

Treatment of Knee Osteoarthritis with Autologous Expanded Bone Marrow Mesenchymal Stem Cells: 50 Cases Clinical and MRI Results at One Year Follow-Up

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Abstract

Knee osteoarthritis is one of the most prevalent joint diseases, causing pain, function loss and disability, leading to a progressive cartilage degeneration induced by biochemical changes in its composition. Current available treatments focus on addressing symptoms and joint replacement is the last treatment option.

Advanced therapies with mesenchymal stem cells build new expectations to improve the results of OA treatments. MSC applied in animal models, show encouraging results in modulating inflammation and joint cartilage repair. Several studies applied autologous mesenchymal stem cells to treat knee osteoarthritis in humans by means of an intra-articular injection.

Our team previously conducted a pilot study applying 40×10^6 autologous bone marrow expanded mesenchymal cells in 12 patients affected with knee osteoarthritis through intra-articular infusion. After 2 years we obtained excellent clinical and quantitative MRI outcome measures, no adverse events reported.

Keywords: Knee osteoarthritis; Autologous expanded mesenchymal cells; Stem cells; Regenerative therapy

Introduction

Osteoarthritis (OA) is the most prevalent chronic joint disease and a frequent cause of joint pain, loss of function, and disability [1]. Knee is one of most affected joints. The pathogenesis of knee OA has been linked to biomechanical and biochemical changes in joint cartilage, e.g. inability to withstand normal mechanical stresses, limited nutrients and oxygen supply, inadequate synthesis of extracellular matrix components, increased synthesis of proteinases and overall apoptosis of chondrocytes [2-5]. Synovial inflammation is a response of synovial macrophages to cartilage debris and catabolic mediators entering the synovial cavity and limits knee cartilage repair [6].

Current treatments for knee OA achieve poor clinical results and fail to modify cartilage. Joint replacement is the last treatment option, bearing enormous effort and expenses [7,8]. Its high prevalence and social impact have promoted the development of many therapeutic options to try to stop or slow down its progression. Diagnosis of OA is typically made late in the disease process and there are no disease modifying osteoarthritis drug (DMOADS) currently available for treatment. Mesenchymal Stromal Cells (MSC) opened new therapeutic perspectives provided their regenerative potential and ability to modulate inflammation [9-12].

MSC were first identified by Friedenstein, and have intrinsic characteristics defined by the International Society for Stem cell Therapy: (a) remain plastic-adherent under standard culture conditions; (b) express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR; (c) differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro* [13]. These cells remain in lethargic status G0, and may be obtained from bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and deciduous teeth [14,15]. Regardless of their origin they have the capacity to differentiate *in vitro* into different cell types

of connective tissue lineages as bone, fat, muscle and cartilage. Many studies show co-cultured MSC to induce chondrocyte proliferation and extracellular matrix protein synthesis, including aggrecan and type II collagen [16,17]. The capacity to differentiate into cells of the chondrogenic lineage and produce extracellular matrix together with their proven anti-inflammatory potential brought to focus MSC as a potential treatment for osteoarthritis [18].

MSC effects in chondrogenic repair have been documented in mice, rabbits, pigs, sheep, and horses [19-21]. These studies report a dose-dependent effect, which requires a lingering time to be apparent. We published a feasibility and safety study in horse and ovine model, with intra-articular infusion of 40×10^6 autologous expanded bone marrow MSC (BM-MSC) with no local or systemic pathologic alterations seen in necropsy after 6 months, and showing clear regenerative findings [22].

These encouraging results in animal model allowed us to attempt translating the procedure to human therapy, with added difficulties when it comes to objectively assess the effect in the whole joint surface. Whilst biopsy is possible, it bears an invasive surgical intervention and limits the analysis to a restricted area of the cartilage.

These limitations of the available treatment tools triggered the search of specific biomarkers for assessing changes in articular

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cartilage. The FDA (Federal Drug Administration) together with the OARSI (Osteoarthritis Research Society International) established the OARSI-FDA Biomarkers Working Group. They published a comprehensive report encompassing the role of biomarkers in the context of clinical trials that charts the course for future research of new therapies and disease modifying drugs for OA [23,24]. Research focuses in the structural components of extracellular matrix, especially type II collagen degradation markers [25].

The most validated MRI image biomarker is T2 mapping. It correlates with the collagen structure and the water interaction with the extracellular matrix. It has been suggested that 60% of T2 variation is due to collagen fibres orientation and organization and the rest could be secondary to water content and other macromolecules [26,27]. Although T2 mapping relation to collagen structure is well known, recent studies suggest that it is also sensible to PG content, since the GAG negative charges affect the water protons interaction [28,29].

Small changes in collagen hydration and organization lead to cartilage degradation and OA early findings may be evident through T2 mapping before any standard procedure such as radiological tests [30-32].

Both animal studies and human clinical trials report increased T2 values in OA patients, thus validating T2 values as hyaline cartilage structure marker for reparative procedures.

Quantitative MRI has meant an important step in OA research and became an essential tool in epidemiologic investigation and development of therapies attempting to modify cartilage structure, allowing us to noninvasively assess cartilage and rendering consistent outcome measures, preventing the need to perform an arthroscopy or a biopsy [33-35].

Previous studies show that patients with OA have a mean cartilage volume decrease of 4% - 6% per year [33]. Studies from the OAI (Osteoarthritis Initiative) stated that T2 values increased at 3 year follow-up in patients with OA risk factors and established OA [36]. Our findings suggest that cell therapy may revert this tendency.

Clinical application of MSC in humans is limited. The European Union (EU) Regulation on advanced therapies considered expanded MSC a medicinal product [37-39]. Therefore, prior to clinical application it is mandatory to conduct a clinical trial in order to prove the potential benefits and the absence of side effects of the investigational new drug.

We reported satisfactory clinical results in a preliminary study applying expanded MSC in degenerative disc disease, with no adverse side effects [40]. Afterwards we published the outcomes of a pilot study for knee OA treated with autologous expanded MSC (EudraCT 2009-017405-11 and NCT01183728). Twelve patients were treated by means of intra-articular infusion of 40×10^6 autologous expanded MSC. Statistically significant changes were observed in cartilage quality, assessed by means of MRI T2 mapping. We also reported excellent results according to pain (VAS) algofunctional and disability tests (Lequesne and WOMAC). No adverse side effects were described [22,41]. Similar results were obtained in a multicentre clinical trial applying allogenic MSC with HA as control [42].

The European Regulation (EC)No1394/2007 and (EC)No 668/2009, as well as the Spanish Regulation RD 477/2014 which set the regulatory framework for advanced therapy, allows the research team, once proven the viability, security and efficacy, to carry on with the treatment under the supervision of the Spanish Medicines Agency (AEMPS).

We present the results at 12 months of the first 50 patients treated

after the previous clinical trial, following the same procedure described in the pilot study [22].

Materials and Methods

The therapeutic procedure started in Centro Médico Teknon in Barcelona, where under sedation and local anaesthetics 100 mL of bone marrow were collected from the iliac crest. The product obtained was shipped to "Instituto de Biología y Genética Molecular" (IBGM), University of Valladolid, for selection and culture under GMP regulation (The distance between Barcelona and Valladolid is 800 Km).

The number of mononuclear cells obtained was $1,13 \pm 0,21 \times 10^9$; expansion time 22 ± 1 days; number of MSCs $40 \pm 1 \times 10^6$ suspended in Ringer-lactate and Albumina at 5×10^6 cells/mL; cells viability $91 \pm 6\%$. Higher cell densities resulted in decreased viability.

After 7 to 10 days in culture, cells became relatively homogeneous and demonstrated a fibroblastic appearance when approaching confluence. In this gap cells went out from G0 status, and came to replication period. This morphology remained unchanged until use [22].

The product is presented in 8 mL suspension and transported by plane to Barcelona, where it is applied by intra-articular infusion in the operating theatre. The time interval between cell release and infusion is less than six hours. The average cell viability in a Neubauer camera measured after application was consistently above 85%.

Clinical evaluation was assessed through physical examination and the validated VAS, Lequesne and WOMAC indices, performed prior to the treatment and at 6 and 12 months.

Cartilage was assessed by T2 mapping average values (ms). 88 regions of interest (ROIs) were well defined, including patellar cartilage (24 ROIs), femoral condyles (32 ROIs) and tibial plateaus (32 ROIs).

T2 relaxation times were averaged for each area, those greater than 50 ms were considered to calculate the Poor Cartilage Index (PCI), expressed as the percentage of all values greater than 50 ms obtained from the 88 ROIs. Values above 99 ms were dismissed for the statistical analysis.

Image

T2 measurements are not absolute and values are MR System dependent, thus the characteristics of the device should be specified when analysing the results. Interpretation of T2 values is challenging due to the range of acquisition parameters and analysis methods used. It is thus important to understand the variables, including MR system components that may influence T2 values.

This technique requires a multichannel coil with a min. 1.5 T scan able to perform a T2 mapping. Sequences for global cartilage assessment and qualitative measures of the cartilage morphology have been validated both *in vitro* and *in vivo* and are available for clinical use through 1,5T and 3T scanners [32,43-48].

For this study, images were acquired on 1.5T MR systems (General Electric) using a 4 channel knee coil with 40 mT/m gradients. We followed the standard knee protocol including sagittal T1, axial T2, sagittal eco gradient and coronal T2 density sequences. We added a sagittal multi-slice, multi-echo spin echo (MSME-SE) acquisition for T2 relaxation time measurement in the axial plane for the study of the femoro-patellar joint and in the sagittal plane for the study of the femoro-tibial joint.

Acquisition parameters of the T2 mapping sequence in the axial plane are: TR 1000, TE:13.1, 26.2, 39.3, 52.4, 65.5, 78.6, 91.7, 105; FOV: 180 × 180 mm; Frequency 288; Phase 288; bandwidth 15.63. 10 minutes acquisition time. MSME-SE acquisition used a 180 mm FOV, 3 mm slice thickness, with in-plane spatial resolution 0.31 mm × 0.45 mm, TR 1375; TE:10.8, 21.6, 32.4, 43.2, 54, 64.8, 75.7, 86.5; Frequency 288; Phase 288; bandwidth 20.83 and was prescribed sagittal to the joint. Acquisition time for this sequence was 13 minutes.

We used specific image processing software to calculate T2 relaxation time in milliseconds (ms) for each cartilage area.

Six regions (MP, LP, MT, LT, cMF, cLF) in each knee were defined by manual cartilage segmentation from the T2 map and intercept images. We defined 88 regions of interest (ROIs) encompassing patella (24 ROI), femoral condyles (32 ROI) and tibial condyles (32 ROI).

After defining the regions, T2 relaxation profiles were generated by projecting the values on a line perpendicular to the subchondral bone. An average T2 relaxation profile for each ROI was created. This value is compared to the same point at 12 months follow-up. Cartilage segmentation as well as T2 value was performed by one person blinded to subject identification.

Reproducibility

Intra-reader reproducibility for T2 measurements of each compartment were determined in baseline T2 maps of 25 randomly selected subjects. Each subject underwent two MR exams. On one day, a test- retest examination was performed. Reproducibility errors for each compartment were calculated as the root mean square error coefficient of variation.

Intra-reader reproducibility for T2 variance was 2.97%. Highest reproducibility errors were observed in the patella, lowest reproducibility errors in the medial femur compartment.

Statistical analysis

The statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL, USA) using a two-sided 0.05 level of significance.

Bonferroni test for paired values and ANOVA were used to compare VAS, Lequesne and WOMAC scores between subjects.

Cartilage quality was assessed by MRI T2 mapping and is quantified as Mean ms (95% IC). Bonferroni test for paired values and ANOVA was used to compare mean ms at baseline and at 12 months and the Poor Cartilage Index (PCI) (computed as the percentage of sample points with a T2 relaxation value >50 ms). The worse possible value for PCI is 100, and healthy cartilage should approach 5.

Paired t-tests were used to determine differences between baseline and 12 month follow-up T2 measurements for each subject. Multivariate linear regression models were used to compare changes in T2 measurements over 12 months.

Results

This study included 50 patients treated between May 2011 and November 2013, 30 male and 20 female (mean age 57,8 ± 14,1 years) who were diagnosed with Kellgren and Lawrence grades II to IV knee osteoarthritis by two independent observers. All selected patients had been unresponsive to conservative treatment (physical and medical) for at least 6 months. No serious adverse events occurred. Transient mild local pain and discomfort in the injected knee during the first 1 to 6 days occurred frequently (50% of patients) and was managed with ibuprofen.

Evolution of pain and disability

Table 1 summarizes the distribution of knee pain and disability indexes throughout the observation period. The starting point was quite homogeneous in the cohort, with mean values of 57 for the Visual Analogue Scale (VAS) and 10,9 for the Lequesne Index. The Lequesne score is one of the most used outcome measures for knee OA. It was created to assess pain and function in patients with knee and hip OA, in order to aid the orthopaedic surgeon when indicating prosthesis [49]. It comprises 10 questions addressing pain, stiffness and function. Scores above 10 support prosthesis indication. We used the Lequesne score before treatment and after cell infusion (6 months, 12 months). At 12 months the score decreased far below prosthesis indication, because bad and very bad values decreased to moderate values (Figure 1).

The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) is a measurement system for knee OA assessing

Test	time	n	Mean	SE
Knee pain VAS -DA(0-100)	0	50	57	22
	12 months	50	23	21
Knee pain VAS- Sp (0-100)	0	32	74	17
	12 months	32	28	22
WOMAC (0-100)				
Pain subscale	0	50	6	3
	12 months	50	3	2.5
Rigidity subscale	0	50	2.2	1.3.
	12 months	50	0.9	1.1
Function loss subscale	0	50	18	8.8
	12 months	50	10	7.8
Total WOMAC scale	0	50	27.15	11.9
	12 months	50	15.14	10.8
Lequesne (0-100)	0	50	10.9	4.9
	12 months	50	5.2	4.1

In all cases, the scale was from 0 to 100%. Measurements were performed before cell transplantation (0) and 12 months afterwards.; VAS-DA, Visual Analogue Scale for pain associated to daily activities; VAS-SP, Visual Analogue Scale for pain associated to sports activities; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

Table 1: Total score sum of VAS, WOMAC, and Lequesne severity indices.

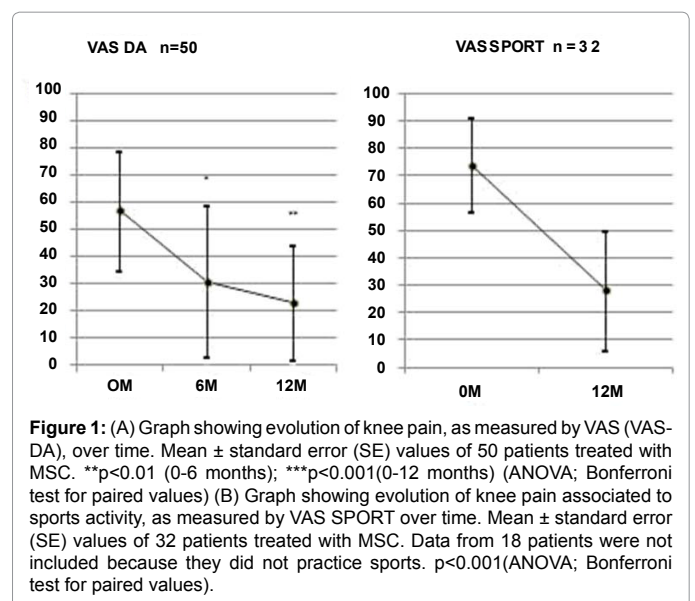


Figure 1: (A) Graph showing evolution of knee pain, as measured by VAS (VAS-DA), over time. Mean ± standard error (SE) values of 50 patients treated with MSC. **p<0.01 (0-6 months); ***p<0.001(0-12 months) (ANOVA; Bonferroni test for paired values) (B) Graph showing evolution of knee pain associated to sports activity, as measured by VAS SPORT over time. Mean ± standard error (SE) values of 32 patients treated with MSC. Data from 18 patients were not included because they did not practice sports. p<0.001(ANOVA; Bonferroni test for paired values).

pain (5 questions, 0-20 points), stiffness (2 questions, 0-8 points) and joint functionality (17 questions, 0-68 points) [50]. The initial mean value was 27.5, with pain dominating over rigidity and function loss. Compared with the basal pain level, improvement was statistically significant at 12 months (Table 1).

The pattern of 1-year improvement was parallel for VAS, WOMAC, and Lequesne indices and resulted in the displacement of the whole distribution toward smaller values, with a strong decrease of median values. Pain relief during sports performance, followed systematically in 32 patients, was even greater (74 to 28 - 63%) (Table 1).

All patients were satisfied with the treatment, and 43 out of 50 (86%) patients reported lasting pain relief greater than 45% throughout 1-year observation period. The median pain reduction was 60% for daily activities and 63% for sport activities. Figure 1 shows knee pain relief at the 1-year follow up assessed by VAS, as a function of the initial pain score. A good positive correlation was observed between the amount of improvement and the initial score ($r=0.55$), indicating that MSC treatment had a clear pain-relieving effect ($P<0.001$). The slope of the line was 0.52 (Figures 2 and 3).

Cartilage MRI

Magnetic resonance imaging (MRI) quantitative T2 mapping was used to assess cartilage quality. T2 relaxation time is sensitive to both changes in cartilage hydration and collagen fibril orientation [51,52].

T2 relaxation time is longer in inflammatory tissue versus hyaline cartilage [53] and increases in osteoarthritis [51]. Consistent with previous results in healthy knee [51,52], the mean TSD T2 value was 37.0 ± 6.8 ms. Because 95% of values should be smaller than (mean \pm SD), 50 was chosen as the threshold above which T2 values were considered inordinately high. To quantify T2 mapping, a Poor Cartilage Index (PCI) was estimated as the percentage of T2 values larger than 50 ms. A PCI of 100 is the worst possible value, and a value near 5 is considered healthy. The mean PCI significantly decreased from 25

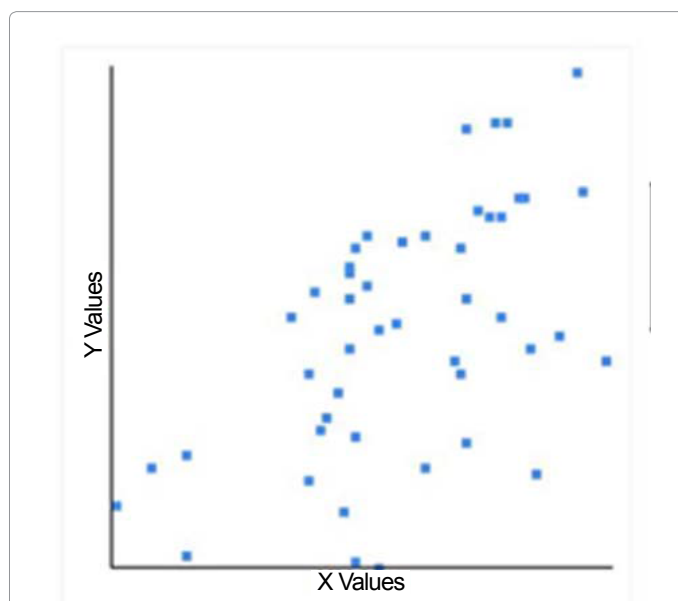


Figure 2: Correlation between improvement of knee pain 1 year after treatment with MSC and initial pain score as measured with VAS-DA for the 50 patients included in this study. The best-fitting line is shown with values for the slope and linear regression coefficient (r) at the right.

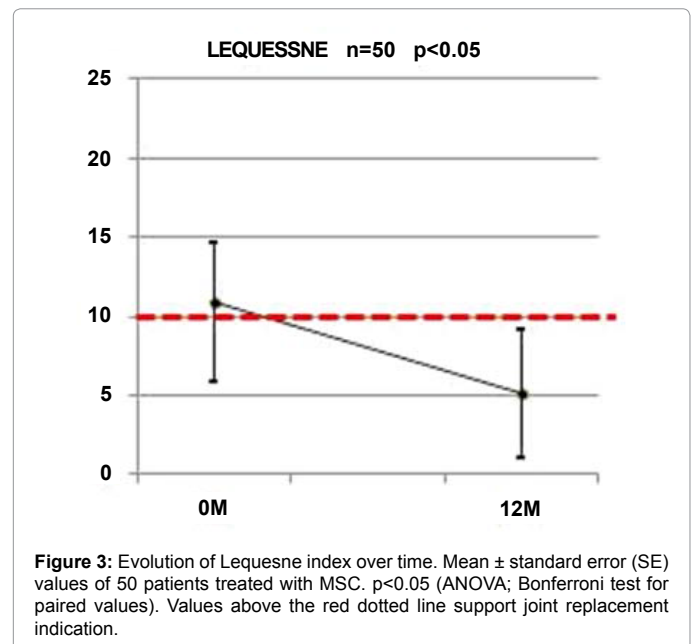


Figure 3: Evolution of Lequesne index over time. Mean \pm standard error (SE) values of 50 patients treated with MSC. $p<0.05$ (ANOVA; Bonferroni test for paired values). Values above the red dotted line support joint replacement indication.

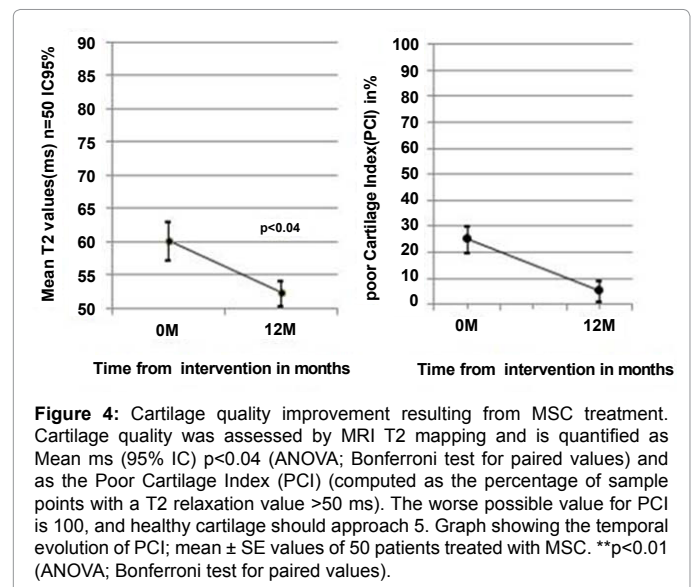


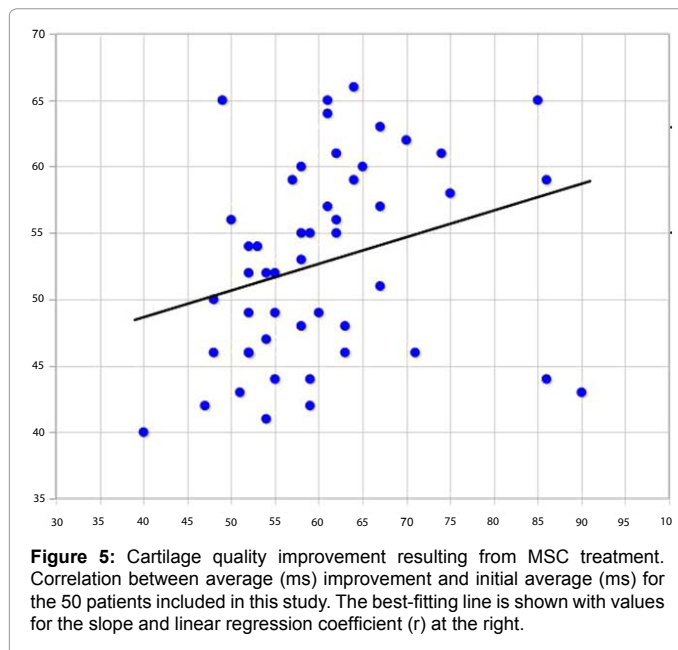
Figure 4: Cartilage quality improvement resulting from MSC treatment. Cartilage quality was assessed by MRI T2 mapping and is quantified as Mean ms (95% IC) $p<0.04$ (ANOVA; Bonferroni test for paired values) and as the Poor Cartilage Index (PCI) (computed as the percentage of sample points with a T2 relaxation value >50 ms). The worse possible value for PCI is 100, and healthy cartilage should approach 5. Graph showing the temporal evolution of PCI; mean \pm SE values of 50 patients treated with MSC. $**p<0.01$ (ANOVA; Bonferroni test for paired values).

to 5 at 12 months after injection (Figure 4). The PCI decreased in 37 of 50 patients (74%), 10 remained the same (20%) and 3 worsened between 7%-10% (6%). Additionally, when PCI improvement was plotted against the initial PCI, a positive correlation ($r=0.38$; $P=0.044$) was noted (Figure 5).

Discussion

We believe that the outcomes obtained support the research path and suggest expanded MSC at a given dose may play an important role in the therapeutic approach of OA.

Cell expansion is a key factor. Cell quality and dose are critical for the healing potential of cellular therapy. The average MSC content of healthy young human BM is about 100 MSC/million MNC, yielding a few thousands (1500-3000) MSC/ml of bone marrow. Furthermore, research on stem-cell transplantation suggests that the results largely



depend on synchronizing donor cells into the G0/G1 phase, and the fraction of mononuclear cells (MNC) in G0 phase is very low (1:10.000 to 1:1.000.000). Cell culture not only induces cell cycle synchronization at G0/G1 stage of mesenchymal stem cells but also facilitates reaching a yield of several millions MSC in a few weeks [54].

Other authors have published studies applying cultured MSC, but using different doses and cell sources.

Wong et al. treated randomly 56 patients with unicompartimental OA knees and genu varum: the cell-recipient group received intra-articular injection of cultured MSC (from bone marrow) with hyaluronic acid 3 weeks after surgery, whereas the control group received hyaluronic acid alone. They described clinical and MRI improvement in the cell-recipient group [55].

Jo et al. treated 18 patients with intra-articular injection of autologous adipose tissue derived MSC for knee OA. They divided patients in 3 groups, administrating 10×10^6 , 50×10^6 and 100×10^6 MSC. The outcomes showed intra-articular knee injection of 100×10^6 autologous MSC improved function and pain as well as cartilage defects by regeneration of hyaline-like articular cartilage [56].

Future studies will have to determine the most accurate dose to achieve the best outcomes. Regarding the cell source we know that, at least *in vitro*, adipose tissue MSC behaviour is different from Bone Marrow MSC, which generate cartilage lineage cells when cultured in TGF- β enriched medium. This is to be taken into account when attempting to regenerate joint cartilage.

Nevertheless, methods involving cell culture under GMP may be considered safe and show clear regenerative findings [57].

There has been concern about MSC inducing neoplasm formation, although MSC lack tumoral epigenome, so they cannot lead to neoplasm formation or transformation of adjacent cells. These cells produced tumors when injected into immunodeficient mice. This situation has never been reported in any cell therapy study: neoplastic transformation of MSC has the same prevalence as the rest of human cells [58].

It seems appropriate to comment that cell products obtained after aspiration and simple centrifugation, which contain a low and undetermined amount of MSC in G0 phase, are being applied as “stem cells” [59-63]. Selection and culture imply supporting MSC with nutrients in a controlled medium. This situation is quite different from the administration of a low and indeterminate number of MSC mixed with other cells which will be disputing for oxygen and nutrients. Moreover, cell products obtained after direct centrifugation contain maximum 5% of MSC, but we shall not forget the unknown effects of the remaining 95%.

Our team, together with other authors, consider important to adapt the nomenclature to the product features of the so called “one-step procedures” [62]. The product obtained from simple aspiration and centrifugation, without culture or cell characterization should not be considered MSC and should not be compared to procedures such as the treatment we herein present.

The availability of non-invasive diagnostic techniques as T2 mapping MRI allows us to accurately determine the grade of disorganization of the extracellular matrix. There is evidence that cell therapy may lead to cartilage reorganization, until recently thought impossible.

Cartilage T2 relaxation time measurements may be a sensitive biomarker for monitoring cartilage quality in subjects with knee OA, since they offer interesting insights in the condition of cartilage matrix beyond morphologically detectable focal knee lesions.

When assessing the outcomes, it becomes important to differentiate weight-bearing cartilage areas. Knee articular cartilage T2 values can vary with plate and coil, with the lateral femoral condyle having the longest value (52 ms), and the lateral tibial plateau having the shortest value (40.6 ms) [64].

In order to accurately evaluate cartilage, it would be necessary to perform a zonal assessment. Normal hyaline cartilage shows an increase of T2 values from deep to superficial cartilage. Deeper zones with dense collagen mesh and greater PG content have shorter T2 values than the transitional zone with greater water content and thus greater T2 values [18].

We assess T2 values for the whole cartilage, assuming a significant difference is enough to establish the tendency towards cartilage organization or degeneration.

The main goal of this therapeutic approach is the osteoarthritic cartilage, which is a diffuse deterioration of different joint areas, not a focal injury. We are approaching the joint as a complex organ where other components (as the synovial membrane) play an important role in normal function. MSC’s anti-inflammatory effect is thus a key factor [65].

Injuries in other knee structures such as the meniscus might be treated with the same procedure. Vangsness et al. published a randomized, double-blind, controlled study showing meniscus regeneration following partial meniscectomy in 55 patients after intra-articular knee injection of 50×10^6 and 150×10^6 MSC compared with control (intra-articular injection of hyaluronic acid). They concluded that patients treated with MSC achieved a significant reduction in pain and partial meniscus regeneration. Our team conducted a study in sheep model achieving complete meniscus regeneration (meniscal free margin injury) [22].

New studies assessing different cell doses and carriers to enhance cell viability and efficacy are indeed necessary, but in the meantime,

and focusing on the outcomes obtained so far, we believe the term “regeneration” should be accepted since the internal structure of cartilage is being modified.

Conclusions

The intra-articular infusion of a dose of 40×10^6 expanded BM- MSC, suspended in 8 mL solution of Ringer-lactate and Albumina at 5×10^6 cells/mL, has no local or systemic adverse effects, may significantly improve symptoms derived from joint inflammation in a short period of time and improve OA cartilage organization assessed by means of T2 mapping MRI.

Future studies are necessary to assess this improvement over time and to optimize results by modifying variables such as dose or medium composition.

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