Intra-articular Injection of Autologous Adipose Derived Mesenchymal Stem Cells in Treatment of Knee Osteoarthritis

Running title: Treatment of Knee OA using AD-MSCs

Duško Spasovski[1,2], Vesna Spasovski[3*], Zoran Baščarević[1,2], Maja Stojiljković[3], Miša Vreća[3], Marina Andjelković[3], Sonja Pavlović[3]

1 Institute for Orthopaedic Surgery "Banjica", Belgrade, Serbia

2 School of Medicine, University of Belgrade, Serbia

3 Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia

*Corresponding author:
Vesna Spasovski
Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia
Vojvode Stepe 444a, 11000 Belgrade, Serbia
Tel: + 381 11 3976 445
Fax: + 381 11 3975 808
E mail: vesna.spasovski@imgge.bg.ac.rs

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jgm.3002

Abstract

Background: Osteoarthritis (OA) is a chronic degenerative joint disease, considered to be the fourth leading cause of disability and the second cause of inability to work in men. Recently, adipose derived mesenchymal stem cells (AD-MSCs) came in focus of regenerative medicine as promising tool for treatment of OA. It has been shown that administration of stem cells into impaired joints results in pain relief and improves quality of life, accompanied with restoration of hyaline articular cartilage.

Methods: In this work, nine patients, two patients with bilateral symptoms, diagnosed with osteoarthritis (IKDC grade B in 5 and grade D in 6 knees) were treated using single injection of AD- MSCs in concentration of $0.5\text{-}1x10^7$ cells, and were followed up for eighteen months. During follow up, all the cases were evaluated clinically (by Knee Society score, Hospital for Special Surgery knee score, Tegner-Lysholm score and VAS of pain), by plain radiography and by MRI visualization with 2D MOCART score assessment.

Results: Significant improvement of all four clinical scores was observed within first six months (KSS for 41.4 points, HSS-KS for 33.9 points, T-L score for 44.8 points, VAS of pain from 54.5 to 9.3), and improvement persisted throughout the rest of the follow-up. MOCART score showed significant cartilage restoration (from 43±7.2 to 63±17.1), while radiography showed neither improvement nor further joint degeneration.

Conclusions: Our results give a good basis for prospective randomized controlled clinical trials regarding the usage of AD-MSCs in the treatment of osteoarthritis.

Keywords: Stem cells, adipose tissue, osteoarthritis, regenerative medicine

Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease characterized by progressive destruction of hyaline articular cartilage, which in severe cases, might lead to wearing out of the cartilage. This results in pain and joint movement limitation, in severe cases greatly impairing ambulation and quality of life. Worldwide, arthritis is considered to be the fourth leading cause of disability[1] and the second cause of inability to work in men[2]. The degeneration of the cartilage is mostly the result of joint dysplasia, cumulative overload or acute trauma, but in some cases, no underlying cause can be found. Various risk factors are identified such as age, obesity, female gender, genetic component[3]. Nonoperative treatments, such as physical therapy[4], viscosupplementation[5], glucosamine and/or chondroitin sulfate[6], arthroscopic surgery[7,8], acupuncture[9,10], and ultrasound[11], failed to demonstrate significant effect. Therefore total joint replacement is considered as a gold standard in the treatment of osteoarthritis.

Biological therapies, including chondrocyte implantation, shown some potential and established guidelines of the future treatments back in early 1990's[12]. Tremendous upgrade in this field came with utilization of stem cells. Several clinical trials were conducted in order to test efficacy and potency of mesenchymal stem cells (MSCs) for the treatment of hip and knee osteoarthritis[2,13-16]. It is known that cartilage in physiological attempt of self -reparation generates fibrous cartilage, biomechanically inferior to hyaline. Application of autologous MSCs grown in cell culture up to certain therapeutic number results in restoration of hyaline cartilage[15].

Stem cells represent unspecialized cells, which have the ability to differentiate into different cell types. Mesenchymal stem cells have been found to be the most promising candidates for the treatment of cartilage defects, as they show good differentiation potential towards cartilage and bone cells. They can be isolated from a number of adult mesenchymal tissues as for example trabecular bone, adipose tissue, bone marrow, synovium, dermis, periodontal ligament, dental pulp, bursa and the umbilical cord[17]. Adipose tissue became a primary source because it contains 500 times more MSCs than the same volume of bone marrow[18] and sampling is performed by minimally invasive surgical procedure, which is safe and cosmetically acceptable[19]. That is why adipose derived mesenchymal stem cells (AD-MSCs) came in focus of regenerative medicine.

In this work, nine patients were treated using AD- MSCs. In two patients, both knees were treated, so totally eleven knee joints were followed up. During follow up, all the patients were evaluated clinically and using radiography and MRI visualization.

Material and methods

Study design

The research was carried out in compliance with the Helsinki Declaration. It was approved by the Serbian Ministry of Health (Licence No. 500-01-01106/2014-03) and the Ethical Committee of the Institute for Orthopaedic Surgery "Banjica" registered it under the number I-67/9 (02/06/2015). Informed Consent was obtained from all participants.

Study included adult patients diagnosed with knee osteoarthritis at the Institute for Orthopaedic Surgery "Banjica" during patient recruitment period (from April-October 2015). Additional inclusion criteria were: intensity of symptoms (Knee Society score <60, duration >3 months, refractory to nonoperative treatment) and absence of any medicamentous or physical therapy related to symptoms at least one month prior to intervention. Exclusion criteria were history or signs of oncologic and systemic metabolic disease (diabetes, thyroid diseases etc.). Nine patients entered the study, three male and six female, with mean age of 63 years (range from 39-78) and with average duration of symptoms of 3,3 years. Two of patients had bilateral disease and both knees were treated, hence 11 knee joints were included in the study. Five joints were classified as IKDC B grade (moderate stage) while six joints were IKDC D grade (severe stage)[20]. We used IKDC classification because of its reliability and good correlation to clinical and arthroscopic data[21]. Baseline patient data are listed in Table 1.

Tissue sampling, isolation and propagation of AD-MSCs

Sampling was done by excision of 5 ml of subcutaneous fat tissue through a small incision from superficial abdominal region in local anesthesia. Sample was transferred to the Laboratory for Molecular Biomedicine, Institute of Molecular Genetics and Genetic Engineering, and left over night at room temperature. After repeated washing in 1xPBS solution, tissue was treated with 0.1% Collagenase (Sigma Aldrich, USA) until tissue was

completely dissolved. Autologous serum was performed by centrifugation of whole blood (without anticoagulants) at 1300rcf for 10 minutes. Obtained serum was collected and filtered through 0.22µm filter. Collagenase solution was neutralized by Low Glucose (DMEM, low glucose, GlutaMAX™, Gibco, Life technologies, USA), supplemented with 10% autologous serum and 1X antibiotic/antimycotic solution (Gibco, Life technologies, USA). Cells were filtered through 100µm filter (BD, USA), counted and seeded in number of $6x10^4/cm^2$ in DMEM/10% autologous serum/1X antibiotic/antimycotic. After one week, floating cells were washed away and cells were cultured for 2-3 weeks, until they reached number of 0.5-$1x10^7$ (second or third passage).

Safety assessment and cell administration

Cells were tested on bacterial and mycoplasma sterility and the presence of the cell surface markers prior application. After final detachment with 1X trypsin-EDTA (Gibco, Life technologies, USA), cells were checked for viability (>90%) on Countess cell counter (Invitrogen) and resuspended in 1mL 1xPBS (Gibco, Life technologies, USA). Cells were transferred immediately to the hospital, loaded into 2 ml sterile syringes and injected in affected joint within one hour after harvesting. No previous preparation or premedication was given. During the procedure, no joint fluid was aspirated and no additional substances were injected in the knee joint. Patients were not hospitalized for the procedure, and went back home half an hour after cell injection. All patients were recommended not to use analgesic, anti-inflammatory and immunosuppressive drugs, or any kind of physical therapy one month before and 6 months after the stem cell application.

Follow up

During follow up period patients were allowed to have regular daily activities. Clinical and radiologic evaluation was done before the treatment and in regular intervals (3, 6, 12 and 18 months). We used common clinical scores for knee osteoarthritis: Knee Society score[22], Hospital for Special Surgery score[23], and Tegner-Lysholm score[24]. Knee range of motion was measured using digital goniometer and level of pain was self-reported using Visual Analog Scale (VAS). Radiographic data were collected from plain knee radiography in standing position. MRI was performed before the intervention and after 6 and 18 months of follow-up, analysed in sagittal and axial planes and assessed for markers of osteoarthritis (joint space narrowing between femur and tibia, intensity and distribution of subchondral sclerosis,

presence of osteophytes or subchondral cysts), signs of inflammation and amount of joint fluid. Analysis of radiographic images was performed using ImageJ open-source digital image analysis software[25] and cartilage was assessed by modified 2D Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) score[26,27]. Data were statistically analyzed by Student t-test, Wilcoxon sign rank test and Fischer exact test.

Phenotypic characterization of AD-MSCs by flow cytometry

The cell surface markers for the MSCs were examined using a flow cytometer (Partec, Germany). The immunophenotype of the MSCs was characterized using the following mouse monoclonal antibodies: CD34-PE, CD45-PE, CD73-PE, CD90-PE, CD105-PE (R&D Systems, USA). The MSCs between passages 2 and 4 were harvested using 1X trypsin-EDTA and resuspended in 0,5% BSA/ PBS solution. A 3×10^5 cell suspension was incubated at 4°C for 30 min in the dark with mouse anti-human antibodies. Following incubation, the cells were washed twice with cold 0,5% BSA/ PBS solution and centrifuged at 300× g for 5 min at 4°C. Cells were then resuspended in 0.5 mL of the same buffer, and analyzed by flow cytometry (Partec, Germany) using the FlowMax software.

Potential towards osteogenic and chondrogenic differentiation

To determine differentiation potential of isolated MSCs, cells were induced to differentiate toward osteognenic and chondrogenic lineage. To induce osteogenic differentiation, cells were cultured in Low glucose DMEM supplemented with 10% FBS (Gibco, Life technologies), 1X antibiotic/antimycotic, 0,1μM dexamethasone (Sigma Aldrich, USA), 50μM L-ascorbic acid-2-phosphate (Sigma Aldrich, USA) and 10 mM β-glycerophosphate (Sigma-Aldrich). After three weeks, calcium deposition and extracellular matrix mineralization was visualized by Alizarin red staining (Alizarin Red S, Sigma- Aldrich). Chondrogenic differentiation was performed in 3-D cultures. Chondrogenic medium contained High glucose DMEM (Gibco, Life technologies), supplemented with 10 ng/ml TGF-β 3- (TGF-β3 E. coli human recombinant, Sigma-Aldrich), 1X ITS (Sigma-Aldrich), 50μg/ml L-ascorbic acid-2-phosphate (Sigma-Aldrich), 0,1μM dexamethasone (Sigma-Aldrich), 1X antibiotic/antimycotic (Gibco, Life technologies). The cultures were incubated for three weeks, and the deposition of extracellular matrix characteristic for chondorgenesis was assessed via Alcian Blue staining (Alcian Blue 8 GX dye, Sigma- Aldrich).

Results

Clinical Outcomes

Few days after injection some patients reported moderate pain and swelling of the knee. These symptoms vanished within one week after treatment. No other side effects were noticed during follow up. Summarized clinical data are presented in Table 2, and individual patient dataset in Supporting information,Table 1.

The first control visit was 3 months after injection. All patients reported reduction of pain and statistically significant improvement of all clinical scores were seen at this stage. Range of motion improved for 17.3^0, KSS for 28.5 points, HSS-KS for 22.2 points and T-L score for 32.3 points. Average VAS of pain decreased for 33.8 points on average (Table 2, Figure 1). At second visit 6 months after injection, further significant improvement of all clinical parameters was observed. Compared to 3-months results, average knee joint range of motion increased for 7.8^0,. KSS for 12.9 points, HSS-KS for 11.7 points, T-L score for 12.5 points and VAS of pain decreased for 11.4 points. (Figure 1).

At 12 months and 18 months of follow up, all clinical scores retained improved, without statistically significant change compared to 6 months level. Noteworthy, during the whole follow-up period all clinical scores showed statistically highly significant difference compared to baseline. Range of motion, however, showed a decline at 12 months for 16.4^0 followed by slight improvement at 18 months, with levels still above baseline but without significance. No significant differences were observed according to dose applied, stage of joint degeneration or patients' age. During follow-up period, no infections or other adverse local effects appeared in treated joints.

Radiological parameters

When plain radiological parameters are concerned, we did not find any significant changes (improvement or worsening) during the follow-up period compared to the baseline. No formation of new osteophytes or cysts were seen, and no changes in the amount of synovial fluid. Average change of subchondral sclerosis intensity before and at the end of follow up was 0.41±7.76%, with significant individual variations: in some cases it got 9% increased while in others 15.6% decreased (Figures 2A and 2D). Average joint space did not show any improvement either: it was 2.3±1.33mm before stem cell treatment, while after 18 months it was 2.2±1.23mm. No change in IKDC grade was seen in all cases.

MRI Evaluation

On the other hand, MRI findings revealed structural cartilage enhancement in accordance with observed clinical results. Fewer subchondral cysts and oedema are visible on both femoral and tibial condyles, with globally more uniform cartilage signal seen on T2 sequence (Figures 2B and 2D). Average 2D MOCART score before the treatment was 43±7.2 (max=100) and after 18 months it was 63±17.1 points – an improvement with high statistical significance (Fischer exact test, t=0.970, p=0.001) (Figure 3).

MSC characteristics

Characterization of isolated cells showed phenotypic characteristics specific for mesenchymal stem cells, according to of The International Society for Cellular Therapy[28]. Characteristic fibroblast like phenotype (Figure 4A), adherence to plastic surface, potential for differentiation into mesenchymal lineage cell types (chondrogenic and osteogenic), expression of certain mesenchymal surface markers (CD73, CD90, and CD105) and absence of expression of hematopoietic surface markers (CD34 and CD45). Differential staining showed that isolated cells are capable to differentiate toward specific cell types and express extracellular matrix components characteristic for osteo- and chondrogenic lineage (Figure 4B). Flow cytometry analysis showed that CD73, CD90 and CD105 were expressed by more than 82.7%, 92.7%, and 96.5% of the cells, respectively, whereas CD34 and CD45 surface markers were expressed in less than 1.3% and 1.2% of the cells, respectively (all patients were analyzed, representative diagrams presented in Figure 4C).

Discussion

In this paper we report our findings concerning the treatment of 9 patents (11 knees, two patients treated bilaterally) with knee osteoarthritis using *ex vivo* expanded autologous adipose derived mesenchymal stem cells, with 18 months follow up. Except transient moderate swelling reported by few patients, no other side effects were noticed during this period. Despite different stages of OA in our patient's cohort, all patients showed significant improvement of all clinical scores and reported substantial pain relief. Improvement was noticed starting from the first checkpoint (3 months), reaching its top at 6 months control examination. During that period, we did not notice significant changes in X ray, while results on MRI evaluation showed structural cartilage improvements. Results on 12 and 18 month checkpoints preserved the values achieved in 6 months evaluation. During the whole follow-up period all clinical scores showed statistically significant difference compared to baseline. Findings reported by Davatchi *et al*. showed that the beneficial effect starts to decline after 6 months, but is still better at 5 years compared to the baseline[13,29]. Study of Emadedin *et al*., who had one year follow-up, reported duration of improvement during 6 months, afterwards the effects appeared to be slightly decreased[14]. Using the bone marrow as a source of stem cells, as both studies mentioned above, Orozco *et al.* documented duration of pain improvement in follow up of 2 years after treatment[2]. Two studies that used adipose derived MSCs showed improvement of all clinical and histological findings, but showed contradictory results concerning the dose of applied cells[15,16].

The most of the early improvements seen in most of the studies so far might be attributed to anti-inflammatory, immunomodulatory and paracrine effects of stem cells. Regenerative potential of stem cells relies on their capability to differentiate toward certain cell types under the addition of specific factors *in vitro*, or under the influence of specific micro-environmental signals *in vivo*. Substitution of damaged or lost cells is also under the control of soluble factors that they secrete, and stimulation of endogenous progenitor cells through these paracrine effects[30]. Stem cells are capable to promote vascularization, cell proliferation, differentiation and modulate an inflammatory process[31]. These biological processes are complex, so the real outcome of the treatment, which would be in our case the quality and thickness of the cartilage, needs to be monitored long term.

The remaining question is the source and the quantity of the cells used for therapy. Most of the previous reports used bone marrow as a source of MSCs. Our study is one of the first reports that used adipose tissue as a source of mesenchymal stem cells. The advantages of

adipose tissue are numerous, since the sampling procedure is minimally invasive and the amount of tissue needed is small. The therapeutic dose used in our study was $0.5\text{-}1x10^7$, and all patients had improvement in clinical examination. No significant difference in any measured outcome parameter was observed so far according to dose applied. This could be due to small patient group and their different baseline clinical and pathoanatomic parameters. Further follow-up of our patients will be performed in order to evaluate the influence of dose applied on the duration of treatment effects.

Concluding remarks and future perspectives

Our results suggest that injection of proposed dose of AD-MSCs may be a safe and efficient method of osteoarthritis treatment. This treatment has several advantages compared to other available treatments: it is minimally invasive, virtually all joints could be treated, and is repeatable when needed. We believe that our study gives a good basis for sound prospective randomized controlled clinical trials.

Acknowledgements

This work has been funded by grants from the Ministry of Education, Science and Technological Development, Republic of Serbia (III 41004). We thank all the patients for their participation in the study.

Authorship and Disclosures

DS recruited the patients, performed sample collection and clinical management of patients. ZB performed clinical management of the patients and coordinated the research. VS performed laboratory work and wrote the paper. MS, MV and MA participated in experimental work and performed statistical analysis. SP designed the study and was the principal investigator. VS takes the primary responsibility for the paper.

Conflict of interest

The authors have not any conflict of interest or any financial support.

REFERENCES

1. Fransen M, Bridgett L, March L, et al. The epidemiology of osteoarthritis in Asia. *Int J Rheum Dis.* 2011;14:113-121.
2. Orozco L, Munar A, Soler R, et al. Treatment of knee osteoarthritis with autologous mesenchymal stem cells: a pilot study. *Transplantation.* 2013;95:1535-1541.
3. Wang Y, Yuan M, Guo QY, Lu SB,Peng J. Mesenchymal Stem Cells for Treating Articular Cartilage Defects and Osteoarthritis. *Cell Transplant.* 2015;24:1661-1678.
4. Pisters MF, Veenhof C, Schellevis FG, De Bakker DH,Dekker J. Long-term effectiveness of exercise therapy in patients with osteoarthritis of the hip or knee: a randomized controlled trial comparing two different physical therapy interventions. *Osteoarthritis Cartilage.* 2010;18:1019-1026.
5. Rutjes AW, Juni P, da Costa BR, et al. Viscosupplementation for osteoarthritis of the knee: a systematic review and meta-analysis. *Ann Intern Med.* 2012;157:180-191.
6. Sawitzke AD, Shi H, Finco MF, et al. Clinical efficacy and safety of glucosamine, chondroitin sulphate, their combination, celecoxib or placebo taken to treat osteoarthritis of the knee: 2-year results from GAIT. *Ann Rheum Dis.* 2010;69:1459-1464.
7. Moseley JB, O'Malley K, Petersen NJ, et al. A controlled trial of arthroscopic surgery for osteoarthritis of the knee. *N Engl J Med.* 2002;347:81-88.
8. Kirkley A, Birmingham TB, Litchfield RB, et al. A randomized trial of arthroscopic surgery for osteoarthritis of the knee. *N Engl J Med.* 2008;359:1097-1107.
9. Manheimer E, Cheng K, Linde K, et al. Acupuncture for peripheral joint osteoarthritis. *Cochrane Database Syst Rev.* 2010;Cd001977.
10. Witt C, Brinkhaus B, Jena S, et al. Acupuncture in patients with osteoarthritis of the knee: a randomised trial. *Lancet.* 2005;366:136-143.
11. Rutjes AW, Nuesch E, Sterchi R,Juni P. Therapeutic ultrasound for osteoarthritis of the knee or hip. *Cochrane Database Syst Rev.* 2010;Cd003132.
12. Schmitt A, van Griensven M, Imhoff AB,Buchmann S. Application of stem cells in orthopedics. *Stem Cells Int.* 2012;2012:394962.
13. Davatchi F, Abdollahi BS, Mohyeddin M, Shahram F,Nikbin B. Mesenchymal stem cell therapy for knee osteoarthritis. Preliminary report of four patients. *Int J Rheum Dis.* 2011;14:211-215.
14. Emadedin M, Aghdami N, Taghiyar L, et al. Intra-articular injection of autologous mesenchymal stem cells in six patients with knee osteoarthritis. *Arch Iran Med.* 2012;15:422-428.
15. Jo CH, Lee YG, Shin WH, et al. Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: a proof-of-concept clinical trial. *Stem Cells.* 2014;32:1254-1266.
16. Pers YM, Rackwitz L, Ferreira R, et al. Adipose Mesenchymal Stromal Cell-Based Therapy for Severe Osteoarthritis of the Knee: A Phase I Dose-Escalation Trial. *Stem Cells Transl Med.* 2016;5:847-856.
17. Nancarrow-Lei R, Mafi P, Mafi R,Khan W. A Systemic Review of the Sources of Adult Mesenchymal Stem Cells and their Suitability in Musculoskeletal Applications. *Curr Stem Cell Res Ther.* 2017;
18. Hass R, Kasper C, Bohm S,Jacobs R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal.* 2011;9:12.
19. Yarak S,Okamoto OK. Human adipose-derived stem cells: current challenges and clinical perspectives. *An Bras Dermatol.* 2010;85:647-656.

20. Irrgang JJ, Ho H, Harner CD,Fu FH. Use of the International Knee Documentation Committee guidelines to assess outcome following anterior cruciate ligament reconstruction. *Knee Surg Sports Traumatol Arthrosc.* 1998;6:107-114.
21. Wright RW. Osteoarthritis Classification Scales: Interobserver Reliability and Arthroscopic Correlation. *J Bone Joint Surg Am.* 2014;96:1145-1151.
22. Insall JN, Dorr LD, Scott RD,Scott WN. Rationale of the Knee Society clinical rating system. *Clin Orthop Relat Res.* 1989;13-14.
23. Insall JN, Ranawat CS, Aglietti P,Shine J. A comparison of four models of total knee-replacement prostheses. *J Bone Joint Surg Am.* 1976;58:754-765.
24. Lysholm J,Gillquist J. Evaluation of knee ligament surgery results with special emphasis on use of a scoring scale. *Am J Sports Med.* 1982;10:150-154.
25. Schneider CA, Rasband WS,Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012;9:671-675.
26. Marlovits S, Striessnig G, Resinger CT, et al. Definition of pertinent parameters for the evaluation of articular cartilage repair tissue with high-resolution magnetic resonance imaging. *Eur J Radiol.* 2004;52:310-319.
27. Lee KT, Choi YS, Lee YK, Cha SD,Koo HM. Comparison of MRI and arthroscopy in modified MOCART scoring system after autologous chondrocyte implantation for osteochondral lesion of the talus. *Orthopedics.* 2011;34:e356-362.
28. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006;8:315-317.
29. Davatchi F, Sadeghi Abdollahi B, Mohyeddin M,Nikbin B. Mesenchymal stem cell therapy for knee osteoarthritis: 5 years follow-up of three patients. *Int J Rheum Dis.* 2016;19:219-225.
30. Murphy JM, Fink DJ, Hunziker EB,Barry FP. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum.* 2003;48:3464-3474.
31. Meirelles Lda S, Fontes AM, Covas DT,Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev.* 2009;20:419-427.

TABLE 1 Baseline characteristics of OA patients

Age, mean (SD) (years)	63 (10.4)
Sex No, (%)	Males 3 (33.3%), females 6 (66.7%)
Height, mean (SD) (cm)	175.5 (12.82)
Weight, mean (SD) (kg)	90.9 (15.23)
Body-mass index[a], mean (SD), (kg/m^2)	29.5 (3.97)
Symptoms duration, mean (SD) (years)	3.4 (1.36)
Activity level[b] (I-IV), No. (%)	
I	0 (0%)
II	0 (0%)
III	2 (22.2%)
IV	7 (77.8)
Functional status, No. (%)	
Active outdoor unlimited	0 (0%)
Active outdoor limited	6 (66.7%)
Active indoor only	1 (11.1%)
Sedentary indoor	2 (22.2%)
Radiographic status[c], No. (%)[d]	
Grade A	0 (0%)
Grade B	5 (45.5%)
Grade C	0 (0%)
Grade D	6 (54.5%)

a BMI= body weight/(body height)^2

b Activity level I indicates high competitive sportsman/woman; II, well-trained and frequently sporting; III, sporting sometimes; IV, nonsporting [15].

c IKDC grading of knee osteoarthritis

d Both knees were treated in two patients, therefore there are 11 results for this parameter

TABLE 2 Clinical results on treatment with AD-MCSs during 18 months follow-up

	Baseline	3 months	6 months	12 months	18 months
Range of motion (degrees); t value; p value	93.2±17.93	110.5±10.42 (t=4.952, p<0.01)	118.3±12.69 (t=2.104, p<0.05)	101.9.3±13.26 (t=2.923, p<0.05)	104.2±14.55 (t=-0.371, p>0.05)
Knee society score (max=100); t value;p value	42.1±15.71	70.6±17.53 (t=7.103, p<0.01)	83.5±6.36 (t=3.223, p<0.05)	86.8±3.49 (t=1.225, p>0.05)	83.7±11.86 (t=-0.858, p>0.05)
HSS Knee score (max=100); t value*; p value	59.0±12.68	81.2±12.64 (t=9.271, p<0.01)	92.9±5.26 (t=4.146, p<0.01)	94.8±2.09 (t=1.19, p>0.05)	91.6±7.93 (t=-1.11, p>0.05)
Tegner & Lysholm score (max=100); t value*; p value	46.7±20.50	79.0±14.56 (t=6.633, p<0.01)	91.5±10.55 (t=5.239, p<0.01)	94.1±8.42 (t=1.014, p>0.05)	92.9±9.55 (t=-1.08, p>0.05)
Knee VAS pain score (max=100); t value*; p value	54.5±16.5	20.7±13.3 (t=6.271, p<0.01)	9.3±6.5 (t=3.097, p<0.05)	8.0±4.9 (t=0.650, p>0.05)	9.1±7.9 (t=-0.659, p>0.05)

* comparison was performed with previous state (3m vs baseline; 6m vs 3m; 12m vs 6m; 18m vs 12m)

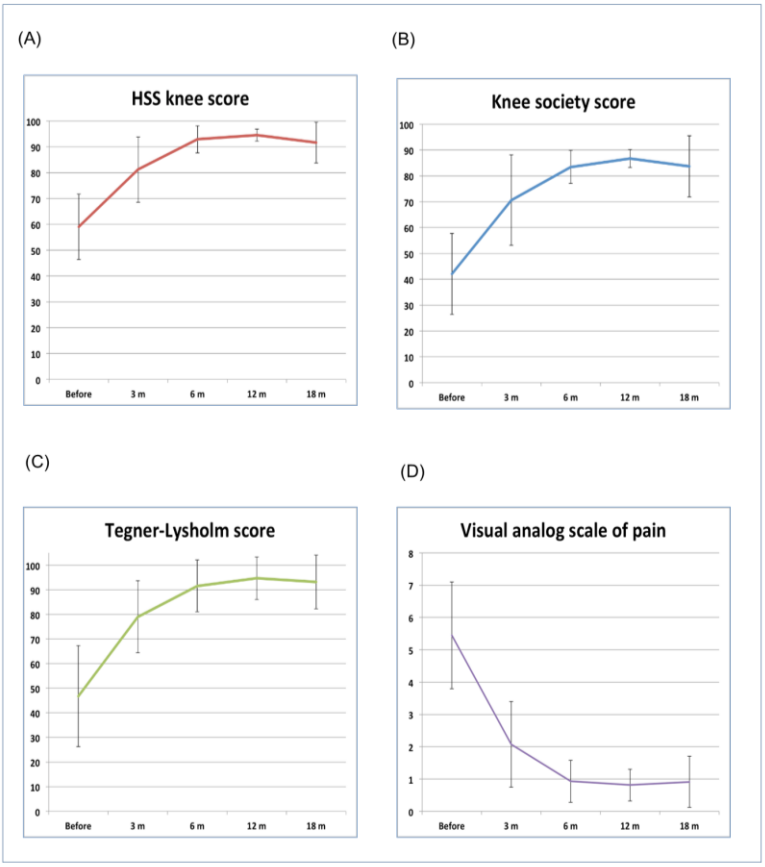

FIGURE 1 Average score results of knee joints treated with AD-MSCs (n=11), assessed in regular intervals (before the treatment and at 3, 6, 12 and 18 months of follow-up) using Hospital for Special Surgery (HSS) knee score (A), Knee society (KS) score (B), Tegner-Lysholm score (C) and Visual analog scale (VAS) of pain (D). Vertical bars show standard deviation

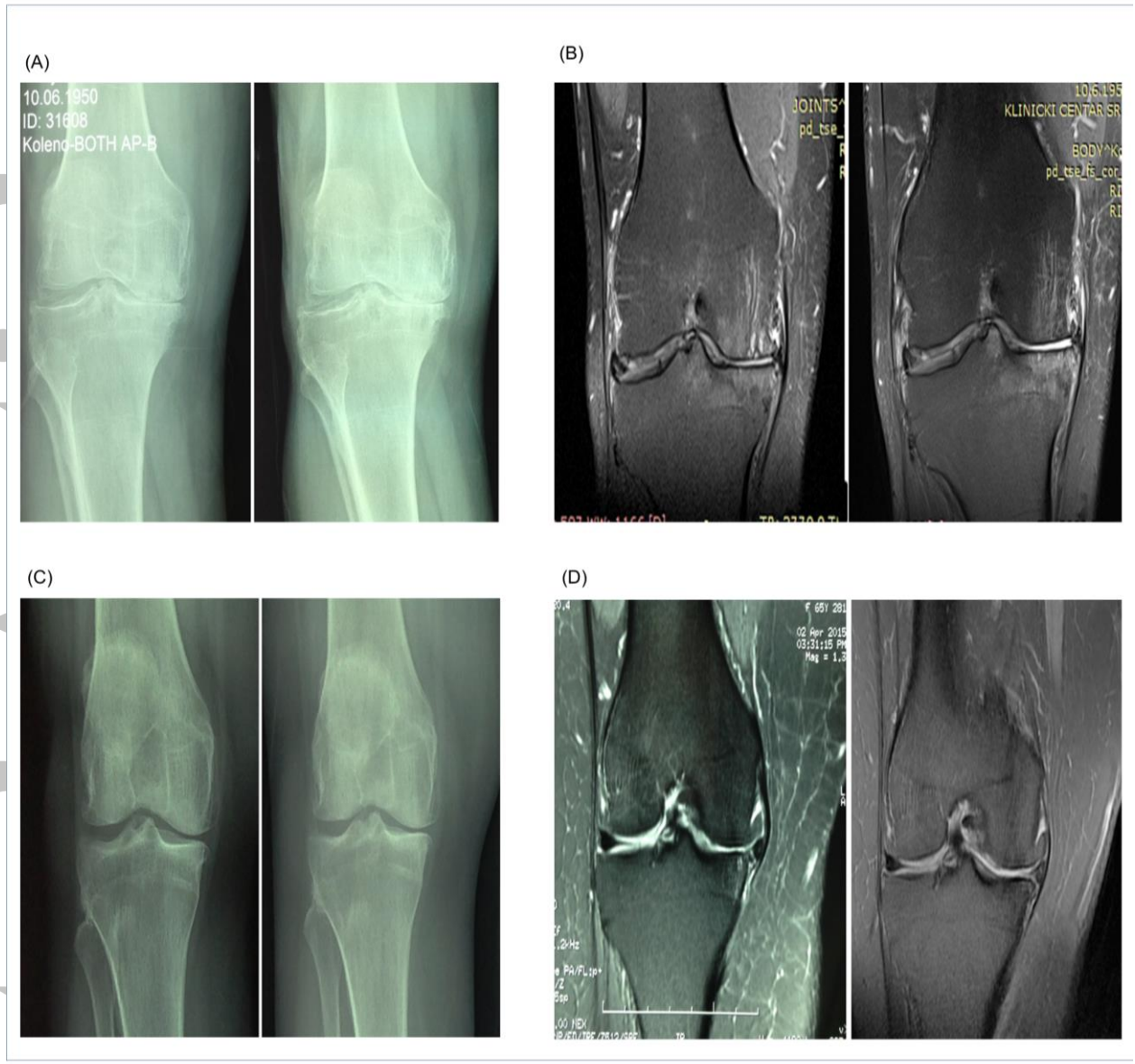

FIGURE 2 Figure shows results on a digital plain radiography and MRI before the treatment and after 18 months of follow-up. Figure 2A. Patient #1, right knee digital plain radiography before the treatment (left) and after 18 months of follow-up (right). Figure 2B. Patient #1, right knee T1 sequence MRI before the treatment (left) and after 18 months of follow-up (right) Figure 2C. Patient #2, right knee digital plain radiography before the treatment (left) and after 18 months of follow-up (right) Figure 2D. Patient #2, right knee T1 sequence MRI before the treatment (left) and after 18 months of follow-up (right)

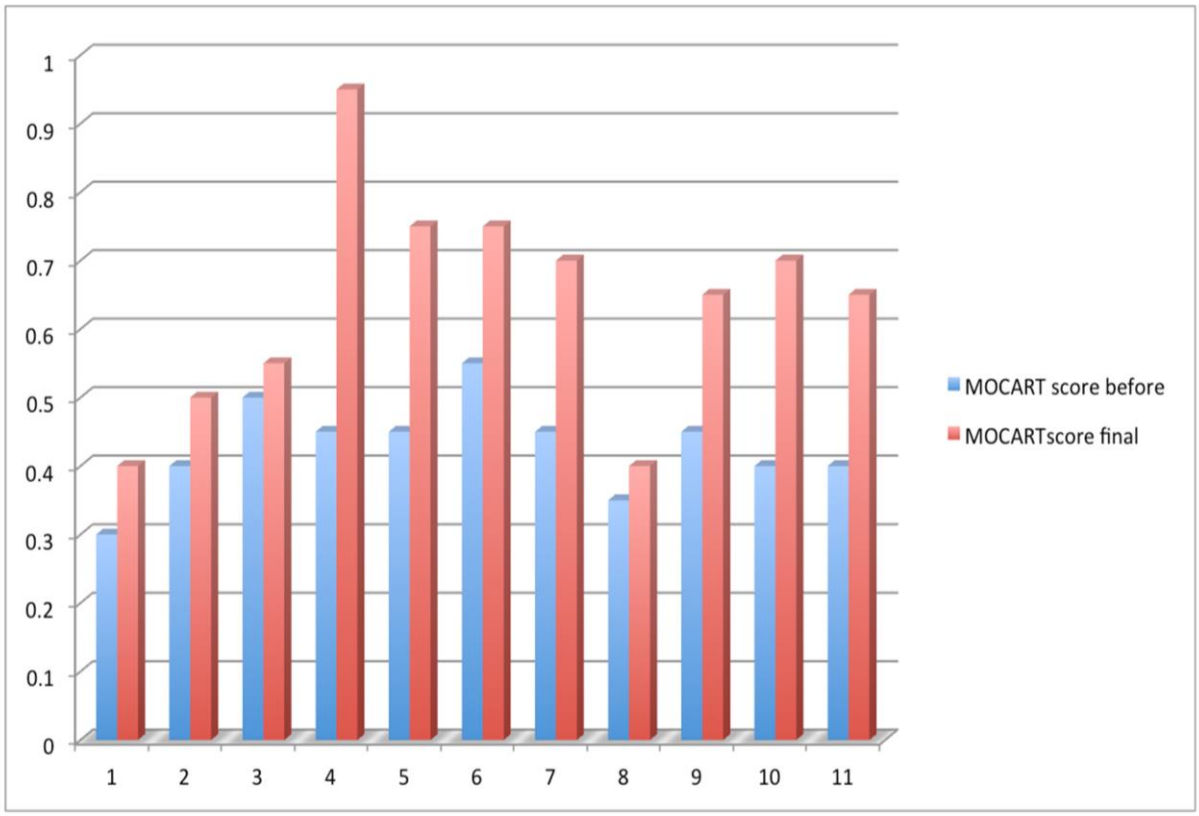

FIGURE 3 Comparison of 2D Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) score assessed before the treatment (blue bars) and after 18 months of follow-up (red bars). Value of the score ranges from 0 (complete destruction of articular cartilage) to 1 (intact articular cartilage)

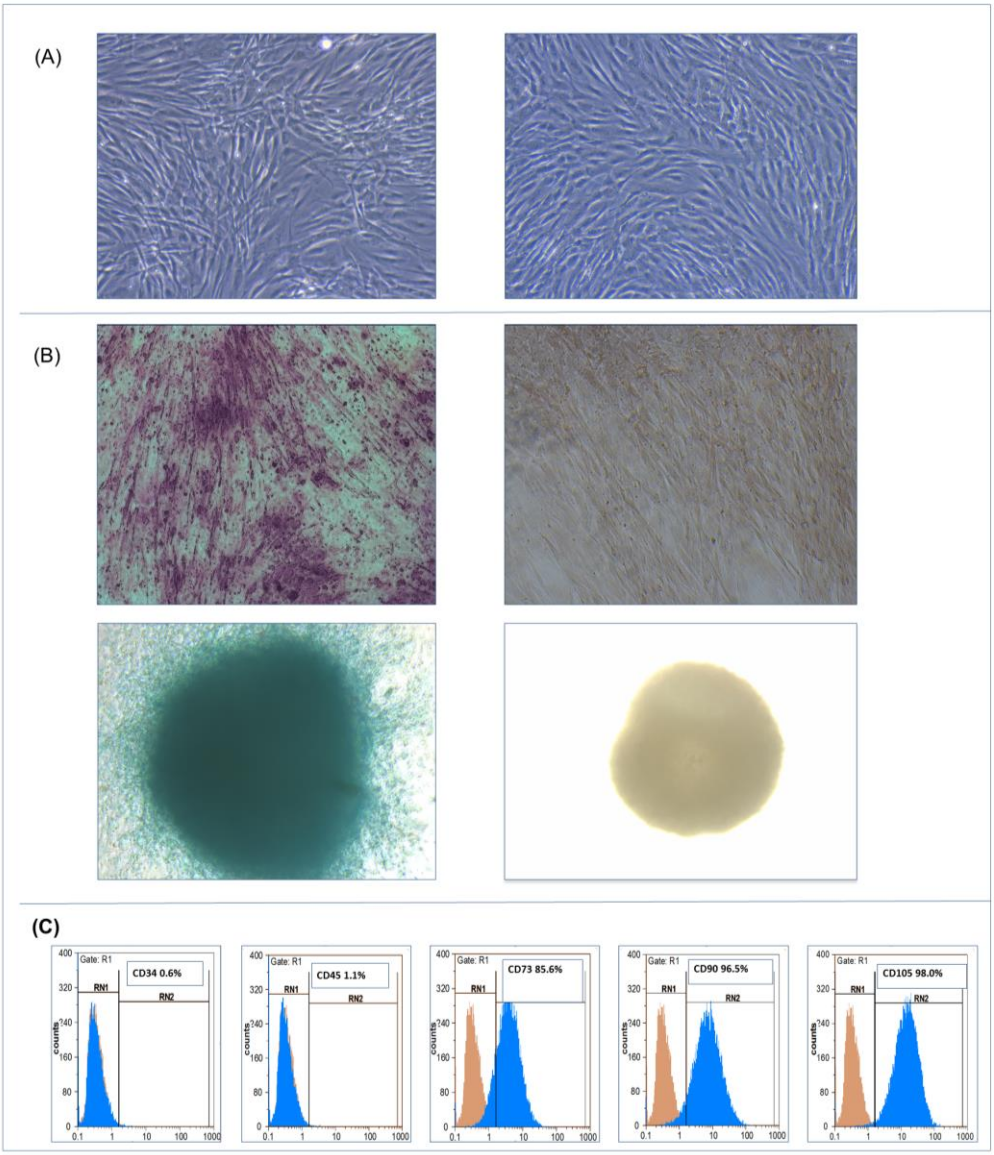

FIGURE 4 Phenotypic characterization of AD-MSCs. A) Characteristic fibroblast like morphology of isolated mesenchymal stem cells (second passage). Representative images showed. B) Extracellular matrix components stained by Alizarin red (upper row) and Alcian blue (lower row) staining show potential for osteogenic and chondrogenic differentiation, respectively. On the left side are differentiated and on the right side are control cells. C) Flow cytometry analysis of surface markers specific for mesenchymal stem cells