Original Article

A Novel, Minimally-Invasive Technique of Cartilage Repair in the Human Knee Using Arthroscopic Microfracture and Injections of Mesenchymal Stem Cells and Hyaluronic Acid—A Prospective Comparative Study on Safety and Short-Term Efficacy

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Abstract

<u>Introduction</u>: Most current cell-based cartilage repair techniques require some form of scaffolds and 2 separate surgical procedures. We propose a novel, scaffold-less technique of cartilage repair in the human knee that combines arthroscopic microfracture and outpatient intra-articular injections of autologous bone marrow-derived mesenchymal stem cells (MSCs) and hyaluronic acid (HA). <u>Materials and Methods</u>: Seventy matched (age, sex, lesion size) knees with symptomatic cartilage defects underwent cartilage repair with the proposed technique (n = 35) or an open technique (n = 35) in which the MSCs were implanted beneath a sutured periosteal patch over the defect. Prospective evaluation of both groups were performed using the International Cartilage Repair Society (ICRS) Cartilage Injury Evaluation Package, which included questions from the Short-Form (SF-36) Health Survey, International Knee Documentation Committee (IKDC) subjective knee evaluation form, Lysholm knee scale, and Tegner activity level scale. Postoperative magnetic resonance imaging (MRI) evaluation was also performed at 1 year for most patients. <u>Results</u>: There were no clinically significant adverse events reported through the course of our study. At the final follow-up (mean = 24.5 months), there was significant improvement in mean IKDC, Lysholm, SF-36 physical component score and visual analogue pain scores in both treatment groups. <u>Conclusion</u>: In the short term, the results of this novel technique are comparable to the open procedure with the added advantages of being minimally invasive and requiring only a single operation under general anaesthesia. Its safety has been validated and its efficacy is currently being evaluated in an ongoing randomised controlled trial.

Ann Acad Med Singapore 2012;41:511-7

Key words: Chondral, Novel, Osteoarthritis, Regeneration

Introduction

Articular cartilage lesions can give rise to potentially crippling symptoms such as pain, swelling and decreased mobility. In addition, such lesions when left untreated can lead to osteoarthritis.[1] Hence, the ultimate goal of treatment is restoration of normal knee function via regeneration of hyaline cartilage in the defect.

To date, numerous procedures have been described and used in the treatment of cartilage injuries. Microfracture[2-4] is a popular first line treatment in cartilage injuries because it is minimally invasive and relatively simple to perform. The main drawback is that the repair tissue is largely fibrocartilage[5] rather than hyaline cartilage, and results are poorer for older patients.[6,7] Some authors have also

reported good early results but deterioration in function over time.[8] We believe the problem is insufficient numbers of mesenchymal stem cells (MSCs) are liberated from the subchondral marrow by this technique to ensure a durable repair.

Other alternatives to the treatment of full thickness cartilage defects have since been described. These include resurfacing techniques with perichondrium, periosteum, osteochondral bone plugs/allografts and cell-based therapies. In the 1990s, cell-based therapy approaches such as autologous chondrocyte implantation (ACI) emerged as potential therapeutic options in the treatment of focal cartilage lesions/injury. Following animal models on the healing of partial thickness chondral defects which had

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not penetrated the subchondral bone plate, the clinical use of ACI was first developed and then described. However, the procedure is not without its limitations which includes the sacrifice of undamaged cartilage within the same joint and the lack of availability of cell numbers (due to degenerative change in cartilage with aging) especially in elderly individuals. In addition, hyaline cartilage is not always found in the repair tissue after ACI.

Over the last few years, MSC therapies for regeneration of cartilage have gained popularity due to various reasons. The ability for MSCs to differentiate into connective tissue including hyaline cartilage plus their easy availability from various tissues such as bone marrow, adipose tissue and trabeculae bone etc. make them easy targets with regards to harvesting of cells. The isolation of MSCs can also be carried out without unnecessary damage to healthy cartilage, a well-known concern in ACIs. In contrast to articular chondrocytes, the expansion of MSCs seemed to confer no higher risk for replicative aging or unlimited growth of MSCs. The above reasons make MSC-based therapy an attractive option in regenerative tissue repair. In principle, the ultimate goal is to induce and expand multi-potent MSCs at the site of interest down a signal pathway into an end-stage phenotype. The MSCs can then be delivered into the knee joint via 2 different approaches. The first is to implant cells directly or via a suitable matrix or scaffold seeded with chondro-progenitor cells and signaling substances and allow the differentiation process to occur in-vivo. The alternative is to differentiate stem cells in vitro and proceed with the implantation of a mature construct.

The ability of stem cells to differentiate and adhere to scaffolds such as matrices of hyaluronan derivatives and gelatin-based resorbable sponge matrices have been investigated and proven. Most studies assume that scaffolds are required for the regeneration of cartilage and these require an invasive arthrotomy. It is assumed that load-bearing and fluids movement would simply prevent cells from thriving where they are needed. However, studies have shown that MSCs can survive in and thrive without scaffold, and injected stem cells have been recovered in viable form in a goat knee with simulated arthritis. An elegant paper from Stanford University using MSCs suggested that are 2 temporarily distinct injury-related signals that first induce MSCs to home in onto the site of injury and then a second local signal induces differentiation of MSCs into the relevant cell type to facilitate repair of the injured tissue.

In a pre-clinical study of ours, Lee et al[9] showed that intra-articular injection of MSCs and hyaluronic acid (HA) was found to be effective in the repair of full-thickness porcine femoral condyle cartilage defects. Moreover, in-vivo tracing of labeled cells confirmed the presence of injected MSCs in the neocartilage.

The purpose of this study was to investigate the safety and clinical results of a novel, minimally invasive technique that combined arthroscopic microfracture with outpatient injections of bone-marrow derived MSCs and HA. Our hypothesis is that the injected MSCs localise and home into the defects and the HA provides a chondroprotective articular environment to facilitate cartilage repair.

Materials and Methods

Preoperative Protocol

This prospective, non-randomised observational cohort study was designed to compare the effectiveness of our proposed technique of cartilage repair (which combined arthroscopic microfracture and intra-articular injections of MSCs and HA) with an open technique in which the MSCs were implanted beneath a sutured periosteal patch over the defect. The inclusion criteria were, at least, one symptomatic full-thickness chondral lesion diagnosed by clinical evaluation and magnetic resonance imaging (MRI) with cartilage sequencing on the femoral condyle, trochlea, or patella and non-existent or correctable concomitant pathologies. Patients with inflammatory arthritis, tri-compartmental osteoarthritis, limited range of motion, in particular fixed flexion deformities and those who were 55 years of age or older were excluded from the study.

Seventy matched (lesion site, age and gender) knees with symptomatic cartilage defects underwent cartilage repair with the proposed technique or the open technique. Institutional review board approval was obtained and all patients gave informed consent. The patients were evaluated prospectively by our physiotherapist collaborators using the International Cartilage Repair Society (ICRS) Cartilage Injury Evaluation Package, which included questions from the Short-Form (SF-36) Health Survey, International Knee Documentation Committee (IKDC) subjective knee evaluation form and Lysholm knee scale. The patients were followed up for a mean of 24.5 months.

Operative Protocol (Study Group)

The surgeries were performed by our senior authors. All subjects in the study group underwent an arthroscopic assessment, debridement and microfracture for their cartilage injury. The procedure[10] involved accurate debridement of all unstable and damaged cartilage in the lesion, including the calcified layer down to subchondral bone plate. All loose or marginally attached cartilage were also debrided from the surrounding rim of the defect to form a stable perpendicular edge of healthy cartilage. An arthroscopic awl was then used to make multiple holes in the defect, 3 mm to 4 mm apart. During the same procedure, the subjects had their MSCs harvested from the iliac crest bone marrow

using disposable Jamshidi bone marrow aspiration needles (11 Gauge 10 cm). This is a safe and quick procedure and added little to the operating time. Also, 60 mL to 80 mL of venous blood sample from the upper limb of the patients were drawn in the same sitting.

Cell culture and expansion was performed in our Health Science Authority (HSA)-approved cGMP tissue engineering laboratory. The heparinised aspirated bone marrow was mixed with a one-fifth volume of 6% (w/v) dextran (molecular weight 100,000; Sigma, St Louis, Missouri, US) and left standing at room temperature for 30 minutes to eliminate erythrocytes. The remaining cells were washed twice with DMEM (Gibco BRL, Grand Island, NY, US). These cells were cultured in T75 cm^2 flasks with initial culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, US) 50 µg/mL L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, St Louis, Missouri, US) and 1% antibiotics-antimycotic (penicillin 100 U/mL, streptomycin 0.1 mg/mL, amphotericin B 0.25 µg/mL) (Sigma, St Louis, Missouri, US) in a humidified atmosphere of 5% CO_2, 37°C. Cells were seeded at a cell density of 10,000 cells per square centimetre. Initial medium changes were done after 5 days, when adherent cells were recognised. Subsequently, culture media without antibiotics were used and changed twice to thrice a week.

Flow cytometry against CD90$^+$, CD105$^+$, CD14$^-$ and CD34$^-$ was used to confirm that cultured cells were mesenchymal stem cells. Aspirated bone marrow and culture media (without antibiotics) were tested for sterility and Mycoplasma hominis contamination. In addition, filtered patients' serum was tested for sterility, anti-HIV and Hepatitis B antigen.

Approximately 3 weeks after bone marrow aspiration, there are approximately 10 million passage 1 cells ready for injection. The cells were transported in 2 mL of their own serum. The patients then received intra-articular injections containing the MSCs followed by 2 mL of HA (Synvisc® -hylan G-F 20) in the outpatient clinic. Two more intra-articular injections of HA alone were then given to the patients in the subsequent 2 weeks at weekly intervals. This is the standard dosing interval for visco-supplementation using HA in the clinical setting.

Operative Protocol (Control Group)

In the control group (open technique), patients underwent bone marrow aspiration under local anaesthesia about 3 to 4 weeks before the actual surgical procedure. Similar to our injectable group, the cells were expanded in culture as described above. Cell sheets (passage 1) were then formed

in the presence of ascorbic acid and for each surgery, at least 4 cell sheets were prepared and around 2 million cells/cm^2 were applied. Similar to the injectable group, flow cytometry was used to confirm that cultured cells were mesenchymal stem cells. The aspirated bone marrow and culture media (without antibiotics) were tested for sterility and Mycoplasma hominis contamination. The filtered patients' serum was also tested for sterility, anti-HIV and Hepatitis B antigen.

The cell sheets were transported to the operating theatre in a sterile container within the patients' own serum. The debrided chondral defect (without damaging subchondral bone) was measured after an arthrotomy. Subsequently, periosteal patch harvesting from the proximal part of the tibia or distal part of femur was done according to the measured size. The cultured cell sheets were implanted onto the defect beneath the patch and very fine stitches (micro suture 7-0) were used to hold the periosteum to the defected site. To avoid cell leakage, fibrin glue was used to create a watertight seal.

Postoperative Rehabilitation

The importance of the postoperative rehabilitation protocol was emphasised to the patients. The rehabilitation protocol was tailored to individual patients and varied according to the location and size of the lesion. For example, patients with patella and trochlea lesions had their knee flexion limited in the first few weeks while patients with condyle lesions were not allowed weight-bearing for the first 6 weeks. There were 4 areas that rehabilitation focused on: walking/weight bearing, range of motion, strength, and cardiovascular capacity.

Postoperative Evaluation

The patients were assessed independently by our physiotherapist collaborators at regular postoperative intervals at 3, 6, 9, 12 and 24 months using the ICRS Cartilage Injury Evaluation Package. As far as possible, a follow up MRI scan was done at 12 months. The mean scores were taken at regular intervals, calculated and tabulated.

Statistical Analysis

Using STATA Version 10, a mixed model analysis was performed by our biostatistician collaborator. This method of analysis appropriately accounts for the possible correlation between repeated measurements of an individual. All statistical evaluations were made, based on an assumption of a 2-sided test at the conventional 5% level of significance.

Results

The results of our technique (injectable group) was compared to a matched control group of 35 knees that were treated with an open technique (open group) using MSC cell sheets. The groups were matched according to age, gender and site of lesions. The mean age in both groups was 44 years. There were 16 males and 19 females in the injectable group and 20 males and 15 females in the open group. In both groups, there were 16 with femoral condyle, 10 with patellofemoral and 9 with multiple lesions. There were 6 patients in the injectable group and 5 patients in the open group who had concomitant high tibial osteotomy to correct coronal alignment. There were also 5 patients in the open group who had concomitant patella realignment and none in the injectable group. The mean follow-up was 24.5 months.

In general, both groups showed significant improvements in the SF-36 Health Survey, IKDC subjective knee evaluation form and the Lysholm knee scale, with a positive time effect demonstrated. As shown in Figures 1 and 2, the injectable group performed better than the open group in terms of the IKDC Sum Score and the Lysholm Score with $P < 0.001$ in both. There was also improvement shown in the Visual Analogue Scale (VAS) Pain Score and the SF-36 (PCS) in both groups over time but the difference between the 2 groups were not statistically significant ($P = 0.230$ and $P = 0.057$ respectively) (Figs. 3 and 4).
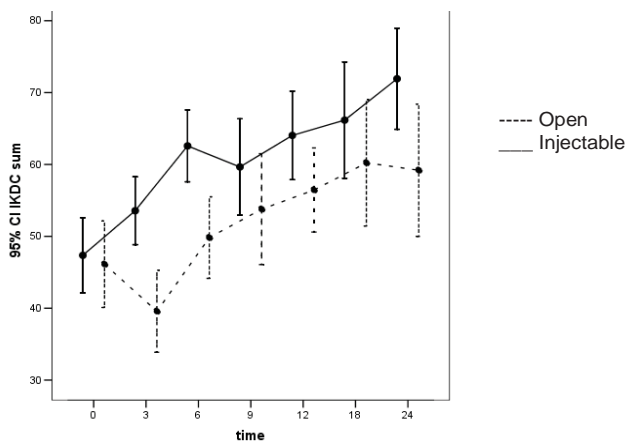
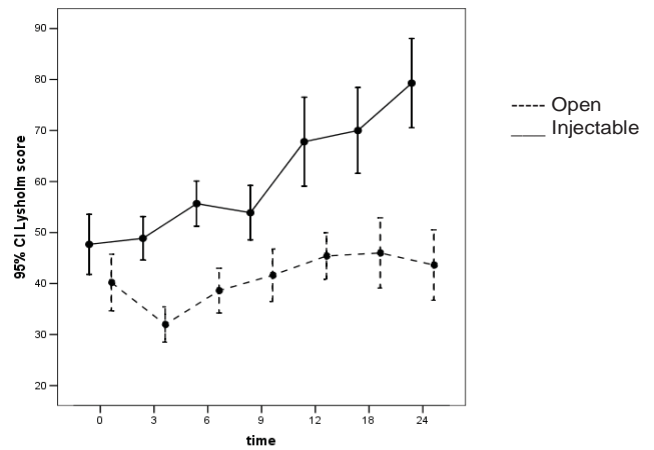In our subgroup analyses, we found that males generally

Fig. 2. A graph showing the Lysholm score: injectable vs open technique.

Fig. 3. A graph showing the visual analogue scale (VAS): injectable vs open technique.

Fig. 1. A graph showing the International Knee Documentation Committee (IKDC) sum score: injectable vs open technique.

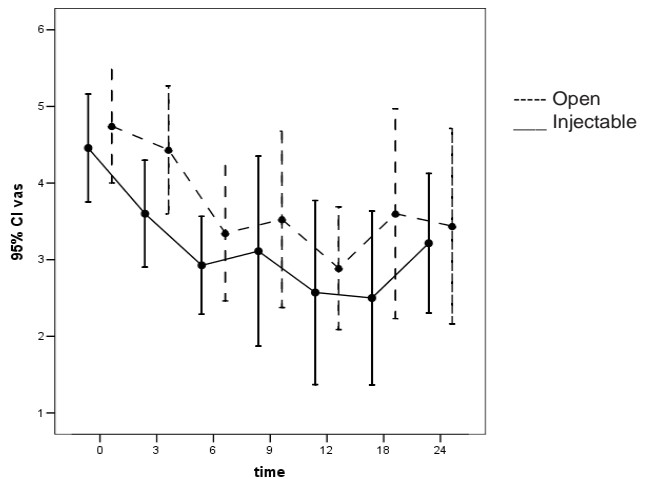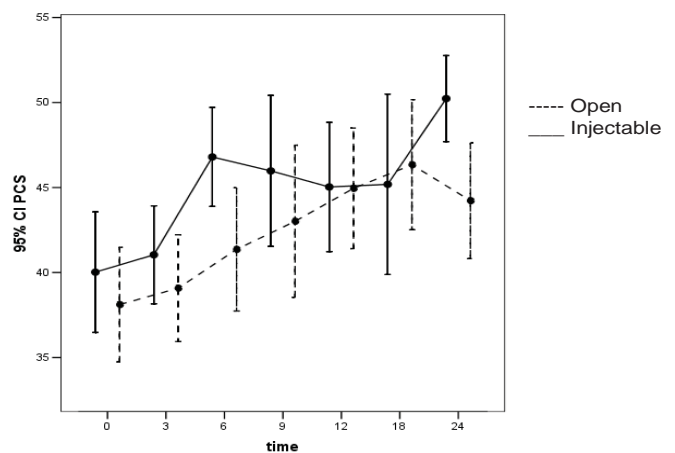Fig. 4. A graph showing SF-36 (PCS): injectable vs open technique.

tend to perform better than females in all 4 scores (IKDC Sum, Lysholm, VAS and SF-36 PCS). However, this difference was only statistically significant (*P* <0.05) in the open technique group in terms of the IKDC Sum, Lysholm and VAS scores. The difference was not statistically significant in the injectable group. There was also no difference in outcomes between different sites of lesions (isolated femoral condyle, patellofemoral or multiple). There were no reports of clinically significant complications such as infections, knee swelling/effusion, allergy or other known adverse events related to the harvesting or arthroscopic procedures.

MRI

An MRI at 1 year post operation was done for all the injectable group patients. As far as possible, an MRI was also done at 1 year post operation for the open group. The MRI findings were encouraging and as illustrated in the examples shown (Figs. 5 and 6), neo-cartilage with good fill and integration were demonstrated in the injectable group of patients. There was also significant reduction in the underlying marrow oedema. However, we still prefer to correlate these positive findings with the clinical outcomes as it has been shown that the sensitivity of MRI for detecting and analysing chondral lesions is only 45%.[11]

Discussion

Cell-based therapy in the form of ACI was first described in 1994.[12] Several studies following this[8,13,14] have also suggested that ACI is an effective procedure for treating cartilage defects in the knee. The disadvantages of this technique include requiring 2 separate surgical procedures, difficulty in obtaining an adequate number of chondrocytes, a slow rate of chondrocyte proliferation and donor site morbidity. In addition, it also requires a formal arthrotomy, even in most cases of second or third generation techniques. In addition, some authors have shown that the clinical results of ACI at 2 and 5 years were no different from that of the relatively simpler microfracture technique.[5,15]

The use of mesenchymal stem cells as an alternative to chondrocytes for cartilage repair in humans has gradually gained some momentum in recent years.[16-20] In a study, Wakitani et al[17] compared 2 groups of patients who had undergone high tibial osteotomy (HTO). The first group received implantation of collagen gel scaffold embedded with bone marrow-derived stem cells while the second group received cell free scaffolds implantation. The author managed to show better arthroscopic and histologic scores in the MSC group.

However, as far as we know, the results of using MSCs have not been compared with other cell sources. In our own recently published study,[21] we compared the clinical outcomes of patients treated with first generation ACI to patients treated with bone marrow-derived MSCs. The latter have been shown to have a better proliferation rate than chondrocytes and have the capacity to differentiate to different tissues, including both bone and cartilage, under the right conditions.[22-24] Our results showed that patients treated by ACI and MSCs had a comparable improvement in quality of life, health, and return to sporting activities. Older patients (above 45 years) treated with MSCs also had
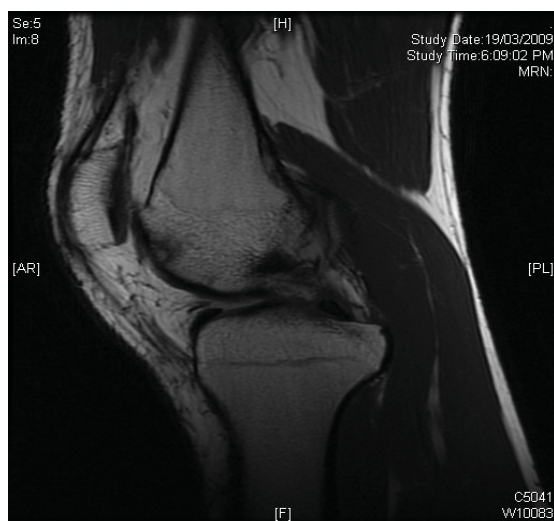

Fig. 5. Preoperation MRI (SAG FSE PD) showing a large full thickness chondral ulcer with discontinuity of the subchondral bone and significant underlying marrow oedema.
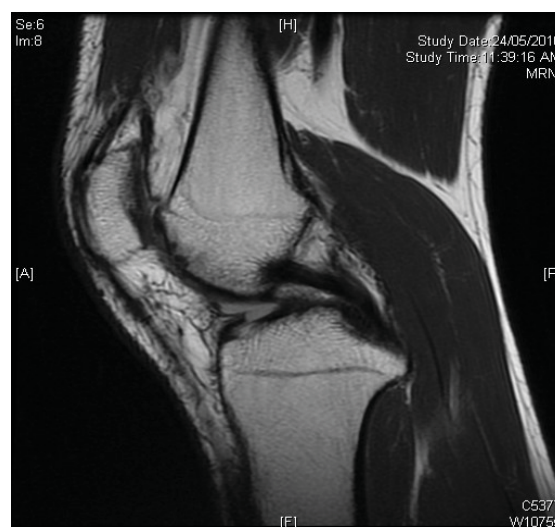

Fig. 6. One year postoperation MRI (SAG FSE PD) showing neo-cartilage formation with good fill and significant reduction in the underlying marrow oedema.

better results compared to those treated by ACI.

The objective of this study was to evaluate a novel, scaffold-less technique of cartilage repair in the human knee that combines arthroscopic microfracture and outpatient injections of autologous bone marrow-derived MSCs and HA. Our reasons for using MSCs over chondrocytes include the potential to treat both chondral and osteochondral injuries, and the potential for better results in older patients. Most studies presume that the use of a scaffold for MSCs to adhere upon before delivery to the site of injury is necessary. Our main hypothesis is that injected MSCs can not only survive in the intra-articular environment, but also home in to the site of injury, adhere to it and repair cartilage.

A study by LaBarge et al[25] suggested that there are 2 temporally distinct injury-related signals that first induce MSCs to home in onto the site of injury and then a second local signal induces differentiation of MSCs into the relevant cell type to facilitate repair of the injured tissue. Injected cells have also been recovered in viable form in a goat knee with simulated arthritis.[26] The homing ability of injected MSCs has been further demonstrated by our own[9] and other pre-clinical studies. In our porcine model, in-vivo tracing of green fluorescent protein (GFP) labeled MSCs showed that these cells localised and formed neo-cartilage at the site of a surgically created full-thickness chondral defect. In a rat model study, Agung et al[27] injected GFP-labeled MSCs into the knees and found that the injected cells mobilised to the sites of surgically created injuries, i.e. the anterior cruciate ligament, the cartilage of the femoral condyles and the medial meniscus. In another rat model, Nishimori et al[28] reported that intra-articular injections of GFP + MSCs along with a bone marrow stimulation procedure was more effective for repairing a chronic osteochondral lesion than bone marrow-stimulating procedure alone. Significantly, GFP cells were present in the specimens up to 4 weeks after treatment and they were localised to the site of osteochondral defect indicating that the injected MSCs "home in" onto the site of injury. They went on to hypothesise that growth factors were induced from the bone marrow and might be attributed to the injected bone marrow stem cells (BMSCs) adhering to the defect, preventing them from escaping and lastly aid in the differentiation to chondrocytes. Currently, we are investigating molecular homing factors and also tracking of injected BMSCs labeled with superparamagnetic iron oxide (SPIO) particles by MRI. This can provide a novel avenue of achieving direct real-time evidences of the temporal spatial localisation of the applied cells in relation to the state of repair of the injured tissues.

HA has both chondroprotective and chondroinductive properties making it a suitable medium for stem cell delivery into the joint. Hyaluronan-based polymers have been shown to enhance the natural healing process of osteochondral defects in animals.[20] These hyaluronan-based materials possess a unique biochemical composition that recreates an embryonic-like environment, which as hypothesised, may be favourable for the regenerative process. In addition, a rabbit study[29] also showed that the rate of synovial cell migration was enhanced with HA alone and that HA increased chondrocyte migration in the presence of basic fibroblast growth factor. The above evidence serves to strengthen our postulation that the anti-inflammatory properties of HA provides a conducive environment for the injected MSCs to migrate, proliferate and differentiate at the site of injury.

The strengths of this prospective study are: (i) selection of patients according to established inclusion and exclusion criteria, (ii) using validated knee cartilage outcomes instruments, (iii) using matched data to decrease the confounding effect of site and age, (iv) using the same outcome evaluation scales from baseline and different time points, and (v) using a trained independent observer for data collection.

The limitations of this study are that possible biases might be introduced since the patients were not randomised and there were variations in patient characteristics between the 2 groups. However, the patients were matched to minimise the variation in lesion location, age and gender among the cohorts and therefore, limit the effect of these important factors on the outcomes. And because of our small numbers, meaningful subgroup analyses were also limited. In our study, we found that there was no difference in outcome between different sites of lesions and we suspect this is due to the small numbers of our subjects. Another limitation in this study is the fact that any improvement in patients' outcome can be due to bone marrow-derived MSCs or MSCs from the subchondral bone. We do realise that a control group with microfracture (MF) will be helpful in addressing this doubt and we have intended to address this issue in our phase 3 trial by including a control group that will receive just MF alone. Practically, however, we realise the difficulty in "double-blinding" the control group and injectable group if we were to have a control group of patients just receiving MF alone. It will be very obvious to the patients from the onset as to which treatment group they belong to. Nonetheless, in our phase 3 trials, we will be attempting to prove the efficacy of our proposed cell based therapy by having a control group of patients receiving MF and intra-articular visco-supplement injection while the treatment group will receive MF and intra-articular injection of visco-supplement in combination with MSCs. Any significance difference between the 2 groups can then be attributed to the effect of MSCs since it is the only variable in the study. Last but not least, we recognise that objective data such as gross morphological findings via a second look arthroscopy and biopsy for histology were not included. This is a limitation that we find very hard to

overcome as it is impossible to convince patients who are asymptomatic to undergo a surgical procedure for research purposes.

Conclusion

In the short term, the results of this novel technique is comparable to the open procedure with the added advantages of being minimally invasive and requiring only a single operation under one general anaesthesia. Its safety has been validated and its efficacy is currently being evaluated in an ongoing randomised controlled trial.

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