MEDICAL SCIENCE MONITOR

ANIMAL STUDY

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 5302-5310
DOI: 10.12659/MSM.907150

Received: 2017.09.15
Accepted: 2017.10.19
Published: 2017.11.07

Cross-Coupling Effects of Silencing of Cyclooxygenase-2 (COX-2)/Aggrecanase-1 and Over-Expressed Insulin-Like Growth Factor 1 (IGF-1) in an Osteoarthritis Animal Model

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Source of support: This project was supported by the National Natural Science Foundation of China (No. 81171774, No. 81272056)

Backgound: This study aimed to observe the effect of lentivirus-mediated cyclooxygenase-2 and aggrecanase-1 silencing and insulin-like growth factor-1 overexpression in human bone marrow mesenchymal stem cells after injection into model osteoarthritic knees.

Material/Methods: Using genetic recombination technique, the genes of cyclooxygenase-2, aggrecanase-1, and insulin-like growth factor-1 were recombined into the lentiviral vectors, and we transfected the human bone marrow stem cells *in vitro*. The BMSC transfected with lentivirus without genes served as a blank-virus group, and saline was used for another control group. One week later, the cytokines PGE2, aggrecanase-1, hIGF-1, and IL-1 were detected and compared between groups.

Results: Compared with blank-virus group, the expression of COX-2 (85.81±5.12 ng/L) and aggrecanase1 (6.256±1.66) were decreased in the virus group ($p<0.05$), while the expression of hIGF-1 (17.46±1.86) was increased ($p<0.05$). The concentrations of PGE2 (85.81±5.12 ng/L), aggrecanase1 (51.34±5.463 ng/L), and IL-1 (82.31±4.321 ng/L) decreased ($p<0.05$) within the knee, but the concentration of hIGF-1 (44.33±0.7194 ng/L) increased ($p<0.05$). Compared with the other groups, the results of histological and immunohistochemical examinations demonstrated that the abrasion of articular cartilage was significantly improved and repaired.

Conclusions: Lentivirus-mediated RNAi can inhibit the expression of COX-2 mRNA and aggrecanase-1mRNA, and enhance the hIGF-1 mRNA expression, thereby influencing the concentration of cytokines in the early osteoarthritic model knee joints.

MeSH Keywords: Bone Marrow Cells • Cyclooxygenase 2 • Insulin-Like Growth Factor Binding Protein 3

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/907150

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Background

Osteoarthritis (OA) is the result of the interaction between mechanical and biological factors that result in the imbalance of chondrocytes and extracellular matrix synthesis and degradation, and present as progressive and severe disease. Its morbidity increases with age, and the incidence in people over 70 years old is as high as 80% [1]. To date, the treatment includes no-drug therapy, drug therapy, and surgical intervention, as well as genetic and biological treatment, which aim to relieve symptoms [2]. Despite the multiple methods of treatment, the outcome is still unsatisfactory, so it is important to curb or reverse the process of OA rather than focusing on alleviating the symptoms. The appearance and development of gene therapy is promising, but experimental research in gene therapy for OA is still in its initial stages. Gene therapy uses normal genes or therapeutic DNA sequences to correct genetic defects or treat diseases after transfecting the genes into target cells. Now, we often choose to enhance protective genes or inhibit destructive ones as a treatment method. If we combine the 2 methods, we can get better outcomes [3]. The advantages are: (1) BMSCs with target gene can express products subtly and persistently; (2) they can maintain this function with cell proliferation, and can improve long-term treatment effect; (3) several genes can be transfected and regulated at the same time; (4) local treatment reduces systemic adverse reactions; (5) BMSCs have multi-directional differentiation ability by carrying the target gene, so BMSCs can differentiate into relatively normal cartilage tissue, so as to treat OA better [4].

OA is a pathological change with senility and degeneration of articular cartilage, and then osteoproliferation present in cartilage, osteophyte, and cartilage fragment can also stimulate the appearance of secondary osteosynovitis [5]. In the process, cyclooxygenase-2 (COX-2) , aggrecanase-1, and insulin-like growth factor 1 (IGF-1) play a relatively important role. COX-2 is the key enzyme for the synthesis of prostaglandin PG (PGE), especially PGE2, which is associated with the occurrence and development of inflammation [6]. COX-2 can promote cartilage cells to synthesize PGE2 and a variety of collagenases, which accelerate the degradation of cartilage matrix [7], and, accompanied by increased PGE2, the free radicals and active oxygen can damage the cartilage cells. Aggrecanase1 and aggrecanase2 are the main protein enzymes that play a major role in the loss of proteoglycans. The expression of aggrecanase1 is very active in the articular cartilage of OA patients, and it may also play an important role in the degeneration of proteoglycans [8]. IGF-1 is the main stimulating factor in serum and joint fluid; it can intensively promote the synthesis of cartilage matrix, and inhibits the degradation of cartilage matrix, and *in vitro* studies show that IGF-1 can promote the synthesis of cartilage and proteoglycan [9].

In this study, we used BMSCs as target cells and Cynomolgus monkeys as the animal model. We transferred lentivirus-mediated cyclooxygenase-2 and aggrecanase-1 silencing gene and insulin-like growth factor-1 overexpression genes into human bone marrow mesenchymal stem cells, then injected the transfected BMSCs into the joint cavity of the osteoarthritic model and observed the changes in inflammatory factor in joint fluid and articular cartilage.

Material and Methods

Osteoarthritis animal model

Male 1-year-old Cynomolgus monkeys were provided by the Experimental Animal Centre of Jinan, Shandong province, China. In view of the cost and experiment demands, we bought 9 monkeys. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Affiliated Hospital of Qingdao University, and all efforts were made to minimize suffering. During the experimentation, no animal got sick or died. The weight of each monkey was about 6±0.3 Kg. The animals were anaesthetized by intramuscular injection of ketamine (1 mg/kg) and diazepam (0.6 mg/kg) and redosed as needed, then a skin incision about 4 cm along anterior median line at right leg was made beginning from the suprapatellar bursa and extending to the level of the tibial plateau. The subcutaneous tissue was incised layer-by-layer, the joint capsule was incised, and the patella was evaginated, and the anterior and posterior cruciate ligament was cut off from its beginning to the attachment, as well as the medial meniscus. Postoperatively, the incision was disinfected with 0.5% iodophor and the sutured layer-by-layer, and all animals were treated with Clindamycin (20 mg/kg) by intramuscular injection twice a day for 3 days. All animals were observed and monitored for 24 h, after which they were placed in an animal house without any limitation. In total, 9 Cynomolgus monkeys were randomly distributed into 3 groups (the virus group, blank-virus groups, saline group). The left legs served as the normal group. Six weeks later, MRI and the same surgery were performed to observe and compare the change in articular cartilage.

Lentiviral vectors construction

Lentiviral vectors were constructed by GenBank. Lentivirus vectors with interference sequences sh-hCOX2, sh-h-aggrecanase-1, and overexpression sequence hIGF-1, named hCOX-2-shRNA, hAggrecanase1-shRNA, and hIGF-1-shRNA, respectively (Table 1), were designed and synthesized by Shanghai GenePharma Co. Ltd. The vectors harbored a green fluorescent protein (GFP) gene, which could be used as an indicator.

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Table 1. Gene sequences of hCOX-2, h-Aggrecanase-1, and hIGF-1.

Oligo name	Oligo sequence
h-COX-2	5'-GAT CCG CTC CGG ACT AGA TGA TAT CAT TCA AGA GAT GAT ATC ATC TAG TCC GGA GCT TTT TTG-3' 5'-AAT TCA AAA AAG CTC CGG ACT AGA TGA TAT CAT CTC TTG AAT GAT ATC ATC TAG TCC GGA GCG-3'
h-Aggrecanase-1	5'-GAT CCG CTA TGG GCA CTG TCT CTT AGT TCA AGA GAC TAA GAG ACA GTG CCC ATA GCT TTT TTG-3' 5'-AAT TCA AAA AAG CTA TGG GCA CTG TCT CTT AGT CTC TTG AAC TAA GAG ACA GTG CCC ATA GCG-3'
hIGF-1	5'-GCC TCG AGG AAG ATG CAC ACC ATG TCC TC-3' 5'-GCG AAT TCC TAC ATC CTG TAG TTC TTG TTT C-3'
Negative control	5'-GAT CCG TTC TCC GAA CGT GTC ACG TTT CAA GAG AAC GTG ACA CGT TCG GAG AAC TTT TTT G-3' 5'-AAT TCA AAA AAG TTC TCC GAA CGT GTC ACG TTC TCT TGA AAC GTG ACA CGT TCG GAG AAC G-3'

Cell culture

Human bone marrow mesenchymal stem cells were isolated from the bone marrow of 10 donors (5 males and 5 females) with a mean age of (54±7.1) years. Bone marrow was obtained from the femur within the framework of total hip replacement. All patients provided informed consent according to the latest version of the Helsinki Declaration. This protocol was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University, China. Five mL of bone marrow mixed with an equal volume of DMEM-L in a 15-mL sterile centrifuge tube and was pipetted to make uniform suspensions. Five mL of suspension was absorbed and plated in 25-cm^2 cell culture flasks, and the cells were maintained in an incubator at 37°C and 5% CO_2, called the primary cells. After 24 h, half of the medium was replaced with 2 mL of fresh configuration culture (DMEM: serum: double antibiotic=89: 10: 1), while the suspension cells and the residue were taken out of the culture flasks, and thereafter the full medium was changed on alternate days. After 7–8 days, when the cells reached 80% confluence, the adherent cells were harvested and passaged (by 1: 2). The passaged cells were plated in culture flasks until the 3rd passage, and then were collected and transfected by virus.

Gene transfection

The third generation of human bone marrow mesenchymal stem cells were digested with 2.5 g/L trypsin, counted, and plated in 6-well culture plates (4×10^5 cells per well) containing serum-free DMEM medium. The cells were cultured in the incubator, and the original medium was changed with fresh medium every other day. The lentivirus was taken out from the freezer (–80°C) and melted. Then, the lentivirus was successively added into the medium, according to multiplicity of infection=40, and was well mixed. Each type of virus occupied 1/3 of the whole volume. The cells were cultured at 37°C, and taken out after 12 h to change the medium containing virus with 10% serum fresh medium, observed after 72 h by an inverted fluorescence microscope. Growth curves were drawn after counting.

Real-time PCR

Using the RNAiso kit (TaKaRa, Japan), with GAPDH as the internal reference, calibrated with the untreated BMSCs, we detected the expression of COX-2, aggrecanse-1, and hIGF-1mRNA of the virus group and blank-virus group using the FTC-3000 RT-qPCR System (Funglyn Biotech Inc, Canada). Then we compared the differences between the groups.

Intra-joint injection

BMSCs were harvested, washed and suspended at a density of 1×10^7/ml after 3-week culture. Animals in each group received intra-joint injection with 1 ml of virus-transfected BMSCs (virus group), 1 ml blank-virus-transfected BMSCs (blank-virus group), or 1 ml NaCl (control group), and were fed under the same condition without activity limitation after surgery. One week later, synovial fluid of animals in each group was extracted, and the density of PGE-2, IL-1, aggrecanase-1, and hIGF-1 were detected using an Enzyme-Linked Immunosorbent Assay Kit (Elabscience, Wuhan, China), which were also detected with the same method at 4 weeks and 6 weeks later.

Histological observation of cartilage

Six weeks later, the animals were sacrificed and cartilage of each group were harvested. Cartilage were observed and compared after HE staining and type II collagen enzyme immunohistochemical staining (Rabbit Anti-Collagen II, Aobesen, Beijing, China). The cartilage in the restoration region was graded under the improved Pineda standard.

Statistical analysis

The results are expressed as mean ± standard deviation (SD) of separate experiments. The paired *t* test was used to value the difference between groups. A *p* value of less than 0.05 was considered significant. Analysis was performed using SPSS version 13 (SPSS Inc, Chicago, IL).

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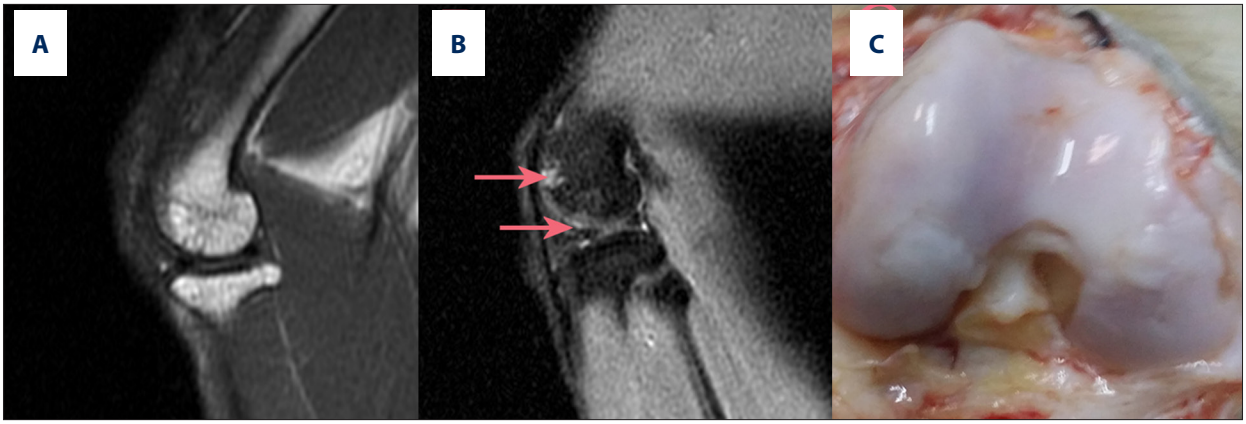

Figure 1. MRI and gross observation 6 weeks later. (**A**) MRI image of normal knee. (**B**) MRI of OA model: an abnormal high-density shadow can be seen (red arrow). (**C**) cartilage defect can be seen in the medial condyle, with osteophyte in lateral condyle. Synovial proliferation was not obvious.

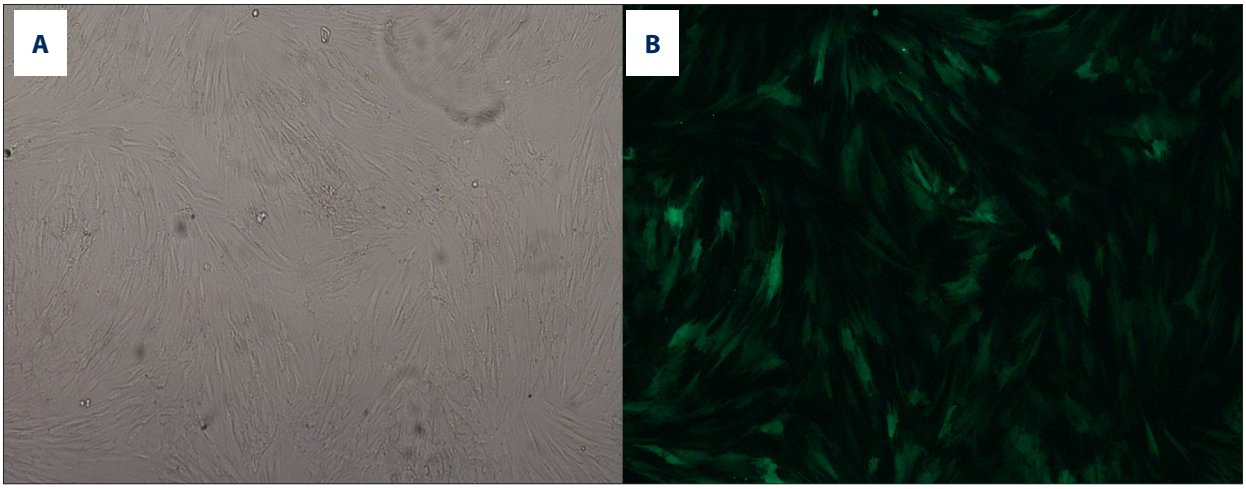

Figure 2. At 72 h after virus transfection (×100). (**A**) Optical microscope. (**B**) Fluorescence microscope.

Results

The building of the osteoarthritis model (Figure 1). Six weeks after surgery, MRI showed an abnormal high-density shadow at the model side, then animals were sacrificed, and both cartilage defect and osteoproliferation can be found at the surgery region. Synovial proliferations were not obvious, and all features were characteristic of early OA.

Cell culture and virus transfection. After 1–2 days of incubation, cells were in exponential growth phase at the 3rd or 4th day, then grew into plateau phase at the 6th to 8th days. The growth curve was nearly parallel between groups. Virus transfection had no obvious adverse effects on cell growth (Figures 2, 3).

RT-PCR detection

Compared with blank-virus and control group, COX-2 (2.867±0.452) and aggrecanse-1 (6.256±1.66) expression

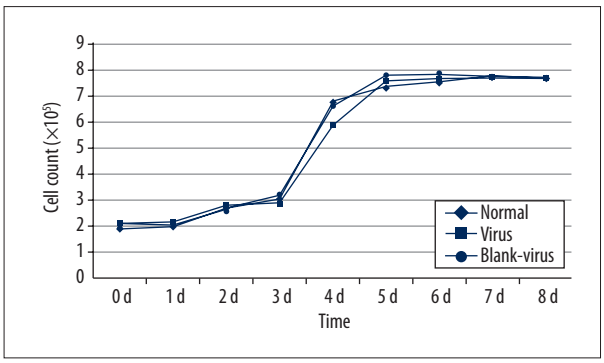

Figure 3. Growth curve.

in the virus group was clearly reduced (P<0.01) and IGF-1 (17.46±1.86) was obviously increased (P<0.01). There was no significant difference between the blank-virus group and control group (P>0.05) (Figure 4; Table 2).

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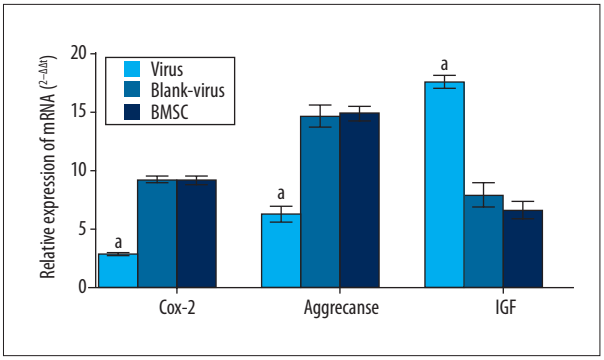

Figure 4. The expression of COX-2, aggrecanse-1, and IGF-1mRNA, [a] P<0.01

Enzyme-linked immunosorbent assay detected synovial fluid

Concentration of PGE2 (Table 3)

Concentration of IL-1 (Table 4)

Concentration of h-aggrecanase-1 (Table 5)

Concentration of h-IGF-1 (Table 6)

Table 2. Relative expressions of COX-2, Aggrecanase-1, and IGF-1 mRNA in each group by RT-PCR(x±s).

Group	Cases	COX-2	Aggreacanase-1	IGF-1
Virus group	3	5.68±0.56#,Δ	7.25±0.52#,Δ	15.27±0.41#,Δ
Blank-virus group	3	10.02±0.17*	12.25±0.38*	8.60±0.53*
Blank control group	3	9.07±0.31*	15.23±0.61*	6.95±0.03*
Normal control group	9	3.88±0.19*,#,Δ	4.99±0.89*,#,Δ	4.95±0.88*,#,Δ
Statistic		F=208.50, P=0.0001	F=164.50, P=0.0001	F=197.20, P=0.0001

* Compared with virus group, P<0.05; # Compared with blank-virus group, P<0.05; Δ Compared with blank contral group, P<0.05.

Table 3. Concentration of PGE2.

Group	Cases	PGE2		
		One week	Four week	Six week
Virus group	3	85.809±5.120#,Δ	90.453±4.774#,Δ	92.746±3.998#,Δ
Blank-virus group	3	106.608±4.907*	110.320±2.641*	112.084±4.726*
Blank control group	3	124.311±5.647*	130.741±4.215*	132.846±3.875*
Normal control group	9	68.297±5.748*,#,Δ	59.294±4.832*,#,Δ	60.733±3.586*,#,Δ
Statistic		F=51.13, P=0.002	F=49.63, P=0.001	F=45.18, P=0.01

* Compared with virus group, P<0.05; # Compared with blank-virus group, P<0.05; Δ Compared with blank contral group, P<0.05.

Table 4. Concentration of IL-1.

Group	Cases	IL-1		
		One week	Four week	Six week
Virus group	3	82.311±4.321#,Δ	90.736±1.042#,Δ	98.937±2.944#,Δ
Blank-virus group	3	105.079±5.411*	114.759±1.874*	114.326±2.045*
Blank control group	3	108.642±4.343*	109.847±1.998*	110.847±2.075*
Normal control group	9	53.656±4.926*,#,Δ	62.746±0.898*,#,Δ	58.339±1.774*,#,Δ
Statistic		F=581.00, P=0.0001	F=49.63, P=0.001	F=50.32, P=0.001

* Compared with virus group, P<0.05; # Compared with blank-virus group, P<0.05; Δ Compared with blank contral group, P<0.05.

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Table 5. Concentration of h-aggrecanase-1.

Group	Cases	Aggrecanase-1		
		One week	Four week	Six week
Virus group	3	51.338±5.463#,Δ	59.736±2.857#,Δ	66.791±1.944#,Δ
Blank-virus group	3	89.985±3.976*	90.635±3.011*	94.226±1.025*
Blank control group	3	93.901±4.915*	95.735±2.009*	99.747±1.975*
Normal control group	9	31.010±1.510*,#,Δ	30.746±1.887*,#,Δ	38.746±1.266*,#,Δ
Statistic		F=115.70, P=0.0002	F=106.10, P=0.0001	F=100.64, P=0.0001

* Compared with virus group, P<0.05; # Compared with blank-virus group, P<0.05; Δ Compared with blank contral group, P<0.05.

Table 6. Concentration of h-IGF-1.

Group	Cases	IGF-1		
		One week	Four week	Six week
Virus group	3	44.329±0.763#,Δ	50.422±3.021#,Δ	56.782±0.854#,Δ
Blank-virus group	3	19.941±2.442*	20.647±1.998*	22.476±1.325*
Blank control group	3	13.401±2.425*	18.630±1.809*	18.937±2.775*
Normal control group	9	11.114±1.610*,#,Δ	14.326±0.997*,#,Δ	13.946±1.651*,#,Δ
Statistic		F=83.91, P=0.001	F=76.10, P=0.001	F=60.67, P=0.002

* Compared with virus group, P<0.05; # Compared with blank-virus group, P<0.05; Δ Compared with blank contral group, P<0.05.

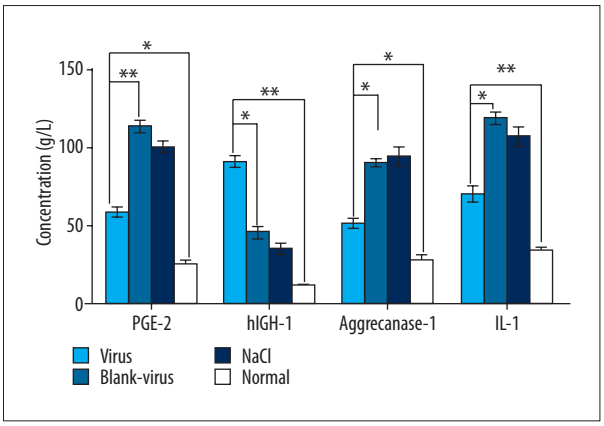

Figure 5. The expressions of COX-2, aggrecanse-1, and IGF-1 mRNA of cartilage in each group. ** P<0.01

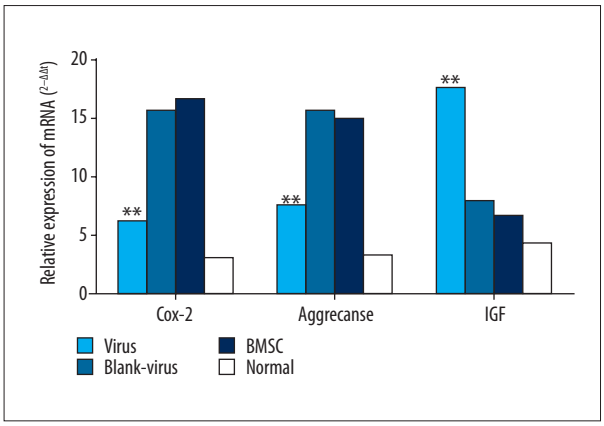

Figure 6. histological observation of cartilage (×200). (**A**) Normal group: uniform size, morphological rules; (**B**) NaCl group: irregular sizes and morphology; (**C**) Blank-virus groups: irregular sizes and local hyperplasia; (**D**) Virus groups: apparent cell hyperplasia, relative densification.

RT-PCR detection of cartilage

Compared with the blank-virus and control group, in the virus group the expression of COX-2 (6.250±0.285) and Aggrecanase-1(7.591±0.674) was clearly decreased (P<0.01), while IGF-1(17.461±0.537) was clearly increased (P<0.01). There was no statistically significant difference between the blank-virus group and control group (P>0.05) (Figure 5).

Histological observation of cartilage

Normal cartilage cells are distributed evenly, arranged evenly with clear hierarchy, no clustered chondrocytes. In NaCl and blank-virus groups, the cartilage accorded with early OA

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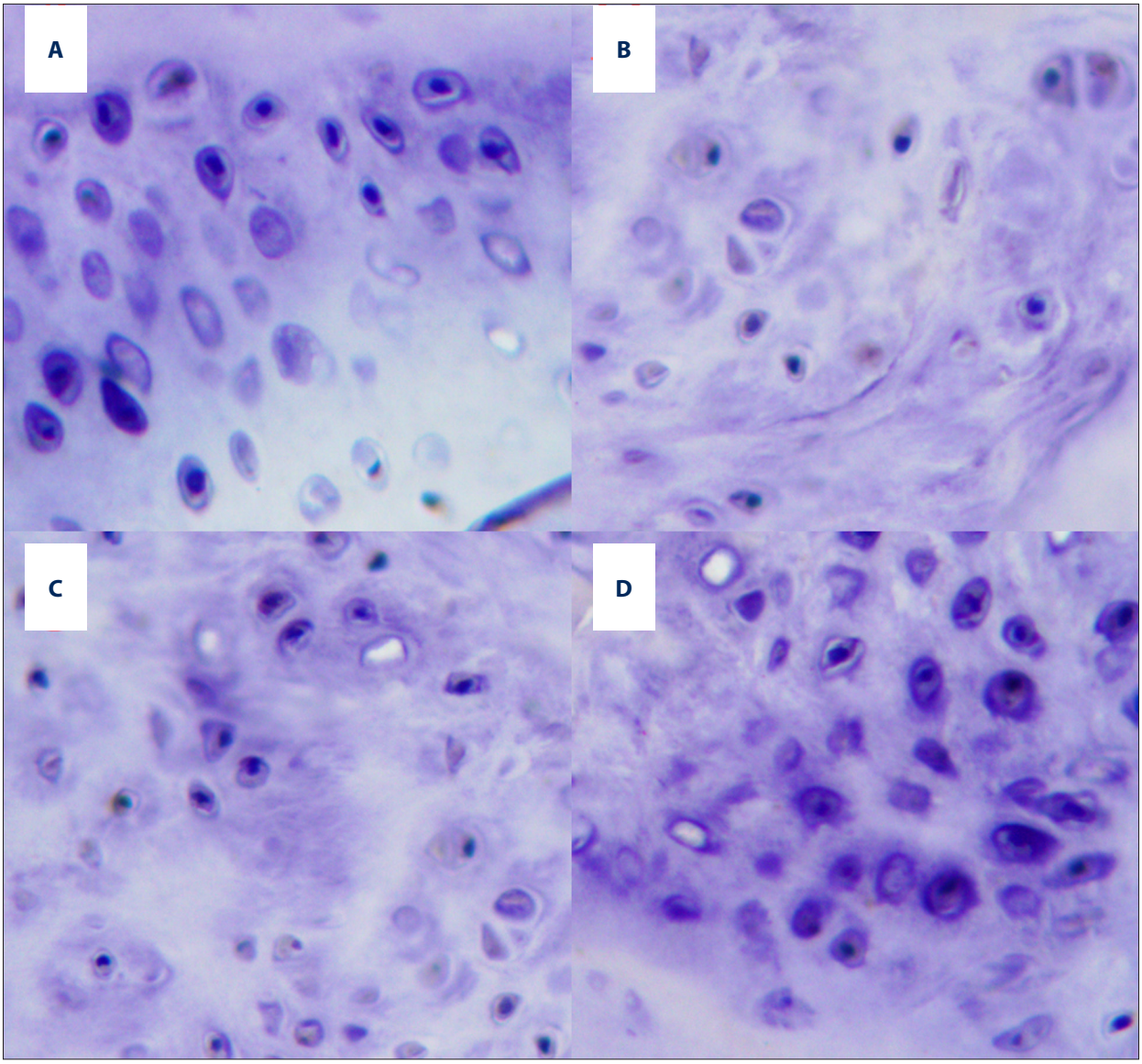

Figure 7. Immunohistochemical dyeing of type II collagen (×200). (**A**) Normal cartilage: cytoplasm stained uniformly brown with blue nuclei; (**B**) NaCl group: cytoplasmic staining is shallow; (**C**) Blank-virus group: cytoplasm uneven with lighter staining than in the normal group; (**D**) Virus group: cytoplasm is deeply stained with occasional brown particles.

Table 7. Scores of cartilage under the improved Pineda standard.

Virus	Blank-virus	NaCL	Normal
3.563±0.34	7.7623±0.65	13.582±0.24	0.000±0.00
3.468±0.61	8.8362±0.89	12.573±0.87	0.000±0.00
2.9832±0.88	7.3575±0.71	12.547±0.61	0.000±0.00

performance reference Mankin [10] articular cartilage standard, the arrangement began to be disordered in cartilage cells, followed by separation and clustering. Cartilage cells of the virus group animal model proliferated significantly, with greater density, larger nuclei, and darker staining (Figures 6, 7).

Scores of cartilage under the improved Pineda standard (Table 7)

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Discussion

Osteoarthritis (OA) is the most common form of arthritic disease, with the manifestations of degeneration of articular cartilage, bone destruction, and hyperosteogeny, which are the main causes of joint pain and dysfunction [11]. OA is a complex pathological process in which a of variety of cytokines participate through a variety of pathological pathway and mechanisms. In OA pathogenesis, many inflammatory cytokines are released into the blood, which aggravates the severity of the illness, and changes of inflammatory cytokines in synovial fluid were similar to the serum [12]. Elevated inflammatory factors of OA patients may be related to synovitis, and there is evidence to support the relationship between synovitis and joint degeneration [13]. At present, the main treatments are conservative treatment and surgical treatment [14], and despite the multiple modalities of treatment, the outcome is still often unsatisfactory. Thus, a revolutionary treatment approach is eagerly awaited.

BMSCs as the seed cells of tissue engineering and transgenic technology have the capacity for multipotential differentiation, which can differentiate into different cells *in vitro*, such as bone, cartilage, fat, and muscle-like cells [15]. Through tissue engineering, arthritis cartilage damage can be repaired, and an experiment showed that stem cells differentiated into bone in a rabbit model of osteoarthritis [16]. In the development of osteoarthritis, COX-2, Aggrecanase-1, and hIGF-1 are critical genes, and increased expression of COX-2 promoted synthesis of PGE2, which ultimately resulted in the development of inflammation and joint damage [17]. The increased activity of aggrecnase1 resulted in more proteoglycans being degraded, which broke the balance between cartilage matrix synthesis and degradation, and led to further cartilage defects and dysfunction of joints [18]. hIGF-1 is an important cytokine that can effectively promote the synthesis of cartilage matrix, enhance mitosis, and inhibit cartilage matrix degradation, so as to maintain the normal function of the articular cartilage [19].

We used BMSCs as target cells, Cynomolgus monkeys as the animal model, transferred lentivirus-mediated cyclooxygenase-2 and aggrecanase-1 silencing gene and insulin-like growth factor-1 overexpression gene into BMSCs, successfully inhibited the expression of COX-2 and aggrecanase-1, enhanced expression of hIGF-1 gene, and sequentially at cellular level reduced the inflammatory cytokine production, and promoted growth factor production. After injecting the gene-transfected stem cells into the knee joint cavity of the osteoarthritis model, the concentrations of inflammatory factors PGE2 and IL-1 in synovial fluid were significantly reduced, and aggrecanase-1 was further decreased, while hIGF-1 level increased. All of these changes were consistent with the changes in gene level, according to *in vitro* study results [20]. Using histological methods, we proved that this approach can better protect the cartilage matrix and promote the generation of cartilage matrix and chondrocytes, thereby proving the operability and effectiveness of the gene therapy for early osteoarthritis.

In the field of gene therapy, genetic research with a single gene or double genes are relatively common, but gene therapy studies with multiple genes are less common. In this study, after 3 genes were simultaneously transfected, they more effectively inhibited the expression of destructive genes, reduced the production of harmful inflammatory cytokines, increased the expression of useful ones, and jointly protected articular cartilage. The Cynomolgus monkey is a primate with physiologic structure similar to humans, and therefore has great scientific value. Through the excision of entire cruciate ligaments, we successfully built the early osteoarthritis model, by multiple gene interference and enhancement, further demonstrating the feasibility of gene therapy for early osteoarthritis.

Conclusions

Due to the high cost of experimental animals, a relatively small sample was used in this study, the observation time was relatively short, and the experimental models in our study were early arthritis ones. Therefore, long-term studies with large sample sizes are required to demonstrate the feasibility and reliability of gene therapy for intermediate and advanced osteoarthritis. We believe that gene therapy, as a new therapeutic tool, will play a unique role in the treatment of osteoarthritis and other arthritic diseases.

Conflict of interest

None.

Acknowledgements

Thanks to the Central Laboratory, Affiliated Hospital of Qingdao University Medical College, for providing research laboratory and technical assistance.

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