JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 764–769

Osteoinduction within PEO/PBT copolymer implants in cranial defects using demineralized bone matrix

R. M. VAN HAASTERT, J. J. GROTE, C. A. VAN BLITTERSWIJK
ENT-department, University Hospital Leiden, and Biomaterials Research Group, University of Leiden, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands

A. B. PREWETT
Osteotech Inc., 1151 Shrewsbury Avenue, Shrewsbury N.J. 07702, USA

This study was undertaken to assess the osteoinductive effect addition of demineralized bone matrix (DBM) gel has, on the behaviour of osteoconductive bone-bonding PEO/PBT copolymer (Polyactive^R) implants. Cranial defects in rats were filled with these composites to study bone formation in comparison with several controls after 2 and 8 weeks survival time. Osteogenesis was qualitatively evaluated by using light- and transmission electron microscopy as well as backscatter electron imaging. Quantification of the amount of bone ingrowth was performed by using a computerized image analysis system. Initially, rapid calcification was observed in the polymer and DBM, followed by formation of new trabecular bone around the demineralized bone fragments. Bone ingrowth in implants consisting of plain copolymer was less than expected based on previous research, but the addition of demineralized bone matrix gel resulted in a significantly greater amount of new bone formation in the defects. We concluded that the application of DBM-gel to Polyactive^R implants had a beneficial effect on the amount of new bone formation in this material. This procedure combines the osteoinductive potential of DBM with the mechanical and bone-bonding properties of a copolymer, thus opening the way to the development of a line of osteoactive composite implants with good surgical handling properties.

1. Introduction

Bone grafts are widely used by orthopaedic, craniofacial and dental surgeons in the repair of osseous defects due to trauma, birth defects, tumor removal or pathological processes like osteomyelitis [1]. Autograft bone, because of its osteogenic potential and inherent biocompatibility, remains the material of choice. Apart from problems related to the limited availability of autogenous bone, specific morbidity may arise as a consequence of the harvesting procedure including donor site pain, infection, blood loss and other post-operative complications [2]. An alternative for autografts is the use of human donor bone (allograft). This bone, however, shows a higher resorption rate, potential host rejection as well as the possibility of disease transmission. These disadvantages of traditional bone grafting have stimulated research into potential bone graft substitutes. Such materials should be osteoactive, which means that they are able to enhance new bone formation [3–5]. This can take place through osteoinduction whereby mesenchymal cells will be stimulated to differentiate into osteogenic cells, and by osteoconduction in which the implanted material acts like a scaffold along which new bone formation can take place.

Bone-bonding biomaterials like calcium phosphates [6] and glass ceramics [7] have shown convincing osteoconductive capabilities. Although recently some calcium phosphates have been seen to induce osteogenesis after intramuscular or subcutaneous implantation [8], these biomaterials are not considered to have a significant osteoinductive potential. Furthermore, their use in surgery has been limited due to non-optimal mechanical properties. These materials are stiffer than bone and relatively brittle, which limits their use to non-loadbearing sites [9]. Polymers possess much better elastomeric properties but unfortunately their osteogenic capacity is mostly poor without the addition of growth factors like bone morphogenetic protein [10–14] or ceramic coatings. Recently, however, a polyethylene oxide/polybutylene terephthalate (PEO/PBT) segmented copolymer (Polyactive^R), which was originally investigated for use as an artificial tympanic membrane [15], was found to bond mechanically tight to bone without prior addition of bone-bonding substrates [16–18]. The exact mechanism behind this bonding remains to be elucidated but it does seem to be related to calcium absorbtion and hydrogel behaviour, characteristics which are directly related to the soft PEO segment of this mater-

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ial. Although research has shown that Polyactive[R] has osteoconductive and bone-bonding properties it does not seem to be osteogenic in itself. The addition of an osteoinductive substrate will therefore be a promising procedure which might optimize the osteoactivity of these implants.

The osteoinductive effect of demineralized bone has been described since 1889 when Senn [19] used decalcified bone for implantation in human osseous defects. This process of bone induction has been attributed to the presence of polypeptide factors in demineralized bone belonging to the TGF-β superfamily called bone morphogenetic proteins. Demineralized bone matrix has been used in craniomaxillofacial reconstruction in the form of blocks or particulates [20], which have a tendency to migrate in the surgical site. Recently glycerol has been explored as a carrier vehicle for preservation, storage and wetting of DBM [21]. Addition of glycerol produces a gel-like material with handling properties far superior to those of particulates.

In this study we qualitatively and quantitatively assessed the effect addition of DBM has on bone formation in porous Polyactive[R] which was implanted in cranial defects in rats, to see whether the osteoconductive properties of this polymer can be supplemented with the osteoinductive potential of DBM.

2. Materials and methods

2.1. Implants

The PEO/PBT copolymer (provided by HC Implants, The Netherlands), used in this study was porous (pore size 150–400 μm) with a PEO/PBT ratio of 80/20 and a molecular weight of the PEO segment of 1000 D. This material was produced as rods with a length of 40 mm and a diameter of 10 mm out of which, after gamma-irradiation, implants were fabricated to fill the 8 mm cranial defects. Because of its hydrogel properties Polyactive[R] will increase in volume after uptake of aqueous solutions, a phenomenon which can be useful in obtaining a tight fit for these implants in defect sites. The rat parietal bone, however, is thin (0.8–1.2 mm), which results in a relatively small contact area between the implant and bone. If this fact is not taken into account prior to surgery, the polymer might be pushed out of the operation site due to swelling after soaking in saline or body fluids. Based on results from a pilot study it was decided that implants with a preoperative diameter of 7.3 mm and 2 mm thickness would be best suited for use in an 8 mm defect. After soaking, the diameter can theoretically increase to about 9.4 mm, which will secure the implant in the defect site without it immediately being pushed out by the swelling pressure.

2.2. DBM-gel

For the preparation of rat demineralized bone matrix, tibiae and femora were harvested from Long-Evans rats (250–300 g) and placed in an iced antibiotic solution (500 000 U of Polymyxin B sulfate and 50 000 U of Bacitracin). After removal of adherent soft tissue and cartilage the bone was morselized to yield cortical chips which were washed, soaked in ethanol and freeze-dried. They were ground further in a water-cooled bone mill, sieved to a particle size of 100–500 μm and decalcified in a solution containing 0.6 N HCL and a non-ionic detergent. After washing and freeze-drying, 50% v/v glycerol was added to act as a carrier and preservative.

2.3. Surgical procedure and experimental design

Forty Long-Evans rats (weight range 250–300 g) were operated on to create 8 mm cranial defects. The animals were anesthetized intraperitoneally using a combination of 1% ketamine, 0.1% xylazine and 0.02% acepromazine. After shaving the skin overlying the parietal bone, a midline incision was made along the saggital suture of the skull and an 8 mm defect was created under copious irrigation, using a trephine mounted in a dental handpiece. After removal of the calvarial disc the defect was filled using the selected implant material. 12 defects were treated with circular porous Polymer implants; to another 12 Polyactive[R] implants we added 100 mgr DBM-gel under sterile conditions. For controls we filled eight defects with just 150 mg of DBM-gel whereas eight defects were left unfilled (see Table I). The healing response was examined after 2 and 8 week periods.

2.4. Microscopy

After sacrifice the implants with a surrounding bone margin were removed from the skulls. For every Polyactive[R] group two implants were fixed in 1.5% glutaraldehyde and post-fixed in 1% osmium ferro (osmium tetraoxide/potassium ferrocyanate 1:1) in 0.14 M sodium cacodylate buffer after which they were embedded in Spurr's resin in order to be processed for transmission electron microscopy (TEM). The other implants were fixed in 10% neutrally buffered formalin followed by dehydration in a graded series of ethanol and subsequent embedding in polymethyl methacrylate.

With the use of a histological diamond saw the defects were first sectioned medially. Next, one half of each sample was sectioned in the coronal plane (Fig. 1) thus yielding four undecalcified sections (10 μm thick) which were stained using methylene blue and basic fuchsine. These four sections were studied with a light microscope coupled to a Vidas Image Analysis System to determine the amount of bone ingrowth into the implants, which was expressed as a percentage of the

TABLE I Experimental design

Implant material	Number of animals	
	2 weeks	8 weeks
Polyactive[R]	6	6
Polyactive[R]/DBM	6	6
DBM	4	4
Unfilled	4	4

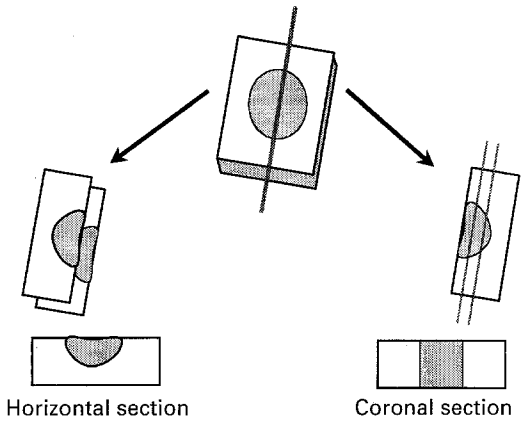

Figure 1 Illustration representing the different directions in which every implant was sectioned for light microscopy.

total defect area in the coronal plane. The remaining MMA-blocks (from which the four sections were taken) were polished, carbon coated and examined by backscatter electron imaging (BSE) using a Philips S525 scanning electron micrscope. The other half of each implant was sectioned in the horizontal plane which gives more information about the distribution of new bone in the defect.

3. Results

3.1. Morphology

On first macroscopical observation it was noticed that five Polyactive^R discs had been partly pushed out of the defect due to swelling pressure. Closer light microscopical evaluation of the copolymer implants after 2 weeks, showed the presence of loosely organized fibrous tissue and some phagocytes as well as ingrowth of new trabecular bone from the edges of the implant into the pores. Some intimate contact between the polymer and bone was observed, but frequently an interposed cellular layer consisting mainly of fibroblasts and collagen was present. At this time we could also see numerous globular structures located within the implant surface. These spots clearly reflected in BSE (Fig. 2), which is suggestive of calcification, and at times showed intimate contact with newly formed bone (Fig. 3). Transmission electron micrographs of decalcified sections of these areas showed the presence of an amorphous electron dense layer with a general thickness of around 200 μm (Fig. 4), which is characteristic of the interface between bone and bone-bonding biomaterials like hydroxyapatite.

Globular structures, which stained red with basic fuchsine, were also observed at the surface of many DBM fragments. These spheres, which closely resemble mineral deposits, were seen to merge, thus forming large areas of apparent re-mineralization. Occasionally groups of spherical cells with large centrally placed nuclei, closely resembling chondrocytes, were seen in proximity of the demineralized bone blocks (Fig. 5).

After 8 weeks the presence of more bone tissue in the pores was observed when compared to 2 weeks post-operatively, although the overall amount of new bone formation was very variable. It was also seen how many fragments of demineralized bone were

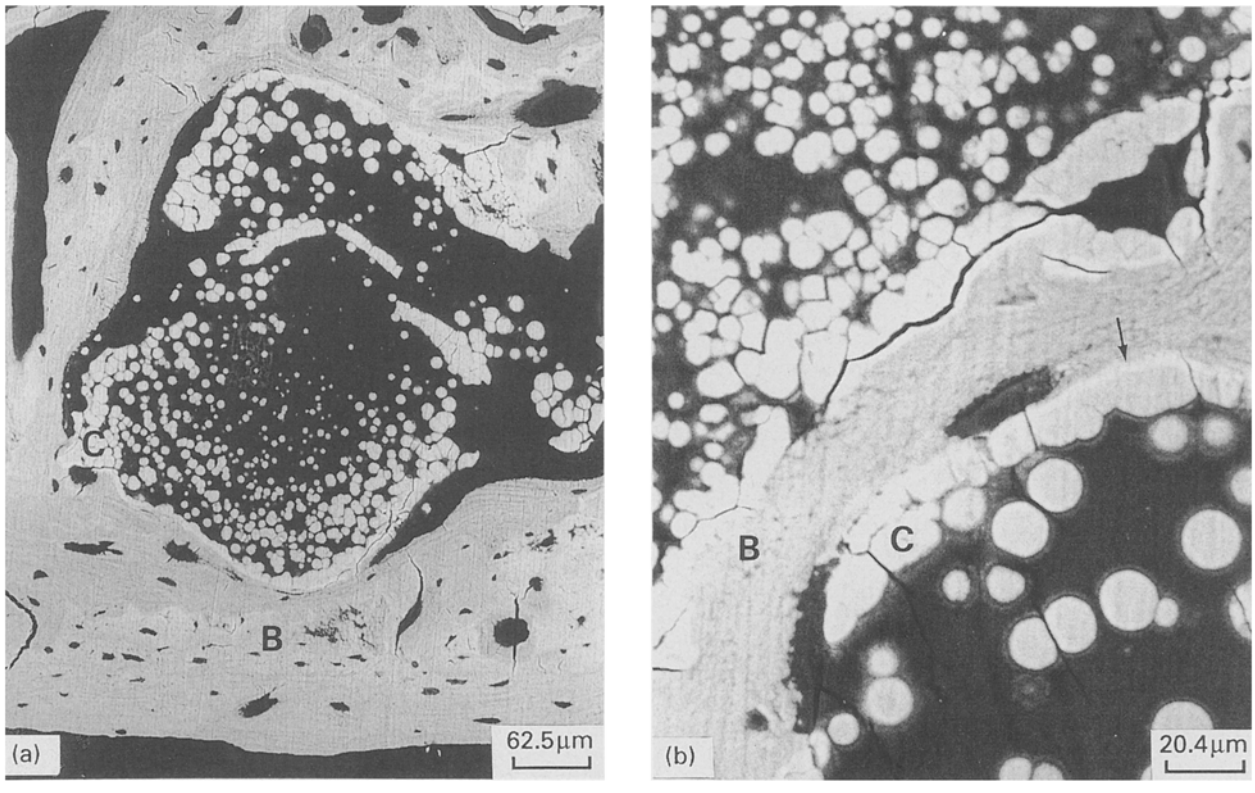

Figure 2 (a) Backscatter electron micrograph of Polyactive^R after 8 weeks displaying extensive calcification (C) within the implant surface which is in close contact with adjacent bone tissue (B). (b) A similar appearance to that in (a) showing the intimate contact (arrows) between globular calcifications (C) and bone (B).

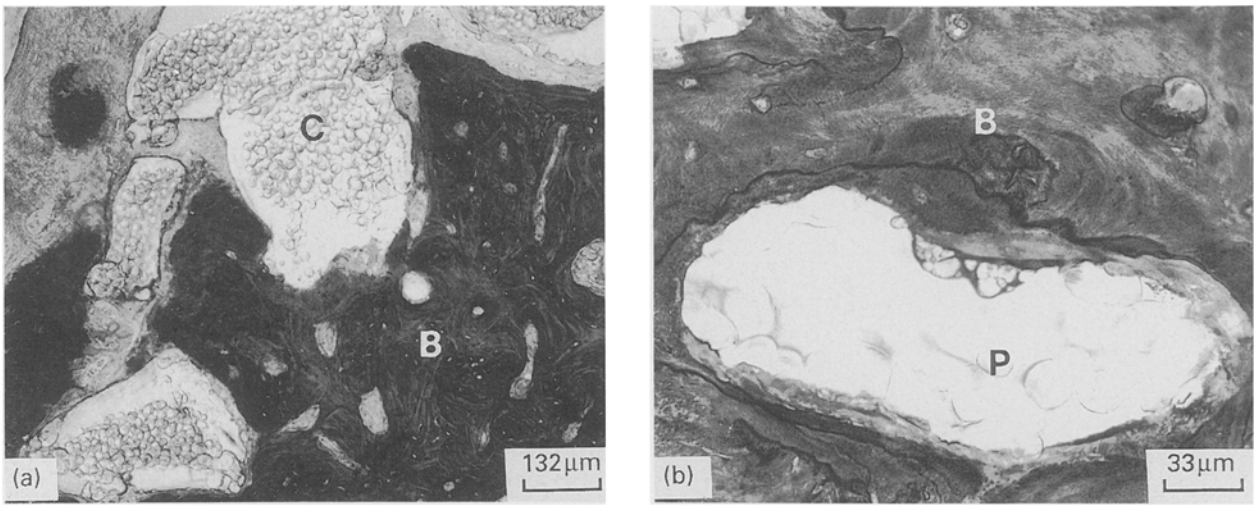

Figure 3 (a) Light micrograph of Polyactive^R implant after 8 weeks implantation time showing bone (B) ingrowth into the pores. Note the extensive calcification (C) of this material in the shape of multiple globular structures. (b) Detail, showing the calcified polymer (P) in close contact with surrounding bone tissue (B).

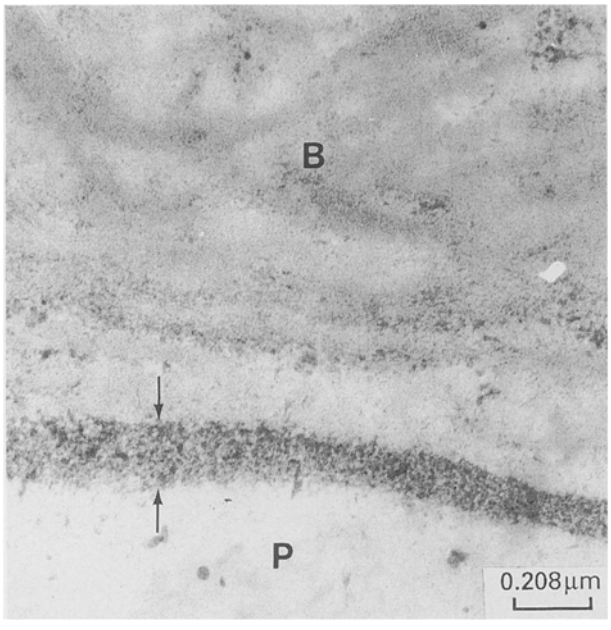

Figure 4 Transmission electron micrograph of the bone (B)–polymer (P) interface showing an electron dense layer (arrows) indicative of bone-bonding.

recalcified and surrounded by trabecular bone, whereas sometimes they were completely incorporated in newly formed bone (Fig. 6).

3.2. Histomorphometry

The amount of bone ingrowth expressed as a percentage of the total defect area (the area that had to be filled with bone), is graphically represented in Fig. 7. After a 2-week period no significant differences in the amount of bone formation were observed between the different treatment groups. After 8 weeks, however, we observed less bone formation in plain Polyactive^R implants (13.5% ingrowth) when compared to polymer implants to which DBM-gel was added (29% ingrowth). This difference was statistically significant notwithstanding considerable standard deviations, which were due to large variations in individual bone ingrowth. Defects which were treated with just DBM-gel or were left unfilled showed 29.2% and 11% bone ingrowth, respectively.

4. Discussion

This study was undertaken to investigate the potential osteoinductive effect of the addition of demineralized bone matrix gel to an osteoconductive bone-bonding copolymer. Apart from some direct bone ingrowth extending from the edges of the bony defect, osteogenesis in the pores of these composite implants took place as has been described for demineralized bone matrix in the literature [22]. In short, acellular mineral deposits [23] were seen on the DBM fragments after 2 weeks which gradually grew and fused together. After 8 weeks these remineralized areas occurred mostly in close contact with newly formed trabecular bone tissue, by which they frequently were incorporated. Sometimes groups of cells, closely resembling chondrocytes, were seen surrounding the DBM blocks which might indicate endochondral ossification taking place, a chain of events triggered by the action of bone morphogenetic protein present in the DBM. These phenomena were observed in the combined Polyactive^R–DBM implants as well as in defects filled with just DBM-gel, showing that the presence of this polymer did not compromise the process of osteoinduction.

The PEO/PBT copolymer qualitatively interacted with bone tissue, as has recently been observed in other research [24]. This material underwent rapid and extensive calcification and locally exhibited intimate contact with newly formed bone. Transmission electron micrographs of the interface showed an electron dense layer, a structure which is usually seen at the bone–hydroxyapatite interface and is often referred to as morphological indication of bone-bonding

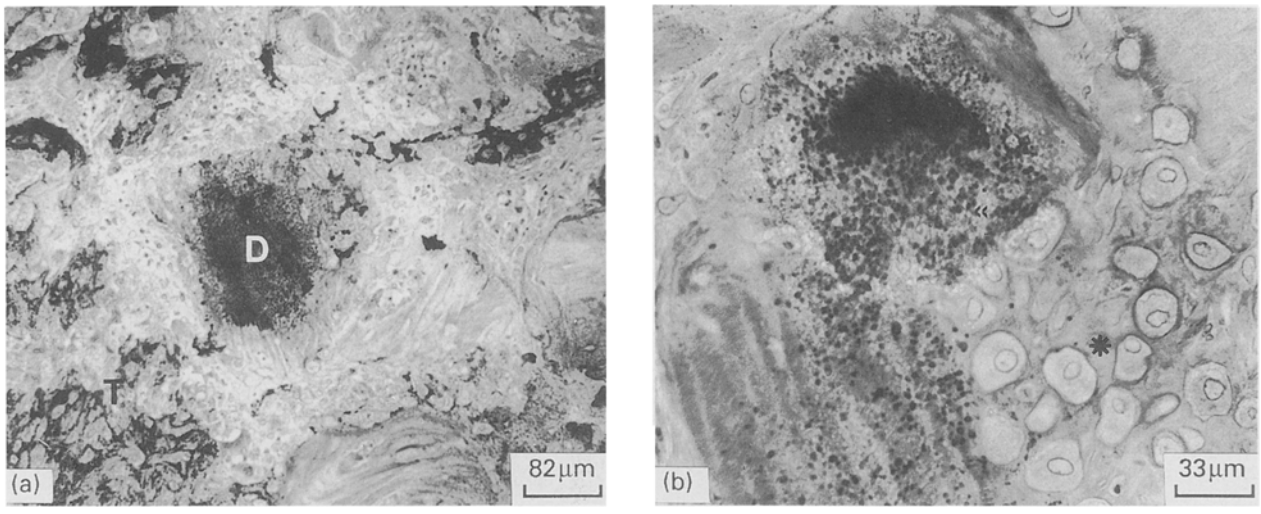

Figure 5 (a)Histology of demineralized bone gel at the 2 week survival time. At the centre a fragment of demineralized bone is visible with multiple acellular deposits (D) on its surface. At some places the formation of new trabecular bone (T) can be seen. (b) Higher magnification of the same section. Fusing acellular deposits on DBM-fragment forming an area of re-mineralization. On the left large cartilage-like cells (*) are clearly visible.

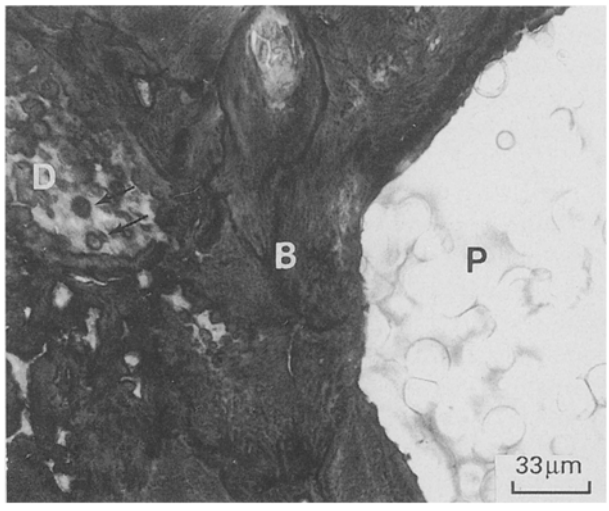

Figure 6 Light micrograph of Polyactive^R implant(P) with DBM-gel after 8 week survival time. A fragment of DBM (D) is incorporated in newly formed bone. Note the residual acellular mineral deposits (arrows).

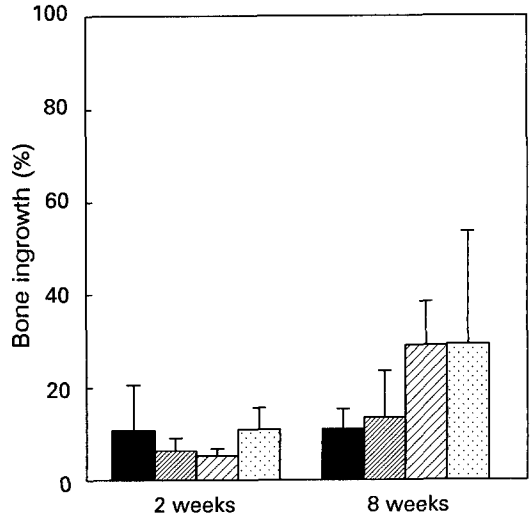

Figure 7 Graphical representation of bone ingrowth expressed as a percentage of the total defect area after 2 and 8 weeks (■ unfilled; ▨ PA; ▨ PA-DBM; ▦ DBM).

[16, 17, 25]. Quantitative data concerning the Polyactive^R differed, however, from previous results. After 8 weeks plain polymer implants showed 14% bone ingrowth as compared to 11% in unfilled defects. This is less than expected based on results by Radder [24] which showed union of 5 mm transcortical defects after 6 weeks implantation time of this polymer in goat femora. Furthermore, Bulstra [26] described faster repair of cortical defects in rabbit femora which were filled with Polyactive^R as opposed to untreated defects. These conflicting findings, besides resulting from differences between test animals and implant locations, could be largely due to the fact that a tight fit between the copolymer and bone, which seems to be a prerequisite for optimal bone-bonding, could not reproducibly be obtained using this experimental model.

The addition of DBM-gel to the Polyactive^R does result in a significantly greater amount of new bone formation (29% ingrowth) in the implants after 8 weeks. This effect must be partly due to the osteoinductive properties of DBM. Defects filled with just DBM-gel also showed 29% bone ingrowth, which is less than reported by Prewett *et al.* [21] who showed 85% bone ingrowth after 8 weeks. It has to be stressed, however, that in the above-mentioned study the mass of demineralized bone particles was included in the calculation of new bone ingrowth, whereas we chose merely to quantify the amount of new trabecular bone formation surrounding the DBM-fragments, thus yielding a relatively smaller percentage of bone ingrowth.

By adding DBM to a PEO/PBT copolymer, we have combined the osteoinductive potential of DBM with the mechanical and bone-bonding properties of

this polymer, thus contributing to the development of composite implants with optimal osteoactive behaviour. These composites could serve as optimal replacements for autogenous bone in bone graft surgery thereby eliminating the morbidity associated with harvest surgery.

Acknowledgements

The authors thank Albert Manrique and Suzanne Collier for their excellent technical assistance, advice and support during this work. Also, thanks to Henk Leenders and Ineke Klamer for their help with the histological work and to Lambert Verschragen for the preparation of the micrographs.

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