A Carboxymethyl Cellulose Bone Graft Carrier Delays Early Bone Healing in an Ovine Model

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ABSTRACT

A limitation in the use of calcium phosphate (CaP) is that in its raw form, it comprises blocks or granules, which are limited in their utility for orthopedic surgery and a number of commercial bone grafts are supplied within an aqueous based carboxymethyl cellulose (CMC) putty. Our hypothesis was that CMC combined with a porous silicate-substituted CaP (SiCaP) scaffold would have no negative effect on bone formation after implantation in an *ovine* femoral condyle. Defects were either (1) empty or filled with (2) SiCaP granules, (3) CMC-SiCaP Putty or (4) a SiCaP press-fit dry block. Scaffolds were identical in composition and remained *in vivo* for 4, 8 and 12 weeks. Bone apposition rates, bone area, %bone-implant contact and graft area were quantified. At 4 and 8 weeks, significantly more new bone and %bone-implant contact was measured within granules when compared with both putty and block scaffolds. At 12 weeks, significantly increased bone was measured for the granules when compared with blocks and no significant difference was found when the granules and putty scaffolds were compared. Results showed the disadvantageous effect that CMC may have on early bone growth and that granules increased new bone formation when compared with a press-fit block composed of the same material.

KEYWORDS: Bone regeneration; calcium phosphate; critical defect; bone substitute materials; carboxymethyl cellulose.

Running title: A Carboxymethyl Carrier

1. INTRODUCTION

Bone substitute materials are increasingly being used, especially in oncologic surgery, traumatology, revision prosthetic surgery and in spinal surgery. Over two million bone grafting procedures are performed each year, and bone substitute materials are often used due to their excellent biocompatibility, improved safety profiles, low cost, time advantages and adaptability to a variety of clinical challenges [1]. These materials can also reduce the use of autologous bone graft where graft harvest can cause donor site morbidity. However, a limitation with current calcium phosphate (CaP) bone substitute materials is that they are provided in granular form and because the granules are difficult to handle, this limits their utility during orthopedic surgery. Recently, injectable and moldable forms of bone substitutes such as pastes and putties have been developed as they offer many advantages including improved handling properties, such as cohesivity, moldability, and resistance to irrigation, yet are suited to filling contained defects of complex geometric shapes. Furthermore, with the development of minimally invasive surgical methods, the need to treat bony defects directly *in situ* with injectable bone grafts is increasing. Many studies have focused on the potential for polymeric cellulose to function as a biologically inert binder and thickener to enhance the handling properties of synthetic bone substitutes [2-8]. Carboxymethyl cellulose (CMC) is a water-soluble, non-toxic polymer derived from cellulose that has been successfully used as a binder for demineralized bone matrix and cancellous bone chips. Research suggests that CMC is well tolerated biologically and supports new bone formation [2, 3]. However, histological data characterizing the effect of CMC on bone regeneration is limited.

This study investigated the effect of using CMC as a bone graft binding agent, referred to as a putty, on bone formation within a porous silicate-substituted CaP (SiCaP) scaffold following implantation in an *ovine* femoral condyle model. CaP scaffolds are available both as granules

and in block form and this study aimed to determine the optimal structure for bone augmentation. Our hypothesis was that CMC would have no negative effect on bone formation and osteoconduction and that granules would provide an increased surface area, promoting earlier and increased new bone formation when compared with a block scaffold composed of the same material.

2. MATERIALS AND METHODS

Seventy-two cylindrical, critical sized defects measuring 8×15 mm were created in the medial condyle of both the left and right femur of 18 skeletally mature commercially crossbred adult female sheep weighing between 65 and 80 kg and aged between 2 and 5 years. This study investigated bone regeneration within three silicate-substituted calcium phosphate scaffolds. Four experimental groups were investigated at three time-points (4, 8 and 12 weeks) post-operatively; (1) empty defects, (2) SiCaP granules, (3) SiCaP granules combined with a CMC carrier (SiCaP Putty) and (4) a SiCaP press fit block. All SiCaP scaffolds investigated were phase pure and had an identical chemical composition and ultra-macroporous morphological structure. The granules investigated in groups 2 and 3 were of the same size and irregularly shaped measuring 1 - 2 mm. The silicate-substituted calcium phosphate (substituted with 2.6 wt % silicate or 0.8 wt % Si) used was phase-pure and materials in all treatment groups had the same total macroporosity of 77.5 – 82.5% with an average macropore diameter of 300 μ m. Scaffolds were manufactured with a strut porosity of $22.5 \pm 2.5\%$ (mean with standard deviation of mean) where micropores within the struts measured < 50 mm in diameter and had a typical diameter of 1 to 10 mm). All implants were provided sterile and pre-packaged by ApaTech Ltd (Baxter Healthcare, Elstree, UK). All scaffolds were manufactured via a foaming route [9] and the phase purity confirmed using X-ray diffraction (XRD) as previously described [9]. The calcium, phosphorus, and silicon content was determined by X-ray fluorescence

(XRF) [9] and total porosity was confirmed according to a water immersion densitometry method [10]. The strut-porosity was confirmed by mercury intrusion porosimetry in combination with helium pycnometry as detailed in our previous study [11]. The putty was prepared using 200 mg of sodium carboxymethyl-cellulose (Hercules, Inc Aqualon Division, Wilmington, DE; Type 7LF PH, 25–50 mPa.s viscosity) and 2 mL sterile saline. The granule packing characteristics were not measured in this study.

2.1 Surgery

Animals were placed in ventral recumbence and an incision ~ 3 cm in length made over the right femoral condyle. The muscle was exposed and parted by blunt dissection and two critical size 8×15 mm holes made. Following irrigation, the appropriate test material was applied and gently pressed into place. In order to prevent migration out of the defect site, the granular specimens without the CMC putty were mixed with venous blood collected from the animal at the time of surgery and the mixture was coagulated prior to insertion. For the block group, a rigid cylindrical plug measuring 8×15 mm was press-fitted into place. For the SiCaP putty group, at least 2 mls of putty was inserted per defect. The wound was then closed layer by layer. Sites allocated with test materials were rotated between animals such that no animal had two test materials of the same type within the same condyle. This procedure was repeated on the contralateral side resulting in a total of four implants per each animal. All procedures were carried out following Ethics approval granted by the Royal Veterinary College and in compliance with the United Kingdom Home Office regulations [Animal Scientific Procedures Act (1986)]. Following surgery, animals were administered with routine prophylactic antibiotics and analgesia and were allowed to mobilize as tolerated. Antibiotic and analgesic prophylaxis was administered daily with subcutaneous injections of Baytril (Enrofloxacin 5 mg/kg; Bayer AG Leverkusen) and Finadyne (Flunixin Meglumine 2 mg/45 kg; Schering-

Plough Ltd) for 3 days post-surgery. Animals were kept in individual pens for 1 week post operatively before being group housed. Two fluorochrome bone markers Oxytetracycline (30 mg/kg) and Calcein Green (30 mg/kg) were administered at weeks 1 and 3 in the four-week group, weeks 4 and 6 in the eight-week group and at 8 and 10 weeks in the twelve-week group. Oxytetracycline and Calcein Green localize to sites of mineralization and when viewed under ultra-violet light, fluoresce an orange and green color respectively. The mean measurable distance between the fluorescent lines provides an assessment of bone apposition rates ($\mu\text{m}/\text{day}$) within defects in each of the groups.

2.2 Histomorphometric Analysis

On retrieval, condyles were placed in 4% paraformaldehyde solution before being processed for undecalcified histology. Following dehydration in serial dilutions of alcohol, specimens were defatted and embedded in hard grade acrylic resin (LR White, London Resin Company, Reading, UK). Thin sections were prepared by making longitudinal cuts through the center of each defect using a diamond saw and then ground and polished to $\sim 70\ \mu\text{m}$ in thickness (EXAKT, Norderstedt, Germany). Samples were stained with Toluidine Blue and Paragon, which stained soft tissue and bone respectively. The sections were examined histologically using light microscopy and in each of the four experimental groups investigated, total new bone area, bone-implant contact and scaffold resorption was quantified. Six random regions of interest (ROI) were image captured using a $5\times$ magnification lens where three ROIs were located at the periphery and three within the center of each defect site (Axiovision Release 4.6, Carl Zeiss, Jena, Germany). A line intercept method was used and a mask of interconnecting lines measuring $10 \times 12\ \text{mm}$ was superimposed on top of each image and the type of material (mineralized bone, soft tissue or scaffold) at the intersection of each line was determined (total of 225 intercept points per image). Assessments were made to evaluate the proportion of

mineralized bone, soft tissue and implant material within each of the 6 regions in each defect. For bone-implant contact, the type of tissue at the interface was determined at the points where lines intersected with the biomaterial surface. Quantification of bone in pores $> 20\mu\text{m}$ were measured. Bone within pores $< 20\mu\text{m}$ was not measured as these sized pores occurred predominantly within the struts of the scaffolds. Data was quantified and compared between groups.

2.3 Statistical Analysis

Analysis of the data was performed using SPSS software (v10.1; SPSS, Chicago, Illinois). A Kolmogorov-Smirnov test showed the data obtained was non-parametric and the Mann-Whitney U test was used for statistical comparison between experimental groups. The Kruskal-Wallis test with post-hoc Mann Whitney U was used to compare data at different time points within one experimental group. p values < 0.05 were considered significant. Mean values $\pm$ standard error of mean are presented in the text.

3. RESULTS

3.1 Bone Apposition Rates

At 4 weeks post surgery, bone markers were seen incorporated within the scaffold with localized areas of new bone growth observed on the surface in all groups (Figure 1). Greatest bone apposition rates were measured in the SiCaP granules group ($1.02 \pm 0.49\mu\text{m/day}^{-1}$), however no significant differences were found when each of the groups were compared (Figure 2). At 8 weeks post surgery, significantly increased rates were measured in the SiCaP putty group ($2.22 \pm 0.22\mu\text{m/day}^{-1}$) when compared with the SiCaP granules group ($1.79 \pm 0.17\mu\text{m/day}^{-1}$; $p = 0.046$). In addition, significantly greater apposition rates were measured in the SiCaP block group ($1.91 \pm 0.39\mu\text{m/day}^{-1}$) when compared with the SiCaP granules group. At 12 weeks post operatively, no significant differences were observed when the granules, putty

and block groups were compared. Longitudinal analysis also showed no significant differences when each of the groups were compared over time.

3.2 New Bone Area

At 4 weeks post surgery, significantly increased bone area was measured within defects containing SiCaP granules ($3.97 \pm 1.11\%$) when compared with both SiCaP putty ($0.09 \pm 0.06\%$; $p = 0.028$) and SiCaP block scaffolds ($0.15 \pm 0.08\%$; $p = 0.028$) (Figure 3). This difference continued with significantly increased bone formation measured within the SiCaP granules group at 8 weeks ($20.14 \pm 4.91\%$) when compared with both the SiCaP putty ($5.47 \pm 1.46\%$; $p = 0.046$) and SiCaP block ($6.69 \pm 2.10\%$; $p = 0.046$) groups. At 12 weeks post surgery, significantly more bone was measured within defects in the SiCaP granules group ($40.56 \pm 3.73\%$) when compared with SiCaP blocks ($15.38 \pm 5.30\%$; $p = 0.028$). No other significant differences were found. Longitudinal analysis showed that in the SiCaP granules, putty and block groups, significantly increased bone was measured within defects when 4 week data was compared with results at 8 weeks ($p = 0.010$, 0.003 and 0.004 respectively). However, only defects in the SiCaP granules group showed a significant increase in bone area when the 8 and 12 week data were compared ($p = 0.010$).

3.3 Bone-Implant Contact

The SiCaP putty appeared to reduce early bone formation in the granules. At 4 weeks, results showed significantly increased bone-implant contact in the SiCaP granules group ($10.25 \pm 3.05\%$) when compared with both the SiCaP putty ($0.63 \pm 0.63\%$; $p = 0.028$) and SiCaP block group ($1.05 \pm 0.45\%$; $p = 0.028$) (Figure 4). At 8 weeks post surgery, significantly increased bone-implant contact was measured in the SiCaP granules ($42.03 \pm 9.46\%$) group when compared with defects containing SiCaP putty ($17.22 \pm 3.61\%$; $p = 0.046$). At 12 weeks

significantly increased bone-implant contact was measured in the SiCaP granules group ($70.84 \pm 6.66\%$) when compared with the SiCaP block specimens ($37.53 \pm 7.73\%$; $p = 0.028$) but no significant difference was seen at this time point when the putty was compared with the granules group.

Longitudinal analysis showed that in the SiCaP granules, putty and block groups, significantly increased bone-implant contact was measured when 4 week data was compared with the results obtained at 8 weeks ($p = 0.016$; 0.003 and 0.004 respectively). However, only defects in the SiCaP granules group showed a significant increase in bone-implant contact when the 8 and 12 week data were compared ($p = 0.016$).

3.4 Graft Area

At 4 weeks, a significantly increased amount of scaffold was measured in the SiCaP granules group ($27.86 \pm 1.64\%$) when compared with the SiCaP putty ($14.74 \pm 1.67\%$; $p = 0.028$) and SiCaP block group ($15.88 \pm 1.64\%$, $p = 0.028$) (Figure 5). No significant difference was found when the putty and block groups were compared. Similarly, at 8 weeks post surgery, a significantly greater amount of scaffold was measured within defects in the SiCaP granules group ($25.01 \pm 1.75\%$) when compared with the SiCaP putty group ($15.77 \pm 1.38\%$, $p = 0.046$) and SiCaP block group ($15.22 \pm 1.39\%$; $p = 0.028$). No significant difference was measured when the putty and block groups were compared at this time-point. At 12 weeks post surgery, the amount of scaffold present had decreased in all groups however a significant difference was found when the SiCaP granules ($20.5 \pm 1.69\%$) and SiCaP block groups were compared ($10.69 \pm 1.43\%$; $p = 0.028$). No significant differences were found when the granules and putty ($14.09 \pm 3.30\%$) and putty and block groups were compared. Results from this study showed that implants gradually resorbed in all groups over time. The highest rate of implant resorption

was measured in the SiCaP granule group. Longitudinal analysis showed that no significant decrease in the amount of the scaffolds was found when each of the groups were compared at the 4 and 8 week time-points. When data was compared at the 8 and 12 week time-point, a significant decrease in scaffold was measured in the SiCaP block group only ($p = 0.045$). Additionally, a significant decrease was measured in the SiCaP granules group when 4 and 12 week data were compared ($p = 0.016$). No other significant differences were found.

3.5 Light Microscopy

Qualitative analysis using light microscopy showed that at 4, 8 and 12 weeks, no bone growth was seen within any of the empty defect samples. At 4 weeks and in all other groups, bone growth was observed on the surface of the graft. In the SiCaP putty group, CMC material was evident with an amorphous lightly stained structure and was interwoven with soft tissue (Figures 6 and 7). Within areas of the CMC carrier, large mononuclear cells and blood vessels were present with no evidence of an adverse cellular reaction. However, only few areas of new bone formation were seen with the majority of the scaffold surface quiescent with little cellular activity. At 8 weeks and in the granules group, new bone formation was seen and in some regions bone bridged individual granules tying them together. Bone formation was seen preferentially forming on the surface of the graft filling in the pores in a centrifugal manner. Osteoblasts were also observed laying down osteoid on the surface of the granules. In the SiCaP block group, less bony bridging was seen and instead bone appeared to conduct itself along the scaffold surface (Figure 7). Large areas of CMC were also evident at this time in the SiCaP putty group however areas of new bone were identified. By 12 weeks post operatively, most of the defect in the SiCaP granules group contained new bone formation with bone in direct contact with the implant surface. An increased amount of bone was also observed in the SiCaP putty group and regions of bony bridging between granules were identified.

4. DISCUSSION

In this study we have shown that use of an injectable bone graft substitute material where CMC is used to compose a putty that holds the bone graft substitute granules together, reduces early bone formation. An injectable SiCaP bone substitute material offers the benefit of a ready-to-use scaffold with improved surgical handling for directed and optimal fill of a bone void. However, a current challenge with their use is the development of carriers that are not detrimental to the osteoconductive and inductive potential of the SiCaP scaffold. The bioactivity of calcium phosphate materials is believed to be associated with material dissolution and the release of calcium and phosphate ions from the implant surface followed by the precipitation of a biological apatite layer. This results in more protein adsorption, osteoblast adhesion and increased bone growth [12]. Ideally, carriers should dissolve within the first few days post operatively, allowing interaction of the CaP surface with the surrounding environment. Continued presence of the carrier may interfere with early bone healing by impeding cell influx, the interaction of connective tissue and vascularization within the porous structure and inter-granular space [13, 14]. In our study we have shown that the CMC carrier is still present 4 weeks after implantation. CMC is known to function as a polymeric binder/thickener that improves the clinical handling properties of bone substitute materials however, histological data characterizing the effect of CMC on bone regeneration is limited. The aim of this study was to investigate use of CMC as the binding agent on bone formation within a porous SiCaP scaffold implanted in an ovine femoral condyle. In this study, use of CMC provided excellent handling properties during surgery however, both quantitative and qualitative results showed reduced early new bone formation and osteoconduction in the CMC putty group at both the 4 and 8 week time-points when compared with control granules without CMC. No adverse cellular reaction was observed at any of the time-points investigated and by

12 weeks after the CMC matrix had appeared to reduce bone formation and bone-implant contact significantly increased to levels comparable to those seen in the dry granules group.

These results are in contrast to studies that have previously compared use of CMC *in vivo* and as a carrier for bone graft materials. A histological study by Reynolds et al. [4] investigated the use of CMC as a binder in the development of an injectable calcium sulfate putty and reported that CMC was well tolerated by tissue and supported bone formation in a critical sized rat calvarial model. Results showed comparable levels of new bone formation when compared with CS graft alone. In fact, several studies have reported the osteoconductive properties of CMC where results showed augmented bone formation at early time-points post-operatively when investigated using *in vivo* bone defect models [5-8]. It is not immediately clear as to why CMC in this study would result in a significant decrease in early bone formation. The SiCaP scaffolds are highly porous and it may be that the CMC binder was able to enter and penetrate throughout the interconnected micro- and macroporous network. This may have increased the time required for dissolution of CMC to below the threshold levels that allow for the influx of biological fluids, proteins and the cells essential for new bone formation. This theory may in part, be supported by the abrupt 6-fold increase in new bone formation measured in the putty group between weeks 8 and 12 compared with the 2-fold increase seen in the granule and block groups at this time. Another factor that may have reduced new bone growth in the putty group is the amount of SiCaP scaffold implanted in the defect at the time of surgery. A limitation of this study is that scaffold area and free space available for bone formation was not measured at time-zero and although defects were completely filled, a portion of the volume of the defect in the putty group was occupied by CMC and not SiCaP scaffold. This difference in scaffold area may be reflected in our results that showed decreased % scaffold area at each of the time-points in the putty group when compared with defects containing dry granules. It is possible

that the reduction in new bone measured in the putty group is due to a lesser volume of bioactive scaffold present within the defect, a challenge that is potentially inherent with all injectable bone substitute products due to the volume occupied by the carrier material.

A critical-sized bone defect is defined as the smallest sized defect that will not spontaneously heal with bone tissue [15]. Results from our study showed no bone healing within the empty defects over the 12-week period demonstrating this model is validated as critically-sized for the investigation of bone growth in response to different bone substitute materials. Our results showed that in all of the experimental groups investigated, the presence of the SiCaP scaffold resulted in significantly increased bone growth at all time-points when compared with empty control. Significantly increased new bone formation was measured in the SiCaP granules group when compared with the SiCaP block group at all of the time-points investigated and in the block group at the 8 and 12 week time-points new bone area and % bone-implant contact rates appeared to decline. This was in contrast to both the granule and putty groups where bone formation and % bone-implant contact continued to significantly increase as time progressed post-surgery. Several studies [16-18] have investigated the effect of CaP granule packing on bone formation both *in vitro* and *in vivo* and results suggest that the 3D environment created by the packing of the granules has a significant influence on increasing bone formation. The packing of granules provides a macroporosity and free space for invasion by body fluids, nutrients, host cell and blood vessels and in our study this may have further augmented bone formation within granules of SiCaP when compared with implantation of a uniform ceramic block. A feature that has been seen previously is the formation of bone in small pores within the struts of the bone graft substitute [11]. In this current study we have identified bone deposition within the scaffold as early as 4 weeks using fluorochrome staining (see figure 1A) and CMC putty may fill in these small pores blocking this bone formation

5. CONCLUSION

The options for moldable synthetic bone graft substitute materials are numerous so this study aimed to improve our knowledge on the use of a CMC carrier on the biological performance of SiCaP scaffolds. For all moldable grafts, rapid dissolution and clearance of their polymer binder is required to allow for optimal bone formation. This study showed that the presence of CMC may have impeded early new bone formation in this *ovine* critically-sized drilled defect model. Results from this study also showed that use of granules significantly increased bone formation within a defined defect size when compared with a press-fit block composed of the same material. Further research is necessary to optimize CMC when used to bind bone graft substitute granules used for the augmentation of bone.

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7. Figure Legends

Figure 1

Photomicrograph taken at 4 weeks post-surgery showing [A] calcein green uptake within the SiCaP scaffold (arrowed) and [B] fluorescently labeled bone growth on the surface of the scaffold. Both images were taken from samples in SiCaP granule group.

Figure 2

Bone apposition rates ($\mu\text{m/day}$) in each of the groups at 4, 8 and 12 weeks post-surgery.

Figure 3

Bone area measured in all groups over the 4, 8 and 12 week study period.

Figure 4

%Bone-implant contact in each group at 4, 8 and 12 weeks post-surgery.

Figure 5

A box and whisker chart comparing SiCaP scaffold resorption in the groups at 4, 8 and 12 weeks.

Figure 6

A photomicrograph showing the CMC carrier present within defects at [A] 4 weeks and [B] at 12 weeks post surgery. At 4 weeks, little new bone formation was seen and at 8 weeks, the CMC carrier was still present however increased amounts of new bone formation was evident adjacent to the granule surface.

Figure 7

A photomicrograph of [A] a SiCaP block specimen showing the osteoconduction of bone along the block surface at 8 weeks post surgery. Image [B] shows extensive new bone formation observed 12 weeks post operatively in the SiCaP granules group.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7