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# Post-implantation Evaluation of the Natural Coral (*Porites* sp.) and Calcium Phosphate Cement in Sheep Femur: A Comparative Study

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**Abstract:** The present study was designed to evaluate and compare the natural coral and Calcium Phosphate Cement (CPC) post-implantation in sheep femoral bone. Twenty one adult, male sheep (weight 15-20 kg) were used in this study and were divided into two groups. Group one consists of 12 animals and implanted with natural coral while group two consists of 9 animals and implanted with CPC material. The large cortical defect (2.5x0.5x0.5 cm) was created surgically on the left proximal femur and replaced by the implant. Radiographs and ultrasonographs were obtained immediately after surgery and at 2, 4, 8 and 12 weeks post-implantation. Both ultrasonographs and radiographs taken at 8 and 12 weeks showed that the implants had been resorbed and left the space that much reduced in size. There was no sign of implant rejection observed in all animals. The sheep were euthanased at 2, 4, 8 and 12 weeks post-implantation and the bone examined grossly. Samples of the implant were taken for histological examination. Microscopically, natural coral exhibited rapid resorption and progressively replaced by new bone. At 8 weeks post-implantation, there was no more coral implant present and by week 12 the implant site was almost completely closed and filled by mature bone. Meanwhile, CPC implant was clearly seen and demonstrated only marginal bone formation at the end of 12 weeks study. The coral implant exhibited good bone substitute, but it has fast resorption rate. Thus, it may suitable for less compact bone with small defect.

**Key words:** Natural coral, CPC, post-implantation

## Introduction

A wide range of materials has been extensively studied for their bone application. Most candidates have been synthetically modified to meet essential requirement of an adequate bone substitute. Natural coral, submitted to rigorous protocols of preparation and purification, can be used as replacement biomaterials for bone graft. Natural coral graft substitutes are derived from the exoskeleton of marine madreporic corals. The structure of coral that commonly used, *Porites* sp. is similar to that of cancellous scaffolds and has been shown to be biocompatible, osteoconductive and biodegradable at variables rates depending on the exoskeleton porosity, the implantation site and the animal species (Demers *et al*., 2002). Previous studies showed that natural coral could be used as biomaterial for onlay grafts in orthopedics and traumatology (Kehk *et al*., 1995), cranio facial (Marchac and Sandor, 1994)

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and dental (Mora and Ouhayoun 1995). Even though there were numerous reports on the clinical used of natural coral and CPC in the literature, the comparison between them is never been studied. Thus this study was designed to evaluate and compare the natural coral and CPC post-implantation in sheep femoral bone.

## Materials and Methods

*Animals and Experimental Design*

Twenty one adult, male sheep (weight between 15-20 kg) were used in this study. The animals were divided into two groups: group one consists of 12 animals while group two consists of 9 animals. Animals in group one were implanted with natural coral while animals in group two were implanted with CPC materials. The animals were housed in an individual pen throughout the post-operative study period. The animals were fed with commercial pellet and grass. Food and water were available *ad libitum*. The experimental animals were evaluated up to 12 weeks post-implantation. The animals were observed daily during post-operative period. For the coral group, the animals were euthanatized at 2, 4, 8 and 12 weeks post-implantation, while for the CPC group, the animals were euthanatized at 4, 8 and 12 weeks post-implantation. The experimental protocol was approved by the Faculty's Ethic Committee.

*Coral Processing*

The natural coral (*Porites* sp.) was used in the present study. Coral blocks were prepared and processed according to the protocol stated by the tissue bank of Malaysian Institute of Nuclear Technologies. By using the band saw, natural corals were cut into blocks of 2.5x0.5x0.5 cm. The blocks were pasteurized at 58°C in shaking water bath for one hour with water being changed three times and left overnight. Coral blocks were then subjected to deep-freezing (-80°C) overnight and then freeze-dried at –40°C for 24 h by using the freeze-drier Christ Loc-1. The condenser temperature, shelf temperature and vacuum used during the freeze-drying process were –40, –30 and 0.12 mbar, respectively. The moisture content for the fiual product was 9.13%. The freeze-dried coral blocks were then sterilized by gamma irradiation at MINTec-Sinagama, using sinagama machine (Model JS8900) at 25 kGy.

Calcium phosphate cement (Rebone Gutai, Shanghai Rebone Biomaterilas Co., Ltd, China) was used as comparison.

*Surgical Procedure*

Prior to surgery, each animal received intramuscularly injection of Norocilin LA and the antibiotic therapy was continued for five days post-operative period. After a premedication with atrophine sulphate (0.1 mg $kg^{-1}$), anesthesia was induced with a combination of ketamine (5 mg $kg^{-1}$) and xylazine (1 mg $kg^{-1}$) and maintained with the 2% halothane gas. The skin of the left femoral region was shaved and disinfected following the standard surgical protocol. A 10 cm longitudiual, slightly curved incision was made over the proximal part of the femur from the point of trochanter major through the skin, subcutaneous tissue and *tensor fasciae latae*. The *vastus lateralis* muscle was held side away and partially removed from the trochanter major. The adductors of the proximal part of the femoral shaft were partially mobilized. Using a surgical saw, two transverse cuts at 2.0 cm apart were made in the proximal third of the femoral shaft just distal to the trochanter major, through the periosteum and reaching into the medullary canal. These cuts were connected with two cuts along the shaft. They were

approximately perpendicular to the cortex and were 0.5 cm apart at the outer circumference of the cortex. Thus, a bony window could be removed from the femur leaving about two-thirds of the cortex circumference intact. Two holes were drilled through the cortex opposite the drill holes of implants. Blocks of coral and CPC were placed in that window in respective animals and secured in position with two sutures. The *vastus lateralis* musculus was reinserted with sutures. The fasciae were closed with a continuing chromic catgut suture and the skin was closed with PDS II. Animals were given post-operative analgesic phenylbutazone (4 mg $kg^{-1}$) and returned to their house. The animals were free to mobilize and weight bearing immediately post-operatively as tolerated. The animals were evaluated clinically and physically twice a week throughout the study period.

*Radiographic Examination*

Radiographs were obtained immediately after surgery and at 2, 4, 8 and 12 weeks post-implantation. The sheep were anaesthesized using the Ketamine (10 mg $kg^{-1}$, intramuscularly) Xylazine (1 mg $kg^{-1}$, intramuscularly). Two radiograph views (lateral and antero posterior) were obtained for left femur from each individual animal using a radiograph machine (Shimadzu Corporation, Model R-20, Kyoto, Japan). The X-ray film (Kodak film, Ltd.) was developed using an automatic developer (Kodak X-OMAT 100 Processor, Kodak Australasia Pty. Ltd., Coburd, Vic., Australia). Qualitative measurements were performed on each of the radiograph.

*Ultrasonographic Examination*

Ultrasound imaging was performed using ultrasound machine (Toshiba Capasee II Justvision 200) connected with 7 MHZ frequency transducer. The scanning was done immediately after surgery and subsequently at 2, 4, 8 and 12 weeks post implantation in both longitudinal and transverse planes of the femur. The machine was linked to a digital still image capture adaptor (SONY Mavicap) that allowed the image to be captured and then transferred into the computer.

*Histological Examination*

For natural coral group, the animals were euthanized at 2, 4, 8 and 12 weeks post-implantation. Meanwhile for the CPC group, the animals were euthanized at 4, 8 and 12 weeks post-implantation. Immediately after euthanasia, the left femur was isolated and the implant site was grossly evaluated. Samples of the femoral bone at the implant site were taken by using the diamond saw (Exakt®, Germany). The bones were cut into small specimens for histological evaluation. The specimens were fixed in 10% neutral buffered formaldehyde. They were then dehydrated in increasing grades of ethanol and subsequently infiltrated in resin (Technovit®7200 VLC). Following embedding in the resin, the blocks were polymerized in plastic fixation medium at 450 um wavelength and sectioned in vertical plane using the Exakt ban cutting machine. The 200-300 μm thick units obtained were further reduced by microgrinding and polishing to a final thickness of about 60-80 μm. The sections were stained in Toluidine blue and Masson-Goldner Trichome. The slides were examined under light microscope.

## Results

*Gross Evaluation*

Gross examination of the retrieved specimens revealed that coral implant was still visible at two weeks post-implantation but has been partially resorbed (Fig. 1a). By week 4 post-implantation, the implant was still visible and at 8 and 12 weeks post-implantation, the whole implant has been resorbed

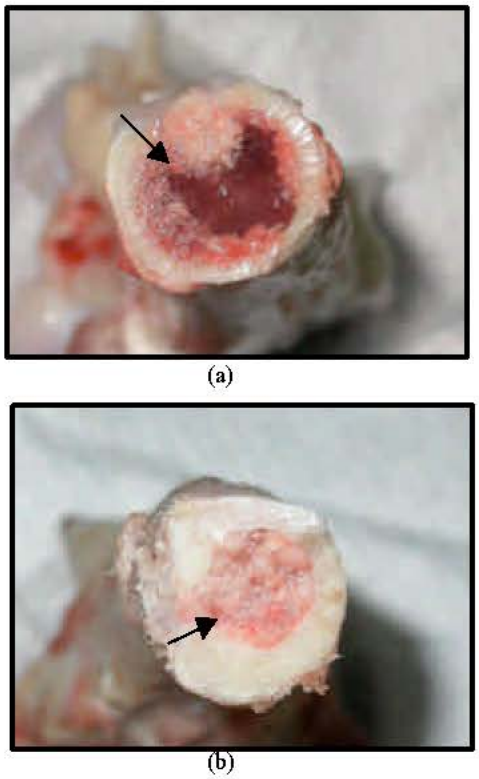



Fig. 1: Gross appearance of the coral implant (a) at week 2 post-implantation and (b) at week 12 post-implantation. Note that the coral implant is still visible (arrow) but partially resorbed in a, and the implant is completely resorbed and size of the defect is much reduced (arrow) in b

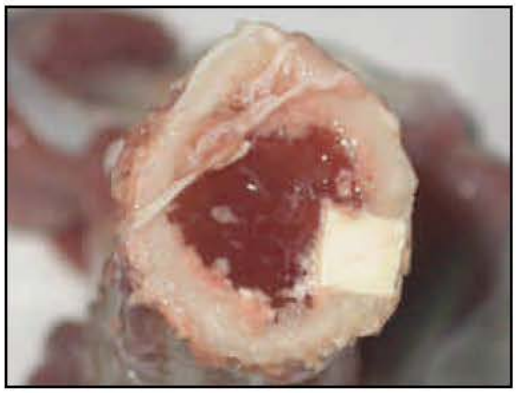


Fig. 2: Gross appearance of the CPC implant at week 12 post-implantation demonstrates the implant that is still clearly visible (arrow)

and soft tissue was observed at the implant site (Fig. 1b). However, for the CPC the implant was still clearly visible at week 12 post-implantation (Fig. 2). All the implants were tolerated well and did not elicit any sign of implant rejection.

*Radiographic Evaluation*

Radiographic evaluation was performed immediately post-operative and at 2, 4, 8 and 12 weeks post-implantation. Radiographs taken immediately post-implantation revealed the bone defect fixed well with the implant in all animals. The coral and CPC implants were clearly visible in plain x-rays immediately post-implantation (Fig. 3). At two weeks post-implantation, the coral implant incorporation was detected and characterized by mild lost of definition of the intrinsic structural pattern, associated with slight indistinctness of margins of the implant (Fig. 4a). At week four post-implantation, implant margins have become indistinct. Intrinsic architecture of the implant has lost and zone of radiolucency has developed in the adjacent bone (Fig. 4b). The central part remained more radio opaque, indicating that resorption was proceeding centripetally. Complete resorption was noted at 8 weeks post-implantation (Fig. 5a) and the size of the defect was observed to be reduced and at 12 weeks (Fig. 5b) post-implantation, radiograph showed similar result as in week 8 post-implantation.

The CPC implant was detected at all time up to week 12 post-implantation with no obvious changed on radiographs up o week 8 post-implantation (Fig. 6a). However, at week 12 post implantation, CPC implants demonstrated development of a radiolucent zone in the adjacent bone indicating some resorption occurred (Fig. 6b).

*Ultrasonographic Examination*

Ultrasonographic examination of the coral implant at week one post-implantation demonstrated the implant that appeared hyperechoic with the implant surface and margins were clearly seen in Fig. 7. At 4 weeks post-implantation, the implant depicted marked decreased in echogenicity and appeared similar to the adjacent bone (Fig. 8). The size of the implant was reduced as indicated by the gap between the implant and bone. There was no adverse tissue reaction detected on ultrasonographs. At 8 and 12 weeks post-implantation, the coral implant was no longer detected and the size of the defect is much reduced (Fig. 9). This indicated that the coral implant has been totally resorbed. Ultrasonographs of the CPC implant at 12 weeks post-implantation demonstrated the implant that was still clearly visible (Fig. 10). The echogenicity and the size of the CPC implant were not obviously changed throughout the study period.

*Histological Evaluation*

The histological examination of the coral implant revealed that at two weeks post-implantation, all the implants showed invasion of granulation tissue accompanied by in growths of blood vessels into pore region (Fig. 11a). Abundant osteoblastic activity was seen directly apposed on the surface of the pores implant (Fig. 11b). Coral implants were successively deformed. Bone began to appear at about 4 week on the surface of the pore regions of the implant. The vacant sites were progressively infiltrated by regenerated bone and new bone was formed at the place of the resorbed implant (Fig. 12a). At 8 weeks, the amount of bone in the pores increased and newly formed bone became matured and showed histologic appearance of trabecular bone (Fig. 12b). Multinucleated giant cells were present on the edges of the coral implant. These cells seemed to phagocytized the pore surface of the coral

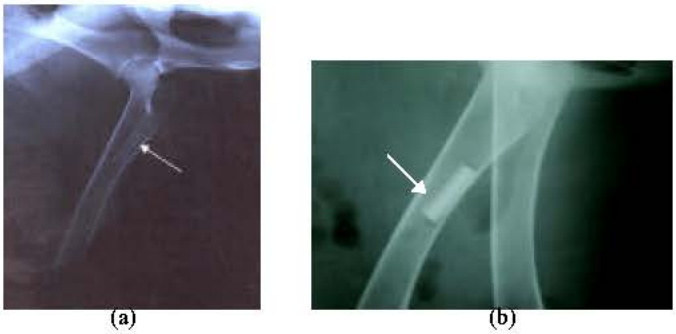



Fig. 3: Immediate post-operative radiographs show a) coral implant and b) CPC implants with distinct margins. Note that the coral and CPC implants architecture is grossly visible

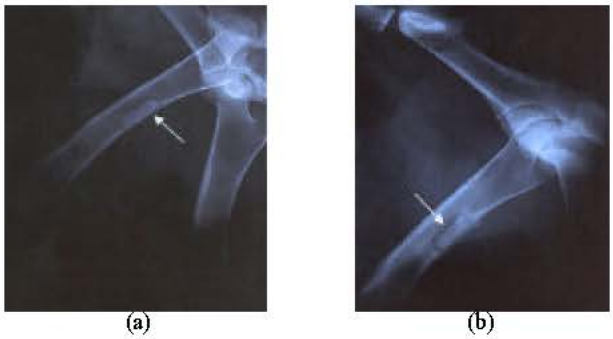
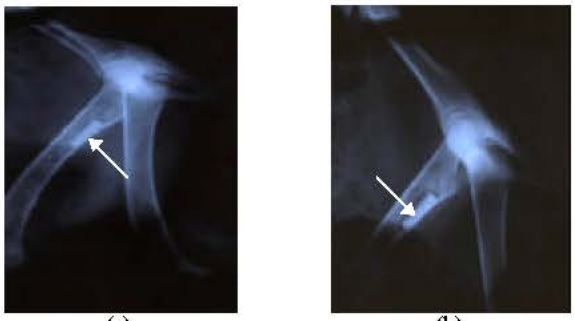



Fig. 4: Radiograph of the left femoral bone taken a) at week 2 post-implantation shows the coral implant margins have become slightly indistinct , b) at week 4 post-implantation shows the coral implant margins have become indistinct, and zone of radiolucency has developed in the adjacent bone. Intrinsic architecture of the implant is lost


Fig. 5: Radiographs of the left femoral bone taken a) at week 8 post-implantation and b) at week 12 post-implantation show the development of radiolucent zone at the coral implant site indicating the implant has been fully absorbed. Note that the size of the defect is much reduced



(a) (b)

Fig. 6: Radiographs of the left femoral bone taken a) at week 8 post-implantation demonstrates the CPC implant is still maintain its radiodensity with distinct margins, b) at week 12 post-implantation demonstrates the margins show an area of radiolucency indicating some resorption occurred

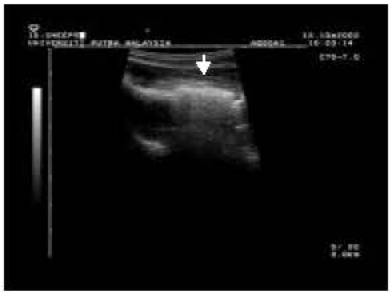


Fig. 7: Ultrasonograph of the coral implant (arrow) at week one post-implantation shows the implant appears hyperechoic with smooth surface and well define margins

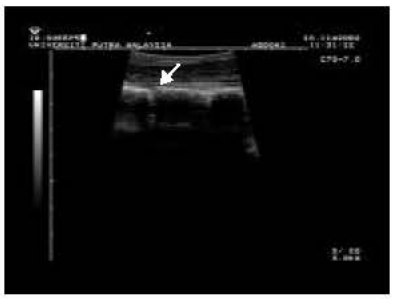


Fig. 8: Ultrasonograph of the coral implant at week 4 post-implantation shows the implant that appears similar echogenicity to the adjacent bone. Note that the size of the implant is reduced as indicated by the gap between the implant and bone (arrow)

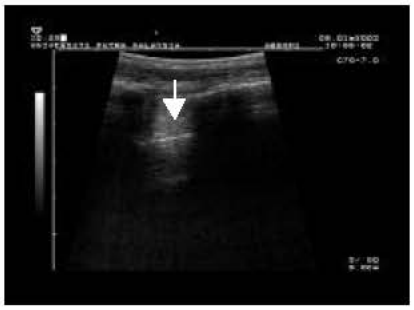


Fig. 9: Ultrasonograph of the coral implant at week 12 post-implantation shows no more implant detected and the size of the defect is much reduced (arrow)

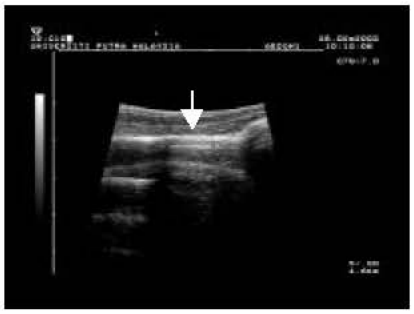
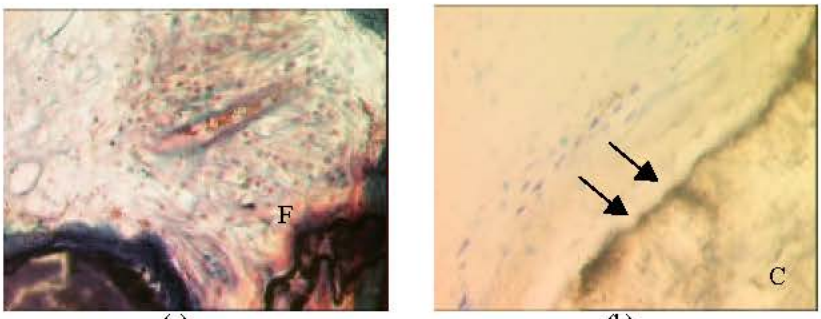


Fig. 10: Ultrasonograph of the CPC implant at week 12 post-implantation (arrow) shows that there is no change in the echogenecity and the size of the implant




(a) (b)

Fig. 11: Histological sections of the coral implant at week two post-implantation demonstrate a) the invasion of the granulation tissue, accompanied by ingrowths of new blood vessel (Masson's Goldner Trichrome), and b) active osteoblasts (arrows) lined on the partially resorbed surface of the coral implant (Toluidine blue). F, fibrovascular tissue, C, coral matrix

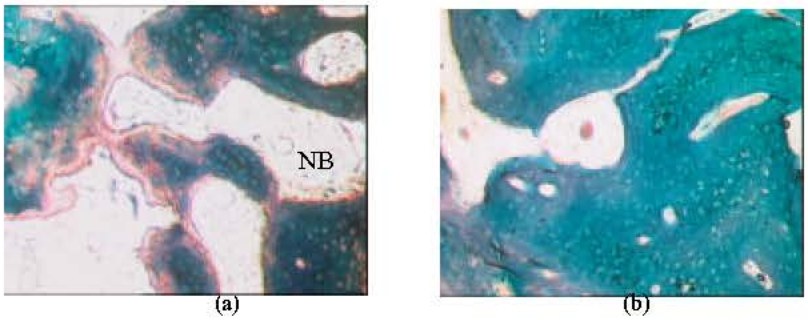



Fig. 12: Histological sections of the coral implant a) at week 4 post-implantation demonstrate the pores of the coral implant are filled with new bone (NB) is placed at the resorbed area, b) at week 8 post-implantation, the amount of bone in the pores increased and newly formed bone became matured (Masson-Goldner's Trichrome)

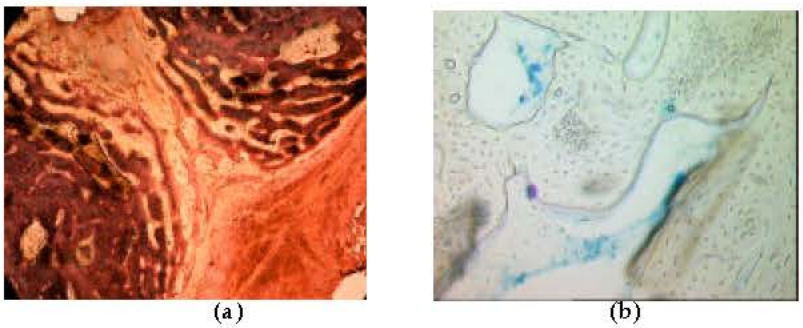



Fig. 13: Histological sections of coral implant site at week 12 post-implantation show a) the defect area that is almost completely filled with bone tissue (Masson-Goldner's Trichrome), b) numerous osteocytes are present at the defect area (Toluidine Blue)

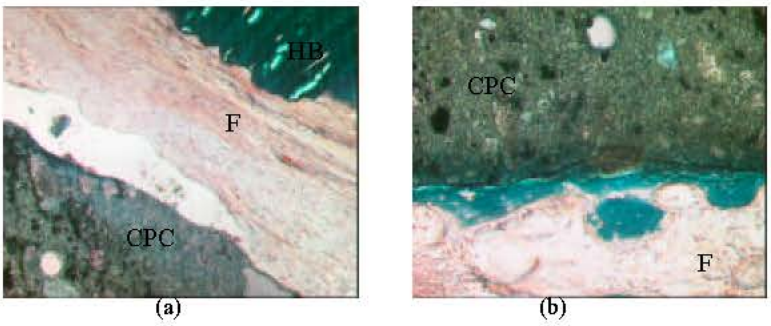



Fig. 14: Histological sections of the CPC implant a) at 4 weeks post-implantation demonstrates the gap between the implant and host bone (HB) that is filled with fibrovascular tissue (F), b) at 8 weeks post-implantation, new bone formation (green area) is deposited directly on the surface of implant

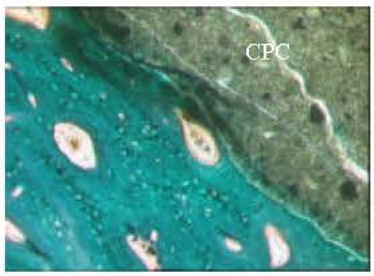



Fig. 15: Histological section of the CPC implant 12 weeks post-implantation reveals the bone apposition from periphery of the CPC implant

implant. At 12 weeks, coral implant was completely resorbed and the defect was almost completely filled with bone (Fig. 13). The center of implant still filled with fibrous. Most of the bone tissue was quite matured and contained abundant osteocytes.

For the CPC implant group, the CPC block appeared in the sections as a dense mass with porosity. At 4 weeks post-implantation, the bone-implant interface was seen in seamless contact with the implant surface (Fig. 14a). There was still gap detected in some areas. At 8 weeks post-implantation, the new bone formation was observed to deposit directly on the surface of implant (Fig. 14b). Extensive bone apposition from the periphery of the implant was observed at 12 weeks post implantation (Fig. 15).

**Discussion**

This study demonstrated that coral block was resorbed centripetally and the implant decreased gradually throughout of the study. Radiographic and ultrasonographic examinations revealed that at 8 weeks post-implantation, all the implants were completely resorbed. The phenomenon of slightly loss of definition involving the intrinsic architecture and margins of the implant on follow-up radiograph is presumably related to bone ingrowths and osteoclastic activity (Sartoris *et al*., 1986). As the implant period prolonged, new bone formation increased in quantity and maturation, resulting in bone formation on almost all surfaces for coral implant. Coral implant has been completely resorbed by the end of experimental period and almost completely replaced by new bone formation. These findings demonstrated that coral have excellent pores size and porosity that facilitate bone in growth, vascular invasion and bone development. The volume of porosity affects the rates of alloplast resorption and bone formation. It has been shown that coral skeletons with higher porosity volume allow larger cellular infiltrate and ion exchange, promoting a faster resorption and bone apposition (Guillemin *et al*., 1989). The CPC implant demonstrated only marginal bone in growth at the end of study period. No interconnection of the porous structure was found to be the major reason for restricted bone in growth. Moreover, the CPC has shallow pores on the surface and no interconnection between them, another drawback compared to open macro porous calcium phosphate. There is no way the CPC can promote fast bone ingrowths and, therefore the CPC degrades from the outside in, layer by layer. The finding in this study is an accord with Bohner (2000). These observations suggest that coral implants present a great advantage as compared to CPC implant in that mineralized tissue almost completely filled the defect area at the end of study period. In our cases, moreover, we observed that the coral had a very high osteoconductive potential because almost all implant were surrounded by mature bone. In addition, histologic examinations also suggest that ingrowth of new bone only occur

in continuity with cortical bone. This implies that coral implant itself does not have any osteogenic capacity, which is in agreement with observation made by Vuola *et al*., (1996). Immediately after implantation of natural coral implant, fibrovascular tissue begins to invade the porosity (Fricain *et al*., 1998). Animal studies have demonstrated fibrovascular ingrowth by 2 weeks post-implantation (Holmes, 1979). This granulation tissue has been shown histologically originates from the bone marrow (Guillemin *et al*., 1987) and is accompanied by ingrowth of blood vessels. The initial invasion of coral by blood and bone marrow cells with subsequent vascularization is a determinant factor for bone regeneration (Demers *et al*., 2002). Our results are an accord with the above findings. The phase of calcium carbonate in the coral is considered to be a biologic asset in rapid initiation of bone formation (Yukna, 1994). A CaP-rich surface layer has been seen to be formed shortly after initial dissolution of the coral implant in vivo and woven osteoid tissue then formed by osteoblasts directly along the coral substratum (Ohgushi *et al*., 1992). In our experiment, there were no sign of intolerance or rejection of the coral implants. In addition, the results also showed that it degradation is due to a phagocytic process mediated by multinucleated giant cells such as osteoclasts like cells. Osteoclasts contain abundant carbonic anhydrase, an enzyme that locally lowers the pH at the osteoclasts-implants, dissolving the calcium carbonate matrix (Chetail and Fournie, 1969).

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