In vivo graft performance of an improved bone substitute composed of poor crystalline hydroxyapatite based biphasic calcium phosphate

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The ability to promote new bone formation of poor crystalline hydroxyapatite (PC-HA) based biphasic calcium phosphate (BCP) bone substitutes were investigated. Various ratios of porous PC-HA/ β -TCP (70/30, 60/40, and 0/100) grafts were fabricated. SEM and XRD measurements were performed to study the morphology and crystalline structure. Cylindrical artificial bone defects (3×6 mm²) were produced in alveolar bone at premolars extraction site and then filled with sterilized bone grafts. Commercial MBCP[®] and unfilled empty defect served as control groups. At 8 weeks postoperation, samples were harvested from each artificial defect site for histological analysis. New bone formation of all the PC-HA/ β -TCP groups was significantly greater than that of the empty control group ($p < 0.05$), but without statistical difference from that of MBCP[®] group. The degree of uniformity of new bone formation within defect region for PC-HA/ β -TCP (60/40) was higher than that for MBCP[®]. The PC-HA/ β -TCP grafts showed enhanced bone regenerations with a more even dispersion of new bone formation than the other materials without causing inflammation, suggesting that these materials may be an alternative choice for bone void fillers in dental applications.

Keywords: Biphasic calcium phosphate, Bone graft, Hydroxyapatite, β -tricalcium phosphate, *In vivo*

INTRODUCTION

Bone grafts are clinically used as a void filler to accelerate the healing of large defects through new bone formation and structural support. Although many types of bone grafts are currently available for bone repair, they all have significant limitations in their chemistry, architecture, and mechanical properties. New materials which more closely mimic the properties of bone itself need to be developed. Autograft, allograft, and synthetic materials comprise the currently available choices in clinics to repair bone in orthopedic, spinal, and craniomaxillofacial (dental) surgeries^{1–3}. Among them, autogenous bone remains the “gold standard” of substitution material which fulfills physicochemical and biological properties, with limitations of post-operative pain and availability. However, the most common alternatives to autogenous bone graft —allografts and xenografts have the disadvantages of infectivity or unfavorable immune response, while synthetic materials have a low osteogenic capacity but are a safe source to apply in clinical use⁴.

Synthetic calcium phosphate ceramics have chemical components and structure similar to those of minerals in bone and teeth. For an ideal bone-grafting material to be replaceable by new bone, it needs to be both biodegradable and osteoconductive. Two of the most widely tested types of materials are hydroxyapatite (HA) and β -tricalcium

phosphate (β -TCP). Both of these osteoconductive bioceramics bond tightly with bone^{5–8}, but HA is less resorbable than β -TCP^{9,10}. A mixture of HA and β -TCP produces biphasic calcium phosphate (BCP) which possesses the reactivity of β -TCP and the stability of HA, providing more bioactivity, involving more new bone growth, and ensuring better resistance of the implants to strain^{11–13}. BCP has an intermediate resorbability which can be controlled by variation of the HA/ β -TCP ratio. Despite the many advantages of BCP ceramic systems, the ideal ratio of HA/ β -TCP for *in vivo* bone replacement remains unclear, and their biodegradation rate range still does not satisfy the requirement for biodegradable biomaterials^{10,14,15}.

Despite recent successes of synthetic calcium phosphates, their performance requires improvement. A well-known strategy is to add biological factors to induce bone formation, to stimulate angiogenesis, or even to make osteogenic hybrids by adding cells. However, rather than adding these costly and complex factors, improvement of the physico-chemical properties so that they instruct their *in vivo* environment would be a better approach. Relatively rapid biodegradation of synthetic bone substitutes is desirable because residual particles that may interfere with bone creeping substitution process¹⁶. Low-crystalline HA shows higher resorption ability^{17,18} which is very different from the theoretical density of HA. This is due to the poor development of the crystal, which results in crystalloid defects that then exhibit different characteristics such as increased

solubility and resorption rate after implantation *in vivo*. By controlling the particulate size and crystallinity of HA and/or calcium phosphate-based bone substitutes, materials with a strong ability to attract bone cells can be developed.

The purpose of this study was to evaluate a new resorption rate enhancing biphasic calcium phosphate bone graft material which contain low-crystalline HA and β -TCP (PC-HA/ β -TCP), and to compare them with a commercially available product (MBCP®). Biological performance was assessed using histological specimens of implants in the alveolar bone of Beagle dogs. Ratio of new bone formation was evaluated histomorphometrically.

MATERIALS AND METHODS

This study was conducted in the animal center affiliated with National Pingtung University of Science and Technology. Four mature Beagle dogs with weight between 8–12 kg and age between 2–3 years old, with healthy general and oral condition served as the experimental animals. They were kept under a standard light-dark schedule and relative humidity. All procedures were approved by the Animal Research Committee of Taipei Medical University.

Preparation of implants

PC-HA and β -TCP powders were synthesized by the wet chemical method: for preparing poor crystalline hydroxyapatite (PC-HA), we added 370 g calcium hydroxide ($\text{Ca}(\text{OH})_2$, J.T. Baker Inc. USA) into 1,000 mL distilled water (DDW). Then, the $\text{Ca}(\text{OH})_2$ aqueous solution was mixed with 13 liters of DDW into a reactor. The 24.3 w/v% phosphoric acid (H_3PO_4 , J.T. Baker Inc. USA) 1,000 mL were dropped slowly into the solution of $\text{Ca}(\text{OH})_2$. The reaction is under a stirring rate of 500–700 rpm at 20°C for 24 hours. After reaction finished, the solution was filtered and dried at 80°C for 24 hours.

β -TCP was prepared by wet-chemical reaction methods. Concentration of CaCO_3 (Riedel de Haen Inc.) and CaHPO_4 (Riedel de Haen Inc.) were prepared as 86.0 w/v% in 1,000 mL DDW and 25 w/v% in 1,000 mL DDW, respectively. They were mixed under stirring rate of 500–700 rpm for 24 hours. Then the solution was filtered and dried at 80°C for 24 hours then sintered at 1150°C and pulverized to become as β -TCP granules.

The PC-HA and β -TCP were chose in different ratio such as 60/40, 70/30. The composites were blended, pressed, sintered 24 hours at 1150°C and cooling down to ambient temperature. The blocks of PC-HA/ β -TCP were pulverized into granules with particle size ranged 500–1,000 μm . The composition of macroporous biphasic calcium phosphate bone substitute (MBCP®, Biomatlante, Vigneux de Bretagne, France) is HA/ β -TCP (60/40). Implants were placed in glass bottles and sterilized by ultraviolet irradiation (wave length: 250–260 nm) before implantation.

Characterization of Materials

1. SEM analysis

Sample surfaces were sputter coated with carbon using an E-1010 Ion Sputter (HITACHI, Japan), and the microstructure was observed by scanning electron microscopy (SEM) (S-2400 HITACHI, Japan) at 15 kV, with a magnification of 1,000 $\times$.

2. X-ray diffraction (XRD) analysis

Implants were dried and ground for XRD (MaxRC, Rigaku, Japan, 10°/min, 2θ from 5° to 60°) studies. The phase composition was checked using Joint Committee on Powder Diffraction standards.

3. Crystallite size analysis

The peak broadening of XRD reflection can be used to estimate the crystallite size in a direction perpendicular to the crystallographic plane based on Scherrer's formula as follows¹⁹⁾:

$$X_s = 0.9\lambda / (\text{FWHM} \times \cos\theta)$$

where X_s is the crystallite size [nm]; λ is the wavelength of monochrome X-ray beam (nm) ($\lambda = 0.15406$ nm for CuK_α radiation); and FWHM is the full width at half maximum for the diffraction angle ($^\circ$). The diffraction peak at $2\theta = 25.9^\circ$ was chosen for calculation of the crystallite size since it is sharper and isolated from the others. This peak assigns to (002) Miller's plane family and shows the crystal growth along the *c*-axis of the HA crystalline structure. The value of X_s was calculated for the PC-HA/ β -TCP powders synthesized with different compositions based on this diffraction peak using Scherrer's formula.

Surgical procedure

Four Beagle dogs were obtained from the National Pingtung University of Science and Technology (Pingtung, Taiwan). Prior to the implantation of bone grafts, all premolars of the Beagle dogs were extracted to create the same edentulous area. After 16 weeks of healing, bone graft implantations were performed on the four animals under general anesthesia. Ten minutes before anesthesia, an intramuscular sedative injection of 0.01 mg/kg Ketalar was given. General anesthesia was induced by intravenous injection of 12.5 mg/kg sodium thiopental and preserved by halothane inhalation. An intravenous injection of 1 g cefalexine was given for pre-operative antibiotic prophylaxis. Cylindrical osseous defects of 3-mm-diameter and 6-mm-length were surgically created in bilateral maxillar (two per animal, one per side) and mandibular (four per animal, two per side) alveolar bone. The implant samples (PC-HA/ β -TCP=70/30, 60/40, 0/100, and MBCP®) were then inserted into the created defects. The same surgical procedure was performed in the control group without insertion of any material.

The implanted sites were conglutinated with resorbable sutures for primary healing. Operated areas and general states of the animals were checked daily according to standard veterinary postoperative care.

Histological evaluation

After the surgery, the operation wound condition, contour of the bone flap, allergy or rejection reaction to the material, and other complications were investigated at time periods of 2, 4, 6, and 8 weeks. Histology specimens were all collected at 8 weeks after the surgery for evaluation of new bone formation, inflammatory reactions, and other complications. 5-mm-diameter and 8-mm-length cylindrical osseous samples were harvested and fixed in 10% buffered formalin, decalcified with rapid decalcification formulation (RDF), embedded, sectioned at 8 μ m thickness, and stained with hematoxylin and eosin (H&E).

Histomorphometry

Histomorphometry was investigated on photomicrographs of four samples from each group. Newly formed bone measurements were made by analysis of light microscope photographs (Nikon, ALPHAPHOT-2/YS2, Japan) exported from a digital camera (Coolpix4500, Nikon, Japan) and quantitatively analyzed by ImageJ 1.37c software (National Institutes

of Health (NIH), Bethesda, MD, USA; freeware from <http://rsb.info.nih.gov/ij>). The contiguity of the microscopy photographs were checked by the juxtaposition of the 9 fields placed side by side at 80 $\times$ magnification. New bone formation rate was calculated as a percentage: (Area of total defect site of vision–Area of total cavities due to bone graft occupation)/Area of total defect site of vision $\times$ 100.

Statistical analysis

Quantitative differences among the explants from each group were assessed by one way ANOVA. Individual differences between controls and explants with materials were determined by Duncan's multiple range tests and CV (coefficient of variation)²⁰. Statistical significance was established at $p < 0.05$.

RESULTS

Material characteristics

1. SEM analysis

In Figure 1, PC-HA/ β -TCP at ratios of 70/30 and 60/40

Fig. 1 Scanning electron microscopy of the implants: (a)PC-HA/ β -TCP=60/40; (b)PC-HA/ β -TCP=70/30; (c) β -TCP=100%; (d)MBCP® (1,000 $\times$).

Fig. 2 X-ray diffraction pattern of poor crystalline-HA (PC-HA), MBCP®, PC-HA/β-TCP=60/40, 70/30, and β-TCP (100%).

Table 1 Crystallite size of different graft materials

	Diffraction angle 2θ (°)	Crystallite size Xs (nm)
PC-HA/β-TCP=70/30	25.87	42.9
PC-HA/β-TCP=60/40	25.96	56.3
MBCP®	25.88	39.5
β-TCP	25.87	40.6

and MBCP® showed typical particle sizes within 500–1,000 μm. PC-HA/β-TCP at ratios of 60/40 and 70/30 showed larger macroporosity than MBCP®, but smaller particle size and macroporosity than pure β-TCP, which may influence the infiltration of the fibrovascular tissue and revascularization.

2. XRD analysis

Analysis of XRD spectra revealed the samples contained a mixture of apatite, HA and β-TCP (Figure 2). Typical diffraction peaks related with HA are 25.90° (0 0 2), 31.86° (2 1 1), 32.20° (1 1 2), and 32.90° (3 0 0); while the diffraction peaks of β-TCP are 27.81° (2 1 4), 31.01° (2 1 10) and 34.31° (2 2 0). A closer look at the peak intensities related with both HA and β-TCP were clearly detectable in MBCP®. For PC-HA along, the HA peak intensity was relatively low. Due to the low crystallinity of PC-HA, only the β-TCP could be detected from XRD for

Fig. 3 Photomicrograph of PC-HA/β-TCP=70/30 at 8 weeks after implantation. (NB: new bone, G: graft)

Fig. 4 Photomicrograph of PC-HA/β-TCP=60/40 at 8 weeks after implantation. (NB: new bone, G: graft)

PC-HA/β-TCP at ratios of 70/30, 60/40 and 0/100.

Further investigation of the crystallite size of the samples using Scherrer's formula showed that the crystallite size in MBCP® was the smallest among the four materials (Table 1).

Clinical observations

Biphasic calcium phosphate bone grafts were implanted and examined using a standardized maxillar and mandibular model. Only minor complications were observed during the surgery. All animals survived without general or localized complications during 8 weeks after the operation. All sites healed uneventfully and no infectious complications or gingival inflammation around implanted area were found during the experimental period.

Fig. 5 Photomicrograph of MBCP® at 8 weeks after implantation. (NB: new bone, G: graft)

Fig. 7 Photomicrograph of empty control at 8 weeks after implantation. (NB: new bone, ST: soft tissue, OB: old bone)

Fig. 6 Photomicrograph of β -TCP at 8 weeks after implantation. (NB: new bone, G: graft)

Histomorphologic study

All implanted and control sites healed uneventfully with no clinical evidence of inflammatory response to the ceramic implant. Extensive evidence of osteoconduction was found in the experimental groups. New bone was deposited directly on the surface of peripheral pores in all PC-HA/ β -TCP and commercial products, and a haversian structure was often apparent.

All of the samples with bone graft materials developed more new bone formation in comparison with the empty control group. The amount of bone regeneration and material replacement appeared to be greater in the specimens with PC-HA/ β -TCP ratios of 70/30 and 60/40 compared with other treatments. Duncan's multiple range test showed that all treatments produced significantly more new bone formation than the empty control group ($p < 0.05$). Two of the "active" treatment

Fig. 8 Histomorphometric data of new bone formation in each group. Blank bar is for empty control group. Data are means $\pm$ standard deviation ($n=4$) $*p < 0.05$ vs. control group determine by one way ANOVA, $\star p < 0.05$ vs. β -TCP group analyzed by Duncan's multiple range test.

groups (PC-HA/ β -TCP=70/30 and 60/40) had significantly more new bone formation than the 100% β -TCP group ($p < 0.05$) (Figure 8).

Figures 3 and 4 illustrate that the new bone formation around the ceramic particles and the trabecular pattern of the newly formed bone in specimens of the groups with PC-HA/ β -TCP ratios of 70/30 and 60/40 was different from that in the MBCP® specimens (Figure 5). Smaller cavities were observed more often in the groups with PC-HA/ β -TCP ratios of 70/30 and 60/40 compared to the MBCP® and β -TCP groups (Figures 5 and 6). These were blood-vessel-like cavities accompanied

Table 2 Percentage of newly formed bone for different kinds of implants

	Ratio of new bone formation Mean (S.D.)	New bone CV (%)
PC-HA/ β -TCP=70/30	0.702 (0.180)	25.56
PC-HA/ β -TCP=60/40	0.681 (0.117)	17.14
MBCP [®]	0.592 (0.242)	40.83
β -TCP	0.494 (0.130)	26.29
Empty control	0.189 (0.086)	45.64

by osteoblasts and trabecular bone characteristic of new bone regeneration.

Most of the ceramic particles were found in larger cavities, and could be observed more easily in the MBCP[®] and β -TCP groups. A greater number of remaining ceramic particles demonstrated a slower resorption rate, which indicates a decrease in osteoblastic activity and delay of new bone replacement.

The mean percentage of newly formed bone for each group is shown in Table 2. The new bone ratio (new bone area/total area) and the coefficient of variation (CV) of the new bone ratio were calculated to analyze the extent of dispersion of new bone in the implanted sites. The histomorphologic results showed the new bone formation ratio in the various groups is as following: PC-HA/ β -TCP=70/30(70.23%)>PC-HA/ β -TCP=60/40(68.10%)>MBCP[®](59.17%)> β -TCP(49.41%)>empty control (18.92%).

DISCUSSION

For biomedical applications, hydroxyapatite (HA) and tricalcium phosphate or the combination of both termed BCP are most frequently used. Based on differences in chemical properties such as calcium to phosphate ratio, crystallinity, sintering, and physical forms such as granules/blocks, coating or cement or geometry like porosity^{21–23}, the subtle differences in these parameters can have a serious effect on the biological outcome. One of the parameters influencing the bone regenerative outcome of a synthetic BCP bone graft substitute is its phase composition. Osteoinductive potential is increased by increasing the specific surface area and by the presence of more soluble calcium phosphate phases^{24,25}.

Important results of Previous BCP *in vivo* studies were as follows: (1) in ceramic bone grafts, the ratio of biphasic HA and β -TCP influenced the healing condition of bone defects²⁶; (2) Sintered BCP with different temperature may change the porosity of the ceramics, and it interact differently with biological fluids, cells and tissues²⁷; (3) the healing effects of mixing 85% HA with 15% β -TCP or 65% HA with 35% β -TCP were better than those obtained using HA or β -TCP alone or mixing 50% HA with 50% β -TCP^{28,29}.

This animal study compared an improved BCP

Table 3 Composition of commercial synthetic calcium phosphate products

Product name	HA (%)	β -TCP (%)
ProOsteon [®] (Interpore)	100	0
Endobon [®] (Merck)	100	0
MBCP [®] (Biomatlante)	60	40
Triosite [®] (Zimmer)	60	40
BCP [®] (Bioland)	60	40
Collagraft [®] (Zimmer)	60	40
Ostilit [®] (Stryker)	20	80
BoneSave [®] (Stryker)	20	80
Vitoss Scaffold [®] (Curasan)	0	100
Cerasorb Ortho [®] (Curasan)	0	100

composed of different ratios of PC-HA with commercial product MBCP[®]. We hypothesized that BCP containing PC-HA could be resorbed and replaced more efficiently than the traditional fully crystallized hydroxyapatite. This study had two important findings which support this hypothesis.

First, SEM study showed that PC-HA/ β -TCP filler had larger pore size and macroporosity than the commercial product MBCP[®], which may make it a better scaffold for the ingrowth of bone. A large macroporosity (*i.e.*, 400–600 μ m) facilitates infiltration by fibrovascular tissue and revascularization, allowing bone reconstruction.

Second, comparison of the crystallinity of the PC-HA and β -TCP ceramics analyzed in this study revealed that β -TCP peaks were clear at the narrow diffraction peaks of 25.61°, 27.81°, 31.01°, and 34.31° in β -TCP and the PC-HA/ β -TCP ratios of 70/30 and 60/40, while peaks of HA were found at the diffraction peaks of 31.86°, 32.20°, and 32.90° in MBCP[®] but not obvious in PC-HA (100%) samples, and were difficult to identify for the PC-HA/ β -TCP ratios of 70/30 and 60/40. The results of XRD revealed that the diagrams for the PC-HA/ β -TCP ratios of 60/40 and 70/30 were more similar to β -TCP (100%) than MBCP[®]. Because the peaks of PC-HA were unremarkable, it was difficult to distinguish between samples with PC-HA/ β -TCP ratios of 60/40, 70/30 and β -TCP (100%), which explains why PC-HA is poor crystallized and can be resorbed more quickly in physiological environments. Although commercial products of HA/ β -TCP are available in many different ratios (Table 3), our analysis revealed no poor-crystalline HA content in these commercial products.

Based on histomorphologic results, the ratios of new bone formation for the PC-HA/ β -TCP ratio of 60/40 and MBCP[®] were 68.10% and 59.17%, respectively. Although there was no significant difference in new bone formation

between PC-HA/ β -TCP groups and MBCP[®] group, the CV for samples of PC-HA/ β -TCP (60/40) group was much lower than that for MBCP[®] group (17.14 and 40.83, respectively), which indicates that the new bone formation of PC-HA/ β -TCP (60/40) group had a higher degree of dispersion and PC-HA/ β -TCP was resorbed more evenly than MBCP[®].

Ideally, the rate of resorption, resulting in a sequentially changing bone-biomaterial interface, is similar to the rate of formation of new bone. The advantages of using a bioresorptive material as a bone substitute are obvious. First, remodeling of the newly formed bone is not influenced by the presence of the ceramic. Second, no foreign body remains in place during the rest of the patient's lifetime. Third, after resorption of the ceramic material the remodeled bone is stronger than the combination of a ceramic and newly formed bone. However, the rate of resorption and the mechanism by which resorption takes place remain to be established.

Oonishi *et al.* reported that the resorption time of HA with a particle size of 100–300 μ m was too long to allow new bone ingrowths, while β -TCP with a similar particle size partially resorbed in 6 weeks and provided suitable space for new bone ingrowth. They also noted that low (poor) crystalline HA had better resorption bioactivity in the bone graft area³⁰. The better bioresorptive ability obtained using PC-HA instead of crystalline HA in this study support these previous findings. We also found that histological specimens of experimental groups of PC-HA/ β -TCP had a higher new bone formation rate as shown by the presence of tinier trabecular bones surrounded by blood-vessel-like cavities. This phenomenon suggests that PC-HA resorbed more evenly and quickly in this animal model, and thus may be a better bone replacement material than fully-crystallized HA.

This study showed that PC-HA induced new bone growth in defects with good biocompatibility and without adverse effects. Our results indicate that incorporation of PC-HA in BCP influenced new bone formation. Although the new bone formation area in this study was not significantly different between treatment groups with different ratios of PC-HA/ β -TCP, histological analysis revealed that the PC-HA/ β -TCP groups showed a tendency to greater gain in new bone formation accompanied by more even new bone distribution than the MBCP[®] and β -TCP groups. Use of a PC-HA component in the biphasic calcium phosphate (BCP) resulted in greater new bone gain than in the groups that received fully crystallized HA. This may be due to the low dissolution rate of fully crystallized HA. Previous studies reported that fully crystallized HA has no osteoinductive capability, and that it has a “benign” reaction with tissues³¹. Our findings that the combination of PC-HA and β -TCP resulted in good new bone regeneration suggests that PC-HA plays a more effective role in inducing cell proliferation, revascularization, and osteogenesis, and may have more potential to dissolve in physiological body fluids during the healing process. Furthermore, to examine the relative variability in

histological analysis, coefficient of variation (CV), a normalized measure of dispersion of a probability distribution, was introduced. The CV value of new bone formation ratio for the PC-HA/ β -TCP of 70/30 and 60/40 groups were much lower than for MBCP[®] suggesting that the osteoconductive materials promoting a consistent healing within the defect area, but the more suitable PC-HA/ β -TCP ratio and sinter environment may need further clinical investigation.

CONCLUSIONS

This *in vivo* study compared the new bone formation rate of different mixing ratios of PC-HA/ β -TCP (70/30, 60/40, 0/100) with commercial products of MBCP[®] and empty controls. The results showed that PC-HA/ β -TCP mixture ratios of 70/30, 60/40 provided better new bone regeneration rate than β -TCP (100%) and empty control groups without any toxic or inflammatory reactions. These findings demonstrate the osteoconductive potential of our synthesized materials. These new BCP bone graft materials may be a more suitable choice for clinical use in bone defects than conventional substitutes, and are worthy of further evaluation.

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