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Intraarticular injection of allogenic chondroprogenitors for treatment of osteoarthritis in rabbit knee model

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1. Introduction

Osteoarthritis (OA) is the most common disabling degenerative joint disease in humans, characterized by breakdown of cartilage and compensatory overgrowth of bone.[1] The prevalent strategies for management of OA include non-operative measures such as life style modification, use of anti-inflammatory drugs, intra-articular injection of lubricants as well as interventional therapies including lavage, debridement, marrow stimulation techniques, transplantation of grafts among others.[2] The varied results with these modalities have prompted the search for other treatment options.

Articular hyaline cartilage is a specialized tissue which comprises of mesenchymal stem cells with chondrogenic potential namely chondroprogenitors (CPs) and chondrocytes within dense extracellular matrix.[3] Cell-based transplantation using chondrocytes and mesenchymal stem cells (MSCs) have been examined for cartilage regeneration.[4,5] However, prospective studies have shown variable results with outcomes including donor site morbidity, post implantation hypertrophic marker expression and formation of fibrocartilage resulting in transplant failure.[6–9] Identification of an appropriate cell population that possesses a stable chondrogenic phenotype and induces hyaline cartilage formation during repair with limited hypertrophy tendency remains a challenge.

Articular cartilage derived CPs have gained popularity in the treatment of osteoarthritis due to their inherent properties and their primed chondrogenic potential. The properties of these cells include differential adhesion to fibronectin with high colony forming efficiency, differential integrin and Notch family expression and the ability to form large number of colonies from an initial small seeding density.[10] This subpopulation of viable cells has been classified as mesenchymal stem cells as it is adherent to plastic, expresses mesenchymal cell surface markers, does not express hematopoietic markers and can undergo tri-lineage differentiation.[11,12] A recent comparative study between equine BM-MSCs and CPs showed that the latter have superior capabilities for cartilage repair as they lacked the expression of hypertrophic markers (Runx-2 and type X collagen).[8,13] Use of cells from the same tissue will also be logical, as they may possess similar characteristics as that of the target tissue. Intra-articular injections of hyaluronic acid

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has been a well-known and accepted treatment option for osteoarthritis.[14] Its chondroinductive and chondroprotective properties would serve as an effective vehicle in the delivery of chondroprogenitors.

Our aim in this study was to assess the efficacy of allogenic injectable chondroprogenitors (CPs) in sodium hyaluronate (HA) in the healing of experimentally created osteoarthritis in rabbit knees.

2. Materials and methods

The study was approved by the Institutional Review Board (IRB) and the Institutional Animal Ethics Committee (IAEC). The procedures were in accordance with the institutional guidelines for care and use of laboratory animals. A total of 13 adult male New Zealand White rabbits with an average age of 7 months (±1month) and weight of 2.19 ± 0.32 kg were used for the study. CP isolation was done from the superficial layer of both knee joints from one rabbit and cultured to create an allogenic cell bank. CPs were subjected to flow cytometry, in vitro differentiation and live dead assay in HA.

The remaining twelve rabbits underwent bilateral knee injection of monosodium iodoacetate (MIA) at a concentration of 4 mg in 250 μl of sterile water for injection to create OA. After a period of 28 days, the rabbits were anaesthetized using intramuscular (IM) injection of 50 mg/kg ketamine and 4 mg/kg of 2% xylazine, arthrotomy was performed and synovial fluid was aspirated to assess the expression of S100A12 protein, a specific biomarker of osteoarthritis. Concurrently the cultured CPs at a concentration of 1 million cells per joint were combined with 250 μl HA and injected into the right knee joints. The corresponding left knee joints were injected with 250 μl of plain HA which served as the control. Six rabbits each were euthanized at the end of 12 weeks and 24 weeks for synovial fluid analysis of S100A12 and the knee joints were harvested for histopathological analysis using Osteoarthritis Research Society International (OARSI) score[15] and Picro-sirius red staining.

2.1. Isolation of rabbit articular derived chondroprogenitor cells, fibronectin adhesion assay and culture

Six well plates (35 mm) were coated with 10 μl/ml bovine fibronectin (FN, Sigma) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ prepared in 0.1 M phosphate buffered saline (pH 7.4) and incubated at 4 °C overnight. The surface zone cartilage shavings obtained were further minced and subjected to overnight sequential digestion with 0.2% pronase (Roche) and 0.04% collagenase II (Worthington). After digestion, the cells were strained through a 40 μm cell strainer (Falcon) and suspended in DMEM-F12 Glutamax (Himedia, India). The cells were added to the precoated fibronectin plates at a concentration of 2000 cells per well and incubated at 37 °C for 20 min. The plates were rinsed to remove the media and non-adherent cells, and replaced with standard growth media, namely DMEM-F12 Glutamax with 10% fetal bovine serum (Invitrogen) plus ascorbic acid 62 μg/ml, L-glutamine 2.5 mM/L, penicillin-streptomycin 100IU/ml and amphotericin-B 2 μg/ml. The medium was replaced every 3 day and cultured cells were maintained in a 5% CO_2 incubator.

2.2. Colony isolation and population doubling

After six days in culture, colonies containing a cluster of 32 cells or more (≈5 population doublings of a single cell) were marked and selected for further cultures (Fig. 1. The marked colonies were bridged using sterile vaseline and isolated by adding 0.05% Trypsin (Himedia) with 0.5 mm EDTA for a period of 3–5 min. The trypsinized colonies were pipetted out and transferred to T-25/T-75 flasks (Tarsons) and cultured up to confluence. The cells obtained from the second passage were harvested and resuspended in 250 μl of HA for intra-articular injections. The Population Doubling (PD) was calculated using the formulae:

$$PD = \log (N) - \log (N_0) / 0.301$$

Where N_0 was the initial number of cells seeded, which was day 1 and N was the number of cells obtained at the end of the passage.

2.3. In vitro adipogenesis and osteogenesis

Passage 2 CPs were subjected to either one of the following differentiation conditions containing Stem Pro adipogenic differentiation medium or Stem Pro osteogenic differentiation medium (Thermo Fischer Scientific). In brief 25,000 cells/cm^2 were cultured to sub-confluence under standard culture conditions and was replaced with adipogenic/osteogenic differentiation medium. The medium was changed every third day for a period of 3 weeks. On day 21, the cells were fixed, both the control and cells maintained in adipogenic medium were stained for Oil Red O and cells in osteogenic medium for Alizarin Red (Fig. 2).

2.4. Flow cytometry

One million passage 2 CPs were washed with phosphate buffered saline (PBS) and incubated for 20 min at room temperature with the following conjugated antibodies: MSC markers CD81-APC and CD 90-FITC, chondroprogenitor specific marker CD49e-PE,[16,17] hematopoietic stem cell markers CD45-FITC and CD34-APC and immune response marker namely HLA-DR-FITC. The labeled cells were subjected to single channel flow cytometric analysis using BD-Celesta and BD-Caliber flow cytometer (Fig. 3). Appropriate IgG controls were run for each antibody.

2.5. HA- CP live dead viability assay

The viability of the CPs in HA was examined qualitatively by a live/dead assay using calcein AM/Ethidium (ThermoFischer Scientific) homodimer after 24 h of CP-HA co-culture under standard culture conditions. The constructs were gently washed thrice in PBS and incubated with 2 μm calcein-AM for 30 min and 4 μm ethidium homodimer for 5 min and observed under an immunofluorescence microscope (Leica) (Fig. 4).

2.6. S100A12 protein analysis

After confirmation of anaesthesia, under sterile precautions the joints were exposed through a midline incision and a medial parapatellar arthrotomy. The synovium was incised, and the synovial fluid was pipetted into cryovials. The level of synovial fluid specific biomarker S100A12 was measured using commercially available rabbit protein S100A12 ELISA kit according to the manufacturers protocol (MyBioSource, Cusabio). Absorbance was detected using a microplate (Bio-Rad) reader at 450 nm, with wavelength correction. Using a standard curve, the readings from the ELISA reader were interpreted and the concentration of S100A12 in each sample was calculated. The samples were run in duplicates and the dilution factor was included (Fig. 5).

2.7. Histological assessment

Six rabbits each at the end of 12 and 24 weeks were euthanized. Synovial fluid for S100A12 analysis from both knees and femoral

condyles with articular cartilage from both knees were harvested and fixed using 10% neutral buffered formalin. The fixed joints were decalcified with 14% EDTA (ethylenediaminetetraacetic) acid solution and paraffin embedded. 5 μm sections in the transverse plane were prepared and stained with Hematoxylin & Eosin, Masson Trichrome and Safranin O. Osteoarthritis severity assessment was done using the OARSI assessment system.[15] The system employs analysis of a standard section assessment by grade and stage of arthritis with subsequent calculation of an arthritis score. The grading methodology includes the following key features which are; grade 0 = surface intact, cartilage morphology intact; grade 1 = surface intact; grade 2 = surface discontinuity; grade 3 = vertical fissures (clefts); grade 4 = erosion; grade 5 = denudation and grade 6 = deformation. Staging considers the percentage involvement of surface, area and volume (0 = no OA activity seen, 1 = <10%, 2 = 10–25%, 3 = 25–50%, 4 = >50%). Higher arthritic score indicated higher the severity of OA. Three blinded investigators performed the histological grading on all the cartilage sections and the mean ± SEM was considered (Figs. 6 and 7).

2.8. Picro-sirius red staining

For specific staining of the extracellular components, the cartilage sections were hydrated and stained with Picro-sirius red followed by Hematoxylin counterstain. A representative area from a uniformly stained section was selected and the intensity of stain uptake in arbitrary units was measured using Image J software.[18] Quantitative analysis of the stain uptake was compared between the corresponding joints at 12 and 24 weeks, the fold change of the test in comparison to the control joints were measured and expressed in mean ± SEM (Figs. 8 and 9).

2.9. Statistical analysis

Analysis and the statistical comparison of the S100A12 protein expression levels at 12 and 24 weeks between the control and test arms were perfomed with Wilcoxon Signed rank test and the histological OARSI score and picro-sirius red staining were performed with non-parametric Mann-Whitney *U* test. Statistical Analysis were done using SPSS 15.0 and a P value < 0.05 was considered significant. All data are expressed as mean and standard error mean.

3. Results

3.1. CP isolation and population doubling

The CPs were successfully isolated from the superficial layer of the articular cartilage following fibronectin adhesion assay, achieving a PD of 13.99 in 9 days (Fig. 1).

3.2. In vitro adipogenesis and osteogenesis

After 21 days of subjecting CPs to adipogenic and osteogenic differentiation using Stem Pro Adipogenesis and Osteogenesis medium, the differentiated cells were subjected to Oil Red O and Alizarin Red staining. Obvious lipid vesicle accumulation was observed in adipocytes differentiated CPs (Fig. 2-C) and positive Alizarin Red staining of the extracellular calcium deposits in mineralized osteocytes differentiated from CPs (Fig. 2-D) but not in parallel controls.

3.3. FACS analysis

Flow cytometric analysis of with the 6 antibodies showed that the clonally expanded passage 2 CPs showed positive expression of CD49e(99.2%), CD81 (90.9%), CD90 (62.6%) and negative expression of hematopoietic markers (CD45 and CD34) and immune response marker (HLA-DR) (Fig. 3).

3.4. Live dead assay

Majority of the entrapped CPs within the HA cultured for 24 h in standard conditions maintained viability (green fluorescence) (Fig. 4).

3.5. S100A12 protein analysis

For confirmation of the creation of OA, prior to intervention, the aspirated synovial fluid from 12 rabbits at 28 days (4 weeks) was estimated for S100A12 protein levels. OA was successfully created bilaterally and the mean S100A12 level in synovial fluid was 3283 ng/ml. At 12 and 24 weeks, the mean S100A12 level was less on the study side (10,822 ng/ml, 3759 ng/ml respectively) compared to the control side (58,184 ng/ml, 4552 mg/ml respectively). However, the difference in synovial fluid levels of S100A12 between the sides at 12 weeks and 24 weeks was not significant (**P = 0.117, P = 0.675**) (Fig. 5).

3.6. Histopathological analysis and OARSI mean score

Representative transverse section microphotographs of Safranin O-stained articular cartilage sections from left knee (HA) and right knee (HA + CP) of two rabbits joint are shown (Fig. 6). Both the left (A1-D1) and right knee joints (A2-D2) showed grades varying from 0 to 4 which ranged from intact uninvolved cartilage to surface irregularities with deformation of articular surface contour with bone remodeling. The OARSI mean scores at 12 and 24 weeks did not show a significant difference between the sides (**P = 0.687**, **P = 0.199**) (Fig. 7).

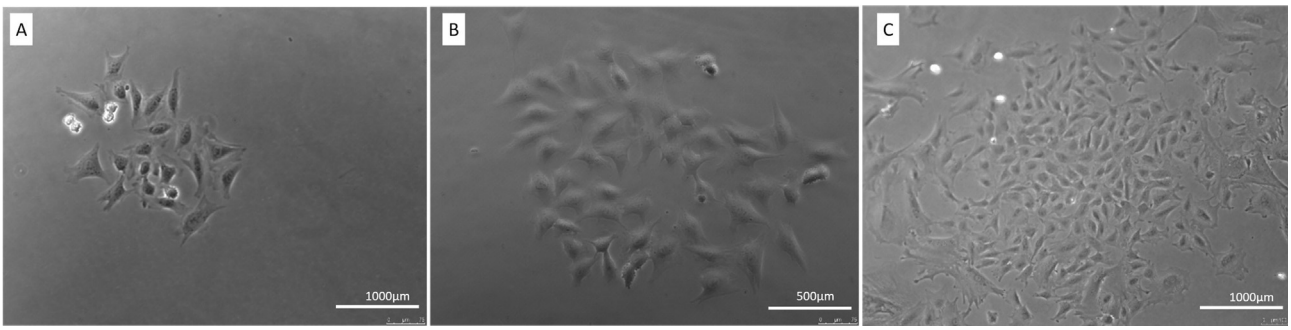

Fig. 1. Isolation of clonally derived articular chondroprogenitor cells from a single rabbit knee joint. A) A clone at day 4 forming a loose cluster of few cells (mag 10x). B) The same clone at day 6 forming a tighter cluster with more number of cells (mag 20x). C) Passage 1 day 9 cells showing a larger cluster reaching confluence (mag 10x).

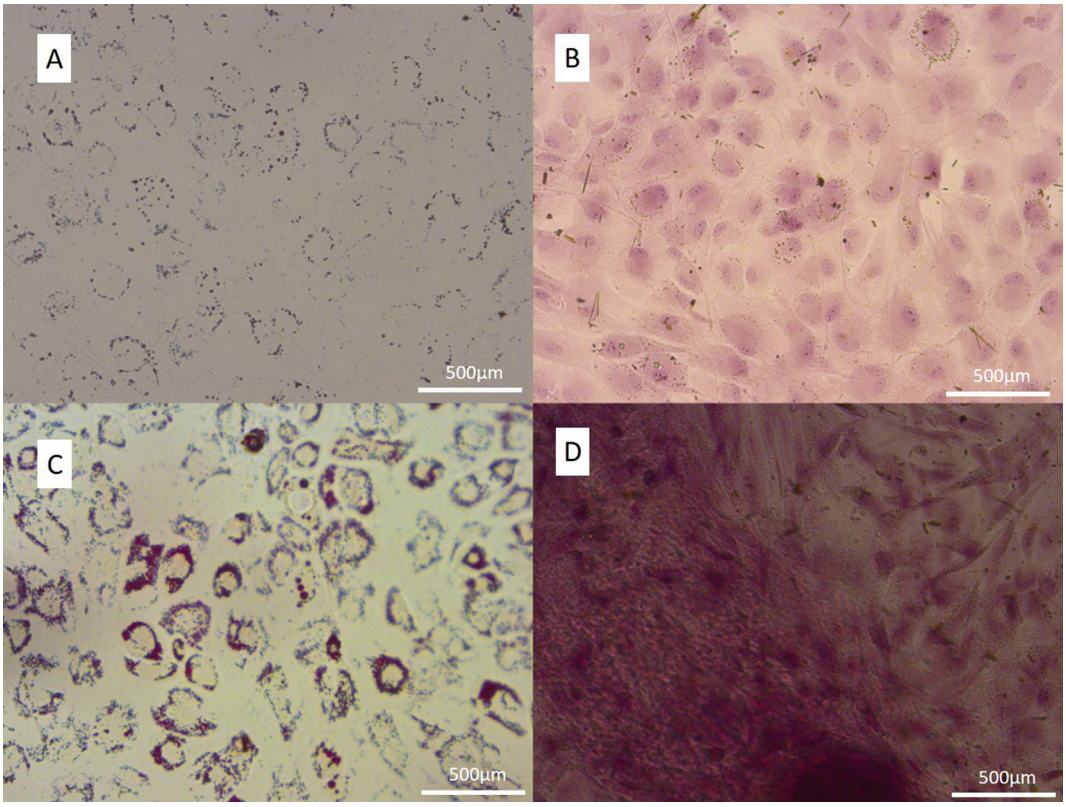

Fig. 2. Differentiation studies of Chondroprogenitors. A (control-adipogenic) and C (test-adipogenic) were with stained Oil Red O. B (control-osteogenic) and D (test-osteogenic) were stained with Alizarin Red (mag 20x).

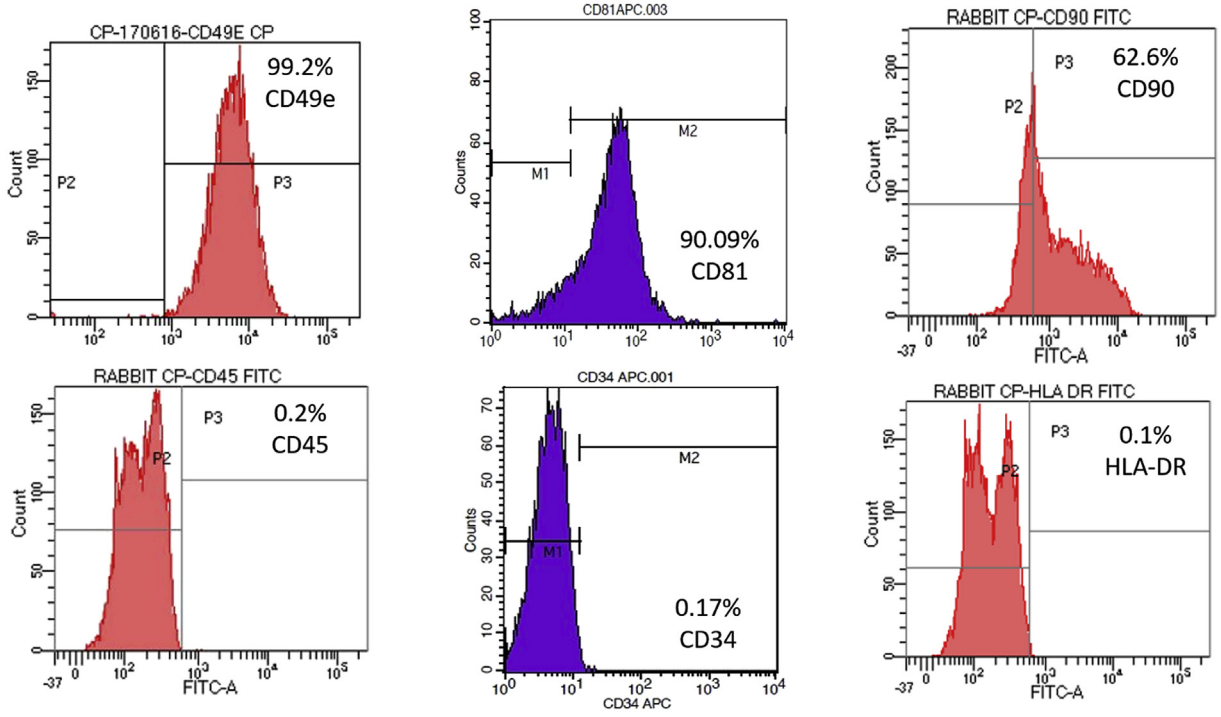

Fig. 3. Flow cytometric analysis of articular cartilage derived chondroprogenitors for stem cells markers CD 49e(A), CD81 (B), CD90(C), CD45(D), CD34(E) and HLA-DR(F).

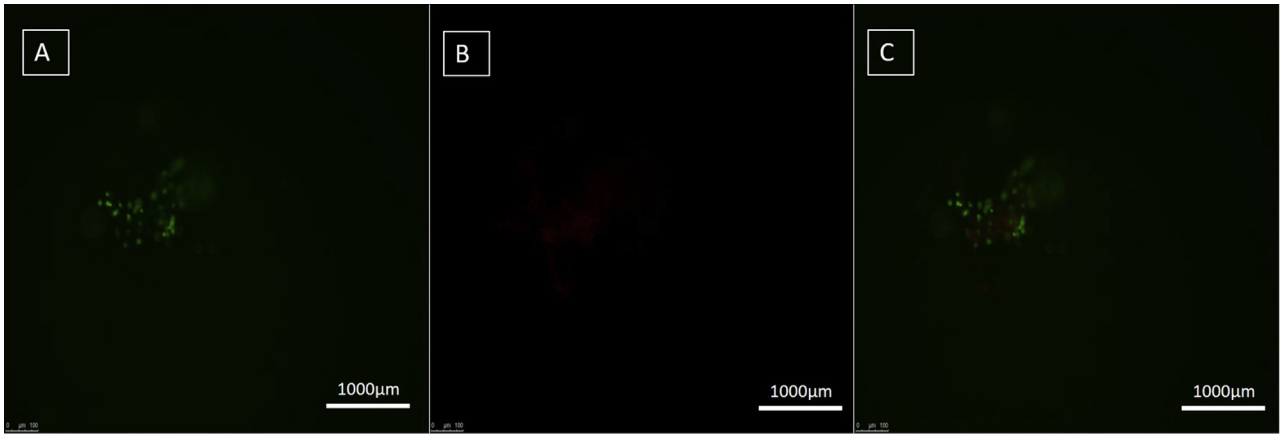

Fig. 4. Live dead assay of chondroprogenitors embedded in Hyaluronic acid scaffold 24 h in cultures. Calcein AM (live cells-green fluorescence) and Ethidium homodimer (dead cells-red fluorescence) (mag 10x).

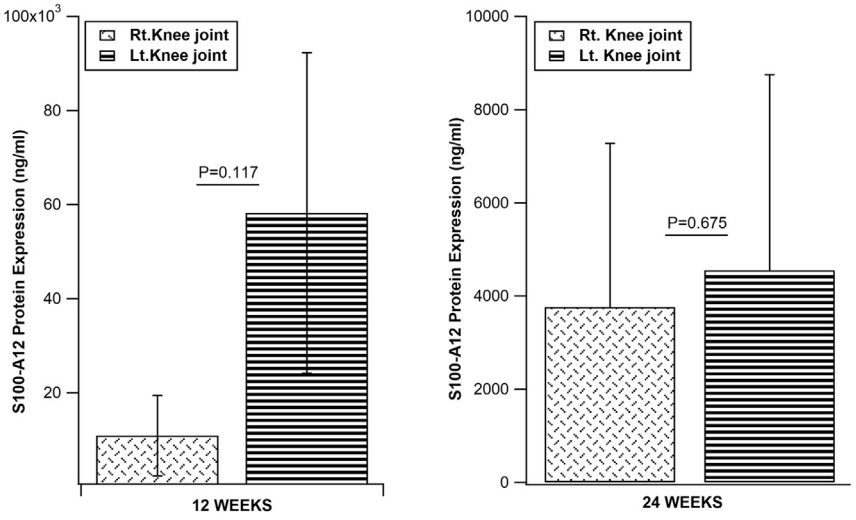

Fig. 5. Comparative synovial fluid S100A12 protein expression levels (ng/µl) between left knee joint (plain HA) and right knee joint (HA + CP). Data expressed are Mean ± SEM, n = 5. *P < 0.05.

3.7. Picro-sirius red

Representative photomicrographs of Picro-sirius red staining from the two groups between the two-time interval are shown (Fig. 8). The intensity of stained Picro-sirius red area obtained was compared between the two groups. The analysis showed that the right knees at 12 and 24 weeks had better stain uptake than the left knees (**P = 0.936, P = 0.689**). The fold change shown was consistent with the OARSI scoring at 12 weeks (Fig. 9).

4. Discussion

Ongoing clinical trials and *in vivo* animal models using bone marrow and adipose derived MSC's have shown promising results in reducing the progression of OA in different animal models.[19–24] In a study by Murphy et al., autologous MSCs have reduced the signs of OA and caused regeneration of meniscus.[21] Similarly autologous MSCs have inhibited synovial thickening and cartilage degradation.[22]

Recent studies have been exploring the use of injectable stem cells in the healing of osteoarthritis.[19,24–26] These strategies aim to restore functionality and defer joint replacement in osteoarthritis. The rationale to do this study was to find out whether allogenic CPs could heal osteoarthritis. This is the first study where articular cartilage derived chondroprogenitors have been used. This is due to their greater chondrogenic potential with positive immunomodulatory properties. The key finding of this study was that intra articular injection of allogenic CPs combined with HA seems to be beneficial however not significant.

The knees which received CP in HA had less S100A12 levels, lower grade in OARSI score and a better collagen staining on Picro-sirius red staining. However, it did not show a significant difference compared to the knees which received HA alone. The lack of significant difference may be attributed in part to suboptimal dosage of cells, higher grade of OA created, lack of multiple doses of injections (CPs in HA) to prolong the effect. Homing/engraftment of cells after injection was also not checked. Previous OA models were created by transecting the Anterior Cruciate Ligament

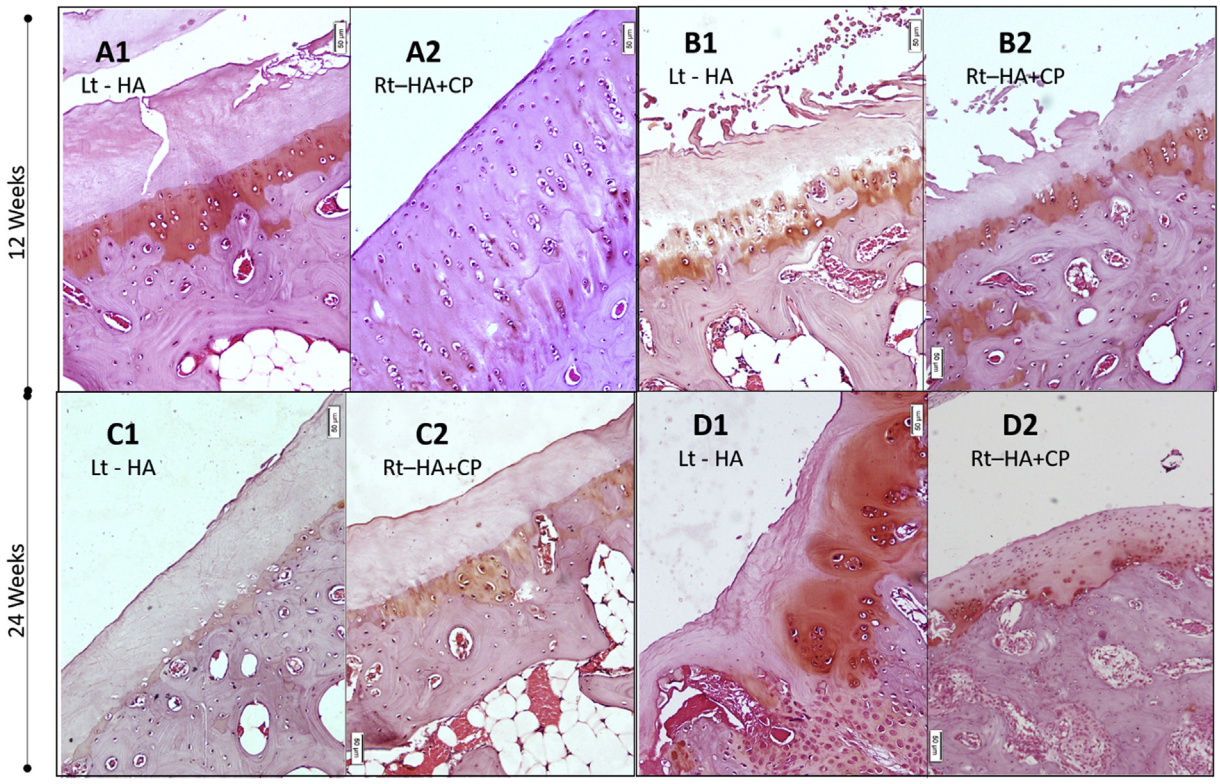

Fig. 6. Histological analysis: Representative images of Safranin O staining of articular cartilage at 12 weeks and 24 weeks (mag 20x).

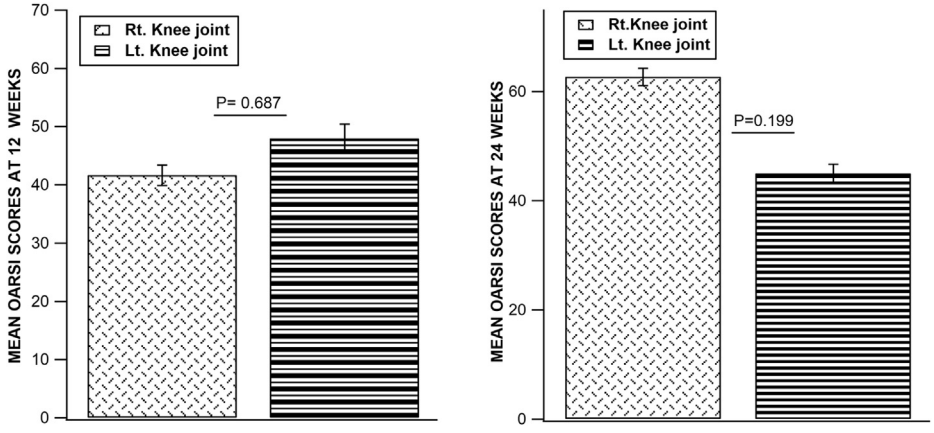

Fig. 7. Histological grading: OARSI mean scores at 12 weeks and 24 weeks between left knee joint (plain HA) and right knee joint (HA plus CP). Data expressed are Mean ± Standard deviation, n = 6.

(ACL).[5,20,21,23] We did not use this model as instability of joints due to transection of ACL may interfere with healing when injectable cells are used. Hence OA was created using MIA. Dosage of MIA should be titrated in the future to carefully monitor the grade of OA created.

Although promising short-term results were observed, we believe that matching the concentration of cells injected to the grade of osteoarthritis created could have made a significant difference in reverting the diseased cartilage towards a healthier architecture. Another concern we would have liked to address is improving the *in vivo* survival of CPs with added growth factors (since the HA was devoid of growth factors which may have been crucial for cell survival *in vivo*) and labeling the cells to identify true engraftment. The findings of this study give a preliminary insight into the therapeutic potential of these cells and more studies need to be carried out, as there is paucity of studies using CPs for treating osteoarthritis.

5. Conclusion

Treatment of osteoarthritis with allogenic CPs in HA seems to be beneficial, however it does not show a significant improvement

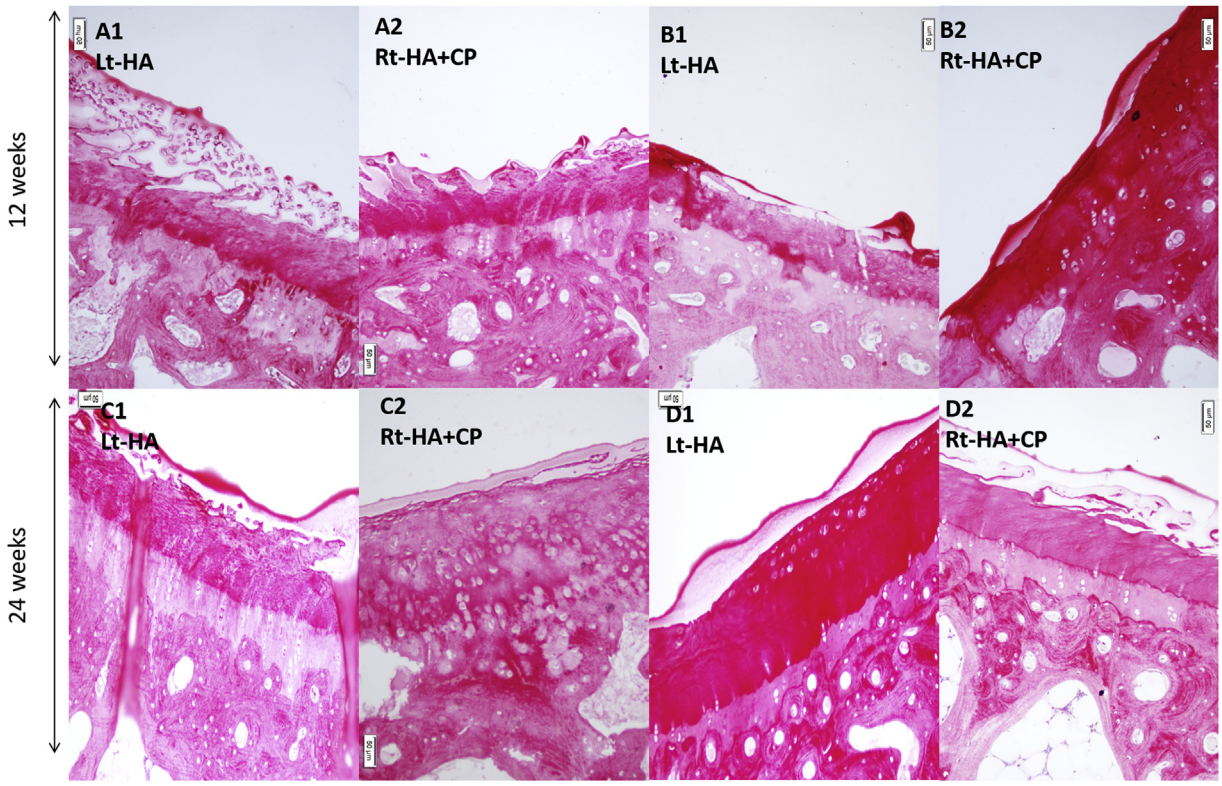

Fig. 8. Histological analysis: Representative images of PicroRed collagen staining of articular cartilage at 12 weeks and 24 weeks (mag 20x).

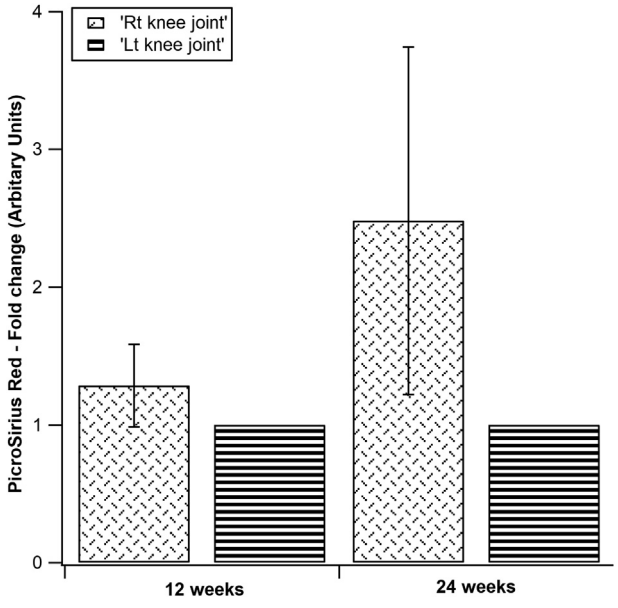

Fig. 9. Quantitative analysis of PicroRed collagen stain at 12 weeks and 24 weeks between left knee joint (plain HA) and right knee joint (HA plus CP). Data expressed are Mean ± Standard deviation.

when compared to HA alone. Further research needs to be done to study the role of CPs in treatment of different grades of osteoarthritis.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jcot.2018.07.003.

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