



■ ARTHRITIS

Sheep condyle model evaluation of bone marrow cell concentrate combined with a scaffold for repair of large osteochondral defects

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Aims

Minimally manipulated cells, such as autologous bone marrow concentrates (BMC), have been investigated in orthopaedics as both a primary therapeutic and augmentation to existing restoration procedures. However, the efficacy of BMC in combination with tissue engineering is still unclear. In this study, we aimed to determine whether the addition of BMC to an osteochondral scaffold is safe and can improve the repair of large osteochondral defects when compared to the scaffold alone.

Methods

The ovine femoral condyle model was used. Bone marrow was aspirated, concentrated, and used intraoperatively with a collagen/hydroxyapatite scaffold to fill the osteochondral defects (n = 6). Tissue regeneration was then assessed versus the scaffold-only group (n = 6). Histological staining of cartilage with alcian blue and safranin-O, changes in chondrogenic gene expression, microCT, peripheral quantitative CT (pQCT), and force-plate gait analyses were performed. Lymph nodes and blood were analyzed for safety.

Results

The results six months postoperatively showed that there were no significant differences in bone regrowth and mineral density between BMC-treated animals and controls. A significant upregulation of messenger RNA (mRNA) for types I and II collagens in the BMC group was observed, but there were no differences in the formation of hyaline-like cartilage between the groups. A trend towards reduced sulphated glycosaminoglycans (sGAG) breakdown was detected in the BMC group but this was not statistically significant. Functional weightbearing was not affected by the inclusion of BMC.

Conclusion

Our results indicated that the addition of BMC to scaffold is safe and has some potentially beneficial effects on osteochondral-tissue regeneration, but not on the functional endpoint of orthopaedic interest.

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Keywords: Bone marrow concentrate, Osteochondral scaffold, Minimally manipulated cells

Article focus

■ This article focuses on exploring the effects of autologous bone marrow concentrate (BMC) together with a collagen/hydroxyapatite scaffold on the regeneration of large osteochondral defects in an in vivo sheep model.

Key messages

■ Addition of BMC to an osteochondral scaffold had some chondroprotective effects in the treatment of large osteochondral defects, but no significant improvements for the functional endpoint of orthopaedic interest were observed.

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Strengths and limitations

- The study presents a comprehensive examination of regenerated bone and cartilage, as well as the gait, in a large animal model to look at the safety and efficacy of BMC augmentation.
- The study did not include an empty defect control in the animals. Following the principles of Arthroplasty, Refinement or Reduction in the use of animals (3Rs), and the UK Home Office regulation on animal study, knowing that according to literature the critical size defects do not fully repair, the local ethical committee recommended against the use of empty defects as control.

Introduction

Repair of osteochondral (OC) defects is one of the biggest challenges in orthopaedics, as untreated defects often lead to the development of osteoarthritis (OA). OC tissue engineering¹ and cell-based therapies² have been studied for the repair of these defects. It is common to employ cells that have been collected from the patient and expanded *in vitro* before transplantation back into the defect, a labour-intensive and costly two-step procedure. In contrast, cell sources such as bone marrow aspirate (BMA) from the iliac crest and its concentrated form (bone marrow concentrate, BMC or BMAC),³ can be used intraoperatively in one-step procedures, which makes them an attractive stem/progenitor cell source for orthopaedics, either as primary therapy or as an augmentation to tissue engineering. An example is Sanghani-Kerai et al⁴ who incorporated BMC into an autologous blood glue, aiming to improve osseointegration in the bone-implant interface.

BMC contains growth factors, anti-inflammatory proteins, and mesenchymal stem cells (MSCs)/progenitor cells,⁵⁻⁸ and has been exploited as a 'point of care' orthobiological product.⁶ BMC's mechanism of action is thought to be twofold. Firstly, through the paracrine effect of MSCs, they secrete chemical messages, microvesicles, or exosomes that act to modulate the local environment and help recruit more stem cells.^{6,9,10} Secondly, through the release of chondroprotective growth factors,¹¹ they promote matrix synthesis and decrease matrix degradation, inducing chondrocyte and MSC replication.

BMC is currently approved by the U.S. Food and Drug Administration for clinical application to obtain progenitor cells and growth factors for homologous use in orthopaedic patients.¹² However, evidence shows that the role of BMC is still inconclusive for the restoration of chondral defects in the knee.¹³ A number of preclinical animal studies have used BMC as part of their treatment, with the results varying from significant improvements in bone and/or cartilage regeneration^{11,14-16} to few or no significant effects^{3,17,18} in osteochondral repair. In addition to preclinical studies, BMC has also been used in several clinical studies as an adjunct to osteochondral allograft

transplantation to improve osseous integration with the host bone. Interestingly, while one study observed superior radiological integration to bone and less sclerosis,¹⁹ another study found that the inclusion of BMC did not improve bone integration or cartilage features.²⁰ BMCs have also been studied clinically^{21,22} as an addition to microfracture or scaffolds for the treatment of full-thickness cartilage defects, with contradictory results.

Comprehensive reviews by Cavinatto et al²³ and Cotter et al⁵ depict the current state of BMC use for single-stage management of chondral defects in the knee. Currently, there is a lack of consensus for or against the use of a scaffold coupled with BMC for clinical applications.¹² Furthermore, data regarding functional orthopaedic assessments and specific safety tests are mostly lacking in the animal studies performed to date.

To address these shortcomings, tackle the current inconsistent outcomes in animal models, and add to the knowledge base for clinical decision-making, we performed an *in vivo* study coupling BMC to an osteochondral scaffold, and hypothesized that the addition of BMC to the osteochondral scaffold in a single-step procedure would be safe and enhance osteochondral regeneration. The safety was measured by assessing the inflammation in the draining lymph nodes and blood biochemistry, while the efficacy was measured by evaluating cartilage and bone regeneration. Any potential chondroprotective effect was determined by looking at the glycosaminoglycan-release into the synovial fluid. The functional weightbearing was measured as a functional endpoint of orthopaedic interest.

Methods

Osteochondral scaffold. A multi-layered porous collagen-based scaffold was used in this study (Figure 1) and prepared as described in the Supplementary Material. The scaffold was designed in the shape of a truncated cone (8 mm upper diameter, 10 mm height, 10° angle) for the ease of implantation and composed of three layers: a collagen type I layer corresponding to cartilage and two layers of collagen-hydroxyapatite (40% and 70% hydroxyapatite (HAp)), corresponding to calcified cartilage and subchondral bone, respectively. The final scaffolds had an upper diameter of 8.3 (standard deviation (SD) 0.3) and a height of 9.6 (SD 0.4). Slight variations in size are due to the shrinkage that occurs during the processing steps. The scaffold pore size and porosity were controlled by the freezing temperature and resulted in porosity of over 80% and pore sizes of 60 µm to 115 µm in diameter. The compressive mechanical properties (storage modulus) were determined using a Dynamic Mechanical Analyzer (PerkinElmer, UK) and resulted in 1.5 MPa, 2MPa, and 3 MPa at 0% strain for the collagen, collagen-40% HAp, and collagen-70% HAp, respectively.

Bone marrow isolation. Bone marrow aspirates were taken from the posterior iliac crest of sheep under general anaesthesia. Using aseptic techniques, bone marrow

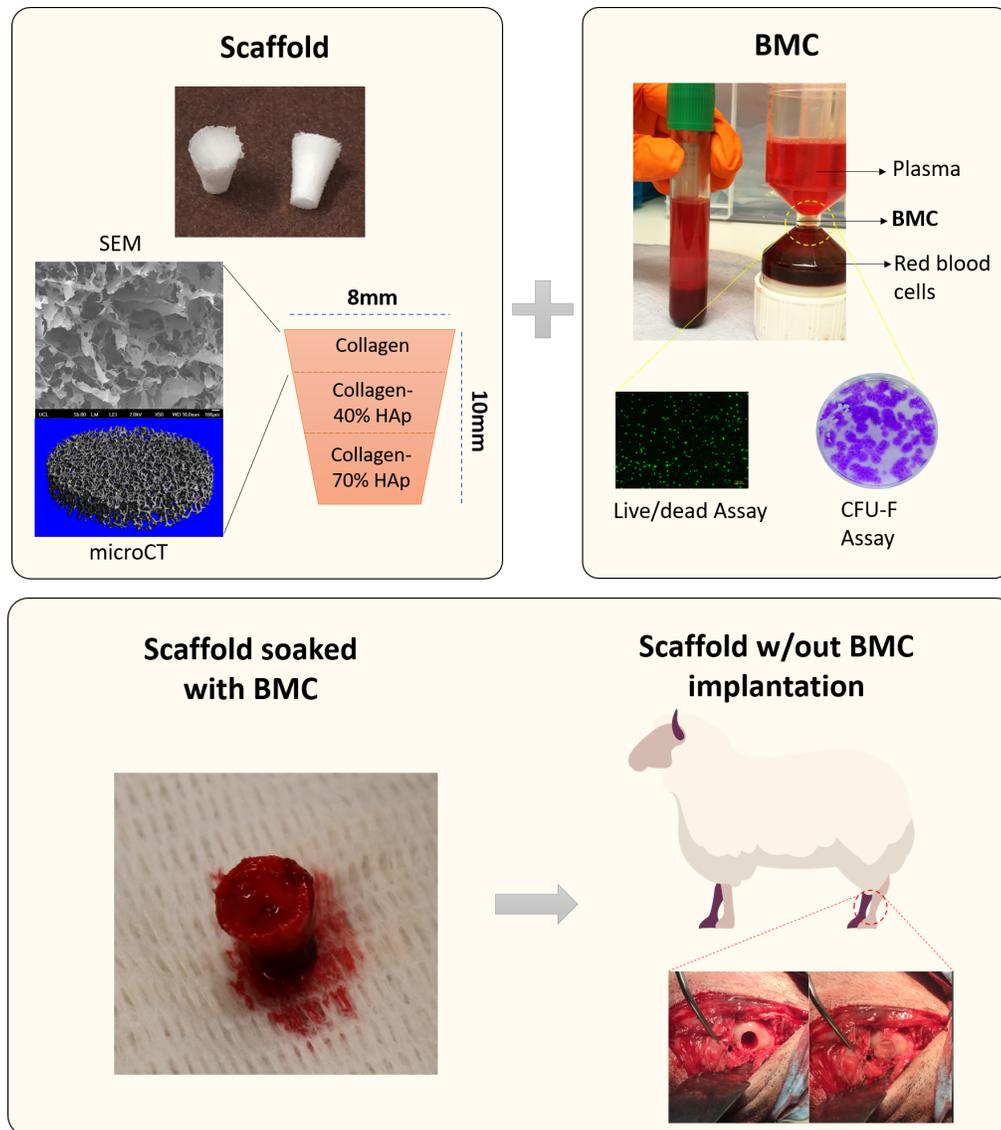


Fig. 1

Surgical procedure, including bone marrow concentrate (BMC) isolation, concentration, and characterization. Collagen-hydroxyapatite (HAp) is soaked in BMC and inserted in the created defect in the femoral condyle of sheep. CFU, colony-forming unit; SEM, standard error of the mean.

was obtained by inserting a Jamshidi needle (Veterinary Instrumentation Limited, UK) into the iliac crest bone. A 30 ml to 50 ml syringe loaded with 1 ml of heparin was used to aspirate the bone marrow. At any one time, only 5 ml of bone marrow was taken from one site. The needle was then withdrawn or repositioned to access a new region of the ilium. Up to 40 ml of bone marrow was obtained. If necessary, the iliac crest on the opposite side was used. A bone marrow isolation and processing kit (NTL Biologica, UK) was used to process the bone marrow. A measure of 20 ml of the bone marrow aspirate was transferred aseptically from the syringe to the kit and this was then centrifuged (4,000 rpm, 20 minutes) to separate the red blood cells from the bone marrow concentrate. During surgery, 1 ml of the concentrate was used by soaking the scaffolds with the BMC for 20 minutes

prior to implantation, where almost all of the BMC was absorbed into the porous scaffold and the rest was retained for characterization.

BMC characterization. Total number of nucleated cells was determined using the recommended protocol by Stemcell Technologies (Canada). Cell suspensions (duplicate per sample) were diluted 50 times in 3% acetic acid with Methylene Blue (Stemcell Technologies) and were counted using a haemocytometer.

Cell viability (duplicate per sample) was assessed by incubating cells with 4 mM Calcein AM and 4 mM ethidium homodimer-1 (Invitrogen, UK) in phosphate-buffered saline (PBS, Invitrogen, UK) for 15 minutes at 37°C, to stain live and dead cells green and red, respectively. Cells in three random regions were imaged with a ZEISS ApoTome.2 Fluorescent Microscope (ZEISS,

Table I. The primer sequences of the targeted genes and the internal control gene.

Primer	Forward	Reverse
<i>GAPDH</i>	GCGGTGGACAGTGGTCATAA	CAGCCTTCTCCATGGTACTGA
<i>COL I</i>	CCT GGA TGC CAT TAA GGT CT	TCT TGT CCT TGC TCT TGC TG
<i>COL II (COL2A1)</i>	CAT GAA AGA CTG CCT CAG CC	GGA GCA CCA TCC TTG ATG TCT
<i>ACAN</i>	TCG GGG TAG GTG GCG AGG AA	GGG CGG TTG GGG AGA CTT CAA
<i>SOX9</i>	GAG GAC AAG TTC CCC GTC TG	GCT CTC GTT CAG CAG TCT CC
<i>RUNX2</i>	CGC CTC ACA AAC AAC CAC AG	GCT TGC AGC CTT AAA TGA CTC T

ACAN, aggrecan; *COL2A1*, collagen, type II, alpha 1; *COL I*, type I collagen; *COL II*, type II collagen; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *RUNX2*, runt-related transcription factor 2.

Table II. Bern scoring system.³²

Uniformity and darkness of Safranin-O Fast Green stain	Score
No stain	0
Weak staining of poorly formed matrix	1
Moderately even staining	2
Even dark stain	3
Distance between cells/ amount of matrix produced	
High cell densities with no matrix in between	0
High cell densities with little matrix in between	1
Moderate cell density with little matrix	2
Low cell density with moderate distance between cells and extensive matrix	3
Cell morphologies represented	
Condensed/necrotic/pycnotic bodies	0
Spindle/fibrous	1
Mixed spindle/fibrous with rounded chondrogenic morphology	2
Majority rounded/chondrogenic	3

Germany). ImageJ was used to quantify the number of live and dead cells.²⁴

Colony-forming unit (CFU-F) assay was performed to functionally evaluate the non-haematopoietic stem/progenitor cell content of BMC.¹² A portion of BMC was diluted 5,000 times in PBS and seeded at a density of 2 µl per well into six-well plates in duplicates (VWR, UK) and incubated at 37°C with 5% CO₂ for ten days. The cells were then fixed in 4% paraformaldehyde for 30 minutes at room temperature and stained with 0.5% crystal violet in methanol for 30 minutes at room temperature. The wells were washed four times with PBS and the plates were scanned using a scanner (Epson, Japan). ImageJ²⁴ and the ColonyArea plugin²⁵ were used to quantify colony areas in each well.

Surgical procedure and implantation of osteochondral scaffold. In vivo assessment was carried out in the sheep medial femoral condyle conducted under the approval of and compliance with UK Home Office requirements (Animals (Scientific Procedures) Act 1986), which included local ethical approval by the local ethics committee. An ARRIVE checklist is included in the Supplementary Material to show that the ARRIVE guidelines were adhered to in this study. A total of 12 skeletally mature adult female sheep of a single breed (mules) and mean weight of 80.7 kg (72 to 88) were used in the study. Anaesthesia was induced with a mixture of ketamine and midazolam and then maintained using gaseous isoflurane at 2.5%. The sheep were given Ceporex antibiotic injections on

days 0 to 3. Each sheep also had fentanyl patches on pre-operatively until day 3.

Under anaesthetic, animals were positioned on their side and an incision was made to gain access to the left femoral condyle. A conical critical-sized osteochondral defect (8 mm diameter, 10 mm height) was created using custom-made surgical tools on the load-bearing area of the medial femoral condyle. Animals were randomly assigned to the control defect group (scaffold only, n = 6) or the BMC group (scaffold and BMC, n = 6). Since the aim of this study is to evaluate the BMC in stimulating tissue regeneration when applied with a scaffold, and not by itself, the control group is the scaffold group and the experimental group is the scaffold and BMC group. According to the principle of Arthroplasty, Refinement or Reduction in the use of animals (3Rs), and the UK Home Office regulation on animal study, knowing these critical size defects do not fully repair, the University Animal Welfare and Ethics committee (AWERB) recommended that it is unethical to use an empty defect group as the control.

For the BMC group, the scaffold was soaked in 1 ml of BMC for 20 minutes, and then press-fitted in the pre-created defect (Figure 1). After the addition of BMC, the wet scaffold was soft in texture. Animals were housed in individual pens for four days post-surgery and then transferred to group pens for the remainder of the study. The joints were radiographed immediately after implantation and at 24 weeks postoperatively after euthanasia.

Force-plate assessment of gait. Gait/functional weight-bearing of the animals was evaluated visually and by force-plate assessment in the preoperative period and at one and six months postoperatively, as this has been demonstrated to be an appropriate marker of normal/abnormal function and functional recovery.²⁶⁻²⁹ The sheep were walked on a pressure pad (Kistler Biomechanics Limited, UK) in a gait analysis laboratory, and results were averaged over 12 walking cycles expressed as the ratio of the ground reaction force of the operated versus nonoperated hind limb. This provided a single percentage value of "Function" over months.

Macroscopic assessment of cartilage regeneration. Post-euthanasia, the joints were opened and the defect site and surrounding joint tissues were examined. Photographs of the defect sites were taken, and one blind assessor (MT) evaluated the quality of cartilage repair according to

Table III. Semiquantitative scoring of lymph nodes by evaluating and scoring the number and size of lymphoid follicles in the cortex, the percentage of lymphoid follicles expressing germinal centres, the presence or absence of secondary follicles, the size of parafollicular and paracortical T cell areas, and noting the content of the sinusoids, including the subcapsular, cortical, and medullary sinusoids.

Analysis	Score
Number of lymphoid follicles visible in the cortex	None = 0; low = 1; moderate = 2; several = 3
Size of lymphoid follicles	Small = 1; medium = 2; large = 3
Proportion of lymphoid follicles with germinal centres	0% = 0; < 25% = 1; 25% to 75% = 2; > 75% = 3
Presence of secondary lymphoid follicles	Yes or No
Thickness of parafollicular zone	Small = 1; medium = 2; large = 3
Thickness of paracortical zone	Small = 1; medium = 2; large = 3
Subcapsular, paracortical, and medullary sinusoidal content	Number of cells: low, +; moderate, ++; large, +++. M = macrophages, H = haemosiderophages, N = neutrophils, E = eosinophils, L = lymphocytes

the International Cartilage Repair Society (ICRS) scoring system.³⁰

Gene expression analysis in regenerated cartilage. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out to analyze specific genes for cartilage-related markers aggrecan (*ACAN*), type-II collagen (*COL2*), and *SOX9* and for bone-related markers *RUNX2* and collagen type-I (*COL1*). Immediately after euthanasia, 1.5 mm biopsies of regenerated cartilage were harvested and flash-frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and complementary DNAs (cDNAs) were synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific, USA). The qRT-PCR reactions were carried out with a Biorad system CFX96 (Biorad, UK). RNA expression was quantified using the Brilliant III SYBR Green QPCR Master Mix (Agilent, USA) kit. The primers of the targeted genes and the internal control gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were purchased from Eurofins (UK), and the sequences are listed in Table I. A difference in Ct values (Δ Ct) was calculated for each gene in duplicate, and the relative quantifications of targeted genes in treatment groups to untreated healthy cartilage were performed using the Δ Ct method.³¹

Histological analysis. A 1.5 mm diameter biopsy punch (Kai Medical, Japan) was used to collect a cartilage core from the regenerated tissue. This was then processed for wax histology. Then 5 μ m thick sections were collected from each sample and stained using routine haematoxylin and eosin (H&E), safranin-O, and alcian blue staining. The quantification was according to the Bern scoring system,³² which evaluates uniformity and darkness of safranin-O stain, amount of matrix produced, and cell morphology (Table II).

Examination of bone regeneration. A peripheral quantitative CT (pQCT) (Stratec XCT 2000; Stratec, Germany)

Table IV. The correlation between colony-forming ability with total nucleated cells, bone ingrowth, trabecular thickness, and trabecular separation was evaluated.

	TNC		BV/TV%		Tb.th		Tb.sp	
	r	p	r	p	r	p	r	p
CFU-f	-0.5	0.342	0.06	0.915	0.3	0.541	0.7	0.163

r = Pearson r correlation.

BV/TV%, percentage bone volume over total volume; CFU-f, colony-forming unit; Tb.sp, trabecular separation; Tb.th, trabecular thickness; TNC, total nucleated cells.

was used to analyze the volumetric bone mineral density (vBMD, mg/cm³) distributions. Each condyle was scanned in eight to ten planes to cover the defect area with steps of 1 mm, voxel size of 0.2 mm \times 0.2 mm, and a scan speed of 20 mm/s. Analysis was performed by the XCT2000 software (version 6.20). In each slice, a region of interest surrounding (and including) the defect was defined for measuring the total bone mineral density and trabecular bone mineral density (threshold 540 mg/cm³). vBMD was averaged across all sections.

MicroCT analysis was performed using a Skyscan 1172 (Bruker, UK) with 100 kV radiograph source, 100 mA (pixel size 16.8 μ m), and an Al + Cu filter to assess the quantity and structure of the newly formed bone. 3D reconstructions were performed using NRecon software (v.1.6.3.2, SkyScan). Subsequent visualization was performed using Bruker CTVOX software and analysis was done with Bruker CTAn software. A circular region of interest (ROI) with a diameter of 10 mm was selected and a threshold of 71 was applied to all samples. Subchondral bone regeneration was expressed as trabecular thickness (mean thickness of trabeculae, Tb.Th, mm), trabecular separation (mean distance between trabeculae, Tb.Sp, mm), and percentage bone volume over the total volume (% BV/TV) in the ROI.

The condyles were then dehydrated through an alcohol series, transferred to LR White Resin (London Resin Company, UK), and cut into sections using a diamond saw microsectioning system (Exakt, Germany). The sections were examined under a digital microscope (Keyence, UK).

Synovial fluid collection and assessment. Upon opening the joint, synovial fluid was collected using syringes. Total protein content was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Total glycosaminoglycans (GAGs) levels were determined using the Blyscan Assay Kit (Biocolor, UK).

Lymph node and inflammation assessment. Popliteal lymph nodes from both legs were collected, trimmed of any fat tissue, photographed, and transferred to Neutral Buffered Formalin (10%) (Thermo Fisher Scientific). The lymph nodes were then weighed and processed for routine histology and analyzed by a pathologist (Table III). Blood samples were collected pre-surgery, and at 90 and 180 days postoperatively, for full haematology and biochemistry tests. Seven biomarkers including red blood cells (RBCs), haemoglobin (HGB), haematocrit (HCT),

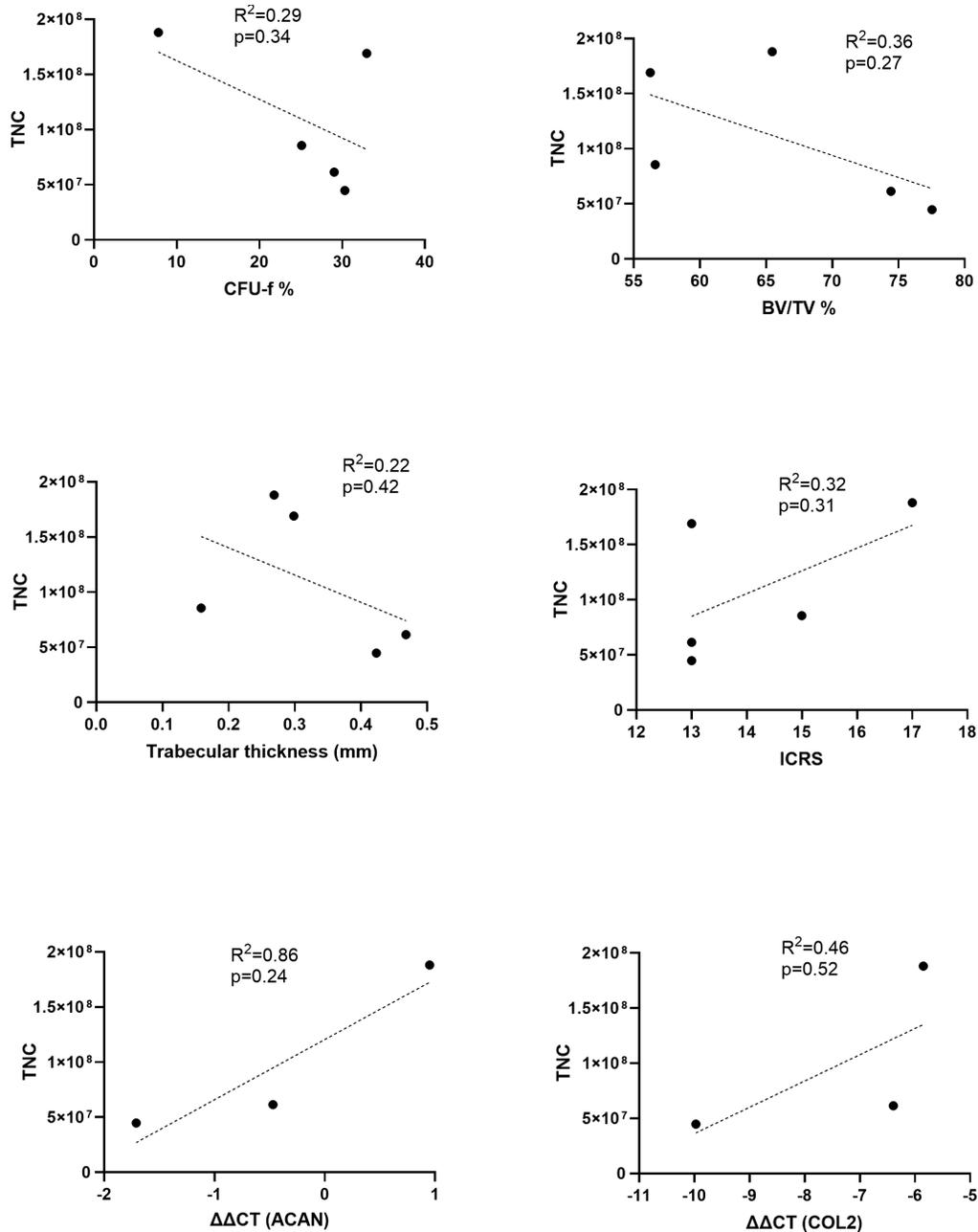


Fig. 2

Correlation between the number of total nucleated cells (TNC) and bone/cartilage regeneration. Pearson R^2 and p -values are shown in each graph. No statistical significance was observed. *ACAN*, aggrecan; BV/TV%, percentage bone volume over total volume; CFU-f, colony-forming unit; *COL2*, type II collagen; ICRS, International Cartilage Regeneration & Joint Preservation Society.

packed cell volume (PCV), sorbitol dehydrogenase (SDH), triglycerides, urea, and creatinine were analyzed further.

Statistical analysis. A randomized block design was performed to group the animals and to rule out interference from age factors. Animals were enrolled in blocks of similar age, and within blocks assigned at random to the treatment group and order of operation. The statistical analysis was performed using Graphpad Prism 8.1 software (GraphPad Software, USA). All quantitative data

were expressed as mean and SD unless specified otherwise. The Shapiro-Wilk test was used to determine the normality of data. For each normally distributed parameter, differences between two groups were analyzed using unpaired t -tests; otherwise, the Mann-Whitney U test was used. For comparisons involving more than two independent groups (qPCR analysis), we used nested one-way analysis of variance (ANOVA) followed by Tukey's test. The Pearson correlation between total nucleated cell

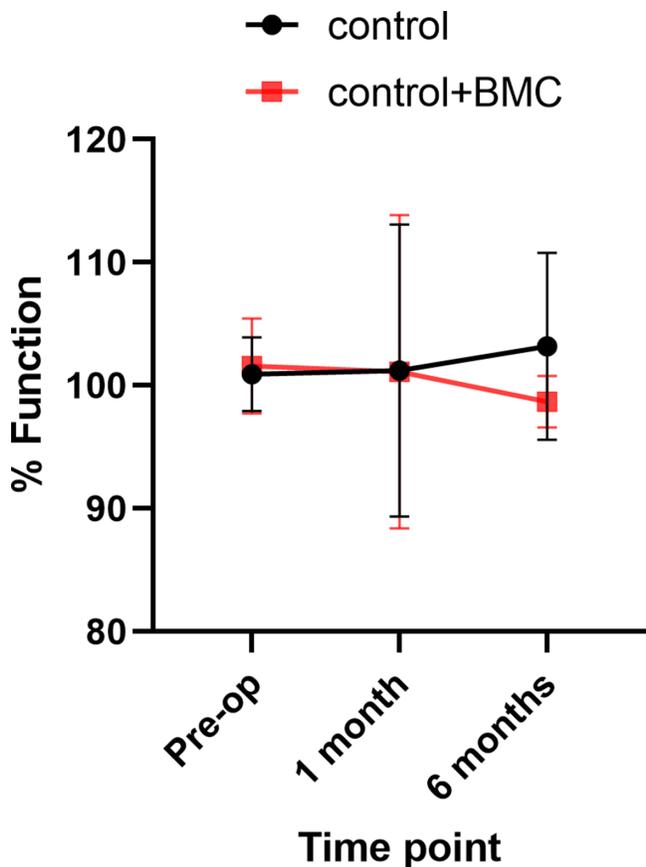


Fig. 3

Force-plate gait analysis: a % limb function (functional weightbearing) over time. No significant differences were observed in the groups with time and between the groups at each timepoint. BMC, bone marrow concentrate.

(TNC) and bone regeneration was calculated after confirmation of normality. The level of statistical significance was established at $p < 0.05$.

Results

BMC characterization. Bone marrow TNCs were measured for all animals (6/6 animals) and ranged from 0.44 to 1.88×10^8 cells/ml with a mean of 1.1×10^8 cells/ml (SD 6.5×10^7 , 95% CI 0.3 to 1.9). The area of the well-plate covered by CFU-f colonies (% area per 0.5×10^6 TNC) was found to be 25.09% (SD 10%, 95% CI 12.5 to 37.5). All BMCs were found to have cell viabilities greater than 90% following density centrifugation and showed colony-forming ability, however this was not correlated significantly with the number of TNCs (Table IV). Given the wide range of TNCs within the group, further analysis of the bone microstructural data was performed. Correlations between the bone regeneration parameter (BV/TV%) and CFU-f/TNC were evaluated, and no significant correlation was observed. The relationship between the number of TNCs and bone and cartilage regeneration was also explored (Figure 2), showing that some parameters such as ACAN chondrogenic gene expression could be correlated with the number of TNCs; however, due to

the large p-values there is no substantial evidence to suggest that the correlation is real.

Force-plate gait analysis. Visual inspection of 6/6 animals in each group showed no limping in either of the groups at all timepoints. Functional recovery was assessed using force-plate analysis. Higher force on the nonoperated leg usually means that the operated leg is less functional. Considering this principle, the % function of the operated limb between the two experimental groups over time was compared (Figure 3). Preoperatively, both groups showed a mean % function of around 100% (control = 100.91 (SD 2.99) and BMC = 101.58 (SD 3)). The mean values at one month stayed at around 100% for both groups (control = 101.22 (SD 11.87) and BMC = 101.11 (SD 12.73)), although larger variation was seen between the animals. At six months, the % function in the control group was 103.17 (SD 7.60), while this value slightly dropped for the BMC group to 98.68 (SD 2.11). None of the differences between the groups were statistically significant at any of the timepoints, meaning that the % function of the limb was not affected by the treatment.

Cartilage regeneration. Macroscopic and histological assessments of the regenerated cartilage are displayed in Figures 4a and 4b. Safranin-O/fast-green and alcian blue stain were used to examine the expression of GAGs (in 3/6 animals in each group) as a guide to extracellular matrix maturity.³² Although developed mostly for in vitro engineered cartilage, Bern scoring was used in this case to semi-quantitatively score the histological images, as most of the other scoring systems require intact osteochondral junction or look at lateral integration of cartilage with surrounding tissue (e.g. Pineda³³ or O'Driscoll scores³⁴). No significant differences were observed in Bern scores between the groups ($p = 0.999$, Mann-Whitney U test), as demonstrated in Figure 3b. H&E staining did not show any inflammatory response in form of mononuclear cell infiltration, however, compared to healthy hyaline cartilage a higher number of cells were present in both groups (Figure 4b).

As shown in Figure 4a, even in the worst cases over 80% of the defect surface was repaired after six months. However, in terms of quality, the response varied from hyaline-like to fibrous cartilage in both control and BMC-treated groups. These results indicate no significant effect of BMC in the quality of regenerated cartilage histologically and macroscopically using ICRS scoring (6/6 animals in each group) (Figure 4a).

The genes pertinent to cartilage (*COL2*, *SOX9*, and *ACAN*) and bone (*COL1*, *RUNX2*) were analyzed in 3/6 animals in each group by qRT-PCR to evaluate the in vivo cartilage formation six months postoperatively (Figure 4c). *COL1* was upregulated in both BMC and control groups compared to the untreated healthy cartilage and *COL2* expression increased significantly in the BMC group compared to the control group ($p = 0.002$, one-way ANOVA followed by Tukey's *t*-test), while no significant differences in *ACAN* expression were observed between the BMC and control groups. At the same

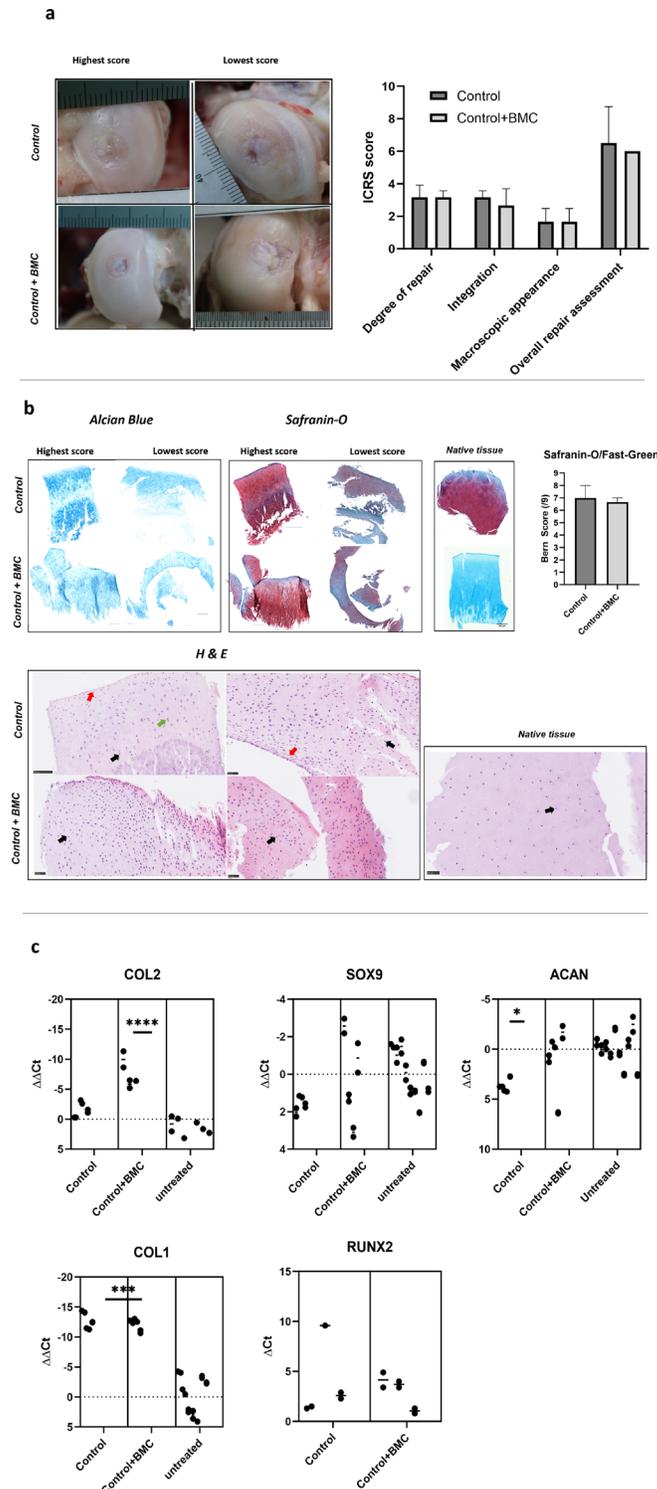


Fig. 4

Evaluation of regenerated cartilage at six months: a) Gross view and macroscopic evaluations showing best and worst cases in each group. ICRS, International Cartilage Regeneration & Joint Preservation Society (ICRS) scoring did not show any macroscopic differences between control and bone marrow concentrate (BMC) groups. b) Histology (alcian blue, Safranin-O, and haematoxylin and eosin (H&E) staining), with best and worst repair tissues shown in each case. Bern score evaluation of Safranin-O staining showed no statistically significant difference between the groups. H&E staining shows chondrocytes in their lacunae (black arrow) in both groups. Flattened chondrocytes (red arrow) and dividing chondrocytes (green arrow) are shown. No inflammatory response in form of mononuclear cell infiltration was observed, however, compared to healthy hyaline cartilage more cells were present in both groups. c) Chondrogenic and osteogenic gene expression analysis. Upregulation of type II collagen (*COL2*) in the BMC group, and downregulation of aggrecan (*ACAN*) in the control group. *COL1* was upregulated and *RUNX2* was expressed in both groups. All comparisons are with untreated normal cartilage indicated as “untreated” in the graph. Ct corresponds to cycle threshold. Scale bar = 200 μ m (alcian blue and Safranin-O), 50 μ m (H&E). Error bars: standard error of means.

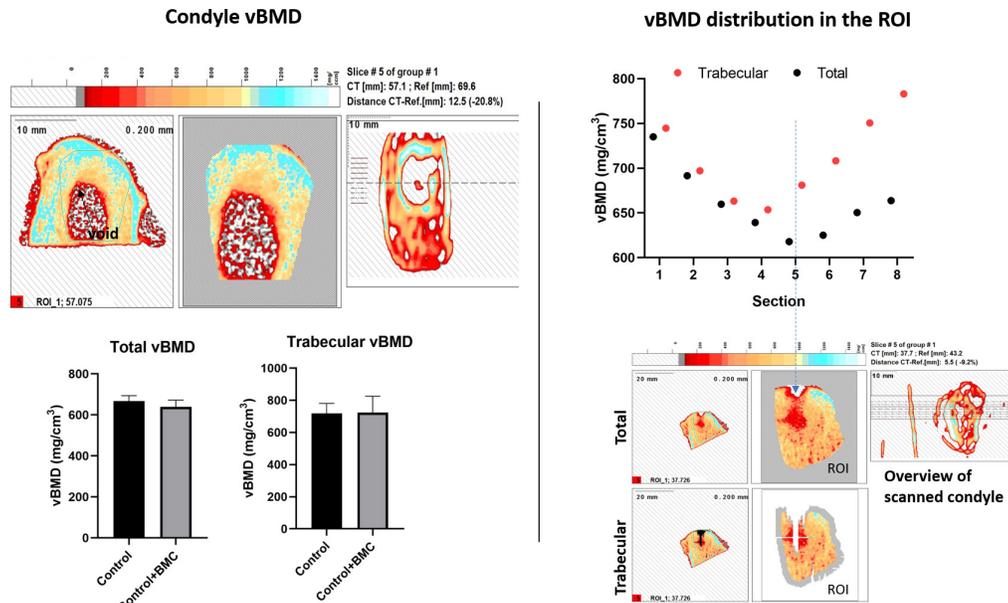


Fig. 5

Peripheral quantitative CT (pQCT) examinations showing a void remaining in the defect site (left); volumetric bone mineral density (vBMD) distribution in the region of interest (ROI) (right), showing a lower vBMD of the regenerated bone (both trabecular and total) in the middle of the defect site. Differences in the calculation of total vBMD and trabecular vBMD are also shown, where only the bone with a mineral density of more than 540 mg/cm³ is considered in trabecular calculations. Total and trabecular volumetric bone mineral densities in the ROI showed no significant differences between groups with and without bone marrow concentrate (BMC). Error bars: standard error of means.

Table V. Volumetric bone mineral density of regenerated bone in the region of interest in control and bone marrow concentrate groups (n = 5).

Mean vBMD, mg/cm ³ (SD)	Control	BMC	p-value
Trabecular	719 (61.5)	724.7 (101)	0.915
Total	667.9 (63)	639.9 (72.1)	0.508

BMC, bone marrow concentrate; SD, standard deviation; vBMD, volumetric bone mineral density.

time in the control group, ACAN was downregulated ($p = 0.048$, one way ANOVA followed by Tukey's t -test) when compared to the untreated healthy cartilage. As expected, *RUNX2* was not expressed in mature untreated cartilage, while it was expressed in all other groups, as shown in Figure 4c. The upregulation of *COL2* messenger RNA (mRNA) in the BMC treated group is consistent with enhanced chondrogenesis.

Bone regeneration. vBMD in 5/6 animals per group was evaluated using pQCT (Figure 5 and Table V). In all samples, the new bone which invaded the defect after six months was usually characterized by a lower mineral density (below 400 mg/cm³). Typically, a bone void was observed in the middle of the tissue (Figure 5). Trabecular and total vBMD did not show any significant differences between control and BMC-treated groups.

The bone microstructures were assessed by microCT in 6/6 animals in each group (Figure 6a). No large differences ($p = 0.568$, unpaired t -test) in new bone formation

between the two groups were noted, however, qualitatively, bone ingrowth in the defect site appeared to be more consistent in the BMC group, as observed in Figure 6a.

Quantitatively, mean bone volume density was not significantly higher in the BMC-treated animals (65.41 (SD 8.9)) compared to the control group (59.8 (SD 21.5)) ($p = 0.568$, unpaired t -test). Similarly, trabeculae were not significantly thicker in the BMC group ($p = 0.429$, unpaired t -test). The new bone formation varied within the groups, therefore samples with the best and worst performances in each group are shown in Figure 6a. Microscopically, there seemed to be no residues of the collagen scaffolds after six months. Optical microscopy of resin embedded sections showed some degree of repair in both groups, however large voids, subchondral bone oedema, and fibrous tissue could be seen in all the samples (Figure 6b), as also shown in radiographs of the joints (Supplementary Figure a).

Changes in synovial fluid. We investigated the total protein content of synovial fluid (SF) in both stifle joints in 4/6 animals from each group. The total protein content in the left joint was normalized to that of the right joint, as presented in Figure 7. The total SF proteins in all groups (705 to 1,900 μ g/ml) were within the reference range (< 18,000 μ g/ml)^{35,36} and below the threshold for inflammatory/abnormal conditions. There was no significant difference between the normalized protein content between the BMC and control groups.

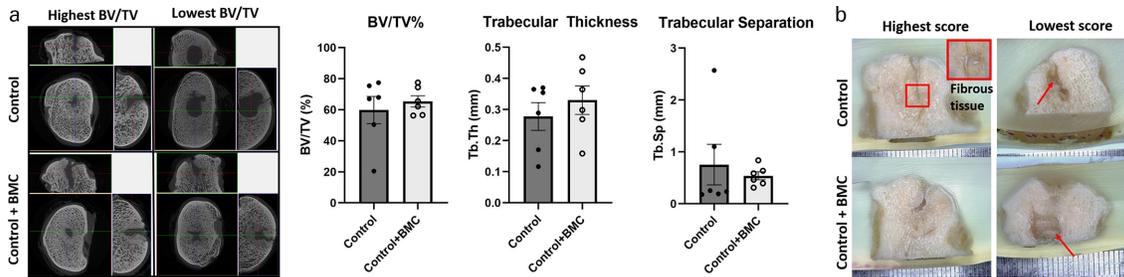


Fig. 6

Evaluation of bone regeneration: a) microCT images show samples with the highest and the lowest bone volume over total volume (BV/TV) in each group (best and worst performance); structural measurements from microCT: percentage trabecular bone volume (BV/TV%), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). Slightly higher bone regeneration and trabecular thickness in bone marrow concentrate (BMC) group, which resulted in slightly lower trabecular separation. b) Fibrous tissue and subchondral bone oedema (red arrows) are seen in both groups. Error bars: standard error of means.

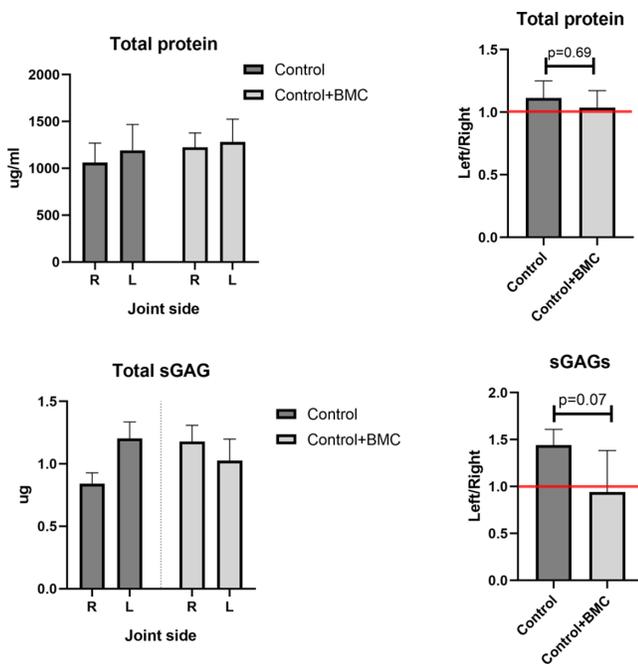


Fig. 7

Synovial fluid measurements: total protein content and sulphated glycosaminoglycans (sGAG). The graphs on the right show the normalized amount of the left leg to the right leg. Error bars: standard error of means. BMC, bone marrow concentrate.

Breakdown of cartilage can release molecular markers of proteoglycan degradation into SF. Therefore, sulphated glycosaminoglycans (sGAG) in SF, which indicate the extent of aggrecan degradation,³⁷ can be used as a probe to monitor cartilage destruction. Comparing the operated and nonoperated joints, the SF in the control group seemed to have a higher concentration of sGAGs in the left joint compared to the nonoperated joint ($p = 0.060$, unpaired *t*-test); this was not the case with the BMC group. When the amount of sGAG in the left joint was

normalized to the right joint, the BMC group showed lower evidence of cartilage breakdown and sGAG release into the SF ($p = 0.079$, unpaired *t*-test).

Lymph nodes and blood biochemistry. The popliteal lymph nodes of 6/6 animals from each group were analyzed and found to be of normal size and colour. However, an enlargement of more than 30% in weight in the left node compared to the right node was observed in three of six cases in the control group and two of six cases in the BMC group. Overall, the changes in these lymph nodes were of mild to moderate reactive hyperplasia and these reactive changes were greater on the left compared to the right. Interestingly, multinucleated giant cells were observed in two out of six nodes in the control group in cortical sinuses, while they were not present in the BMC group. Also, a larger number of lymphocytes were observed in the subcapsular sinuses of the BMC group. In fact, five out of six nodes showed a higher number of lymphocytes in the BMC group compared to only one out of six in the control group. The draining lymph nodes were assessed using a quantitative scoring system, showing that there were significantly fewer germinal centres in the left node compared to the right in the BMC group.

The blood haematology and biochemistry profile of four sheep in each group at three timepoints (preoperatively, three months, and six months) were compared, and the analysis for seven of the biomarkers, are presented in Supplementary Figure b. Two of the biomarkers, red blood cells (RBCs) and triglycerides, had significantly different preoperative levels ($p = 0.038$ and 0.026 , respectively, two-way ANOVA followed by Sidak multiple comparison test), however, over time no statistically significant differences were observed between the groups. None of the measured biomarker concentrations were outside the normal range according to Dimauro et al.³⁸

Discussion

Point-of-care biological products such as BMCs are autologous products that can be easily delivered to bone/cartilage defects by osteochondral scaffolds. In this study,

we have examined the effect of BMC addition to a multi-layered scaffold for the treatment of large osteochondral injuries in an ovine model.

We compared cartilage and bone regeneration in the critical-sized defects treated with a scaffold with/without BMC. In terms of cartilage, no statistically significant differences were noted in the gross cartilage repair score or the quantitative histology score, however, significant upregulation of *COL2* gene expression was observed in the BMC group over the control group, while *ACAN* was significantly downregulated in the control group. This shows that BMC may be beneficial to cartilage repair by enhancing the expression of the chondrogenic transcription factors such as *COL2* and maintaining *ACAN* expression. However, it was also noted that *COL1* expression and *RUNX2* expression, which were absent in healthy cartilage, were increased in both groups. *RUNX2* plays a vital role in chondrocyte maturation, hypertrophy, and vascularization.³⁹ *RUNX2* is also one of the genes responsible for the pathogenesis of OA as it is upregulated in chondrocytes of OA cartilage.⁴⁰ Expression of this gene in both groups indicates that BMC is perhaps not enough to induce hyaline cartilage formation, and results in a hyaline-like tissue or a fibrocartilaginous tissue. Qualitative assessment of the repair tissue with Safranin-O and alcian blue confirms this mixed response: in the best repairs, there was strong positive staining, indicating proteoglycan deposition and the formation of hyaline cartilage, and in the worst cases there was a lack of strong staining using alcian blue, but positive staining with fast green, indicating the formation of a fibrous/fibrocartilaginous tissue. Evidence of less cartilage breakdown, shown by the lower release of sGAGs into SF in BMC group, may indicate that BMC could act as a chondroprotective agent.

In addition to the effects of BMC on cartilage repair, the presence of BMC on the scaffold affected subchondral bone formation, albeit minimally. The amount of new bone formation (BV/TV%) in the BMC group was slightly higher (65 vs 59%) than the control group, however this was not statistically significant. A similar trend has been observed in some of the large animal studies¹⁸ where bony defects were smaller in groups treated with BMC, but not significantly. This contrasted with other studies that looked at the effects of BMC on bone regeneration. In one study, it was observed that the BV/TV % in the group with BMC was significantly higher (51 vs 29%) at the six-month timepoint when compared with the scaffold alone.¹⁴ The reasons for this are unknown, however we speculate that different methods of bone marrow concentration and individual variability in the final BMC could contribute to this contrasting result. Another explanation could also be the differences in the scaffolds used across these studies, since the materials¹⁸ or structure could affect the tissue regeneration.

Subchondral bone cysts are often detected in osteochondral repair models; however, it was expected that the addition of BMC would reduce the occurrence of these cysts as noted in several studies.^{17,41,42} An interesting

observation in this study was the presence of subchondral bone cysts in most of the defects, regardless of the treatment group. It is possible that the larger defect size in our model could not heal to the same extent in six months, or this could indicate that even after six months the cancellous bone adjacent to the implanted material is still undergoing a remodelling process.¹⁶

Microscopically, there seemed to be almost no residues of the scaffolds after six months, and the defects were filled with newly formed bone/cartilage or fibrous tissues. In our previous experience of this scaffold in sheep, most of the resorption seemed to have been completed by three months. This is in agreement with some of the studies conducted on collagenous scaffolds showing extensive scaffold arthroplasty.^{43,44} No/minimal unusual cell behaviour, such as polymorphonuclear/mononuclear cells infiltration, was observed after six months, which is in line with the observations by Jiang et al.⁴⁵ Histologically, we noticed more cells within the regenerated matrix in both groups when compared to normal mature cartilage, although large variations between animals were seen (Figure 4b).

BMC treatments are generally considered to be safe. Most clinical studies have looked at the safety of this procedure in terms of “adverse events”,⁴⁶⁻⁴⁹ however, a literature search showed that hardly any other data are reported for safety measurements. Here, we attempted to understand the effects of BMC on the draining lymph nodes. We observed that five out of six nodes showed a higher number of lymphocytes in BMC compared to only one out of six in non-BMC. It was interesting to note that the lymph nodes of the operated leg were on average 25% bigger than the unoperated leg in the control group, whereas this was reduced to only about 5% in the BMC group.

The above findings only partly supported our hypothesis that BMC improves osteochondral tissue regeneration. The results suggest that the addition of BMC combined with a collagen-HAp scaffold improves cartilage regeneration by upregulation of *COL2* and lowering GAGs breakdown. Furthermore, a better bone regeneration was observed with BMC, however the improvements were incremental. Clinically, in terms of cost, BMC is relatively cheap compared with using expanded cells, and therefore clinicians may want to perform an economic cost analysis to decide on the clinical use of BMC for osteochondral tissue regeneration.

Supplementary material



Scaffold fabrication methodology, and supplementary figures (radiographs and blood tests).

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