Original Paper

N-Cadherin Attenuates High Glucose-Induced Nucleus Pulposus Cell Senescence Through Regulation of the ROS/NF- κ B Pathway

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Key Words

Diabetes mellitus • Nucleus pulposus • Cell senescence • Glucose • Reactive oxygen species • NF- κ B

Abstract

Background/Aims: Diabetes mellitus (DM) is a potential etiology of disc degeneration. N-cadherin (N-CDH) helps maintain the cell viability, cell phenotype and matrix biosynthesis of nucleus pulposus (NP) cells. Here, we mainly aimed to investigate whether N-CDH can attenuate high glucose-induced NP cell senescence and its potential mechanism. **Methods:** Rat NP cells were cultured in a base culture medium and base culture medium with a 0.2 M glucose concentration. Recombinant lentiviral vectors were used to enhance N-CDH expression in NP cells. Senescence-associated β -galactosidase (SA- β -Gal) activity was measured by SA- β -Gal staining. NP cell proliferation was evaluated by CCK-8 assay. Telomerase activity and intracellular reactive oxygen species (ROS) content were tested by specific chemical kits according to the manufacturer's instructions. G0/G1 cell cycle arrest was evaluated by flow cytometry. Real-time PCR and Western blotting were used to analyze mRNA and protein expressions of senescence markers (p16 and p53) and matrix macromolecules (aggrecan and collagen II). Additionally, p-NF- κ B expression was also analyzed by Western blotting to evaluate NF- κ B pathway activity. **Results:** High glucose significantly decreased N-CDH expression, increased ROS generation and NF- κ B pathway activity, and promoted NP cell senescence, which was reflected in the increase in SA- β -Gal activity and senescence marker (p16 and p53) expression, compared to the control group. High glucose decreased telomerase activity and cell proliferation potency. However, N-CDH overexpression partially attenuated NP cell senescence, decreased ROS content and inhibited the activation of the NF- κ B pathway under the high glucose condition. **Conclusion:** High glucose decreases N-CDH expression and

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promotes NP cell senescence. N-CDH overexpression can attenuate high glucose-induced NP cell senescence through the regulation of the ROS/ NF- κ B pathway. This study suggests that N-CDH is a potential therapeutic target to slow DM-mediated disc NP degeneration.

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Introduction

Intervertebral disc degeneration (IDD) closely correlates with lower back and leg pain. It causes enormous socioeconomic loss and seriously affects the patient's quality of life [1, 2]. The systematic disease diabetes mellitus (DM) was recently identified as a potential etiology of disc degeneration [3, 4]. However, the underlying mechanism of disc degeneration in DM patients is not known.

The central gelatinous nucleus pulposus (NP) first exhibits degenerative changes during disc degeneration, such as degradation of the extracellular matrix, decreased matrix synthesis and reduced cell density [5]. NP cell senescence is also a classical cellular feature in degenerative disc NP tissue, which decreases NP matrix content and ultimately attenuates disc function [6]. Previous epidemiological studies reported that DM patients exhibit a higher incidence of disc degenerative diseases than patients without DM, and DM patients with disc degenerative diseases are much younger than non-DM patients [4, 7, 8]. Notably, several basic researchers demonstrated that high glucose accelerated disc cell senescence *in vitro* [9, 10]. These findings suggest that a high glucose environment reduces the healthy status of disc NP cell biology.

N-cadherin (N-CDH) is an adhesion molecule that was first identified in the nervous system [11, 12]. N-CDH is also highly expressed in normal NP cells, but it is down-regulated with advancing disc degeneration [12, 13]. Previous studies demonstrated that N-CDH played an important role in maintaining normal NP cell phenotype and NP matrix biosynthesis [14, 15]. N-CDH-mediated signaling can protect against NP cell apoptosis under mechanical loading [16, 17]. However, whether N-CDH can protect against NP cell senescence under high glucose conditions is not clear.

Therefore, the present study primarily aimed to investigate the effects of N-CDH on NP cell senescence under a high glucose condition and the potential mechanism of this process. NP cell senescence is evaluated using direct and indirect parameters, including senescence-associated β -galactosidase (SA- β -Gal) activity, telomerase activity, NP cell proliferation, G0/G1 cell cycle arrest, senescence marker (p16 and p53) expression and matrix homeostatic phenotype.

Materials and Methods

Ethics statement

All experiments were performed in compliance with the relevant guidelines [SYXK (YU) 2012-0012] of the Ethics Committee at Southwest Hospital affiliated with the Third Military Medical University.

Disc harvesting and NP cell isolation

Thirty-four healthy New Zealand rats (250-280 g, 6-8 weeks old) were sacrificed via excessive carbon dioxide. Briefly, the thoracic and lumbar discs were harvested under sterile conditions, and central NP tissues were separated and digested with type I collagenase (0.25%, Sigma, USA) for 15-17 minutes. The collected NP cell pellets were cultured in a base DMEM/F12 medium containing 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) under standard conditions (37°C, 20% O₂ and 5% CO₂).

NP cell transfection

NP cells were seeded in a 24-well plate and grown to 40-50% confluency. Culture medium (500 μ L) supplemented with 50 μ L of recombinant lentiviral vectors (GenePharma, Shanghai, China) was added to the culture wells for 48 hours to enhance N-CDH expression in NP cells (NP-N-CDH). NP cells transfected

with 500 μ L of culture medium supplemented with 50 μ L of negative vectors were used as controls (NP-N-CDH-NC). All transfected cells were further selected via application of puromycin in culture medium for 3-4 days. N-CDH overexpression in NP cells was verified using real-time PCR and western blotting.

Grouping

We cultured NP cells in 10% FBS culture medium (control group) and 10% FBS culture medium with a high glucose concentration (0.2 M, high glucose group) for 3 days to examine the effects of high glucose on N-CDH expression and cellular senescence of NP cells. NP cells (NP group), NP-N-CDH-NC cells (NP-N-CDH-NC group) and NP-N-CDH cells (NP-N-CDH group) were cultured in 10% FBS culture medium with 0.2 M glucose for 3 days under standard conditions (37°C, 20% O₂ and 5% CO₂) to investigate the effects of N-CDH overexpression on NP cell senescence under the high glucose condition. The high glucose concentration used was based on previous studies [9, 10].

SA- β -Gal activity

Briefly, NP cells (seeded in 6-well plate, 1×10^4 cells/well) were washed three times with a phosphate buffer solution (PBS). An SA- β -Gal staining assay was performed using a chemical reagent kit according to the manufacturer's instructions (Beyotime, China). NP cells were observed under a light microscope (Olympus BX51, Japan). SA- β -Gal activity is expressed as the ratio of positively stained cells to total NP cells.

Cell proliferation assay

Briefly, NP cells (seeded in 6-well plate, 1×10^4 cells/well) were cultured in conditioned culture medium for 24 and 48 hours, and cell proliferation (OD 450 value) was analyzed using a Cell Counting Kit-8 (CCK-8, Beyotime, China).

Telomerase activity detection

Cultured NP cells were lysed using RIPA lysis buffer (Beyotime, China) and centrifuged to collect the supernatant. Telomerase activity (IU/L) was measured using a telomerase (TE) ELISA kit (Mlbio, China).

Cell cycle analysis

NP cell pellets were fixed in 75% ethanol overnight at 4°C, stained with a propidium iodide (PI) solution (50 μ g/ml, Beyotime, China) and incubated with RNase A (100 μ g/ml, Beyotime, China) for 30 minutes. NP cells were subjected to flow cytometry to analyze the fraction of cells in each cell cycle phase (G0/G1, G2/M and S).

Intracellular reactive oxygen species (ROS) measurement

Briefly, NP cells were cultured with conditioned medium and washed three times with PBS. Cells were incubated with DCFH-DA (10 μ M) for 40 minutes and washed with FBS to remove the uncombined DCFH-DA. NP cells (1×10^5 cells in each group) were used to measure the intracellular ROS content using a reactive oxygen species assay kit (Nanjing Jiancheng Bioengineering Institute, China). ROS content is expressed as the fluorescence intensity at an excitation/emission wavelength of 490/585 nm.

Real-time polymerase chain reaction (PCR) analysis

Briefly, total RNA was extracted using Trizol reagent (Invitrogen, USA) and reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany). The cDNA, SYBR Green Mix (TOYOBO) and primers (Table 1) were mixed together and subjected to real-time PCR (Thermal, USA). The following thermal cycling conditions were used: 3 min at 95°C, followed by 30 amplification cycles of 30 seconds at 95°C, 15 seconds at 56°C and 15 seconds at 72°C. The relative gene expression of target genes was normalized to the internal reference β -actin and calculated using $2^{-\Delta\Delta Ct}$.

Table 1. Primers of target genes

Gene	Accession number	Forward (5'-3')	Reverse (5'-3')
β -actin	NM_031144.3	CCGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC
Aggrecan	XM_002723376.1	ATGGCATTGAGGACAGCGAA	GCTCGGTCAAAGTCCAGTGT
Collagen II	NM_012929.1	GCCAGGATGCCCGAAAATTAG	CCAGCCTTCTCGTCAAATCCT
p53	XM_008767773.1	CCTTAAGATCCGTGGGCGT	GCTAGCAGTTTGGGCTTTCC
p16	NM_031550.1	TACCCGATACAGGTGATGA	TACCGCAAATACCGCACGA

Western blotting analysis

Briefly, the total protein in each group was extracted using a RIPA lysis solution (Beyotime, China). Protein concentration was determined using a BCA kit (Beyotime, China). Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. The PVDF membrane was blocked with 5% FBS for 1 hour at the room temperature and incubated with primary antibodies (β -actin: Proteintech, 60008-1-Ig; p16: Novus, NBP2-37740; p53: Proteintech, 10442-1-AP; aggrecan: Santa Cruz, sc-16492; collagen II: Abcam, ab34712; NF- κ B p65: Beyotime, AV365; and phosphor-NF- κ B p65: Beyotime, AV371) at 4°C overnight. The PVDF membrane was washed three times with Tris-buffered saline plus Tween (TBST) and incubated with corresponding secondary antibodies (ZSGB-BIO, China, diluted 1:2000) at 37°C for 2 hours. The SuperSignal West Pico Trial Kit (Thermo) was used to develop protein bands, and gray values were measured using ImageJ software (National Institutes of Health, USA).

Statistical analysis

All numerical data are expressed as the means $\pm$ SD and analyzed using SPSS 13.0 software. The homogeneity test for variance was performed, and intergroup comparisons were performed using one-way analysis of variance (ANOVA). A p-value < 0.05 was considered significant.

Results

Effects of high glucose on N-CDH expression and cellular senescence of NP cells:

High glucose down-regulated N-CDH expression in NP cells

N-CDH expression in NP cells was analyzed using real-time PCR and western blotting. The results demonstrated that N-CDH gene (Fig. 1A) and protein (Fig. 1B) expression decreased significantly in the high glucose group compared to the control group.

High glucose promoted NP cell senescence

NP cell senescence was evaluated using SA- β -Gal activity, telomerase activity and senescence marker (p16 and p53) expression. The results revealed that SA- β -Gal activity in the high glucose group was significantly increased compared to the control group

Fig. 1. High glucose significantly decreased N-cadherin (N-CDH) expression in nucleus pulposus (NP) cells. A: Real-time PCR analysis of N-CDH mRNA expression. B: Western blotting analysis of N-CDH protein expression. Data are expressed as the means $\pm$ SD, n=3. #: Indicates a significant difference compared to the control group.

Fig. 2. High glucose promoted nucleus pulposus (NP) cell senescence. A: SA- β -Gal activity analysis. Magnification: 200 $\times$, Scale=100 μ m, n=3. B: Telomerase activity measurement. C: Cell cycle analysis. D: Cell proliferation potency evaluated using the CCK-8 assay. E: Real-time PCR analysis of senescence marker (p16 and p53) gene expression. F: Western blotting analysis of senescence marker (p16 and p53) protein expression. Data are expressed as the means $\pm$ SD, n=3. #: Indicates a significant difference compared to the control group.

(Fig. 2A). However, telomerase activity (Fig. 2B) and cell proliferation potency exhibited an opposite trend (Fig. 2C). The gene and protein expression of senescence markers (p16 and p53) in the high glucose group was higher than in the control group (Fig. 2D-E).

High glucose attenuated NP cell matrix biosynthesis

Matrix homeostasis is an indirect parameter of cellular senescence [18]. Aggrecan and collagen II are two primary NP matrix macromolecules [19]. We analyzed the matrix homeostatic phenotype in the high glucose group. The results demonstrated that high glucose significantly down-regulated the expression of aggrecan and collagen II at the gene (Fig. 3A) and protein (Fig. 3B) levels.

High glucose increased ROS generation and NF- κ B pathway activity in NP cells

Previous studies reported that high glucose induced an oxidative stress reaction. High glucose significantly increased intracellular ROS generation (Fig. 4A) and NF- κ B pathway activity (Fig. 4B) compared to the control group.

Effects of N-CDH overexpression on high glucose-induced NP cell senescence: N-CDH overexpression attenuated NP cell senescence under the high glucose condition

The results of part 1 indicated a negative relationship between N-CDH expression and NP cell senescence under a high glucose condition. Therefore, we enhanced N-CDH expression using a recombinant lentiviral vector (Fig. 5A). Further analysis showed that N-CDH overexpression increased NP cell proliferation potency (Fig. 5B), decreased the fraction of cells in the G0/G1 cell cycle (Fig. 5C), increased telomerase activity (Fig. 5D), and down-regulated p16 and p53 expression at the gene (Fig. 5E) and protein (Fig. 5F) levels under the high glucose condition.

Fig. 3. High glucose decreased matrix molecule expression in nucleus pulposus (NP) cells. A: Real-time PCR analysis of aggrecan mRNA and collagen II mRNA. B: Western blotting analysis of aggrecan and collagen II proteins. Data are expressed as the means $\pm$ SD, n=3. #: Indicates a significant difference compared to the control group.

Fig. 4. High glucose increased reactive oxygen species (ROS) generation and NF- κ B pathway activity in nucleus pulposus (NP) cells. A: ROS generation measurement. B: Western blotting analysis of NF- κ B pathway activity. Data are expressed as the means $\pm$ SD, n=3. #: Indicates a significant difference compared to the control group.

N-CDH overexpression increased NP matrix biosynthesis under the high glucose condition

Our results revealed that N-CDH overexpression significantly up-regulated aggrecan and collagen II expression at the gene (Fig. 6A) and protein (Fig. 6B) levels. This result indicates that N-CDH overexpression can promote NP cell matrix biosynthesis under the high glucose condition.

Fig. 5. N-cadherin (N-CDH) overexpression attenuated nucleus pulposus (NP) cell senescence under the high glucose condition. A: Verification of the efficacy of recombinant lentiviral vector transfection at the gene and protein levels. B: NP cell proliferation assay. C: Measurement of cell fraction in each cell cycle (G0/G1, S and G2/M). D: Telomerase activity measurement. E-F: Real-time PCR analysis and western blotting analysis of the expression of senescence markers (p16 and p53). Data are expressed as the means $\pm$ SD, n=3. *: Indicates a significant difference between two groups.

Fig. 6. N-cadherin (N-CDH) overexpression increased matrix synthesis of nucleus pulposus (NP) cell senescence under the high glucose condition. A: Real-time PCR analysis of aggrecan mRNA and collagen II mRNA expression. B: Western blotting analysis of aggrecan and collagen II protein expression. Data are expressed as the means $\pm$ SD, n=3. *: Indicates a significant difference between the two groups.

Fig. 7. N-cadherin (N-CDH) overexpression decreased reactive oxygen species (ROS) generation and NF- κ B pathway activity in nucleus pulposus (NP) cell senescence under the

high glucose condition. A: ROS generation measurement. B: Western blotting analysis of NF- κ B pathway activity. Data are expressed as the means $\pm$ SD, $n=3$. *: Indicates a significant difference between the two groups.

N-CDH overexpression decreased ROS generation and NF- κ B pathway activity in NP cells under the high glucose condition

N-CDH overexpression obviously decreased ROS generation (Fig. 7A) and NF- κ B pathway activity (Fig. 7B) in NP cells under the high glucose condition.

Discussion

DM is a major public health problem worldwide [20]. Recent epidemiological and basic studies demonstrated that DM is a potential etiological factor for disc degeneration [3, 4]. Hyperglycemia is a causative factor in the development of senescence-associated diseases in DM patients [21-24]. Cellular senescence is one classical pathological feature within the degenerative disc tissue [6], but DM-associated disc degeneration was not fully investigated. The present study confirmed that high glucose accelerated disc NP cell senescence and demonstrated for the first time that N-CDH attenuated disc NP cell senescence via inhibition of the ROS/NF- κ B pathway under a high glucose condition. This study directly elucidated the mechanism of DM-associated disc degeneration and suggests that N-CDH may be a potential therapeutic target for retarding disc degeneration.

Cellular senescence correlates with numerous degenerative diseases [25]. Senescence influences cell biological activities and causes these cells to arrest the cell cycle and decrease matrix production [6]. Gruber H.E. et al. and Le M. et al. reported a dramatic increase in senescent cells in degenerative disc tissue that positively correlated with disc degeneration [26, 27]. Several basic studies demonstrated that disc cell senescence was associated with disc degeneration [28, 29]. The present study demonstrated that high glucose significantly increased SA- β -Gal activity, decreased telomerase activity and up-regulated the expression of senescence markers (p16 and p53), which suggests that high glucose promoted disc NP cell senescence. These results are consistent with previous studies [9, 10]. These findings improve our understanding of DM-associated disc degeneration.

N-CDH is highly expressed in disc NP cells [12, 13]. Previous studies demonstrated that N-CDH maintained disc NP cell viability and matrix synthesis [14-17]. The present study found that high glucose significantly decreased N-CDH expression in disc NP cells. Therefore, we hypothesized that the down-regulation of N-CDH was related with NP cell senescence under a high glucose condition. We enhanced N-CDH expression in disc NP cells to verify this hypothesis. Further analysis revealed that the senescence of N-CDH overexpressed NP cells was attenuated under the high glucose condition, which is reflected in the decrease in SA- β -Gal activity and the fraction of cells in the G0/G1 cell cycle, increased telomerase activity, and down-regulated expression of p16 and p53. Matrix homeostasis phenotype is an indirect parameter to evaluate cellular senescence [18]. Our results demonstrated that N-CDH overexpression attenuated NP cell senescence and obviously increased matrix biosynthesis ability under the high glucose condition. This finding is consistent with previous studies [14, 17].

Oxidative stress induces cell senescence *in vitro*, and numerous *in vivo* studies reported age-related oxidative stress in many tissues [30, 31]. Oxidative stress markers, including carboxy methyl lysine (CML), advanced glycosylation end products (AGEs), and nitrotyrosine (NT), are significantly increased in degenerative tissue [32]. The present study found that high glucose obviously increased intracellular ROS generation and activated the downstream NF- κ B pathway in NP cells, which indicates that high glucose induced an oxidative stress reaction in NP cells. This result is consistent with previous studies that demonstrated that oxidative stress may be a potential mechanism for high glucose-related disc degenerative changes [9, 33]. However, we found that N-CDH overexpression decreased ROS generation and NF- κ B pathway activity in NP cells under the high glucose condition, which indicates that N-CDH may suppress high glucose-induced cellular oxidative stress in NP cells. The protective effects of N-CDH overexpression against NP cell senescence suggest that the suppression of the ROS/NF- κ B pathway is the mechanism underlying the protective effects of N-CDH on NP cell senescence under the high glucose condition.

This research has several limitations. First, rat NP tissue contains many notochordal cells, and there are no specific cellular makers to differentiate notochordal cells from NP cells. The existence of notochordal cells may interfere with the present results. Second, we verified the protective effects of N-CDH against high glucose-induced NP cell senescence *in vitro*, but an *in vivo* animal model will strengthen our findings.

These findings support the conclusion that high glucose decreased N-CDH expression in disc NP cells, and N-CDH may attenuate high glucose-induced disc NP cell senescence via the regulation of the ROS/NF- κ B pathway. This study improves our understanding of DM-associated disc degeneration.

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Disclosure Statement

The authors declare to have no conflict of interests related to this work.

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