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CXCR3 mediates chondrocyte injury through regulating nitric oxide

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Abstract. – OBJECTIVE: As a common joint disease, osteoarthritis exhibits increasing trend in recent years. C-X-C motif chemokine receptor 3 (CXCR3) is a kind of chemokine with the characteristic of recruiting inflammatory cells. Its function in osteoarthritis has not been clarified. This study aims to explore the role of CXCR3 in cartilage injury by affecting unfolded protein response (UPR) pathway.

PATIENTS AND METHODS: The sample was obtained from osteoarthritis patients to test CXCR3 expression by Real-time polymerase chain reaction (PCR). Chondrocyte apoptosis model was established *in vitro* induced by interleukin 1β (IL-1β) and sodium nitroprusside (SNP). CXCR3 level was downregulated by using siRNA. Cell apoptosis was determined by using transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay. UPR pathway related factors C/EBP homology protein (CHOP) and glucose regulated protein 78 (GRP78) protein expressions were tested by using Western blot.

RESULTS: CXCR3 protein level significantly increased in osteoarthritis patients (2.66 ± 0.25 vs. 1.00 ± 0.05, $p<0.05$). CXCR3 siRNA significantly reduced nitrate level in chondrocytes induced by IL-β (35.22 ± 1.76 vs. 17.82 ± 0.89, $p<0.05$) without affecting cell apoptosis (1.13 ± 0.05 vs. 0.859 ± 0.04, $p>0.05$). CXCR3 siRNA markedly downregulated nitrate level in chondrocytes (50.63 ± 2.53 vs. 30.63 ± 1.63, $p<0.05$) and alleviated cell apoptosis induced by SNP (1.98 ± 0.10 vs. 1.25 ± 0.06, $p<0.05$). UPR pathway C/EBP homology protein (CHOP) and glucose regulated protein 78 (GRP78) participated in the process of chondrocyte apoptosis.

CONCLUSIONS: Endoplasmic reticulum (ER) stress signaling pathway CHOP and GRP78 are involved in CXCR3 receptor attenuating chondrocyte apoptosis induced by SNP.

Key Words:
CXCR3, Chondrocyte, Apoptosis, Endoplasmic reticulum stress.

Introduction

Osteoarthritis is a common joint disease that commonly occurs in senile patients. There are about 3 million patients diagnosed as osteoarthritis each year[1,2]. Joint replacement is usually used in clinic for the patients with advanced osteoarthritis[3]. However, the pathological process of osteoarthritis is still unclear. Moreover, limited methods were found to effectively treat osteoarthritis. Thus, it is of great significance to explore the mechanism of osteoarthritis and provide reminder for its treatment. Chondrocyte apoptosis and extra-cellular matrix destroy are considered as characteristics of osteoarthritis[4,5]. In addition, mechanical injury, age, and inflammation also participate in the process of osteoarthritis. Chondrocyte apoptosis is one of the signs of osteoarticular degeneration. It is showed that endoplasmic reticulum (ER) stress is one of the main pathways in inducing cell apoptosis[6]. ER stress can activate a series signaling pathway named unfolded protein response (UPR). UPR signaling pathway induces cell death by dissociating chaperone glucose regulated protein 78 (GRP78) and promoting transcriptional factor C/EBP homologous protein (CHOP) expression[7,8]. URP pathway is activated in chondrocytes treated by inflammatory factors or other stimulus such as nitric oxide, and attenuates protein translation through phosphorylating protein, thus stressing ER[9]. Released GRP78 selectively cut mRNA to produce protein spliceosome with high transcription activity[10]. These spliceosome further accelerate ER degeneration to induce cell apoptosis[10]. It is found that multiple factors are involved in chondrocyte apoptosis, including tumor necrosis factor α, interleukin 1β, and nitric oxide[11-13]. However, its specific mechanism still needs further investigation. We aimed at clarifing the role of C-X-C motif chemokine receptor 3 (CXCR3) in cartilage injury by affecting UPR pathway.

Patients and Methods

Patients

Cartilage tissue was collected from 10 patients received total knee arthroplasty in our hospital.

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Normal cartilage tissue was obtained from 10 patients without osteoarthritis-received surgery because of accident. All the patients had signed informed consent. This study was approved by the Ethics Committee of our hospital. The basic information was listed in Table I.

Materials

Inflammatory factor IL-1β and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) medium, chondrocyte medium, serum, and related reagents were bought from Gibco (Grand Island, NY, USA). Rabbit anti human CXCR3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and horseradish-peroxidase (HRP) conjugated goat anti-rabbit antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Cell apoptosis detection kit was provided by R&D systems Inc. (Minneapolis, MN, USA).

Cell Culture

Human chondrocytes were purchased from Cell Library, Fudan University (Shanghai, China). The cells were seeded at $1 \times 10^4/cm^2$ and maintained in medium containing chondrocyte growth factor, penicillin, and streptomycin. The cells were digested and passaged when their fusion reached 70%. The cells were treated by inflammatory factor IL-1β at 5 ng/mL or SNP at 1 mM for 24 h when the cell fusion reached 70-80%.

siRNA Interference

CXCR3 siRNA (GGAUUUCAGCCUGAACUUUUU) or control siRNA (AAAGUCGACCUUCAGUAAGGA) was transfected to chondrocytes by using lentivirus. The lentivirus was bought from Hanbio (Shanghai, China). The cells in 6-well plate were incubated in 1 ml serum free medium and 50 μl lentivirus for 6 h. Then, the cells were further incubated for 18 h after adding another 1 ml medium. After changing the medium, they were cultured for 48 h to test the silence efficiency.

Nitrate Detection

Nitrate analysis kit (Cayman, CA, USA) was used to test nitrate level according to the manual. The chondrocyte medium was incubated in nitrate reductase and related factors at room temperature for 1 h. After Griess reagent was added, the sample was tested at 540 nm.

Cell Apoptosis Detection

The cells were cracked and incubated in anti-histone-biotin and anti-DNA peroxidase antibodies (Abcam Biotech., Cambridge, MA, USA). Total protein was quantified by bicinchoninic acid (BCA) kit (Beyotime Biotech., Shanghai, China). Cell apoptosis was calculated as absorbance value in treatment group/absorbance value in control.

Real-time PCR

Total RNA was extracted by TRIzol and reverse transcribed to cDNA by PrimeScript RT (Tiangen Biotech Co. Ltd., Beijing, China). The reverse transcription system contained 2 μl 5× PrimerScript Buffer (Tiangen Biotech Co. Ltd., Beijing, China), 0.5 μl PrimerScrip RT Enzyme Mix I, 0.5 μl Oligo dT Primer (50 μM), 0.5 μl Randome 6 mers (100 μM), 500 ng total DNA, and Rnase free dH_2O. The reverse transcription was performed at 37°C for 15 min and 85°C for 5 s. PCR reaction system contained 3.2 μl Rnase free H_2O, 0.4 μl primer (10 μM), 1 μl cDNA, and 5 μl SYBR Green Real-time PCR Master Mix (Tiangen Biotech Co. Ltd., Beijing, China). The PCR reaction was performed at 94°C for 4 min, followed by 40 cycles of 94°C for 40 s, 52°C for 40 s, and 72°C for 40 s. SYBR Premix ExTaq was performed on ABI 7500. The result was analyzed using $2^{-\Delta\Delta Ct}$ method. GAPDH was selected as loading control. The primers were designed and synthetized by Sangon (Table II).

Western Blot

The cells were collected and washed by PBS for twice. After that, the cells were cracked by ristocetin-induced platelet aggregation (RIPA) buffer containing protease inhibitor and quanti-

Table I. Clinical information of osteoarthritis patients and normal control.

Characteristics	Osteoarthritis (n=10)	Control (n=10)	*p*-value
Age (year)	39.8 ± 4.35	37.0 ± 5.32	0.21
Gender (male, %)	5 (50%)	5 (50%)	1

Table II. Primer sequences.

Gene	Forward	Reverse
CXCR3	AAAACAGCACCTCTCCCTA	TCTGAACTTCACTCCCACA
GRP78	CAACTCACGTCCAACCCGGAGAA	TGTCTTGGTTTGCCCACCTCCG
CHOP	AAGAATCAAAAACCTTCACTACTCTTGACC	TGGGAGGTGCTTGTGACCTCTGC
GAPDH	TGACAACTTTGGTATCGTGGAAGG	AGGCAGGGATGATGTTCTGGAGAG

fied by BCA method. After the protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it was transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked by Tris-buffered saline and Tween-20 (TBST-20) containing 5% skim milk and incubated in corresponding primary antibody (1:1000) at 4°C overnight. The membrane was incubated in horseradish-peroxidase (HRP) conjugated antibody and developed by enhanced chemiluminiscence (ECL, Amersham Biosciences, Piscataway, NJ, USA). The optical density was measured by Image J Scion Image (version 2.0, Frederick, MD, USA).

Statistical Analysis

All data analyses were performed on SPSS 19.0 software (IBM, Armonk, NY, USA). The measurement data were presented as mean ± standard deviation. The Student's *t*-test was used to compare the differences between two groups. Tukey's post hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was considered as statistical significance.

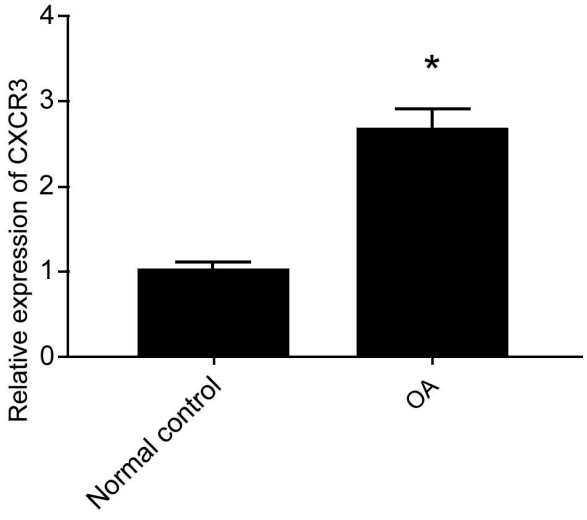

Figure 1. CXCR3 expression significantly increased in osteoarthritis patients. Real-time PCR detection of CXCR3 expression in cartilage tissue. $^{*}p<0.05$, compared with normal control.

Results

CXCR3 Expression Significantly Increased in Osteoarthritis Patients

Real-time PCR was applied to test CXCR3 expression in cartilage tissue from 10 cases of osteoarthritis patients and normal control. CXCR3 level significantly increased in osteoarthritis patients (2.66 ± 0.25 *vs.* 1.00 ± 0.05, $p<0.05$, Figure 1).

CXCR3 Level Elevated in Chondrocytes Treated by IL-1β and SNP

Chondrocytes cultured *in vitro* were treated by IL-1β and SNP, respectively. Western blot was adopted to detect CXCR3 level in chondrocytes. IL-1β and SNP treatment significantly up-regulated CXCR3 expression in chondrocytes compared to the control (Figure 2).

The Effects of CXCR3 Intervention on Chondrocyte Function Treated by IL-1β

The results showed that CXCR3 level was significantly elevated in chondrocytes after IL-1β treatment ($p<0.05$). We selected siRNA to down-regulate CXCR3 expression. As shown in Figure 3, CXCR3 siRNA significantly reduced nitrate level in chondrocytes induced by IL-β (35.22 ± 1.76 *vs.* 17.82 ± 0.89, $p<0.05$) without affecting cell apoptosis (1.13 ± 0.05 *vs.* 0.859 ± 0.04, $p>0.05$).

The Influence of CXCR3 siRNA on Chondrocyte Function treated by SNP

It was demonstrated that CXCR3 level apparently elevated in chondrocytes after IL-1β treatment. We used siRNA to down-regulate CXCR3 expression. CXCR3 siRNA markedly downregulated nitrate level in chondrocytes (50.63 ± 2.53

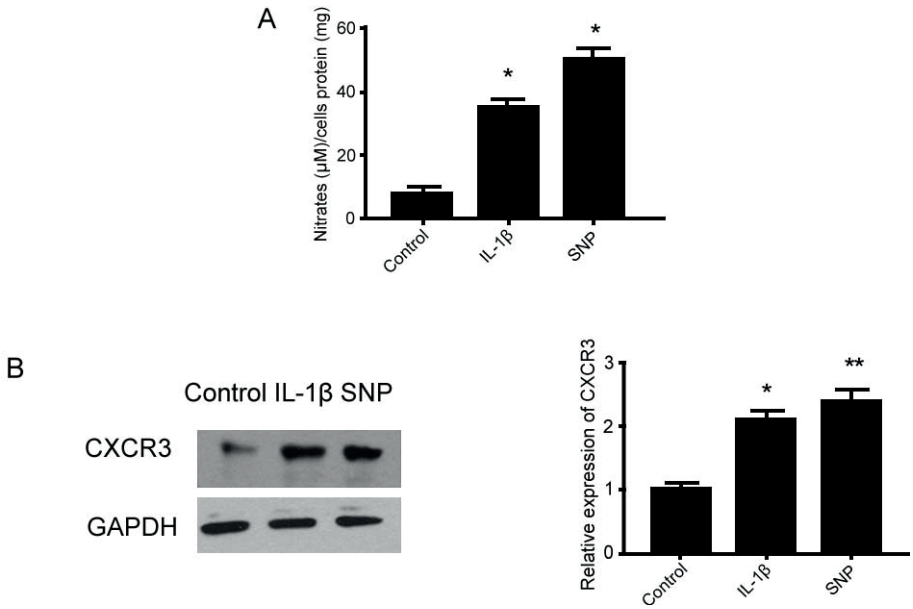

Figure 2. CXCR3 level elevated in chondrocytes treated by IL-1β and SNP. A, Nitrate detection suggesting successful modeling. B, Western blot detection of CXCR3 level. $^{*}p<0.05$, $^{**}p<0.01$, compared with normal control.

vs. 30.63 ± 1.63, $p<0.05$) and alleviated cell apoptosis induced by SNP (1.98 ± 0.10 *vs.* 1.25 ± 0.06, $p<0.05$) (Figure 4).

Mechanism of CXCR3 siRNA on Chondrocyte Induced by SNP

Since ER stress protein was considered to be involved in chondrocyte apoptosis, we performed Real-time PCR to test the effect of ER stress protein CHOP and GRP78 on chondrocyte apoptosis induced by SNP. CXCR3 siRNA significantly affected CHOP and GRP78 to participate in chondrocyte apoptosis.

Discussion

We collected specimens from osteoarthritis patients and tested CXCR3 expression by Real-time PCR. We further established chondrocyte apoptosis model induced by IL-β or SNP[11-13]. We also transfected CXCR3 siRNA to chondrocytes to down-regulate CXCR3 expression and detect cell apoptosis. Furthermore, we used Real-time PCR to assess UPR related pathway CHOP and GRP78 protein expressions to reveal the related mechanism. Our data demonstrated that CXCR3 protein significantly upregulated in osteoarthri-

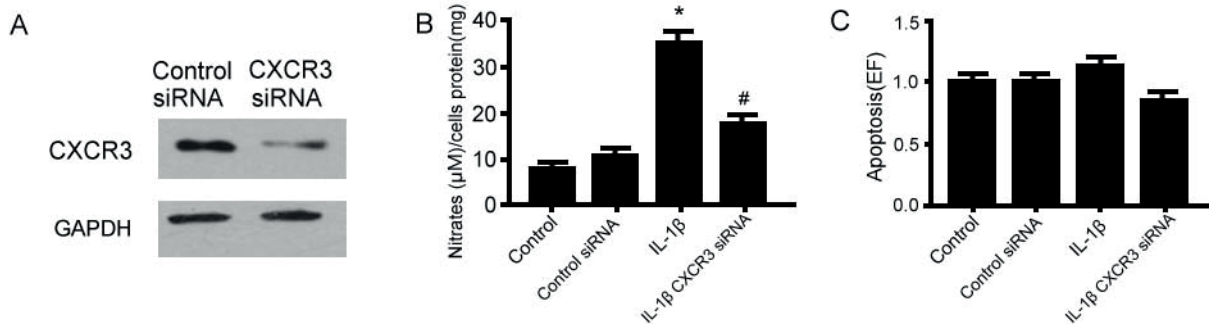

Figure 3. The impact of CXCR3 intervention on chondrocyte function treated by IL-1β. ***A,*** Western blot detection of CXCR3 siRNA. ***B,*** CXCR3 siRNA significantly reduced nitrate level. ***C,*** CXCR3 siRNA failed to affect chondrocyte apoptosis. $^{*}p<0.05$, compared with normal control. $^{\#}p<0.05$, compared with IL-1β group.

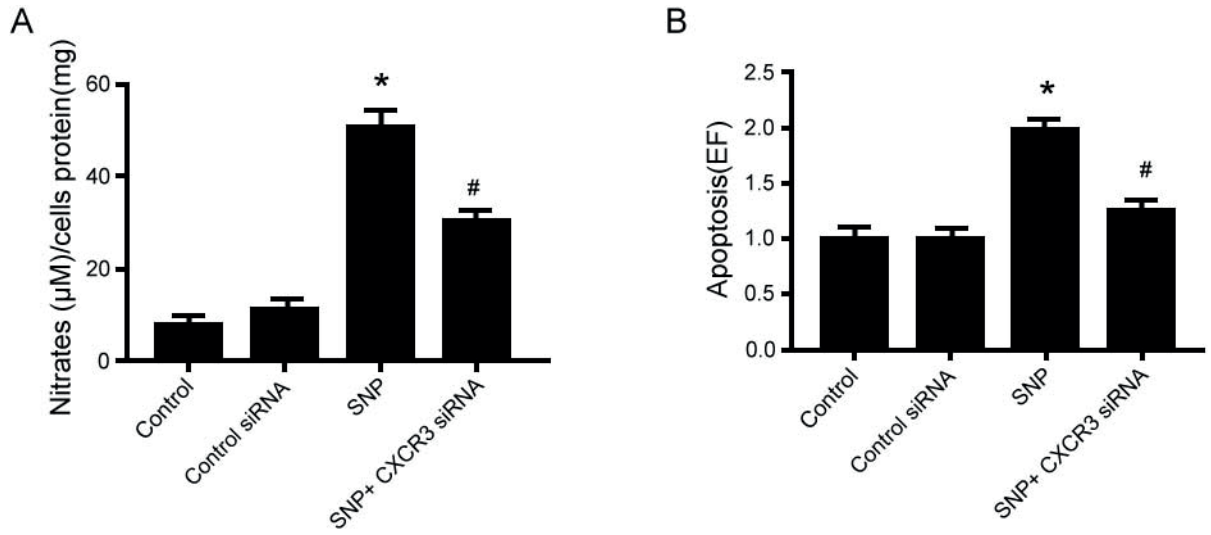

Figure 4. CXCR3 siRNA markedly downregulated nitrate level in chondrocytes ***(A)*** and alleviated cell apoptosis induced by SNP ***(B)***. $^{*}p<0.05$, compared with normal control. $^{\#}p<0.05$, compared with SNP group.

tis patients. CXCR3 siRNA significantly reduced nitrate level in chondrocytes induced by IL-β without affecting cell apoptosis. CXCR3 siRNA markedly downregulated nitrate level in chondrocytes and alleviated cell apoptosis induced by SNP. UPR pathway CHOP and GRP78 participated in the process of chondrocyte apoptosis. To our knowledge, this is the first study exploring the regulatory role of CXCR3 on chondrocyte apoptosis.

Chemokine is a kind of protein molecule guiding cell chemotactic migration. They are thought to be associated with the pathophysiological process of a variety of diseases, especially the inflammatory disease. Essentially, osteoarthritis is also a type of inflammatory disease. Therefore, we speculated that chemokines may affect the pathophysiological process of osteoarthritis. As a kind of chemokine receptor, CXCR3 can interact with numerous cytokines, including CXCL[9], CXCL[10], and CXCL[11]. Previous studies showed that CXCR3 expresses in the activated $CD4^{+}$ and $CD8^{+}$ T cells, NK cells, glial cells, monocytes, and dendritic cells. Moreover, it is highly expressed in Th1 polarized T cells[14]. CXCR3 is considered to participate in T cell migration. Further researches exhibited that CXCR3 is also expressed in chondrocytes and plays an important role in activating metabolic pathway and chondrocyte hypertrophy[15]. In addition, it was demonstrated that chemotactic factor elevated in osteoarthritis patients and joint injury patients[16]. Thus, we enrolled 10 cases of osteoarthritis patients and 10 normal controls, and adopted Real-time PCR to test CXCR3 expression. CXCR3 level significantly increased in osteoarthritis patients compared with control.

Various investigations showed that the chondrocyte apoptosis is one of the important reasons of osteoarthritis occurrence and development. Multiple factors were thought to be involved in the chondrocytes apoptosis process of osteoarthritis, including IL-1β, TNF-α, and nitric oxide[17-19]. Exploration of the factors restraining chondrocyte apoptosis is a crucial idea in the treatment of osteoarthritis. We applied IL-1β and SNP to establish chondrocyte apoptosis model, so as to provide basis to investigate the impact of CXCR3 on chondrocyte apoptosis. Our results indicated that CXCR3 siRNA significantly reduced nitrate level in chondrocytes induced by IL-β without affecting cell apoptosis. Since CXCR3 siRNA markedly downregulated nitrate level in chondrocytes, we proposed that CXCR3 may participate in chondrocyte apoptosis induced by SNP.

Similarly, CXCR3 siRNA declined chondrocyte apoptosis induced by SNP. Moreover, previous work revealed that ER stress is induced by the imbalance between unfolded protein load and ER load, which may cause the accumulation of unfolded or mis-folding protein on ER. In mammal cells, it can restore and maintain ER function by alleviating protein translation. Multi-

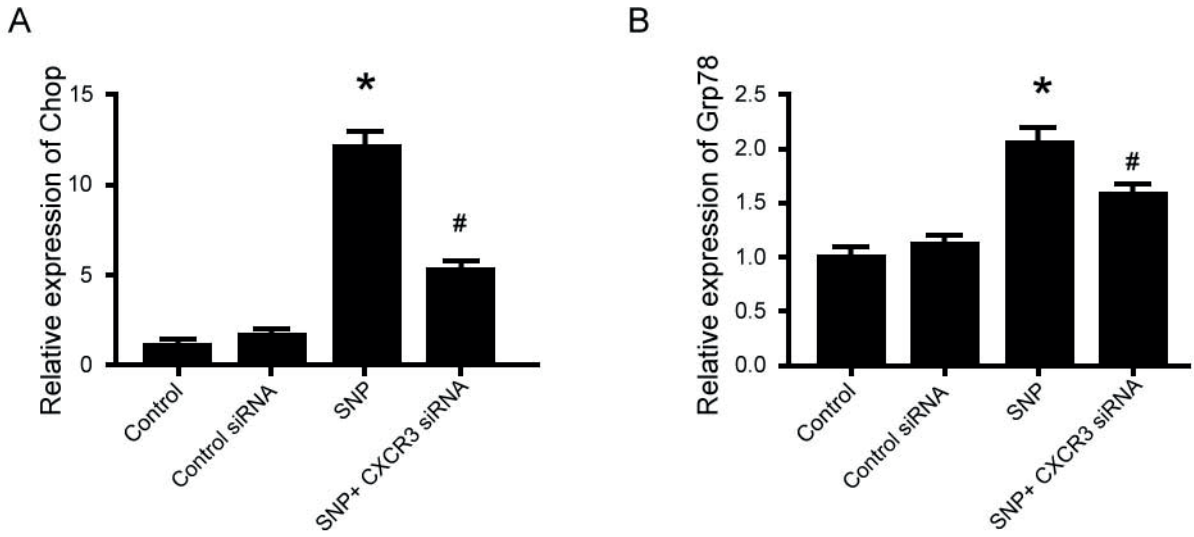

Figure 5. GRP78 ***(A)*** and CHOP ***(B)*** participated in CXCR3 in regulating chondrocyte apoptosis induced by SNP. $^{*}p<0.05$, compared with normal control. $^{\#}p<0.05$, compared with SNP group.

ple proteins participate in this protective mechanism, including GRP78 that is considered to play a role through ER related protein degradation system[20-22]. Down-regulation of protective response or sustained ER stress may activate CHOP to eliminate the damaged cells[23-26]. It was indicated that ER stress related molecules are involved in chondrocyte apoptosis mediated by nitric oxide. Therefore, we tested GRP78 and CHOP expressions. It was showed that reduction of CXCR3 decreased GRP78 and CHOP levels in chondrocyte apoptosis mediated by nitric oxide. It suggested that CXCR3 can affect ER stress related molecules GRP78 and CHOP expressions.

Conclusions

We showed that ER stress signaling pathway CHOP and GRP78 are involved in CXCR3 receptor regulating chondrocyte apoptosis induced by nitric oxide.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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