The effects of bovine trabecular bone matrix particulates on cortical bone repair

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This paper reports the effects of a synthetic bone substitute and bone allograft on cortical bone repair in an experimental model. To test the hypothesis that bovine trabecular bone matrix, BBM, can enhance the repair rate of cortical bone, osteotomies were created in the rabbit fibula and filled with either allograft or BBM particulates or left empty as controls. At five weeks post-surgery, mechanical tests and histological evaluations were performed. No significant differences were observed in the mechanical properties of the healing bone in the three animal groups ($n=6$). Histologically, the medullary cavity was obstructed and the cross-sectional area ratio of the osteotomies to intact bone was approximately 3 : 1. Highly significant area differences were observed between the intact bone group and both the BBM and the allograft groups ($p<0.001$). At the junction between the original bone and the newly formed bone, both woven and lamellar bone microstructures were prevalent. However, in the BBM filled defects, the woven bone microstructure was not ostentatious. It is concluded that failure to demonstrate significantly differences between the treatments were due to the small sample sizes and or the efficacy of the tensile analysis.

1. Introduction

To minimize the time a patient is incapacitated following a fracture, bone continuity and fracture stiffness must be restored as soon as possible. Since the stiffness of the healing fracture is a good indicator of the repair process [1], this property must be achieved early. Axial or bending stiffness is attributed to the amount of callus formation, i.e. the areal moment of inertia, particularly periosteal callus [2]. Several authors [2–5] have demonstrated that by permitting a limited amount of interfragmentary motion at the fracture site, the amount of external callus is augmented. Using external skeletal fixation Goodship and Kenwright [6] observed that, when micromotion was induced in osteotomies created in the sheep tibiae, proliferative external callus formation was evident at the fracture site within one week, whereas in osteotomies that were not mechanically stimulated axially, callus was visible three weeks after surgery. Although external devices aid in the repair rate, the technique has one major disadvantage; it is invasive and has a continuous link to a non-sterile outside. Additionally, healthy tissue is traumatized during fracture management and infection may be a problem [7–9]. Less invasive management of bone fractures is clinically a viable alternative. Pastes [10–13] and particulates [14–17] of calcium phosphates have been observed to influence the processes of bone repair. Whilst loose particulates reduce the gap size and so enhance the invasion of the fracture site by the fibrovascular stroma, they also allow bone to grow without the restrictions imposed by porous block material implants [18, 19]. Particulate migration can be controled [16], for instance by injecting pastes that set immediately *in situ* [10, 11, 13] and the precision of the deposition of the biomaterials can be refined by using an endoscope.

2. Materials and methods

2.1. Material preparation

Bovine trabecular bone matrix (BBM) (E. Merck, Germany) is prepared through a hydrothermal conversion process of bovine cancellous bone [20]. To prepare BBM microparticles for implantation, porous blocks (apparent density 0.51–1.38 Mg m^{-3}) of the bioceramic were crushed using a pestle and mortar. The crushed

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BBM particles were then sieved through standard wire cloth sieves (106 and 150 μm) and the microparticles within this range were collected for implantation. The particulates were sterilized by autoclaving at 121 °C for 20 min.

Allograft microparticulates were obtained from the distal femoral cancellous bone of New Zealand white rabbits. The bone tissue was cleaned in a sterile environment using sterile petri dishes. Sterile instruments were used to remove all soft tissue and the red marrow was flushed-out by stirring in sterile endotoxin free distilled water for more than 2 h. The cleaned bone was then stirred in 100% ethanol for 30 min followed by 15 min in ethyl ether before pulverization using a pestle and mortar. Liquid nitrogen was used to freeze the bone during crushing. The crushed particulates were sieved through standard wire cloth sieves of 106 and 150 μm, and the particulates within this range were used for implantation. After cleaning and rinsing with copious sterile distilled water, the particulates were sterilized in a cell culture-cabinet using 70% ethanol for over 2 min, Hanks' balanced salt solution with the antibacterial actimyociton for 1 min and two solution changes before drainage and storage at -20 °C.

2.2. Surgery

The guidelines for the care and use of laboratory animals (Animals (Scientific Procedures) Act 1986) were observed and before implantation. Thirty female, New Zealand white rabbits, 2.88 kg weight average, (2.4–3.5 kg range) were operated unilaterally. After anaesthesia, the fibula was located by palpation and a 25–40 mm longitudinal incision was made on the postero-lateral side of the lower limb through the skin to expose the underlying muscles. The fibula was approached by separating the *soleus* and *tibialis cranialis* muscles using two metal dental probes, and exposure of the fibula was achieved without incising the muscles. A 1 mm wide osteotomy in the mid-diaphysis was created using stainless steel circular saw blades mounted on an electrical dental drill.

Twenty osteotomies were filled with either BBM particulates or allograft bone particulates and the rest were left empty as controls. Using interrupted suturing, the incisions were closed in two parts. After surgery, the animals were kept in groups of five and allowed to weight-bear on all four limbs. Low fat dry pellet, SDS rabma (Essex, UK), hay and water *ad libitum* were provided. At one, two, three and four weeks post-surgery, tetracycline, alizarin red, calcein green and xylenol orange were administered once at 25 mg (kg body weight)$^{-1}$, respectively. Five weeks post-surgery, the animals were sacrificed by intravenous administration of an overdose (5 ml) of pentobarbitone sodium BP (Expiral; 200 mg ml^{-1}). The proximal tibia and the entire fibula with the surrounding soft tissue were dissected and macroradiographs were taken on AGFA films at 32 kV, 2 mA and 3 min of exposure using a Faxitron X-ray cabinet system (Vinten Instruments Ltd, England). From each group, six and four fibulae were selected for biomechanical and histological analyses, respectively. Specimens for mechanical analysis were wrapped with cotton-wool soaked in distilled water and stored in air-tight plastic bags. Histology specimens were stored in alcoholic formaldehyde.

2.3. Mechanical testing

Using a high-power electric saw (Minigraft, UK) the tibia was cut at the tibio-fibular syndesmosis and on the proximal end, the fibular was separated along the epiphyseal growth plate. The bone ends were then potted into brass tubes (25 mm long by 30 mm diameter) with Technovit 4000 and Pulver resin mixture (Kulzer and Co., Dammwold, Germany) and loaded in tension using the Instron 4464 testing machine (Instron, UK). During preparation, mounting and testing, the specimens were kept fully moistened. To allow axial orientation of the specimen during testing, the metal pots were attached to the testing machine with universal joints. At 5 mm min^{-1}, with continuous recording of load with a 2 kN-capacity load cell and displacement by the in-built devices, the specimens were loaded to failure. In total, eighteen fibulae with healing osteotomies and fifteen intact fibulae from the contra-lateral limbs were tested. After mechanical testing, the cross-sectional area of the fracture surface was determined using a Quantimet 570 image analyzer (Cambridge Instruments, UK).

2.4. Histological and roentgenographic evaluations

After fixation in alcoholic formaldehyde for two weeks, the 12 dissected fibulae were dehydrated in 100% industrial methylated spirit, IMS, as follows; 48 h on a stirring plate under 600 mBar vacuum pressure, then 24 h in fresh IMS, followed by 24 h of de-fatting in chloroform and finally 24 h in fresh IMS. Sectioning of the specimen block was performed parallel to the longitudinal axis of the fibula using a diamond band saw and an Exakt system for grinding thin sections (Exakt Corp., Hamburg, Germany). Toluidine blue stain on 15 μm thick sections was used for light microscopy and macroradiographs were used to categorize the osteotomies into four groups, namely excessive external callus, little external callus, atrophic non-union and hypertrophic non-union. Unstained sections, also 15 μm thick, were examined using a Zeiss ultraviolet microscope to detect fluorescent bone labels.

2.5. Mechanical and statistical analysis

The load-displacement curve was used to determine the stiffness and the failure load of the specimen. Kruskal–Wallis and Mann–Whitney tests were used to compare the biomechanical data. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Morphological of particulates

BBM particulates were shorter and more angular than the allograft particulates (Fig. 1). However, the smallest dimensions of the two material particulates were closely

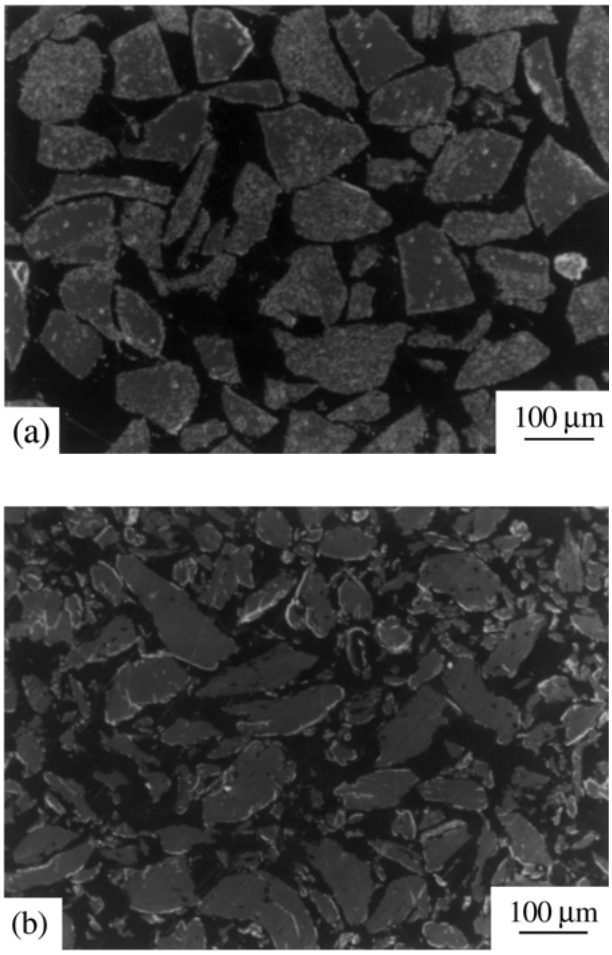

Figure 1 Photomicrographs of polished BBM (a) and cancellous bone allograft (b) particulates.

matched. The allograft particulates exhibited lamellae and ubiquitous empty lacunae.

3.2. Mechanical results

As shown in Fig. 2, all the osteotomized fibulae were loaded to failure and generally each curve exhibited a toe-in region, a linear elastic region and a non-linear plastic region. The sizes of these regions varied considerably, and both brittle and ductile failure modes were displayed. The allograft treated fibulae exhibited the greatest peak load (Fig. 2a) and in contrast to the control group (Fig. 2b), the BBM group (Fig. 2c) showed the greatest scatter in elongation to failure. The intact bone group exhibited the highest median peak load value (Table I). However, there were no significant differences between the peak loads of the experimental groups. The only significant difference was observed between the BBM group and the intact bone group ($p < 0.05$). The BBM group had the highest median stiffness (Table I), but differences with the other osteotomized groups were not significant.

3.3. Cross-sectional area

The cross-sectional area of intact bone was approximately a third of the osteotomized fibulae (Table I). Compared to the intact fibulae, the size differences were

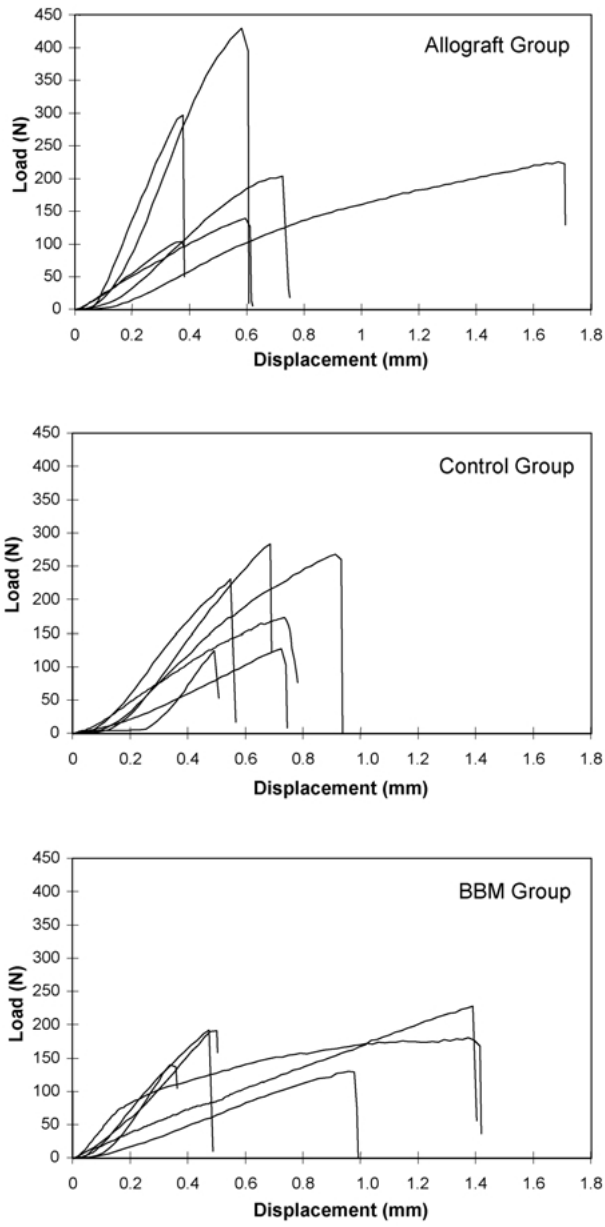

Figure 2 Load-displacement curves of the osteotomized fibulae.

significant for the control group ($p < 0.05$), but highly significant for both the allograft and BBM filled groups ($p < 0.001$). Clearly, at five weeks after surgery the osteotomized fibulae had not attained structural normality.

3.4. Roentgenographic evaluation and histology results

Fig. 3 shows typical radiographs taken at five weeks post-surgery. It is evident that callus formed across the osteotomy gap in some fibulae. In others consolidation was poor and non-union was evident. The callus was of two sizes and non-union was classified as being atrophic or hypertrophic. Table II shows that the degree of consolidation depended on the treatment regime employed. Although the control osteotomies produced the most excessive external callus, the allograft implanted osteotomies, which produced the least external callus, healed best. The healing rate amongst the remaining allograft filled animals at five weeks after

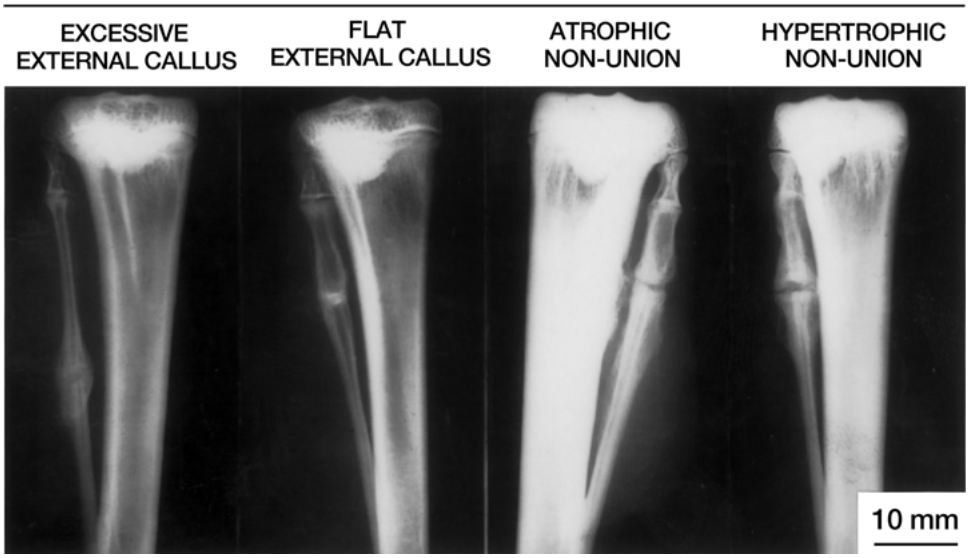

Figure 3 Typical radiographs of the healing osteotomies illustrating the four different categories used in the roentogenograhic evaluation of the osteotomies.

TABLE I The median and range of mechanical properties and cross-sectional area values of the osteotomized ($n = 6$) and intact fibulae ($n = 15$) at five weeks after surgery

Observation group	Stiffness (kN m^{-1})	Peak load (N)	Cross-sectional area (mm^2)
Allograft	375 (232–1105)	214 (104–433)	13.8 (6.51–17.9)
Control	506 (210–624)	202 (124–284)	11.4 (3.23–21.7)
BBM	510 (165–642)	186 (130–227)	12.3 (10.5–13.6)
Intact bone	422 (130–802)	232 (118–387)	4.12 (2.83–8.68)

TABLE II Number of fibulae exhibiting the four types of repair five weeks post-surgery

	Osteotomy repair type				
Observation group	Excessive external callus	Flat external callus	Atrophic non-union	Hypertrophic non-union	Total
Allograft	3	6	1*	0	10
Control	7	1	1	1	10
BBM	6	2	0	2	10
Total	16	9	2	3	30

*Animal was sacrificed 16 days post-surgery.

surgery was 100%. Twenty per cent of the BBM filled osteotomies suffered hypertrophic non-union and the same percentage of the control osteotomies suffered non-union, equally divided into the two types. Approximately 55% of all the animals killed at five weeks post-operatively healed with excessive callus and 44% of those belonged to the control group. Of the nine animals that exhibited flat external callus, approximately 67% had been treated with allograft particulates and 11% were controls. Four animals failed to heal by the end of the fifth week.

3.5. Light and UV microscopy

Bone repair involved callus and at five weeks post-surgery the medullary cavity was obstructed (Fig. 4). Unmineralized and mineralized tissue occupied the osteotomy site as revealed by the gaps between the diffuse tetracycline and alizarin red labels which where present on either side of the osteotomy (Fig. 5). At the junction between the osteotomy and original bone osteoclasts could be seen resorbing bone and woven and lamellar bone types co-existed (Fig. 6). Osteointegration existed between the newly formed osseous tissue and the particulates. Woven and lamellar bones, which are demonstrated as diffuse tetracycline and linear alizarin and calcein green bone labels, grew adjacent to the particulates (Fig. 7).

4. Discussion and conclusion

An osteotomy was created in the diaphyseal portion of the fibula, and BBM and allograft bone particulates were implanted in the gap to investigate their effects on cortical bone repair. The rabbit fibula model permitted a comprehensive study of the healing of the osteotomy without the need to immobilize the bone ends [1, 21, 22].

All osteotomies were located in the mid-diaphysis region of the fibula and only one animal was killed before the designated time for reasons unrelated to the presence of particulates. At five weeks after surgery, sixteen of the twenty-nine experimental animals exhibited excessive external or periosteal callus whilst 30% produced flat or little external callus. Seventy per cent of the control

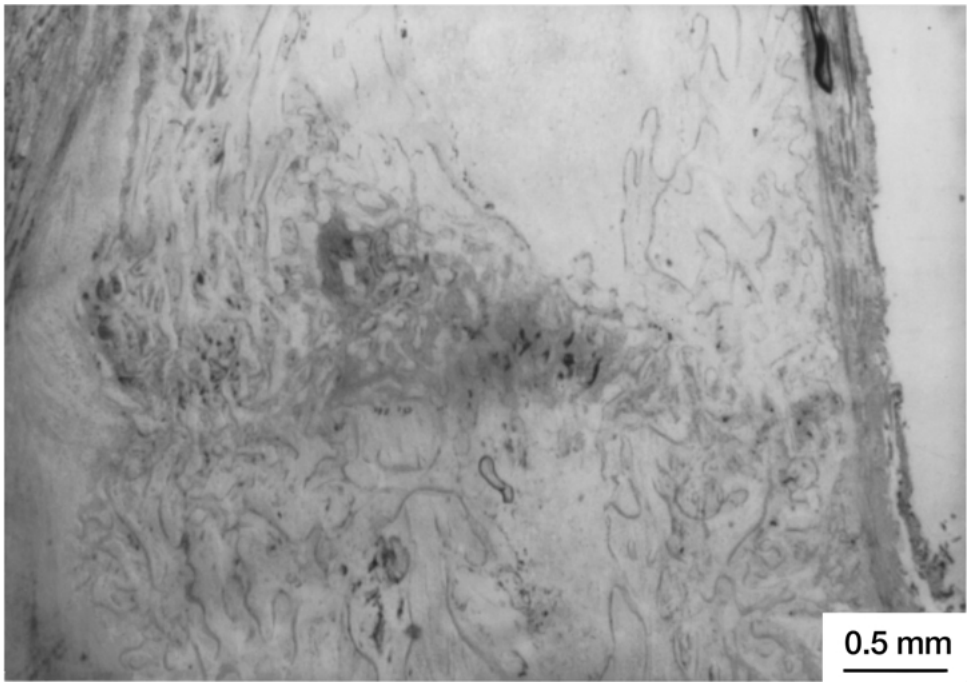

Figure 4 A control osteotomy depicting the filled medullary cavity at five weeks after surgery. Toluidine blue stain.

animals produced excessive external callus which is in agreement with the observations of Yamagishi and Yoshimura [3]. Yamagishi and Yoshimura [3] observed that when micromotion was allowed during the healing of rabbit tibial fractures, 70% of the twenty-nine experimental animals developed excessive external callus and there were no incidences of failed-union or non-union. In this current work however, at five weeks after surgery, one animal from the control group exhibited atrophic non-union and three animals (one from the control and two from the BBM group) exhibited hypertrophic non-union. The healing of the transverse osteotomies followed the same sequence observed in fracture healing (endochondral ossification). Bridging external callus and medullary callus were evident in twenty-five animals. Therefore tibio-fibular syndesmosis did not prevent micromotion across the osteotomy site which is considered essential for the progress of indirect healing [2, 6, 23, 24]. Kenwright and Goodship [4] observed that controled micromotion across a sheep tibial fracture increased the bone mineral content or periosteal callus, hence the increased cross section and stiffness.

At five weeks post-surgery, the greatest values of stiffness were displayed by two allograft filled osteotomies, nevertheless, no significant differences were observed between the observation groups. The failure to distinguish between the osteotomies may partly be explained by the observations of Black *et al*. [1] and Pienkowski *et al*. [22]. At 16 days post-surgery, Black *et al*. [1] observed that the stiffness of healing osteotomies did not differ from that obtained at 18 days post-surgery. Pienkowski *et al*. [22] observed that osteotomies created by a paediatric bone-cutting forceps were most responsive to the effects of stimulation at approximately 16 days post-operatively. At three weeks, because the changes in the stiffness were too small the effects of stimulation were equally too small to be mechanically discernible. This observation was also noted by Aro *et al*. [5] who observed that the bone mineral content in closed rat tibial fractures was unaffected by healing time three weeks after fracture. In dogs, Markel *et al*. [24] reported that new bone formation in the callus peaked at four weeks and yet the formation of radiographic callus peaked at six weeks. Using the animal sizes to approximate the peak time in rabbits, four to five weeks post-surgery would be reasonable. In fact, at five weeks post-surgery, all the osteotomies were observed to be considerably larger in cross-sectional area than in intact bone. On average, the cross-sectional area ratio of intact bone to healing osteotomies was approximately 35%. Markel *et al*. [24] reported that remodeling in dogs may take up to 36 months, hence in rabbits which have a higher appositional rate, $3.1 \pm 0.2\,\mu m\ day^{-1}$, [20] versus $1.9 \pm 0.8\,\mu m\ day^{-1}$, [25] the time could be up to nine months.

At five weeks post-surgery the osteotomies were in the process of remodeling. Histological analysis revealed that in some osteotomies, cartilaginous tissue was prevalent within the osteotomy gap. Therefore as remodeling progressed the mineralized cartilage was replaced by woven bone and the woven bone in turn, was replaced with lamellar bone as callus was removed from the obstructed medullary cavity. The variability in the material within the interfragment gaps was also indicated by the biomechanical behavioral patterns of the osteotomies, that is both brittle and ductile properties were depicted.

Further work is required to ascertain the effects of BBM particulates on the repair of cortical bone defects, although in cancellous bone, the effects of BBM were unequivocal [17]. It is suggested that a reduction in the observation time and the use of an alternative mechanical testing method, such as torsion, are employed in the future.

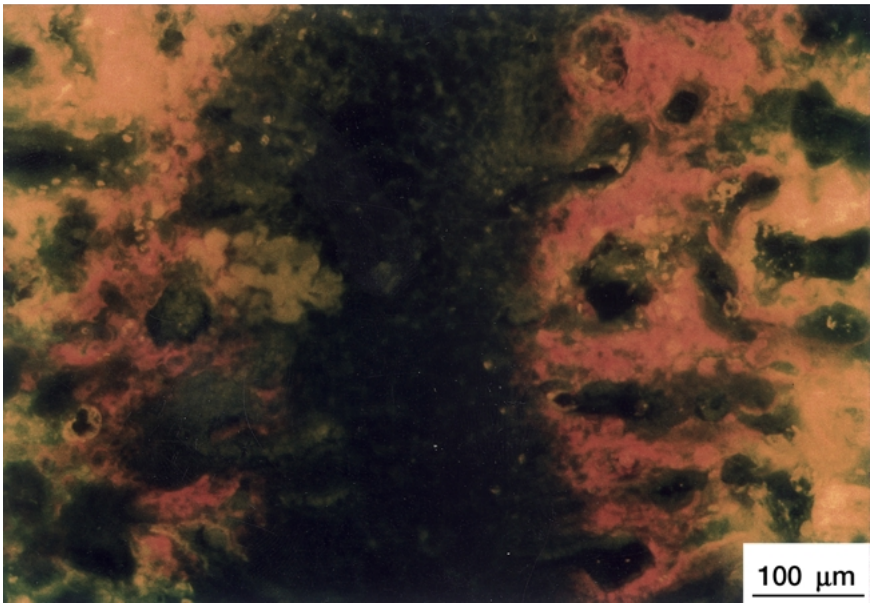

Figure 5 Fluorochrome labeled control osteotomy showing diffuse tetracycline and alizarin red indicating woven bone emerging from either side of the gap.

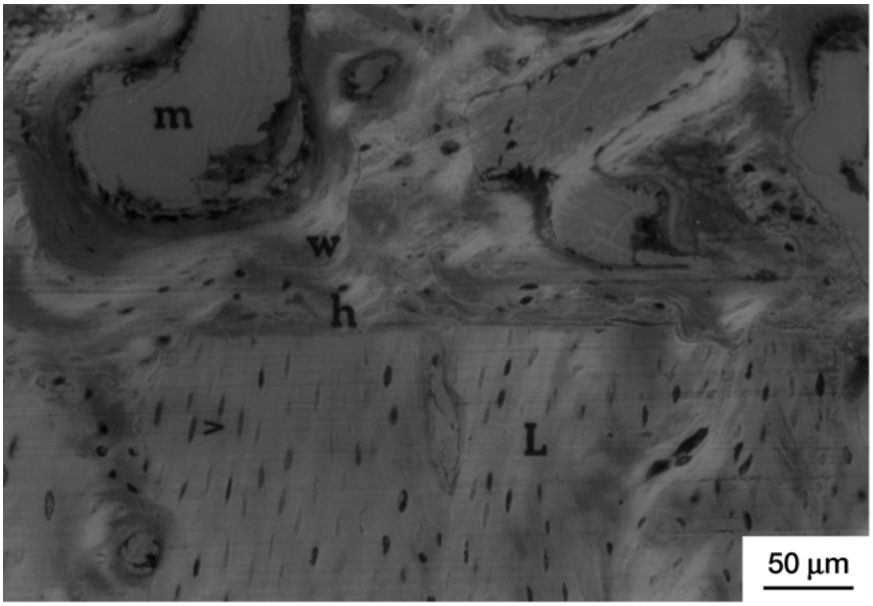

Figure 6 Polarized photomicrographs of the junction between new woven bone (w) and old lamellar bone (L) in a control osteotomy showing resorptive Howship's lacunae (h), the bone marrow spaces (m) and osteocytes (o).

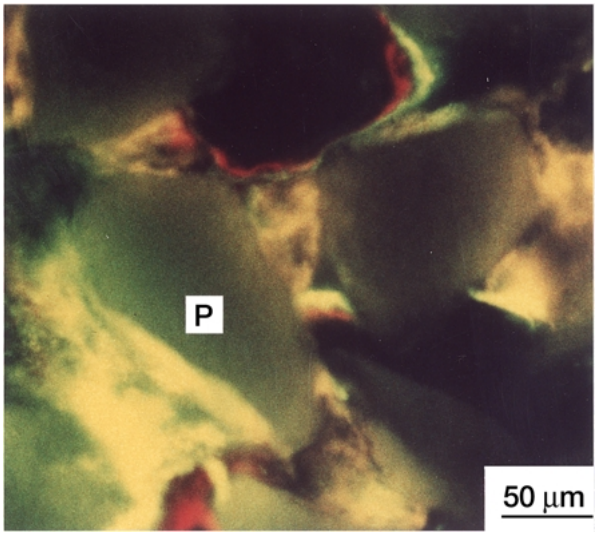

Figure 7 Osteointegration of BBM particulates (p) with newly formed woven (diffuse bone labels) and lamellar bone (linear bone labels).

Acknowledgment

The Beit Trust and the ORS for their support of MTM and EPSRC for the provision of the core grant of the IRC in Biomedical Materials. Dr K. A. Hing is also acknowledged for supplying the BBM.

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Received on 19 April 2000
and accepted 27 February 2001