

Cell therapy of a knee osteoarthritis rat model using precartilaginous stem cells

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Abstract. – OBJECTIVE: To explore the effect and mechanism of precartilaginous stem cells (PSCs) engraftment-inducing tissue repair in a knee osteoarthritis (OA) rat model.

MATERIALS AND METHODS: Knee osteoarthritis (OA) model was constructed in Sprague Dawley (SD) rats by partial removal of the medial meniscus of the right knee. PSCs were engrafted by injecting precartilaginous stem cells (PSCs) into the right knee cavity. At 4 and 8 weeks after model construction, the serum levels of interleukine (IL)-1 β , tumor necrosis factor (TNF)- α , and IL-6 were assessed using enzyme-linked immunosorbent assay (ELISA). Hematoxylin-eosin (HE) staining was performed to assess the histopathology of synovial membrane and cartilage. Western blot analysis was used to assess Notch1, Bcl-2 and Bax levels in the articular cartilage.

RESULTS: At 4 and 8 weeks, OA rats demonstrated significantly higher IL-1 β , TNF- α , and IL-6 levels than normal rats ($p < 0.05$), whereas PSCs treatment prominently attenuated IL-1 β upregulation ($p < 0.05$). In OA rats, the number of chondrocytes dramatically decreased over time in OA rats, with disruption of chondrocytes organization and cell layers. PSCs alleviated the deterioration of cartilage, as evidenced by the relatively smooth articular surface, distinct tidemark and clear cell layers. The model and treatment groups demonstrated substantially higher Notch1 expression. The Bcl-2/Bax value in the OA rats was lower than the control group, while PSCs treatment led to increase in Bcl-2/Bax value.

CONCLUSIONS: PSCs treatment downregulated the expression of inflammatory cytokines, alleviating osteoarthritis in the knee of rats. Notch1 signaling pathway plays an important role in this ameliorating effect of PSCs treatment.

Key Words:

Cell therapy, Knee osteoarthritis, Precartilaginous stem cell, Notch, inflammation.

Abbreviations

OA = osteoarthritis; MSCs = Mesenchymal stem cells; PSCs = precartilaginous stem cells; FBS = fetal bovine serum; GVHD-graft-versus-host disease; ELISA = enzyme-linked immunosorbent assay; HE = hematoxylin-eosin; PBS = phosphate-buffered saline; IL = interleukine; TNF = tumor necrosis factor; DMEM = Dulbecco's Modified Eagle Medium.

Introduction

Osteoarthritis (OA) of the knee joint is a chronic devastating disease. It is a major public health problem among elderly population¹. OA is characteristic of degeneration of the articular cartilage, fibrillation, loss of matrix, and formation of fissures. Current clinical tools of OA management mainly include medication and/or physiotherapy, as well as surgical methods, such as tibial osteotomy² and total knee arthroplasty³. To date, very few effective surgical or non-surgical therapies are available for OA, which demands development of novel treatment regimen to impede the pathobiology course of the disease.

Mesenchymal stem cells (MSCs) are a class of cells that can differentiate into a plethora of tissue cells, including cartilage, bone, muscle, tendon, etc⁴. MSCs can easily be isolated from bone marrow and further expanded in culture, retaining the ability to differentiate in response to external signals. This gives rise to opportunities for cellular therapies with no ethical issues associated with embryonic stem cells usage^{5,6}. Due to their multipotentiality, self-renewal ability, immunosuppressive activities, and limited immunogenicity, MSCs hold promise to regenerate damaged tissues as a result of disease or trauma⁴. In addition, MSCs are autol-

ogenous source of cells, which eliminate concerns of rejection and disease transmission, possessing less tumorigenicity than their embryonic counterparts. Thus far, bone marrow⁷, adipose tissue⁸, and synovium⁹ are predominantly used to derive MSCs. Precartilaginous stem cells (PSCs) are a reservoir of stem cells that control limb growth¹⁰. They can be used as seed cells for reconstructive tissue therapy of cartilage and bone defects. With advances in isolation and culturing techniques of PSCs¹⁰, the therapeutic potential of PSCs has been increasingly tested. MSC therapy is simple and does not require hospitalization or surgery. Recent works¹¹⁻¹³ in transplanting MSCs into articular cartilage for osteoarthritis treatment are encouraging. As PSCs are the progenitor cells of cartilage tissues, it is worth testing whether PSCs alleviates symptoms of knee osteoarthritis by facilitating cartilage repair.

Herein, we strive to explore cell therapy with PSCs in the repair of damaged tissues. We first constructed a knee OA model in rat by partial removal of the medial meniscus of the right knee, and performed direct intra-articular injection of PSCs. We demonstrated that cell engraftment resulted in a marked regeneration of the cartilage tissue. We also confirmed that cell therapy with MSCs led to a decrease in inflammatory factors, along with attenuation of Notch 1 signaling. Notch signaling is widely accepted as a marker of MSCs. The data presented in current study not only demonstrate the feasibility of cell therapy using PSCs for knee OA, but also shed light on the role of Notch signaling in PSCs treatment in alleviating knee OA.

Materials and Methods

Animals and Materials

All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Yantaishan Hospital. Healthy male Sprague Dawley (SD) rats with the age of 8 weeks and body weight of 22-24 g were purchased from Guandong Medical Experiment Animal Resource Center (No. 44007200021628). Dulbecco's Modified Eagle Medium (DMEM) medium, ampicillin, 0.25% trypsin with EDTA, and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). Hematoxylin-eosin (HE) was purchased from Sigma-Aldrich (St. Louis, MO,

USA). The anti-rat Notch1 monoclonal antibody, anti-rat Bax polyclonal antibody, and anti-rat Bcl-2 polyclonal antibody were acquired from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rat IgG antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Extraction of Precartilaginous Stem Cells

The precartilaginous stem cells were acquired from the cartilage tissue of rat using the enzymic methods. The mesenchymal stem cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/mL ampicillin, and 100 U/mL streptomycin, and cultured in an incubator maintained at 37°C with 5% CO₂. Cells were passaged at 1:5 dilution when they reached 30-60% confluency using trypsin. Cells of generation 2-4 were used for further studies.

Knee OA Model Construction

30 rats were randomly assigned to model group, control group and treatment groups, 10 mice for each group. The OA model was constructed by partial removal of the medial meniscus of the right knee. The surgery was performed under 1% sodium pentobarbital anesthesia and iodine sterilization. Under microscope, a lateral parapatellar skin incision was made longitudinally with ophthalmic scissors in the right knee joint at a level of 1 cm proximal to the patella. The patella was turned outward and fixed to expose joint capsule. The medial meniscus and tibial ligament was disconnected, followed by removing partial medial meniscus. The patella was then placed in the original position and sealed with a scalpel. After surgery, ampicillin (1.5 mg/kg) was injected intramuscularly for 3 days to prevent infection. Rats were then allowed to feed freely. 3 days after surgery, the treatment was performed by injecting 5×10⁶ precartilaginous stem cells. Model group and control group were injected at the same position with 100 μL phosphate-buffered saline (PBS).

Cytokine Measurement

Blood was collected at 4 and 8 weeks after surgery and it was centrifuged to separate serum in the supernatant. Enzyme-linked immunosorbent assay (ELISA) kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to assess the levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6.

Histological Analysis

At 4 and 8 weeks after cell implantation, rats were sacrificed and the distal femur along with peripheral cartilage was dissected. The bone tissue was fixed in 4% paraformaldehyde and decalcified, followed by being embedded in paraffin. The tissue blocks were then sectioned. Consequently, hematoxylin-eosin (HE) staining was used to assess cartilage and synovial membrane.

Western Blot

Cartilage lysate of 30 μ g was added to 50 μ L Laemmli buffer and boiled for 5 min. The lysate was loaded to 10-20% ready gels. Electrophoresis was performed at 20 V for 3 h to resolve the proteins. Proteins were electro-transferred to polyvinylidene-difluoride (PVDF) membranes, which were subsequently washed and blocked with 5% non-fat milk. Primary antibodies against Notch 1, Bcl-2 and Bax (1:1000 dilution) were applied to the membrane and washed three times with phosphate-buffered saline (PBS). Next, goat anti-rat IgG antibodies were used to incubate the membrane for 1 h with shaking. Protein bands were visualized with the chemiluminescence method.

Statistical Analysis

SPSS19.0 (SPSS Inc., Armonk, NY, USA) was used to analyze the data. All data were represented as mean \pm standard deviation (SD). $p < 0.05$ was considered statistically significant.

Results

Mesenchymal Stem Cell Therapy Decreases Inflammatory Factors

We first constructed knee OA models in rats by partial removal of the medial meniscus of the right knee. These OA rats were then treated with PSCs by direct infusion into the knee joint cavity. Serum inflammatory factors were analyzed in OA rats. As expected, IL-1 β , TNF- α , and IL-6

levels in model groups were greatly upregulated, suggesting increased inflammatory activity (Table 1). Interestingly, at 4 weeks after treatment, IL-1 β , TNF- α , and IL-6 levels in treated rats were significantly lower than the model groups ($p < 0.05$). At 8 weeks, the level of inflammatory factors further escalated in the model group, but treated rats demonstrated pronouncedly suppressed expression of those inflammatory factors. The levels of IL-1 β , TNF- α , and IL-6 levels in treated OA rats at 8 weeks were even lower than those at 4 weeks. This evidence indicated that PSCs engraftment in the knee-alleviated inflammation resulted from OA, which is an important step toward a better outcome of OA treatment.

Cell Therapy With Mesenchymal Stem Cells Protects Cartilage from OA Damage

To corroborate whether PSCs therapy decreases damage to knee joint cartilage, we conducted HE staining to examine the cartilage histopathology. The characteristics of OA mainly include erosion of articular cartilage, formation of periarticular osteophytes, and disrupted trabecular organization of the subchondral bone. As shown in Figure 1, at 4 weeks after model construction, noticeable cracks and shallow ulcers were present in the model group. Articular surface demonstrated substantial fibrillation and a larger area of osteophytic remodeling. In contrast, at 4 weeks after treatment, cartilage destruction and osteophyte formation were reduced. The tidemark and cell layers became clarified. Although the synovium membrane was still disrupted, chondrocytes were more uniformly organized. At 8 weeks after model construction, chondrocytes dramatically decreased, with the disappearance of tidemark and cell layers (Figure 1). Conversely, the 8-week cell therapy greatly improved the cartilage morphology. Synovium membrane became much smoother and tidemarks and cell layers turned more distinct (Figure 1).

Table 1. Levels of inflammatory factors under cartilage mesenchymal stem cell treatment.

(pg/mL)	4 weeks			8 weeks		
	Control	Model	Treatment	Control	Model	Treatment
IL-1 β	12.09 \pm 2.31	32.42 \pm 4.12*	22.43 \pm 3.21*#	11.21 \pm 2.43	38.97 \pm 4.52*	17.21 \pm 2.67*#
TNF- α	10.42 \pm 2.34	25.35 \pm 4.31*	19.31 \pm 3.45*#	12.07 \pm 3.01	32.34 \pm 3.12*	15.31 \pm 2.13*#
IL-6	8.03 \pm 2.15	34.04 \pm 5.65*	25.45 \pm 4.67*#	9.76 \pm 2.34	46.23 \pm 5.23*	20.78 \pm 2.16*#

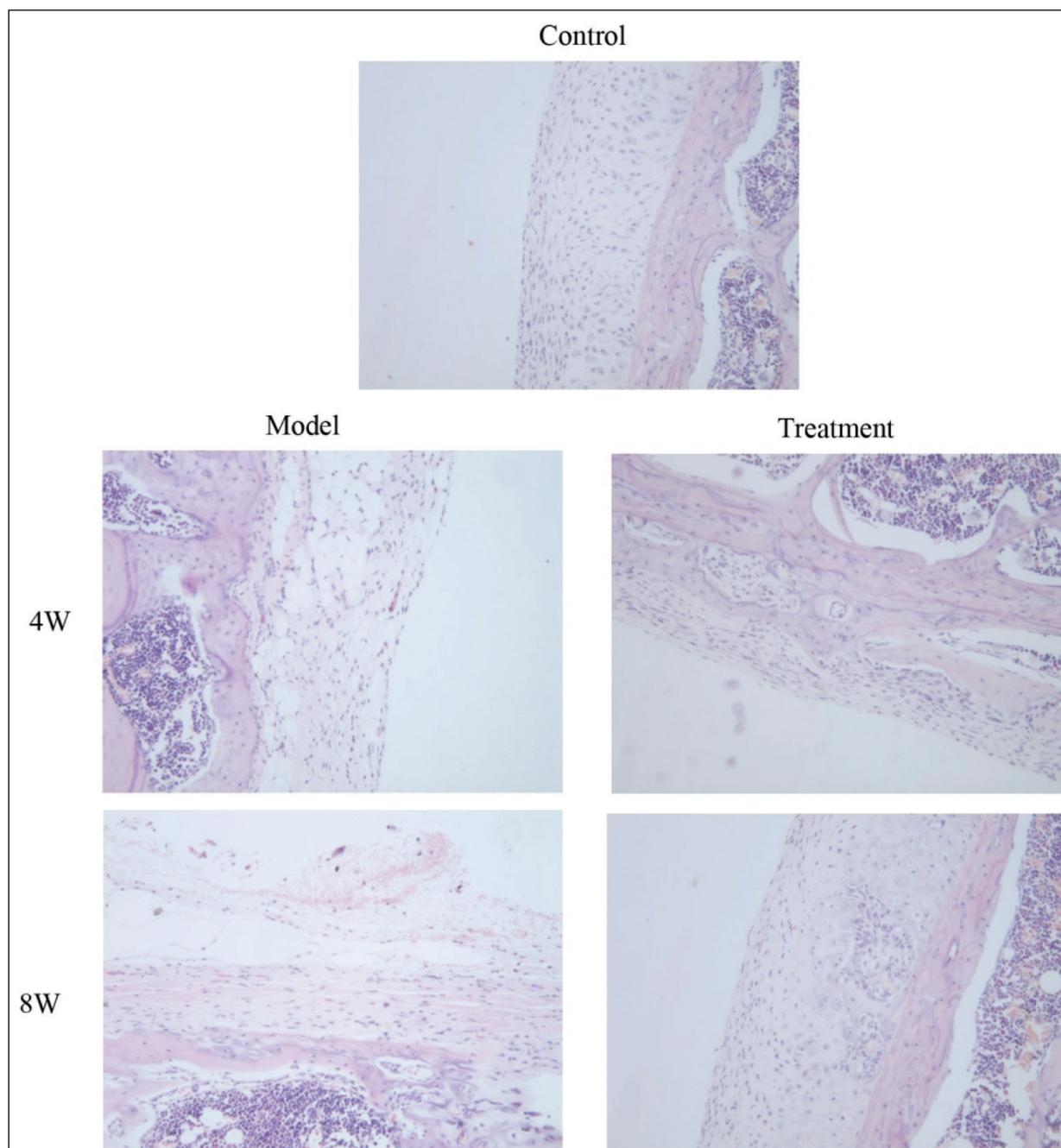


Figure 1. HE staining of cartilage tissue collected from control group, OA group, and treatment group ($\times 200$).

Cartilage Stem Cell Therapy Attenuates Notch 1 Signaling in OA Rats

To provide molecular basis on how PSC therapy protects knee joint from OA damage, we performed Western blot to analyze the expression of key proteins in the Notch signaling. Notch signaling is a putative signaling pathway involved in the inflammation and regulation of

knee joint OA. In our study, we showed that Notch 1 expression was significantly upregulated in model group ($p < 0.05$). This upregulation became more prominent at 8 weeks (Figure 2a-b). PSC therapy significantly attenuated the upregulation of Notch 1 in OA rats. Similarly, in model and treatment groups, Bcl-2 and Bax expressions were also higher than the control

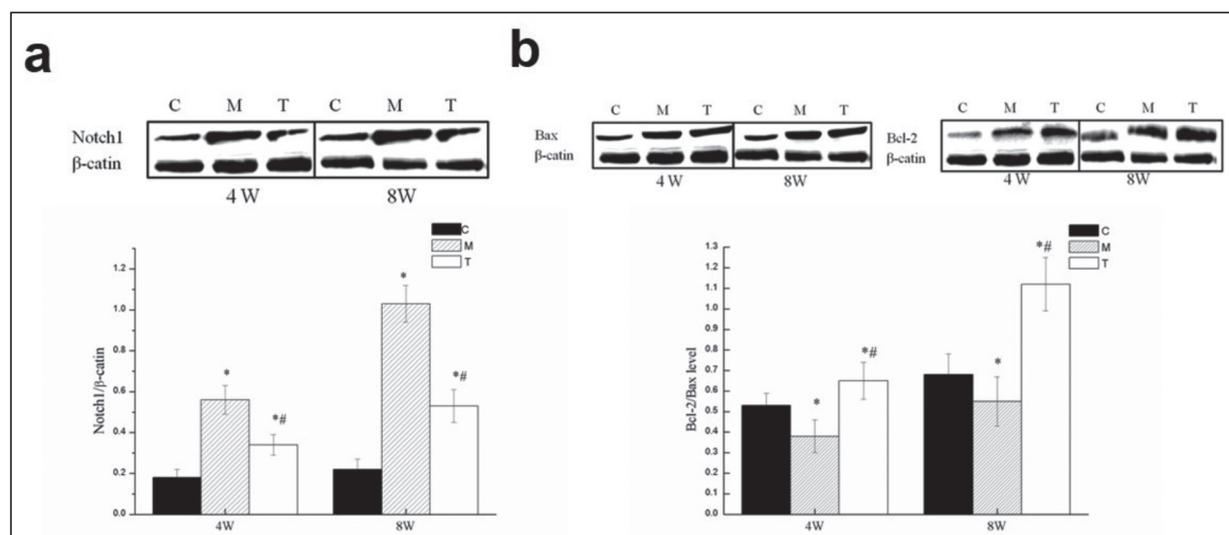


Figure 2. Expression of Notch1 **(a)**, Bcl-2 and Bax **(b)** in knee joint cartilage in osteoarthritis rat model. C: control group; M: model group; T: treatment group. * $p < 0.05$ compared to control group. # $p < 0.05$ compared to model group.

groups. Importantly, while the Bcl-2/Bax value in the model group was lower than the control group, Bcl-2/Bax value in the treatment group was not only higher than the model group ($p < 0.05$), but also higher than the control group ($p < 0.05$) (Figure 2b). Taken together, cell therapy with PSCs downregulates Notch signaling and intriguingly alters the balance between Bcl-2 and Bax expression to alleviate knee OA.

Discussion

In recent years, the ability of MSCs in differentiating into multiple different cell lineages, producing therapeutic growth factors and cytokines has drawn increasing interest to facilitate repair of various damaged tissues. PSCs are a class of MSCs that have rarely been used for cell therapy. Our study was enabled by recent advances in isolation and purification of precartilaginous cells¹⁰. In the current work, we evaluated the effect of PSCs engraftment in alleviating knee joint OA in a rat model. Inflammation is a natural consequence of knee OA. We first showed that PSCs were able to exert potent immunosuppressive effects by downregulating the expression of immunocytokines, including IL-1 β , TNF- α , and IL-6. This result mirrors previous studies showing that MSCs modulates immune responses through the production of immunoregulatory molecules, such as CD28,

PDL-1, and a panel of cytokines.⁶ The inhibited expression of immunological cytokines further induced suppression of T and B-lymphocytes proliferation. In support of this, evidence indicated immunoregulatory function of MSCs *in vivo*. It was shown that MSCs treatment improved clinical outcome of allogeneic transplantation in human by stimulating hematopoietic engraftment and dampening graft-vs.-host disease (GVHD)^{14,15}. In animal models, MSCs also demonstrated efficacy in preventing autoimmunity in lupus-prone mice and ameliorating experimental autoimmune encephalomyelitis^{16,17}. Here, we provided for the first time direct evidence that engraftment of PSCs also suppresses inflammation in OA rat model, which is important for ameliorating the devastating effects of OA facilitating bone tissue repair.

We examined the histopathology of cartilage harvested from OA rats and treated rats. It was clear that cartilage from PSCs treated rats restored relatively normal histopathology pattern compared to those without treatment. This was manifested by the clarification of tidemark and cell layers, as well as reduction of osteocytes. Notably, the treatment was initiated at 4 weeks after model construction. Considerable damage has been done at this stage. Therefore, a single engraftment of PSCs in the knee cavity served as a reconstructive therapy that potently alleviated damage by OA. Our results are paralleled by previous reports showing that a single injec-

tion of allogenic MSCs could effectively prevent the occurrence of severe, irreversible damage to bone and cartilage. The use of MSCs from pre-cartilaginous tissues, which is of the same origin as cartilage tissues, could possibly improve the therapeutic outcome.

In our attempt to elucidate the mechanism of PSCs treatment, we evaluated how PSCs treatment altered Notch signaling. Notch signaling pathway is aberrantly activated in cancers¹⁸, and is also highly involved in the endochondral ossification process, which is indispensable for OA development¹⁹. We showed that the OA rats demonstrated pronouncedly higher Notch 1 protein levels, indicating that Notch signaling is highly upregulated. The downstream targets of Notch signaling pathway, Bax and Bcl-2, are pro-apoptotic and anti-apoptotic proteins, respectively²⁰. Compared with the control group, both model group and treatment group demonstrated Bax and Bcl-2 upregulation. However, model group displayed a lower Bcl-2/Bax ratio, and treatment with PSCs significantly upregulated this ratio. This is in agreement to the decrease in the apoptotic activity observed in histopathological analysis of cartilage. Previous studies have indicated that decreased Bcl-2/Bax ratio is associated with osteoblast and osteocyte apoptosis²¹. Some existing drugs, for example Naringenin, are thought to provide ameliorating effects in arthritis by regulating the Bcl-2/Bax balance²². In cancer, the ratio of Bcl-2 and Bax is positively correlated to cancer resistance^{20,23}, in which mesenchymal cells greatly contribute to these malignant phenotypes. Indeed, cell therapy with MSCs introduces mesenchymal cells characterized by higher Bax/Bcl-2 ratio²⁴. This downregulation of Notch signaling is consistent with the decreased inflammation as Notch signaling and inflammation is closely intervened^{19,25}, and the engrafted PSCs served as immunosuppressants in arthritis.

Conclusions

We demonstrated that cell therapy with PSCs, is capable of downregulating inflammatory factors and alleviating knee osteoarthritis by mediating Notch signaling. Considering that reconstructive cell therapy is becoming recognized in clinics, particularly in orthopedics, our study justified the potential use of PSCs as novel therapeutic modality to impede the pathologic course of knee OA.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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