Testing bone substitutes in a small animal model of revision arthroplasty

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This study evaluated a modification of the rat-pin model to enable testing of bone substitute materials. The model was characterized using the ceramic, β-tricalcium phosphate (βTCP) as a filler.

A 1 mm wide, 3.6 mm deep defect was created around a stainless steel (SS) implant in the proximal tibia of a rat. This defect was filled with a ceramic powder. Large particles (90–312μm) of βTCP were mixed with Gelfoam® to form a paste which was then molded around the proximal end of either an uncoated SS pin or a pin coated with hydroxyapatite (HA). The pin with its ceramic collar was then implanted into the proximal tibia of 16 male Sprague Dawley rats. Two animals with coated implants and two with uncoated implants were sacrificed at 3, 6, 14 and 26 weeks. Longitudinal sections of each tibia were stained with toluidine blue and labeled for tartrate resistant acid phosphatase (TRAP).

There was initial fibrous tissue interposition around the implants which was completely remodeled around the HA coated pins but which persisted in apposition to the SS pins. The remodeling process peaked at 3 weeks around the HA coated pins and at 6 weeks around the uncoated implants. There was little remodeling around either implant by 26 weeks. There was considerable residual βTCP present which was well tolerated as the particles were often encased in bone.

The model has several characteristics of revision arthroplasty and the results demonstrate the suitability of this model for testing bone substitutes.

Introduction

Revision arthroplasty is more difficult and less successful than primary joint replacement owing to the loss of bone stock. These defects are frequently filled with autograft or allograft but neither of these are ideal. Autograft produces the best results but it is difficult to find a suitable donor site to harvest adequate autograft and there are further problems with donor site morbidity [1, 2]. The disadvantages of using allograft include concerns about its immunogenicity and a clinical failure rate of 20–30% [3]. There is, therefore, a need for a synthetic bone substitute which is easy to work with and will be as effective *in situ* as autograft.

Many experimental bone substitutes involve a group of calcium phosphate ceramics that have a mineral composition similar to that of bone and are generally well tolerated by bone [4]. There are numerous formulations of these ceramics that have different calcium : phosphate ratios and porosities, both of which can affect their resorbability and biocompatibility [5, 6].

Potential bone substitutes have been tested *in vivo* using various animal models. Many of these models involve large animals such as dog [7] and goat [5] or rabbit [2, 8–10] and most involve testing the bone substitute by filling a critical sized defect in a long bone [11–13]. Few have tested the material with an implant *in situ* [7].

To address this, we have adapted a rat pin model, previously used to study periprosthetic loosening [14, 15], to incorporate a defect around the implant which can be filled with experimental bone substitute materials. For the purposes of this study, the model has been characterized using a filler of β-tricalcium phosphate (βTCP) and Gelfoam®.

Materials and methods

Experimental groups

Sixteen male Sprague Dawley rats (mean body weight: 430 g) (Charles Rivers, Margate, UK) were assigned to eight groups (Table I). Each group of two animals received either an uncoated stainless steel implant or an implant which had been plasma sprayed with hydroxyapatite (HA) and were sacrificed at 3, 6, 14 or 26 weeks.

Experimental model

The dimensions of the stainless steel pin are shown in Fig. 1 (manufactured by R. J. Layland, Rayleigh, UK). The dimensions of the pins to be coated were adjusted to

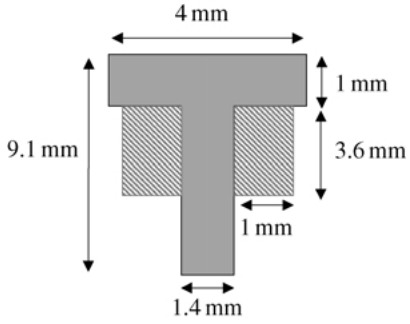

Figure 1 Dimensions of the tibial implant with its βTCP/Gelfoam® collar in place. Striped area represents βTCP/Gelfoam®.

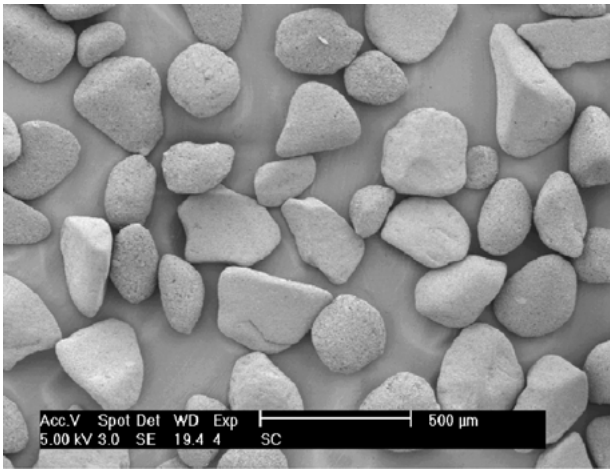

Figure 2 SEM of βTCP particles.

allow for a 50 µm thick coating of HA (Plasma Biotal, Tideswell, UK) such that the final size of the coated and uncoated pins were identical.

Particles of βTCP (Plasma Biotal) with 5–8% porosity were sequentially sieved to obtain a fraction with a mean equivalent circle diameter of 166 µm (range 90–312 µm) (Fig. 2). Energy dispersive X-ray analysis was performed on the particle sample to ensure there was no metal contamination from the sieving process.

A defect of the dimensions shown in Fig. 1 would be created around the pin in the proximal tibia. Rather than filling the defect during surgery, the ceramic powder was mixed with an agent to form a paste which was then molded around the pin to create a single implantable entity. This ensured that an equal amount of ceramic was placed into each defect and was the most reproducible way of filling the defect. 27.8 mg of βTCP was mixed with 5.6 mg of Gelfoam®, a medical grade absorbable gelatin powder (Pharmacia and Upjohn, Kalamazoo, MI) and warmed to 37 °C. A few drops of warm saline (0.9% w/v) were added to the powder mixture in a beaker and mixed to a paste. The paste was then pressed into a specially designed mold and a pin was inserted. The prosthesis was left undisturbed in the mold for 4 h after which time the mold was opened and the βTCP collar was allowed to cure for a further 1 h. The pins, βTCP and all utensils were autoclaved prior to use and the procedure was carried out using aseptic technique in a laminar flow cabinet. The total volume of the ceramic collar was 108.6 mm^3.

Operative procedure

All procedures underwent ethical review and were carried out in accordance with the regulations as laid down in the Animals (Scientific Procedures) Act 1986. All drugs were obtained from National Veterinary Services, Stoke-on-Trent, UK. The operative procedure was as previously described [15]. Briefly, animals were anaesthetised with 1 : 1 : 2 fentanyl-fluanisone (Hypnorm), midazolam (Hypnovel) and sterile water at a dose of 2.3 ml/kg body weight. The right hindlimb was clipped, draped and sprayed with chlorhexidine spray. A 1 cm incision was made lateral to the patella and a lateral capsulotomy performed allowing medial dislocation of the patella. The tibial plateau was visualized and a hole drilled centrally using a 2 mm hand drill. A defect of the exact dimensions of the implant with the proximal βTCP collar in place was then reamed using a custom made counterbore. The prosthesis was inserted so that the top of the pin lay flush with the tibial plateau (Fig. 3). The patella was reduced and the incision closed with interrupted sutures. The animals received postoperative antibiotic cover (Synulox, 150 mg/kg, i.m.) and both preoperative (Carprofen 5 mg/kg, s.c.) and postoperative analgesia (Buprenorphine, 0.15 mg/kg, s.c.). The animals were weight bearing within 12 h postoperatively. They were housed in IVC caging systems (Techniplast UK Ltd, Northhants, UK) exposed to a 12 h light/dark cycle and had access to food and water ad libitum.

Fluorochrome injections

To label the calcification front, each animal was given a fluorochrome injection by i.p. at weekly intervals up to a maximum of four injections (Table I). The labels were

TABLE I Description of the groups in the study and the schedule of fluorochrome injections

Group	Implant	Label 1 (XO)	Label 2 (CG)	Label 3 (AC)	Label 4 (XB)	Sacrifice
1	SS	wk 1	wk 2	—	—	wk 3
2	HA	wk 1	wk 2	—	—	wk 3
3	SS	wk 3	wk 4	wk 5	—	wk 6
4	HA	wk 3	wk 4	wk 5	—	wk 6
5	SS	wk 10	wk 11	wk 12	wk 13	wk 14
6	HA	wk 10	wk 11	wk 12	wk 13	wk 14
7	SS	wk 22	wk 23	wk 24	wk 25	wk 26
8	HA	wk 22	wk 23	wk 24	wk 25	wk 26

SS: uncoated stainless steel; HA: hydroxyapatite coated; XO: xylenol orange; CG: calcein green; AC: alizarin complexone; XB: xylenol blue.

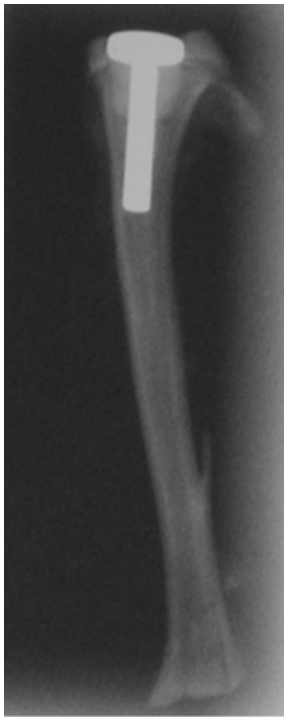

Figure 3 Radiograph of rat tibia showing the placement of the implant with its ceramic collar.

xylenol orange (90 mg/kg), calcein green (5 mg/kg), alizarin complexone (30 mg/kg) and xylenol blue (40 mg/kg). All fluorochromes were obtained from BDH, Dorset, UK.

Tissue processing

Following sacrifice, the right tibia was dissected out and placed in cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 with 0.1% w/v sucrose and 0.05% v/v glutaraldehyde, for 4 h. The specimens were then washed in phosphate buffered saline (PBS) and dehydrated and de-fatted in increasing concentrations of alcohol (50–100%) followed by acetone under vacuum with several changes of each solution. The specimens were embedded in Technovit® 9100 (TAAB, Aldermaston, UK) following the manufacturers instructions.

Three hundred micrometer longitudinal sections were cut using a low speed diamond saw (Accutom 5, Struers, Glasgow, UK) then ground and polished to 100 μm with a graded series of silicon carbide polishing discs using a Rotopol 21 (Struers).

Histological analysis

One section from each case was surface stained with toluidine blue (pH 9, at 56 °C) and examined under a light microscope (Dialux 20, Leica, Milton Keynes, UK). Images were grabbed from around the perimeter of the pin (see Fig. 10) using a 3-chip color camera (JVC) and dedicated software (Aquis, Synoptics, Cambridge, UK). Bone, marrow, βTCP, fibrous tissue and growth plate were identified in each section by their characteristic staining and the area of each was measured using Adobe Photoshop 5.0 and the freeware image analysis software, Scion Image (Scion Corporation, www.scioncorp.com). The area of each of these parameters under the head of the pin and along the length of its stalk, is expressed as a percentage of the total area.

Fluorescence analysis

A section from each tibia was examined under epifluorescence using a DMRB microscope (Leica) using both FITC and rhodamine filters. Sequential images were captured from around the perimeter of the pin using a LC 100C low light camera (Seescan, Cambridge, UK) and the area of fluorescent label was measured using Scion Image. These measurements are expressed as the sum of the area of fluorescence seen with each filter, in the tissue surrounding the pin (tissue contained within the area under the head and along the stalk of the pin) as a percentage of the total area of tissue.

Images were also taken from the areas in this region where two or more labels were visible. The mineral apposition rate (MAR) was calculated from these images using a semiautomatic image analysis package with a digitising tablet, cursor and drawing tube attachment (SUMMASKETCH II, Summagraphics, Fairfield, CT).

TRAP analysis

One section from each sample was stained for tartrate resistant acid phosphatase (TRAP). All reagents were obtained from Sigma Aldrich, Dorset, UK, unless otherwise stated. The methacrylate was removed from each section using acetone and the sections were rehydrated through graded alcohols to distilled water. They were placed in a reaction mixture (0.1 M citrate buffer pH 4.5 containing 1 mM napthol AS-BI phosphate and 10 mM sodium tartrate) for 7 min at 37 °C. The reaction was stopped by washing in cold 50 mmol sodium fluoride and sections were incubated with post coupler (0.1 mM acetate buffer pH 6.2 containing 2.2 mM Fast Garnet GBC) for 5 min at 4 °C, rinsed in distilled water and mounted using Aquamount (BDH, Dorset, UK).

Images were grabbed from around the perimeter of the pin using Aquis and the total area of TRAP staining was measured using Adobe Photoshop 5.0 and Scion Image.

Statistics

Although the groups were too small to test for any significant differences between the coated and the uncoated implants, univariate analysis of variance was performed using a general linear model to test for significance in the changes with time.

Results

One animal from the 3-week, coated pin group died postoperatively of unknown causes. All others were included in the study.

Histology

Macroscopic examination revealed that, in several of the samples, the βTCP had migrated down the length of the pin and was not contained within the original dimensions

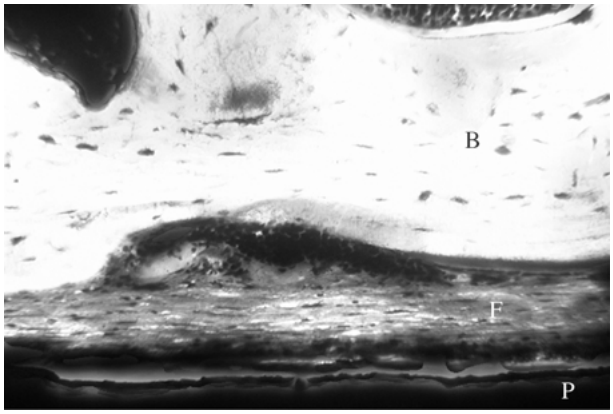

Figure 4 Section from week 14/uncoated pin group stained with toluidine blue showing fibrous tissue (F) interposition between the pin (P) and the bone (B) (original mag. × 100).

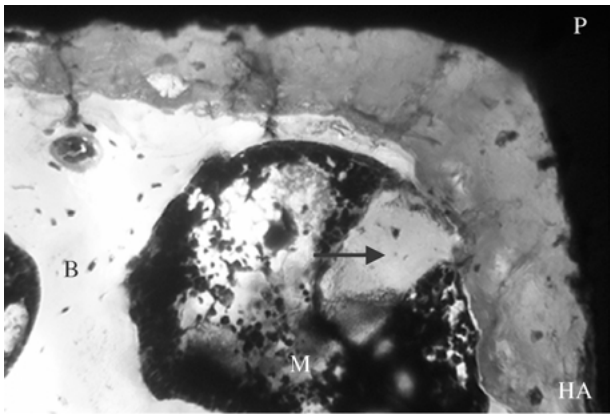

Figure 5 Section from week six/coated pin group showing bone (B) apposition to the HA coating (HA) of the pin (P). There is a particle of βTCP (arrow) contained within marrow (M) (original mag. × 100).

of the collar. βTCP particles were never found at, or below, the bottom of the pin.

At the early time points, fibrous tissue was found below the head of the pin, in both the coated and the uncoated groups. Fibrous tissue formed a sheath between the SS pin and the bone that was not seen around HA coated pins, where bone was in close apposition with the implant (Figs. 4 and 5).

At week 3, the particles of βTCP were embedded in fibrous tissue or surrounded by marrow. By weeks 14 and 26, however, the particles were encased in bone (Fig. 6). Image analysis of the sections showed that, while the percentage of the total area that was bone increased with time, the amount of marrow peaked at six weeks (Fig. 7). The increase in both of these parameters with time was significant, however (bone: $r^2 = 0.692$, $p = 0.001$; marrow: $r^2 = 0.515$, $p = 0.021$). When the areas of bone and marrow around each pin were added together, there was more bone and marrow around coated than uncoated implants at all time points from 6 weeks onwards (Fig. 7). Again the increase with time was significant ($r^2 = 0.808$, $p < 0.001$).

Conversely, from six weeks, there was more fibrous tissue around SS uncoated implants than around coated and there was a decrease in the amount of fibrous tissue around both types of implant with time ($r^2 = 0.814$, $p < 0.001$) (Fig. 7). There remained a substantial amount of fibrous tissue around SS implants at 26 weeks. The

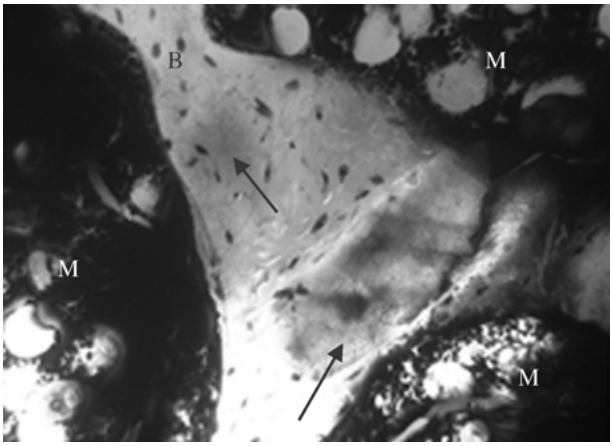

Figure 6 Section from week 14/coated pin group showing βTCP particles (arrows) encased in bone (B) (M = Marrow) (original mag. × 100).

amount of growth plate seen in each section decreased with time around both types of pin ($r^2 = 0.768$, $p < 0.001$) but the amount of growth plate remodeling was greater around HA coated implants (Fig. 7).

The amount of βTCP in the samples decreased significantly with time ($r^2 = 0.621$, $p = 0.005$) (Fig. 8). The area of βTCP in the sections at 26 weeks was approximately one half that found in the 3-week samples. Similarly, there was a reduction in the mean particle size seen in each sample (reduction of approximately one third from 3 to 26 weeks) that was also significantly correlated with time ($r^2 = 0.832$, $p < 0.001$). There was little difference between coated and uncoated implants (Fig. 8).

Fluorescence

At week 3, there was more fluorescence around the HA coated pin than the uncoated but at week six the opposite was true (Fig. 9). There remained more fluorescent label around the SS pin than the HA coated pin at week 14. The amount of fluorescence decreased with time ($r^2 = 0.595$, $p = 0.007$) and there was very little fluorescent label present by week 26 (Fig. 9).

Mineral apposition rate

The MAR during weeks 2 and 4 was greater around HA coated pins than uncoated SS pins but was at a similar rate around the coated and uncoated implants at subsequent time points (Table II). Although there was a

TABLE II Mineral apposition rate following weekly fluorochrome injections around uncoated and coated implants, μm/day (SD)

	Uncoated SS pins	HA Coated pins
wk 2	2.55 (1.16)	3.45
wk 4	1.34	2.33
wk 5	2.34 (0.22)	2.34 (0.58)
wk 11	1.75 (0.47)	1.42 (0.15)
wk 12	1.47 (0.17)	1.29 (0.15)
wk 13	1.57 (0.30)	1.62 (0.32)
wk 23	1.04 (0.14)	1.17 (0.07)
wk 24	0.95 (0.23)	1.17 (0.10)

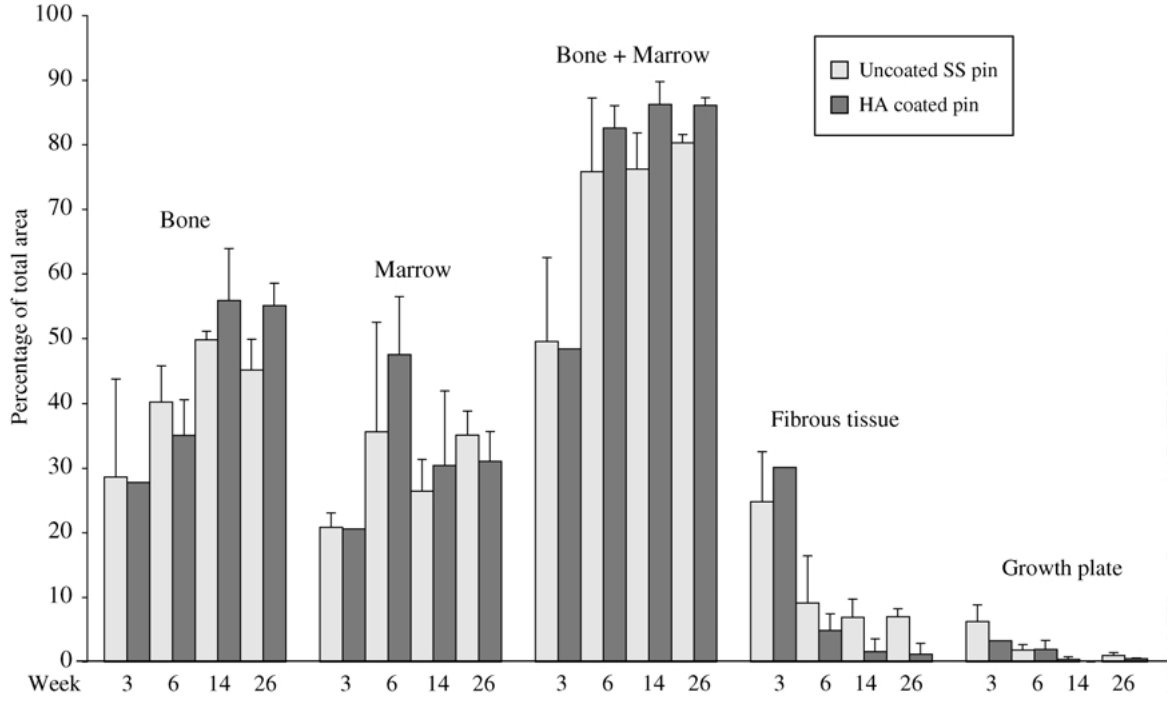

Figure 7 The percentage of the total area around the implanted pin that is bone, marrow, bone + marrow, fibrous tissue or growth plate ($n = 2$).

general decrease in the MAR with time ($r^2 = 0.856$, $p = 0.007$) this was not of the magnitude seen in the total area of fluorescent label.

TRAP

The total amount of TRAP staining around the pin followed a similar pattern to that seen with the levels of fluorescence. There were more TRAP positive cells around HA coated pins than uncoated SS pins at week 3 but this relationship was reversed by week 6 (Fig. 9). Around SS uncoated pins, the amount of TRAP staining peaked at 6 weeks but the peak in staining was seen at week 3 around HA coated pins. TRAP levels in the tissue at week 14 were lower than those seen at week 6 and by week 26 there were few TRAP positive cells around either coated or uncoated pins.

On closer examination, it could be seen that the majority of the TRAP staining was in the area immediately under the head of the pin, in the region containing particles of βTCP (Fig. 10). TRAP positive cells were often associated with these βTCP particles (Fig. 11).

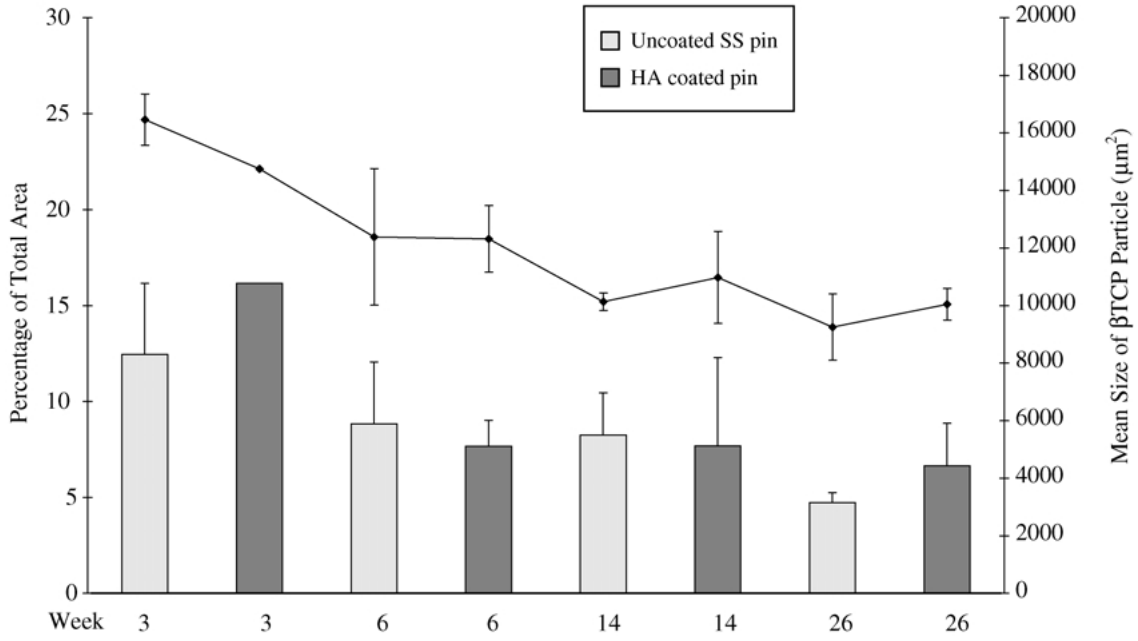

Figure 8 The area around the implanted pin taken up by particles of βTCP, expressed as a percentage of the total area (bars), and the mean size of these particles (line) ($n = 2$).

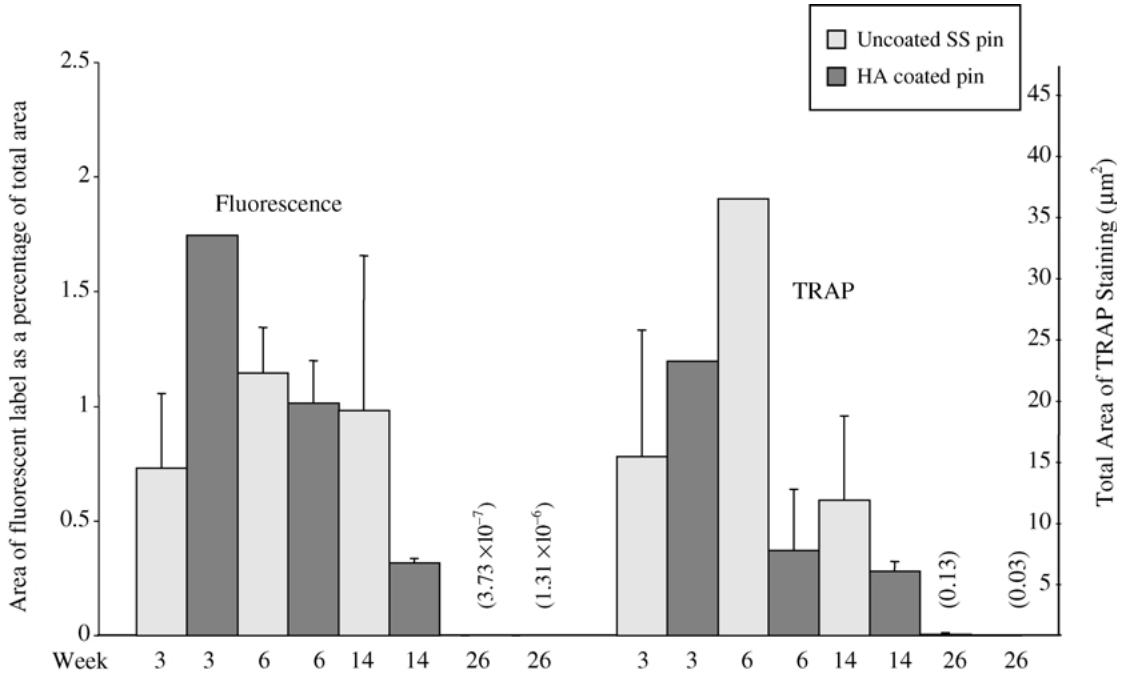

Figure 9 The total amount of fluorescent label under both a FITC and a rhodamine light source, in the area under the head of the pin as a percentage of the total tissue area and the total area of TRAP staining around each pin ($n = 2$ except those without error bars where $n = 1$).

Discussion

The development of a synthetic bone substitute that is at least as effective as autograft clinically and that can quickly be replaced by bone to allow early weight bearing by the patient is a major goal of orthopedic research. It is necessary to test experimental bone substitutes in vivo and there are numerous animal models that have been used in the past. However, these models often involve larger animals such as dog or goat and the smaller animal models generally involve filling a defect in a long bone with the material. The model described here has all the advantages associated with using a small animal, such as cost and convenience, and it allows bone substitutes to be tested around an implant that is open to the joint space and is probably load bearing. The prosthesis is seated on cortical bone and there is some trabecular bone present therefore the model has several features of a revision arthroplasty.

The model was characterized using βTCP. Calcium phosphates as bone substitutes and osteoconductors, have been used in many different compositions, formulations and porosities. Hydroxyapatite, βTCP and biphasic calcium phosphates (BCP; HA and βTCP mixtures) are the three most commonly used ceramics. HA is considered to be almost completely insoluble and βTCP is more soluble, therefore the ratio of HA to βTCP

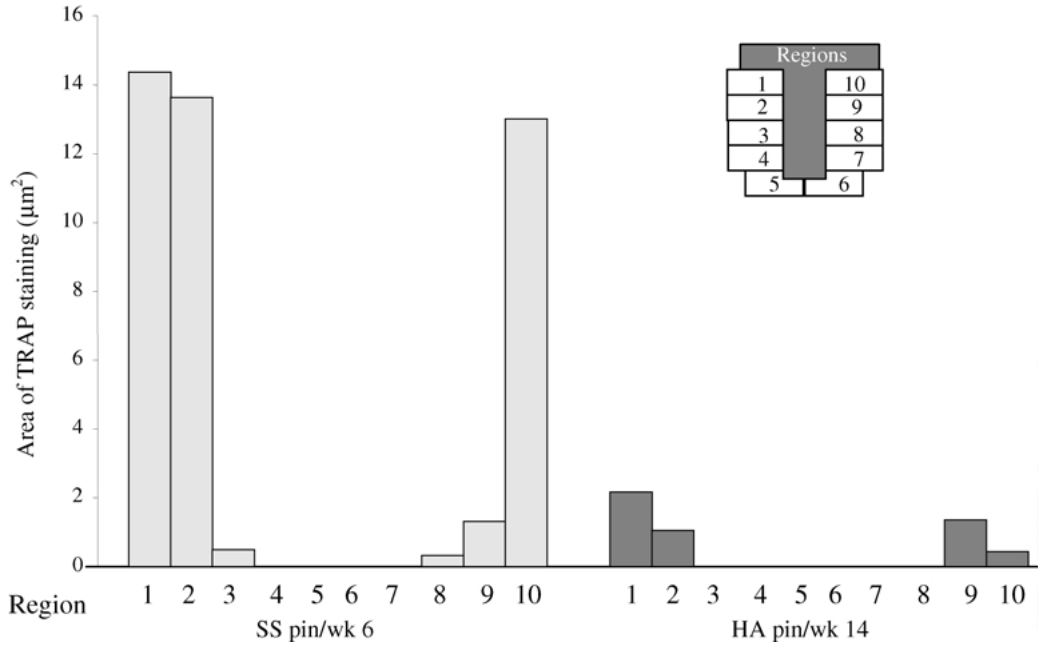

Figure 10 Amount of TRAP staining in each area from two representative cases illustrating that the TRAP positive cells were found in the areas directly under the head of the pin. Regions 1–3 and 8–10 contained the collar of βTCP and Gelfoam®.

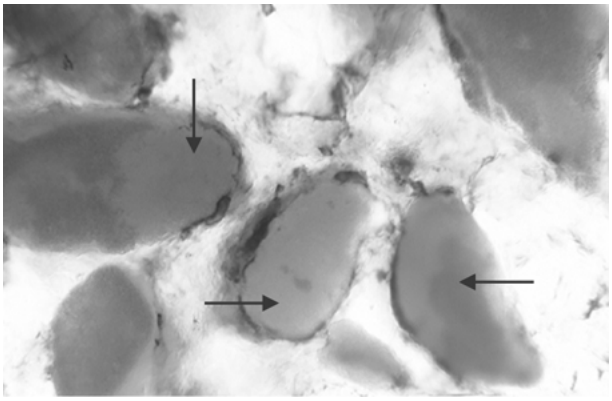

Figure 11 Section from week 3/uncoated pin group showing TRAP positive cells (red) around particles of βTCP (arrows) (original mag. × 100).

in the BCP determines its solubility. This in turn determines the bioactivity of the ceramic [16], in terms of its osteoconductive abilities. If the filler dissolves too rapidly there will be no scaffold to support cell attachment and growth, conversely an insoluble filler will never be replaced by bone. Furthermore, calcium ions released during the dissolution of the ceramic are incorporated into new bone [17], but a high local concentration of calcium may adversely affect osteoclast resorption [6] and osteoblast attachment and proliferation [18].

In the present study, precisely how much of the βTCP is removed through dissolution and how much through active resorption is unknown but the presence of large numbers of TRAP positive cells, specifically in the area of the βTCP collar and around the particles, suggest a degree of resorptive activity. Although remodeling began earlier around the coated pins than the uncoated, shown by the amount of TRAP and fluorescent label and by the MAR, there were more and larger particles of βTCP remaining around the HA coated implants by 26 weeks. This may be due to the prolonged resorptive period around the SS pins.

There was minimal remodeling around both coated and uncoated pins by 26 weeks, demonstrated by the lack of fluorochrome incorporation and TRAP positive cells, yet approximately 50% of the βTCP remained. Others, who have used similar formulations of βTCP with a binder but used it to fill a segmental defect in a long bone or a defect in proximal femur, have also reported residual βTCP after 8 weeks [8, 9]. This residual ceramic is well tolerated and the histological analysis showed bone in contact with the particles with no evidence of inflammatory cells.

The importance of porosity in a bone substitute has been demonstrated repeatedly [16] particularly when the ceramic is implanted in a cylindrical form, as it allows colonization by osteoblasts [19, 20]. The βTCP used in this study was particularly dense, with only 5% porosity compared to some which are up to 70% porous [5, 19]. However, as it was used in a particulate form with a water soluble binder (Gelfoam®), cellular access is still possible. Daculsi suggests that this type of formulation (i.e. particles in a soluble matrix) allows bone ingrowth at the expense of ceramic resorption [16]. A βTCP formulation with a greater porosity may be more easily removed in the current model and may, therefore, allow better substitution with bone, however this may also release greater concentrations of calcium ions into the surrounding milieu, with possible detrimental effects.

The residual βTCP particles appear to be well tolerated by the bone but, as mechanical tests were not carried out, the effect of these particles on the bone strength is unknown. Others have suggested that the bond formed between βTCP (in a cement formulation) and bone is weaker when compared with bioactive bone cements containing other ceramics [21] but some have observed that βTCP particles incorporated into bone are mechanically stable [16].

In summary, there was early fibrous interposition around both types of implant which was quickly remodeled around the HA coated pins. Around the coated pins there was a peak in the number of TRAP-positive cells, the area of fluorescent label and the MAR at the earliest time point of 3 weeks. This peak in remodeling was not seen around the uncoated SS pins until 6 weeks. By 26 weeks there was little activity around either type of implant.

The results demonstrate the suitability of this model for testing bone substitute materials for revision arthroplasty. The implant is loaded and the pin head is within the joint space and therefore exposed to factors which could modify the response of periprosthetic tissue, such as changes in intra-articular pressure and cytokine levels. The model allows quantitative measurements of several parameters giving information on the osteoconductive properties of the material within a workable time frame. Furthermore, the absence of remodeling at 26 weeks will allow mechanical testing on these specimens to test the stability of the implant.

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