Osteoinductive and Antibacterial Biomaterials for Bone Tissue Engineering

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Chapter 2

Bone regeneration in critical-sized bone defect enhanced by introducing osteoinductivity to biphasic calcium phosphate granules

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Abstract

Objectives: Biphasic calcium phosphate (BCP) is frequently used as bone substitute and often needs to be combined with autologous bone to gain an osteoinductive property for guided bone regeneration in implant dentistry. Given the limitations of using autologous bone, a bone morphogenetic protein-2 (BMP2)-coprecipitated, layer-by-layer assembled biomimetic calcium phosphate particles (BMP2-cop.BioCaP) has been developed as a potential osteoinducer. In this study, we hypothesized that BMP2-cop.BioCaP could introduce osteoinductivity to BCP and so could function as effectively as autologous bone for the repair of a critical-sized bone defect.

Materials and methods: We prepared BMP2-cop.BioCaP and monitored the loading and release kinetics of BMP2 from it *in vitro*. Seven groups (n=6 animals/group) were established: 1)Empty defect; 2)BCP; 3) BCP mixed with biomimetic calcium phosphate particles (BioCaP); 4) BCP mixed with BMP2-cop.BioCaP; 5) BioCaP;6) BMP2-cop.BioCaP; 7) BCP mixed with autologous bone. They were implanted into 8mm diameter rat cranial critical-sized bone defects for an *in vivo* evaluation. Autologous bone served as a positive control. The osteoinductive efficacy and degradability of materials were evaluated using micro-CT, histology and histomorphometry.

Results: The combined application of BCP and BMP2-cop.BioCaP resulted in significantly more new bone formation than BCP alone. The osteoinductive efficacy of BMP2-cop.BioCaP was comparable with the golden standard use autologous bone. Compared with BCP alone, significantly more BCP degradation was found when mixed with BMP2-cop.BioCaP.

Conclusion: The combination of BCP and BMP2-cop.BioCaP showed a promising potential for guided bone regeneration clinically in the future.

Keywords: Animal experiments; Biomaterials; Guided bone regeneration; Growth factor

1. Introduction

The major concern in oral implantology is often the presence of insufficient alveolar bone to facilitate the insertion of dental implants according to planned prosthetic position. Recent development in guided bone regeneration has made it possible to install dental implants in regions which were previously considered unsuitable due to presence of insufficient bone. Various grafting materials, including autografts, allografts, xenografts, and synthetic materials, have been used in implant dentistry for guided bone regeneration, however with varying degree of success [1]. Although autografts are regarded as the “gold standards” for bone regeneration [2, 3], their application is limited due to the low availability as well as pain and morbidity from the donor site [4]. Synthetic calcium phosphate (CaP)-based materials are therefore used clinically for the repair of bone defects [5, 6]. Biphasic calcium phosphate (BCP), a mixture of hydroxyapatite and β tricalcium phosphate, is one of the most commonly used synthetic CaP materials due to its excellent osteoconductivity [7-9]— the ability to facilitate the migration and proliferation of osteoblasts and progenitor cells [10]. In the progress of bone regeneration, growth factors are further needed to differentiate progenitor cell into osteoblasts and subsequently to form new bone. Basing on this theory, there is an increasing interest in combining osteogenic and/or vasculogenic growth factors with osteoconductive materials to enhance bone regeneration. This kind of materials are considered to possessing osteoinductivity — the ability to induce progenitor cells to differentiate down osteogenic lineages [10].

To endowing materials with osteoinductivity, bone morphogenetic protein-2 (BMP2) is an often used osteogenic growth factor due to not only its powerful capacity of inducing osteogenesis but also its saftey. Its application has been approved by the Food and Drug Administration (FDA). The positive influence of BMP2 on bone regeneration has been shown to induce bone formation in animal studies [11, 12] and clinical trials [13, 14]. However, the present way of delivering BMP2 clinically, the superficial adsorption of BMP2 onto bone filling materials [15], causes burst release and consequently the transient high local concentration of BMP2. This kind of delivery of BMP2 is often associated with various potential side effects such as an excessive stimulation of bone resorption and the induction of bone formation at unintentional sites [16]. To maximize its osteoinductivity, BMP2 needs to be continuously delivered to target sites at a low concentration [17]. Our research group has recently developed BMP2-coprecipitated layer-by-layer assembled biomimetic calcium phosphate (BioCaP) particles (BMP2-cop.BioCaP). It could serve as an independent osteoinducer when mixed with osteoconductive biomaterials [18]. BMP2 was incorporated into the outermost layer of an inorganic crystalline latticework of BMP2-cop.BioCaP. Very low doses of the incorporated BMP2 were released slowly and steadily as the BMP2-cop.BioCaP particles underwent degradation at the site of the implantation. These low doses (at μg level) were proved to induce bone formation

efficiently in a pro-fibrotic environment and to suppress the foreign body reaction to a clinically-used bone substitute in rat ectopic sites [18]. Compared with the high dose (at mg level) of BMP2 used clinically (INFUSE® Bone Graft, 1.5mg BMP-2 per ml collagen sponge), the BMP2-cop.BioCaP granules (about 150μg BMP2 per ml granule) achieved a satisfactory bone regeneration with 10 times less BMP2. BMP2-cop.BioCaP was proved to be not only an effective, but also an efficient method to deliver BMP2 for the repair of critical-sized bone defects.

In the present study, we combined BMP2-cop.BioCaP with BCP to heal critical-sized cranial defects in rats. The bone regeneration was evaluated 4 and 12 weeks after the operation using micro-CT, histological and histomorphometrical analysis. Our hypothesis was BMP2-cop.BioCaP could introduce osteoinductivity to BCP and it could function as effectively as autologous bone for the repair of critical-sized bone defects.

2. Materials and Methods

2.1 Study design

Biomimetic calcium phosphate (BioCaP) and BMP2-coprecipitated layer-by-layer assembled biomimetic calcium phosphate (BMP2-cop.BioCaP) granules were prepared and evaluated both *in vitro* and *in vivo*. BioCaP and BMP2-cop.BioCaP were characterized *in vitro* by using confocal laser scanning fluorescence microscopy (CLFM) and scanning electron microscopy (SEM). The loading and release kinetics of BMP2 in each group was also assessed. For the *in vivo* experiments, we adopted a rat critical-sized cranial defect model for assessing the formation of bone tissue and degradation of the materials assessed using micro-CT evaluation, histological and histomorphometrical analysis.

2.2. Fabrication of BioCaP and BMP2-cop.BioCaP granules

The BioCaP granules were prepared using the well-established protocol developed by our group[18]. Briefly, 2000ml 5-fold-concentrated simulated body fluid (684mM NaCl, 12.5mM $CaCl_2 \cdot 2H_2O$, 5mM $Na_2HPO_4.2H_2O$, 21mM $NaHCO_3$, 7.5mM $MgCl_2.6H_2O$ (Sigma, St. Louis, USA)) was incubated for 24 hours at 37°C to obtain amorphous calcium phosphate (ACP) particles which served as a core of the particles. Subsequently, crystalline octacalcium phosphate (OCP) was deposited on the core. This was produced by immersing ACP granules into 1000 ml supersaturated calcium phosphate solution (40mM HCl, 4mM $CaCl_2.2H_2O$, 2mM $Na_2HPO_4 \cdot 2H_2O$(Sigma, St. Louis, USA)), which was buffered to pH 7.4 with 50mM TRIS, for 48 hours at 37°C. ACP layer and OCP layer were obtained alternately three times to obtain the final granules. To produce BMP2-cop.BioCaP, the final crystalline layer was functionalized by the incorporation of BMP2 (INFUSE® Bone Graft, Medtronic,

Minneapolis, MN, USA) into this solution at a concentration of 2 μg/ml. The entire procedure was conducted under sterile conditions.

2.3 Characterization of BioCaP and BMP2-cop.BioCaP granules

Structure characterization of BioCaP using CLFM

To investigate the inner structure of BioCaP granules, bovine serum albumin (BSA) labelled with fuorescei-isothiocyanate (FITC-BSA, siga, St. Louis MO, USA) (0.1mg/ml) was coprecipitated into ACP layer and Rhodamine B (0.1mg/ml) was co-precipitated into OCP layer. After freeze drying, the granules were embedded in methyl methacrylate. 600μm-thick sections were prepared and affixed to Plexiglas holders. These sections were then ground down to a thickness of 80 mm for an inspection in a fluorescence microscope.

Surface characterization of BioCaP and BMP2-cop.BioCaP using scanning SEM

The morphology of BMP2-cop.BioCaP granules was evaluated using a scanning electron microscope (XL 30, Philips, the Netherlands). For this purpose, samples of the material were mounted on aluminum stubs and sputtered with gold particles to a thickness of 10-15nm.

2.4 Quantification of the amount of the incorporated BMP2

The amount of incorporated BMP2 was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (PeproTech, London, UK). 0.05g of BMP2-cop.BioCaP (n=6) was dissolved in 1ml 0.5M EDTA (pH 8.0) and vortexed twice for 5 minutes. The supernatants were withdrawn for analysis of the total loading of BMP2.

2.5 Release kinetics of BMP2 from BMP2-cop.BioCaP

To study the release kinetics of BMP2 from BMP2-cop.BioCaPgranules, FITC-BSA was used as cost effective alternative for BMP2. Previous studies have indicated the similarity between the release kinetics of BSA and BMP2 [19-21]. Six samples of FITC-BSA-cop BioCaP were incubated in a sealed 10ml glass tubes containing 2ml of phosphate-buffered 0.9% saline (pH 7.4). The tubes were incubated for up to 35 days in a shaking waterbath at 37°C (60agitations/min). The supernatant of each sample was withdrawn for spectrophotometric analysis in a fluorimeter (Spectrama M2, Molecular Devices, CA, USA).The temporal release of FITC-BSA was expressed as a percentage of the total amount that had been coprecipitated into the crystalline layer of the BioCaP.

2.6 Experimental groups for animal study

A rat cranial defect (8 mm in diameter), which was considered as a critical-sized bone defect [22] was used for the experimental animal model in this study. Seven groups (n=6) were established for treating critical-sized bone defects (Table 1).

A) Empty defect (Blank control)
B) BioCaP alone
C) BCP alone (Negative control for the effects of BMP2-cop.BioCaP)
D) BMP2-cop.BioCaP alone
E) BCP mixed with BioCaP (Negative control for the effects of BMP2)
F) BCP mixed with BMP2-cop.BioCaP (Experimental groups)
G) BCP mixed with autologous bone (Positive control)

Straumann® BoneCeramic™ (Straumann, Basel, Switzerland) was applied as BCP. In group C and D, volume ratio of BCP to BioCaP/BMP2-cop.BioCaP was 4:1. 0.28ml of BCP (size: 0.25-1.00mm) and 0.07ml of BioCaP/BMP2-cop.BioCaP (size: 0.25-1.00mm) per sample were placed into 1ml Eppendorf tube and homogenously vortexed. Autologous bone in group G was harvested from the cranial defects in rats, ground to chips and then mixed with BCP under sterile conditions. The amount of the materials in each group and the corresponding volume ratio was determined as in our previous study [18].

Table 1. Composition and BMP2 loading dose of groups

Groups	Abbreviation	Volume of BCP(ml)	Volume of BioCaP with/without BMP2(ml)	BMP2 loading dose (μg)
Empty defect	Empty defect	-	-	-
BioCaP alone	BioCaP	-	0.35	-
BCP alone	BCP	0.35	-	-
BCP mixed with BioCaP	BCP+BioCaP	0.28	0.07	-
BMP2-cop.BioCaP alone	BMP2-cop.BioCaP	-	0.35	51.13 ± 9.68
BCP mixed with BMP2-cop.BioCaP	BCP+ BMP2-cop.BioCaI	0.28	0.07	10.29 ± 1.94
BCP mixed with autologous bone	BCP+autologous bone	0.28	0.07*	-

* 0.07ml autologous bone, instead of BioCaP

2.7 Surgical procedure

Fourty two male Sprague-Dawley rats (12 weeks and weighing~500g) were randomly divided into 7 groups mentioned above at each time point. In total, 84 rats were used for two time points in the animal experiments, 4 or 12 weeks after surgery. The animal care was performed in accordance with the guidelines of the Ethical Committee of Zhejiang Chinese Medical University. All animal experiments were carried out according to the ethic laws and regulations of China. Critical-sized cranial defects (8 mm in diameter) were created in these rats [22]. Briefly, the rats were anaesthetized with an intraperitoneal injection of pentobarbital (Nembutal 3.5 mg/100 g). A subcutaneous injection of 0.5 ml of 1% lidocaine as a local anesthetic was given along the sagittal midline of the skull. Following this, a sagittal incision was made over the scalp from the nasal bone to the middle sagittal

crest and the periosteum was dissected (Fig. 1A). The 8mm defect was created using a dental surgical drill with a trephine which was constantly cooled with sterile saline solution (Fig. 1B and C). Subsequently, the calvarial disk was carefully removed to avoid tearing the dura (Fig. 1D). After thoroughly rinsing with physiological saline to wash out any bone fragments (Fig. 1E), samples from various groups were implanted randomly into these defects (Fig.1F). Afterwards, a collagen membrane (Bio-Gide®, Geistlich Biomaterials, Wolhusen, Switzerland) was used to cover the defect (Fig. 1G). The periosteum and the scalp were closed in layers with interrupted 4-0 Vicryl resorbable sutures (Fig. 1H). The rats were sacrificed with an overdose of Pentobarbital® (Merck, Darmstadt, Germany) 4 and 12 weeks after the operation, and samples with the surrounding tissues were taken.

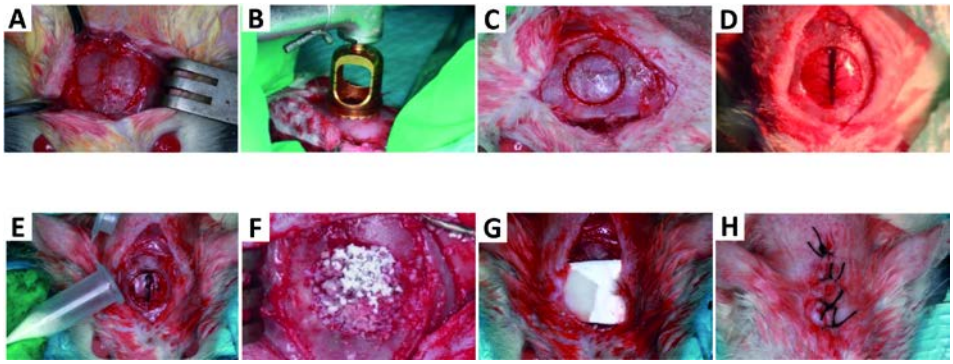

Fig 1. Surgical procedure: 8mm cranial defect was created with intersection of sagittal suture and coronal suture as center of round defect

2.8 Micro-CT evaluation

Samples with surrounding tissues were fixed chemically, and embedded in methylmethacrylate (MMA) as previously reported [23]. After MMA got hardened, samples were fixed in synthetic foam and placed vertically in a polyetherimide holder and scanned at a 18µm isotropic voxel size, 70kV source voltage and 113µA current using a high resolution micro-CT system (µCT 40, Scanco Medical AG, Bassersdorf, Switzerland). Grey values, depending on radiopacity of the scanned material, were converted into the corresponding degrees of mineralization by the analysis software (Scanco Medical AG)[24]. They were used to distinguish BCP, BioCaP/BMP2-cop.BioCaP and newly formed bone as well as to measure their volumes.

2.9 Histological and histomorphometrical analysis

By applying a systematic random sampling strategy [25], samples were sawn vertically to sagittal suture of rats into 6 or 7 slices of 600 µm thickness 1 mm apart. Slices of each

sample were mounted on Plexiglas holders and polished. Subsequently, the surfaces of the slices were stained with McNeal's tetrachrome, basic fuchsine, and toluidine blue O. Images were recorded in a stereo-microscope at a final magnification of 30 times and printed in color for the histomorphometric analysis of various parameters. The volume of the newly formed bone and the remaining material were determined stereologically from its area density on tissue sections using the point counting technique [26]. The space within the original bone defect was taken as the reference space [26]. The volume density of the newly formed bone in each group was calculated as the new bone area in the defect divided by corresponding reference area of each sample. There were two kinds of new bone observed in the histological sections. The new bone found on the periphery of the bone defect in contact with the host bone was named as osteoconductive bone, while the new bone found in the center of the bone defect, without any contact with the host bone, was named as osteoinductive bone (Fig. 2). Their volume density was again calculated as their area in the defect divided by the corresponding reference area of each sample. The relative volume of the remaining BCP granules was estimated by dividing the absolute volume of the remaining BCP by the corresponding original volume of the BCP. The original volume of BCP in group of pure BCP (0.35 ml) was considered as 1 unit, and the original volume of BCP in other group (0.28ml) was considered as 0.8 unit.

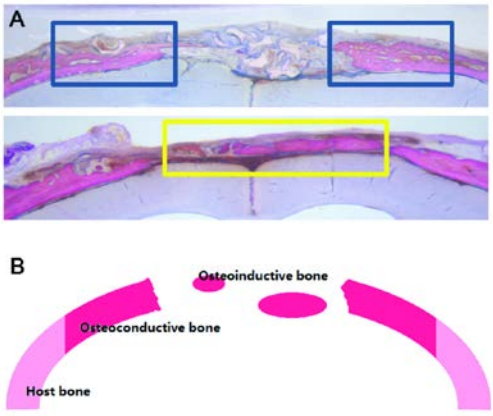

Fig 2. Representative histological sections (A) of osteoconductive bone (Blue frame) and osteoinductive bone (Yellow frame). The histological sections were stained with McNeal's Tetrachrome, basic Fuchsine and Toluidine Blue O. The schematic diagram is shown in B.

The volume density of the foreign body giant cell (FBGC) was determined to evaluate the foreign body reaction to the BCP in the different groups. Due to small size of the FBGCs, a higher magnification was needed for point counting strategy. About 20 images of each sample were recorded at a final magnification of 320 times with a Leica DMRA microscope (Leica, Wetzlar, Germany) and printed in color for the histomorphometric analysis.

Multinucleated giant cells on surface of the BCP were counted as FBGC and its volume density was normalized to the corresponding volume density of BCP.

2.10 Statistical analysis

All data were presented as the mean values with the standard deviation (median ± standard deviation). Data were compared using the Kruskal-Wallis one-way analysis of variance with significance level 0.05. Post hoc comparisons were made using Dunn's test.

3 Results

3.1 Preparation and characterization of BioCaP and BMP2-cop.BioCaP granules

In this study, we produced BioCaP granules using biomimetic layer-by-layer assembling technique and incorporate BMP2 into the most outer layer of BioCaP granules to obtain BMP2-cop.BioCaP granules. The CLFM images of cross sections of BioCaP granules confirmed the layer-by-layer structure in that an amorphous CaP layer (Fig. 3A) and a crystalline CaP layer (Fig. 3B) were deposited alternately onto each other three times to form the final granules─BioCaP (Fig. 3C). BMP2-cop.BioCaP granules were seen with a scanning electron microscope as irregular clusters of microspheres ranging from 100 to 1000μm (Fig. 3D). The outermost of BMP2-cop.BioCaP granules exhibited a uniform crystalline surface (Fig. 3E). In BMP2-cop.BioCaP granules, the total loading of BMP2 in $0.35cm^3$ (size of each sample) was 51.13±9.68μg with a co-precipitation rate of 30.1±5.7%. Protein coprecipitated into BioCaP was released gradually and at a steady rate from the 3rd day until 35th day [18].

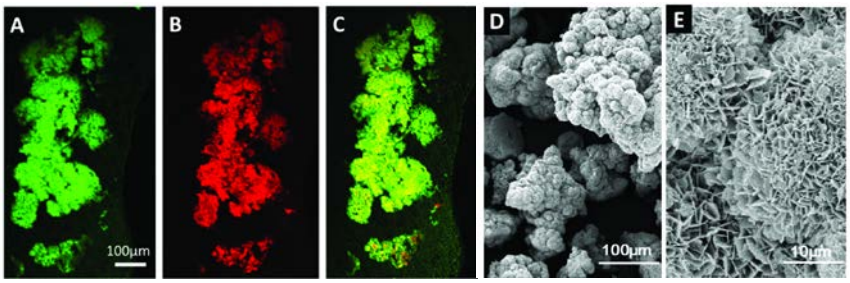

Fig 3. Laser-scanning fluorescence micrographs of cross-sections BioCaP granules depicting that amorphous (A, green signal) and crystalline (B, red signal) calcium phosphate were layer-by-layer assembled. The images in A and B are merged in image C. Scanning electron micrographs depicting the morphology of BMP2-cop. BioCaP granules (D) and crystalline outer layer of BMP2-cop. BioCaP granule (E).

3.2 Clinical and micro-CT observations

All 84 rats remained healthy and all the surgical sites healed without any complications. No visual sign of inflammation or adverse tissue reaction was observed. All specimens were retrieved after implantation periods of 4 and 12 weeks. BCP could be recognized in the micro-CT examination because its mineralization was significantly higher than in the rest of the material or tissue. Newly formed bone and BioCaP/BMP2-cop.BioCaP can hardly be distinguished from one another because their grey values were too close (Fig. 4).

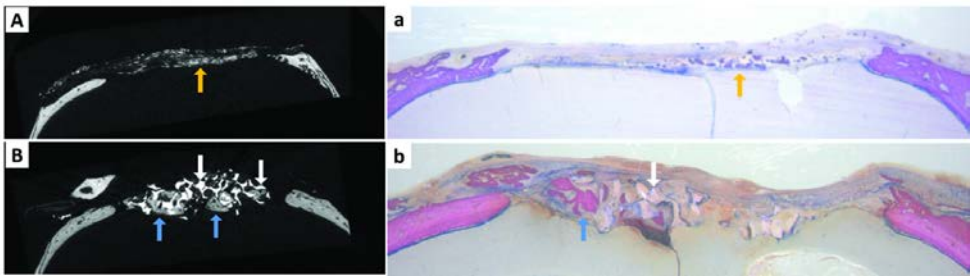

Fig 4. Micro-CT images and corresponding histological images of group containing only BioCaP (A, a) and group containing BCP with BMP2-cop.BioCaP (B, b). In micro-CT image, BCP (white arrow) showed highest grey value due to its strongest radiopacity; BioCaP(yellow arrow) and newly formed bone (blue arrow) showed quite similar grey values which can hardly be distinguished the micro-CT machine.

3.3 Histological results

An empty defect was not completely healed even 12 weeks after implantation, which proved that the 8mm diameter rat cranial defect was a critical-sized defect (Fig. 5 A, a).

In the groups with BCP alone or with BCP mixed with BioCaP, bone formation was found along the borders of the bone defect, which was considered as osteoconductive bone. After 4 weeks, it showed as deep pink stained woven bone (Fig. 5B, 5C), while after 12 weeks, the new bone had grown into lamellar bone and light pink stained. BCP granules were distributed uniformly in the bone defect and no significant degradation of the granules was seen after 12 weeks (Fig. 5b, 5c).

When BCP was mixed with BMP2-cop.BioCaP, a higher amount of new bone was observed as compared to other groups. After 4 weeks, both osteoconductive and osteoinductive bone were observed in the defect (Fig. 5D). After 12 weeks, these two kinds of new bone had merged into bone bridge, which fulfilled most of the defect (Fig. 5d). In the area of new bone, BCP granules were encapsulated by newly formed bone. The outline of the embedded BCP granules was not as sharp as for the original granules. The granules

were gradually degraded and replaced by new bone.

In group of BioCaP alone, the amount and distribution of new bone formation was found to be similar to that of the empty defect. After 4 weeks, BioCaP granules were distributed uniformly in the defect (Fig. 5E). After 12 weeks, no BioCaP granules were observed any more (Fig. 5e).

In group of BMP2-cop.BioCaP alone, osteoconductive and osteoinductive bone were both found after 4 (Fig. 5F) and 12 weeks (Fig. 5f). They were similar to those found the group of BCP mixed with BMP2-cop.BioCaP, but with a relatively smaller volume. BMP2-cop.BioCaP particles cannot be found any more 4 weeks after the operation.

In the group of BCP mixed with autologous bone, obvious osteoconductive bone and a relatively small piece of osteoinductive bone were observed at 4 weeks. Pieces of autologous bone cannot be found anymore (Fig. 5G). After 12 weeks, osteoinductive bone had merged into a bigger block of new bone. It decreased dramatically after 12 weeks compared the amount of BCP on the 4 week histological sections (Fig. 5g).

In summary, in all the groups without BMP2 or autologous bone, only osteoconductive bone was observed. The amount of osteoconductive bone did not increase with time, but the structure of it became more mature. In the groups with BMP2 or autologous bone, not only osteoconductive bone but also osteoinductive bone were found in the defects and more newly formed bone could be expected in comparison with groups without BMP2.

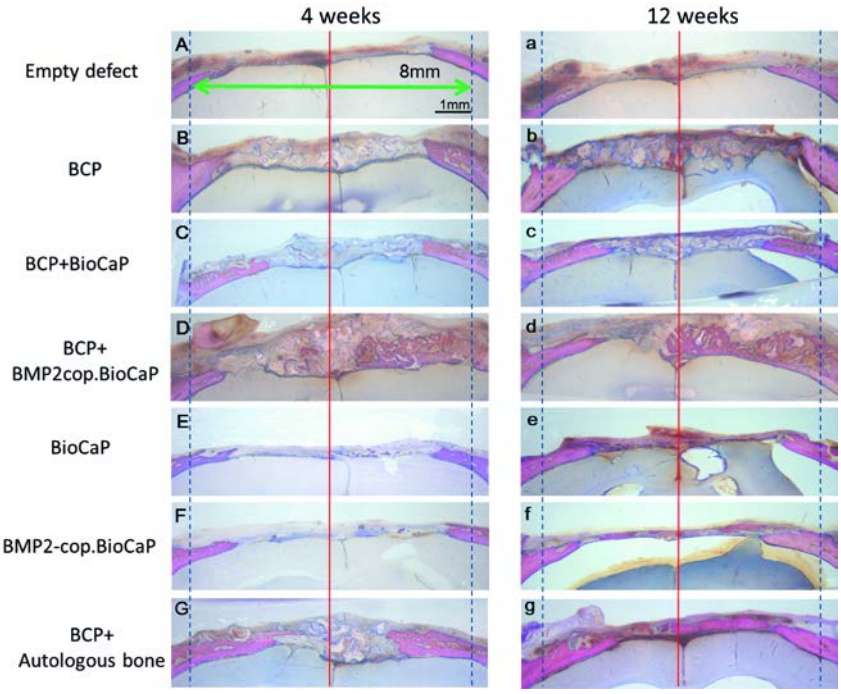

Fig 5. Representative histological images of each group after 4 and 12 weeks at a low magnification (30×).The sections were stained with McNeal's Tetrachrome, basic Fuchsine and Toluidine Blue O.

3.4 Histolomorphometric results

New bone formation

The descriptive measurements of the total new bone, including osteoconductive bone and osteoinductive bone, 4 and 12 weeks after implantation was shown in table 2. Itrevealed that the volume density of the newly formed bone was significantly higher when BCP was mixed with BMP2-cop.BioCaP compared with BCP alone after 4 weeks and 12 weeks. The volume density of newly formed bone was the same when BCP was mixed with BMP2-cop.BioCaP or with autologous bone. The volume density of the newly formed bone in the group with BMP2-cop.BioCaP was significantly less at both 4 and 12 weeks compared to group of BCP mixed with BMP2-cop.BioCaP (Fig. 6).

Table 2. Descriptive measurements of volume density (mm^3/mm^3) of total newly formed bone, including osteoconductive and osteoinductive bone, in all groups at 4 and 12 weeks after implantation (Median±Standard deviation).

	4 weeks			12 weeks		
	Osteoconductive bone	Osteoinductive bone	Total new bone	Osteoconductive bone	Osteoinductive bone	Total new bone
Empty defect	0.04±0.01	-	0.04±0.01	0.04±0.01	-	0.04±0.01
BioCaP	0.05±0.02	-	0.05±0.02	0.06±0.02	-	0.06±0.02
BCP	0.23±0.04	-	0.23±0.04	0.25±0.05	-	0.25±0.05
BCP+BioCaP	0.24±0.04	-	0.24±0.04	0.27±0.06	-	0.27±0.06
BMP2-cop.BioCaP	0.18±0.03	0.08±0.02	0.26±0.07	0.19±0.03	0.15±0.06	0.38±0.04
BCP+BMP2-cop.BioCaP	0.30±0.02	0.17±0.03	0.43±0.04	0.30±0.04	0.28±0.04	0.58±0.05
BCP+autologous bone	0.30±0.02	0.15±0.03	0.44±0.04	0.30±0.04	0.26±0.04	0.59±0.02

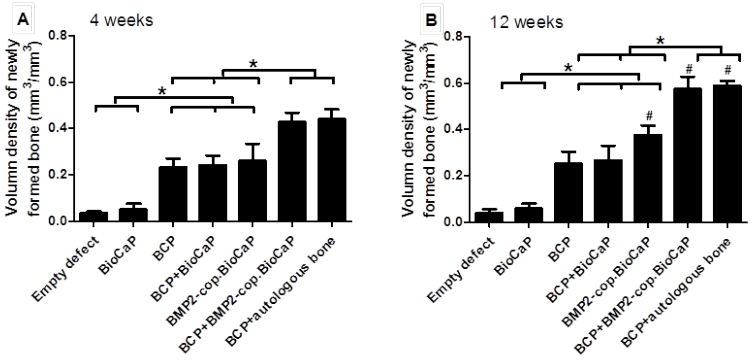

Fig 6. Histomorphometric measurements of volume density of total newly formed bone including osteoconductive bone and osteoinductive bone at 4 (A) and 12 (B) weeks after implantation. Values are shown as median± standard deviation. * $p < 0.05$, indicating significant difference between groups at the same time point; # $p < 0.05$, indicating significant difference between 4 weeks and 12 weeks of the same group.

The amount of newly formed bone displayed different growth profiles with time. When the bone defect was filled with BCP alone, the newly formed bone did not increase much from 4 weeks to 12 weeks. Whereas, BCP mixed with BMP2-cop.BioCaP induced a statistically significant increase in bone formation after 12 weeks compared with after 4 weeks. A similar phenomenon was also observed in positive control group of BCP mixed with autologous bone. As mentioned above, the new bone was divided into two types namely osteoconductive and osteoinductive bone. The amount of osteoconductive bone did not increase with time (Fig. 7A). On the other hand, the amount of osteoinductive bone did increase with time in the groups with BMP2-cop.BioCaP or with autologous bone (Fig. 7B). To sum up, the amount of new bone in the group with BCP alone did not increase because only osteoconductive bone formed, which did not show an obvious increase with time. In contrast, when defects were implanted with BCP mixed with BMP2-copBioCaP, both osteoconductive and osteoinductive bone were observed and a significant increase in osteoinductive bone was observed from 4 weeks to12 weeks.

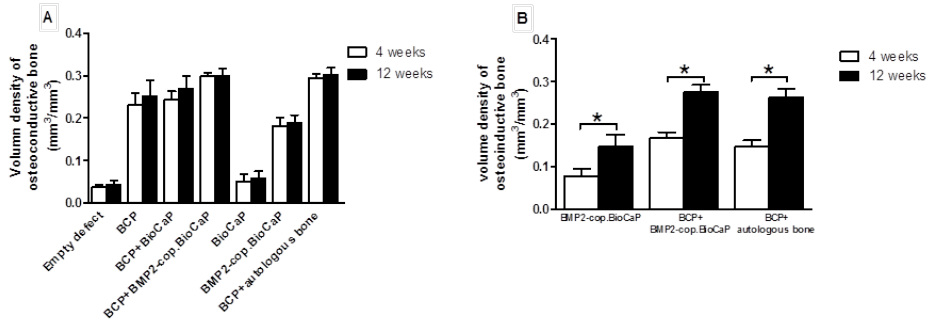

Fig 7. Histomorphometric measurements of volume density of osteoconductive bone (A) and osteoinductive bone (B) at 4 and 12 weeks after implantation. Values are shown as median± standard deviation. * $p < 0.05$.

Material degradation

The degradation of BCP was associated with the bone formation. BCP did not degrade much when it was implanted alone or mixed with BioCaP. These were the groups with an unfavorable new bone formation. When it was mixed with BMP2-cop.BioCaP or autologous bone, more new bone formation and more BCP degradation were observed simultaneously. This phenomenon was even more obvious after 12 weeks (Fig. 8A).

At 4 weeks, the volume density of BioCaP in the group containing BioCaP alone was 0.06±0.02 $mm^{3/}mm^3$. Interestingly, BioCaP was hardly found in the group of BCP mixed with BioCaP. Whereas, BMP2-cop.BioCaP degraded completely no matter if it was with or without BCP. After 12 weeks, no BioCaP could be observed in groups with BioCaP alone or with BCP mixed with BioCaP.

Foreign body reaction

The FBGCs at 12 weeks were too few to count. Therefore, only data at 4 weeks were obtained (Fig. 8B). The volume density of FBGCs to BCP was significantly lower when BCP was mixed with BMP2-cop.BioCaP than it was either alone or mixed with BioCaP. No statistically significant differences were found between BCP mixed with BMP2-cop.BioCaP and autologous bone.

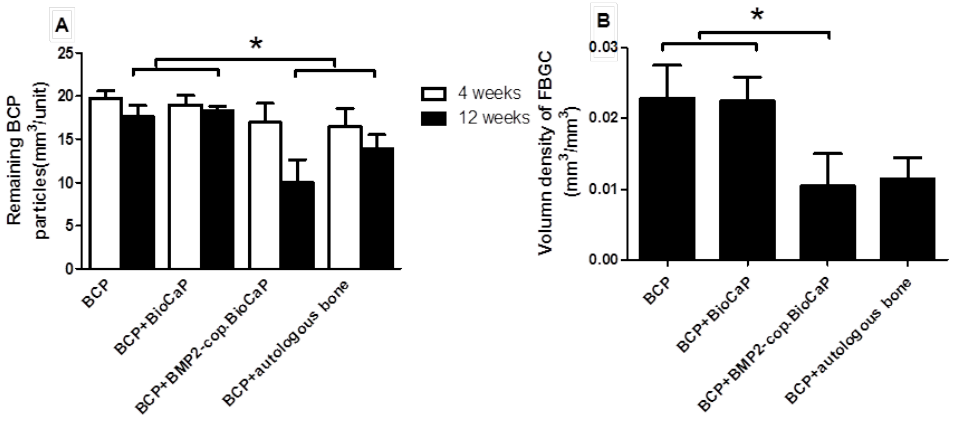

Fig 8. Histomorphometric measurements of remaining BCP granules at 4 and 12 weeks after implantation (A) and volume density of FBGC at 4 weeks after implantation (B). Values are shown as median± standard deviation. * p <0.05.

4 Discussion

The aim of this study was to investigate if BMP2-cop.BioCaP could replace autologous bone by functioning as an effective osteoinducer for BCP and enhancing bone regeneration in a critical-sized bone defect. Our results supported the positive effect of BMP2-cop.BioCaP on bone formation. BCP mixed with BMP2-cop.BioCaP induced more new bone in rat cranial critical-sized defects than BCP alone and it was as effective as BCP mixed with autologous bone. It indicated that BMP2-cop.BioCaP could be considered as an effective method of introducing osteoinductivity into bone substitutes. Doctors and dentists would not have to rely on autologous bone and the complications caused by autologous bone transplantation could be avoided. What's more, the outstanding benefit of this osteoinducer was that it could be mixed with any commercially available bone substitute granules without altering their original property. Thus BMP2-cop.BioCaP could be made into an off-the-shelf product. Compare to other approaches of conferring bone substitutes with osteoinductivity, adding BMP2-cop.BioCaP to clinically available bone substitutes should be easier for clinical application.

BMP2-cop.BioCaP is a kind of biomimetic calcium phosphate granule on which an amorphous calcium phosphate layer and a crystalline calcium phosphate layer are deposited alternately on the granule. When these two kinds of calcium phosphate layers were stained with different fluorescent dyes, their emerged confocal images showed the two overlapping fluorescent colors (Fig. 3C), which confirmed the layer-by-layer structure of BMP2-cop.BioCaP granules.

Rat cranial critical size defect (8mm in diameter) [22] was used as an implantation model to evaluate the osteoinductive efficacy of materials. This kind of preclinical testing has been widely used for large skeletally mature animals including dogs, goats, sheep and pigs. Compared to rodents, these animals were considered to have more similar structure and composition of bone tissue as humans. However, there was no substantiation that one model was proved to be better than any other when it was used to address clinical translation of assayed therapeutic approaches [27]. What is more, rats are inexpensive, easy to house and to manipulate. In addition, the reduced lifespan of rats avoids the influence of animals' ageing on the bone metabolism and regeneration processes.

BMP2-cop.BioCaP improved bone formation because it could induce osteoinductive bone that was new bone formed in the center of bone defect. The formation of osteoinductive bone produced a new bone regenerating center besides the one at the periphery of host bone. According to the understanding of bone defect healing process in animals, bone regeneration starts from the borders of the defect, moves centripetally to fill the defect and so tends to heal the defects finally [28]. This does not happen for critical sized bone defects and the growth to the center stops at a certain point. This phenomenon could be observed in the empty defect in our study. To some extent, BCP filled in the defect enhanced the healing but it did not change the conventional healing pattern. Whereas, the addition of BMP2-cop.BioCaP altered the healing by introducing a new center of regenerating bone—osteoinductive bone. Consequently, the two centers of bone regeneration accelerated and enhanced the healing of the defect.

The efficacy of BMP2-cop.BioCaP in improving bone formation was comparable with the most often used osteoinducer in clinical practice─autologous bone. Although the distribution, composition and the amount of new bone in these two groups were similar as the histological sections showed, the mechanism of osteoinductive bone formation in these two groups was quite different. Autologous bone is osteogenic, including hydroxyapatite functioning as scaffold, osteogenic cells and a bunch of bioactive agents, which could form new bone by itself. Therefore it can be applied to almost all bone defects independently or combined with other bone grafting materials. Whereas, BMP2-cop.BioCaP needed an extra resource of osteogenic cells and scaffold to heal the critical-sized bone defect. Therefore, although our results support the use of BMP2-cop.BioCaP for replacing autologous bone, further studies with other animal models and human beings are still needed to evaluate this hypothesis.

Osteoinductive bone was able to grow on its own even in the absence of BMP2. According to our results, BMP2-cop.BioCaP stimulated new bone formation within 4 weeks. After 4 weeks, the BMP2-cop.BioCaP particles were completely degraded. Theoretically it could not then stimulate bone formation any more. However, we found osteoinductive bone

growing bigger from 4 weeks to 12 weeks, indicating the ability of the osteoinductive bone to grow by itself. But it could not heal the complete bone defect even after 12 weeks, which contradicted other studies using the same animal model [29]. There could be two possible explanations for the unhealed bone defects in the present study: one was that osteoinducer (BMP2-cop.BioCaP) only needed to form osteoinductive bone at the early stage of bone regeneration to stimulate bone regeneration. Even after the BMP2-cop.BioCaP was degraded, the existence of osteoinductive bone was enough to completely heal the defect since it could grow on its own. The incomplete healing was only because the observing period was not long enough; the other one was that during the entire healing period the existence of osteoinducer was needed, the BMP2-cop.BioCaP particles degraded too fast to ensure a complete healing of the defect. To find the real reason we will need to study extra groups of BMP2-cop.BioCaP with a slower degradation profile or groups of BioCaP loaded with more BMP2 and to measure for a longer time to assess if the results will be different from the current ones.

The sustained release of BMP2 guarantees that BMP2-cop.BioCaP functions as an effective osteoinducer [30]. It has been proved *in vitro* that protein coprecipitated into BioCaP was released gradually and at a steady rate after the 3rd day until the 35th day, at which juncture the initial depot had been depleted by no more than 50.1% [18]. Although there are no direct *in-vivo* supporting experiments, it is logic to speculate that the release of BMP2 from BMP2-cop.BioCaP *in vivo* would be slow and sustained, because BMP2-cop.BioCaP is derived from biomimetic calcium phosphate coating and they used the same technique to carry BMP2—incorporating BMP2 into the inorganic lattice network work of crystalline calcium phosphate. Our previous experiment proved that calcium phosphate coating degraded slowly *in vivo* and BMP2 was liberated as the crystalline calcium phosphate degrades [23]. Titanium alloy discs with a biomimetic BMP2-incorporated calcium phosphate coating were implanted subcutaneously in rats and retrieved for histomorphomtrical analysis at 7 day intervals over a period of 5 weeks. From the first to the fifth week, volume of coating remaining was 9.22, 9.21, 5.78, 5.54, 2.04 mm^3. It proved directly that the coating degraded slowly and supported indirectly the *in vivo* slow release of BMP2 from BMP2-cop.BioCaP. Compared with the degradation profile *in vitro*, the degradation of BMP2-cop.BioCaP *in vivo* was much faster due to the acceleration by many types of cells (e.g. fibroblasts, monocytes/macrophages) through phagocytotic mechanism [31].

A bone substitute should have proper degradation profile to match the bone growth rate [32]. It cannot degraded too fast since it has to function as a scaffold for the osteoblast to attach, proliferate, differentiate and finally form new bone. Also it cannot degrade too slowly since the newly formed bone needs finally to replace it. As a bone substitute, BCP does not degrade easily. It was reported that 26.6% of BCP granules were still not degraded

after 6 to 8 months in a treated maxillary sinus in a clinical trial [33], which needed to be accelerated. The present study showed that in the group with BCP alone, the volume of BCP did not change much even after 12 weeks. Significantly more BCP granules degraded in the groups containing BCP mixed with BMP2-cop.BioCaP or autologous bone than in the group with BCP alone. This indicated that BMP2-cop.BioCaP stimulated the degradation of BCP because in the process of osteogenesis, BCP served as a scaffold and a resource of calcium and phosphate ions for the new bone formation. With osteogenesis going on, BCP would be gradually degraded and replaced by new bone. The more actively the bone regenerated, the more BCP granules were degraded [34].

One major concern of biomaterials is their biocompatibility. Unfavorable biocompatibility is often characterized by a foreign body reaction to biomaterials [35, 36], which histologically is seen by the local accumulation of macrophages, their fusion to form FBGCs, and the deposition of dense fibrous connective tissue. Therefore, the FBGCs shown in histological sections could be a reliable parameter to evaluate the foreign body reaction. Some studies [23, 37] applied tartrate resistant acid phosphatase (TRAP) reaction to recognize osteoclasts (TRAP-positive) and subtracted them from the total of multinucleated giant cells found on the histological images to calculate the number of FBGCs. The undecalcified sections we used in this study were not optimal for the histochemical demonstration of TRAP reactivity. The poor staining reactivity could potentially raise the possibility that the population of TRAP-negative cells included not only FBGCs but also osteoclasts registering falsely negative. Therefore, TRAP staining was not applied for evaluating foreign body reaction in the present study. Instead we counted multinucleated giant cells on the surface of BCP as FBGC to evaluate the foreign body reaction caused by different materials. This was based on the theory that FGBC should appear on the surface of foreign material and that the other multinucleated giant cells on the newly formed bone are osteoclasts for bone remodeling. In the present study, we recognized that the volume ratio of FBGCs to BCP was significantly lower in the group with BCP mixed with BMP2-cop.BioCaP as compared to that in the group of either BCP alone or BCP with BioCaP. It showed that BMP2-cop.BioCaP was not only able to induce bone formation efficiently but it could also inhibit the host foreign body reaction to BCP. The inhibition to foreign body reaction was probably caused by the extensive osteogenesis [38]. During bone regeneration the level of osteopontin increases. Furthermore, osteopontin was previously shown to suppress the fusion of macrophages into FBGCs both *in vitro* and *in vivo* [39]. Therefore, it was reasonable to speculate that the extensive osteogenesis, stimulated by BMP2-cop.BioCaP, helped to inhibit foreign body reaction.

BMP2-cop.BioCaP could function as an effective osteoinducer to introduce osteoinductivity to BCP, however it could hardly be applied alone as a bone substitute material. The result of new bone formation in group with BMP2-cop.BioCaP confirmed this

speculation. This was because if the CaP-based materials degraded too quickly, it could not function as scaffold long enough for the osteoblasts to form new bone. Apparently, 4 weeks was too short for BMP2-cop.BioCaP to heal critical-sized bone defect. To optimize the degradation rate of BMP2-cop.BioCaP, it may be helpful to understand the possible factors involved: Firstly, the incorporation of BMP2 played a role in the on degradation of BioCaP granules. We found that the degradation rate of BMP2-cop.BioCaP increased significantly in comparison with BioCaP. Interestingly, the suppression of FBGCs to CaP coatings in the presence of coprecipitated BMP2 could be found from 2 to 3 weeks [23]. Therefore, the degradation of BMP2-cop.BioCaP could hardly be attributed to phagocytic activity. In contrast, mineralization mediated by osteoblasts may possibly affect its degradation. This mineralization process generated many protons [40]. Therefore, an extra cellular buffering system was needed to neutralize them so as to prevent their accumulation, otherwise it would influence the activity of osteoblasts negatively. Calcium and phosphate ions dissolved from BMP2-cop.BioCaP and BioCaP granules could directly neutralized the protons. The more osteogenesis there was, the more the protons were generated and the more calcium and phosphate ions were needed to neutralize them. As we learnt from results, BMP2-cop.BioCaP showed more osteogenic efficacy than BioCaP. It degraded faster to release more calcium and phosphate ions to neutralize protons. Secondly, the mixture with BCP did not affect significantly the degradation rate of either BioCaP or BMP2-cop.BioCaP, which suggested that the degradation of BMP2-cop.BioCaP was not impacted by the materials used for bone grafting. Thirdly, the impact of the environment on the degradation rate of BMP2-cop.BioCaP and BioCaP granules should also considered. In the present study, the BMP2-cop.BioCaP completely degraded after 4weeks in a rat cranial bone defect. A previous study showed that BMP2-cop.BioCaP had not been completely degraded during a 5 week period at an ectopic site[18]. The granules may result in a faster degradation in the orthotopic site compared with the ectopic site. The possible reasons for this could be that compared with a connective tissue environment, the osteogenic environment was more suitable for bone formation. Enhanced bone formation needed more calcium and phosphate ions from BioCaP degradation.

BMP2-cop.BioCaP was based on the technique of producing lay-by-layer assembled calcium phosphate carrier loaded with BMP2. These BioCaP particles could also be incorporated with other proteins to serve as a carrier for the slow release of those proteins. Given this valuable property, we can add many specific functions by coprecipitating with different agents, such as those against inflammation and cancer.

4 Conclusion

With the limitation of the animal model and the timeframe used in this study, we conclude that BMP2-cop.BioCaP successfully introduced osteoinductivity to BCP and function as

effective as the golden standard－autologous bone. Our findings further showed that the excellent osteoinductivity of BMP2-cop.BioCaP resulted from its slow and sustained delivery of BMP2, BCP combined with BMP2-cop.BioCaP has shown a promising potential for guided bone regeneration clinically in the future.

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