressive deformation) compression, respectively. C and D: Real-time PCR and western blotting analysis of pro-apoptosis (Bax and caspase-3/cleaved caspase-3) and anti-apoptosis (Bcl-2) molecules under high-magnitude (20% compressive deformation) compression, respectively. Data are expressed as mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups. #: Indicates a significant difference when compared with the control group.

Fig. 6. Inhibition of PI3K/Akt pathway in N-CDH overexpressed NP cells suppressed GSK-3 β phosphorylation but had no effects on N-CDH expression under high-magnitude compression. Western blotting assay showed that LY294002 successfully inhibited activation of PI3K/Akt pathway and attenuated phosphorylation of the downstream GSK-3 β in N-CDH overexpressed NP cells under high-magnitude (20% compressive deformation) compression, but had no obvious effects on N-CDH expression. Data are expressed as mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups.

Inhibition of PI3K/Akt pathway promoted cellular apoptosis of N-CDH overexpressed NP cells under high-magnitude compression

Results showed that the rate of cell apoptosis (Fig. 7A) and caspase-3 activity (Fig. 7B) of N-CDH overexpressed NP cells were increased under high-magnitude compression

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Fig. 7. Inhibition of PI3K/Akt pathway promoted cellular apoptosis of N-CDH overexpressed NP cells under high-magnitude compression. A and B: Flow cytometry analysis of cell apoptosis rate and caspase-3 activity of N-CDH overexpressed NP cells under high-magnitude (20% compressive deformation) compression, respectively. C: Western blotting analysis of pro-apoptosis (Bax and cleaved caspase-3) and anti-apoptosis (Bcl-2) molecules under high-magnitude (20% compressive deformation) compression. Data are expressed as mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups.

after PI3K/Akt pathway was inhibited. Similarly, PI3K/Akt pathway inhibition up-regulated protein expression of pro-apoptotic molecules (Bax and cleaved caspase-3), whereas down-regulated protein expression of anti-apoptotic molecule (Bcl-2) in N-CDH overexpressed NP cells under high-magnitude compression (Fig. 7C). These results indicate that inhibition of PI3K/Akt pathway in N-CDH overexpressed NP cells promoted cell apoptosis under high-magnitude compression.

Discussion

Disc degeneration largely contributes to low back and leg pain [29]. The chronic and progressive matrix degradation is the main pathological character during disc degeneration [30, 31]. Cell apoptosis-induced decrease in NP cell number is the direct reason for the decrease in the NP matrix synthesis [6, 9, 32]. Therefore, deep investigation on the mechanism behind NP cell apoptosis will be helpful to understand pathogenesis of disc degeneration. Under *in vivo* conditions, disc NP tissue experiences various types of mechanical compression [10, 33]. Previous studies demonstrated that physiological/low compressive stress maintains NP cell viability and NP matrix synthesis, whereas un-physiological/high compressive stress promote NP cell death and decrease NP matrix synthesis [34–36]. Hence, inhibition of high compressive stress-induced NP cell death may be helpful to retarding disc degeneration caused by un-physiological/high compressive stress.

N-CDH is first discovered in nerve system and reported to function in nerve development [17, 18]. Recently, N-CDH is found to be a specific marker that differentiates normal NP cells from degenerative NP cells [19]. Importantly, N-CDH expression decreases with disc degeneration [20], whereas apoptotic NP cells increases with disc degeneration [37]. This suggests that there may be a negative relationship between N-CDH expression and NP cell apoptosis susceptibility during disc degeneration. In the present study, we investigated for the first time that the relationship between N-CDH expression and NP cell apoptosis under high-magnitude compression and the potential signal transduction in this process. Our results showed N-CDH down-regulation and the resulting inhibited activation of PI3K/Akt-GSK-3 β signaling may contribute to NP cell apoptosis under high-magnitude compression.

This study provides theoretical basis for understanding the role of N-CDH down-regulation in promoting NP cell apoptosis under high-magnitude compression.

Previous studies demonstrated that external stimuli-induced N-CDH down-regulation caused cell apoptosis in various types of cells [24, 25]. In this study, high-magnitude compression promoted NP cell apoptosis and down-regulated N-CDH expression compared with the low-magnitude compression. This is in line with previous studies reporting that high-magnitude compression promoted decrease in NP cell viability [34, 35, 38]. Meanwhile, this also indicates that high-magnitude compression may promote NP cell apoptosis through down-regulating N-CDH expression. Importantly, N-CDH overexpression attenuated NP cell apoptosis under high-magnitude compression, further verifying the above speculation and indirectly demonstrating the protective role of N-CDH in attenuating NP cell apoptosis under high-magnitude compression.

N-CDH is a transmembrane protein that constitutes of extracellular domain, transmembrane region and cytoplasmic domain [17]. The cytoplasmic domain of N-CDH can bind various proteins including catenins, kinases and phosphates [17]. In line with this, a previous study demonstrated that the cytoplasmic domain of N-CDH can bind PI3K which can activate its downstream substrate Akt [39]. Additionally, Akt can decrease GSK-3 β activity by phosphorylating it at residue serine 9 (Ser9) [40]. Furthermore, increased GSK-3 β activity is reported to be implicated in cell death [41-43]. In this study, we found that N-CDH down-regulation, promoted NP cell apoptosis and decreased activity of PI3K/Akt-GSK-3 β signaling concurrently happened under high-magnitude compression. However, N-CDH overexpression attenuated NP cell apoptosis and increased activity of PI3K/Akt-GSK-3 β signaling under high-magnitude compression. These results indicate that N-CDH may be able to protect NP cell from apoptosis through activating the PI3K/Akt-GSK-3 β signaling under high-magnitude compression.

To further verify the role of PI3K/Akt in this regulatory process, LY294002 was used to inhibit activation of PI3K/Akt pathway in the N-CDH overexpressed NP cells under high-magnitude compression. Though LY294002 had no obvious effects on N-CDH expression, it significantly increased GSK-3 β activity and promoted NP cell apoptosis in N-CDH overexpressed NP cells under high-magnitude compression, indicating that N-CDH has a regulatory effect on PI3K/Akt pathway whose activation was negatively related with NP cell apoptosis susceptibility under high-magnitude compression.

Several limitations were also existed in this study. First, NP cells were cultured under normoxic condition in this study. This differs from the physiological conditions in which NP cells are contained in a three-dimensional (3D) environment under hypoxic condition. Second, currently, due to the difficulty in developing a device that can accurately apply dynamic compression to intervertebral disc *in vivo*, we did not verify our findings in an *in vivo* animal model. If possible, we will develop this kind of device to perform similar experiments in the *in vivo* animal model in the future. In conclusion, this study studied N-CDH expression of NP cells under mechanical compression and verified the protective effects of N-CDH on inhibiting NP cell apoptosis under high-magnitude compression. Moreover, the PI3K/Akt-GSK-3 β signaling participated in this regulatory process. This study will ultimately help us to understand the mechanism behind mechanical overloading-induced disc degeneration, and provide us a new strategy to attenuate NP cell apoptosis-induced by mechanical overloading.

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Disclosure Statement

The authors do not have any conflicts of interest related to this work.

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