Original Article

Intra-articular Injection of Autologous Mesenchymal Stem Cells in Six Patients with Knee Osteoarthritis

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Abstract

Background: Osteoarthritis (OA) is a progressive disorder of the joints caused by gradual loss of articular cartilage, which naturally possesses a limited regenerative capacity. In the present study, the potential of intra-articular injection of mesenchymal stem cells (MSCs) has been evaluated in six osteoarthritic patients.

Methods: Six female volunteers, average age of 54.56 years, with radiologic evidence of knee OA that required joint replacement surgery were selected for this study. About 50 ml bone marrow was aspirated from each patient and taken to the cell laboratory, where MSCs were isolated and characterized in terms of some surface markers. About 20-24×10^6 passaged-2 cells were prepared and tested for microbial contamination prior to intra-articular injection.

Results: During a one-year follow-up period, we found no local or systemic adverse events. All patients were partly satisfied with the results of the study. Pain, functional status of the knee, and walking distance tended to be improved up to six months post-injection, after which pain appeared to be slightly increased and patients' walking abilities slightly decreased. Comparison of magnetic resonance images (MRI) at baseline and six months post-stem cell injection displayed an increase in cartilage thickness, extension of the repair tissue over the subchondral bone and a considerable decrease in the size of edematous subchondral patches in three out of six patients.

Conclusion: The results indicated satisfactory effects of intra-articular injection of MSCs in patients with knee OA.

Keywords: Cell therapy, mesenchymal stem cells, osteoarthritis

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Introduction

Many people worldwide suffer from a cartilage tissue loss referred to as degenerative articular cartilage. This disorder, if not treated, eventually leads to degeneration of the cartilage layer on the joint and exposure of underlying bone which is referred to as osteoarthritis (OA) and is more painful than degenerative articular cartilage.[1,2] Since cartilage naturally possesses a limited capacity for regeneration,[3] its defects are considered problematic. It has been generally accepted that articular cartilage injuries that do not penetrate the subchondral bone are not repaired while those that penetrate the subchondral bone are repaired with formation of fibrous tissue or fibrocartilage. Such reparative tissue lacks the biochemical capability of hyaline cartilage that occurs in articular cartilage.[4] Traditional methods to regenerate defects of articular cartilage include micro-fracture, multiple perforations, abrasions and mosaicplasty, the results of which are not satisfactory.[5–11]

The modern approach to cartilage regeneration has been implantation of cartilage-forming cells into the defect. Autologous chondrocytes implantation (ACI) could be one approach to regenerate an articular cartilage defect.[12–14] Nowadays, small cartilage defects can be repaired using this technique, although its effectiveness is still controversial. According to some authors, even after ACI, some defects continue to persist in the articular cartilage although not in the main weight-bearing portions of the joint. Indeed, in ACI no evidence of effectiveness has been reported thus far.[15,16] Preparation of chondrocytes for ACI is associated with several limitations, which include the limited number of chondrocytic cells and their dedifferentiation during the culture period for propagation.[14–17] For this reason, an alternative cell source should be found.

Mesenchymal stem cells (MSCs) are another alternative that can be used to regenerate articular cartilage defects. These cells have gained considerable attention since they possess two unique potentials: the ability to differentiate into skeletal cell lineages and the capacity to self-renew for a relatively long period of time. Easy accessibility of MSCs from multiple sources, including bone marrow aspirates, and low immunogenicity of the cells adds to their value as cellular candidates for cartilage regeneration.[18–22] Multiple investigations have indicated that MSCs could be effective in promoting regeneration of cartilage defects in animal models.[23] There are several reports regarding successful application of MSCs on the regeneration of human cartilage defects. In this context, a report by Wakitani et al. in 2002 on patients with knee osteoarthritis (OA) is remarkable. In this study, researchers have evaluated the knees of 24 patients. Adherent cells from bone marrow aspirate were embedded in collagen gel and transplanted into articular cartilage defects in the medial femoral condyle of 12 patients. The

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Table 1. Enrolled patients

Patient	Age	BMI	Marrow volume	MNC Number	Colony/100 cells
1	62	31.1	45 ml	272×10^6	30
2	64	35.4	55 ml	360×10^6	27
3	52	35.1	40 ml	234×10^6	25
4	49	35	50 ml	425×10^6	40
5	40	26.9	55 ml	320×10^6	20
6	56	26.3	50 ml	257×10^6	35

Table 2. Mean changes in measures of physical function, pain, walking distance, gelling time, patellar crepitus, and joint flexion.

	Pre-procedure	2 weeks post- procedure	1 month Post-procedure	2 months post-procedure	6 months post-procedure	12 months post-procedure
VAS (mm)	57±33	34±29	27±31	16±23	1±4	11.6±24
WOMAC Index	2.91±0.37	2.37±0.46	2.22±0.68	2.1±0.8	1.82±0.66	1.89±0.3
Walking distance (m)	88.3±93.2	88.3±93.2	140±162	306±417	406±467	377±449
Time to gelling (min)	8.1±4.9	18±21	14±6	30±26	19±13	15±12
Patellar crepitus	4	3.3±0.5	2.8±0.4	2.6±0.5	2.7±0.5	3.3±0.8
Knee flexion	88°±23	91°±23	100°±15	102°±19	106°±18	106°±26

remaining 12 subjects served as cell-free controls.[24] According to their findings, although clinical improvement was not significantly different, the arthroscopic and histologic grading score was better in the cell-transplanted group. Two years later, the same authors transplanted autologous MSC combined with collagen gel into two patients with full thickness articular cartilage defects in their patellae and reported a significant improvement of patient clinical symptoms (pain and walking ability) six months post-transplantation.[25] Wakitani et al. have also evaluated the effectiveness of such an approach on regenerating cartilage defects in patello-femoral joints of three other patients.[26] Other investigators have also used autologous MSCs to repair full-thickness cartilage defects and found these cells to be effective.[27]

In the above-mentioned studies, MSCs were introduced through an invasive approach (surgery) into the defect. Introduction of the cells by injection would be another strategy associated with less invasiveness. Using this approach, in 2007 Lee et al. introduced autologous MSCs into porcine knees to regenerate the experimentally-created defects in cartilage tissue. According to their reports, repair was better in the experimental compared to the control group.[28] In another study by Horie et al., the injection strategy was reported to be effective at promoting regeneration in rat meniscal defects.[29] Injection strategy has been applied in humans by Centeno et al. who have culture-expanded autologous MSCs and transplanted the cells through an intra-articular injection into a 46-year-old patient's knee with OA. They reported that 90% of the patient's pain was reduced two years post-injection.[30] Furthermore, according to Davatchi et al., trials in four OA patients have reported to be encouraging but not excellent.[31] In the present study, the effect of MSC injections have further been evaluated in six volunteer OA patients in terms of pain, joint function and walking ability, as well as articular cartilage thickness before and after transplantation.

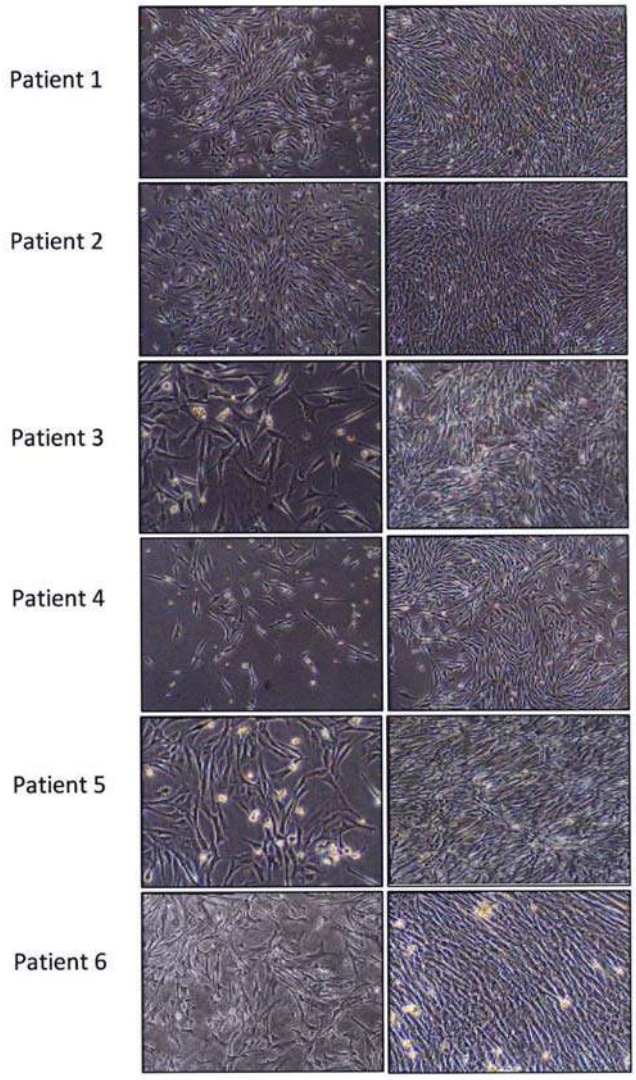
Materials and Methods

Patients

After obtaining approval from the Ethics Committee of Royan Institute and informed written consent from patients, volunteers with radiologic evidence of knee OA that necessitated joint replacement surgery were recruited. Six female patients (Table 1) who met the study inclusion criteria were entered into the study. The following were inclusion criteria for the study: either male or female; ages 18 to 65; and OA diagnosed based on magnetic resonance imaging (MRI). Patients with histories of taking corticosteroids or NSAIDs were only eligible for enrollment if this treatment was suspended for one month prior and six months after the study procedure. Exclusion criteria included: diagnosis of malignancy; pregnancy or lactating in female patients; active neurologic disorder; active endocrine disorder (i.e., hypothyroidism and diabetes); active cardiac or respiratory disease in need of medication; presence of infection with hepatitis B, C, or HIV; and a history of allergic reaction to the component of the study treatment. We enrolled six patients (F = 6, mean age: 54.5 years) with advanced knee OA classified as stage IV according to the Kellgren and Lawrence classification.

Bone marrow aspiration and culture

Patients were placed on an operating table in the prone position. The indicated area was numbed with 1% lidocaine and we collected about 50 ml of bone marrow from each patient's iliac crest. The samples were transferred to a clean room for cell isolation. Bone marrow aspirate was added to 50 ml phosphate buffer saline (PBS, Clinimax, Germany), then loaded onto a Lymphodex (Inno-Train, Germany) and centrifuged at 1500 g for 20 minutes. Mononuclear cells were then gently collected and counted using a nucleocounter (Chemometec, USA). Bone marrow volumes as well as the amount of mononuclear cells harvested from each sample are shown in Table 1. Mononuclear cells were washed with PBS and plated at 10^6 cells/cm^2 in 150-cm^2 culture flask in 15 ml alpha modified eagle medium (alpha MEM, Gibco, Germany) supplemented with 100 IU penicillin and 100 IU streptomycin (Gibco, Germany) and 10% hyclon bovine serum (Thermo Scientific, USA). Seven days after culture initiation, floating cells were removed by medium replacement. The cells were expanded through subcultures and passaged-2 cells were prepared for injection. For each patient, the cells were characterized in terms of colonogenic activity and expression of some surface markers. Prior to injection the cells were tested for possible microbial contamination.



Fiigure 1. Culture of marrow cells from patients.

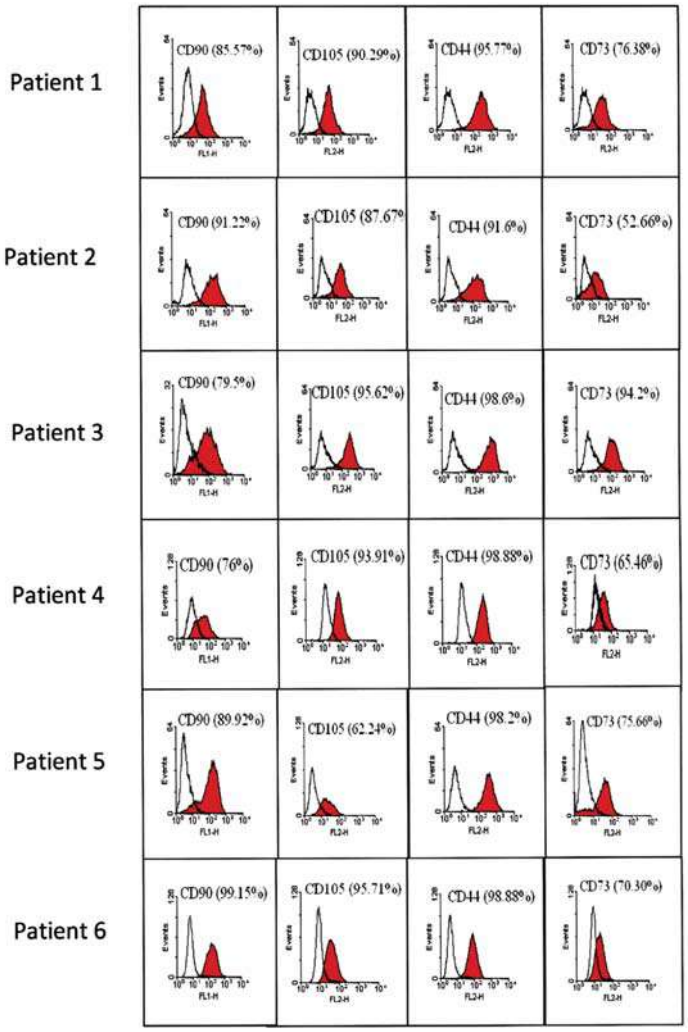


Figure 2. Expression of surface markers for marrow cells from each patient.

Colony-forming unit (CFU) assay

To evaluate proliferation potential of the isolated cells, the colony-forming unit (CFU)-fibroblast assay was performed. About 1000 cells from passage 1 were plated in 60-mm dishes and allowed to proliferate for one week. The cultures were then fixed and stained by crystal violet for 10 minutes and colonies were counted under an invert phase contrast microscope.

Flow cytometry

About 2×10^5 cells from passaged-2 cultures were placed into flow cytometry vials, washed with PBS and centrifuged at 1500 g for 5 minutes. Five µl of antibodies that included phycoerythrin (PE)-conjugated CD105 , CD44, CD73 (Becton Dickinson, USA), and fluorescein isothiocyanate (FITC)-conjugated CD 90 (Dako) were added to the cells and incubated in the dark for 20 minutes, followed by washing with PBS. As negative controls, cells were stained with murine FITC-conjugated IgG1 (eBioscience) and PE-conjugated IgG2b (eBioscience). All samples were analyzed by flow cytometry (BD FACS Caliber, BD Biosciences, San Jose CA, USA) and winMDI software.

Microbial test

One ml of blood from each patient taken during bone marrow aspiration along with one ml of culture medium before cell injection were tested to ensure that there was no bacterial or fungal contamination.

Preparation of cells for injection

Passaged-2 cultures of MSCs were washed with PBS and trypsinized with trypsin/EDTA (0.2%). The cells were then suspended in physiological serum (Gibco, Germany) at a density of 5×10^6 and loaded into 10 ml sterile syringes. For each patient, about $20 - 24 \times 10^6$ cells were prepared and taken to the hospital in a portable incubator. Under fluoroscopy, cells were injected into the patients' knees.

Follow-up

All patients were requested to not use any oral or intra-articular pain relieving drugs (including NSAIDs, corticosteroids, glycosaminoglycan, etc.) before and during one-year follow-up. Clinical

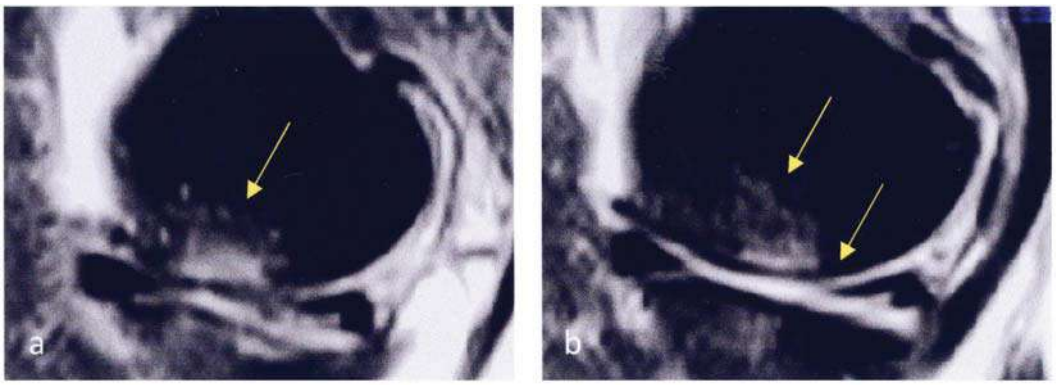


Figure 3. a) MRI of the knee in the sagittal plane before stem cell injection in a 56-year-old female patient with OA. Arrow shows region of increased T2-weighted signal reflective of subchondral edema. **b)** Six months post-stem cell injection illustrating the reduction in the increased signal intensity (upper arrow). The arrow below shows extension of the cartilage surface over the subchondral bone.

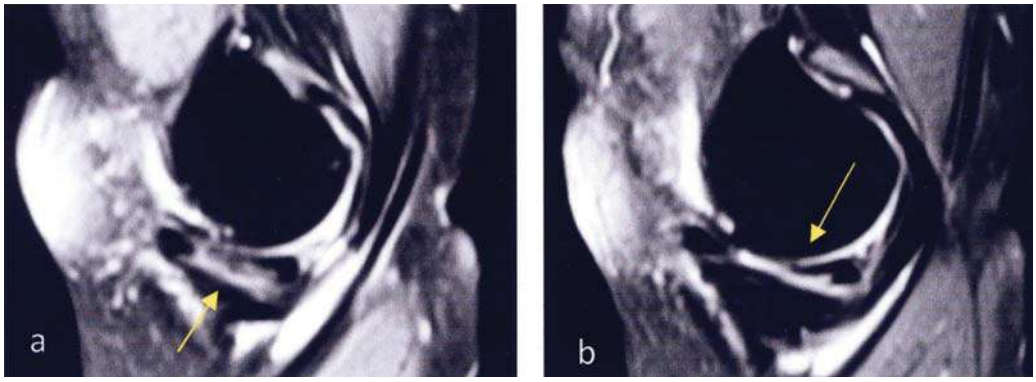


Figure 4. a) MRI in the sagittal plane of the same patient depicted in Figure 1 showing increased signal intensity due to subchondral edema before stem cell injection. **b)** Six months after stem cell injection demonstrating disappearance of signal changes. Arrow`s head shows a slight increase in cartilage thickness.

and radiological assessments were performed before the procedure and during the one-year follow-up, at determined time intervals. Pain intensity was scored with a 0 – 100 mm Visual Analogue Scale (VAS) which is a subjective assessment that represents patient`s perception of the current pain state with a higher score reflecting more severe pain. Functional status of the knee was assessed by Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index. This index evaluates pain, joint stiffness, physical and social function of patients with OA of the knee. The time until the appearance of gelling was recorded in all patients before and after the procedure. Walking ability was determined in terms of the distance (meters) the patient could walk before and after the cell injection. MRI of the affected knee was obtained preoperatively and six months after treatment on a GE 1.5 T MR system in the sagittal and axial planes.

Results

Bone marrow culture

Two to three days after culture initiation, some adherent cells with fibroblastic morphology emerged. These cells proliferated and formed a small colony that later grew larger and became confluent. Fibroblastic morphology was maintained throughout the primary culture as well as subsequent subcultures. Figure 1 indicates the culture of bone marrow cells for each patient.

Figure 1, Left column, indicates bone marrow cells of each patient at primary culture while the right column shows the cells after passage.

MSC characteristics

According to our findings, an average of 20 – 40 colonies was observed for each 1000 cells that were plated. Table 2 indicates the colony number for each of the marrow-derived MSCs isolated from the patients. According to flow cytometric results for all cases, CD44 expressed in more than 95% of the cells, whereas we observed that CD 105 expressed in about 87% of the cells. The percentages of CD90 and CD73 were 86.5% and 73%, respectively. No contamination was observed in cell specimens prepared for transplantation.

Follow-up

During the one-year follow-up period, we found no local or systemic adverse events. All patients were partly satisfied with the results at the end of the study. Table 1 presents all baseline parameters and the results during the one-year follow-up period. As shown in Table 1, there was an obvious decrease in mean pain intensity evaluated by VAS, as well as improvements in joint functioning and walking distance from baseline to the end of the study. The other parameters of walking distance, time to gelling, patellar crepitus, and knee flexion all tended to improve after cell injection.

Magnetic resonance images (MRI)

MRI films (before and after treatment) were reviewed by an independent radiologist who was not aware of the treatment procedure. Patient`s weight-bearing surface of the knee in sagittal and axial planes are shown in Figures 3 – 6. Comparison of MRI images at baseline and six months post-stem cell injection displayed an increase in cartilage thickness and extension of the repair tissue

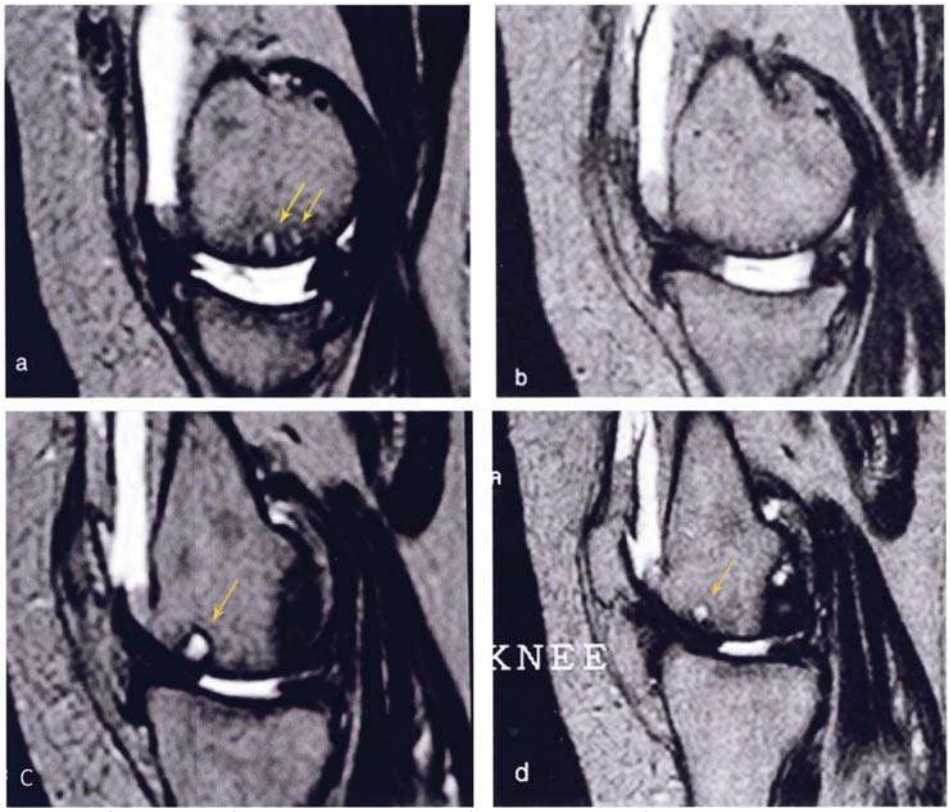


Figure 5. Sagittal MRI of the knee in a 65-year-old female patient with OA. Arrows in a and c show areas of increased signal intensity related to subchondral edema. Six months post-injection, MRI shows disappearance (b) or distinct decrease (d) of the size of these areas.

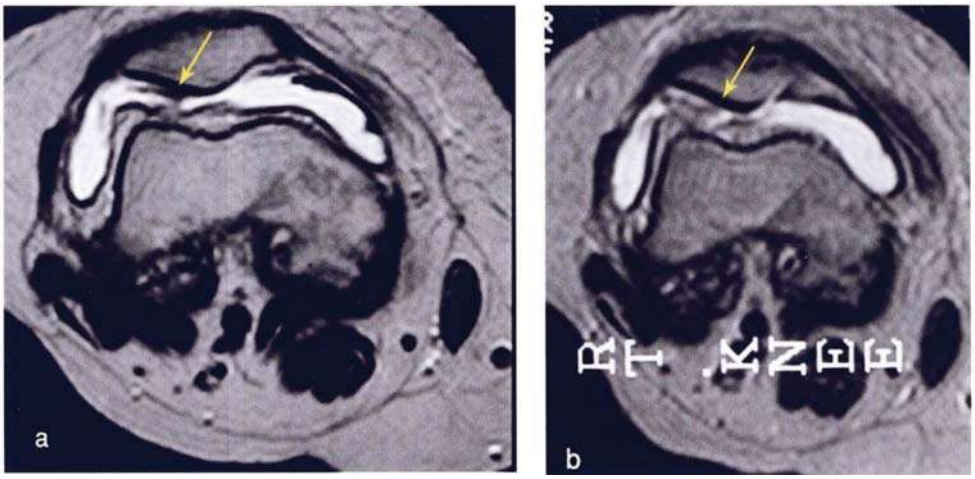


Figure 6. a) Axial knee MRI of the same patient illustrated in Figure 3. Note the discontinuous cartilage surface. **b)** Six months after stem cell injection, repair tissue shows integration with the surrounding cartilage surface.

over the subchondral bone in three out of six patients (Figures 3 – 6); in addition to a considerable decrease in the size of edematous subchondral patches (Figures 3 – 6). Interestingly, the best MRI results correlated with improvements in the corresponding WOMAC scores.

Discussion

Although the potential for marrow-derived MSCs to regenerate articular cartilage has long been recognized, in this regard clinical trials have rarely been performed. In most clinical trials, the mode of introduction of MSCs into affected joints was by surgery and therefore, invasive. The other route of MSC introduction could be intra-articular injection of the cells. In this context, Centeno et al. was the first to have performed a clinical trial on one patient and reported the intra-articular injection of expanded MSCs as a safe procedure without any complications.[30] The second report that has emphasized the safety of this method belonged to Davatchi et al. who examined the expanded MSC potential on four OA patients with six months follow-up after cell injection.[31] The present study was the third clinical trial on MSC intra-articular injection in six OA knees that has confirmed the findings by the previously performed trials. In the present investigation, we reported the results of a one-year follow-up study. According to our findings, patient

pain tended to be reduced up to six months post-injection; afterwards it appeared to be slightly increased. Regarding patient ability for walking distance, although there was a significant improvement until six months post-injection, it appeared to decrease from months 6 to 12 post-injection. These findings suggest that the injection would be effective for six months, then a second injection would probably be necessary.

Davatchi et al. have reported a high improvement in subjective parameters.[31] According to their findings, physical parameters showed much less improvement (i.e., X-ray images). In the present study, we examined the patient's articular cartilage with MRI images which was not performed in the previously mentioned study. According to MRI images, cartilage thickness appeared to be increased in three out of six patients, which indicated that injected MSC participates in repair of damaged cartilage in OA knees. To find out the exact nature of the repair tissue, histologic examination of a biopsy was necessary. This was not performed in the present trials due to the ethical issues associated with human studies. In three patients this effect was not seen, perhaps due to the unique condition of OA in each patient.

The other interesting finding was the decrease in size of edematous subchondral patches following intra-articular injection of MSC. This result was obvious in the MRI images and not mentioned in either former trial. Such effects can be attributed to anti-inflammatory influences of MSCs, which have been reported by previous investigations.[32]

According to Davatchi et al., it was concluded that MSC injections in four OA patients were encouraging but not excellent.[31] Our results were much better than the outcomes by Davatchi et al. The difference would be attributable to the amount of cells injected. While we injected about $20 – 24 \times 10^6$ cells, Davatchi et al. have transplanted about $8 – 9 \times 10^6$ cells.

This study was a phase one clinical trial in which six patients with radiologic evidence of knee OA that required joint replacement surgery were recruited. The main objective of this phase was to evaluate treatment safety. A large controlled trial, however, is necessary to compare intra-articular MSC injection with standard of care.

In conclusion, it could be said that intra-articular injection of culture-expanded MSCs in OA knees would be a promising way to reduce the signs of this disorder and lead to patient satisfaction. Furthermore, this therapy possesses the potential of regenerating destructed articular cartilage in an osteoarthritic knee. According to our results, all evaluated parameters appeared to progressively improve up to six months post-injection. This value was slightly reduced until 12 months post-injection. For this reason, it can be concluded that a second injection would be needed six months after the first injection.

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