

Intraarticular Injection of Allogenic Mesenchymal Stem Cells has a Protective Role for the Osteoarthritis

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Abstract

Background: Researchers initially proposed the substitution of apoptotic chondrocytes in the superficial cartilage by injecting mesenchymal stem cells (MSCs) intraarticularly. This effect was termed as bio-resurfacing. Little evidence supporting the treatment of osteoarthritis (OA) by the delivery of a MSC suspension exists. The aim of this study was to investigate the effects of injecting allogenic MSCs intraarticularly in a rat OA model and to evaluate the influence of immobility on the effects of this treatment.

Methods: We established a rat knee OA model after 4 and 6 weeks and cultured primary bone marrow MSCs. A MSC suspension was injected into the articular space once per week for 3 weeks. A subgroup of knee joints was immobilized for 3 days after each injection, while the remaining joints were nonimmobilized. We used toluidine blue staining, Mankin scores, and TdT-mediated dUTP-biotin nick end labeling staining to evaluate the therapeutic effect of the injections. Comparisons between the therapy side and the control side of the knee joint were made using paired *t*-test, and comparisons between the immobilized and nonimmobilized subgroups were made using the unpaired *t*-test. A *P* value < 0.05 was considered significant.

Results: The three investigative approaches revealed less degeneration on the therapy sides of the knee joints than the control sides in both the 4- and 6-week groups (*P* < 0.05), regardless of immobilization. No significant differences were observed between the immobilized and nonimmobilized subgroups (*P* > 0.05).

Conclusions: Therapy involving the intraarticular injection of allogenic MSCs promoted cartilage repair in a rat arthritis model, and 3-day immobility after injection had little effect on this therapy.

Key words: Intraarticular Injection; Mesenchymal Stem Cell; Osteoarthritis

INTRODUCTION

Osteoarthritis (OA) is treated primarily by knee arthroplasty during the last stage, and even the early therapies of oral glucosamine and hyaluronic acid intraarticular injections only postpone OA progression rather than reverse the disease. OA therapy is becoming more important with the increasing morbidity of this disease.

Some studies have reported that excess apoptosis of chondrocytes and mesenchymal stem cells (MSCs), and the decreased abilities of these cells to replicate and differentiate contribute to OA.^[1-6] MSCs have been applied for the treatment of bone loss and for the induction of cartilage repair due to their multi-directional differentiation ability.^[7-13] The matrix-guided delivery of MSCs has been applied to therapy for cartilage defects.^[14-20] Some studies have observed that injected MSCs could attach to the injured cartilage and promote repair.^[21-24]

Murphy *et al.*^[9] reported that the intraarticular injection of MSCs retarded the progressive destruction of OA. Primarily, these studies involved a single implantation of MSCs without other adjuvant therapies for cartilage injury. We hypothesized that MSC implantation would have a therapeutic effect on OA; this effect is termed bio-resurfacing. Based on the time of cell attachment and the nutritional requirements of the joint space, we proposed that multiple intraarticular injections of high-dose MSCs with joint immobilization would produce better effects

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during the early stage of OA. We aimed to investigate the effects of multiple intraarticular injections of MSCs on early OA and to evaluate the influence of immobilization on the effects of this treatment. Our study was performed using male Sprague–Dawley (SD) rats, which were divided into two groups based on OA severity; each group included immobilized and nonimmobilized subgroups. After every MSC injection, we immobilized two legs of each rat in the immobilized subgroups. Using this approach, we evaluated the effects of both multiple injections and immobilization on MSC therapy for OA.

METHODS

Preparation of rat mesenchymal stem cells

The femur and tibia were harvested from male SD rats on a super-clean bench. The bone cavity was washed with low-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Beijing, China) containing 1% antibiotic-antimycotic and centrifuged. Then, the precipitated cells were suspended in medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, Beijing, China). The cells were seeded in the same medium in 75 ml culture flasks and maintained at 37°C with 95% humidity and 5% CO₂. After 2 days, the red blood cells were washed away with phosphate-buffered saline (PBS), and fresh medium was added. Colonies of adherent cells formed within 12 days. The colonies were trypsinized from the flasks when the colonies covered 60–90% of the plate and were seeded into fresh flasks. Passage three cells were harvested for each set of experiments.

Characteristics of rat mesenchymal stem cells

Cell viability was examined using a Cell Counting Kit-8 (CCK-8) (KeyGen BioTech, Nanjing, China) according to the manufacturer's protocol. The cells were seeded in a 96-well plate at a density of 1×10^4 cells per well. In total, 10 μ l of CCK-8 reagent was added to each well, and the samples were incubated for 4 h. The absorbance of the medium was recorded at 450 nm using a microplate spectrophotometer. The examination was performed in quintuplicate for 9 days. The rat MSCs were characterized by flow cytometry (FC). The cells were harvested and resuspended in FC buffer (50 μ l of PBS supplemented with 1% bovine serum albumin and 0.1% NaN₃) in 4 tubes at a concentration of 1×10^6 cell. The cells were separately stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34, anti-CD44, anti-CD45, and anti-CD90 antibodies at 4°C for 45 min in a dark room. After the cells were washed 3 times with FC buffer, they were fixed in 2% paraformaldehyde. Then, the MSCs were washed with FC buffer and analyzed using a FACScan flow cytometer and BD FACSDiva software (Becton Dickinson, NJ, USA).

Differentiation assay

Chondrogenesis was performed with 50 μ g/ml ascorbic acid (Sigma, St. Louis, MO, USA), 4×10^{-5} mg/ml dexamethasone (Sigma, St. Louis, MO, USA) and 10 ng/ml recombinant human transforming growth factor-3 (Calbiochem, USA). After the cells were cultured

for 14 days, they were fixed in 10% formalin and stained with periodic acid-Schiff (PAS) (Baso, Taiwan, China). The cells were incubated with goat anti-rat type II collagen antibody (Santa Cruz, USA) at 4°C overnight, followed by incubation with rabbit anti-goat FITC-IgG (Zhongshan Gold Bridge Bio, Beijing, China). The cells were observed by immunofluorescence microscopy. For osteogenic differentiation, the cells were cultured in 10 nmol/L dexamethasone, 10 nmol/L β -glycerophosphate (Sigma, St. Louis, MO, USA), and 50 μ mol/L ascorbic acid. The cells were stained with alizarin red after 14 days. Adipogenic differentiation was induced by culturing the MSCs in adipogenic medium (DMEM containing 10% FBS, 10 μ mol/L dexamethasone, 10 nmol/L 3-isobutyl-1-methylxanthine, 5 μ g/ml insulin, and 60 μ mol/L indomethacin) for 2 weeks, and the cells were assessed using oil red O stain.

Surgical protocol

The Institutional Animal Care and Use Committee approved all procedures used in this study. Forty male SD rats (weighing 120 g, aged 7 weeks) were used. The animals were treated with 5% pentobarbital (1 ml/kg) via abdominal cavity injection. For combined anterior cruciate ligament (ACL) transaction and medial meniscectomy, a lateral parapatellar skin incision was made. The lateral aspect of the vastus lateralis and the joint capsule were incised, and the patella was luxated laterally to expose the femoral condyles and the tibial plateau. ACL excision was performed first, followed by the medial meniscectomy. After patella reduction, the incision was carefully washed and sutured routinely.

Study design

Forty male rats were randomized into a 4-week group and a 6-week group ($n = 20$). Each group was randomized equally into an immobilized subgroup ($n = 10$) and a nonimmobilized subgroup ($n = 10$). OA was induced bilaterally in the knee joints of donor rats via complete excision of the medial meniscus and resection of the ACL as described above. All rats were allowed free movement after the operation. The rats in the 4-week group received the first intraarticular injection therapy at 4 weeks after the operation, and the rats in the 6-week group received the first therapy at 6 weeks after the operation. The control and therapy sides of the knee joints were labeled on each rat. The injections were administered once per week for 3 weeks [Figure 1a]. The rats were euthanized at 3 weeks after the last injection. The therapy side of the knee joint received an intraarticular injection of 0.1 ml of PBS containing 5×10^5 allogenic MSCs, and the control side of the knee joint received 0.1 ml of PBS alone [Figure 1b]. After the injections, the knee joints in the immobilized subgroup were immobilized with a cast for 3 days [Figure 1b and 1c]. The rats in the nonimmobilized subgroup were allowed free movement.

Intraarticular injection of allogenic mesenchymal stem cells

Frozen cells were thawed rapidly at 37°C, washed with culture medium and PBS, centrifuged, and resuspended in PBS at a

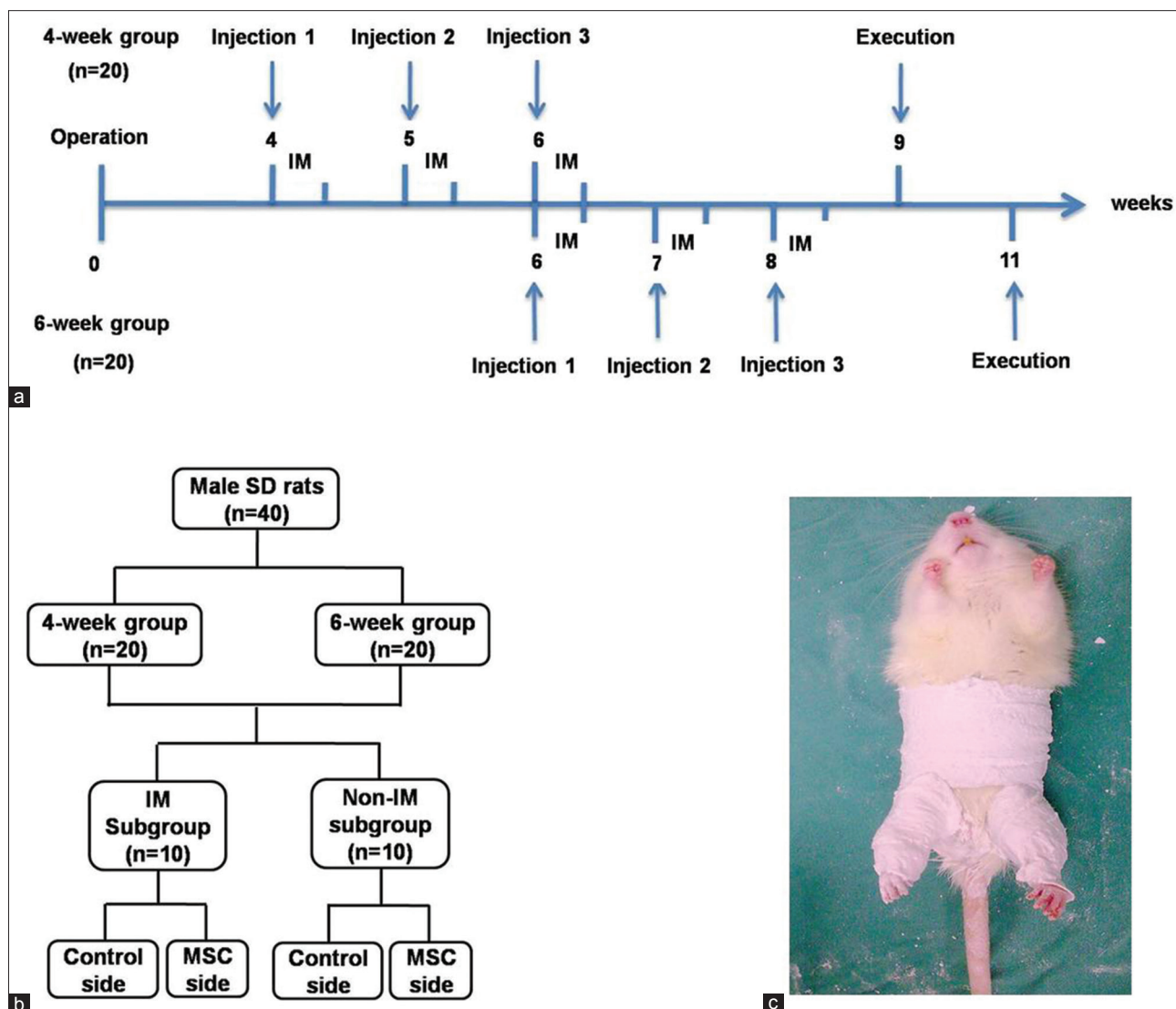


Figure 1: Study design – (a) The study groups of male rats with the time of intraarticular injection and immobilization (intramuscular); (b) Forty rats were divided into 4- and 6-week groups. Immobilized and nonimmobilized subgroups in each group and intraarticular injection of mesenchymal stem cell suspension for the therapy side, phosphate-buffered saline for the control side; (c) Immobilized rat by the cast.

density of 5×10^6 cells/ml. The rats were anesthetized and placed in dorsal recumbency. The needle of a 1 ml syringe was inserted posterior to the medial edge of the patellar ligament. In total, 0.1 ml of the cell suspension was injected into the medial compartment of the operated joint. Following injection, the joint was repeatedly flexed and extended to disperse the suspension throughout the intraarticular space.

Histological analysis of cartilage

Immediately after the rats were euthanized, the distal head of the femur and the proximal tibial plateau were removed, fixed in 4% paraformaldehyde for 2 days, and decalcified in 10% EDTA for 2 months. Then, the knee joints were embedded in wax. Serial sagittal sections (5 μ m thick) were cut from both the therapy and control joints. The sections were stained with toluidine blue and safranin O. The sections were graded using the Mankin grading scheme:^[25] (1) Articular cartilage structure, (2) reduced articular cartilage matrix staining, (3) alignment of chondrocytes,

and (4) the integrity of the tidal line. The articular cartilage structure was graded on a scale ranging from 0 (normal) to 14 (most severe cartilage injury).

Immunohistochemical analysis of cartilage

The TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using a TUNEL-POD kit according to the manufacturer's recommendations (Roche, Germany). Briefly, the sections were dewaxed and rehydrated according to standard protocols (by heating at 60°C, followed by washing in xylene and rehydration through a graded series of ethanol and double distilled water). The sections were incubated with 15 g/ml proteinase K for 15 min at room temperature and then washed in PBS. Then, the sections were immersed in buffer containing terminal deoxynucleotidyl transferase and incubated for 90 min at 37°C in a humid atmosphere. After the sections were washed again in PBS, they were incubated with anti-digoxigenin conjugate for 30 min at room temperature. After the sections were

washed in PBS and the color of the peroxidase substrate was developed with diaminobenzidine, the signals were examined by microscopy. Labeled nuclei were counted in 10 independent, randomly selected fields.

Statistical analysis

Comparisons between the therapy side and the control side of the knee joint were made using paired *t*-test, and comparisons between the immobilized and nonimmobilized subgroups were made using the unpaired *t*-test. $P < 0.05$ was considered significant.

RESULTS

Characterization of rat mesenchymal stem cells

MSCs were isolated from the bone marrow that was washed from the femur and tibia shafts of SD rats and expanded to form confluent cultures of adherent cells with a fibroblastic morphology [Figure 2a]. The MSCs grew continuously on day 1 and reached a plateau on day 6 [Figure 2b]. The phenotypic profile of the rat MSCs was determined by FC analysis. The rat MSCs were 96.3% positive for CD44, 98.5% positive for CD90, 95.91% negative for CD34, and 92.62% negative for CD45 [Figure 2c].

Differentiation assay

The capacity of the MSCs to differentiate into chondrocytes, osteocytes, and adipocytes was demonstrated *in vitro*.

After the chondrocytic induction of the cultures, the MSCs were PAS-positive [Figure 3a] and exhibited abundant type II collagen in the cytoplasm [Figure 3b] as indicated by immunofluorescence microscopy. For osteogenic differentiation, the cells were stained with alizarin red. Calcium deposition was detected in this culture [Figure 3c]. The cells that were maintained in adipogenesis medium for 2 weeks exhibited lipid vacuoles in the colonies as indicated by oil red O stain [Figure 3d].

Effect of allogenic mesenchymal stem cell therapy on cartilage degradation

After allogenic MSCs were injected intraarticularly into the rat knee joints, toluidine blue staining in the 4-week group revealed less cartilage degradation, including chondrocyte aggregation on the concave surface of the cartilage, cartilage fissure, and reduced cartilage matrix staining on the therapy sides of the knee joint in both the immobilized and nonimmobilized subgroups [Figure 4a1 and 4a3] compared with the control sides, which exhibited reduced cartilage thickness and superficial cartilage erosion [Figure 4a2 and 4a4]. On the therapy sides of the knee joints in the 6-week group, reduced thickness, fissure, and erosion of cartilage were observed in the immobilized and nonimmobilized subgroups [Figure 4b1 and 4b3]. In contrast, more severe arthritic changes including full thickness cartilage abrasion, total matrix loss, chondrocyte

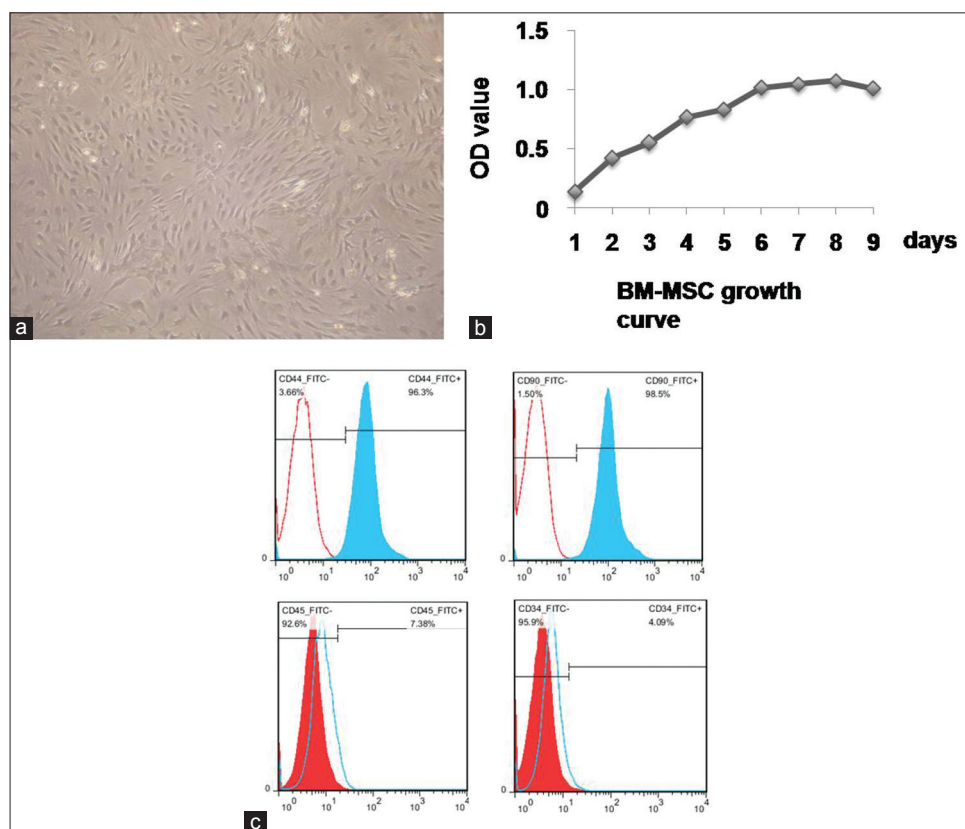


Figure 2: Primary culture and phenotypic characterization of rat mesenchymal stem cells. (a) Mesenchymal stem cells in the primary culture; (b) The growth rate of mesenchymal stem cells; (c) The phenotypic profile of mesenchymal stem cells. The mesenchymal stem cells were positive for CD44 and CD90, negative for CD34 and CD45.

disappearance, and subchondral bone sclerosis were obvious on the control sides of the knee joints [Figure 4b2 and 4b4].

The Mankin scores of the therapy and control sides of the knee joints in the immobilized subgroup of the 4-week group were 4.75 ± 1.67 and 8.00 ± 1.85 , respectively [Figure 5a]. The therapy side had a lower score than the control side ($P < 0.05$). The Mankin score of the therapy side of the knee joint in the nonimmobilized subgroup was 6.00 ± 1.69 , which was lower than that of the control side (8.77 ± 2.22) ($P < 0.05$) [Figure 5a]. In the 6-week group, the Mankin score of the therapy side (5.13 ± 1.46) in the immobilized subgroup was lower than that of

the control side (9.78 ± 1.20) ($P < 0.05$) [Figure 5b]. The same results were found in the nonimmobilized subgroup ($P < 0.05$), which had Mankin scores of 5.33 ± 1.21 and 9.42 ± 1.81 for the therapy and control sides, respectively ($P < 0.05$) [Figure 5b]. No significant differences between the immobilized and nonimmobilized subgroups were observed in the 4- and 6-week groups ($P > 0.05$).

Effects of allogenic mesenchymal stem cell therapy on chondrocyte apoptosis

After allogenic MSCs were injected intraarticularly into the rat knee joints, TUNEL-positive chondrocytes were found in the superficial cartilage on the therapy sides of the knee joints in the immobilized and nonimmobilized subgroups of the 4-week group [Figure 6a1 and 6a3], and small increase in TUNEL-positive chondrocytes was observed on the control sides [Figure 6a2 and 6a4]. The 6-week group showed a cluster of TUNEL-positive chondrocytes. The control sides of the knee joints in the immobilized and nonimmobilized subgroups [Figure 6b2 and 6b4] exhibited large increases in TUNEL-positive chondrocytes compared to the therapy sides [Figure 6b1 and 6b3].

After allogenic MSCs were injected intraarticularly into the rat knee joints, the ratios of apoptotic chondrocytes on the therapy and control sides in the immobilized subgroup of the 4-week group were $(36.64 \pm 7.69)\%$ and $(51.75 \pm 8.56)\%$, respectively [Figure 7a]. The therapy sides had fewer apoptotic chondrocytes than the control sides ($P < 0.05$). In the nonimmobilized subgroup, the chondrocyte apoptosis ratio on the therapy side was $(35.50 \pm 13.89)\%$, which was less than that of the control side ($50.56 \pm 10.76)\%$ ($P < 0.05$) [Figure 7a]. In the 6-week group, on the therapy side of the knee joints in

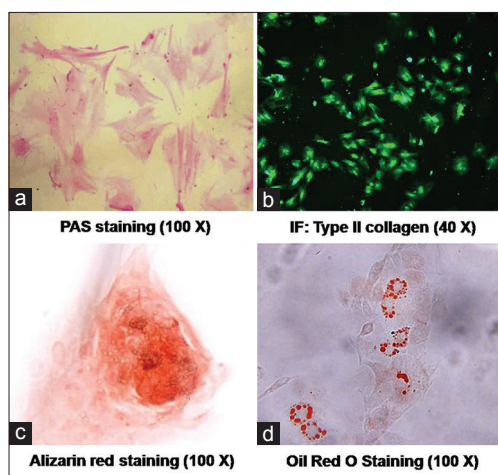


Figure 3: Differentiation capacity of rat mesenchymal stem cells. (a) Positive of periodic acid-Schiff staining for chondrogenic differentiation; (b) Positive immunofluorescence of type II collagen; (c) Alizarin red staining showed calcium deposition for osteogenic differentiation; (d) Oil red O staining showed lipid vacuoles for adipogenic differentiation.

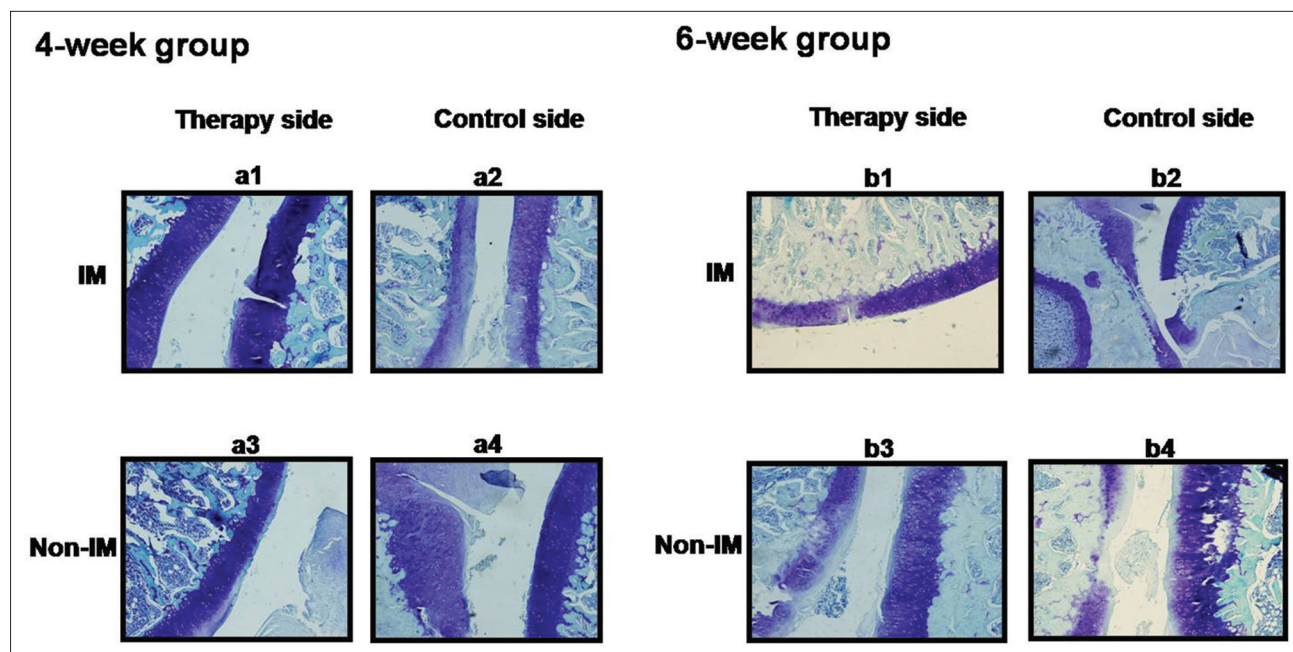


Figure 4: Toluidine blue staining evaluation The therapy sides (a1 and a3) showed less cartilage degeneration than the control sides (a2 and a4) in 4-week groups, the similar result to the 6-week group (b1 vs. b2, b3 vs. b4).

the immobilized subgroup, the chondrocyte apoptosis ratio ($49.42 \pm 15.32\%$) was lower than that of the control side ($69.75 \pm 13.17\%$) ($P < 0.05$) [Figure 7b]. The same result was found in the nonimmobilized subgroup ($P < 0.05$). The chondrocyte apoptosis ratios were ($47.00 \pm 11.05\%$) and

($67.17 \pm 15.02\%$) on the therapy side and the control side, respectively [Figure 7b], and the control side had many more apoptotic chondrocytes ($P < 0.05$). No significant difference was observed between the immobilized and nonimmobilized subgroups in the 4- and 6-week groups ($P > 0.05$).

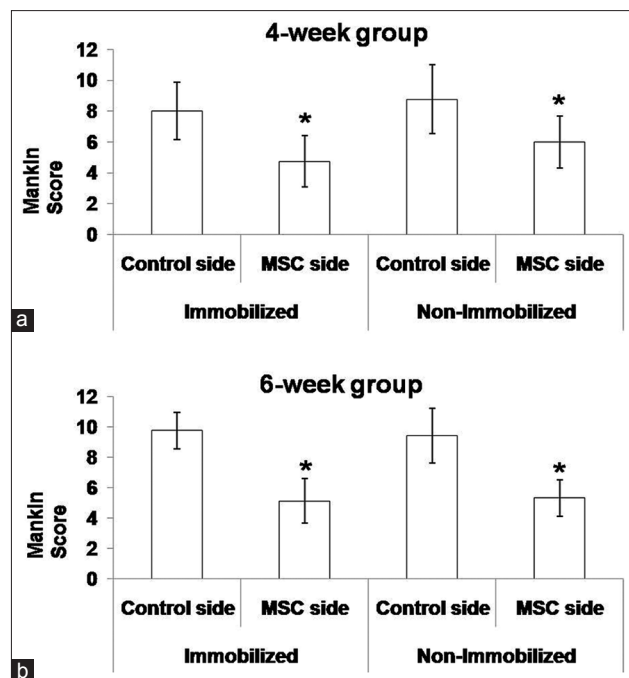


Figure 5: Mankin score evaluation The Mankin score of the therapy side was less than the control side in the 4-week group (a) and 6-week group (b). There was no significant difference between the immobilized and nonimmobilized subgroups. * $P < 0.05$.

DISCUSSION

Several studies have reported that MSCs injected intraarticularly could attach to the surface of articular cartilage and differentiate into chondrocytes to repair cartilage defects.^[17-19,21,26,27] A few studies regarding the use of the intraarticular injection of MSCs for OA therapy were conducted on animals and humans; the results of these studies indicated that the effect of this therapy was beneficial.^[9,28,29] In our study, we evaluated the therapeutic effects of MSCs injected intraarticularly for 3 consecutive weeks in 4- and 6-week OA groups. Our study increased the time course of injection compared to previous studies.^[9,21,26,28,29] Murphy *et al*^[9] reported that the long-term effect of this treatment was reduced by OA progression due to ACL rupture. Thus, we set the end-point at 3 weeks after the last injection to avoid the effect of knee joint instability.

Some researchers have raised the possibility that MSCs might deposit in the joint space without attaching to the surface of the injured cartilage. Furthermore, the mobility of the joint, such as crushing and friction, might damage MSCs and reduce cartilage repair.^[17] No immobility study regarding the intraarticular injection of MSCs has been published. To determine whether immobility

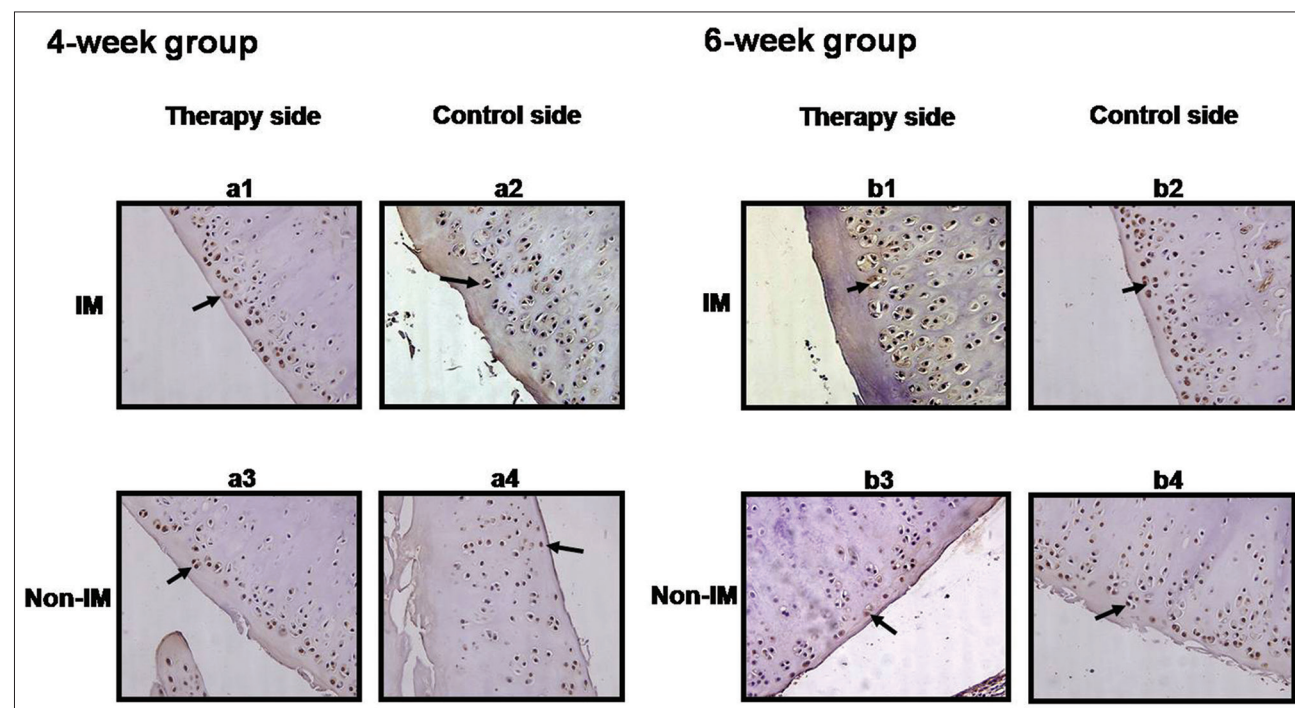


Figure 6: TdT-mediated dUTP-biotin nick end labeling staining Less TdT-mediated dUTP-biotin nick end labeling positive chondrocytes were found in the therapy sides of the 4-week group (a1 and a3) than the control sides (a2 and a4). The similar result to the 6-week group (b1 vs. b2, b3 vs. b4) (arrowheads: TdT-mediated dUTP-biotin nick end labeling positive chondrocyte).

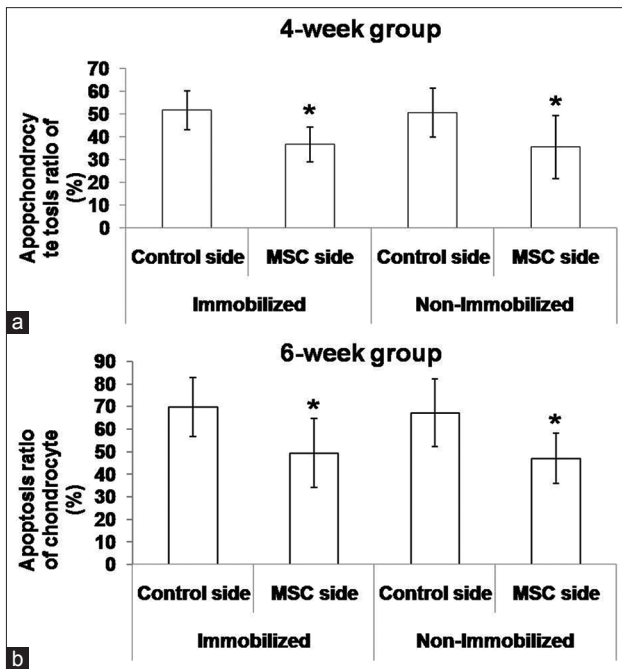


Figure 7: Chondrocyte apoptosis evaluation The therapy side had smaller ratios of apoptotic chondrocyte than the control side in the 4-week group (a) and 6-week group (b). There was no significant difference between the immobilized and nonimmobilized subgroups. * $P < 0.05$.

would facilitate MSC attachment, the therapy and control sides of the knee joints in the immobilized subgroup were immobilized with a cast in our study. However, long-term immobilization itself can injure the cartilage and induce OA.^[30] Langenskiöld *et al.*^[31] reported that 5–6-week immobilization of rabbit knee joints caused moderate or severe OA, including articular cartilage loss and osteophyte formation as observed in human OA. Short-term immobilization could also cause some physiological changes. Suliman *et al.*^[30] reported that 5-day immobilization of the lower limbs could reduce the level of insulin growth factor-1 in the spinal cord and increase the level of the cholinergic receptor in the soleus and tibialis anterior muscle. The physiological influence of immobilization and the time of MSC attachment must be considered when selecting the immobilization time. Therefore, we limited the joint immobilization time to 3 days after each intraarticular injection in our study.

Murphy *et al.*^[9] studied the effect of the intraarticular injection of BM-MSCs with hyaluronic acid on goat OA. After 6 weeks, the therapy group had less cartilage rupture, fewer osteophytes, and less subchondral bone sclerosis than the control group. A neomeniscus was observed in the medial compartment of the knee joint, which contained green fluorescent protein-transduced MSCs and type II collagen. However, after 20 weeks, both groups had severe cartilage injury. To limit the long-term impact of instability on the knees, we evaluated the therapeutic effect of the intraarticular injection of MSCs for early-stage OA at 4 and 6 weeks after the operation. In both the 4- and 6-week

groups, the histological results showed that the therapy sides of the knee joints exhibited a smaller reduction of cartilage thickness, a reduced loss of cartilage matrix, and less erosion of superficial cartilage than the control sides, regardless of immobilization. Similar results were found for the Mankin scores, for which significant differences were observed between the therapy and control sides of the knee joints. Our results indicated that the intraarticular injection of MSCs had a therapeutic effect during the early stage of OA.

Because chondrocytes are the only cells in the articular cartilage, chondrocyte apoptosis plays an important role in cartilage degradation. Johnson *et al.*^[3] and Goggs *et al.*^[32] recommended that the ratio of apoptotic chondrocytes could be considered as an evaluation criterion for OA. Matsumoto *et al.*^[27] evaluated the effect of MSCs on OA using TUNEL and found that the therapy group had less apoptotic chondrocytes than the control group. This finding provided evidence that the MSC therapy attenuated OA. Our study had results similar to those of previous articles. Less apoptotic chondrocytes were found on the therapy sides of the knee joints than on the control sides in both the 4- and 6-week groups. The difference in the apoptotic chondrocyte ratios between the two sides was significant. Thus, we generated additional immunohistochemical evidence to support the therapeutic effects of MSCs on rat OA.

We made an interesting observation concerning the therapy sides of the knee joints in the immobilized and nonimmobilized subgroups. The therapy sides of the knee joints in the immobilized and nonimmobilized subgroups of both groups had Mankin scores and ratios of apoptotic chondrocyte that differed significantly compared with those of the control sides. Notably, immobilization had little effect on the therapeutic effects of the intraarticular injection of MSCs based on our study results. These findings offer a reference for clinical therapy involving the intraarticular injection of MSCs.

The therapeutic use of MSCs for OA is controversial due to uncertainties regarding many issues. However, an increasing number of articles regarding basic research, animal experiments, and clinical trials have been published to support the bio-resurfacing effect of MSCs on OA. Grogan *et al.*^[33] noted that the highest number of cells positive for stem cell markers occurred in the superficial zone of normal cartilage. The presence of such molecules increased in the cartilage during OA, implicating the involvement of progenitor cells in the modulation of OA progression. Kim *et al.*^[34] injected MSCs encapsulated in self-assembled peptide hydrogels into the rat articular cavity in an OA model and reported evidence of chondroprotection, as well as of decreased expression of biochemical markers of inflammation and apoptosis. Singh *et al.*^[29] directly injected scaffold-free MSCs obtained from the bone marrow into the rabbit knee articular cavity in an OA model. The result suggested that MSC culture and scaffolds were not necessary for a favorable outcome. Our study also supports the use of MSCs for OA therapy. However, a number of

unclear mechanisms require further study, such as the MSC implantation mechanism, the MSC anti-inflammatory mechanism, and the MSC differentiation mechanism. We will continue to focus on these problems.

This study suggests that a therapeutic benefit is associated with the intraarticular injection of allogenic MSCs after traumatic injury to the knees. Our results demonstrate that the intraarticular injection of MSCs may be applied for the clinical treatment of OA.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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