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IL-6 Reversing Degenerative Properties of Nucleus Pulposus Cells through Dedifferentiation

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Abstract

The viability and number of nucleus pulposus cells are the key to maintain the normal physiological function of the intervertebral disc. We found that after treatment with low dose of IL-6, the proliferative potency of nucleus pulposus cells derived from the low back pain patients increased, the apoptosis decreased, the expression levels of pluripotency markers (OCT4, Nanog), MSC specific markers (CD29, CD105) increased, and mature nucleus pulposus cell markers (ACAN, COL2A1) decreased, indicating that IL-6 may play an effective therapeutic role in intervertebral disc degeneration by inducing dedifferentiation of degenerative nucleus pulposus cells.

Keywords

Interleukin 6; Intervertebral disc degeneration; Nucleus pulposus cells; Dedifferentiation.

1. INTRODUCTION

With the aggravation of population aging, low back pain mainly caused by age-related intervertebral disc degeneration (IDD) has become a major socio-economic problem affecting the health and quality of life of the global population[1, 2].

A healthy intervertebral disc (IVD) consists of peripheral annulus fibrosus (AF), central nucleus pulposus (NP) and cartilage endplate (EP) adjacent to the vertebral body. Healthy nucleus pulposus tissue is gelatinous and consists mainly of proteoglycans and type II collagen forming an irregular loose network, which forms a high osmotic pressure that allows the IVD to resist compressive loads and be reversibly deformed[3, 4]. Nucleus pulposus cells are highly heterogeneous. Apart from mature nucleus pulposus cells, the NP tissue contains nucleus pulposus stem/progenitor cells with the characteristic like mesenchymal stem cells, which own the potentiality of self-renewal and differentiation[5].

Intervertebral disc degeneration begins in nucleus pulposus, where nucleus pulposus stem/progenitor cells play a major role in maintaining cellular homeostasis and regeneration after aging damage, and the decline in their biological activity is the critical factors of disc degeneration[6]. With the progression of intervertebral disc degeneration, the number of intervertebral disc cells decreases, the cell phenotype and function transforms, metabolic activity increases, synthetic activity decreases, and the production of extracellular matrix (ECM) of the intervertebral disc decreases. Moreover, many reports have been demonstrated that the expression of classical inflammatory cytokines[7], like tumor necrosis factor (TNF)-α, IL

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(interleukin)-1β, and Interleukin 6 (IL-6), are up-regulated in degenerated disc tissue, playing important roles in the occurrence and progression of IDD[8-10].

While considering the role of inflammatory cytokines in inducing NP degeneration, IL-6 which be considered not so 'classical', receives less attention than IL-1β and TNF-α because the expression levels of IL-6 are demonstrated to be affected by IL-1β or TNF-α, especially when the other two are applied to induce NPC degeneration. Interleukin 6, a cytokine with a wide variety of biological functions in immunity, tissue regeneration, and metabolism. A reported recently has demonstrated that IL-6 as a positive regulator of chondrogenic cell proliferation and maintenance through IL-6/gp130/STAT3 signaling[11]. The nucleus pulposus tissue has cartilage properties, and in this study, we found that IL-6 could promote proliferation, inhibit apoptosis and up-regulate the expression of pluripotent gene and MSC specific markers of nucleus pulposus cell, indicating IL-6 may exert beneficial effects in reversion the progress of IDD.

2. METHOD

2.1. Human nucleus pulposus tissues collection

This study was subject to approval by the medical ethics committee of Jinan University. Informed consent was obtained from each patient and all experiments with human samples conformed to human ethical guidelines. The NP tissues were collected from patients (n=8) who underwent surgery for lumbar disc herniation, and were evaluated with the Pfirrmann classification system.

Table.1 Characteristics details of the patients enrolled in the study. BP: back pain; RP: radicular pain.

Case no.	Age (years)	Gender	Symptoms	Disc level	Pfirrmann grading
1	45	M	BP	L4/5	IV
2	48	M	BP-RP	L5/S1	III
3	42	M	BP-RP	L5/S1	IV
4	49	F	BP	L4/5	IV
5	41	M	BP	L5/S1	III
6	38	F	BP	L5/S1	III
7	34	F	BP	L5/6	IV
8	49	F	BP	L4/5	III

2.2. NP cells isolation and culture

NP tissues were mechanically chopped into pieces (1mm3) and digested with 0.2 mg/mL collagenase II (Sigma, USA) for 4 hours at 37℃. After passing cells through a 70 mm cell strainer, the isolated NP cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12, Sigma, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Sigma, USA) under 5% O_2 and 5% CO_2 at 37℃. Passages 2-4 of NP cells were used for all the experiments.

2.3. Cell Counting Kit-8 assay

Cell viability was determined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan) following the manufacturer's instruction. The absorbance at 450nm was measured with a microplate reader (Bio-Rad Laboratories, USA).

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2.4. Flow cytometry assay

NP cells were seeded in 6-well plates and fixed with or without 5ng/ml IL-6 for 72 h. All experiments mentioned below were analyzed using flow cytometry (CellQuest software pro, BD Biosciences, USA).

Cell cycle was determined using Cell cycle detection kit (keygen, China) according to the instructions.

Cell apoptosis was evaluated by flow cytometry using Annexin V-FITC/PI Apoptosis Detection Kit (keygen, China).

For the dectection of MSC cell surface markers expression, NP cells were stained with fluorescence-tagged antibodies and detected with antibodies against human CD29 (1:100 dilution, Abcam), CD44 (1:100 dilution, Abcam), CD73 (1:100 dilution, Abcam), CD105 (1:20 dilution, Abcam) and appropriate isotype control - mouse IgG2A-FITC (1:100 dilution; Miltenyi Biotech) or IgG1-PE (1:100 dilution, Life Technologies).

2.5. Real-time quantitative PCR

Total RNA was isolated from NPCs using the TRIzol (Invitrogen, USA) and reversed transcribed using All-in-one cDNA synthesis supermix (biomake, USA). The cDNA ptoductions were amplified using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA) with 2xSYBR Green qPCR Master Mix (biomake, USA). Relative gene expression levels were normalized to GAPDH values and calculated by the comparative 2-△△Ct method. The specific primers used for qRT-PCR are shown in Table. 2.

Table 2. Primers used for this study

Gene name	Forward Primer(5'to3')	Reverse Primer(5'to3')
ACAN	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
COL2A1	GGCAATAGCAGGTCACGTACA	CGATAACAGTCTTGCCCCACTT
NANOG	GATTTGTGGGCCTGAACAAA	CAGATCCATGGAGGAAGGAA
OCT4	GAGAAGGAGAAGCTGGAGCA	AATAGAACCCCCAGGGTGAG
SOX2	TTTGTCGGAGACGGAGAAGC	TAACTGTCCATGCGCTGGTT
IL-1β	ACAGGATATGGAGCAACAAGTGG	CACGCAGGACAGGTACAGATTC
TNF-α	CAATGGCGTGGAGCTGAGAG	TCTGGTAGGAGACGGCGATG
IL-6	CTTCGGTCCAGTTGCCTTCTC	ATTCGTTCTGAAGAGGTGAGTGG
GAPDH	CAGCGACACCCACTCCTC	TGAGGTCCACCACCCTGT

2.6. Statistical analysis

The data are expressed as mean±standard deviation of at least three independent experiments. Comparisons between two groups were performed by T test and the experiments with more than two groups were analyzed by one-way analysis of variance (ANOVA). $P<0.05$ was considered significant difference.

3. RESULT

3.1. The impact of IL-6 to the cell viability of nucleus pulposus cells

To investigate the effect of IL-6 on the viability of nucleus pulposus cells, NPCs were exposed to different concentrations of 0, 5, 10, 20, 40, 80ng/ml IL-6 for 72h respectively. The CCK-8 result as shown in Figure 1, the concentrations of 5 ng/ml and 10 ng/ml IL-6 had no significant effect on the cell viability of nucleus pulposus cell, while the concentrations increased to 20 ng/ml or greater, cell viability was significantly inhibited (*, $P<0.005$).

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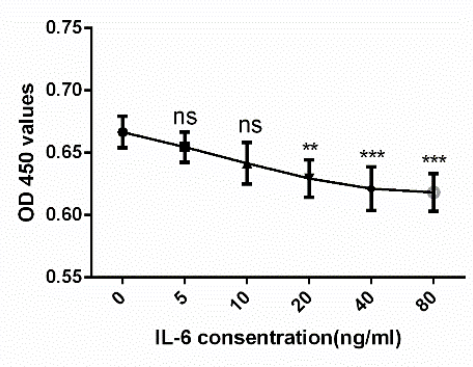

Figure 1. IL-6 declines the cell viability of nucleus pulposus cells in a high concentration

NP cells were exposed to different concentrations of IL-6 for 72 h respectively and and cell viability was detected by CCK-8 assay.All the data are displayed as the mean ± SD of three independent experiments, ns means means not significant, ** means p-value < 0.01 and *** means p-value < 0.001, compared with the control group.

3.2. IL-6 could promotes proliferation and inhibits apoptosis of nucleus pulposus cells

To further verify that low doses of IL-6 had no effect on promoting apoptosis, nucleus pulposus cell were treated with 5ng/ml IL-6 for 72 h and Annexin V/PI double staining assay was performed to examine the apoptosis activity. The result (Figure 2A) showed that the proportion of apoptosis cells treated with 5ng/ml IL-6 was significantly lower than those of the control group. Moreover, as shown in Figure 2B, the percentage of cells in the G1 phase after IL-6 treatment for 72 h was lower than that in the control group, while the S phase and G2 phase were higher than those in the control group, indicating that IL-6 can promote more nucleus pulposus cells to enter the cell cycle division phase, which in turn promotes cell proliferation.

Taken together, these results suggested that low doses of IL-6 could stimulate proliferation and inhibit apoptosis of nucleus pulposus cells.

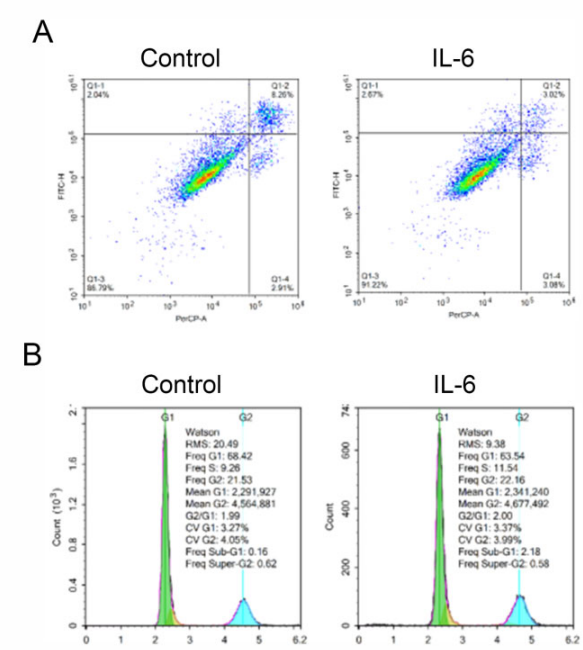

Figure 2. Low dose IL-6 exhibits a good proliferation inhibitory effect on nucleus pulposus cells.

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NP cells were treated with IL-6 (5 ng/ml, 72 h) and flow cytometry was performed to detect cell cycle distribution fractions. The percentage of cell cycle distribution fractions is displayed as the figure.

3.3. IL-6 promotes the expression of pluripotent and MSC markers of nucleus pulposus cells.

NPSCs have similar phenotypes of mesenchymal stem cells. We further detected flow cytometry finding that IL-6 could increase the proportion of the MSCs specific markers, CD29 and CD105 positive cells in nucleus pulposus cells (Figure 4A).

In order to further investigate whether IL-6 could promote the pluripotency of degenerated NPCs, the expression of transcription level of pluripotency markers and nucleus pulposus cell-related genes were detected by qRT-PCR. The results (Figure 4B) showed that after 5ng/ml IL-6 treatment, although the expression of pluripotent gene SOX2 was no significant differences, but the OCT4, NANOG were significant up-regulated in nucleus pulposus cell. Moreover, terminal differentiated mature nucleus pulposus cells related marker Aggrecan(ACAN) and type II of collagen (COL II, COL2A1) were significantly down-regulated.

These results were indicating that in heterogeneity nucleus pulposus cells, the percentage of NPSCs increased while differentiated NP cells decreased after treated with IL-6.

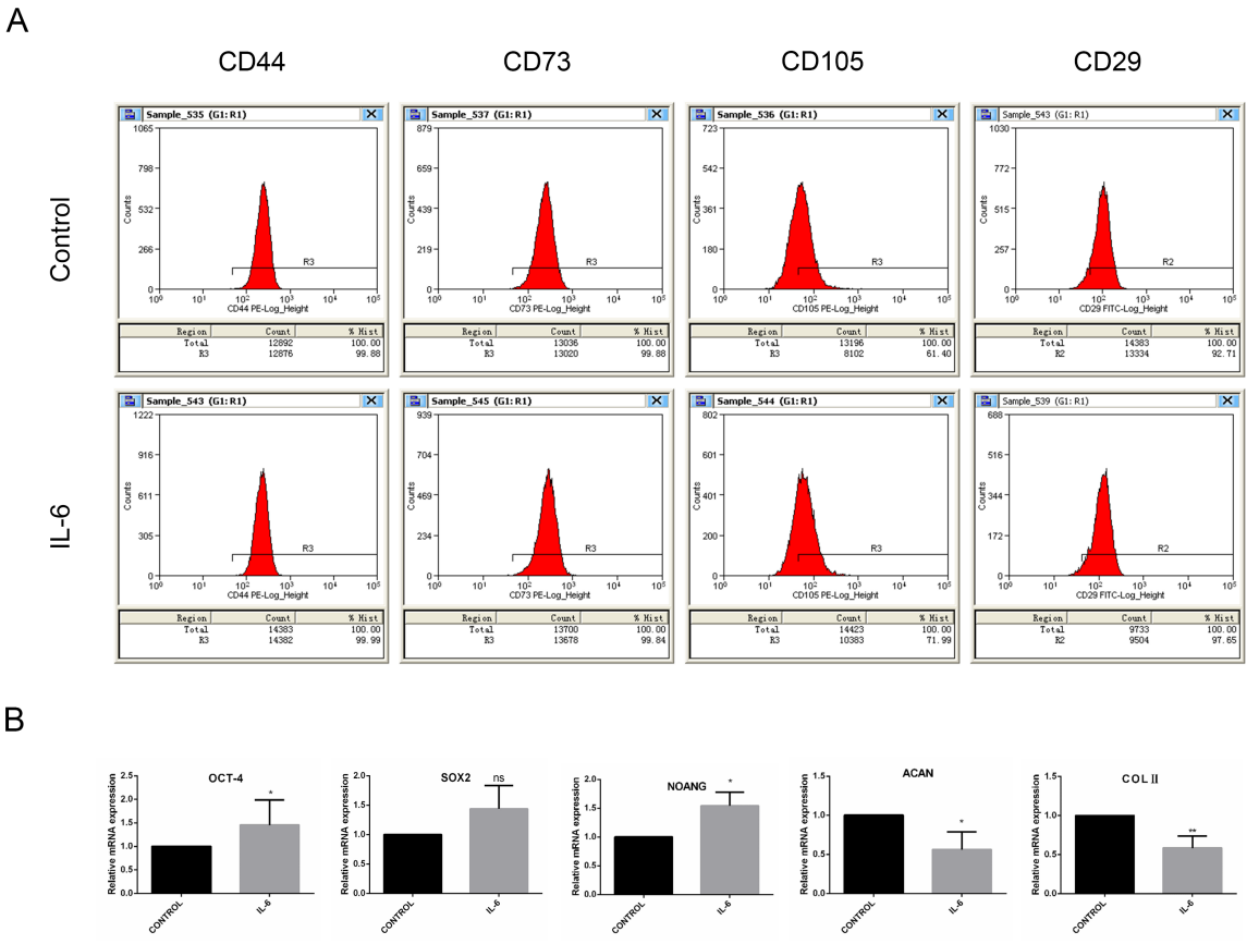

Figure 3. IL-6 promotes the expression of pluripotent and MSC markers of nucleus pulposus cells

A. NP cells were exposed to 5ng/ml IL-6 for 72h and subjected to flow cytometry analysis

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B. NP cells were pretreated with 5ng/ml IL-6 for 72h, and qRT-PCR analysis was performed to detect the mRNA levels changes of genes associated with pluripotency markers and nucleus pulposus cell-related genes, and GAPDH as a reference gene. All the data are displayed as the mean ± SD of three independent experiments, ns means not significant, * means p-value < 0.05 compared with the control group.

3.4. IL-6 reduces the expression of inflammatory factors in nucleus pulposus cells

Further, we investigated the expression of inflammatory factors in nucleus pulposus cells by qRT-PCR after treated with IL-6.

Interestingly, we found that 5ng/ml IL-6 treatment significantly down-regulated the expression of IL-1β and IL-6 itself in nucleus pulposus cell while the expression of TNF-α had no change as showed in Figure 5. The result indicating that insteading of promoting pro-inflammatory factors expression, low dose of IL-6 could reduce the expression of pro-inflammatory factors and alleviates the inflammatory response of cells.

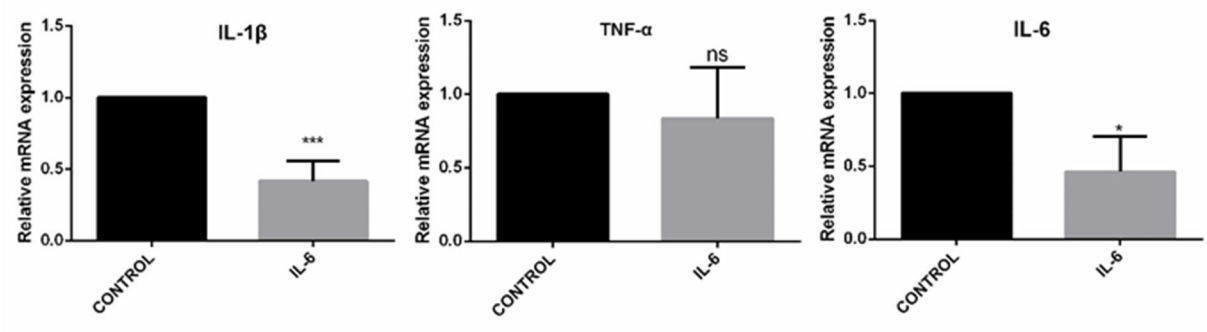

Figure 4. IL-6 decreases the mRNA expression levels of genes associated with the inflammatory factors in degenerative nucleus pulposus cells.

NP cells were treated with 5ng/ml IL-6 for 72h, and qRT-PCR assay was performed to detect the mRNA level changes of IL-6, TNF-α and IL-1β, and GAPDH as a reference gene. The data are the mean ± SD of three independent experiments. * means p-value < 0.05, *** means p-value < 0.001, and ns means not significant.

4. DISCUSSION

IL-6, a 26 kDa protein of 184 amino acids, that forms both monomers and dimers, signals through a type I cytokine receptor complex comprising the ligand-binding IL-6Rαchain (CD126), and the signal-transducing component gp130 (CD130)[12].

Classically, IL-6 has been viewed as a harmful accelerator of the process of intervertebral disc degeneration. IL-6 is secreted by intervertebral disc cells[13] and raised expression levels were present in herniation discs[10]. Studer et al. showed that IL-6 potentiates the catabolic actions of IL-1 and TNF-α on NP cells[14]. A human clinical studies founded that epidural application of tocilizumab, an anti-IL-6R monoclonal antibody, to the spinal nerve produced pain relief[15]. Beside from as a proinflammatory cytokine, IL-6 could as a anti-inflammatory cytokine playing a positive role in the regeneration of various tissues like wound skin[16] and damaged liver through the activation of IL-6/STAT3 signaling[17].

In our study, we found that IL-6 may also exert a positive role in regeneration of nucleus pulposus tissue. CCK8 assay found that IL-6 could dose-dependently inhibit the viability of degenerative nucleus pulposus cells, but the concentration of 5 ng/ml had no significant effect on the cell viability was used uniformly to carry out subsequent experiments. Cell activity and

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number of nucleus pulposus cells are key to maintain the normal physiological function of the intervertebral disc[6].

Interestingly, after treated with a low dose of IL-6, we found that the nucleus pulposus cell in S and G2 phase of the cell cycle were increased. In addition, IL-6 could inhibit nucleus pulposus cell apoptosis indicating that IL-6 may be able to alleviation the phenomenon of massive nucleus pulposus cell reduction during the IDD process.

Nucleus pulposus cells are heterogeneous, nucleus pulposus stem/progenitor cells play a major role in maintaining cellular homeostasis and regeneration after aging damage, and the decline in their biological activity is the critical factors of disc degeneration[6]. Our previous research has demonstrated the presence of NPSCs which high expression of MSC surface markers CD29, CD44, CD73, CD90 and CD105 at rates >95% in healthy nucleus pulposus tissue, and the expression of CD29 and CD105 were down-regulated with age reflecting the decline in regenerative capacity[18].After treated with low dose of IL-6,the expression of transcription level of pluripotent gene OCT-4, NANOG and MSC specific surface markers CD29 and CD105 were significantly up-regulated in nucleus pulposus cell while terminal differentiated mature nucleus pulposus cells phenotypic markers, ACAN and COL2A1 were significantly down-regulated. These results demonstrated that after treated with IL-6, the percentage of NPSCs increased while differentiated NP cells decreased in heterogeneity nucleus pulposus cells, indicating the differentiated cells may undergo de-differentiate and gain stemness.

After IL-6 treatment, IL-6 and IL-1β expression in degenerative nucleus pulposus cells were significantly down-regulated. Moreover, it was shown that IL-6 may have a regulatory process of negative feedback after promoting the regeneration of degenerative nucleus pulposus cells, further alleviating the inflammatory environment.

Our results suggest that IL-6 may exert therapeutic benefits in reversing the degenerative properties of nucleus pulposus cells by promoting proliferation and inhibiting apoptosis through dedifferentiation, but the mechanism of IL-6 exert function still needs to be further explored.

5. AVAILABILITY OF DATA AND MATERIALS

All the data and materials were presented in the main paper.

6. ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures performed for the present work were approved by the medical ethics committee of Jinan University. Specific informed consent was obtained in all cases.

7. DISCLOSURE STATEMENT

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

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