**In vivo** evaluation of poorly crystalline hydroxyapatite-based biphasic calcium phosphate bone substitutes for treating dental bony defects

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**Background/purpose:** Poorly crystalline hydroxyapatite was improved so that it has better cell affinity *in vitro*. We studied the efficacy of a novel resorbable poorly crystalline hydroxyapatite-based biphasic calcium phosphate, BonaGraft, for bone regeneration *in vivo*.

**Materials and methods:** The beagle was used as an animal model, and cylindrical artificial bone defects (3 mm in diameter and 6 mm long) were produced in the alveolar bone. The BonaGraft (ratio of poorly crystalline hydroxyapatite to β-tricalcium phosphate, 60:40) was used to fill in the defect, and unfilled defects served as a control group. At 5, 8 and 10 weeks after the operation, the size of the residual graft and new bone formation were evaluation by a histomorphometric analysis. In a clinical trial, 33 enrolled patients included 15 males and 18 females with ages ranging from 35 to 54 years. The main indications were ridge augmentation (n=12), sinus lifting (n=2), repair of periodontal disease (n=14), and repair of radicular cysts (n=5). The clinical outcomes of the surgery were primarily evaluated by clinical radiographs.

**Results:** In the animal study, implanting BonaGraft produced greater new-bone formation (74.5%±1.0%) at 10 weeks postoperatively than that of the control (40.2%±0.3%). BonaGraft particles were gradually resorbed and substituted by bone. The *in vivo* graft resorption time and bone healing time of 12.1 weeks were mathematically determined by the least squares method. In the clinical test, all patients implanted with BonaGraft reported satisfactory clinical outcomes without major material-related side effects. According to the radiographic pictures, implantation of BonaGraft enhanced bone formation.
Introduction

For several decades, typical graft materials include both transplanted bone and bioceramics for bone regeneration. Autotransplanted bone is the gold standard in clinical practice because of its osteoinductive and osteogenesis properties. However, autologous bone is limited in terms of availability as well as owing to secondary harm caused to patients. Allotransplanted bone is usually taken from disinfected cadaveric bones. Potential immune responses can be induced by antigens of the allograft with an associated risk of disease transmission, especially in cases of human immunodeficiency virus and hepatitis. To minimize donor site morbidity, artificial bone substitutes exhibiting good biocompatibility and osteoconduction are widely used in orthopedic and dental fields.

Calcium phosphate is widely used as a bone graft substitute owing to biomimicry of similar inorganic components of bones and teeth. After implanting a bioceramic material, new bone gradually replaces the implant together with graft resorption; accordingly, the periods of graft absorption and bone regeneration should be matched to achieve the optimal bone regeneration efficiency. Hydroxyapatite (HAp), β-tricalcium phosphate (β-TCP), and calcium sulfate are most widely used in the clinic for bone regeneration. In conventional products, the resorption times for HAp, β-TCP, and calcium sulfate are about 1−3 years, 0.5−1 year, and 1−2 months, respectively. In 2006, Rack et al. reported that unabsorbed bone graft deposits may influence treatment of the implant bed. Knabe et al. concluded that the resorption time for bone grafts used in ridge preservation contexts should be less than 6 months, or the grafts may hinder new bone formation and delay the healing process. Ordinarily, bone tissue needs 3−6 months to mature under unloaded conditions. For this reason, the use of calcium sulfate (with a resorption time of 1−2 months) may lead to soft-tissue invasion that subsequently hinders bone regeneration. For the aforementioned reasons, grafts with a 3−6 month resorption period may be appropriate for dental applications.

In general, β-TCP has a median resorption period of around 6−12 months, but its cell affinity is lower than that of HAp. Jalota et al. reported that the addition of HAp into β-TCP may enhance cell attachment. Nery et al. also concluded that biphasic calcium phosphate (BCP) with a high HAp content may be desirable for repairing periodontal defects. For this reason, most ceramic bone grafts tend to be formulated with BCP. Various commercial biphasic bone grafts and their compositions are listed in Table 1.

The absorption times of β-TCP and HAp are beyond those clinically advisable. Achieving an appropriate balance between biologic activity and the duration of the degradation period is a key challenge in developing graft materials for dental applications. The preferred resorption time for dental bone grafts is within 3−6 months. According to the degradation mechanisms, bioceramics are gradually dissolved by body fluids. Usually, the material with a larger surface area or a lower crystalline degree tends to exhibit a more rapid dissolution rate. In fact, the mineral in natural bone is poorly crystalline HAp (PC-HAp). To achieve suitable degradation rates and biomimetic properties, the porous BonaGraft with poorly crystalline properties was designed. Clinical data were retrospectively analyzed and are presented in this article.

Table 1. Composition of commercial synthetic calcium phosphate products

<table>
<thead>
<tr>
<th>Product name</th>
<th>PC-HAp (%)</th>
<th>β-TCP (%)</th>
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<tbody>
<tr>
<td>Pro Osteon (Biomer, WAR)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Endobon (Merck, Darmstadt, Germany)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>MBCP (Biomatlan, Vigneux de Bretagne, France)</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Triosite (Zimmer, WAR, USA)</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>BCP (Bioland, Toulouse, France)</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Collagraft (Zimmer)</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Ostilite (Stryker, Kalamazoo, MI, USA)</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>BoneSave (Stryker)</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>Vitoss (Orthovita, Malvern, PA, USA)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Cerasorb (Curasan, Kleinostheim, Germany)</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

PC-HAp=poorly crystalline hydroxyapatite; β-TCP=β-tricalcium phosphate.

Conclusion: According to the animal study results, BonaGraft has a suitable resorption period and satisfactory outcomes of new bone formation. The clinical study produced high satisfaction with clinical results both objectively and subjectively. For this reason, BonaGraft seems to be an alternative choice for a bone substitute in dental applications.
Materials and methods

Animal study

Twelve beagles were obtained from the National Pingtung University of Science and Technology, Pingtung, Taiwan, for the animal study. In this study, three time points (5, 8 and 10 weeks) were used with four dogs at each time point. Prior to implantation of the bone grafts, the first, second and third premolars were extracted to create an edentulous area, and six teeth were extracted from each dog. After 16 weeks, when all extraction sockets had healed, cylindrical osseous defects of 3 mm in diameter and 6 mm depth were surgically created in the alveolar bone within the mandible by a trephine bur (with a diameter of 3 mm) under general anesthesia, and two defects were created in each dog (one for the control and one for the implant). The implant samples of BonaGraft (Biotech One, Taipei, Taiwan; PC-Hap:β-TCP ratio of 60:40) were then installed within the resulting defects (Fig. 1). The same surgical procedure was performed in the control group without inserting any material.

Histologic evaluation

Histologic specimens were collected at 5, 8 and 10 weeks after the surgery to evaluate new bone formation and the resorption rate of the graft. Cylindrical osseous samples of 5 mm in diameter and 8 mm long were harvested, soaked in 10% buffered formalin for fixation, and then sectioned using a low-speed cutter (TechCut 4; Allied High Tech Products, Rancho Dominguez, CA, USA). Formic acid (40%) with a 10% sodium citrate solution was used for decalcification until the specimen had become flexible, and then the slide of a specimen was stained with hematoxylin and eosin. Histologic analyses were conducted using an optical microscope (Olympus EX51; Olympus, Tokyo, Japan) connected to a personal computer equipped with an image analysis system. For the histomorphometric analysis, areas of new bone, soft tissue and the residual graft were delineated by personal judgment of the histology, and selected areas of the images (residual graft and new bone) were quantified using the ImageJ analysis package (National Institutes of Health, Bethesda, MD, USA).

The new bone formation ratio was calculated as follows: new bone formation (%) = (new bone area/total area of section) × 100.

Clinical test

The study was performed at Taipei Medical University Hospital, Taipei, Taiwan. The clinical study was approved by the hospital’s institutional review board (approval no. P960306). Patients who were pregnant, or had a medical history of bone infection, cancer, diabetes, tuberculosis, sickle cell anemia or immunologic deficits were excluded from the study. Thirty-three healthy patients were systemically enrolled in the study. The indications of patients are listed in Table 2 (ridge augmentation, sinus lifting, repair of periodontal disease, and repair of radicular cyst).

Table 2. Summary of sex, diagnosis, and results of 33 patients

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Operation</th>
<th>Results</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Outcome</td>
</tr>
<tr>
<td>12</td>
<td>Ridge augmentation</td>
<td>Good</td>
</tr>
<tr>
<td>2</td>
<td>Sinus lifting</td>
<td>Good</td>
</tr>
<tr>
<td>14</td>
<td>Repair of periodontal disease</td>
<td>Good</td>
</tr>
<tr>
<td>5</td>
<td>Repair of radicular cyst</td>
<td>Good</td>
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cysts). All bony defects and treatment sites were filled with granular BonaGraft with a particle size of 500–1000 μm. In all cases, a broad-spectrum antimicrobial drug was prescribed for 5–7 days after the operation. Clinical radiographs were obtained in all cases pre- and postoperatively to evaluate the efficacy of the implanted grafts.

Results

In vivo study (beagle model)

A model of beagles with alveolar bone defects (columnar defects with a diameter of 3 mm and a depth of 6 mm) was used in this study. Specimens (BonaGraft) were implanted in the defects and harvested 5, 8 and 10 weeks later. Histomorphometric values were quantified by an image analysis. Filled defects were compared with unfilled defects (controls). All experimental dogs survived the experimental period. Few wounds, inflammatory reactions or other complications were observed. Fig. 2 shows the histologic results at various harvesting times, and no immune or inflammatory response was evident at any implant site. The control group was filled with connective tissue, and no bone was found 5 weeks after the operation. However, in the BonaGraft group, a lot of new bone had formed in the defect; the new bone formation ratio in the BonaGraft group was 49.5% ± 0.7% within 5 weeks (Table 3). Eight weeks postoperatively, some new bone had formed in the control group, and the new bone formation ratio was 18.9 ± 8.6%. The defects filled with BonaGraft contained a lot of regenerated bone, and the new bone formation was 68.1% ± 11.7%. Ten weeks after the operation, new bone formation of the control and BonaGraft had increased to 40.2% ± 0.3% and 74.5% ± 1.0%, respectively. According to the above results, implanting BonaGraft in bony defects indeed enhanced bone regeneration. The resorption rate of the graft was also evaluated by a histomorphometric method.10 The histomorphometric results showed that the graft was gradually resorbed with the implanted time, and the average particle size decreased from 750 ± 250 μm to 100 ± 75 μm within 10 weeks (Fig. 3). According to the histomorphometric results, the resorption period of the mathematical prediction was 12.1 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>New bone formation (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
</tr>
<tr>
<td>BonaGraft</td>
<td>49.5 ± 0.7</td>
</tr>
</tbody>
</table>

*Values reported are an average of n=4. ND = not detected.

Fig. 2 Histologic evaluation of the control and BonaGraft for 5, 8 and 10 weeks of implantation (hematoxylin and eosin, 100×). (A) Control (5 weeks), (B) control (8 weeks), (C) control (10 weeks), (D) BonaGraft (5 weeks), (E) BonaGraft (8 weeks), (F) BonaGraft (10 weeks). ST = soft tissue; NB = new bone.
Clinical study

According to the above-described results, the efficiency of BonaGraft was remarkable. In the preclinical study, BonaGraft was used as a bone substitute to enhance bone regeneration. Thirty-three patients were recruited for this study. The patients were enrolled because of ridge augmentation (n=12), sinus lifting (n=2), repair of periodontal disease (n=14), and repair of radicular cysts (n=5) (Table 2). All treatment sites were covered with collagen membranes, except in the case of the patients with radicular cysts. All patients reported satisfactory outcomes, including occlusion. None of the patients exhibited postoperative problems besides normal swelling and inflammation at the surgical site as determined during the follow-up clinical and radiographic examinations.

In patients with ridge augmentation, the grafts were covered with collagen membranes following placement (Fig. 4C). Regenerated ridges healed uneventfully; no postoperative complications were recorded after the ridge augmentation procedure. By comparing the radiographs before and after surgery (Fig. 5), the graft could be observed postoperatively with radiographs (Fig. 5B). After 2 months, the ridge heights had significantly increased, and the graft was almost invisible on the radiograph (Fig. 5C). Most patients had satisfactory outcomes, and after 2 months of implantation, the radiopacity of the graft had decreased. It was difficult to distinguish between the new bone and graft, and this may indicate the graft had been resorbed and replaced by bone tissue.

In cases of sinus lifting, the grafts were directly filled under the sinus membranes, and the open windows were covered with a collagen membrane (Fig. 6). Postoperatively, the stitches were taken out after 2 weeks. During the healing period, no flap dehiscence or membrane exposure was observed. The length of patient follow-up was 3–6 months. The degree of bone augmentation in each patient was determined by radiographs. As shown in Fig. 7B, substantial bone formation was observed in the sinus. After the operation, all patients had satisfactory outcomes, but the case number was still insufficient; for this reason, more clinical cases should be collected in further studies. In cases due to periodontal disease, no patients had radicular lesions.
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Fig. 5 Panoramic radiographs of ridge augmentation: (A) preoperatively, (B) postoperatively (BonaGraft), and (C) after 2 months.

Fig. 6 Surgical procedure for sinus floor augmentation. (A) Open window, (B) BonaGraft under the sinus membrane, and (C) covering by a membrane.

Fig. 7 Panoramic radiographs of sinus lifting: (A) preoperatively and (B) 3 months postoperatively.

After open-flap debridement, grafts were used to fill the bony defects, which were then covered by collagen membranes (Fig. 8). After the operation, little membrane exposure and graft loss were seen with clinical observations. The outcome of surgery was confirmed by evaluating clinical radiographs. According to Fig. 9B, the alveolus bone had regenerated after 3 months. In the surgical procedure for radicular cysts, a full thickness flap was reflected, and a thin piece of cortical bone was removed to create the bony window. Endodontic therapy was implemented after curettage of the cysts. The bony defects at the lesion sites were filled with BonaGraft (Fig. 10). Wound healing was uneventful after 1 week. A postoperative radiograph was obtained to evaluate the regeneration of bone at the lesion site. After 6 months, the bony defects caused by the cyst had regenerated (Fig. 11), and no other clinical symptoms were evident in the follow-up examinations, which confirmed that apical healing was uneventful. According to the above results, we concluded that BonaGraft exhibited good efficiency, and no delayed graft resorption hampered bone regeneration.

Discussion

In order to measure the efficiency and absorption times of BonaGraft, a beagle model, which is one of the more frequently used large animals for orthopedic and dental research, was used in this study, as its bone composition is most similar to that of human bone. In the animal study, the harvest times were 5, 8 and 10 weeks. Pearce et al.\textsuperscript{11} reported
Fig. 8 Surgical procedure for periodontal disease. (A) Before operation, (B) after debridement, (C) placement of BonaGraft in the defect, (D) covering by a membrane, and (E) after operation.

Fig. 9 Radiographs of periodontal disease: (A) preoperatively and (B) 3 months postoperatively.

Fig. 10 Surgical procedure for radicular cysts: (A) surgical site after debridement, and (B) placement of BonaGraft in the defect.
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that the bone regeneration rate and metabolism in beagles were 1.5 times those of human bones, and for this reason, the durations of the animal study approximated 2, 3 and 6 months in humans. Results of the animal study imply that all grafts could help enhance new bone growth in defects with good biocompatibility and without adverse effects.

BonaGraft has interconnected pore structures; these may facilitate osteocyte and neovascularized ingrowths. Tamai et al. reported that abundant interconnected pores may promote the ingrowth of bone tissue, bone marrow, and blood vessels. This promotes bone remodeling throughout the material. Certain other papers reported that calcium phosphate with macropores and micropores may exhibit intrinsic osteoinductivity. Such a geometry is believed to allow the entrapment and concentration of circulating growth factors and osteoprogenitor cells that may be responsible for bone formation.

As to the resorption rate, the interconnected porous structure can enhance the surface area of the bone graft, and a graft with a larger surface area would theoretically exhibit faster dissolution rates under physiologic conditions.

BonaGraft is composed of PC-HAp and $\beta$-TCP. Oonishi et al. reported that PC-HAp exhibits suitable resorption rates and better bioactivity in bone graft areas. Our histologic results also showed that specimens of BonaGraft had high new bone formation rates, as evidenced by the presence of smaller trabecular bones surrounded by blood vessel-like cavities.

According to the histomorphometric results and a linear regression, we conjectured that the residual graft should gradually be resorbed within 3 months in canines (Figs. 2 and 3). According to the relationship of the canine and human metabolic rates, we estimated that the resorption period of BonaGraft may be approximately 4–5 months in humans. For this reason, PC-HAp added to a graft may be resorbed more evenly and quickly in vivo, and thus it may be a better bone replacement material than fully crystallized HAp.

In light of the above-described results, we chose BonaGraft for the preclinical study because of its superior performance in an animal study. The results of the clinical study revealed a favorable solubility and biocompatibility of the tested bone graft as evidenced by the almost complete bone regeneration without a foreign body reaction. During the surgery, the graft was mixed with blood to form a slurry that was easy to shape and position onto treatment sites. In cases of ridge augmentation, the patients needed bone augmentation owing to the lack of alveolus bone during implantation procedures. As shown in Fig. 5A, the patient’s ridge was absorbed because of long-term hypodontia. After implanting BonaGraft, a lot of granular grafts were observed on a radiograph (Fig. 5B). Two months later, the ridge heights had significantly improved. From the radiographic picture (Fig. 5C), an image of the residual graft can be found in the regenerated bone by radiography. It seems that grafts may gradually be resorbed and substituted by new bone.

Fig. 11 Radiograph of a radicular cyst: (A) preoperatively and (B) 3 months postoperatively.
membrane side to make a space, a mixture of BonaGraft and platelet-rich plasma was filled in the middle layer for new bone formation, and autogenous bone was placed around the implant to encourage early osseointegration. After 1 year, the implants had become imbedded with new bone. The authors mentioned that BonaGraft exhibited a high affinity for platelet-rich plasma; the mixture decreased the use of autografts and allografts in complicated cases of sinus lifting. In cystic bone lesions that are often self-healing or can be cured by decompression, filling the lesions with bone graft material may help stabilize blood clots in defects and consequently lead to advanced bone regeneration. In this study, cystic lesions were filled with the graft after debridement. The lesions had almost fully healed after 3 weeks, and no adverse reactions were observed. In the periodontal defect cases, BonaGraft also helped with alveolar bone regeneration. Nery et al. reported that BCP with a higher HA:PC content appeared to demonstrate improved attachment levels and bone regeneration when treating periodontal osseous defects. Sun et al. also reported that non-crystalline calcium phosphate may significantly increase the adhesion and proliferation of periodontal ligament cells compared with bare glass slides, and suggested that non-crystalline calcium phosphate not only initiates mineralization but also plays a role in the regulating cell physiology. According to the aforementioned reference, decreased crystallinity of calcium phosphate or an increased ratio of HA in BCP may be more suitable for periodontal operations. In summary, we concluded that low-crystalline and porous BCP should be efficacious for bone formation in dental applications. However, long-term follow-up and additional cases are needed to confirm our results.

Acknowledgments

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References