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Effects of Cyclic Compressive Loading on Chondrogenesis of Rabbit Bone-Marrow Derived Mesenchymal Stem Cells

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ABSTRACT

The objective of this study was to examine the effects of cyclic compressive loading on chondrogenic differentiation of rabbit bone-marrow mesenchymal stem cells (BM-MSCs) in agarose cultures. Rabbit BM-MSCs were obtained from the tibias and femurs of New Zealand white rabbits. After the chondrogenic potential of BM-MSCs was verified by pellet cultures, cell-agarose constructs were made by suspending BM-MSCs in 2% agarose (10^7 cells/ml) for a cyclic, unconfined compression test performed in a custom-made bioreactor. Specimens were divided into four groups: control; transforming growth factor (TGF-β) (with TGF-β1 treatment); loading (with stimulation of cyclic, unconfined compressive loading); and TGF-β loading (with TGF-β1 treatment and loading stimulation) groups. In the loading experiment, specimens were subjected to sinusoidal loading with a 10% strain magnitude at a frequency of 1 Hz for 4 hours a day. Experiments were conducted for 3, 7, and 14 consecutive days. While the experimental groups (TGF-β, loading, and TGF-β loading) exhibited significantly higher levels of expressions of chondrogenic markers (collagen II and aggrecan) at three time periods, there were no differences among the experimental groups after an extra 5-day culture. This suggests that compressive loading alone induces chondrogenic differentiation of rabbit BM-MSCs as effectively as TGF-β or TGF-β plus loading treatment. Moreover, both the compressive loading and the TGF-β1 treatment were found to promote the TGF-β1 gene expression of rabbit BM-MSCs. These findings suggest that cyclic compressive loading can promote the chondrogenesis of rabbit BM-MSCs by inducing the synthesis of TGF-β1, which can stimulate the BM-MSCs to differentiate into chondrocytes. ***Stem Cells*** *2004;22:313-323*

INTRODUCTION

Injured articular cartilage has a limited capacity of self-repair provided damage does not extend beyond the subchondral bone. However, when injuries penetrate the subchondral bone, mesenchymal stem cells (MSCs) from the bone marrow migrate toward the injured area and form new cartilage-like reparative tissue [1-6]. This clinical finding indicates that local biomechanical and/or biochemical stimuli at the injured site of articular cartilage can induce chondrogenic differentiation of MSCs.

Recent in vitro studies demonstrated that chondrogenesis of bone marrow-derived MSCs (BM-MSCs) can be

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induced with the treatment of cytokines such as transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) [7-11]. While combining BMP and TGF-β treatments, proteoglycan biosynthesis can be upregulated during chondrogenesis of BM-MSCs [12]. In addition, *O'Driscoll et al*. demonstrated that continuous passive motion helped to heal injured rabbit knee joint articular cartilage repaired with autogenous periosteal grafts containing MSCs [13]. Recently, *Wakitani et al*. transplanted BM-MSCs into full-thickness cartilage defects of rabbit knee joints. They showed that 6 months after the implantation, different local mechanical environments resulted in substantial differences in mechanical properties of reparative tissues on the posterior and anterior aspects of the repair area [14]. These previous studies suggested that chondrogenic differentiation of MSCs is influenced by mechanical stimuli.

The effects of mechanical loading on the biosynthetic activities of chondrocytes have been extensively studied using agarose and cartilage explant cultures. Compressive loading has been shown to modulate the cartilage-specific macromolecule biosynthesis and pericellular matrix deposition of mature chondrocytes [15-21]. Additionally, static and dynamic compressive loadings were found to promote chondrogenic differentiation of embryonic limb-bud mesenchymal cells [22-24]. More recently, *Angele et al*. showed that cyclic hydrostatic pressure enhanced the extracellular matrix deposition of human BM-MSCs, which underwent chondrogenesis in pellet cultures [25]. However, the effects of physical stimuli associated with the mechanical environment of articular cartilage on chondrogenic differentiation of BM-MSCs still remain unclear. The hypothesis of this study is that dynamic compressive loading can promote chondrogenesis of BM-MSCs. Therefore, the objective of this study is to examine the effects of cyclic compressive loading on chondrogenic differentiation of rabbit BM-MSCs in agarose cultures.

Materials and Methods

Isolation of Rabbit BM-MSCs

The procedure for isolating rabbit BM-MSCs from BM has been described in previous studies [7, 10], which showed that cells isolated from the same procedure demonstrated chondrogenesis in pellet specimens after culture in serum-free medium with the treatment of ascorbic acid, dexamethasone, and TGF-β1 for 21 days [10]. The tibias and femurs were harvested from 10, 3-month old New Zealand white rabbits. A 10-ml syringe containing 1 ml of heparin (3,000 U/ml) with a 16-gauge needle was used to aspirate or flush out the BM from the tibial or femoral shaft. Marrow was placed in a 50-ml tube containing 5 ml of low-glucose Dulbecco's-modified Eagle's medium (DMEM; GIBCO/BRL; Grand Island, NY; http://www.invitrogen.com) and centrifuged at 600 *g* for 10 minutes. After removing the supernatant, cells were resuspended in 7 ml of low-glucose DMEM. A small aliquot of this cell suspension was mixed with an equal volume of 4% acetic acid to lyse the red blood cells. The nucleated cells were then counted with a hemocytometer. The cells were plated in 10-cm dishes at a density of 1×10^5 cells and cultured with low-glucose DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 5 days of culture, nonadherent cells were removed by changing the culture medium. After 2 weeks of primary culture, each dish of cells was passaged into three 10-cm culture dishes every 7 days.

Chondrogenic potential of the rabbit BM-MSCs was initially examined by pellet cultures. Approximately 2.5×10^5 cells were trypsinized and centrifuged in a 15-ml polypropylene tube to form a pellet. The pellets of the control group ($n = 30$) were cultured in serum-free medium consisting of high-glucose DMEM, 1% Insulin-Transferrin-Selenium supplements (GIBCO/BRL; final concentrations: 10 μg/ml bovine insulin, 5.5 μg/ml transferrin, 6.7 μg/ml sodium selenite), 1.25 mg/ml bovine albumin, 5.33 μg/ml linoleic acid, 40 μg/ml proline, 50 μg/ml ascorbic acid, and 10^{-7} M dexamethasone (Sigma; St. Louis, MO; http://www.sigmaaldrich.com), while the same serum-free medium, supplemented with 10 ng/ml of recombinant human TGF-β1 (R&D Systems; Minneapolis, MN; http://www.rndsystems.com), was used for the treated group ($n = 30$). The concentration of TGF-β1 and the serum-free culture medium were chosen based on the previous study [7]. Nine pellets in each group were used for histological and immunohistochemical evaluations, while the expression of the chondrogenic genes (collagen type II and aggrecan) and collagen type I gene was examined on the remaining pellets. All pellet cultures were performed in a humidified incubator maintained at 37°C in 5% CO_2 for 21 days. The culture medium was changed every 2-3 days.

Preparation of Cell-Agarose Constructs

After trypsinizing and cell counting, rabbit BM-MSCs of the fourth passage were suspended in a commercial serum-free medium (UltraCulture, Biowhittaker; Walkersville, MD; http://www.cambrex.com/redirect), and then mixed with an equal volume of 4% (wt/vol) agarose solution at 37°C to produce mixtures of 10^7 cells/ml. The cell-agarose constructs (8 mm in diameter and 1.5 mm thick) were formed by casting the cell-agarose mixture in a custom-designed mold and gelling for 10 minutes at room temperature.

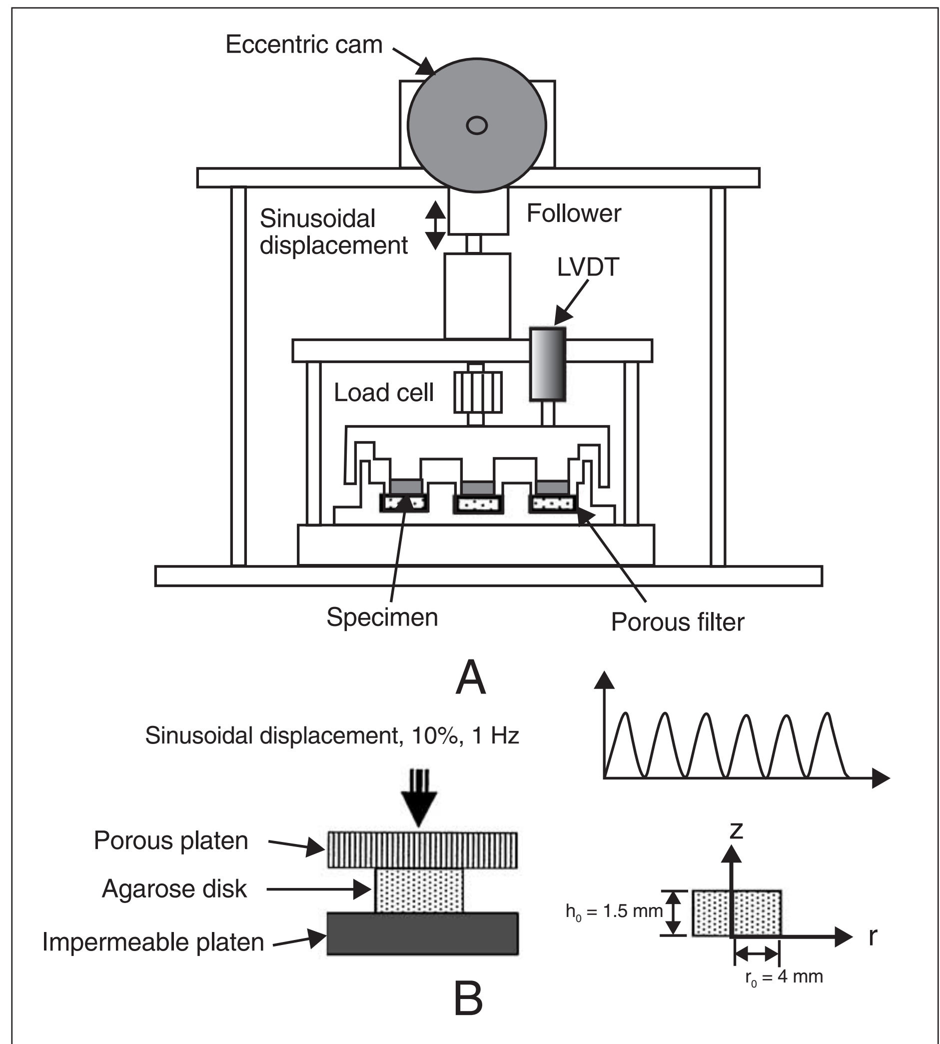


Figure 1. A) Schematics of the bioreactor. *B) Loading configuration of the unconfined compression test.*

Bioreactor System

A bioreactor was developed to promote mass transport and induce mechanical stimuli within agarose specimens under dynamic, unconfined compression (Fig. 1A). In the bioreactor, 12 specimens, placed between porous filters (pore size = 20 μm) and an impermeable platen, can be subjected to dynamic compressive loading simultaneously. A load cell and a linear variable differential transformer (LVDT) measured the load response of specimens and the imposed displacement, respectively (Fig. 1A). A cam-follower system was used to produce sinusoidal displacements with different magnitudes and frequencies. In order to maintain the viability of cells, the testing chamber was filled with culture medium while the bioreactor performed the experiments in a humidified incubator maintained at 37°C in 5% CO_2.

Dynamic Unconfined Compression Tests

After construct preparation, specimens were cultured in 24-well culture plates containing a basic serum-free medium consisting of UltraCulture, 1% antibiotics, and 0.29 mg/ml L-glutamine. After a preliminary culture of 20 to 24 hours, specimens were divided into four groups: control (n = 18; three specimens per rabbit × 6 rabbits), TGF-β (n = 18), loading (n = 18), and TGF-β loading (n = 18) groups. The specimens of the TGF-β and control groups were cultured in serum-free medium with and without the supplement of 10 ng/ml TGF-β1, respectively. Specimens of the loading group were placed into the testing chamber of the bioreactor filled with serum-free medium. After preloading the specimens with 5% static strain for 300 seconds, sinusoidal compressive loading was applied with 10% magnitude at a frequency of 1 Hz for 4 hours. After the unconfined compression test, specimens were unloaded and incubated for 20 hours to allow constructs to recover. For the TGF-β loading group, both the TGF-β1 treatment and the cyclic compressive loading were applied to the specimens. The culture medium was changed every day. For each group that underwent compression tests, the culture medium was changed following the 4-hour period of compressive testing. Experiments were conducted for 3, 7, and 14 consecutive days. After experiments were completed, the gene expressions (aggrecan and collagen types I, II, and X) and DNA contents of the specimens were analyzed.

The TGF-β1 gene expression was also evaluated, since TGF-β1 treatment was shown to promote its gene expression in MSCs that resided in rabbit periosteal grafts [26]. Since compressive loading can transiently modulate the chondrocyte biosynthesis of cartilage-specific macromolecules, a set of specimens (n = 36: four groups per rabbit per time period × 3 rabbits × 3 time periods) were cultured in serum-free medium for 5 extra days after experimentation to account for the transient effects. Finally, the same gene expressions of all specimens were analyzed. Furthermore, in order to allow cells to produce and deposit more extracellular macromolecules, three specimens (from three

rabbits) in each group of the 14-day experiment were cultured in high-glucose DMEM supplemented with 10% FBS and 1% antibiotics for two extra weeks after the experiment and then analyzed histologically and immunohistochemically.

Theoretical Prediction of Mechanical Events Within Agarose Construct

The finite element formulation of the biphasic model [27, 28] was used to analyze the mechanical events within the agarose disk under the dynamic, unconfined compression. In the dynamic, unconfined compression experiment conducted in this study, an agarose disk (thickness h_0 = 1.5 mm and radius r_0 = 4 mm) was placed between an adhesive permeable filter and a frictionless impermeable platen and subjected to a sinusoidal displacement with a magnitude of 10% strain and a frequency of 1 Hz (Fig. 1B). Using the material properties of 2% agarose gels, which were reported in our previous study [29], with the assumption of Poisson's ratio of 0.45 [30], three major mechanical events (strain, axial fluid velocity, and fluid pressure) within the agarose disks were calculated.

RNA and DNA Extraction

The total RNA was extracted from the cell-agarose constructs using the reagent Trizol (GIBCO/BRL), according to the manufacturer's instructions. The sample was briefly homogenized in Trizol using a glass homogenizer and then incubated for 5 minutes at room temperature. After mixing vigorously with chloroform for 30 seconds, the mixture was separated into a lower phenol-chloroform phase and an upper aqueous phase by centrifuging at 12,000 *g* for 15 minutes at 4°C. After the aqueous phase was transferred into a fresh tube, the RNA was precipitated by adding isopropyl alcohol and centrifuging at 12,000 *g* for 10 minutes at 4°C. The RNA was used for analysis of gene expression after washing with 75% ethanol. The aqueous solution containing the DNA was separated from the lower phenol-chloroform phase by mixing vigorously with a back extraction solution (4 M guanidine thiocyanate, 50 mM sodium citrate, 1 M Tris) for 30 seconds and then centrifuging at 12,000 *g* for 20 minutes at 4°C. The aqueous DNA solution was used for the measurement of the DNA content.

Reverse Transcription Polymerase Chain Reaction (RT–PCR) Analysis

The gene expressions of the specimens were analyzed using RT-PCR analysis. The RT-PCR was performed in a GeneAmp PCR system (9600, Perkin Elmer Ceyus; Norwalk, CT; http://instruments.perkinelmer.com/index.asp) using the ThermoScript RT-PCR system (GIBCO/BRL). The cDNA synthesis was performed over a 60-minute incubation at 50°C, with an avian RNase H-minus reverse transcriptase and Oligo$(dT)_{20}$ primer, followed by enzyme inactivation at 85°C for 5 minutes. PCR amplifications for the resulting cDNA samples were carried out for 35 cycles by denaturing at 95°C for 30 seconds, annealing at 58°C for 45 seconds, and extending at 72°C for 45 seconds, and were finally extended at 72°C for 10 minutes. PCR primers are shown in Table 1. As an internal control, 353 bp of the constitutively expressed housekeeping gene, β-actin, were also synthesized. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and photographed using a low light image system (ChemiImager 4000, Alpha Innotech Corporation; San Leandro, CA; http://www.alphainnotech.com). The integrated density value (IDV) of each PCR product from the electrophoresis image was measured by the AlphaEase software (Alpha Innotech Corporation) and normalized with the IDV of the β-actin PCR product.

Table 1. Sequences of PCR primers

Gene		Sequence	Size	Reference
Collagen I	(Sense)	5′-CGTGGTGACAAGGGTGAGAC-3′	827 bp	Genbank
	(Antisense)	5′-TAGGTGATGTTCTGGGAGGC-3′		Z74615
Collagen II	(Sense)	5′-GCACCCATGGACATTGGAGGG-3′	366 bp	Genbank
	(Antisense)	5′-GACACGGAGTAGCACCATCG-3′		S83370
Collagen X	(Sense)	5′-ATAAAGAGTAAAGGTATACC-3′	342 bp	Genbank
	(Antisense)	5′-CTGTTGTCCAGGTTTTCCTG-3′		AF247705
Aggrecan	(Sense)	5′-ACATCCCAGAAAACTTCTTT-3′	276 bp	Genbank
	(Antisense)	5′-CGGCTTCGTCAGCAAAGCCA-3′		L38480
TGF-β1	(Sense)	5′-CTTCCGCAAGGACCTGGG-3′	142 bp	[26]
	(Antisense)	5′-CGGGGTTGTGCTGGTTGTAC-3′		
β-actin	(Sense)	5′-GCTCGTCGTCGACAACGGCTC-3′	353 bp	GIBCO/BRL
	(Antisense)	5′-CAAACATGATCTGGGTCATCTTCTC-3′		

Measurement of DNA Content

The DNA content of the samples was measured using the Hoechst 33258 fluorometric assay described in the previous study [31]. The stock solution (1 mg/ml) of Hoechst 33258 dye was made by dissolving Hoechst 33258 dye in distilled water. A working buffer was prepared by diluting the stock solution to 0.15 mg/ml in 100 mM Tris, 1 mM EDTA, and 0.2 M NaCl (pH = 7.4) immediately before use. One hundred microliters of the DNA solution were mixed with 2 ml working buffer in a disposal cuvette. The fluorescence measurement of Hoechst 33258 dye was performed using a spectrofluorometer (RF-1501, Shimadzu Scientific Instruments Inc.; Norcross, GA; http://www.iscpubs.com) with an excitation wavelength of 365 nm and an emission wavelength of 458 nm. A standard curve was generated using 50-400 ng of calf thymus DNA.

Histological and Immunohistochemical Analysis

Pellets and cell-agarose constructs were fixed in 10% buffered formalin for 2 hours at 4°C. After washing in phosphate-buffered saline (PBS) (GIBCO/BRL), the specimens were dehydrated in a graded series of increasing concentrations of ethanol, cleaned with xylene, embedded in paraffin, and cut into 5-μm sections. Proteoglycans were detected by staining sections with alcian blue solution. The deposition of collagen type II protein was identified by immunohistochemical analysis. After deparaffinizing, sections were incubated in 0.3% hydrogen peroxide in distilled water for 30 minutes to remove endogenous peroxidase. The samples were rinsed with distilled water and PBS, and then 10% normal horse serum was placed on the sections to block nonspecific background, and the sections were incubated with mouse monoclonal anti-human collagen type II antibody (II-4CII, 500 μg/ml; ICN Biomedical; Aurora, OH http://www.icnbiomed.com) at 4°C overnight. Following extensive washing with PBS to remove the primary antibody, immunoactivity was detected by incubating the sections with biotinylated horse anti-mouse antibody, followed by incubation with avidin-biotin-peroxidase complex (ImmunoPure ABC peroxidase staining kits; Pierce; Rockford, IL; http://www.piercenet.com). Peroxidase activity was visualized using 3-3′-diaminobenzidine (DAB) as the substrate. The sections were incubated with 0.06% DAB in 0.1 M Tris-HCL (pH 7.5) containing 0.03% H_2O_2, followed by counterstaining with hematoxylin. All incubations were performed in a humidified chamber.

Statistical Analysis

Differences in chondrogenic gene expressions and DNA content between the four experimental groups at the three time periods were analyzed statistically using a one-way analysis of variance as well as a student *t*-test when necessary. Significance was assumed for $p < 0.05$. Statistical analyses were performed using Excel (Microsoft, Inc.).

Results

Based on the calculations from the biphasic model, the mechanical responses of the agarose disks can be considered to reach equilibrium after 200 cycles of loading in the configuration described in Figure 1B. The predicted axial strain, axial fluid flow, and fluid pressure of the three zones within the agarose disks, shown in Figure 2, are at the peak of the two hundredth cycle. Generally, the distributions of the strain, fluid flow, and fluid pressure were not uniform throughout the specimen. The axial strain (in compression) increased from about 4% at the region attached to the porous filter to 14% at the region attached to the impermeable platen (Fig. 2A) while the radial strain (in tension) increased from 1% to 7%. The axial fluid velocity decreased with increasing radius and decreasing depth (from the porous filter side to the impermeable platen side) with a maximal velocity of 9 μm/second (Fig. 2B), while the radial fluid velocity was near zero except near the edge. The profile of fluid pressure decreased with increasing radius and had a maximal pressure of 0.1 kPa. This profile remained consistent along each depth, except at the region close to the porous filter (Fig. 2C).

Chondrogenic potential of rabbit BM-MSCs was confirmed in pellet cultures, as illustrated in Figure 3. After a 21-day culture, the TGF-β1-treated specimens exhibited stronger expressions of collagen type II and aggrecan than the specimens without the treatment of TGF-β1 (Fig. 3A). The specimens of the TGF-β1-treated group exhibited stronger alcian blue staining of proteoglycans (Fig. 3B-3E), while the immunohistochemical assay revealed intense dark brown staining of collagen type II on the TGF-β1-treated specimens (Fig. 3F-3I).

Figure 4 shows the comparison of DNA content among the four groups at each time period. The DNA content of the specimens increased an average of 40% after the 14-day experiment. The TGF-β1-treated specimens tended to have more DNA content than the specimens without the treatment. However, no significant differences were found in the DNA content between the four groups at each of the three time periods.

The typical gene expressions of the four groups at the three time periods are shown in Figure 5. Generally, the control group exhibited weaker chondrogenic gene expressions than the other three groups at all time periods. The collagen II gene expression of the TGF-β, loading, and TGF-β loading groups slightly increased with increasing time of culture, whereas their aggrecan gene expressions remained similar for every time period. Four groups expressed the collagen

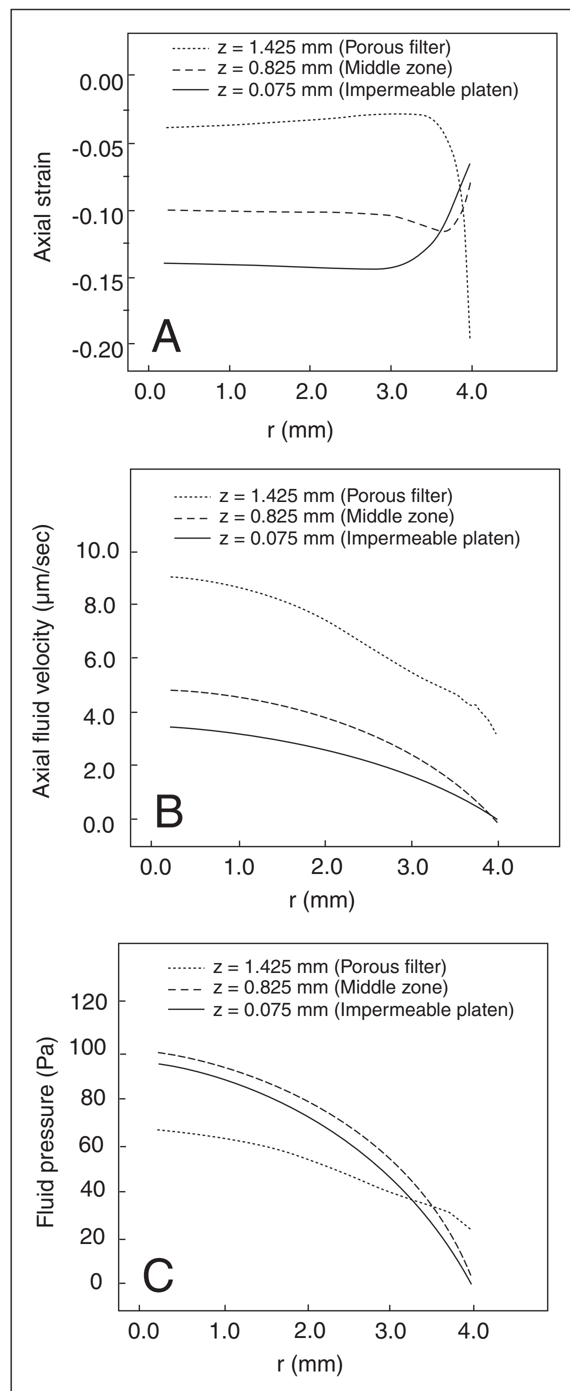


Figure 2. The predictions of the (A) axial strain, (B) fluid flow, and (C) fluid pressure for three zones within the agarose disk at the peak of the two hundredth cycle in the unconfined compression test.

type I expression at a similar level except the 14-day experiment in which the collagen type I expressions of the TGF-β, loading, and TGF-β loading groups were decreased and were weaker than that of the control group. However, none of the experimental group exhibited the expression of collagen type X at all time periods.

Statistical analyses of the chondrogenic gene expressions of the control and experimental groups are shown in Figures 6. Significant differences were found in both collagen II and aggrecan expressions among the four groups at each time period, with the control group having the lowest production of both chondrogenic genes while compressive loading and TGF-β1 were similar in their abilities to induce chondrogenesis. For the 14-day experiment, compressive loading in the presence of TGF-β1 promoted the collagen type II expression better than TGF-β1 alone. After five extra days of culture in serum-free medium following each of the three experiments, the chondrogenic gene expressions of the TGF-β, loading, and TGF-β loading groups only slightly decreased, whereas the specimens of the control groups still exhibited less production of collagen type II and aggrecan than those of the other groups in which no significant differences were found (Fig. 7).

Figure 8 shows typical gene expressions of TGF-β1 in all 4 groups, as well as its statistical comparison among the groups, for each time period. The control group significantly exhibited less TGF-β1 gene expression than the other three groups for the 3-day and 7-day experiments, whereas no significant difference was found for the 14-day experiment. The TGF-β1 gene expression of the TGF-β, loading, and TGF-β loading groups appeared to be inversely related to the time of culture.

The histological and immunohistochemical analyses demonstrated that the rabbit BM-MSCs of the TGF-β group, the loading group (Fig. 9), and the TGF-β loading group were able to proliferate and deposit more extracellular macromolecules (proteoglycan and collagen type II protein) to form larger cellular aggregates than those of the control group.

Discussion

Previous in vivo studies indicate that the mechanical environment can influence cartilage healing in the rabbit joint using the transplantation of MSCs from autogenous periosteal grafts and bone marrow [13, 14]. These data may suggest that mechanical forces associated with the articular joint stimulate chondrocyte differentiation of MSCs as well as cartilage-specific macromolecule production and deposition of differentiated MSCs. In fact, it has been demonstrated that compressive loading modulates the cartilage matrix biosynthesis of mature chondrocytes [15-21], while cyclic hydrostatic pressure enhances the extracellular matrix productions of human BM-MSCs while undergoing chondrogenesis [25]. However, the present study offers new insight to the effects of cyclic compressive loading on chondrogenic differentiation of BM-MSCs.

This study found that the specimens subjected to cyclic compressive loading exhibited more chondrogenic gene expressions than those of the control group, suggesting that this mechanical stimulation promotes chondrogenesis of rabbit BM-MSCs in the absence of cytokines. The histological and immunohistochemical analyses also demonstrated that the loading group exhibited more deposition of cartilage-specific macromolecules (collagen type II protein and proteoglycans) than the control group. In addition, combining cyclic compressive loading with the TGF-β1 treatment promoted the collagen type II gene expression of rabbit BM-MSCs more effectively than TGF-β1 alone. These findings strongly support the premise of this study. After an extra 5-day culture in serum-free medium, the profiles of chondrogenic gene expressions for the four experimental groups remained similar to those processed without the additional culture period. This proves that the compressive loading protocol used in this study prevents major transient effects from altering any chondrogenic gene expression. The aggrecan and collagen type II expression of the treated samples did not return to the control level after the extra 5-day culture period. Therefore, the rabbit BM-MSCs differentiated into chondrocyte-like cells after the TGF-β1 treatment or the stimulation of cyclic compressive loading. Furthermore, since no significant differences were found among the DNA contents, it can be inferred that the cyclic compressive loading used in the study did not affect the proliferation of rabbit BM-MSCs.

An important finding in this study is that the effects of TGF-β1 treatment and the stimulation of cyclic compressive loading on chondrogenic gene expressions of rabbit

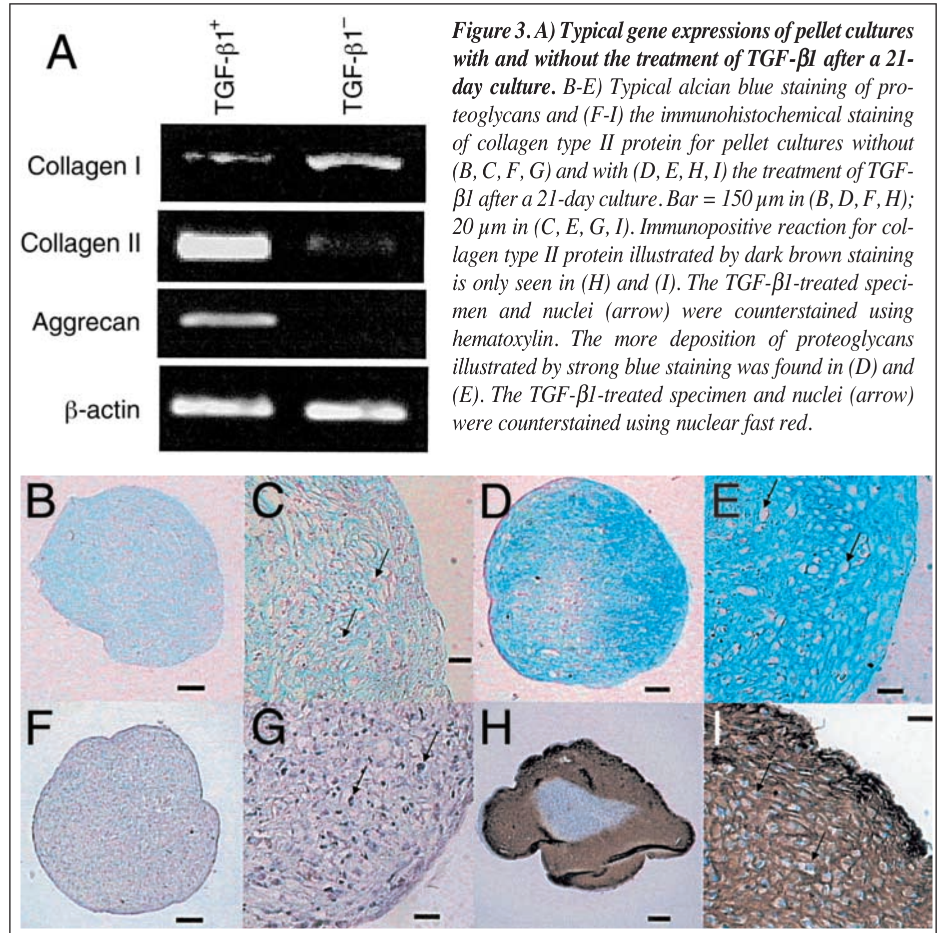


Figure 3. A) Typical gene expressions of pellet cultures with and without the treatment of TGF-β1 after a 21-day culture. *B-E) Typical alcian blue staining of proteoglycans and (F-I) the immunohistochemical staining of collagen type II protein for pellet cultures without (B, C, F, G) and with (D, E, H, I) the treatment of TGF-β1 after a 21-day culture. Bar = 150 μm in (B, D, F, H); 20 μm in (C, E, G, I). Immunopositive reaction for collagen type II protein illustrated by dark brown staining is only seen in (H) and (I). The TGF-β1-treated specimen and nuclei (arrow) were counterstained using hematoxylin. The more deposition of proteoglycans illustrated by strong blue staining was found in (D) and (E). The TGF-β1-treated specimen and nuclei (arrow) were counterstained using nuclear fast red.*

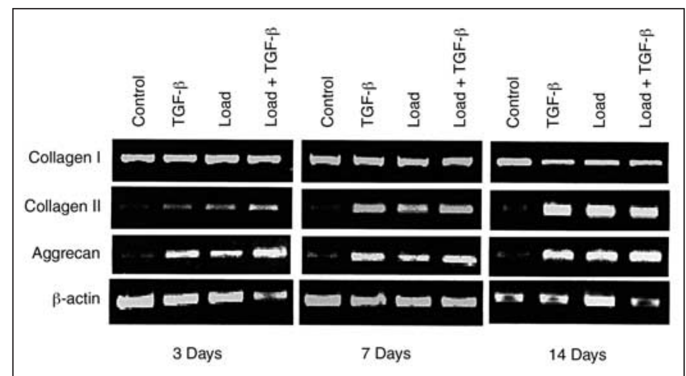


Figure 5. Typical RT-PCR analyses of chondrogenic genes for the four experimental groups at three time periods.

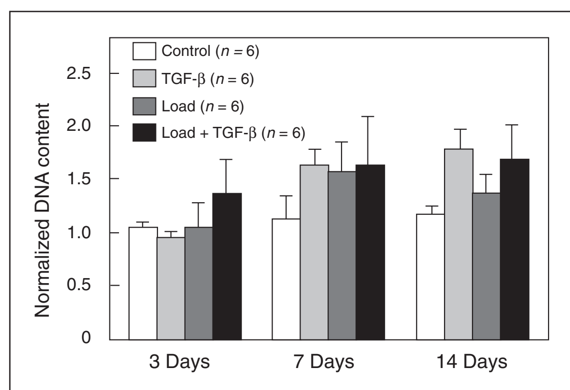


Figure 4. Comparison of normalized DNA content among the four experimental groups at three time periods.

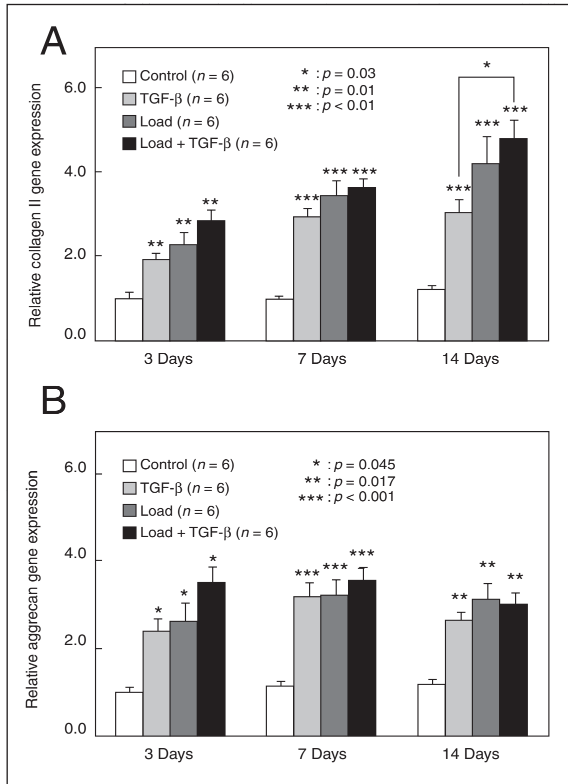
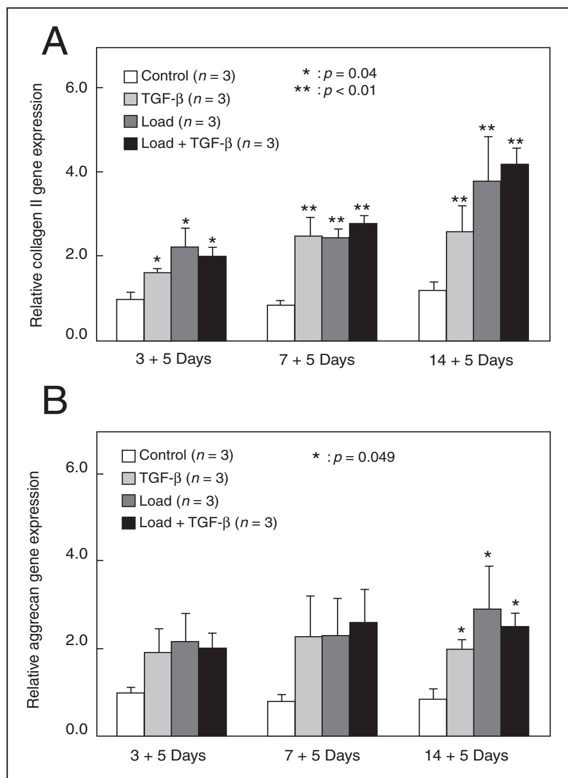


Figure 6. Comparison of gene expression of (A) collage type II and (B) aggrecan among the four experimental groups at three time periods.



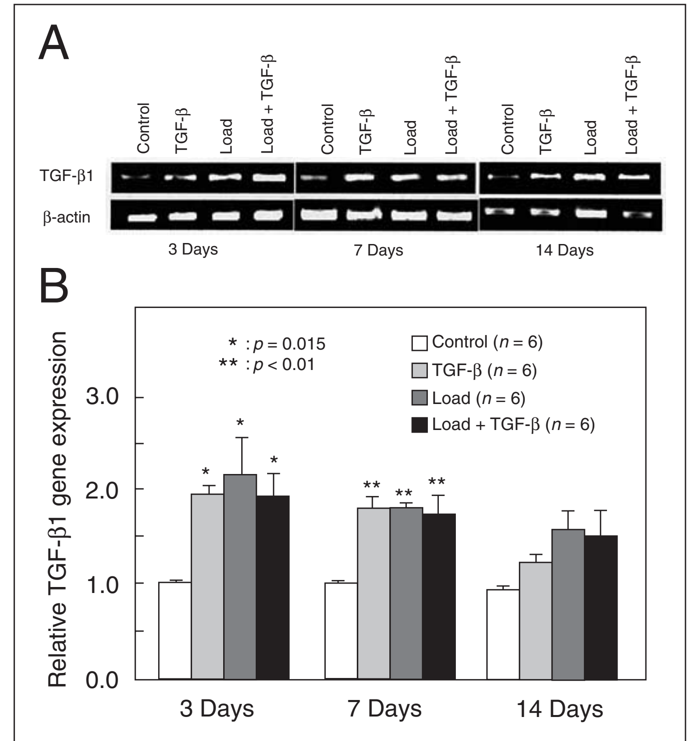


Figure 8. A) Typical RT-PCR analysis of TGF-β1 gene for the four experimental groups at three time periods. *B) Comparison of TGF-β1 gene expression among the four experimental groups at three time periods.*

BM-MSCs follow a similar trend. Hence, it is a possibility that both stimuli induce chondrogenesis of rabbit BM-MSCs through a similar signal pathway. This finding is further supported by the observation that TGF-β1 treatment and cyclic compressive loading promoted TGF-β1 gene expression in rabbit BM-MSCs. The effects of TGF-β1 treatment on the endogenous TGF-β1 gene expression found in this study were consistent with a recent finding, which showed that the TGF-β1 gene expression of rabbit periosteal explants increased after the TGF-β1 treatments of 7 and 42 days [26]. Previous studies have also demonstrated that mechanical loading can promote TGF-β1 production in cell cultures [32] and tissues [33, 34]. The gene expression of TGF-β1 for rat tibial periosteum was found to be 2-fold higher after 4 hours of mechanical loading [33], while the medium of cultured periosteal cells, stimulated by intermittent hydrostatic compression for 3 days, exhibited a 20-fold increase of TGF-β1 activity [32]. The findings of this study are consistent with the study of *Robbins et al.*,

Figure 7. Comparison of gene expression of (A) collage type II and (B) aggrecan among the four experimental groups after five extra days of culture in serum-free medium following each of the three experiments.

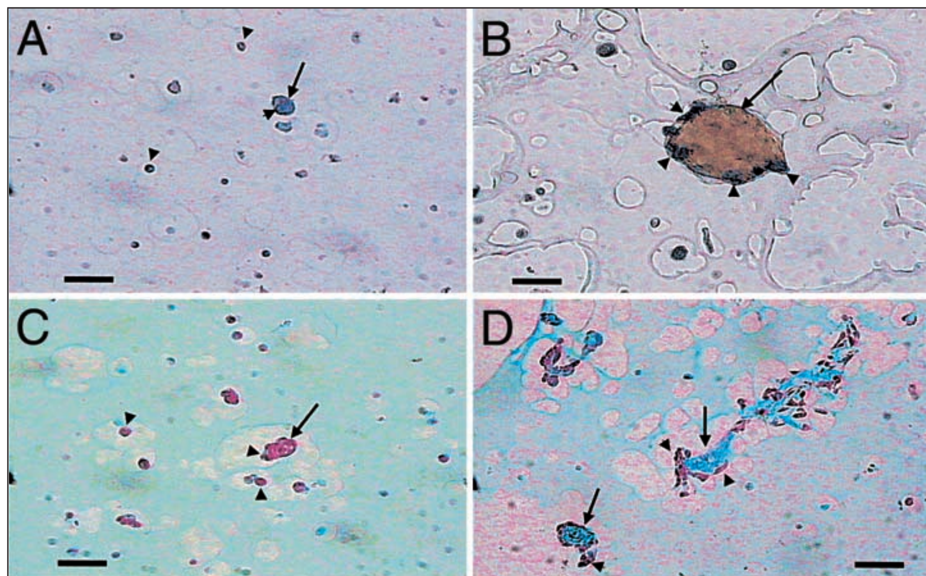


Figure 9. Typical immunohistochemical staining of collagen type II protein (A and B) and alcian blue staining (C and D) of proteoglycans on the cell-agarose constructs of the control (A and C) and loading (B and D) groups, which were cultured for two extra weeks after a 14-day experiment (bar = 20 μm). *The rabbit BM-MSCs were able to proliferate and deposit more extracellular matrix to form larger cellular aggregates (arrow) within the agarose constructs of the loading group (B and D) than the control group (A and C). The loading group exhibited deposition of collagen type II protein (brown staining in B) and proteoglycans (strong blue staining in D). Nuclei (arrow) were counterstained using (blue-violet) in (A) and (B) and nuclear fast red (red-pink) in (C) and (D).*

who found that mechanical loading not only stimulated proteoglycan synthesis and gene expression of the bovine flexor tendon, but also promoted TGF-β1 synthesis, leading toward fibrocartilage formation [34]. Cyclic, compressive loading presents a more innovative and lucrative substitute for the use of TGF-β1 in promoting chondrogenesis of BM-MSCs. In order to further confirm the effects of loading on endogenous TGF-β1 production, the TGF-β1 synthesis of rabbit BM-MSCs will be studied in the future.

The collagen type X gene expression was not detected by RT-PCR analysis for all four groups during experimental time periods, suggesting that the cells did not progress into hypertrophy under the culture conditions used in this study. Since TGF-β1 is the major cytokine in regulating BM-MSC chondrogenic differentiation, this finding is supported by the previous studies that have shown that TGF-β1 can inhibit chondrocyte hypertrophic differentiation [35, 36].

This study used the finite element method based on the biphasic model to predict strain, fluid flow, and fluid pressure within an agarose disk. Fluid flow around cells can generate shear stress that may affect cellular activity. Predicted fluid rate was found to be within the range of 0 and 9 μm/seconds, which is the same order of magnitude as the fluid rate in articular cartilage, yet several orders of magnitude less than those observed to stimulate chondrocytes in previous in vitro studies [37, 38]. However, fluid flow, induced by dynamic compression, facilitated the transport of nutrients [16] and cytokines [39], thus the biosynthesis of chondrocytes was increased. This explains the finding that cyclic loading in the presence of TGF-β1 further promoted the expression of collagen type II in rabbit BM-MSCs. The maximal fluid pressure was found to be 0.1 kPa, which was much lower than that found to affect chondrocyte differentiation of embryonic limb-bud cells [22-24], the extracellular matrix deposition of differentiated human BM-MSCs [25], and the physiological level of articular joint [40]. Therefore, fluid pressure may not serve as a mechanical stimulus to cells in this study. The range of magnitude of predicted axial strain was between 4% and 14%, the same level of compressive strain observed to modulate the biosynthetic activity of mature chondrocytes [15-21] and to stimulate the chondrogenic differentiation of embryonic limb-bud cells [22, 23]. Moreover, cells are known to deform in agarose gels under compression [41, 42]. Based on theoretical predictions, cell deformation plays an important role in promoting chondrogenesis of rabbit BM-MSCs in this study.

The bioreactor developed in this study provided a simple cam-follower system to generate cyclic displacements of different magnitudes and frequencies by adjusting the center offset of the eccentric cam and the frequency of the driving motor. Since this study was to examine whether dynamic loading influences chondrogenesis of BM-MSCs, only one combination of three loading factors (frequency, magnitude, and duration) was chosen based on the protocols used in several mechanical loading studies [15-21]. These methods indicate that a magnitude from 3%-15%, a frequency from 0.3-1.0 Hz, and a duration from 2-24 hours were observed to promote chondrogenesis of chondrocytes and embryonic limb-bud mesenchymal cells in agarose cultures. The effects of different combinations of the three loading factors will be systematically investigated in future studies.

This study served to establish an in vitro, three-dimensional culture system that supports chondrogenesis of BM-MSCs and

allows application of compressive loading to cells. By demonstrating that the accumulation of cartilage-specific macromolecules was seen in pellet cultures and not in monolayer cultures treated with TGF-β1, the study by *Yoo et al.* [11] indicated that suspension culture contributed to promoting the chondrogenesis of human BM-MSCs. Preliminary research for this study has shown that chondrogenesis of human BM-MSCs can be induced in agarose gels with the treatment of TGF-β3 [43]. In addition, agarose culture was found to maintain the phenotypic expression of chondrocytes [44, 45] and is widely used in the mechanical loading studies of chondrocytes [15, 18-20]. Thus, agarose culture was adopted for the compression experiments throughout this study. Future investigations will examine the application of this study using human BM-MSCs in hopes of creating an implantable construct, modeling cartilage, for clinical use.

This study demonstrated that cyclic compressive loading was indeed able to stimulate chondrogenic differentiation of rabbit BM-MSCs in the absence of cytokines. Additionally, combining cyclic compressive loading and the TGF-β1 treatment promoted collagen type II gene expression of rabbit BM-MSCs more effectively than TGF-β1 alone. The influence of cyclic compressive loading on chondrogenic differentiation of rabbit BM-MSCs was found to be similar to the TGF-β1 treatment. This interesting observation was supported by the finding that cyclic compressive loading promoted the endogenous TGF-β1 gene expression of rabbit BM-MSCs, suggesting that cyclic, compressive loading can become a beneficial alternative for the use of TGF-β1 in promoting chondrogenesis of BM-MSCs. In accordance with previous research, the results of this study indicated that characteristics of the mechanical environment affect the chondrogenesis of BM-MSCs during cartilage healing.

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