



■ RESEARCH

Canine articular cartilage regeneration using mesenchymal stem cells seeded on platelet rich fibrin

MACROSCOPIC AND HISTOLOGICAL ASSESSMENTS

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Objectives

Mesenchymal stem cells have the ability to differentiate into various cell types, and thus have emerged as promising alternatives to chondrocytes in cell-based cartilage repair methods. The aim of this experimental study was to investigate the effect of bone marrow derived mesenchymal stem cells combined with platelet rich fibrin on osteochondral defect repair and articular cartilage regeneration in a canine model.

Methods

Osteochondral defects were created on the medial femoral condyles of 12 adult male mixed breed dogs. They were either treated with stem cells seeded on platelet rich fibrin or left empty. Macroscopic and histological evaluation of the repair tissue was conducted after four, 16 and 24 weeks using the International Cartilage Repair Society macroscopic and the O'Driscoll histological grading systems. Results were reported as mean and standard deviation (SD) and compared at different time points between the two groups using the Mann-Whitney U test, with a value < 0.05 considered statistically significant.

Results

Higher cumulative macroscopic and histological scores were observed in stem cell treated defects throughout the study period with significant differences noted at four and 24 weeks (9.25, SD 0.5 vs 7.25, SD 0.95, and 10, SD 0.81 vs 7.5, SD 0.57; $p < 0.05$) and 16 weeks (16.5, SD 4.04 vs 11, SD 1.15; $p < 0.05$), respectively. Superior gross and histological characteristics were also observed in stem cell treated defects.

Conclusion

The use of autologous culture expanded bone marrow derived mesenchymal stem cells on platelet rich fibrin is a novel method for articular cartilage regeneration. It is postulated that platelet rich fibrin creates a suitable environment for proliferation and differentiation of stem cells by releasing endogenous growth factors resulting in creation of a hyaline-like reparative tissue.

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Keywords: Mesenchymal stem cells, Platelet rich fibrin, Articular cartilage

Article focus

- The aim of this study was to assess osteochondral defect repair and articular cartilage regeneration in a canine model.
- Autologous bone marrow derived mesenchymal stem cells were seeded onto platelet rich fibrin.
- Macroscopic and histological assessments of the repair tissue were assessed for the treated and control defects at various time points.

Key messages

- Higher cumulative macroscopic and histological scores were observed in treated defects.
- We determined the feasibility of using this novel technique in osteochondral defect repair and articular cartilage regeneration.

Strengths and limitations

- To our knowledge, this is the first study to report the combined use of stem cells and platelet rich fibrin in cartilage repair.

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- Lack of biomechanical and immunohistochemical assessments.
- Lack of comparative assessment between active treatment groups.

Introduction

Successful treatment of articular cartilage injuries remains a major challenge for orthopaedic clinicians, with untreated lesions ultimately leading to osteoarthritis and degenerative joint disease. Articular cartilage is devoid of blood vessels, nerves and lymphatics and contains a minimum number of cells in the form of chondrocytes. Due to these structural characteristics, this highly specialised form of connective tissue has a very limited capacity for self-repair following injury. Among the various techniques of articular cartilage repair, cell-based therapies have emerged as an important means of treating focal cartilage lesions. Autologous chondrocyte implantation (ACI) is a form of cell therapy which is currently considered as the mainstay for regeneration of articular cartilage. During ACI, healthy chondrocytes are first harvested from a non-weight-bearing area of the joint and then transplanted back into cartilage lesions following *in vitro* isolation and culture expansion.¹⁻⁶

Although the results of ACI treatment have been encouraging indicating the formation of hyaline like tissue,^{1,5,6} obtaining chondrocytes in sufficiently high cell densities and maintenance of their differentiation state is technically difficult.^{2,3,7} Hence, alternative cell sources are sought to replace chondrocytes. Currently, mesenchymal stem cells (MSCs) are considered as the potential replacements to chondrocytes in cell-based therapies. MSCs can easily be isolated, and culture expanded in sufficient numbers. They have also shown the ability to differentiate into various cell types, including chondrocytes, under laboratory conditions.¹⁻⁵ Studies concerning the application of MSCs in cartilage repair have mainly focused on the use of bone marrow derived MSCs (BM-MSCs) and it has been shown that chondrogenic differentiation of these cells is induced by growth factors, in particular the transforming growth factor β (TGF- β) family.⁸⁻¹⁰ Wakitani et al¹¹ were the first to demonstrate the efficacy of BM-MSCs in cartilage repair. Subsequently, numerous pre-clinical and clinical studies have documented the positive influence of BM-MSCs in articular cartilage repair and regeneration as reviewed by Bornes et al.¹² Seeding of MSCs onto various types of scaffolds and the placement of these cell-laden structures inside cartilage lesions is a method employed for the delivery and implantation of MSCs into cartilage defects. Aside from containment of implanted MSCs inside cartilage lesions, the ideal scaffold should provide the bioactive molecules necessary for promotion of cellular differentiation and maturation.¹³ Growth factors are among the bioactive molecules that can induce chondrogenic differentiation of MSCs.¹⁴

Platelet-rich fibrin (PRF) is a platelet concentrate containing a multitude of growth factors including TGF- β .^{15,16} *In vitro* studies have indicated that PRF has the ability to support the proliferation of MSCs and various other cell types.¹⁷⁻²⁰ PRF consists of a three-dimensional fibrin network favouring cytokine enmeshment and cellular migration. Its growth factors are slowly released over an extended period of time, making this biomaterial an attractive choice to be used as scaffold for the delivery of stem cells in tissue regeneration.²¹ Combination of MSCs and PRF has been used for dental, myocardial and bone regeneration and adipose tissue transplantation.²¹⁻²⁸ Although it has been shown that PRF alone positively influences cartilage repair,²⁹ the effect of MSCs combined with PRF on cartilage repair have not previously been studied.

The aim of the present study was to evaluate the effect of BM-MSCs seeded onto PRF for articular cartilage regeneration in a canine model. It was hypothesised that the combined use of MSCs and a biological source of growth factors in the form of PRF would enhance osteochondral repair and cartilage regeneration in comparison with untreated control defects.

Materials and Methods

Animals and study design. This experimental study was approved by the research council and experimentation ethics committee of our institution (research project number 2-17-5-16154 approved on 30.6.2012). All the experiments were conducted with adherence to institutional guidelines for the care and use of laboratory animals in research.

A total of 12 clinically healthy adult mixed breed male dogs with a body weight of between 18 kg and 40 kg were used in this study. They were judged to be healthy based on physical examination findings and results of laboratory tests (complete blood cell count, blood biochemistry profiles and urinalysis). The stifle joint of each animal was carefully examined to rule out any joint instability. Skeletal maturity was determined by radiography prior to the initiation of the experiment.

A total of 24 osteochondral defects were created on the medial femoral condyles of the stifle joint (two defects per dog, one on each limb). The stifle joint is the homologue for the human knee joint. In each dog, the defects were randomly treated by implantation of BM-MSCs seeded onto PRF or left empty as a control. Simple randomisation by flipping a coin was used to determine whether to treat the left (heads) or right (tails) stifle defect. The animals were killed at four, 16 and 24 weeks following creation of the defects, and the resultant repair tissue was evaluated by macroscopic and histological means.

Isolation, expansion and characterisation of BM-MSCs. Autologous BM-MSCs were isolated from the animals and culture expanded for later use according to the established

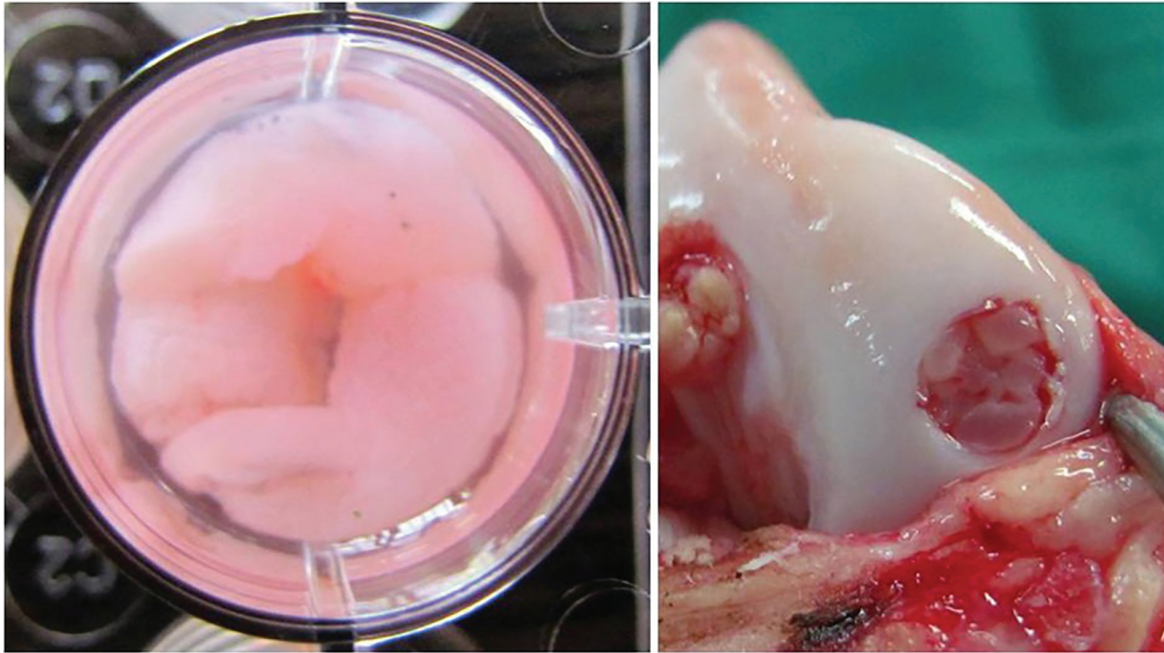


Fig. 1

Platelet-rich fibrin (PRF) seeded with stem cells. Bone marrow derived mesenchymal stem cells were seeded onto PRF in the laboratory and incubated for 6 hours (left) before being press fitted inside the cartilage defects (right).

protocols.³⁰⁻³² The animals were pre-medicated with 0.05 mg/kg of intramuscular acepromazine and anaesthesia was induced intravenously 15 to 20 minutes later with 10 mg/kg thiopental. Using a sterile aspiration needle attached to a pre-heparinised syringe, approximately 12 ml of bone marrow aspirate was collected from the proximal humerus. The aspirates were immediately transported on ice to the laboratory and processed within the next two hours. Bone marrow mononuclear cells were separated by Ficoll (Histopaque-1077 density 1.077 g/ml, Sigma-Aldrich, St Louis, Missouri) density centrifugation at 4°C with 800 g for 35 minutes. Mononuclear cells (1×10^5 /cm²) were cultured in 25 cm² culture flasks containing 5 ml of low glucose Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% foetal bovine serum, 2 mM L-Glutamine, 10000 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in the presence of CO₂. After four days, the non-adherent cells were removed by washing the culture dishes with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) and the culture medium was changed every two days. Adherent cells or BM-MSCs were cultured (passage 0) until they reached over 75% confluence. The cells were re-suspended by washing twice with DPBS and digested with 0.125% trypsin in ethylene diamine tetraacetic acid (EDTA) for two minutes. The enzyme was inactivated by 5 ml of foetal bovine serum in DMEM. The cell suspension was subsequently centrifuged at 4°C and 1000 rpm for 10 minutes and used for further passages. Passage 3 cells were seeded onto PRF and transplanted in osteochondral defects. Before transplantation they were characterised

by flow cytometry and evaluation of cell surface markers (CD34, CD44, CD45 and CD105).

Preparation of PRF. Autologous PRF was prepared based on the method previously described by Dohan et al.³³ 24 hours prior to surgery and creation of osteochondral defects, 20 ml of venous blood was withdrawn from the jugular vein of each dog and transferred into two sterile glass test tubes without any anticoagulant. The samples were immediately centrifuged at 3000 rpm (400 g) for ten minutes. The resultant PRF clot located in the middle layer of the test tube was removed and the red blood cells at the bottom and acellular plasma at the top of the tube were discarded. The PRF clots were transferred into 12 well flasks and 1 ml of DPBS was added to prevent drying and kept inside an incubator at 37°C for BM-MSC seeding later on.

Seeding of BM-MSCs onto PRF. On the day of the surgery, 1×10^6 cells of passage 3 BM-MSCs were added to PRF containing wells and incubated at 37°C for at least six hours before being transplanted into osteochondral defects during surgery (Fig. 1).

Surgical procedure and creation of cartilage defects. Food was withheld from the animals for 12 hours before the anaesthesia. Each dog was pre-medicated by intramuscular injection of xylazine (1 mg/kg) and atropine (0.04 mg/kg). Anaesthesia was induced by intravenous injection of 2.5% solution of thiopental (10 mg/kg) and maintained with Isoflurane in oxygen following endotracheal intubation. Cefazolin (20 mg/kg) was given as a pre-operative antibiotic immediately following induction and lactated ringer's solution (10 ml/kg/h) was infused during the

Table I. International cartilage repair society macroscopic evaluation of cartilage repair³⁴

Categories	Score
Degree of defect repair	
In level with surrounding cartilage	4
75% repair of defect depth	3
50% repair of defect depth	2
25% repair of defect depth	1
0% repair of defect depth	0
Integration to border zone	
Complete integration with surrounding cartilage	4
Demarcating border < 1 mm	3
¾ of graft integrated, ¼ with a notable border > 1 mm width	2
½ of graft integrated with surrounding cartilage, ½ with a notable border > 1 mm	1
From no contact to ¼ of graft integrated with surrounding cartilage	0
Macroscopic appearance	
Intact smooth surface	4
Fibrillated surface	3
Small, scattered fissures or cracks	2
Several, small or few but large fissures	1
Total degeneration of grafted area	0
Overall repair assessment	
Grade I: normal	12
Grade II: nearly normal	11-8
Grade III: abnormal	7-4
Grade IV: severely abnormal	3-1

operation. The animal was placed in dorsal recumbency and under aseptic conditions. The medial approach to the stifle joint with lateral patellar luxation was used to access inside each of the right and left joints. The joint was fully flexed to access the weight-bearing areas of the femoral condyles. Osteochondral defects with a diameter of 6 mm and depth of 5 mm were created in the weight bearing area of each medial femoral condyle using a drill equipped with a 6 mm drill bit. Bleeding was observed in all the defects confirming the involvement of subchondral bone and the full thickness nature of the injury. The defects were thoroughly lavaged with normal saline solution (Shahid Ghazi Pharmaceutical Co., Tabriz, Iran). From a total of two defects created in each dog, one was press fitted with PRF seeded with BM-MSCs (Fig. 1) and the other left empty as a control. After completion of the procedure, the patella was returned to its normal anatomic location and the joint capsule, subcutaneous tissues and skin sutured routinely to close the wound. Post-operatively, penicillin (40000 IU/kg for five days) and ketoprofen (2.2 mg/kg for three days) were administered to the dogs. The animals were allowed to walk freely without any restrictions following recovery. No bandages, splints or casts were used to immobilise the limbs and full weight bearing and exercise was allowed as tolerated by the dogs within their confinement.

Tissue sampling. The animals were killed using an overdose of thiopental injection at predetermined time intervals and the distal femurs were harvested for evaluation of the resultant repair tissue. Four dogs were randomly assigned to each of the sampling periods using QuickCalcs software (GraphPad Software Inc., La Jolla,

California), therefore the number of defects which were treated by BM-MSCs and left empty as a control was four at each time point.

Macroscopic evaluations. Immediately after death, the International Cartilage Repair Society (ICRS) evaluation score (Table I)³⁴ was used for macroscopic assessment of the repair tissue based on gross observation. Digital photographs of the defects were taken for documentation purposes. The assessment was blinded to treatment allocation.

Histological (microscopic) evaluations. Following macroscopic assessment, each femoral condyle was fixed in 10% buffered neutral formalin, decalcified and embedded in paraffin for routine histological sectioning. Sagittal sections (5 µm thick) were cut from the centre of each defect and stained with haematoxylin-eosin and safranin O and examined under a light microscope. Sections were blindly examined and scored according to the O'Driscoll histological grading scale (Table II).³⁵

Statistical analysis. Mann-Whitney U test was used to compare macroscopic and microscopic scores at different time points between the two treatment groups. A p-value < 0.05 was considered statistically significant. Graph Pad Prism 6 software package (Graph Pad Software Inc.) was used for data analysis.

Results

Characteristics of BM-MSCs. The criteria used for characterisation of BM-MSCs in this study were plastic adherence of cells which was observed during culture and expression of cell surface markers quantified by flow cytometry. The results of flow cytometric analysis indicated the positive expression of mesenchymal markers CD44 and CD105 by BM-MSCs while the non-mesenchymal markers CD34 and CD45 were negative.

Macroscopic findings. All animals survived the surgery and recovered uneventfully. Signs of post-operative synovitis, joint infection, osteophyte formation and degenerative joint disease were not observed in any of the joints during the sampling procedure. The defects were readily identifiable from the surrounding normal cartilage in both treatment groups at all sampling times.

At four weeks after surgery, the full thickness defects of the BM-MSC-treated group were filled with a brightly red coloured repair tissue with remnants of PRF observed in the central areas of the repair tissue in two defects (Fig. 2). The repair tissue which had filled the defects of the control group had a darker red colour, and in the central areas, a slight depression was also evident. At 16 weeks after surgery, the reparative tissue filling the defects in both groups had an opaque white colour resembling the normal surrounding cartilage. The repair tissue had integrated well with the native cartilage at the edges of the defects in both treatment groups. The BM-MSC-treated defects were filled with an opaque white repair tissue very similar to the native cartilage and

Table II. O'Driscoll histological cartilage repair score³⁵

Characteristics	Score
Nature of predominant tissue	
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue or bone	0
Safranin-O staining of the matrix	
Normal or nearly normal	3
Moderate	2
Slight	1
None	0
Structural characteristics	
Surface regularity	
Smooth and intact	3
Superficial horizontal lamination	2
Fissures 25% to 100% of the thickness	1
Severe disruption including fibrillation	0
Structural integrity	
Normal	2
Slight disruption including cysts	1
Severe disintegration	0
Thickness	
100% of normal adjacent cartilage	2
50% to 100% of normal cartilage	1
0% to 50% of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end or partially at both ends	1
Not bonded	0
Freedom from cellular changes of degeneration	
Hypocellularity	
Normal cellularity	3
Slight hypocellularity	2
Moderate hypocellularity	1
Severe hypocellularity	0
Chondrocyte clustering	
No clusters	2
< 25% of the cells	1
25% to 100% of the cells	0
Freedom from degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, slight staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0
Total	24

a slightly depressed central region 24 weeks post-operatively. The repair tissue filling the control defects at the same time had a dark red or purple colour with a larger and deeper central depression in comparison with the BM-MSC-treated defects. Although defect margins were still identifiable from the surrounding native cartilage in both groups, superior tissue integration of the repair tissue at the edges of the defect was observed in the BM-MSC-treated defects compared with the empty controls at 24 weeks post-operatively.

The results of macroscopic scoring of repair tissue indicated consistently higher scores in BM-MSC-treated defects in comparison with the controls at all sampling times (Fig. 3). The mean scores of BM-MSCs treated defects were significantly higher than the control defects at four and 24 weeks after surgery (9.25, SD 0.5 vs 7.25, SD 0.95 and 10, SD 0.81 vs 7.5, SD 0.57, respectively). The macroscopic scores of BM-MSCs treated defects at 16

**Fig. 2**

Macroscopic appearance of defects in the condylar regions at four (left), 16 (middle) and 24 (right) weeks after surgery. Control as well as stem cell treated (bone-marrow derived mesenchymal stem cell) defects are represented (scale bar, 3 mm).

and 24 weeks had increased compared with the four-week time point corresponding with the increased quality of reparative tissue, and its resemblance to native cartilage, which was observed macroscopically. The increase in macroscopic scores of the control defects at 16 weeks post-operatively in comparison with the four-week time point was also due to the improved quality of the repair tissue. There was a decrease in mean macroscopic scores of control defects at 24 weeks following surgery corresponding with the disintegration and loss of the quality of repair tissue which was observed macroscopically at this time point.

Histological findings. The results of histological findings at four weeks after surgery indicated that defects treated with BM-MSCs were filled by fibrous tissue containing numerous fibroblasts and blood vessels (Fig. 4). Remnants of PRF were seen at the central portions of the defects as dark red stained areas. The repair tissue had integrated almost completely with the host cartilage at the edges of the defect and the surface of the repair tissue was smooth. The untreated control defects were also filled by a fibrous tissue. However, fewer fibroblasts were present, particularly near the surface of the defect and larger blood vessels were present. No obvious cartilaginous extracellular matrix was identified by Safranin O staining in either group at four weeks after surgery.

At 16 weeks after surgery, major parts of the defects in both treatment groups were filled by fibrous tissue, but parts of the defects in BM-MSCs treated group, particularly at the edges of the defect and near the subchondral bone had chondrocyte-like oval cells inside lacunae resembling normal articular cartilage (Fig. 5). These cells were organised in columnar fashion emanating from the

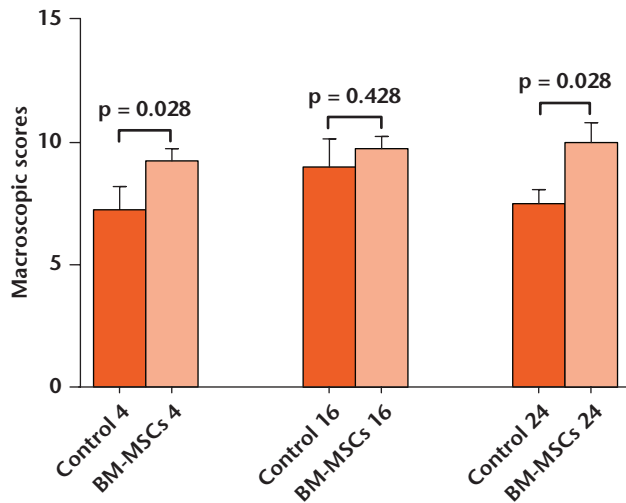


Fig. 3

Mean International Cartilage Repair Society macroscopic scores for control and stem cell treated (bone-marrow derived mesenchymal stem cell (BM-MSC)) groups at four, 16 and 24 weeks after surgery. Error bars indicate standard deviation and the p-values (Mann Whitney U test) represent the statistical differences between the two treatment groups.

deeper parts of the defect towards the surface. They were stained positively with Safranin O indicating the presence of cartilaginous extracellular matrix in the regenerated tissue. These findings were also observed in BM-MSCs treated defects at 24 weeks, although the centre of the

defects was filled by fibrous tissue (Fig. 6). Intense clustering of the chondrocytes was a constant feature observed at the native edges of the defect in the untreated control group at 16 and 24 weeks following surgery (Figs 5 and 6).

The histological observations described above are also reflected in terms of histological scoring of the repair tissue. The mean histological scores of BM-MSC-treated group were consistently higher than the untreated controls (Fig. 7) although statistically significant difference between the two groups was only observed at 16 weeks after surgery (16.5, SD 4.04 vs 11, SD 1.15, respectively).

Discussion

This study has successfully demonstrated the feasibility of using a combination of stem cells originating from bone marrow with a type of platelet concentrate as a novel strategy in cartilage repair. The quality of repair tissue observed in osteochondral defects treated by this combined method of therapy was superior to untreated defects in terms of both macroscopic and histological criteria, and even partial articular cartilage regeneration was evident. The suitability of PRF as an autologous biomaterial scaffold for implantation of stem cells in cartilage defects was also demonstrated in this study.

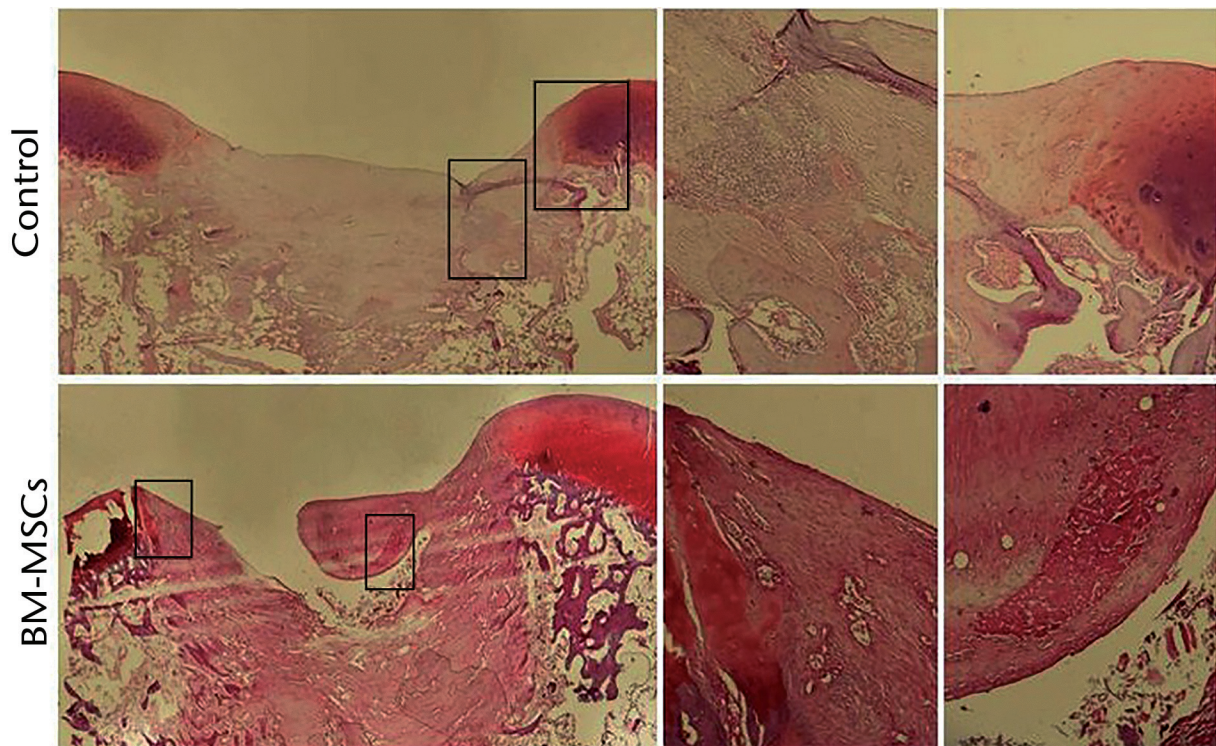


Fig. 4

Histological appearance of defects in the condylar region at four weeks after surgery. Control as well as stem cell treated (bone-marrow derived mesenchymal stem cell) defects are represented. Magnified views of the boxes are presented in the middle and right side. SafraninO staining; original magnification 40x (left side) and 100x (middle and right side).

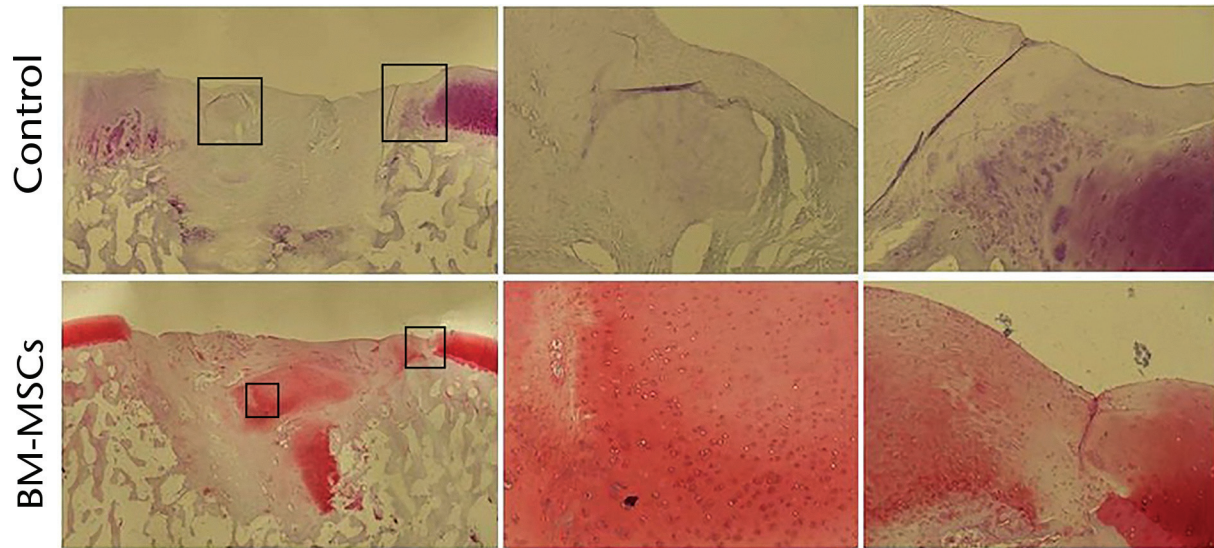


Fig. 5

Histological appearance of defects in the condylar region at 16 weeks after surgery. Control as well as stem cell treated (bone-marrow derived mesenchymal stem cell) defects are represented. Magnified views of the boxes are presented in the middle and right side. Safranin O staining; original magnification 40x (left side) and 100x (middle and right side).

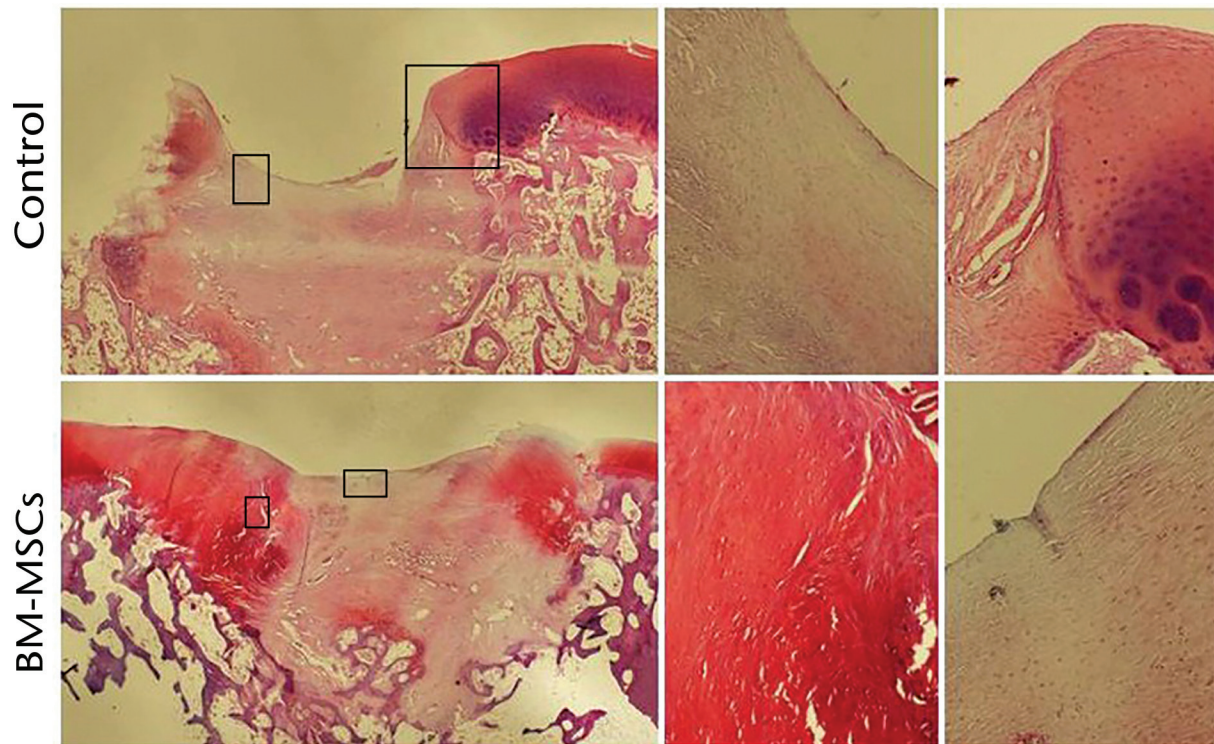


Fig. 6

Histological appearance of defects in condylar region at 24 weeks after surgery. Control as well as stem cell treated (bone-marrow derived mesenchymal stem cell) defects are represented. Magnified views of the boxes are presented in the middle and right side. Safranin O staining; original magnification 40x (left side) and 100x (middle and right side).

The ICRS macroscopic grading was used in this study in order to grade the repair tissue. Macroscopic evaluation is a frequent feature of animal studies in the field of cartilage repair because the whole joint is accessible, and this

method provides the first impressions of the quality of repair tissue. The ICRS macroscopic grading system is validated and reliable.³⁶ The benchmark for evaluation of the success of various cartilage repair procedures is Histological

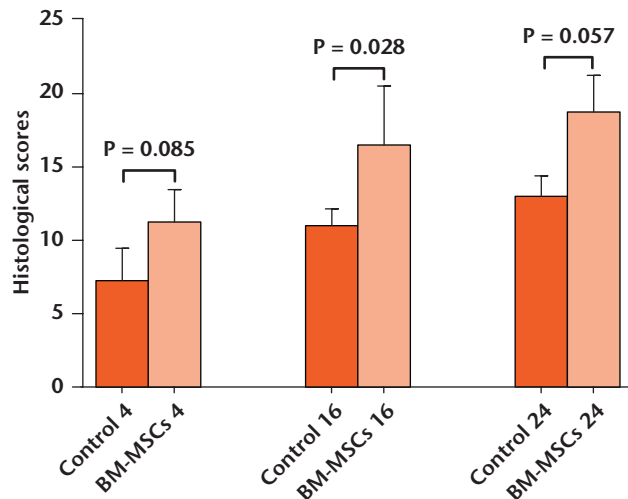


Fig. 7

Mean O'Driscoll histological scores for control and stem cell treated (bone marrow derived mesenchymal stem cell (BM-MSC)) groups at four, 16 and 24 weeks after surgery. Error bars indicate standard deviation and the p-values (Mann Whitney U test) represent the statistical differences between the two treatment groups.

assessment, and the O'Driscoll scoring system, which was used in this study, is recommended for histological analysis of cartilage repair in animal studies.^{37,38}

Apart from the fact that this species is considered an acceptable animal model for cartilage repair studies³⁹⁻⁴² dogs were used in this study for several reasons. It has been recommended that studies involving the use of PRF should be carried out in large animals like dogs in order to obtain true PRF as opposed to the poor quality PRF like fibrin often produced from smaller laboratory animals.⁴³ Moreover, dogs are considered as a large animal model in translational stem cell studies⁴⁴ and because they are frequently affected by cartilage disorders, the results of this study could pave the way for clinical use of this particular method of treatment in canine articular cartilage defects.

Osteochondral defects were created in the weight-bearing areas of the medial femoral condyles to closely resemble clinical cartilage lesions which are usually observed on these anatomic locations. The diameter and depth of the defects were chosen as such to create a critically-sized defect based on recommendations for the canine species.⁴² A critical size cartilage defect is defined as the minimum defect dimension in diameter that the animal is incapable of repairing without intervention. For the canine species, the critical size of the defect is 4 mm, although defects of larger than 5 mm are generally considered critical and the depth of the surgically created defect ranges from 3 mm to 12 mm depending on the type of defect, i.e., chondral or osteochondral.⁴²

The ultimate goal of all cartilage repair techniques is to produce a repair tissue with similar structural, biochemical and biomechanical characteristics to normal hyaline

cartilage. Implantation of BM-MSCs in osteochondral defects is an exogenous form of cell therapy.⁴⁵ BM-MSCs have the potential to differentiate into chondrocytes and regenerate articular cartilage. Growth factors, particularly TGF- β family, are strong inducers of chondrogenesis. In addition, homeostasis and the repair of articular cartilage is regulated by a number of growth factors including: TGF- β , fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF).⁴⁶ Considered as a natural bioscaffold; PRF is a rich source of these growth factors.^{15,16} PRF is termed second-generation platelet concentrate to distinguish it from platelet rich plasma (PRP), which is classified as first generation platelet concentrate. All platelet concentrates have evolved from fibrin sealants or fibrin glues which are prepared from whole plasma and essentially contain fibrinogen. The addition of calcium chloride or bovine thrombin to fibrin sealants at the time of application results in the formation of fibrin clot. Despite having a similar origin, there are numerous differences between PRF and PRP,³³ which are briefly summarised in Table III. PRF does not dissolve quickly after application because of its unique structural properties and the solid consistency of fibrin which results in slow release of growth factors over an extended period of time.⁴⁷ Previous studies have indicated that numerous growth factors are involved in the process of chondrogenesis, and the use of multiple growth factors could better stimulate cartilage regeneration as opposed to a single factor and application. PRF fulfils this concept of multiple growth factor delivery.⁴⁶ Based on the above assumptions, we hypothesised that the combined use of BM-MSCs and PRF could have regenerative effects on articular cartilage.

To the best of our knowledge, this is the first study to examine the effect of BM-MSCs transplanted onto PRF in cartilage repair and regeneration. Studies concerning tissues other than articular cartilage have shown that the combination of MSCs and PRF has resulted in significantly more tissue repair and regeneration compared with either MSCs or PRF therapy alone or no treatment.²¹⁻²⁸ Chen et al⁴⁸ have investigated the effect of PRF on proliferation and osteogenic differentiation of canine BM-MSCs. It was found that the proliferation of stem cells was stimulated, but PRF alone had no effect on their osteogenic differentiation. The authors concluded that the proliferative effects were likely due to the release of growth factors from PRF. In a similar *in vitro* study, Dohan Ehrenfest et al⁴⁹ and colleagues demonstrated that PRF had a significant stimulatory effect on both proliferation and differentiation of human oral bone MSCs. As stated previously, the combined use of stem cells and PRF has not been reported in the literature, but the application of stem cells plus PRP for cartilage repair has been reported in three studies. The clinical use of autologous culture expanded BM-MSCs transplanted onto platelet rich fibrin

Table III. Differences between platelet rich plasma (PRP) and platelet rich fibrin (PRF)

PRP	PRF
First generation platelet concentrate	Second generation platelet concentrate
Prepared from venous blood containing anticoagulant in a two-step centrifugation process	Prepared from venous blood without any anticoagulant in a single step centrifugation process
Requires activation using calcium chloride and bovine thrombin at the time of application to form a gel	Does not require any activation prior to application
Rigid three-dimensional fibrin structure does not favour cytokine enmeshment and cellular migration	Fine and flexible three dimensional fibrin network suitable to support cytokine enmeshment and cellular migration
The rigid three dimensional structure is appropriate to firmly seal biological tissues	The elastic and flexible three dimensional structure is appropriate for application at various surgical procedures

glue was first reported by Haleem et al.⁵⁰ Significant functional and diagnostic imaging improvements were observed in human patients with second look arthroscopy indicating the presence of nearly normal hyaline cartilage. It must be noted that despite the use of the term PRF by the authors, the platelet concentrate used by Haleem et al was actually PRP. In another study conducted by Xie et al⁵¹ on rabbits, treatment of full thickness cartilage defects with MSCs plus PRP resulted in significantly higher macroscopic and histological scores at six and 12 weeks after surgery. The authors concluded that PRP may be a suitable scaffold for cell based cartilage repair as it is capable of releasing growth factors and inducing the differentiation of stem cells to chondrocytes. Application of synovial membrane derived MSCs and PRP also resulted in successful treatment of experimental cartilage defects of rabbits with the formation of hyaline cartilage and significantly higher histological scores 24 weeks after surgery.⁵² These studies indicate that PRP is capable of creating a beneficial environment to promote *in vivo* proliferation and differentiation of stem cells. PRF, used in our study, could also create this favourable environment as it also contains growth factors and releases them in a longer period of time compared with PRP.

This study does have limitations. Collagen type characterisation by immunohistochemical staining methods and stiffness tests to determine the biomechanical properties of the repair tissue were not carried out. Furthermore, it was not possible to extend the study period to evaluate the repair tissue over an even longer period of time, for example up to one year after surgery. The main objective of the present study was to evaluate the feasibility of using the combination of BM-MSCs and PRF as a novel treatment method in cartilage repair and regeneration. To accomplish this, osteochondral defects treated with BM-MSCs seeded on PRF were compared with the empty control defects. Therefore, it was not possible to compare the regenerative effects of PRF or BM-MSCs alone with that of the combination of BM-MSCs and PRF. This could also be considered as another limitation of this study. The main factor for the above limitations was financial constraints. Above all, it must be noted that the stem cell seeded PRF was press fitted inside relatively deep osteochondral defects in the present study. This

technique could be used in a similar fashion for treatment of large osteochondral defects in a clinical setting but it is not clear whether the stem cell seeded PRF would remain in place inside shallower partial thickness or full thickness cartilage defects without using any patches or membranes. The treatment of partial thickness or full thickness cartilage defects using combination of MSCs and PRF requires further investigations.

Our findings have demonstrated that transplantation of autologous BM-MSCs onto PRF is a promising, novel, method of osteochondral repair and articular cartilage regeneration within the context of cell therapy. Further studies are required in this subject area. The use of PRF creates a suitable environment for proliferation and differentiation of BM-MSCs into chondrocytes with the appearance of a hyaline like tissue with improved macroscopic and histological characteristics.

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Author Contribution

- D. Kazemi: Conceived and designed the study, carried out the animal experiments, acquired and analysed data and drafted the manuscript.
- K. Shams Asenjan: Conducted the laboratory experiments and participated in data acquisition and drafting of the manuscript.
- N. Dehdilani: Conducted the laboratory experiments and participated in data acquisition and drafting of the manuscript.
- H. Parsa: Conducted the laboratory experiments and participated in data acquisition and drafting of the manuscript.

ICMJE Conflicts of Interest

- None declared

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