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Effect of siRNA interference on nerve growth factor in intervertebral disc inflammation rats

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ABSTRACT

Objective: To investigate the inhibition effect of siRNA interference on NGF induced by inflammatory factor IL-6, and IL-1 so as to provide novel targets for clinical treatment of discogenic low back pain. **Methods:** The intervertebral disc nucleus and annulus fibrosus cells of rats were separated .The cells were co-cultured with different concentrations (10 nmol/L, 20 nmol/L, 50 nmol/L, 100 nmol/L) of IL-6 and IL-1 β . The NGF- siRNA was leaded into the co-cultured cells with its import ability assessed by flow cytometry instrument tests,before and after which the NGF mRNA expression was detected by real-time Q-PCR and the NGF content was detected by ELISA. **Results:** Flow cytometry instrument test results showed that the NGF-siRNA cell conversion rate was 99.8%. Real-time Q-PCR detection results showed that compared with negative control group, the NGF mRNA expression of co-cultured cells treated by 10 nmol/L, 20 nmol/L, 50 nmol/L, 100 nmol/L IL-6 and IL-1 β were respectively raised 3.4, 3.7, 4.7, 3.7 times which were all significantly down-regulated after the import of NGF- siRNA. EILSA detection results showed that compared with negative control group, the NGF content of co-cultured medium treated by 10 nmol/L, 20 nmol/L, 50 nmol/L, 100 nmol/L I-L6 and IL-1 β were respectively raised 2.9, 3.3, 4.5, 7.4 times which were all significantly decreased after the import of NGF- siRNA. **Conclusions:** These molecular biological results suggest that inflammatory factor IL-6 and IL-1 β could stimulate NGF on intervertebral disc cells in vitro culture model and its efficiency is concentration dependent, while siRNA interference can inhibit the stimulation effect of IL-6 and IL-1 β on intervertebral disc cell, which provides a new targets for the clinical treatment of discogenic low back pain.

1. Introduction

Discogenic low back pain (DLBP) is the hot spot of modern orthopedic research with its complex etiology, unclear pathogenesis and absence of effective clinical treatment[1]. Epidemiological survey results showed an increasing incidence of DLBP as the upgrading of people's life pace and work intensity[2,3]. Electrophysiological studies indicated that, under normal physiological conditions, nerve fiber only exists at the outer layer of the fibrous ring of intervertebral disc, while constitutively distributed in the nucleus pulposus and the annulus inner layer in DLBP patients. This evidence suggests that the ingrowth of nerve fibers is the important pathogenesis mechanism associated with DLBP[4,5], which provide theoretical bases for the clinical treatment at molecular biology level. Nerve growth factor (NGF) overexpression and excessive secretion of inflammatory factors are the key in the DLBP pathogenesis[6,7]. This study aims to investigate the effect of siRNA interference on NGF in rats with inflammatory disc.

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2. Materials and methods

2.1. Experimental animals

Sixty Sprague-Dawley (SD) rats of specific pathogen free level were selected, which were half male and half female, weighing 180-240 g, with mean weight as (223±12.4) g, aged 2.5-3.5 months, with mean age of (2.94±0.73) months. They were provided by the Animal Center of Shandong University School of Medicine, China with license No. SYXK (Lu) 2007-0081.

2.2. Main instruments and reagents

Main instruments: PCR instrument (C1000 Thermal cycler, BIO-RAD); fluorescence quantitative instrument (IQ5, BIO-RAD); ice machine (AF100, Scotsman); cryogenic overweight centrifuge (Scanspeed 1730, Labogene); micro-oscillator (QL-901, Haimen Kirin Medical Instrument Factor, Haimen, China); flow cytometry (EPICS®ALTRA™, Beckman). Reagents: Lipofectamine™ 2000 (Invitrogen); Trizol total RNA extraction kit (Invitrogen); ELISA measurement kit (Chemicon); Oligo dT Primer, Random Primer, 5× PrimeScriptTM Buffer and other PCR reagents (TAKARA); ethanol and chloroform (domestic analytical pure); interleukin-6 and interleukin-1 β (Peprotechec Company); DMEM/F12 media and fetal bovine serum (Gibco).

2.3. Methods

2.3.1. In vitro culture of cells

Cells were cultured in vitro as the previously described methods[8]. The intervertebral disc was rinsed with ice-cold PBS three times immediately after removal, the nucleus pulposus tissue was isolated and cut into pieces in DMEM/F12 medium, then the pieces were centrifuged at 4 000 r/min, 4 ℃ and resuspended, incubated at 37 ℃ with 5% CO_2 for 8 h. The supernatant was removed after centrifugation at 4 000 r/min, 4 ℃, the cells were rinsed with PBS and digested with 0.25 % trypsin and 2% Ⅱ collagenase. After another 4 ℃ centrifugation at 4 000 r/min, the supernatant was removed and the cells was resuspended in DMEM/F12 medium, then the resuspended cells were counted to determine the number of cells up to 10^4/L. Subsequently the cells at 1× 10^4/L were transferred to 5% CO_2 incubator and cultured 37 ℃. After cells were cultured for 7 d, the cells were transferred to serum-free medium for additional 24 h. The cells were divided into a control group and an experimental group, culturing in DMEM/F12 medium containing 0.3% fetal bovine serum, experimental group was added with 10 nmol/L, 20 nmol/L, 50 nmol/L, 100 nmol/L of interleukin-6 and interleukin-1 β for 48 h.

2.3.2. Import and identification of NGF-siRNA

Cells at the logarithmic growth phase were incubated onto the six-well culture plate at the density of 2.5×10^5/mL. In accordance with the kit instructions, DEPC H_2O and Lipofectamine™ 2000 were diluted and then mixed with NGF-siRNA, the mixture was incubated for 10 minutes and added to the culture plate (2.5×10^5/hole) in 37 ℃, 5% CO_2 incubator. The culture medium was changed promptly. The conversion rate of the cells was determined with flow cytometry to assess transfer efficiency.

2.3.3. Determination of NGF mRNA

Q-PCR primer sequence of NGF mRNA was as follows. Forward: 5′-ACT TCA GCA TTC CCT TGA CAC A-3′, Reverse: 5′-ACG GGC AGC TAT TGG TTC AG-3′, Probe: 5′-FAM-CCC TCC GCA GAG CCC GCA-TAMRA-3′; GAPDH, the reference gene, primer sequence: Forward: 5′-AGG GCT GCC TTC TCT TGT GA-3′, reverse 5′-AAC TTG CCG TGG GTA GAG TCA-3′, probe: 5′-FAM-CCA TCA ACG ACC CCT TCA TTG ACC TC-TAMRA-3′. Reverse transcription and PCR amplification was automatically performed with by PCR instrument, and paired using a conventional system. Reaction conditions: Reverse transcription: 7 ℃ for 15 min, 85 ℃ for 5 s; amplification: 93 ℃ for 2 min; 93 ℃ for 15 s, 55 ℃ for 25 s, 72 ℃ for 25 s, totally 40 cycles; 72 ℃ for 10 min.

Ct value (the number of copies/μL cDNA) refers to the number of cycles when fluorescence signal reaches the preset threshold value, Ct values for each template has a log-linear relationship with the initial copy number, *ie*., the higher the initial copy number is, the smaller Ct value is. Due to the difference of total RNA concentration in different samples, NGF mRNA expression is calculated according to the following formula: $C_{ttarget\ gene}/Ct_{reference\ gene}$.

2.3.4. Determination of NGF

Cell culture medium 100 μL were collected to measure NGF levels in strict accordance with the ELISA kit instructions.

2.4. Statistical analysis

Data were analyzed using SPSS 17.0 software. NGF mRNA expression and NGF levels were normally distributed and expressed as mean±SD. The difference before and after the introduction NGF-siRNA was compared with paired *t*-test. Multiple-group comparison was performed using one-way

analysis of variance. $\alpha = 0.05$ was considered as significant difference.

3. Results

3.1. Identification of cells

Toluidine blue staining showed that the nucleus pulposus cells stained were strongly positive (Figure 1A); fibrous ring cells were purple in nuclei and dark blue in cytoplasm, most of the stained cells were fusiform shaped, a large number of vacuoles were visible in the cytoplasm (Figure 1B). Safranin O staining showed that, nucleus pulposus cells stained were positive, the cytoplasm was pink, and brownish red perinuclear granules were visible (Figure 2A); fibrous ring cells were lightly stained (Figure 2B). Cells were successfully cultured in vitro, nucleus pulposus cells and fibrous annulus cells were obtained.

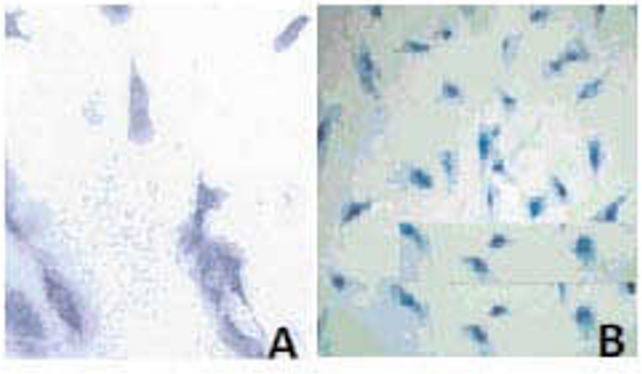

Figure 1. Dyeing appraisal results of intervertebral disc nucleus pulposus cells and annulus fibrosus cells of rats.
NOTE: A, nucleus pulposus cells, B, annulus fibrosus cells; dyed with toluidine blue, ×200.

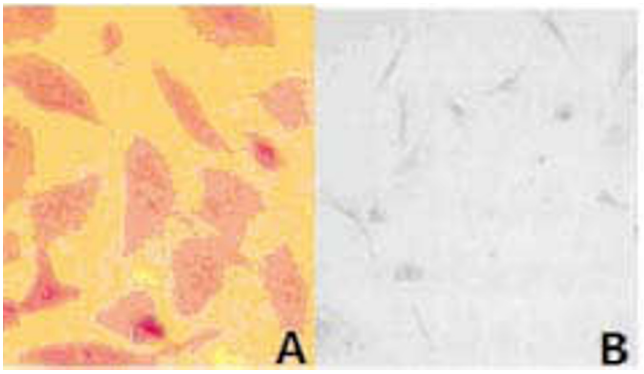

Figure 2. Dyeing appraisal results of intervertebral disc nucleus pulposus cells and annulus fibrosus cells of rats.
NOTE: A, nucleus pulposus cells, B, annulus fibrosus cells; dyed with sarranine, ×200.

3.2. Conversion rate of cells

Flow cytometry analysis showed that, NGF-siRNA cell conversion rate was up to 99.8% (Figure 3).

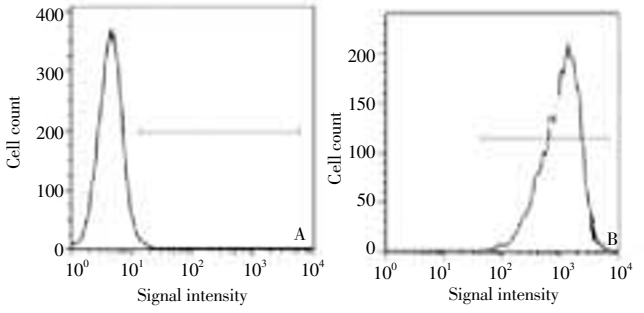

Figure 3. Flow cytometry results of cultured cells before and after NGF-siRNA.
(A) Before NGF-siRNA; (B) After NGF-siRNA; marked by Cy5.

3.3. NGF mRNA relative expression

Q-PCR results of GAPDH and NFG mRNA are shown in

Table 1
Compare of NGF mRNA expression before and after NGF-siRNA interference (mean±sd, nmol/L).

Time	Control group	10 nmol/L group	20 nmol/L group	50 nmol/L group	100 nmol/L group	*F* value	*P* value
Preimplementation	0.114±0.021	0.388±0.032*	0.422±0.037*	0.536±0.041*	0.912±0.064*	7.714	0.000
After import	0.069±0.014	0.224±0.017*	0.231±0.024*	0.322±0.031*	0.476±0.039*	4.121	0.007
Difference	0.045±0.013	0.164±0.016	0.191±0.021	0.214±0.027	0.436±0.041	5.772	0.001
t value	2.644	2.214	2.384	2.319	2.654		
P value	0.012	0.023	0.017	0.018	0.011		

Table 2
Compare of NGF contents before and after NGF-siRNA interference (man±sd, ng/L)

Time	Control group	10 nmol/L group	20 nmol/L group	50 nmol/L group	100 nmol/L group	*F* value	*P* value
Preimpleme	21.40±2.36	62.10±11.30*	70.60±14.70*	96.30±21.40*	158.00±33.40*	11.240	0.000
After import	11.30±2.03	41.10±21.30	45.60±22.70	54.90±24.70	71.30±31.60	6.774	0.000
Difference	10.10±1.94	21.00±16.20	25.10±16.40	41.40±17.30	86.70±33.40	7.254	0.000
t value	2.453	2.382	2.315	2.514	2.653		
P value	0.014	0.017	0.019	0.013	0.011		

Note: compared with control group,*$P<0.05$.

Figure 4. A and B are respectively the standard amplification curve of GAPDH and NFG mRNA. The standard amplification curve at different concentrations showed good parallelism and surface smooth, indicating that the amplification conditions were accurate with a high specificity. C and D are respectively the standard linear regression graphs of GAPDH and NFG mRNA. The linear regression graphs have a good regression and high reproducibility, and the regression coefficient R^2 was>0.99. E and F are respectively the specimen amplification curve of GAPDH and NFG mRNA. Before the NGF-siRNA intervention, NGF mRNA expression in 10 nmol/L, 20 nmol/L, 50 nmol/L, 100 nmol/L groups was increased 3.4, 3.7, 4.7, 8.0 times compared with the control group. After NGF-siRNA intervention, NGF-mRNA relative expression in each group was decreased 39.5%, 45.5%, 45.3%, 39.9%, 47.8% compared with before intervention (P<0.05; Table 1).

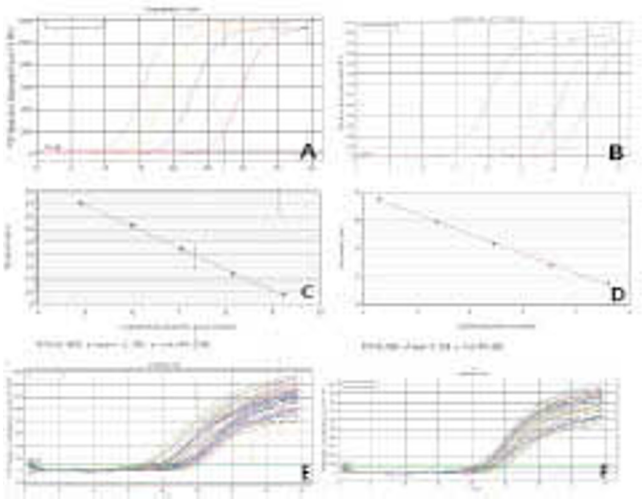

Figure 4. Q-PCR results of GAPDH and NFG mRNA.
A and B represent standard amplification curve for GAPDH and NFG mRNA; C and D represent standard regression line for GAPDH and NFG mRNA; E and F represent sample amplification curve for GAPDH and NFG mRNA.

3.4. Determination of NGF levels

Before NGF-siRNA intervention, NGF levels in 10 nmol/L, 20 nmol/L, 50 nmol/L, 100 nmol/L groups were increased 2.9, 3.3, 4.5, 7.4 times compared with the control group; after NGF-siRNA intervention, NGF levels in each group were decreased 47.2% , 33.8% , 35.4%, 43.0%, 54.9% compared with before intervention (P<0.05; Table 2).

4. Discussion

DLBP is also known as intradiscal disorders, showing a progressive degeneration. As the progress of the disease, adjacent segments may be affected, eventually involving the whole vertebral system[9]. From the view of morphological pathology, DLBP patients are accompanied with multiple fractures and damage in intervertebral disc and nucleus pulposus, as well as loss of bone density, leading to abnormal lordosis, motion segment instability and neurological mechanical injury; from the view of neuropathology, increased innervation density, lower nociceptor threshold value, and a wide range of pain-sensitive reaction are detected in DLBP patients; from the view of biological pathology, the mechanical injury and pain-sensitive response in DLBP patients are mediated by inflammatory reaction, they are mutually complementary and interdetermined.

Previous studies[11,12] have shown that, a large number of inflammatory mediators such as tumor necrosis factor-α, interleukin-1β, interleukin-6 are involved in the process of intervertebral disc damage, and their contents in intervertebral disc are greatly changed at acute onset period. This evidence suggests that inflammatory mediators are an important incentive for the exacerbations of DLBP, but the underlying mechanism remains unclear. In this study, inflammatory intervertebral disc cells were treated with interleukin-1β and interleukin-6. The results showed that, before the intervention of interleukin-1β and interleukin-6, there were low NGF levels and weakly positive mRNA expression in the model of intervertebral disc inflammation established using Freund's adjuvant; after the intervention, NGF levels and NGF mRNA expression were significantly up-regulated, in a dose-dependent manner. Along with the increase of interleukin-1β and interleukin-6 concentrations, the NGF levels and NGF mRNA expression was gradually increased. This evidence suggested that secretion of inflammatory cytokines interleukin-1β and interleukin-6 is the important cause of NGF activation in the rat intervertebral disc. NGF supplies one of the basic nutrients for autologous neurons and may participate in a series of fundamental physiological functions of the nerve cells, such as growth, development, repair, and regeneration. NGF is ubiquitously distributed in the rat embryo intervertebral discs and can maintain the spinal nerve cells differentiation and proliferation[13]. In healthy adult rats, NGF is mainly located at the outer edge of intervertebral disc, and scarcely seen in nucleus pulposus; their abnormal expression is closely related to the initiation of self-repair of damaged nerve cells, thus having been widely concerned in bone and neurosurgery injury research[14]. In addition, NGF abnormal expression is considered the major pathological mechanism of nerve

ingrowth in DLBP, and inflammatory pain signaling in DLBP is mainly mediated by NGF-dependent neurons[15]. NGF can not only induce the nerve ingrowth in DLBP, but also involves inflammatory pain sensitization. Therefore, we hypothesized that NGF abnormal expression caused by interleukin-1β and interleukin-6 is an important mechanism of intervertebral disc injury. Based on the above speculation, we preliminarily determined the NGF abnormal expression using siRNA interference, the results showed that NGF content and mRNA expression levels were significantly reduced after the intervention of NGF-siRNA. This finding indicates that, NGF siRNA interference can inhibit the stimulation effect of proinflammatory cytokines interleukin-6 and interleukin-1β on NGF in intervertebral disc cells, which provide a novel approach for the treatment of DLBP.

The present study revealed the possible mechanism of proinflammatory cytokines interleukin-6 and interleukin-1β-induced DLBP from the in vitro cell levels, and provided crucial data for the feasibility of NGF-siRNA interference in the treatment of DLBP. However, the limitations of this study lies on the use of cultured rat intervertebral disc cells in vitro. On the one hand, we prepared the models of inflammatory intervertebral disc using Freund's adjuvant according to the principle of that, direct stimulation of 65 kD heat shock protein, an arthritis-induced antigen in Mycobacterium tuberculosis, could trigger inflammatory reaction within 10-20 days and reach the peal at 20 days. The exact mechanism associated with DLBP is very complex, and most of affected patients suffer from a long-term, chronic lesion. Therefore Freund's adjuvant-caused model of inflammatory disc only partially mimics inflammatory reaction in the DLBP, and the establishment of models needs further improvement. On the other hand, the present study only confirmed the in vitro applicability of NGF-siRNA interference in the treatment of DLBP, and further investigations are needed for in vivo tests.

Conflict of interest statement

We declare that we have no conflict of interest.

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