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In vitro testing of Advanced JAX™ Bone Void Filler System: species differences in the response of bone marrow stromal cells to *β* tri-calcium phosphate and carboxymethylcellulose gel

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Abstract The Advanced JAX™ Bone Void Filler System (AJBVFS) is a novel bone graft material manufactured by Smith and Nephew Orthopaedics Ltd. and comprises *β* tri-calcium phosphate granules with carboxymethylcellulose (CMC) gel as a handling agent. This study investigated the potential, in vitro, of the AJBVFS to function as a delivery system for cell therapy to enhance healing of bone defects. The attachment of rabbit bone marrow stromal cells (rbBMSCs), human BMSCs (hBMSCs) and human bone-derived cells (hBDCs) to JAX™ granules and the effect of CMC gel on cell proliferation and differentiation were investigated. There were slight species differences in the number and morphology of cells attached on the JAX™ granules with less rbBMSC attachment than human. All cells tolerated the presence of CMC gel and a reduction in cell number was only seen after longer exposure to higher gel concentrations. Low concentrations of CMC gel enhanced proliferation, alkaline phosphatase (ALP) expression and ALP activity in human cells but had no effect on rbBMSC. This study suggests that AJBVFS is an appropriate scaffold for the delivery of osteogenic cells and the addition of CMC gel as a handling agent promotes osteogenic proliferation and differentiation and is therefore likely to encourage bone healing.

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Introduction

The Advanced JAX™ Bone Void Filler System (AJBVFS) is manufactured by Smith and Nephew Orthopaedics for use as a bone graft material. It comprises *β* tri-calcium phosphate (TCP), in the form of 6-armed granules (see Fig. 2) that interlock to provide an osteoconductive scaffold with 55% intergranular porosity. Carboxymethylcellulose (CMC) gel is included as a handling agent and is designed to be mixed with JAX™ in a ratio of 1:1 to aid retention of granules in the defect site. JAX™ granules have been approved for clinical use and CMC gel has been used both clinically and experimentally for a variety of purposes such as wound healing [1] and drug delivery [2–6].

Current bone substitutes provide an osteoconductive scaffold but have little or no osteoinductive properties making their clinical performance inferior to autograft [7]. Attempts to remedy this by adding osteogenic growth factors absorbed onto a biomaterial [8–12], injected locally [13–15] or delivered systemically [14] have met with varying degrees of success in terms of improving bone healing in experimental models-although some products are now commercially available such as INFUSE® which is bone morphogenetic protein-2 (BMP-2) on a collagen sponge, and OP-1 (BMP-7).

More recently, scaffolds have been placed in bone defects supplemented with osteogenic cells either in the form of bone marrow aspirate [16–19] or ex vivo expanded bone marrow stromal cells (BMSCs) [20–26]. Again, there are commercially available products in this area such as Healos® bone graft replacement—an hydroxyapatite coated scaffold designed to be used with bone marrow aspirate. The rationale for using bone marrow is based on the presence of mesenchymal stem cells (MSCs) within the mononuclear cell fraction which give rise to multiple cell types including osteoblasts and chondrocytes [27]. The process of using culture expanded autologous cells is known as cell therapy and has been trialed in patients for treatment of fracture nonunions (unpublished data) and myocardial infarction [28].

This project investigated the potential, in vitro, of the AJBVFS to function as a delivery system for cell therapy to enhance healing of bone defects. To do this we examined cell attachment to JAX™ granules and the effect of CMC gel on cell proliferation and differentiation. In vivo product testing was to be carried out in a rabbit defect model (Bone 2007; 40(4):939–947) therefore we compared the response of rabbit BMSCs (rbBMSCs) with human BMSCs (hBMSCs). Furthermore, as the product is intended for use as a scaffold for osteogenic cells but will also come into contact with osteoblasts at the defect site, hBMSCs were compared with bone derived cells (BDCs) from the same donors.

Materials and methods

JAX™ advanced bone void filler system

JABVFS is a two-component system composed of β tricalcium phosphate granules and a carboxymethylcellulose hydrogel. JAX™ granules are 4 mm tip to tip and the interlocking granule arms provide 55% intergranular porosity in the defect site. It has a resorption time of 9–12 months and is indicated for non load bearing defect sites less than 4–5 cm. The CMC hydrogel consists of 1.75% (max) CMC and 10% glycerol in water. This is equivalent to 0.0175 g/mL of CMC.

Patient details

The number of human samples available was not large enough to allow strict exclusion criteria to be set however, age, sex and current medication for each donor were noted to inform analysis (Table 1). All procedures were performed with informed consent following approval from the Local Research Ethics Committee.

Cell preparation

Bone marrow was obtained from both femora and tibiae of cadaveric New Zealand White rabbits (male, mean age: 17 weeks) within an hour of death. Bone marrow was harvested into 5 mL of PBS containing 300 μL of heparin and stored on ice until use. Human bone marrow was harvested from the iliac crest during elective surgery. Up to 8 mL of aspirate was taken from a single puncture site, placed in 500 μL of heparin and stored on ice until use. The mononuclear cell fraction of bone marrow (both species) was isolated by density centrifugation using Lymphoprep according to the manufacturer's instructions (Sigma, UK). Cells were plated at 3×10^5 cells/cm^2 in complete medium [αMEM + 20% FBS + 100 μM ascorbate-2-phosphate + 2 mM L-glutamine + 100 U/mL Pen/strep + 2.5 μg/mL fungizone (all Gibco except ascorbate-2-phosphate:Sigma)], left undisturbed for 7 days then fed biweekly thereafter. Cells were passaged (1 flask: 4 flasks) when approximately 90% confluent using 0.25% trypsin/EDTA (Gibco).

Trabecular bone (approximately 1 cm^3), was harvested from the iliac crest of patients during elective surgery placed in PBS and stored on ice. Using aseptic technique, the bone was stripped of soft tissue, chopped into small fragments and washed several times in PBS until the fragments were white [29]. These fragments were then placed in a T75 cm^2 tissue culture flask, with complete medium and left undisturbed for 7 days. Thereafter, medium was replenished biweekly and cells were passaged (1 flask: 4 flasks) when approximately 90% confluent using 0.25% trypsin/EDTA (Gibco).

All cell types were expanded until there were sufficient cells to allow all experimental conditions to be performed on cells from each donor animal or patient.

Cell attachment to JAX™ granules

Six JAX™ granules were placed in each well of a non-tissue culture, 96 well plate. Cells (average passage 3 (P3) for BMSC, average explant 1[1] (E1), P2 for BDC) were seeded onto these granules at 1×10^4 and 1×10^5 cells/well and cultured for 1, 7, 14 and 21 days. Medium was changed biweekly. At each time point, the JAX™ from one well were processed for SEM (see below). The remaining 4 wells were washed with carbonate buffer (pH 10.2) several times, then 150 μL of 0.1% Triton-X 100 (Sigma) in carbonate buffer was added to each well and the plate was freeze/thawed (x4) to lyse the cells. The quantity of double stranded (ds) DNA in each sample—which relates to the cell number—was then calculated using a

[1] Explant 1 is the first flask of cells to grow from fresh bone chips.

Table 1 Details of human donors

ID	Age	Sex	Surgery	Drugs
2	49	M	Spinal fusion	Steroid inhaler
3	70	F	Foot arthrodesis	None
4	73	F	Foot arthrodesis	Atorvastatin
5	23	F	Foot arthrodesis	None
8	77	F	Foot arthrodesis	None
9	59	M	Foot arthrodesis	Pravastatin Steroid inhaler

picogreen assay according to the manufacturer's instructions (Molecular Probes).

Scanning electron microscopy

Briefly, JAX^{TM} granules were washed (x3) with sterile PBS and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C. The granules were washed in 0.1 M sodium cacodylate buffer, dehydrated through increasing alcohols and dried in a critical point drier. After mounting onto stubs and gold sputter coating in a Polaron SEM coating system and argon atmosphere, the samples were viewed in a Jeol JSM-840A scanning electron microscope.

Effect of CMC gel on cell number

Each of the three cell types were plated into 96 well plates (average P3 for BMSC, average E1, P2 for BDC) at 1×10^5 cells/cm^2 in complete medium. The cells were allowed to adhere for 2 h after which time the medium was removed and medium containing increasing amounts of gel were added. Medium:gel (v/v) ratios used were: no gel, 100:1, 10:1, 5:1, 2:1 and 1:1 (equivalent to CMC doses of 0, 0.2, 1.6, 2.9, 5.8 and 8.75 g/L respectively). Up to four time points (d1, d4, d7 and d14) and six repeats per treatment were performed depending on cell yield. Cells were fed biweekly with continuous exposure to gel. At the end of each experiment, cells were washed (x6) with carbonate buffer to completely remove gel, 150 µL of 0.1% Triton-X 100 in carbonate buffer was added to each well and the cells were lysed as before. The total amount of dsDNA in each lysate was determined using the picogreen assay.

Effect of CMC gel on alkaline phosphatase

The effect of CMC gel on alkaline phosphatase (ALP) activity and expression was examined. ALP activity was measured for all three cell types using the same lysates prepared above following exposure to medium:gel ratios of 0, 100:1, 10:1, 5:1, 2:1 and 1:1 and ALP expression was investigated using a colony forming Unit-fibroblastic assay (CFU-F assay).

ALP activity was determined in cell lysates using a colourimetric assay. Briefly, 50 µL of samples and *p*-nitrophenol standards (Sigma) were placed in a non-tissue culture 96 well plate. 200 µL of 4-nitrophenol phosphate (pNPP; Sigma) in 1.5 M alkaline buffer solution (Sigma) was added to each well and incubated for 30 min in the dark. The reaction was stopped by the addition of 50 µL of 1 M NaOH and read at 405 nm wavelength [30]. The results were normalized for the amount of DNA in each lysate using results from the picogreen assay.

CFU-F assay was performed as follows. Freshly aspirated bone marrow (human and rabbit) was passed several times through a 21 gauge needle to homogenize the sample. Following red cell lysis with 2% acetic acid, cell number and viability in the sample were determined using a modified Neubauer haemocytometer and trypan blue exclusion assay [31]. Cells were then plated at 1×10^5 cells/cm^2 in 24 well plates and exposed immediately to medium:gel ratios of 0, 100:1, 10:1, 5:1, 2:1 and 1:1 without a period for cell attachment. Time points were d7, d14 and d21 with three repeats per treatment. Following treatment, the wells were washed several times with PBS to remove the gel, fixed in 90% alcohol for 30 min and allowed to air dry. Cells were then stained for alkaline phosphatase (Sigma kit 86-c), photographed on a light box, then restained with toluidine blue (0.1% for 1 h) to stain all colonies. Colony number and area for both total colonies and ALP-positive colonies were measured semi-automatically using Scion Image freeware (available as a free download from http://www.scioncorp.com).

Statistics

The data was not normally distributed therefore analysis of differences between treatments was performed using Kruskal–Wallis with Mann–Whitney *U* post hoc testing on data from individual donors.

Results

Cell attachment to JAX^{TM} granules

SEM micrographs (Fig. 1a–d) showed rbBMSC with a fibroblast-like, stellate morphology attached to TCP JAX^{TM}, similar to that seen on tissue culture plastic, however some cells remained rounded. With time, cell number increased and cells appeared to proliferate and migrate on the JAX^{TM} surface.

hBMSCs (Fig. 2a–f) and hBDCs (Fig. 3a–d) on JAX^{TM} had an elongated morphology but, unlike rbBMSC, there were no rounded cells seen on the granules. All cells spread out on the JAX^{TM} surface and even spanned cracks. Again

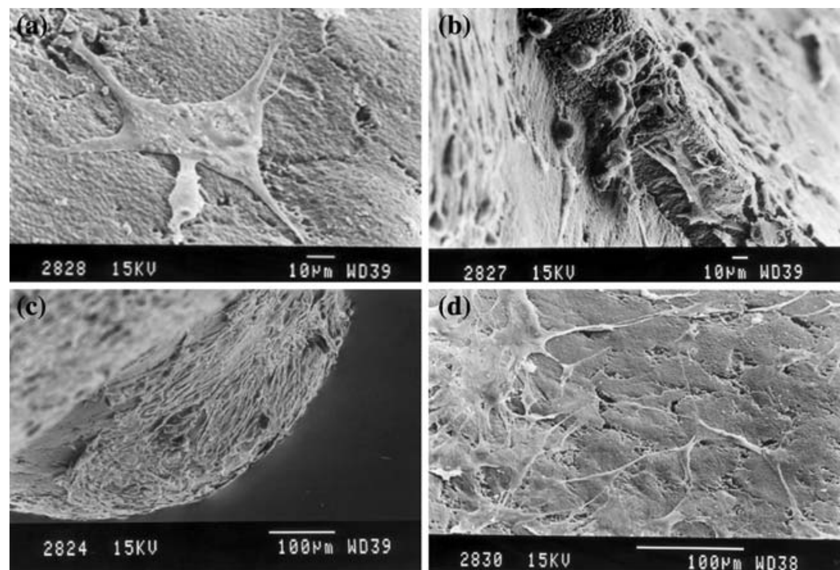

Fig. 1 SEM micrograph: Rabbit BMSC cultured on TCP JAX™ showed that some cells displayed a fibroblast-like morphology (**a**) while others remained rounded up even after 7d in culture (**b**). There is an apparent increase in cell number with time at d7 (**c**) and the cells appear to be proliferating and migrating on the JAX™ surface (**d**)

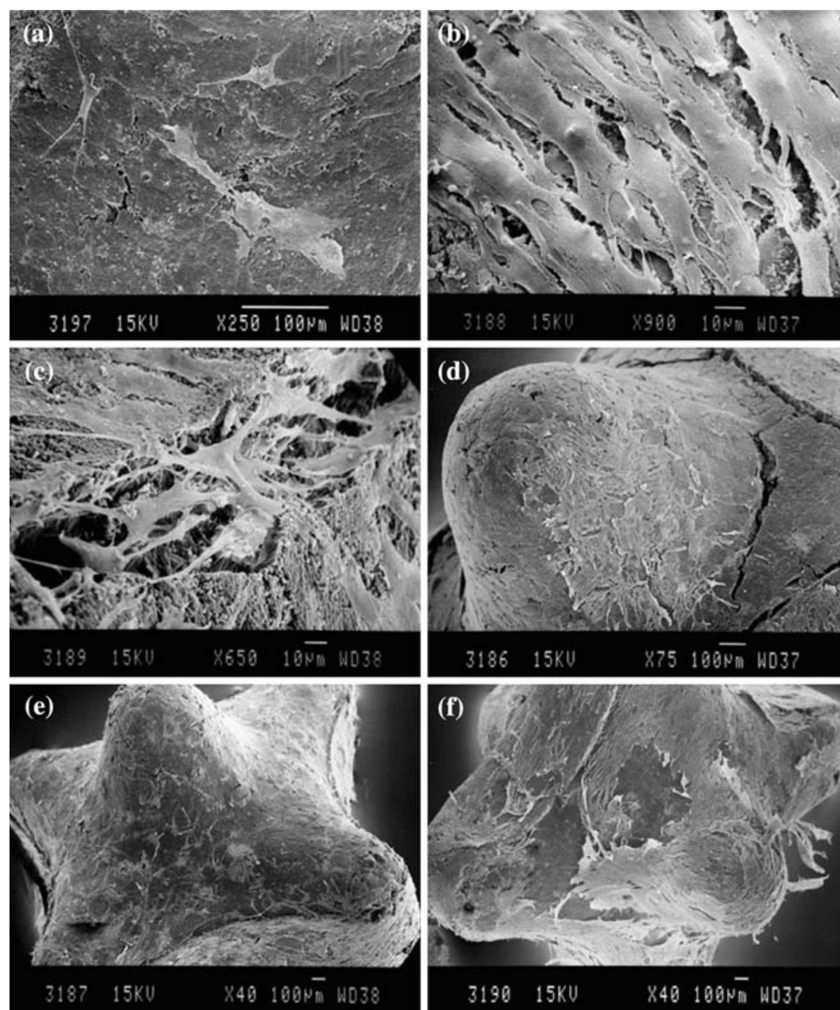

Fig. 2 SEM micrograph Human BMSC on TCP JAX™ shown by SEM. All cells were fibroblast-like shaped (**a**, **b**) and even spanned cracks in the JAX™ surface (**c**). There was an increase in cell number from d1 (**d**) to d7 (**e**) to d14 (**f**)

there was an increase in cell number with time and at the higher cell concentration at d14 confluency caused the cells to detach. For all cell types the best coverage was seen on the 'up' surface of the granule but by d7 and d14 there was good coverage over most of the surface indicating that the cells proliferate and migrate on the JAX™.

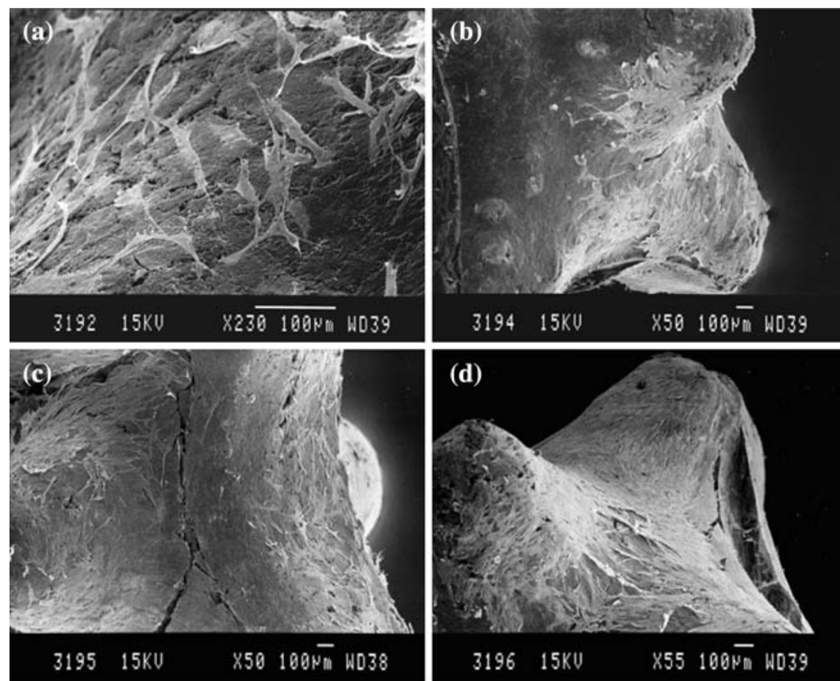

Fig. 3 SEM micrograph Human BDC on TCP JAX™. Cells are fibroblast-like shaped (**a**) and there is an increase in cell number from d1 (**b**) to d7 (**c**) until confluency at d14 (**d**) caused them to detach

DNA analysis confirmed that more cells attached to JAX™ at the higher cell concentration (data not shown). There was a time dependent increase in cell number of hBDCs but not rbBMSCs or hBMSCs. Absolute amounts of DNA showed that there were more human cells attached to JAX™ granules than rabbit at equivalent cell concentrations and times.

Effect of CMC gel on cell number

rbBMSC: At earlier time points, CMC gel had no effect on cell number but by d7 and d14, the two highest concentrations of gel reduced the number of cells in each well ($n = 4$ except at d14 where $n = 2$; Fig. 4a). The lower concentrations of gel continued to have no effect on rbBMSC number even after 2 weeks in culture. The effect of CMC dose on cell number was significant on cells from all rabbits at d7 ($p < 0.001$, $p = 0.001$, 0.014, 0.007) and d14 ($p < 0.001$, p = 0.045).

hBMSC: At d1 there is no effect of the gel on cell number and at the later time points there is a reduction in cell number in the presence of the highest two concentrations of gel ($n = 6$; Fig. 4b). Unlike rbBMSC however, the lower concentrations of gel at d4 and d7 increased cell number. This is consistent for each donor and is lost by d14. There was a statistically significant effect of gel concentration on hBMSCs from 2/6 donors, 5/6 donors and 4/6 donors at d4, d7 and d14 respectively. Post hoc testing using the Mann–Whitney *U* test found that gel concentrations of 100:1 and 10:1 had a significant effect on cell number at d4 and d7 in a third of cases.

HBDC: CMC gel had a similar effect on cell number. No effects were seen at d1 but at the later time points there was a reduction in hBDC number with the higher concentrations of gel ($n = 5$; Fig. 4c). An increase in cell number with lower gel concentrations was also seen, with a peak at the 10:1 dose. There was a significant difference between treatments in 5/5, 4/5 and 4/5 donors at d4, d7 and d14 respectively. Post hoc testing showed that there was a significant difference in cell number between control and lower gel concentrations in 4/5, 2/5 and 2/5 donors at d4, d7 and d14 respectively.

Effect of CMC gel on alkaline phosphatase

rbBMSCs: CMC gel had no effect on the ALP activity at any time point ($n = 4$; Fig. 4d). hBMSC: There was no effect of the gel on ALP activity at d1 but an increase in ALP activity with increasing gel concentration at the later time points was seen ($n = 6$; Fig. 4e). This effect was significant in at least one case at all time points.

hBDCs: There was an increase in ALP activity with lower gel concentrations at later time points similar to that seen with the cell number results above (Fig. 4f). This treatment effect was significant in 4/5 and 5/5 cases at d7 and d14 respectively.

Effect of CMC gel on colony forming efficiency

rbBMSCs (Fig. 5a–c): There was a reduction in both the total number of colonies and number of ALP-positive colonies with higher gel concentrations (statistically

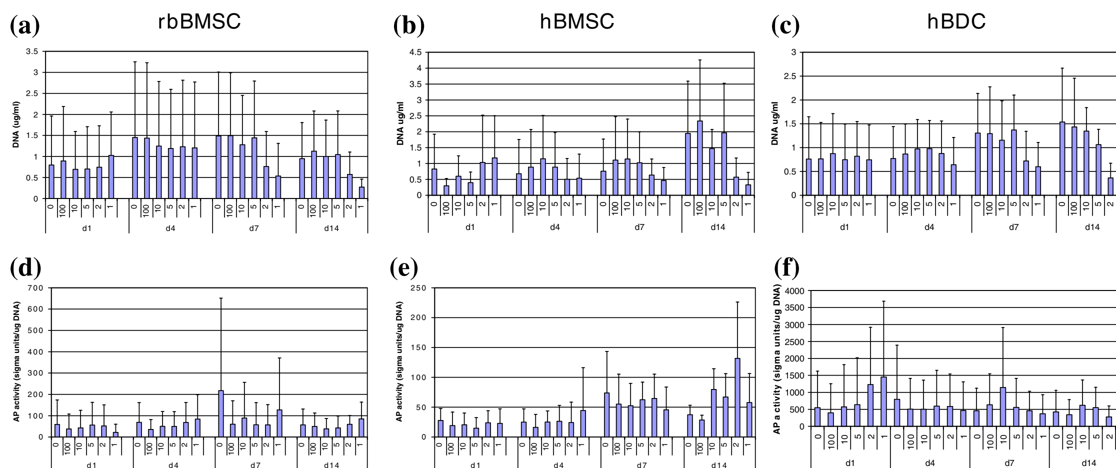

Fig. 4 Results from experiments using rbBMSC (**a** and **d**), hBMSC (**b** and **e**) and hBDC (**c** and **f**); total dsDNA in cell lysates following exposure to CMC gel (**a**, **b** and **c**) and alkaline phosphatase activity in lysates following exposure to CMC gel normalised to DNA content (**d**, **e** and **f**). (mean + std dev)

significant at d21). When number of ALP-positive colonies was normalized as a percentage of the total number, there was no dose dependent effect on ALP expression. Colonies exposed to the higher concentrations (2:1; 1:1) of gel were smaller. ALP-positive colonies were smaller than ALP negative and showed a similar response to the gel. There was no effect on colony area at the lower doses of gel.

hBMSC (Fig. 5d–f): Lower concentrations of gel had no effect on overall colony number but increased the number of colonies that were ALP-positive (Fig. 5d). The positive effect on ALP expression is still apparent when the ALP-positive colonies are normalized as a percentage of total colony number (Fig. 5f). Lower concentrations of gel (100:1; 10:1; increased colony area Fig. 5e).

Discussion

This study investigated the potential of the Advanced JAX^{TM} Bone Void Filler System (βTCP granules and CMC gel) to support cell growth in vitro. Three different cell types were used; rabbit BMSC, human BMSC and human BDC.

SEM showed clearly that cells attached and spread on the granule surface and both SEM and DNA results (not shown) suggest that human cells (hBMSC and hBDC) attached better than rabbit BMSCs.

Statistical analysis was performed on data from individual donors. Inter-individual differences in measurements were large therefore many more human and rabbit samples would be required to achieve statistical significance on pooled data. Such large numbers of samples were not available for this study. However each experiment was carried out with sufficient repeats to allow statistical analysis on each data set and effects that were repeated across donors were deemed noteworthy.

Higher concentrations of CMC gel resulted in a reduction in cell number with all cell types although it is not known if this is due to direct cytotoxicity, an inhibition of proliferation or a combination of the two. All three cell types tolerated lower concentrations of CMC gel well and other studies have also reported that CMC gel has no effect on viability of the fibroblast cell line, 3T3, the osteoblast-like cell line, MG63 [32] and a murine neural stem cell line [33]. In the latter study, CMC gel had no affect on viability until the concentration reached 10 g/L.

In the current study, it was only at later time points that higher concentrations of gel had a detrimental effect, suggesting that short-term exposure to the gel would have little effect. The gel is intended for use in a bone defect site where it could be diluted almost immediately with blood, extracellular fluid and inflammatory exudates. If this is the case it is likely that CMC gel used in the clinical situation would not be detrimental to cell viability even if used in its undiluted formulation, but this needs to be verified by in vivo testing. The gel is quite viscous and its clearance from the defect site will depend on many factors.

Analysis of individual data showed that in most cases low concentrations of CMC gel enhanced both cell proliferation and differentiation (shown by ALP expression and activity) of human BMSCs and BDCs. This finding is supported by others who found an increase in ALP activity from human bone marrow derived fibroblasts in the presence of CMC gel [5]. This effect was not seen with rabbit BMSCs, although others have reported enhanced healing of defects stimulated by the CMC carrier alone in a rabbit model [4] when investigating the use of CMC gel as a delivery system for osteogenic growth factors.

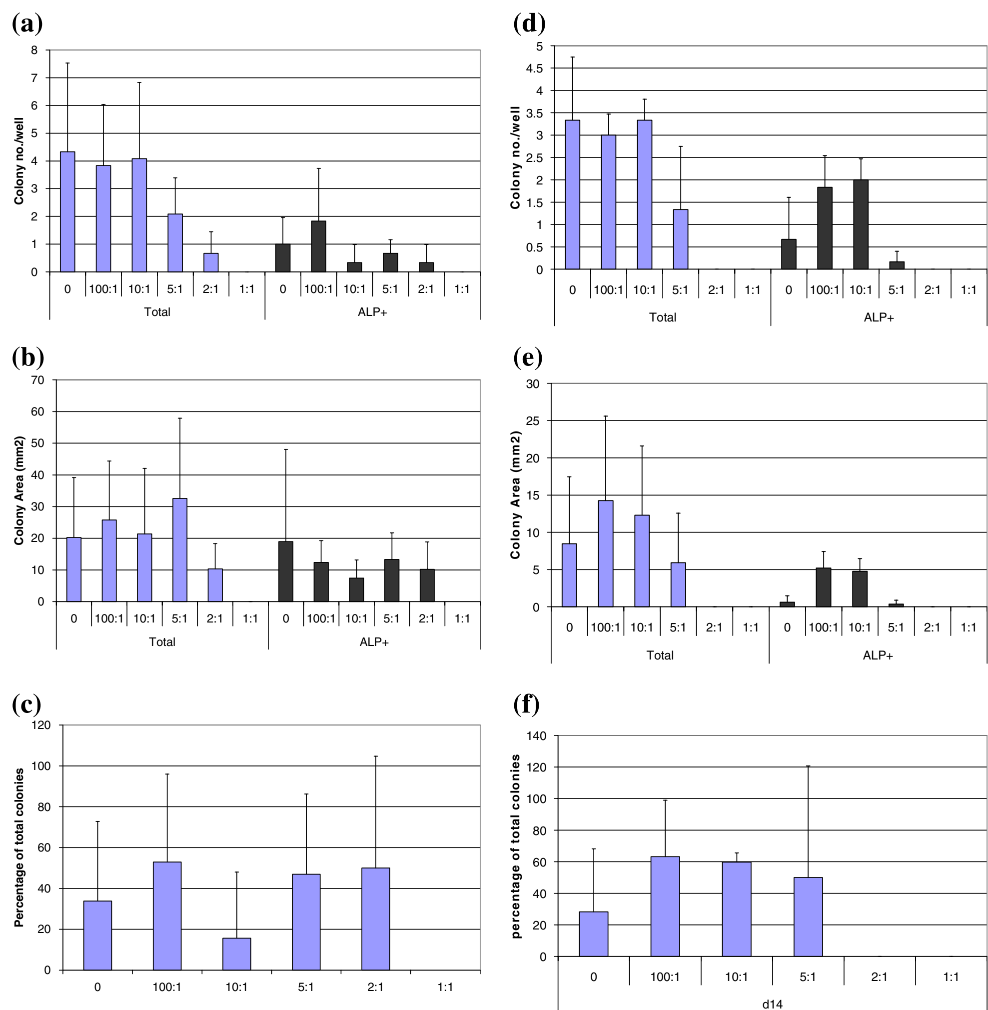

Fig. 5 CFU-F assay using rabbit (n = 3; **a–c**) and human (n = 2; **d–f**) bone marrow cells exposed to CMC gel for 21d and 14d respectively. (**a** and **d**) number of total and ALP-positive colonies, (**b** and **e**) mean area of total and ALP-positive colonies, (**c** and **f**) ALP-positive colonies as a percentage of total colonies. (mean + std dev)

In the CFU-F assay, colony size gives an indication of the proliferative rate of the cell population and colony number is an indicator of the number of stem cells or progenitors in the population [34]; those that are ALP-positive have osteogenic potential. The results from the CFU-F assays using fresh bone marrow cells therefore are similar to those using culture expanded BMSCs; there was a reduction in cell number at higher concentrations of gel for both species but with human cells there was also an increase in proliferation and ALP expression in the presence of low gel concentrations that was not seen with rabbit. Again this highlights a species difference in response to CMC gel.

An increase in ALP activity was also found when human bone marrow cells were exposed in vitro to both low concentrations of gel and JAX^{TM} granules combined (results not shown—assays for cell number and ALP activity performed as above). Therefore it seems likely that the addition of CMC gel to the AJBVFS would have beneficial effects beyond its use as a handling agent and may actually enhance healing by increasing cell proliferation and osteogenic differentiation. In vivo testing must be performed to confirm this hypothesis but the species difference in both cell attachment to β tricalcium phosphate and response to CMC gel must be considered. In this instance the most appropriate model for in vivo testing of AJBVFS (JAX^{TM} plus gel) may be to use human cells in an athymic animal model.

In summary, CMC gel was well tolerated by all cell types and only reduced cell numbers after prolonged exposure to higher concentrations. There were species differences in both cell attachment to βTCP JAX^{TM} granules and in response to the CMC gel. Low concentrations of the gel increased proliferation and differentiation of human cells (both hBMSCs and hBDCs) and more human cells attached to JAX^{TM} in culture when compared to rbBMSCs. This study suggests that AJBVFS is an appropriate scaffold for the delivery of osteogenic cells and the addition of CMC gel as a handling agent is likely to further enhance bone healing through its positive effects on proliferation and osteogenic differentiation.

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