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Prevascularized bone graft cultured in sintered porous β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ addition ceramic chamber

Chi-Chang Lin, Chun-Jen Liao, Jui-Sheng Sun, Hwa-Chang Liu and Feng-Huei Lin

Centre for Biomedical Engineering, Department of Orthopaedics, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC

Autogenous bone transfer is an important part of reconstructive plastic surgery. Presently available techniques have the disadvantages of limitation of available donor site, loss of donor tissue and the possibility of donor defect or deformity. In the present study, a vascularized bone graft was created and cultured in the groin area of the New Zealand rabbit. The cylindrical ceramic chambers, 15 mm in length, 6 mm in outer diameter and 3 mm in inner diameter, were prepared by the addition of sintered porous β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$. In the first group, the chambers impregnated with autogenous bone fragments and allogenous demineralized bone matrix with volume ratio 1:1 were cultured in the rabbit's groin area with saphenous vessels passing through. In the second group, the chambers were treated by the same procedures as the first group but without saphenous vessels passing through. In the third group, the chambers were not impregnated, and were cultured in the groin area with saphenous vessels. After 2, 4, 6, 8 and 12 wk of operation, the animals were killed with an overdose of intravenous pentobarbital. The viability of the osseous tissue in the chamber was evaluated by histological examination, microangiograms and fluorochrome incorporation for the three groups. The autogenous bone chips could survive and retain their osteogenic properties while packed into the sintered porous β-$Ca_2P_2O_7$ (with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ addition) ceramic chamber and implanted in the rabbit groin area up to 12 wk. However, even at the longest time periods, considerable amounts of dead bone were present in the chambers. In addition, we observed bone resorption in the three groups up to 12 wk, which might be attributed to lack of physiological stress. There were significant differences in new bone formation and osseous cell viability among the three groups. The prevascularized vessels and autogenous bone chips were both necessary for the formation of new bone and osteogenic property in the chamber under these heterotopic circumstances. The biodegradable ceramic used in this study was gradually absorbed and dissolved in the physiological environment. However, the degradation debris of the ceramic caused no injury to the new bone formation. These findings support the concept of creating a preformed vascularized bone graft to reconstruct segmental bone defects. © 1996 Elsevier Science Limited

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The history of bone transplantation to replace defective bone tissue subjected to trauma, infection, degenerative or neoplastic disease, or congenital anomalies covers several centuries. When the defect is small, autogeneic bone grafting is the best choice, although its use involves an additional operation. However, when the defect is large, as in wide resection of an osteosarcoma, allogeneic bone grafting or use of a synthetic prosthesis with tissue-compatible alloys or ceramics is sometimes considered. There are, however, problems with bone alternatives. Antigen removed by physical or chemical treatment and a bone bank system are possible solutions to the problems of allogeneic bone grafting, but the bone bank system has problems of limited resources and high cost. Artificial or synthetic substitutes have been tried in recent decades, but did not achieve great success owing to the lack of osteogeneic and osteoinductive properties[1–4].

When bone is fractured or when bone grafts are used to reconstruct skeletal defects, several mechanisms

Correspondence to Dr Feng-Huei Lin.

that generate new bone formation are initiated: osteogenesis, osteoinduction and osteoconduction[5]. It would be ideal in circumstances requiring bone replacement if the patient could be stimulated to produce new bone that would fill the defect or permanently replace any foreign material that was used as a temporary space filler. This is impossible for the metals, fine ceramics and polymers currently used, but might be achieved with a biodegradable ceramic.

Sintered porous β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ addition ceramic has been reported to aid osteoconduction, to withstand severe mechanical loading and to have optimum biodegradability in the physiological environment[6]. Autogenous bone chips and allogenous demineralized bone matrix have been reported, which could transfer viable bone-producing cells within the graft to a new anatomical location[5–7]. It would be clinically relevant to design a β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ addition ceramic chamber impregnated with autogenous bone chips and allogenous demineralized bone matrix, so that the new generated bone could be stimulated and formed within the porous structure of the ceramic, and slowly replaced by a form of 'creeping substitute'.

Vascularized bone grafts, by virtue of their intrinsic blood supply, can obtain more rapid union and tolerate infection better than conventional bone grafts, in a recipient site devoid of adequate blood supply[3,7–10]. In the present study, a biodegradable ceramic chamber filled with autogenous bone chips and allogenous demineralized bone matrix was prepared. The packed ceramic chamber would be positioned in the groin area with the saphenous vessels passing through the chamber longitudinally in contact with the packed bone fragments. By combining the tissue-compatible characteristics of the biodegradable ceramic with the osteogeneic and osteoinductive capacity of autogenous bone, and utilizing the vessel-sprouting capability of a transposed vessel, we wish to investigate whether autogenous bone tissue and cells could be bone generative under these heterotopic conditions, whether autogenous bone cells, known to be osteogenic after transplantation to a groin site, retain their viability if placed in contact with the biodegradable ceramic materials, and whether the ceramic material chosen in this study is histocompatible and non-injurious to the free tissues into which it is implanted.

MATERIALS AND METHODS

Ceramic chamber and allogeneic demineralized bone matrix preparation

Ceramic chamber preparation[6,11]

Five grams of $Na_4P_2O_7 \cdot 10H_2O$ powder was dissolved in distilled water and well mixed with 95 g β-$Ca_2P_2O_7$ powder as a slurry condition. The well-mixed slurry was then dried at 50°C for 3 d. The dried cake was ground into powder form and sieved into a processing particle in a range of 40–60 mesh by the addition of binders and lubricants, which facilitate pressing and are burned off in a later firing process. The sieved particles were then compacted into a stainless steel die under a hydrostatic pressure of 270 MPa. The cylindrical compacts, measuring 6 mm in outer diameter, 3 mm in inner diameter and 15 mm in length, were made as a porous type with 48% porosity, which was mixed with polyethylene glycol 4000 in particle sizes of 5 and 500 μm to produce macropore and micropore structures after decomposition. The prepared green body was placed on a platinum sheet and heated to 910°C with a heating rate of 3°C min^{-1} in a conventional Ni–Cr coiled furnace, and then maintained for about 1 h for sintering. The sintered porous β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ addition ceramic chambers were cut longitudinally into two halves along the central line of the chamber.

Allogeneic demineralized bone matrix preparation[7,12,13]

Allogeneic demineralized bone matrix was prepared from the diaphysis of donor rabbit femur, tibiae and humeri by acid demineralization in 0.6 N HCl for 72 h and defatting in 70% ethanol for 72 h. Dry matrix was ground to 50–250 μm using a mill and Nylon mesh filters. Ten New Zealand white rabbits were used for this purpose.

Surgical procedure and implantation

Sixty male, 6-month-old, New Zealand white rabbits (3–3.5 kg in weight) were divided into three groups, according to the chamber with or without impregnation with materials inside and with or without saphenous vessels passing through. Rearing of these animals and the animal experiments were carried out at the Institute of Laboratory Animals, College of Medicine, National Taiwan University. They were delivered to the laboratory no later than 1 wk before the start of the test, and acclimatized to the housing condition, tap water and standard dry feed diet. The animals were kept in single stainless steel cages at 22°C and 60% relative humidity in natural daylight with a day–night rhythm.

All animals were anaesthetized with an intramuscular injection of a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg). General anaesthesia was maintained by inhalation of oxygen, nitrous oxide and halothane. Ringer's solution was infused continuously through an intravenous catheter placed in the ear. Antibiotic prophylaxis with 400 000 units procaine penicillin and 500 mg streptomycin was administered 30 min prior to skin incision and was continued 2 d postoperatively. The animals were shaved dorsally over both iliac crest and medially in the groin area of the right leg. Small autogeneic bone fragments were harvested from the iliac crest and washed in isotonic saline. Allogeneic demineralized bone matrix, which had been soaked previously for 1 h in normal saline, was well mixed with autogeneic bone fragments and then placed in the ceramic chambers.

The saphenous vascular bundle (consisting of the artery and vein) was exposed in the right groin and

dissected free from the surrounding tissues. The saphenous vessels of the first group of animals were elevated, and one half of the ceramic chamber, previously filled with autogeneic bone chips and allogeneic demineralized bone matrix, was positioned beneath the vascular bundle. The second half of the ceramic chamber was placed on the top of the first half, permitting the vessels to pass through the enclosed chamber. A single silk suture surrounded the chamber to prevent it from opening and the silk was closed with 5-0 Vicryl sutures. Animals in the second group were treated as described previously, but without saphenous vessels passing through the chamber. Animals in the third group had the same operation with saphenous vessels passing through the chamber, but without bone chips and demineralized bone matrix inside the chamber. The rabbits were returned to their cages, where they were permitted unrestrained mobility. After 2, 4, 6, 8 and 12 wk of operation, the animals were killed with an overdose of intravenous pentobarbial.

Measurement and analysis

Fluorochrome bone labelling

Fluorochrome bone markers were administered to indicate the characteristics of bone growth. Double labelling was performed in all rabbits with oxytetracycline (30 mg/kg) and calcine green (35 mg/kg) by means of subcutaneous injections, which were alternatively injected for every other week after operation[7,12].

Microangiography

To confirm blood flow in the vascular bundle, rabbits from the vascularized group were killed for microangiography. Immediately after killing the animals, the abdominal cavity was opened and the abdominal aorta was exposed. The proximal portion of the aorta was ligated, and a Teflon catheter was inserted distally from the ligation and fixed tightly with silk thread. The tip of the catheter was placed at least 5 mm proximally from the bifurcation. An incision was made on the inferior vena cana, and heparinized saline at 37°C was infused at a pressure of 150 mmHg. Infusion was continued until the toes of both legs became completely white. Then a 5% gelation and 5% carmine solution at 37°C was infused at 150 mmHg. The infusion was continued for about 30 min after toes became pink and backflow of the dye from the inferior vena cana was observed. Immediately after infusion, the implants (ceramic chambers) were harvested with surrounding soft tissues and placed in ice-cold formalin solution (20%)[14,15].

Histology

After solidification of the infused gelatin solution, ceramic chambers were carefully separated from the surrounding tissue and fixed further in 10% phosphate-buffered formalin solution. The harvested ceramic chambers were bisected transversely and the remainder longitudinally with a microbone saw. One half of each bisected chamber was fixed in formalin, decalcified in EDTA and sectioned in paraffin for staining with haematoxylin and eosin. The other half was fixed in 70% ethanol, dehydrated in an alcohol series and embedded in methylmethacrylate. These undecalcified tissue sections were observed under a fluoromicroscope with reflected light[16,17].

Quantitative evaluation

Quantitative evaluation was then performed using a semiautomatic histomorphometric method. The system consisted of a microscope with cross-polarizing filters, digitizing plate, digitizer, printer and microcomputer with a mini-floppy disk drive. The microscope was equipped with a drawing tube, through which the image of the digitizing plate was projected over the optical field. By moving a cursor on the digitizing plate, which was visible by its projection over the histological field, newly grown bone in the ceramic chamber was traced and its area was calculated by the microcomputer. The area of newly grown bone, soft tissue and blood vessel in each section was calculated, and expressed as a percentage of the area of the ceramic chamber central hollow. Each section was analysed statistically for significance, using multiple Student's *t*-tests to compare individual groups[14,17].

RESULTS

Three rabbits died unexpectedly during the study; however, additional operations were performed to complete the experimental groups. At the time of being killed, the saphenous artery was consistently found to be filled with gelatin/carmine solution on both sides of the chamber, indicating that the vascular pedicle remained patent. Where there were structural defects in the ceramic or irregularities in the surface, bone or fibrous tissue filled the gap. Whether such irregularities had been produced by osteoclastic resorption or by enzymatic or other chemical dissolution could not be determined (*Figure 1*).

Histological study

In the first group (the chambers with saphenous vessels passing through were impregnated with autogenous bone chips and allogenous demineralized bone matrix), the chambers were covered by thinner layers of granulation tissue and were less firmly anchored to the host tissue after 2 wk of operation. A large number of small vessels was observed in fibrous tissue surrounding the bone fragments at 4 wk. New bone formation was present on the surface of acellular bone fragments adjacent to the implanted vessels (*Figure 2*). At 6 wk, osteocytes and new bone formation were more widespread, and bony union between bone chips was observed (*Figure 3a*). Newly formed small blood vessels with a smooth muscular were often noted (*Figure 3b*). However, wide acellular areas were noted in the centre of the fragments. In addition, numerous osteoclasts were seen on the surface of the bone. The bone infrequently demonstrated basophilic nuclear staining with the lacunae. However, minimal

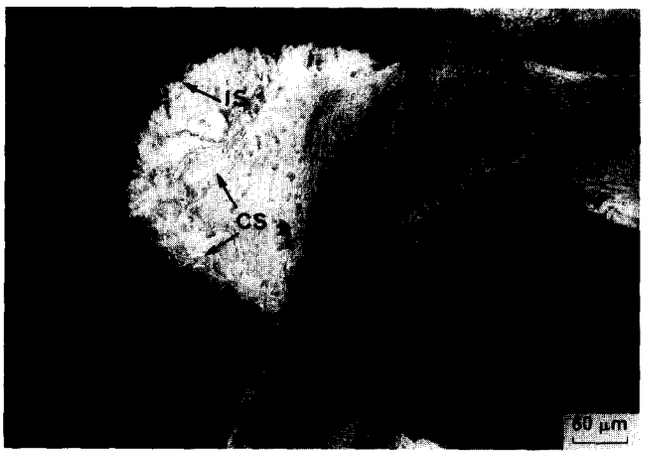

Figure 1 The fibrous tissue and ostoid contain gaps where there are structural defects in the ceramic or irregularities in the surface. CM, ceramic (sintered β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ ceramic); GP, gap; FT, fibrous tissue; IS, irregularities in the surface; CS, ostoid or bone-like structure.

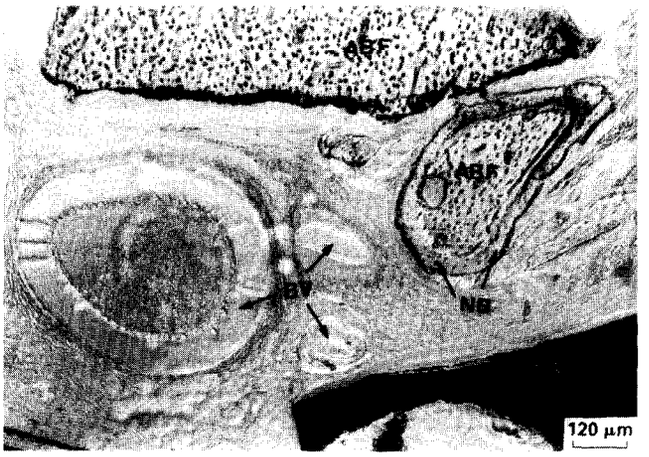

Figure 2 The histological section of the first group was examined under an optical microscope after 4 wk of operation. New bone formation was present on the surface of the autogenous bone fragment adjacent to the implanted vessel. BV, blood vessel; NB, new bone; ABF, autogenous bone fragment; CM, ceramic (sintered β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ ceramic).

areas in the vicinity of the saphenous vessels had intensely stained osteocytes. Osteocytes appeared to be more numerous in 6-week specimens, although more than half their visible lacunae were empty. Areas with stainable osteocyte nuclei always appeared to be located along the peripheral rim of the osseous fragments, while the acellular areas were more centrally located. The osseous matrix in the cellular area showed a slightly less dense structure, taking the eosin stain differently to the acellular portions.

By 8 wk, stainable osteocytes were more widespread, with viable bone elements outlining and sometimes bridging between the dead bone fragments. The new bone was attached towards the surface of the ceramic internal pores (*Figure 4*). Fluorescent microscopy demonstrated uptake of the last five bone labels

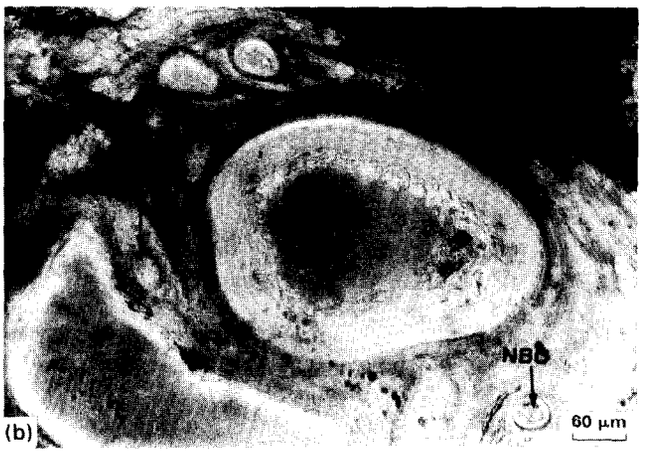

Figure 3 The histological section of the first group examined under an optical microscope after 6 wk of operation. **a**, Osteocytes and new bone were growing around the surface of the bone chips, and bony union between bone chips was occasionally observed. **b**, Newly formed vessels with a smooth muscular were often observed. NB, new bone; BU, bone union; ABF, autogenous bone fragment; NBV, new blood vessel; CM, ceramic (sintered β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ ceramic).

administrated, although calcine green was sparse (*Figure 5*). The fluorescence in periosteal specimens had more of a parallel appearance than normally occurs in osseous tissue.

At 12 wk, the chamber's central hollow area was occupied by increased amounts of lamellar bone and fatty marrow tissue. The laminar bone formation appeared with very few plump osteoblasts on its surface (*Figure 6a* and *b*). Most of the demineralized bone matrix had resorbed, and was replaced by loose adipose tissue or bone marrow tissue.

In the first group, newly formed bone was found within the chambers 2 wk after the operation. Such bone was visible at all times up to 12 wk. The demineralized bone matrix absorption was apparently observed at 6 wk.

In the second group (the chamber without vessels passing through was impregnated with autogenous bone chips and allogenous demineralized bone matrix), necrosis of some of the bone chips always occurred at 2 wk after the operation; frequently, only the fat spaces were retained peripherally. Small, well-

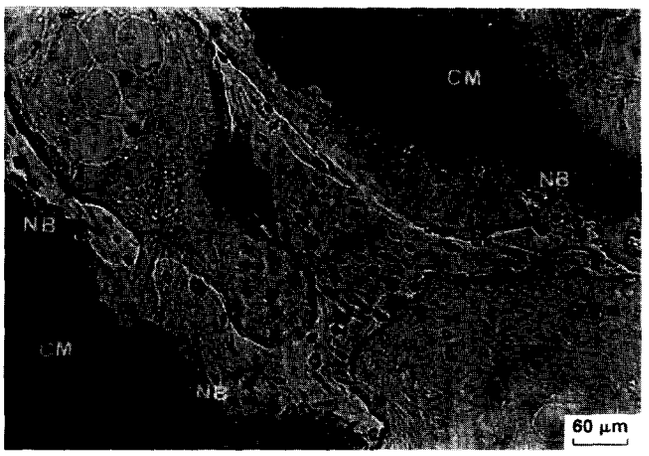

Figure 4 At 8 wk in the first group, the new bone sometimes bridged the dead bone fragments and was attached toward the surface of the ceramic internal pores. CM, ceramic (sintered β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ ceramic); ABF, autogenous bone fragment; NB, new generated bone.

Figure 5 Fluorescent microscopy demonstrated uptake of the last five bone labels administered. Tc, tetracycline; Cg, calcine green.

defined resorptive lacunae were noted; these may represent osteoclastic activity (*Figure 7*). The osteocytic lacunae throughout the bone were empty at 4 wk. At 8 wk, large numbers of osteoclasts were still present at the periphery and resorptive bays, representing phagocytosis, could be seen eroding the bone chips and demineralized bone matrix. At 12 wk, there was a strong vascular response around the margin of the chip with early vascular ingress, but bone formation was not seen at the periphery of the bone chips and demineralized bone matrix (*Figure 8*).

In the third group (the chamber with saphenous vessels passing through was not packed with autogenous bone chips and allogenous demineralized bone matrix), all the spaces were filled with fibrous fatty tissue, without evidence of new bone formation. In the experimental period, in all chambers multinucleated giant cells lined the interface between the chamber and surrounding soft tissue. The presence of the giant cells suggested a foreign body reaction, and may be related to degradation or resorption of the ceramic. There was no large-scale

Figure 6 At 12 wk in the first group, the chamber's central hollow area was occupied by increased amounts of **a**, lamellar bone, and **b**, very few plump osteoblasts on its surface. SABF, surviving autogenous bone fragment; OHV, old Haversian system; NHV, new Haversian system; OB, osteoblast; NB, new generated bone; BF, bone fragment.

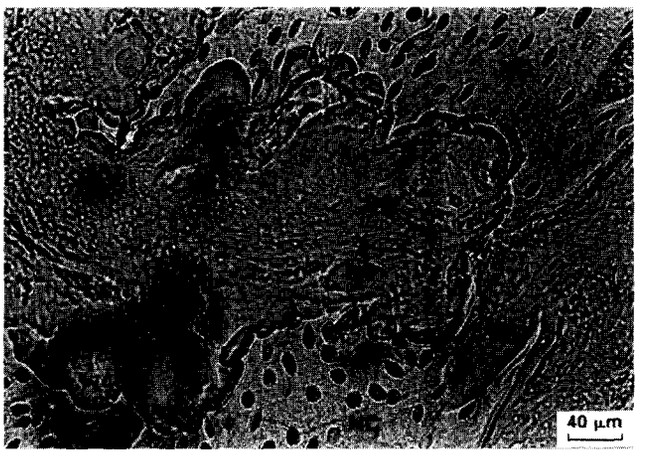

Figure 7 Necrotic bone chips always occurred at 2 wk after operation. Small and well-defined resorptive lacunae were noted. NC, necrotic bone fragment; RL, resorptive lacunae; RB, resorptive bay.

inflammatory reaction around the chamber; it was simply encapsulated within a sheath composed of elongated fibroblastic cells arranged in a multilayer (*Figure 9*).

Figure 8 At 12 wk in the second group, bone formation was not seen at the periphery of the bone chips and demineralized bone matrix. ABF, autogenous bone fragment; DBM, allogenous demineralized bone matrix.

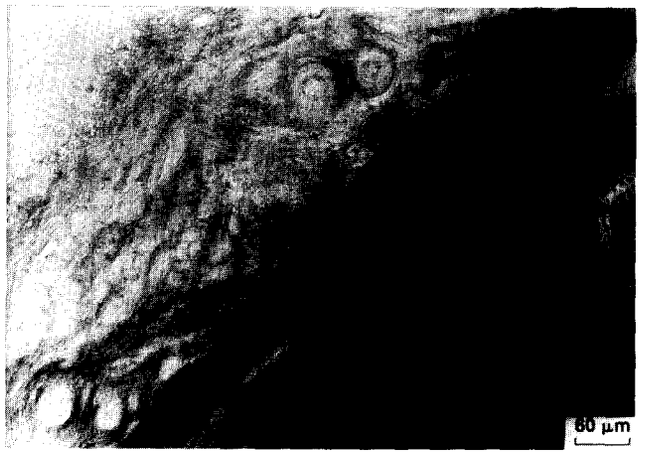

Figure 9 In the third group, all spaces were filled with fibrous tissue without evidence of new bone formation. It was simply encapsulated within a sheath composed of elongated fibroblastic cells arranged in a multilayer. CM, ceramic; FT, fibrous tissue.

Histomorphometric assessment

Most porous calcium implants currently being investigated have been fabricated to have interconnecting macropores in the range of 100–400 μm to meet the requirements for bone ingrowth which emerged from the studies of Klawitter and Hulbert[18]. One interesting suggestion, put forward by K. de Groot[18,19], is that it is the micropores in these structures which determine the rate of bioresorption. In the designed ceramic chamber, interconnected micropores (about 5 μm) and macropores (100–400 μm) were prepared by adding two different sizes of polyethylene glycol particles as foaming agent. The pores were left in the matrix after the foaming agent decomposed at the volatization temperature; they were uniformly distributed with the gross polycrystalline structure and frequently localized at the grain boundaries which connected the individual crystallites (*Figure 10*).

The amounts of newly grown bone, autogenous bone chips and pores were expressed as a percentage of their area to the area of the chamber's central hollow; the area of the ceramic chamber's central hollow was defined as 100%. The area occupied by pores included areas of soft tissue, connective tissue, bone marrow, osteoid and pores.

Figure 11 summarizes the results of the quantitative assessment of the new bone formation for the three groups. The newly grown bone of the first group increased with the implantation time. However, there was no significant evidence of new bone formation for the second and the third groups. *Figure 12* shows the areas of autogenous bone chips and allogenous demineralized bone matrix with the implantation time of the three groups. The areas of the autogenous bone chips and allogenous demineralized bone matrix steadily decreased from 36% at 2 wk to 22% at 12 wk for the first group. This phenomenon was caused by autogenous bone chips gradually being replaced by the new bone, as a 'creeping substitute'[7,20]. In the second group, there was a sharp decrease in the area of autogenous bone chips and allogenous bone chips and

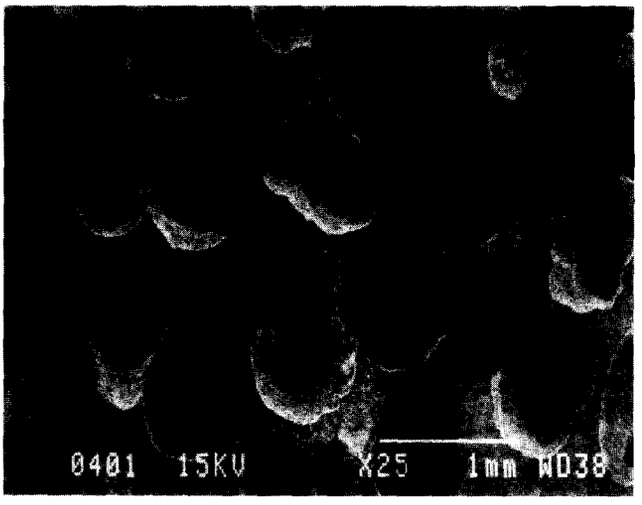

Figure 10 Macrostructure of the sintered β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ ceramic. The interconnected micropores and macropores were prepared by adding two different sizes of polyethylene glycol particles as foaming agents.

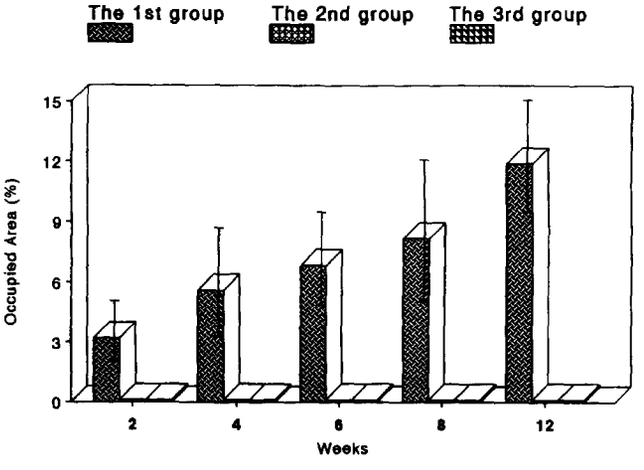

Figure 11 Summarized results of the quantitative assessment of the new bone formation for the three groups.

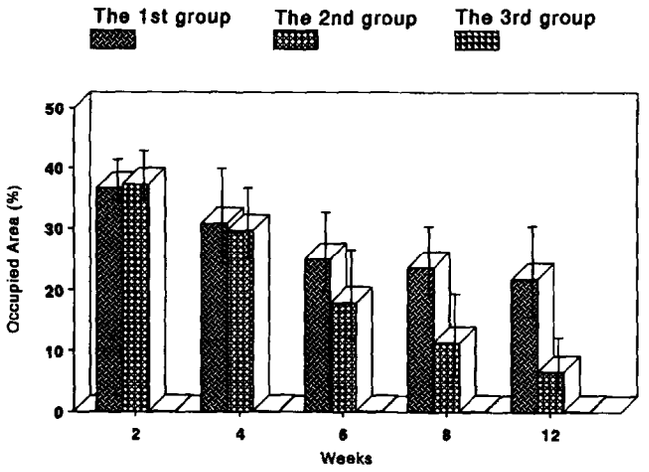

Figure 12 Summarized results of the areas of autogenous bone fragments and allogenous demineralized bone matrix changed with the implantation time of the three groups.

allogenous demineralized bone matrix, from 38% at 2 wk to only 6% at 12 wk. There was no area occupied by the autogenous bone chips and allogenous demineralized bone matrix throughout the experimental period for the third group, because the ceramic chambers were not impregnated with autogenous bone chips and allogenous demineralized bone matrix before implantation. There was little change in the pore area for the first group. However, the pore area of the second group increased with implantation time.

DISCUSSION

From the results of the present study, there was a significant difference in the new bone area at each time period between the first group and the second group. Also, there was a large discrepancy in osteocytic, osteoclastic and osteoblastic activities between the two groups. In the first group, new bone formation stated at 2 wk postoperation and continued vigorously during the experimental periods. There was no strong evidence to support new bone formation in the second group, although it was packed with autogenous bone fragments and allogenous demineralized bone matrix.

Currently, vascularized bone grafts are used extensively and clinically. Clinical and experimental studies indicate that vascularized bone grafts are superior to conventional bone grafts as far as bony incorporation, osteoproliferation, mechanical strength and other aspects are concerned[9,10,21,22]. However, the application of the technique is limited by the number of donor sites available, donor site morbidity and use of microsurgical techniques. Alternatives to conventional vascularized bone grafts have been investigated by several authors[21–24]. They described creating a free composite osteocartilaginous graft, using a staged microvascular transfer of a vascular pedicle with attached soft tissue. They demonstrated the potential of creating a vascularized bone graft nourished by an implanted vascular bundle. The reports concluded that the vascularized vessels provided nutrition and growth factors for the bone grafts to stimulate or induce new bone formation. The authors supposed that the saphenous vessels passing through the chamber contributed to new bone formation and caused the large difference in histological examination between the two groups.

They also revealed that no new formation was observed in the histological sections of the third group, where the saphenous vessels passed through the chamber without impregnation of autogenous and allogenous materials. Autogenous bone fragments were used in this study because of their well-known bone osteogenic capacity[12,13,20,21]. The bone-forming property was enhanced by the saphenous vessels. It might act as an embryo to vascular sprouting from the saphenous vessels to the chambers. It is possible that some of the osseous cells in the autogenous bone fragments survived, and that the bone cells observed in the chamber were formed from mesenchymal cells through induction. In the third group, there were no impregnated autogenous bone fragments, and there was a lack of bone osteogenic capacity in the chamber. This caused the third group to have no new bone formation. The use of other cell-free inducers such as allogenous demineralized bone matrix would alleviate the quantity of the autogenous graft-harvesting. The efficiency of these substances for the induction of moulded vascularized bone needs to be investigated.

The viability of the osseous tissue in the chamber was increased with prolonged implantation time, judging from histological evidence, microangiograms and fluorochrome incorporation. However, even at the longest time periods, considerable amounts of dead bone were present in the chambers. In addition, we observed bone resorption in the three groups up to 12 wk, which could be attributed to a lack of physiological stress[25].

The pore area of the second group showed a negative tendency with the implantation time, because of the osteoclastic activity and phagocytosis process in the chamber. Although similar activities happened in the first group, the rate of new bone formation was assumed to maintain the pace, and was balanced with absorption rate.

CONCLUSIONS

The autogenous bone chips survived and retained their osteogenic properties while packed into the sintered porous β-$Ca_2P_2O_7$ (with 5 wt% $Na_4P_2O_7 \cdot 10H_2O$ addition) ceramic chamber and implanted into the rabbit groin for up to 12 wk. There were significant differences in new bone formation and osseous cell viability among the three groups. The prevascularized vessels and autogenous bone chips were both necessary for new bone formation and osteogenic property in the chamber under such heterotopic conditions. The biodegradable ceramic used in this study was gradually absorbed and dissolved in the physiological environment. However, the degradation debris of the ceramic caused no injury to the new bone formation.

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