Original Paper

Dynamic Compression Promotes the Matrix Synthesis of Nucleus Pulposus Cells Through Up-Regulating N-CDH Expression in a Perfusion Bioreactor Culture

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Key Words

Nucleus pulposus • Dynamic • Static • Compression • Matrix • Regeneration

Abstract

Background/Aims: An adequate matrix production of nucleus pulposus (NP) cells is an important tissue engineering-based strategy to regenerate degenerative discs. Here, we mainly aimed to investigate the effects and mechanism of mechanical compression (i.e., static compression vs. dynamic compression) on the matrix synthesis of three-dimensional (3D) cultured NP cells *in vitro*. **Methods:** Rat NP cells seeded on small intestinal submucosa (SIS) cryogel scaffolds were cultured in the chambers of a self-developed, mechanically active bioreactor for 10 days. Meanwhile, the NP cells were subjected to compression (static compression or dynamic compression at a 10% scaffold deformation) for 6 hours once per day. Unloaded NP cells were used as controls. The cellular phenotype and matrix biosynthesis of NP cells were investigated by real-time PCR and Western blotting assays. Lentivirus-mediated N-cadherin (N-CDH) knockdown and an inhibitor, LY294002, were used to further investigate the role of N-CDH and the PI3K/Akt pathway in this process. **Results:** Dynamic compression better maintained the expression of cell-specific markers (keratin-19, FOXF1 and PAX1) and matrix macromolecules (aggrecan and collagen II), as well as N-CDH expression and the activity of the PI3K/Akt pathway, in the 3D-cultured NP cells compared with those expression levels and activity in the cells grown under static compression. Further analysis showed that the N-CDH knockdown significantly down-regulated the expression of NP cell-specific markers and matrix macromolecules and inhibited the activation of the PI3K/Akt pathway under dynamic compression. However, inhibition of the PI3K/Akt pathway had no effects on N-CDH expression but down-regulated the expression of NP cell-specific markers and matrix macromolecules under dynamic compression. **Conclusion:** Dynamic compression increases the matrix synthesis of 3D-cultured NP cells compared with that of the cells under static

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compression, and the N-CDH-PI3K/Akt pathway is involved in this regulatory process. This study provides a promising strategy to promote the matrix deposition of tissue-engineered NP tissue *in vitro* prior to clinical transplantation.

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Introduction

Intervertebral disc degeneration (IDD) is a progressive condition which leads to functional deficiency in patients [1-4]. Existing therapies including spinal fusion and discectomy are mainly aimed to alleviate pain symptoms but not to biologically regenerate disc function. Currently, considerable efforts have been made toward biologically regenerating degenerative discs using tissue engineering approaches [5-9].

The intervertebral disc (IVD) is a cartilaginous structure consisting of the nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP) [10]. IVDs play an important role in load bearing, absorption and transmission between two adjacent vertebral bones [11], which mainly depends on the matrix homeostasis within the NP region. IDD is proposed to originate in the NP region, which is characterized by a decrease in the NP cell density and normal extracellular matrix content (i.e., proteoglycan and collagen II) [12, 13]. All these cellular and molecular changes can lead to NP dehydration and thus weaken the disc capacity to absorb compression [14]. Based on the previous understanding of disc degeneration pathogenesis, a widely acceptable consensus has been reached that increasing the quantity and enhancing the biosynthesis of NP cells encapsulated in a biocompatible scaffold are important to regenerate discs using a tissue engineering approach [1, 15, 16].

Under physiological conditions, the disc NP cells are subjected to various kinds of mechanical compression [17], which have a great influence on disc NP cell biology, including cell phenotype and matrix biosynthesis [18-21]. Among the types of mechanical loads, static compression and dynamic compression have usually been applied in the research field of disc degeneration [22-24]. Though a general consensus has been reached that dynamic compression is more physiologically relevant than static compression *in vivo* [24] and that static compression often causes negative effects on healthy disc biology compared with those in dynamic compression [23], the potential mechanisms need to be further studied. N-cadherin (N-CDH) is one of the classical cadherin transmembrane glycoproteins [25]. It was first identified in the neural system and is responsible for the development of the neural crest and the formation of neuronal connections [25, 26]. Furthermore, N-CDH is highly expressed in normal disc NP cells compared with the expression in degenerative NP cells, adjacent disc AF cells and CEP cells [27, 28]. In addition, N-CDH-mediated signaling is helpful to maintain the normal NP cell phenotype and NP matrix synthesis *in vitro* [29-32]. However, whether N-CDH-mediated signaling is responsible for the positive effects of dynamic compression on NP cell biology remains unknown.

In this study, we hypothesize that dynamic compression may enhance NP matrix biosynthesis and maintain the NP cell phenotype through N-CDH-mediated signaling. The main objective of this study is to compare the effects of static and dynamic compression on the matrix synthesis of three-dimensional (3D) cultured NP cells *in vitro* using a self-developed perfusion bioreactor and to investigate the role and underlying mechanism of N-CDH in regulating this process.

Materials and Methods

Ethical statement

All experiments in this study were approved by the Ethics Committee at the Third Affiliated Hospital of Sun Yat-sen University and were performed in accordance with the relevant guidelines [SYMN (YUE) 2009-0014].

Rat NP cell isolation and culture

Thirteen healthy Sprague-Dawley rats (male, 230-250 g and 6-8 weeks old) were used in this study. Briefly, after the thoracic and lumbar discs were collected, the innermost NP tissue was separated under a dissecting microscope. Next, the tissue was subjected to enzymatic digestion with 0.25% trypsin (Gibco, USA) for 2-3 minutes followed by 0.25% Type I collagenase (Sigma, USA) for 8-10 minutes. All collected NP cell pellets were re-suspended in a monolayer culture with DMEM/F12 medium (HyClone, USA) containing 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and 1% (v/v) penicillin-streptomycin (Gibco, USA). Culture medium was refreshed every two days.

NP cell transfection

The recombinant lentiviral vector LV3-N-CDH (Genepharma, Shanghai, China) was used to silence N-CDH expression in NP cells (NP-shN-CDH). NP cells transfected with the negative vector (LV3-N-CDH-NC) were used as controls (NP-shN-CDH-NC). According to the manufacturer's instructions, the transfected cells were further selected via puromycin. Transfection efficacy was verified by real-time polymerase chain reaction (PCR) and Western blotting assays.

NP cell seeding and compressive loading application

NP cells (5×10^6 cells per scaffold, 500 μ L) were seeded onto a lyophilized porcine small intestinal submucosa (SIS) cryogel. The SIS cryogel was freely provided by Dr. Wenping Wang (Department of Cosmetic Plastic Surgery, Southwest Hospital, Third Military Medical University). After each cell-seeded scaffold was pre-cultured for 3 days in an incubator (37°C, 5% CO₂), the scaffolds were put into a culture chamber of a self-developed perfusion bioreactor and further bioreactor-cultured for 10 days. The NP cells seeded on scaffolds were assigned into the following groups: the control group (non-compression), the static compression group (10% deformation for 6 hours per day) and the dynamic compression group (10% deformation compression at a frequency of 1.0 Hz for 6 hours per day). The culture medium was circulated at 15 mL/min and changed every 3 days.

Cytocompatibility test of the scaffold

To evaluate the cytocompatibility of the SIS cryogel, we tested NP cell proliferation and viability when the cells were seeded onto the SIS cryogels. The sterilized SIS cryogel was made into a cylinder-like formation (7 mm in diameter and 5 mm in height) using a homemade mold. Then, NP cells (1×10^6 cells per scaffold, 500 μ L) were seeded onto the SIS cryogel as described. Next, the cell-seeded SIS cryogels were placed in an incubator (37°C, 5% CO₂) for 3 hours to allow for cell adhesion. NP cell proliferation potency was determined using a Cell Counting Kit-8 (CCK-8, Beyotime, China). Briefly, on days 1, 3, 5 and 7, the CCK-8 working solution was added into the fresh culture medium, and then incubated for another 2 hours. Then, 200 μ L of reaction mixture was used to analyze the cell proliferation potency, which was reflected by the absorbance value at a wavelength of 450 nm that was measured using a microplate reader (Model 550, Bio-Rad, USA).

NP cell viability was analyzed using a LIVE/DEAD Viability/Cytotoxicity® Kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, on days 3 and 7, a SIS cryogel slice was prepared using a homemade tool and washed with PBS 2-3 times. Then, NP cells on the scaffolds were stained with 2 μ M calcein green (green fluorescence when metabolized by live cells, 495 nm/515 nm excitation/emission) and 4 μ M ethidium homodimer (red fluorescence when metabolized by dead cells, 495 nm/635 nm excitation/emission) for 40 minutes at room temperature. Finally, the live or dead NP cells were viewed under a laser scanning confocal microscope (LSCM; Carl Zeiss, LSM 780, Germany).

Real-time PCR analysis

Briefly, total RNA was extracted using the TriPure Isolation Reagent (Roche) and synthesized into cDNA with a reverse transcription

Table 1. Primers of target genes

Gene	Accession number	Forward (5'-3')	Reverse (5'-3')
β -actin	NM_031144.3	CGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC
Aggrecan	XM_002723376.1	ATGGCATTGAGGACAGCGAA	GCTCGGTCAAAGTCCAGTGT
Collagen II	NM_012929.1	GCCAGGATGCCCCAAAATTAG	CCAGCCTTCTCGTCAAAATCCT
Keratin-19	NM_199498.2	ATGAGCTGAACCCAGGTC	ATGCTGAGCTGAGACTGCAA
FOXF1	XM_006255724.3	CGCTCAGCGAGATCTACCA	GGGCAGTTTGATGAAGCACT
PAX1	NM_001107787.1	GACTCGAGAAACCTGCCTTG	ATACTCCGTGCTGTTGGAA

kit (Roche). Then, cDNA, primers and SYBR Green Mix (Dongsheng Biotech, China) were mixed together and loaded into a real-time PCR instrument (CFX96 Real-Time System, Bio-Rad). Primers of the target genes are shown in Table 1. β -actin was used as the reference gene, and the relative gene expression was calculated as $2^{-\Delta\Delta C_t}$.

Western blotting assay

Briefly, the cell-scaffold constructs were cut into small pieces (1 mm×1 mm×1 mm). Then, total protein was extracted from the cellular component using a RIPA reagent (Beyotime, China), and protein concentration was measured using a protein BCA detection kit (Beyotime, China). Protein samples in each group were subjected to SDS-PAGE and transferred to PVDF membranes. Then, incubation with primary antibodies (β -actin: Proteintech, 60008-1-Ig; N-CDH: Abcam, ab18203; aggrecan: Santa Cruz, sc-16492; collagen II: Abcam, ab34712; Akt: Cell Signaling Technology, #9272; p-Akt: Cell Signaling Technology, #9271; all diluted 1:1000) and corresponding HRP-conjugated secondary antibodies was performed. Finally, protein bands on the PVDF membranes were visualized using the SuperSignal West Pico Trial Kit (Thermo, USA). Protein expression normalized to β -actin was analyzed using ImageJ software (National Institutes of Health, USA).

Statistics

All numerical data expressed as the mean±SD were analyzed using SPSS version 13.0 software in this study. Each experiment in this work was performed in triplicate. After homogeneity testing for variance was completed, one-way analysis of variance (ANOVA) was performed and then post hoc LSD tests were carried out. A significant difference was indicated when p-value<0.05.

Results

Cytocompatibility test

Though previous studies have demonstrated that this SIS cryogel has good biocompatibility, we still simply evaluated the cytotoxicity of the SIS cryogel here. The results showed that NP cells could normally proliferate in the SIS cryogel, which was indicated by an increase in the OD value at 450 nm with increasing experiment duration (Fig. 1A). Then, we evaluated the cell viability of NP cells seeded on the scaffold. The results showed that almost all NP cells were stained with green fluorescence and few NP cells were stained with red fluorescence, suggesting that the NP cells can remain viable in the SIS cryogel (Fig. 1B).

Dynamic compression up-regulated gene expression of NP cell-specific markers compared with those in the static compression condition

The maintenance of a normal cell phenotype is important in constructing an engineered tissue. Previous studies have demonstrated that keratin-19, FOXF1 and PAX1 are three cellular markers of normal NP cells [33-35]. In this study, the results showed that the mRNA expression of keratin-19, FOXF1 and PAX1 in the dynamic compression group was higher than that in the static compression and control groups, and static compression significantly down-regulated the expression of FOXF1 and PAX1 compared with that in the control group (Fig. 2).

Dynamic compression increased NP matrix biosynthesis compared with that in the static compression condition

Because the main matrix protein molecules within

Fig. 1. Cytocompatibility evaluation of the small intestinal submucosa (SIS) cryogel. A: Cell proliferation of the nucleus pulposus (NP) cells encapsulated in the SIS cryogel. B: Cell viability of the NP cells encapsulated in the SIS cryogel by LIVE/DEAD staining. All numerical data are expressed as the mean±SD (n=3).

the natural NP tissue are aggrecan and collagen II [36], the expression of NP matrix-related macromolecules was investigated. Here, we found that dynamic compression significantly up-regulated the gene expression of aggrecan and collagen II compared with that in the static compression and control groups (Fig. 3A). Consistently, the protein expression of aggrecan and collagen II in the dynamic compression group was increased compared with that in the static compression and control groups (Fig. 3B).

Dynamic compression increased N-CDH expression and the activity of the PI3K/Akt pathway compared with the expression and activity in the static compression group

As an important molecule that is helpful to maintain a normal NP cell phenotype and NP matrix synthesis *in vitro* [29], results showed that N-CDH expression in the dynamic compression group was increased both at gene and protein levels compared with those in the static compression group and the control group

Fig. 2. Dynamic compression up-regulated the gene expression of nucleus pulposus (NP) cell-specific markers compared with that in the static compression group. Gene expression of keratin-19, FOXF1 and PAX1 was analyzed by real-time PCR. All data are expressed as the mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups; #: indicates a significant difference (p<0.05) compared with the control group.

Fig. 3. Dynamic compression increased the matrix biosynthesis compared with that under static compression in nucleus pulposus (NP) cells encapsulated in the small intestinal submucosa (SIS) cryogel. A: Real-time PCR analysis of the mRNA expression of aggrecan and collagen II in NP cells. B: Western blotting analysis of the protein expression of aggrecan and collagen II in NP cells. All data are expressed as the mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups; #: indicates a significant difference (p<0.05) compared with the control group.

Fig. 4. Dynamic compression increased N-cadherin (N-CDH) expression and the activity of the PI3K/Akt pathway compared with the expression and activity under static compression in nucleus pulposus (NP) cells encapsulated in the small intestinal submucosa (SIS) cryogel. A: Real-time PCR and Western blotting analysis of N-CDH expression, respectively. B: Western blotting analysis of the activation of the PI3K/Akt pathway. All data are expressed as the mean $\pm$ SD (n=3). *: Indicates a significant difference (p<0.05) between two groups; #: indicates a significant difference (p<0.05) compared with the control group.

(Fig. 4A). Moreover, N-CDH expression in the static compression group was lower than that in the control group. In addition, the activity of the PI3K/Akt pathway showed a similar trend among these groups (Fig. 4B).

N-CDH knockdown attenuated the activation of the PI3K/Akt pathway, NP cell phenotype and matrix biosynthesis under dynamic compression

To investigate whether N-CDH up-regulation is responsible for the maintenance of the normal NP cell phenotype and NP matrix biosynthesis under dynamic compression, N-CDH expression was silenced using recombinant lentiviral vectors (Fig. 5). Subsequently, we found that the N-CDH knockdown inhibited the activation of the PI3K/Akt pathway and decreased N-CDH expression under dynamic compression (Fig. 6A). Additionally, the N-CDH knockdown decreased mRNA expression of NP cell-specific markers (keratin-19, FOXF1 and PAX1) (Fig. 6B) and suppressed the protein expression of the NP matrix macromolecules (aggrecan and collagen II) under dynamic compression (Fig. 6C).

Inhibition of the PI3K/Akt pathway attenuated matrix biosynthesis and NP cell phenotype under dynamic compression

To further investigate the role of the PI3K/Akt pathway and determine the relationship between N-CDH and the PI3K/Akt pathway, we used the inhibitor LY294002 (10 μ M) to inhibit the activation of the PI3K/Akt pathway in NP cells under dynamic compression (Fig. 7A). The results showed that the inhibitor had no effects on N-CDH expression in NP cells under

Fig. 5. Verification of N-cadherin (N-CDH) knockdown in nucleus pulposus (NP) cells. A: Real-time PCR analysis of N-CDH gene expression. B: Western blotting analysis of N-CDH protein expression.

Fig. 6. N-cadherin (N-CDH) knockdown attenuated the activation of the PI3K/Akt pathway, NP cell phenotype and matrix biosynthesis of nucleus pulposus (NP) cells encapsulated in the SIS cryogel under dynamic compression. A: Western blotting analysis of the expression of N-CDH and the activity of the PI3K/Akt pathway under dynamic compression after N-CDH knockdown in NP cells. B: Real-time PCR analysis of the expression of NP cell-specific markers (keratin-19, FOXF1 and PAX1) under dynamic compression after N-CDH knockdown in NP cells. C: Western blotting analysis of the expression of NP matrix macromolecules (aggrecan and collagen II) under dynamic compression after N-CDH knockdown in NP cells. All data are expressed as the mean $\pm$ SD (n=3). *: Indicates a significant difference ($p < 0.05$) between two groups; #: indicates a significant difference ($p < 0.05$) compared with the control NP cells or the Dyn group.

dynamic compression, but it significantly down-regulated the protein expression of NP matrix macromolecules (aggrecan and collagen II; Fig. 7B) and decreased the gene expression of NP cell-specific makers (keratin-19, FOXF1 and PAX1; Fig. 7C) under dynamic compression.

Discussion

Intervertebral disc degeneration is a disease that is positively correlated with aging [37, 38]. With the increase in the population of elderly people around the world, disc degeneration has attracted more and more attention [39]. Traditional surgical treatments are able to alleviate pain symptoms in most instances but are not able to biologically restore normal disc functions. It is well established that biological therapies for disc degeneration are better able to restore normal disc function than previous traditional treatments [2]. Importantly, the normal biological function of NP cells (i.e., matrix biosynthesis) is important for the efficacy of the tissue-engineered NP tissue in regenerating disc degeneration [40]. In the present study, we demonstrated that dynamic compression is more beneficial than static compression to increase the matrix synthesis of NP cells and that the N-CDH-PI3K/Akt pathway is involved in this regulatory process.

Degenerative discs often have a limited number of viable cells, which directly contributes to the decrease in normal disc NP matrix components and results in attenuated disc function [41]. During the past decades, various kinds of cell sources were adopted in constructing cell-based scaffolds to regenerate degenerative discs, such as autologous or xenogenous NP cells and mesenchymal stem cells (MSCs) [42, 43]. Apart from the cell source, superior biocompatibility is another necessary requirement for cell-based scaffolds to regenerate degenerative discs. In this study, we evaluated the cytotoxicity of SIS cryogels on NP cells and found that NP cells seeded on the SIS cryogel maintained proliferation and a high level of viability during the experiment duration, suggesting that this SIS cryogel has excellent cytocompatibility. This result is consistent with our previous experience and previous studies [44, 45].

Under physiological conditions, disc NP cells are subjected to various types of mechanical compression [17]. Previous studies and our own investigations have demonstrated that mechanical compression has an important role in regulating disc NP cell biology [18-21]. To investigate effects of mechanical compression on the matrix production of 3D-cultured NP cells, we analyzed for the first time the expression of NP cell-specific markers (keratin-19, FOXF1 and PAX1) under mechanical compression. Generally, the expression of these NP cell-specific markers was higher in the dynamic compression group than that in the static

Fig. 7. Inhibition of the PI3K/Akt pathway attenuated the matrix biosynthesis and NP cell phenotype but had no effects on N-cadherin (N-CDH) expression in nucleus pulposus (NP) cells under dynamic compression. A: Verification of the inhibitory effect of LY294002 on PI3K/Akt pathway activation. B: Western blotting analysis of the expression of N-CDH and NP matrix macromolecules (aggrecan and collagen II) under dynamic compression after inhibiting the PI3K/Akt pathway. C: Real-time PCR analysis of the expression of NP cell-specific markers (keratin-19, FOXF1 and PAX1) under dynamic compression after inhibiting the PI3K/Akt pathway. All data are expressed as the mean $\pm$ SD ($n=3$). *: Indicates a significant difference ($p<0.05$) between two groups.

compression group, indicating that dynamic compression is beneficial to maintaining a normal NP cell phenotype compared with the ability of static compression to maintain the desired phenotype. Maintenance of a normal NP cell phenotype is important for synthesizing and secreting a native tissue extracellular matrix. Collagen and proteoglycan are the main macromolecules within the NP matrix [41]. Here, dynamic compression also promoted the gene and protein expression of the NP matrix molecules (aggrecan and collagen II) compared with the expression levels in the cells under static compression, indicating that dynamic compression is helpful to promote the matrix biosynthesis of NP cells compared with the ability of static compression to do so. In line with this, several previous studies demonstrated that dynamic compression is more helpful than static compression in maintaining a healthy disc biology [11, 23, 46].

N-CDH-mediated signaling is helpful to maintain a normal NP cell phenotype and NP matrix synthesis *in vitro* [29, 30]. In this study, N-CDH up-regulation coincided with better maintenance of the NP cell phenotype and NP matrix synthesis in the dynamic compression group. However, N-CDH knockdown decreased the expression of NP cell-specific markers (keratin-19, FOXF1 and PAX1) and NP matrix macromolecules (aggrecan and collagen II) under dynamic compression, indicating that N-CDH up-regulation may be responsible for the positive effects of dynamic compression on the maintenance of the NP cell phenotype and NP matrix biosynthesis. Moreover, N-CDH knockdown decreased the activity of the PI3K/Akt pathway in the cells under dynamic compression, whereas inhibition of the PI3K/Akt pathway did not affect N-CDH expression but attenuated the maintenance of the NP cell phenotype and NP matrix synthesis under dynamic compression, further indicating that N-CDH sustains the NP cell phenotype and matrix biosynthesis through activating the PI3K/Akt pathway under dynamic compression.

This study also has several limitations. First, though we evaluated the effects of mechanical compression on the matrix synthesis of 3D-cultured NP cells, an *in vivo* animal model was not performed due to the difficulty in developing a device that can accurately apply dynamic compression to IVDs *in vivo*. Second, an *in vivo* animal repair model may be more helpful to investigate the efficacy of this kind of tissue-engineered NP tissue in regenerating degenerate discs after it is matured under dynamic compression stimulation. In the future, we will plan to perform an *in vivo* animal experiment to verify this point.

In conclusion, we studied the effects and potential mechanism of mechanical compression on the matrix production of 3D-cultured NP cells *in vitro*. Based on our findings, we demonstrate that dynamic compression can promote matrix biosynthesis in 3D-cultured NP cells *in vitro* through regulating the N-CDH-PI3K/Akt pathway.

Disclosure Statement

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

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