Preliminary Communication

Regenerative Medicine

Human adipose-derived mesenchymal stem cells for osteoarthritis: a pilot study with long-term follow-up and repeated injections

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Aim: This study aimed to evaluate the safety and therapeutic potential of autologous human adipose-derived mesenchymal stem cells (haMSCs) in patients with osteoarthritis. **Materials & methods:** Safety and efficacy of haMSCs were preclinically assessed *in vitro* and in BALB/c-nu nude mice. 18 patients were enrolled and divided into three dose groups: the low-dose, mid-dose and high-dose group (1×10^7, 2×10^7 and 5×10^7 cells, respectively), provided three injections and followed up for 96 weeks. **Results & Conclusion:** The preclinical study established the safety and efficacy of haMSCs. Intra-articular injections of haMSCs were safe and improved pain, function and cartilage volume of the knee joint, rendering them a promising novel treatment for knee osteoarthritis. The dosage of 5×10^7 haMSCs exhibited the highest improvement (ClinicalTrials.gov Identifier: NCT01809769).

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Osteoarthritis (OA) is the most common arthritis among adults and is a frequent source of joint pain, the loss of function and disability. It is characterized by the destruction of the articular cartilage, altered material properties of the subchondral bone, inflammation of the synovial membrane, degeneration of ligaments and hypertrophy of the joint capsule [1]. Although several treatments have been proposed for OA, majority of these exhibit modest efficacy and are often limited by poor adverse event (AE) profiles. Several prevalent therapeutic options, such as manual therapy, valgus-directing force brace, glucosamine, chondroitin and intra-articular corticosteroids, exhibit limited efficacy and none of these are recommended by the American Academy of Orthopaedic Surgeons in their updated guidelines [2].

Mesenchymal stem cells (MSCs), also known as mesenchymal progenitor cells, are stem cells of the mesodermal origin that can differentiate into connective tissues, including bone, cartilage, tendon, ligament and fat [3], and have demonstrated anti-inflammatory and immunomodulatory effects [4]. Autogenous human adipose tissue offers various advantages as a therapeutic cell source. First, being autogenous, adipose evades immunogenic and ethical concerns. Second, adipose tissue facilitates harvesting substantial amounts with simple, repeatable and minimally invasive methods. Finally, and most importantly, the amount and quality of MSCs are significantly higher in adipose tissue than in other tissues [5–7]. Thus, these advantages render autogenous adipose tissue an accessible, abundant and reliable source for the isolation of adult MSCs suitable for tissue engineering and regenerative medicine applications.

Several preclinical animal studies have demonstrated promising therapeutic effects of MSCs for OA. MSCs isolated from different tissues, administered either directly through an intra-articular injection or via transplantation

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on scaffolds, have exhibited improvements for rescuing local defects or minimizing generalized cartilage loss in OA animal models [8–15]. Despite this promising preclinical data, limited evidence exists regarding the safety and efficacy of human adipose-derived MSCs (haMSCs) for the treatment of knee OA in humans, the optimal administration dosage and frequency and the safety and efficacy of haMSCs in the long-term follow-up. We have previously demonstrated that the intra-articular injection of haMSCs promote the regeneration of the articular cartilage in a rabbit OA model [16]. Therefore, this pilot study investigated the therapeutic potential of the direct injection of haMSCs in patients with knee OA. After conducting *in vitro* and *in vivo* preclinical tests, we conducted a randomized Phase I/IIa clinical trial with a 96-week follow-up to assess the safety and therapeutic potential of haMSCs in patients with knee OA.

Materials & methods

Patient eligibility

The inclusion criteria for patients were as follows: those aged 40–70 years; those with a definite diagnosis of knee OA according to the American College of Rheumatology Clinical classification criteria for knee OA [17]; those who were graded $\geq$2 by the Kellgren–Lawrence criteria; and those with an average pain intensity of grade $\geq$4 on a 10-point visual analog scale for at least 4 months. The exclusion criteria were as follows: those who were administered anti-inflammatory drugs in the preceding 14 days; those with concomitant severe infection, malignant tumor, coagulation disorder or uncontrolled or unmanageable systemic diseases; those with other variants of arthritis, except OA; those who were injected with intra-articular hyaluronic acid or corticosteroid in the preceding 2 months; those who were pregnant or were breast-feeding; and those participating in another clinical trial or treatment with a different investigational product within 3 months before inclusion. This study was approved by the Institutional Review Board of the Shanghai Jiao Tong University and Ren Ji Hospital and registered at ClinicalTrials.gov with identifier NCT01809769. All procedures were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients after the physicians illustrated detailed relevant information, such as the purpose and overall procedure of the study, potential risks and benefits.

HaMSC preparation, flow cytometry & trilineage differentiation *in vitro*

In this study, we used purified haMSCs, and culture-expanded cells at passage 4 were used (see Supplementary Appendix for detailed information).

Preclinical toxicity & chronic tumorigenicity *in vivo*

We conducted preclinical studies after patient enrollment and liposuction.

The toxicity and tumorigenicity studies were conducted under the Good Laboratory Practice conditions. We maintained mice under high-barrier conditions and carefully monitored for the presence of mouse pathogens. In addition, food, water, bedding, cages and anything in contact with mice were sterilized or disinfected. Water was acidified to pH 2.5–3.0. All mice were randomly assigned to different groups using the table of the random number (see Supplementary Appendix for detailed information).

Study participants & intervention

We conducted this single-center, randomized, Phase I/IIa clinical trial from March 2013 to July 2015 at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. All participants were enrolled following the inclusion and exclusion criteria detailed in the patient eligibility section.

In Phase I, we randomly categorized participants into three groups as follows: low-dose (1×10^7 cells); mid-dose (2×10^7 cells) and high-dose (5×10^7 cells) groups; patients in each dose group received cells in 3-ml cell suspension into both knee joints through the medial portal under the guidance of B-mode ultrasound in the 3rd and 6th week after liposuction and were followed up at 12th, 24th and 48th week after injection. In addition, we conducted a safety review before moving to the next phase. In this phase, both patients and researchers were blinded to the group and cell doses administered.

Then, a subsequent open clinical research (Phase IIa) was conducted following Phase I to assess the long-term safety and potentially benefits of haMSCs. In this phase, the third injection was provided at the discretion of patients. The third injection of MSCs was administered at 48 weeks after injection in patients who signed informed consent again with the high-dose consulting and no treatment-related serious adverse events (SAEs) in Phase I.

All patients were invited for follow-up interviews at 48, 72 and 96 weeks after the injection. In our study, participants did not receive other special treatments after they received an intra-articular injection of haMSCs.

Randomization

An independent allocator randomly allocated cards using computer-generated random numbers, with a 1:1:1 allocation using a random block size of 6. The original random allocation sequences and corresponding information were recorded, put into each envelope and kept in an inaccessible third place and worked with a copy.

The syringes with haMSCs of low-, middle- and high-dose groups were prepared based on the allocation orders. In addition, syringes could not be distinguished because they contained the same colored liquid with the same volume. Researchers administered the injection as ordered and recorded patients' ID, date, time and other relevant details. Both researchers and patients were blinded to the content of the injection.

Outcome measures

We set safety as the primary outcome in Phase I and assessed with AEs and SAEs, electrocardiography, vital signs, physical examination and laboratory tests (including routine blood and urine tests, hepatic and renal functions tests, blood lipid and glucose tests and immunological tests). The assessment of safety was performed after enrollment (baseline) and every 2 weeks during this phase. We recorded concomitant medications along with AEs and SAEs. In Phase IIa, the Western Ontario and McMaster Universities Osteoarthritis index (WOMAC) [18], which is a validated, self-administered outcome measure designed to assess knee and hip OA, was set as the primary outcome, and higher scores indicated increased pain, stiffness and decreased function, with a significant proportion of the total WOMAC score for function.

The numerical pain rating scale (NRS)-11 [19], short form 36 (SF-36) health survey questionnaire [20] and MRI were set as secondary outcomes and were assessed in both Phase I and Phase IIa at the baseline, 12, 24, 48, 72 and 96 weeks of the follow-up, together with WOMAC. NRS-11 was evaluated on a frequently used instrument measured on the 11-point pain intensity numerical rating scale, where 0 represented no pain and 10 represented worst possible pain. The SF-36 questionnaire was used to evaluate the quality of life of participants, where higher score implied the lower quality of life. In addition, an MRI was performed to calculate the knee cartilage volume (including the femur, tibia and patella) and was assessed using a semi-automated segmentation method [21] blindly by two independent researchers. The cartilage volume of knee comprised three distinct regions, the femur, tibia and patella. Furthermore, the improvement rate was evaluated for additional assessment of clinical effectiveness for the WOMAC, NRS-11 and SF-36, which was reported as the percentage of change of score in each time-point of follow-up compared with the baseline.

Statistical analysis

In this study, statistical analyses were performed on the intent-to-treat population, defined as all patients randomized to treatment and receiving at least one injection. All data presented were on an intent-to-treat/last observation carried forward basis. Given the lack of safety and efficacy data of intra-articular haMSCs in patients with knee OA at the time of this pilot study, the sample size was based on other MSCs clinical trials for other indications and decided in consultation with the Chinese FDA, so did the sample size of mice in the preclinical study.

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS Inc., IL, USA) and GraphPad Instat3 package software version 3.06 (GraphPad Software, CA, USA). Data are reported as mean (arithmetic mean) ± standard deviation (SD) or numbers of patients and percentage. Paired Student's *t*-test or Wilcoxon signed-rank test was used according to the statistical distribution by the Normality test to assess differences between two groups, and the general linear model was used to assess differences between three groups. $p < 0.05$ (two-tailed) was considered statistically significant. All data on which the conclusions of the report rely are available on request.

Results

Flow cytometry & trilineage differentiation *in vitro*

Adipose tissue-derived MSCs were spindle-shaped cells with a fibroblast-like morphology and attached to the plate during cell culture; these characteristics were well-preserved during repeated subculture. For immunophenotypic characterization of haMSCs, culture-expanded cells at passage 4 were collected and examined by flow cytometry.

The findings revealed that haMSCs were positive for CD90, CD73, CD29 and CD49d, whereas negative for actin, CD14, CD34, CD45 and HLA-DR (Supplementary Figure 1).

Regarding chondrogenic differentiation, we observed regional condensations on day 7 of the differentiation period, which was more evident after 14 days when stained for Alcian blue compared with the faint background Alcian blue staining in the negative control medium. When cells were under osteogenic conditions, matrix deposition and Alizarin red staining were observed in the presence of ascorbic acid and β-glycerol phosphate, whereas no matrix or mineral deposition was detected when cells were grown under nonosteogenic conditions. Regarding adipogenic differentiation, large lipid vesicles were detected by oil red O staining, whereas cells grown in the absence of adipogenic inducers demonstrated no large lipid vesicles or oil red O staining (Supplementary Figure 2).

Preclinical toxicity & chronic tumorigenicity in BALB/c-nu nude mice

None of the mice became severely ill or died at any time before the experimental end point. We observed no abnormal reaction during the study period. The body weight, temperature and food consumption were similar after the haMSC injection among three groups with no significant difference (Supplementary Table 1 in the Supplementary Appendix). The results of routine blood counts and biochemical tests revealed no significant differences among three groups after injection. In addition, we observed no significant morphological differences after histology hematoxylin–eosin staining among the phosphate-buffered saline control, low-dose and high-dose MSC groups in the liver, kidney, spleen, testis or ovary (Supplementary Figure 3). One week after subcutaneous inoculation, we observed tumor nodules (mean size = 10 mm) in the neck of the A549 mice group. The tumor formation rate was 100% in 2 weeks. The results of multiple sites of biopsy of the dorsal skin confirmed the existence of a tumor, and the survival rate was 37.5% on day 120 from transplantation. In the haMSC group, no macroscopical or morphological changes were observed, and biopsy results revealed standard skin structures (Supplementary Figure 4).

Patient profiles

Figure 1 presents patient disposition in this study. Of 21 patients screened at the study entry, 18 patients fulfilled the criteria for inclusion and were randomized to six in each group. Of 18 participants, 14 (77.8%) completed the final study visit. One patient in the low-dose group withdrew because of vertebral fracture, and two in the middle-dose group and one in the high-dose group lost follow-up for unknown reasons. Furthermore, all 14 patients voluntarily chose to receive the third injection at the 48th week after the first injection.

Most patients enrolled were females (66.7, 83.3 and 83.3% in the low-, mid- and high-dose group, respectively) aged approximately 55 years, with an average BMI of approximately 24 kg/m^2, and suffered from symptomatic knee OA for >6 years despite conservative treatments. Patients in each group exhibited similar baseline characteristics for age, height, weight, BMI, radiographic grade of OA, cartilage volume of both knees by MRI, previous treatment history and concomitant disease (Table 1). No significant differences were observed among the three groups regarding vital signs and physical examination at baseline.

Safety of haMSCs

All 18 patients completed the follow-up for safety assessment. During the study period, no death or SAEs was reported. In addition, no significant changes were noted in the results of an electrocardiogram, vital signs, physical examination and laboratory tests. AEs occurred in a similar proportion among the three groups with eight (66.67%) in the low-dose group, seven (58.33%) in the mid-dose group and six (50%) in the high-dose group, with no significant differences ($p = 0.864$). The most common AEs were transient pain and swelling of joints, which were mild to moderate and were spontaneously relieved within 7 days without special treatment. One patient experienced mild edema and cramps of bilateral lower extremities, which were relieved in 21 days without treatment and not related to the MSC treatment. Furthermore, no AEs led to treatment discontinuation, study termination or change of the treatment dose (Table 2).

Clinical outcomes

Overall, the WOMAC score reduced from 34.75 ± 17.05 at baseline to 25.94 ± 16.09 ($p < 0.0001$), 20.38 ± 19.89 ($p = 0.0002$), 22.77 ± 22.72 ($p = 0.0044$), 15.00 ± 11.36 ($p = 0.0034$) and 12.44 ± 8.99 ($p = 0.0009$) in the 12th, 24th, 48th, 72nd and 96th week with a mean improvement rate of 27.81, 48.63, 39.07, 47.95 and 53.29%, respectively (Figure 2A). In the 12th week after the first injection, significant differences were observed in the

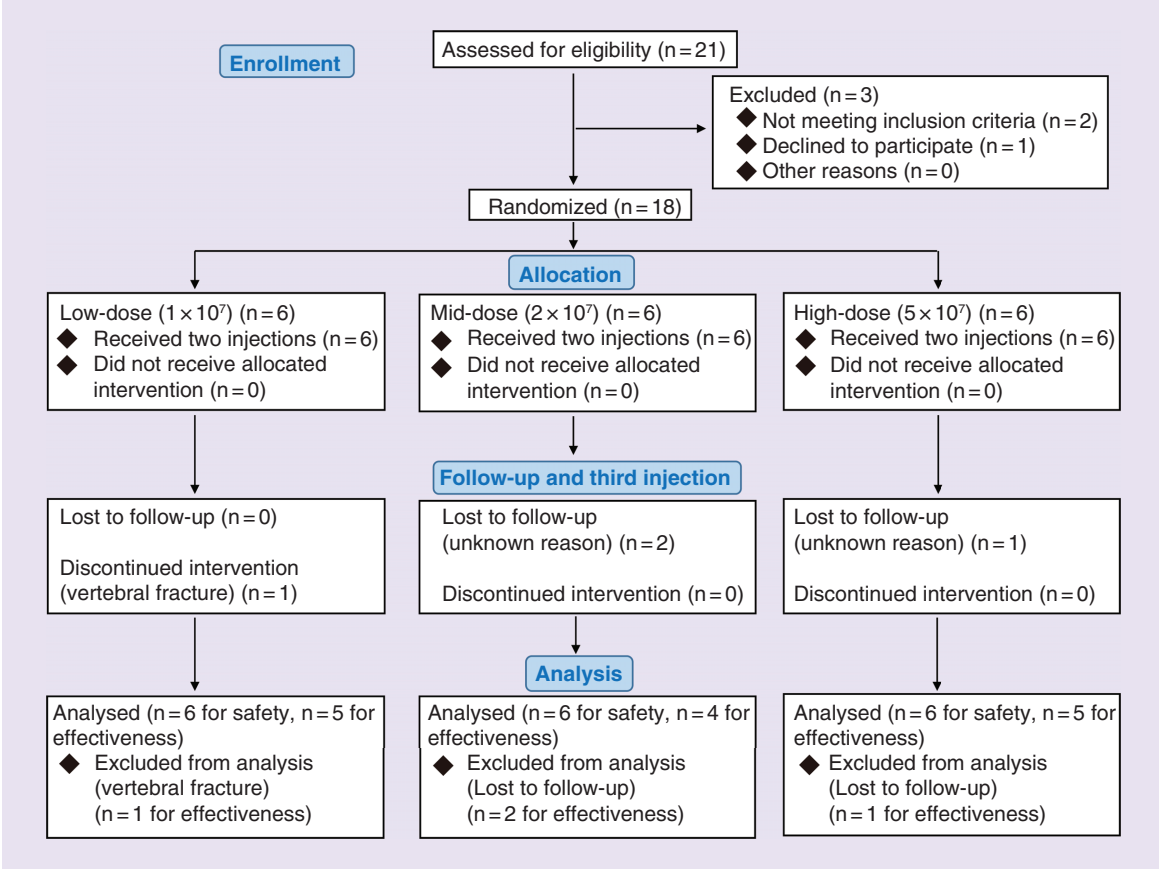

Figure 1. Flowchart of the clinical trial.

low- and middle-dose groups, with a mean improvement rate of 31.08% ($p = 0.0109$) and 18.06% ($p = 0.0239$), respectively, compared with the baseline. In addition, the decreasing tendency of WOMAC was maintained in all groups in the latter time-points, and statistical significance was determined in the 24th week in the high-dose group with a mean improvement rate of 43.16% ($p = 0.0213$) and in the 48th week in the low- and high-dose groups with a mean improvement rate of 36.24% ($p = 0.0440$) and 44.40% ($p = 0.0178$), respectively, compared with that at baseline (Figure 2B & C).

The MSC injections correlated with a significant reduction of the NRS-11 score in the 12th, 24th, 48th, 72nd and 96th week compared with the baseline. The mean NRS-11 score reduced from 4.94 ± 2.29 at the baseline to 2.19 ± 1.17 ($p < 0.0001$), 2.62 ± 1.71 ($p = 0.0016$), 3.62 ± 2.10 ($p = 0.0017$), 2.94 ± 1.57 ($p = 0.0043$) and 3.17 ± 2.08 ($p = 0.0070$) in the 12th, 24th, 48th, 72nd and 96th week, with a mean improvement rate of 54.85, 35.74, 16.77, 33.22 and 23.94% (Figure 2D), respectively. In the 12th week after the first injection, we observed significance in the low- and high-dose groups, with a mean improvement rate of 59.52% ($p = 0.0055$) and 66.33% ($p = 0.0193$), respectively, compared with the baseline. Regarding the latter time-points, the statistical significance was only determined in the high-dose group with a decrease of 61.67% ($p = 0.0110$), 51.25% ($p = 0.0305$), 46.77% ($p = 0.0138$) and 49.38% ($p = 0.0061$) in the 24th, 48th, 72nd and 96th week, respectively, compared with the baseline (Figure 2E & F).

The SF-36 score demonstrated a tendency of reduction after the three injections and during the whole follow-up. However, a significant reduction was only observed in the 12th week (from 80.44 ± 17.98 to 72.06 ± 17.94; $p = 0.0002$) and 96th week (from 80.44 ± 17.98 to 63.33 ± 9.21; $p = 0.0092$; Figure 2G). In addition, a tendency to further increase was observed after the third injection, as demonstrated by the improvement rate. Likewise, a tendency to improvement was observed in all dose groups; however, only the difference in the low-dose group in the 12th week and the middle-dose group in the 96th week reached significance compared with the baseline (from 72.33 ± 14.40 to 62.33 ± 8.45; $p = 0.0115$; 64.00 ± 9.90, $p = 0.0236$; Figure 2H & I).

Table 1. Clinical and demographic characteristics of the patients in the low-, mid-, and high-dose groups.

Variable	Low dose	Mid dose	High dose	p-value
Number of patients	6	6	6	
Age, mean (SD), years	52.1 (11.6)	59.6 (10.2)	52.7 (8.7)	0.4096
Sex, n (%)†:				0.4821
– Male	2 (33.3)	1 (16.7)	1 (16.7)	
– Female	4 (66.7)	5 (83.3)	5 (83.3)	
Height, mean (SD), cm	166.5 (6.4)	159.8 (6.8)	163.6 (4.1)	0.1917
Weight, mean (SD), kg	71.2 (8.8)	60.8 (10.2)	64.7 (5.1)	0.1427
BMI, mean (SD)	25.6 (1.9)	23.7 (2.9)	24.1 (1.4)	0.3288
Symptom duration in months, mean (SD)	74.3 (85.1)	106.2 (89.4)	38.2 (52.1)	0.3880
Kellgren–Lawrence grade, n (%)†:				0.8290
– Grade 0	0 (0.0)	0 (0.0)	0 (0.0)	
– Grade 1	0 (0.0)	0 (0.0)	0 (0.0)	
– Grade 2	5 (83.3)	2 (33.3)	4 (66.7)	
– Grade 3	1 (16.7)	4 (66.7)	2 (33.3)	
– Grade 4	0 (0.0)	0 (0.0)	0 (0.0)	
Baseline NRS-11 score, mean (SD)	3.8 (1.9)	5.2 (1.5)	6.1 (3.0)	0.2375
Baseline WOMAC score, mean (SD)	25.8 (10.6)	49.0 (15.7)	31.2 (17.8)	0.0570
Baseline SF-36 score, mean (SD)	72.3 (14.4)	91.4 (19.1)	79.2 (18.4)	0.2218
Baseline cartilage volume of left knee, mean (SD), mm^3	10,574.7 (7901)	3981.8 (478)	13,619.5 (8333)	0.0962
Baseline cartilage volume of right knee, mean (SD), mm^3	10,321 (7593)	3959.9 (614.2)	13,294.4 (8174)	0.0972
Previous treatment history, number (%)†:				0.551
– Yes	1 (16.7)	6 (100.0)	5 (83.3)	
– No	5 (83.3)	0 (0.0)	1 (16.7)	
Concomitant diseases, number (%)†:				0.551
– Yes	1 (16.7)	0 (0.0)	1 (16.7)	
– No	5 (83.3)	6 (100.0)	5 (83.3)	

n = 18.
†Statistics were calculated by analysis of variance or χ^2 test.
NRS-11: Numerical pain rating scale-11; SD: Standard deviation; SF-36: Short Form 36 Health Survey questionnaire; WOMAC: Western Ontario and McMaster Universities Osteoarthritis index.

Table 2. Adverse and severe adverse events of osteoarthritis patients after treatment of mesenchymal stem cells.

Variable	Low	Middle	High	Total
Swelling of injection-site joint, n (%)†	7 (58.3)	5 (41.7)	4 (33.3)	15 (44.4)
Pain at injection-site joint, n (%)†	0 (0)	2 (15.7)	2 (16.7)	4 (11.1)
Edema and cramps, n (%)†	1 (8.3)	0 (0)	0 (0)	1 (2.8)
Total AEs, n (%)†	8 (66.7)	7 (58.3)	6 (50)	16 (88.8)
Outcome	Relief	Relief	Relief	Relief
SAEs, n (%)†	0 (0)	0 (0)	0 (0)	0 (0)

n = 18.
†'n' denotes the frequency of AEs or SAEs and '(%)' denotes incidence rate (the ratio of frequency of AEs or SAEs to times of injection).
AE: Adverse event; SAE: Serious adverse event.

Radiological outcomes

We observed an increase in the thickness of the articular cartilage after MSC therapy by MRI (Figure 3A). Overall, the volume of the cartilage of the bilateral knee persistently increased during the entire follow-up. In addition, a significant increase was noted in the 24th, 48th and 72nd week after therapy for both knees, with a total cartilage volume increasing by 30.06 ± 128.64 mm^3 (p = 0.0269), 56.24 ± 92.87 mm^3 (p = 0.0024) and 125.03 ± 106.66 mm^3 (p = 0.0277) for left knees (Figure 3B) and 23.01 ± 33.47 mm^3 (p = 0.012), 45.76 ± 42.11 mm^3 (p = 0.0002) and 120.22 ± 123.01 mm^3 (p = 0.0189) for right knees compared with that at baseline, respectively (Figure 3C).

When calculated separately in different dose groups, the persistent increasing tendency was more apparent in the high-dose group (Figure 3C). In addition, we observed a significant difference in the left knee in the mid-dose group in the 48th week (increased volume: 26.83 ± 15.39; p = 0.0398). For the right knee, the significance was

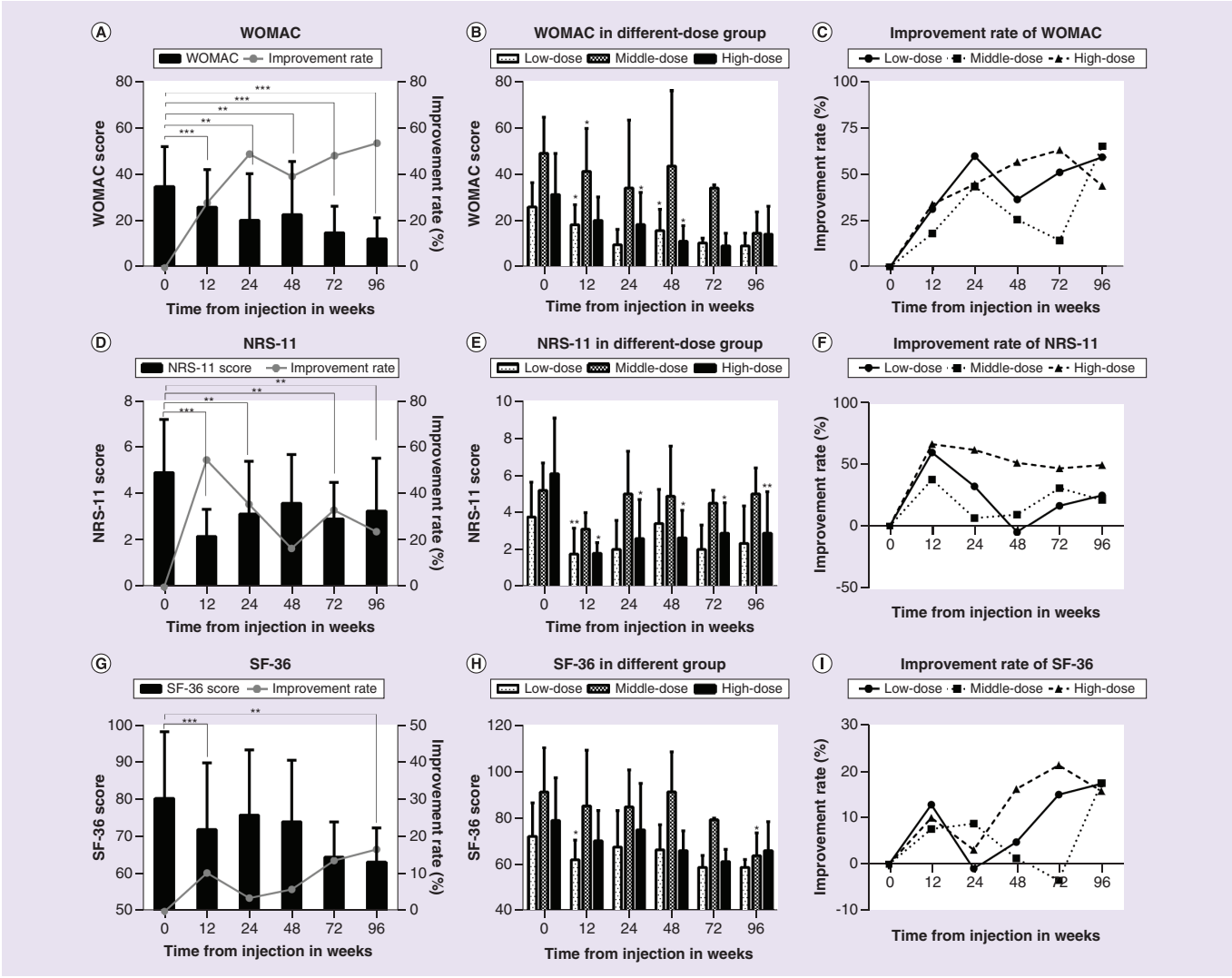

Figure 2. Changes in the Western Ontario and McMaster Universities Osteoarthritis index, numerical pain rating scale-11 and short form-36 score during 96 weeks after the intra-articular injection of human adipose-derived mesenchymal stem cells. (A,D &G) The score and mean improvement rate of the WOMAC, NRS-11 and SF-36 in all dose groups, respectively. **(B,E & H)** The score of the WOMAC, NRS-11 and SF-36 in different dose groups, respectively. **(C, F &I)** The mean improvement rate of the WOMAC, NRS-11 and SF-36 compared with the baseline in different dose groups, respectively. The score is shown in mean ± standard deviation. The mean improvement rate was shown as the percentage of the change of score at each follow-up time-point compared with that at the baseline. Statistics were determined by a paired *t*-test.
*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$.
NRS-11: Numerical pain rating scale-11; SF-36: Short form 36; WOMAC: Western Ontario and McMaster Universities Osteoarthritis index.

observed in the 24th week (increased volume: 20.56 ± 5.71; p = 0.0055) and in the 48th week (increased volume: 43.76 ± 15.69; p = 0.0114) in the middle-dose group (Figure 3C).

In addition, a change in the cartilage increased to a higher degree after the third injection compared with that after the first two injections within the same interval from injection to the follow-up time-point (an increase of 93.04 ± 141.95 and 91.76 ± 120.14 for left and right knees when comparing 72nd and 24th weeks, representing intervals from baseline to the 24th week and from the 48th week to the 72nd week), although this increase was not statistically significant. This tendency suggested a therapeutic benefit of repeated injections.

We measured the cartilage volume and calculated separately by the femur, tibia and patella to investigate the impact of haMSCs on the different anatomical locus of the knee. Overall, the cartilage volume of most anatomical

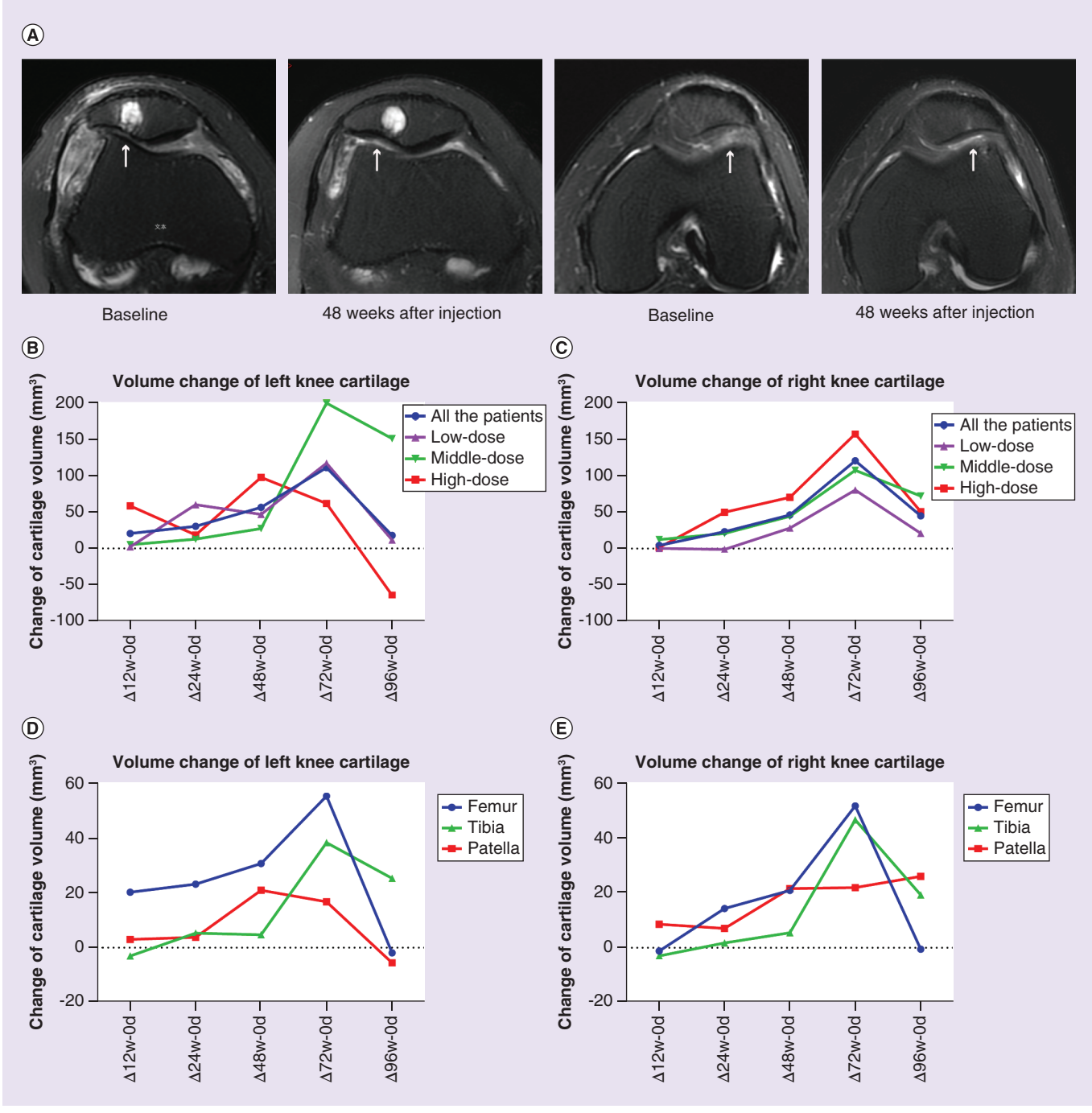

Figure 3. An increase in the articular cartilage volume by MRI. (A) The articular cartilage in two patients of the high-dose group. MRI demonstrated that the thickness of the articular cartilage of the lateral femoral condyle significantly increased at 24 weeks after therapy (arrow). **(B)** Changes in the left knee cartilage volume by MRI in different dose groups. **(C)** Changes in the right knee cartilage volume by MRI in different dose groups. **(D)** Changes in the left knee cartilage volume by MRI at different anatomy locations. **(E)** Changes in the right knee cartilage volume by MRI at different anatomy locations. The score is shown in mean ± standard deviation. Statistics were determined by the Wilcoxon signed-rank test. Baseline is shown as 0 d changes in the knee cartilage volume are shown as Δ12 w–0 d, Δ24 w–0 d, Δ48 w–0 d, Δ72 w–0 d and Δ96 w–0 d.
*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$.
d: Day; w: Week.

locus persistently increased in the 72nd week and decreased in the 96th week. In addition, a significant increase in the cartilage volume of the left femur was observed in the 48th week, with an increased volume of 30.53 ± 77.25 mm^3 (p = 0.013) compared with the baseline. The increase in the left tibia cartilage volume was statistically significant in the 72nd and 96th weeks after the first injection with a volume of 38.31 ± 33.07 (p = 0.0084) and 25.31 ± 29.66 (p = 0.0465), respectively. In addition, a significantly increased volume of the left patella occurred in the 24th week after the first injection (increased volume: 3.63 ± 63.99; p = 0.034), and this significant difference was also observed in the 48th week (increased volume: 20.98 ± 27.08; p = 0.006) compared with the baseline (Figure 3D). For the right knee, we observed a significant increase in the 24th (increased volume: 14.35 ± 30.24; p = 0.0269) and 48th week (increased volume: 20.88 ± 26.89; p = 0.0007) in the femur, in the 72nd week (increased volume: 46.61 ± 34.54; p = 0.0037) in the tibia and in the 48th week (increased volume: 21.66 ± 14.72; p = 0.002) in the patella (Figure 3E).

Likewise, comparing the first two injections with the third, an increase occurred when the interval from the injection to the follow-up time-point was settled (24 weeks, which represents intervals from baseline to the 24th week and from the 48th week to the 72nd week); this increase was statistically significant in the left and right tibia with increased volume 34.94 ± 28.76 (p = 0.0065) and 40.05 ± 27.84 (p = 0.0026) in the 72nd week compared with the 24th week, respectively (Figure 3D & E).

Discussion

haMSCs were first identified and reported in the early 2000s and have been since shown to possess self-renewal ability and multilineage differentiation potential [15,22–24]. Besides the advantages of adipose tissue described earlier, haMSCs offer several benefits, including easier and faster expansion in culture, more passage cells that retain stem cell phenotypes and pluripotency [25], less susceptibility to age and less morbidity of patients [26–30]. In addition, isolated haMSCs can be easily cryopreserved [31].

Despite these advantages of haMSCs, limited data are available regarding the direct administration of haMSCs into the knees of patients with OA to a few animal experiments and case reports [32–34]. One proof-of-concept clinical trial conducted by Jo *et al.* reported the safety and efficacy of intra-articular injections of haMSCs for the treatment of knee OA [35]. However, their follow-up period was only 24 weeks, without long-term assessment. Although Pers *et al.* reported adipose MSC therapy for severe knee OA with a study design similar to ours [36], their follow-up period was also only 24 weeks, and no significant differences were reported in the MRI evaluation. Furthermore, one injection of MSCs in the above studies led to several limitations, and as mentioned by researchers of the study, which could alternatively be broken into several injections [35]. To the best of our knowledge, our study is the first to demonstrate the safety and potential efficacy of haMSCs therapy with long-term follow-up of 96 weeks with repeated injections.

In addition, clinical studies combined growth factors, including platelet-rich plasma (PRP) or arthroscopic microfracture, to enhance the therapeutic efficacy of adipose MSCs for the treatment of knee OA [37–39]. Stem cells therapy, which was combined with PRP, improved pain and function [37,38]. These two studies reported no significant differences in the cartilage volume and did not compare stem cells combined with PRP versus stem cells or PRP. The clinical outcomes of Freitag *et al.* were not reported, which planned to compare stem cells combined with arthroscopic microfracture versus arthroscopic microfracture [39].

During *in vitro* culture, haMSCs remained preserved with stable characteristics during the repeated subculture for immunophenotype and ability to differentiate into the triple lineage, which conformed to the minimal criteria for defining multipotent MSCs by the International Society for Cellular Therapy position statement [40]. Considering the cell quality and quantity, culture-expanded cells at passage 4 were used in our study.

In addition, the safety of haMSCs *in vivo* was acceptable because the transplantation of up to 1.0×10^7/kg haMSCs in immunodeficient mice did not reach the no-observed-effect level for toxicity, and subcutaneously inoculated haMSCs did not induce tumor formation and death (refer to Supplementary Appendix for more details). Furthermore, toxicity and chronic tumorigenicity assay were repeated with NOD-SCID mice (data not shown), considering that BALB/c-nu mice (also used in this study) exhibited an immune response in several studies. In addition, mice were fed with acidified water with pH range of 2.5–3 in our study. Acidified water are regularly used in the period of feeding mice, as it could inhibit the growth of bacteriums and remained sterile for a long time [41]. Thus, acidified water might be beneficial to mice, especially immune deficient mice in our study, and mice were fed with pH range of 2.5–3 in other studies [42,43]. In the toxicity study, we selected the liver, kidney, spleen, testis and ovary, which are all vital organs and often detected in the toxicity assay.

This clinical trial obtained its predetermined primary outcomes in both Phase I and Phase IIa studies. Despite different dosages, the intra-articular injection of haMSCs into the osteoarthritic knee was not related to apparent AEs or SAEs, but the improved function of the knee measured with the WOMAC over the 96-week follow-up. In addition, physical examination and laboratory tests revealed no significant difference in vital signs or organ system involvement compared with the baseline. Furthermore, haMSCs significantly improved pain and quality of life, as indicated by the NRS-11 and SF-36 score. The high dose (5×10^7 cells) of haMSCs exhibited more significant effects compared with those of the other two groups on pain relief and the improvement of knee function by the prolonged time of improvement, and this dosage was used for all patients in the Phase IIa study. Notably, the statistically significant improvement was also be observed in the low- and middle-dose groups, which mostly occurred in 12 weeks after the injection (not reported in previous trials). Perhaps, this difference could be attributed to age and race of enrolled patients, potentially contributing to the quality of haMSCs, especially the paracrine effects that are crucial and potential mechanisms of MSCs for the treatment of OA [44,45] and could be triggered by very few MSCs [46]. The phenomenon that the SF-36 score tended toward significance could only be reached in the 12th week and, surprisingly, in the low-dose group might be because of the small sample size and a large SD. In addition, the WOMAC and NRS-11 scores revealed a slight increase in 48 weeks (Figure 2). During the study period, two patients, one in the low-dose and one in the middle-dose group, experienced severe pain at 48 weeks. In addition, the mean score increased because of the small sample size.

After injecting haMSCs, the cartilage volume increased, which was exciting and expected to reverse the process of OA; this is superior to other noninvasive drugs and traditional treatments. In this study, the cartilage volume decreased in 96 weeks, which is consistent with the study that the knee cartilage volume was significantly associated with age and decreased per annum [47–50]. Hence, the decrease in the cartilage volume in 96 weeks might be the natural loss of the cartilage volume. Furthermore, more increase in the cartilage volume was observed in the high-dose group (Figure 3C), even though not all increment reached significance; this could be because of the small sample size and, thus, a relatively larger SD. At present, our team is conducting a Phase IIb clinical trial with a larger sample size that could probably solve this problem. An increase in the cartilage volume was statistically significant in the 48th or 72nd week after the injection in the femur, tibia or patella, suggesting that the improvement in clinical outcomes of haMSCs might differ between different regions of the knee cartilage.

Although it would be interesting to detect injected cells in the MRI evaluation after cell labeling, we did not execute a cell labeling strategy as it was not suitable for our clinical study. Furthermore, intra-articular injections of haMSCs correlated with the significantly increased thickness of the articular cartilage (Figure 3), suggesting a lineage-specific differentiation of the injected cells.

To the best of our knowledge, this is the first attempt at repeated injection of MSCs in a clinical trial. With an interval of 48 weeks between the first two and the third injection, haMSCs maintained the long-term improvement on symptomatic relief, function and quality of life. After the first two injections, a substantial improvement in the function, pain and quality of life was reported in the 12th week, whereas a decreasing tendency was observed, as indicated in the line chart illustrating the improvement rate of the WOMAC, NRS-11 and SF-36 (Figure 2A, D & G). Of note, the third injection generated another increase in the improvement rate, especially in the low- and middle-dose groups, highlighting a time- and dose-dependent effect. Regarding the thickness of the cartilage, an increase was more significant after the third injection compared with the first two injections, as indicated by MRI results, suggesting enhanced benefits of repeated injections with an interval of 48 weeks. Of note, this improvement of repeated injections in the cartilage increase was most significant in the tibia. However, a decrease occurred in the 96th week in the femur, patella and the total volume of the knee cartilage, which could be attributed to the single injection at the 48th week and two repeated injections similar to the first two in the Phase I trial and repeated injections per year were probably required for long-term benefits.

This study had some limitations. First, the sample size was relatively small and no control groups were used. Since this is a pilot study, it primarily evaluates the safety of haMSCs in OA and preliminarily explores its therapeutic potential. At present, we are conducting a multicentered study with appropriate control, which hopefully will provide more evidence for haMSCs in OA. Second, patients enrolled in this study were all below grade 4 by the Kellgren–Lawrence grade. The effect of haMSCs in patients with severe OA remains unknown and warrants further research. Finally, we did not recommend the period of nonweight bearing after injection for the decreased and delayed recovery of the knee function as reported by Jo *et al.* [35]. However, it might cause discomfort, such as swelling and pain, which, though transient and could relieve spontaneously, could exert some negative impact on the treatment compliance, especially when repeated treatment was needed.

Conclusion

This study suggested that intra-articular injections of haMSCs improved the pain, function and cartilage volume of the knee joint without causing treatment-related SAEs. In addition, the improvement was superior in a dosage of 5×10^7 cells combined with repeated injections. These results are promising to encourage more extensive and randomized clinical trials, and we are cautiously optimistic regarding the articular injection of haMSCs as a putative treatment for knee OA.

Executive summary

- Human adipose-derived mesenchymal stem cells (haMSCs) preserved the characteristics of MSCs in cell phenotype and the ability of the trilineage differentiation *in vitro*.
- haMSCs led to no abnormal manifestation, organ damage or death in BALB/c-nu nude mice.
- Intra-articular injections of haMSCs did not correlate with treatment-related serious adverse events.
- Intra-articular injections of haMSCs correlated with a significant reduction of the Western Ontario and McMaster Universities Osteoarthritis index and improvement in the numerical pain rating scale-11 and short form 36 score.
- Intra-articular injections of haMSCs were associated with a significantly increased thickness of the articular cartilage, which was superior in the dosage of 5×10^7 haMSCs.
- Intra-articular injections of haMSCs were safe and improved the pain, function and cartilage volume of the knee joint, suggesting a potential novel treatment for knee osteoarthritis.
- This study was the first to demonstrate the safety and potential benefits of haMSCs therapy with a long-term follow-up of 96 weeks with repeated injections.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/rme-2017-0152

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Ethical conduct of research

This study was approved by the Institutional Review Board of the Shanghai Jiao Tong University and Ren Ji Hospital and registered at ClinicalTrials.gov with identifier NCT01809769. All procedures were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients after the physicians illustrated detailed relevant information, such as the purpose and overall procedure of the study, potential risks and benefits.

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